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**HOMOGENEOUS AND HETEROGENEOUS
ENZYMEIMMUNOASSAYS
FOR THE HOME DETECTION OF FERTILITY**

A thesis presented in partial fulfilment of
the requirements of the degree of
Doctor of Philosophy in Biochemistry
at Massey University

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March, 2000

ABSTRACT

The physiology of the menstrual cycle has been reviewed and the suitability of the ovarian steroid urinary metabolites estrone glucuronide and pregnanediol glucuronide as markers of fertility and as utilised by the home Ovarian Monitor fertility assay was discussed. The biomaterials for the homogeneous enzymeimmunoassay which forms the basis of the Ovarian Monitor home fertility assay were prepared. One of the major difficulties in preparing a signal generator for use in home tests is the separation of the unconjugated enzyme material from the desired signal generators. In this thesis a new procedure was developed for the purification and isolation of the complex range of signal generators formed during acylation of hen egg white lysozyme with estrone glucuronide. A cation exchange column in the presence of 7 M urea allowed the separation to be carried out in the absence of the hydrophobic effects which complicate other schemes. Even under these conditions complex behaviour was seen which could be rationalised in terms of the tertiary structures of the conjugates and their electrostatic fields. A second step involving hydrophobic interaction chromatography gave two pure mono conjugated estrone glucuronide lysozyme conjugates in good yield the activities of which were highly inhibited (>90%) by anti-estrone glucuronide antibodies. The availability of the pure conjugates allowed the effect of tertiary structure on the immunoassays to be evaluated. The results showed that both mono acylated hen egg white lysozymes could be used to give good standard curves for use in monitoring menstrual cycles for the naturally occurring periods of fertility and infertility. Since the specific activity of human lysozyme is three times that of hen egg white lysozyme, in an attempt to provide a more rapid test for fertility human lysozyme estrone glucuronide conjugates were synthesised with estrone glucuronide for the first time. However, despite the fact that these two enzymes had very similar tertiary structures they behaved completely differently in the protein chemistry and immunological experiments reported in this thesis. The human enzyme was more easily acylated to give pure mono acylated conjugates in high yield and the conjugates were more easily purified to give highly inhibitable conjugates (>95%). The differences in behaviour could be accounted for in terms of the sequence differences between the two lysozymes and the relative exposure of the lysine residues. A fast assay (1-2 minutes) was developed for urinary estrone glucuronide using the three new signal generators. However, the sensitivity of the assays was less than half that of the hen egg white conjugates making them unsuitable for use in home assays for fertility. The assays could be useful for women using fertility drugs such as clomiphene. The lack of sensitivity of the assays and other binding behaviour indicated a much tighter binding to the antibody than with the hen egg white conjugates. This important difference was accountable on the basis of the extra extension of the lysine residues in the human enzyme. A new method for producing estrone glucuronide conjugates of the active enzyme horse radish peroxidase was evaluated. The mono substituted hemin conjugates reconstituted with the apo protein to give active peroxidases with good specific activities (~50%) and good stability. The procedure is such that any small molecule can be attached to the enzyme using the same procedures and a large range of signal generators can be formed for immunoassays. However, both assay formats examined failed to produce an assay in this thesis. The reconstituted enzymes, although binding to the immobilised anti- estrone glucuronide antibodies as required did not produce the necessary colours. The reasons for this were discussed.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Associate Professor Len Blackwell, who has been likened to a Saint and a fool by my family for his fortitude in putting up with me over the years. It truly is the end of an era.

I would also like to thank Dr Mark Smales for the very efficient and free electrospray mass spectral service he has provided from England, Dr Todd Kagawa for the very late night graphic sessions, and Andrew Baldwin for expert technical assistance. I am also grateful to the now disbanded Separation Science unit for the extensive use of their FPLC, to Zhandong Zhao and Dr Yinqi Wu who between them provided all the hemin compounds upon which Chapter Five was based, and to Dr Keith Henderson of AgResearch at Wallaceville who has generously provided our research group with both antisera and information of his ELISA assay.

For the extended writing up phase of this thesis I am indebted to my father who gave up his new computer without ever getting to use it and everyone who kept the system working, and in particular Bob Lambourne and the people at Canon.

I also wish to thank all my friends and past flatmates who kept a low profile during the final stages, and especially Anja my current flatmate.

Finally a special thanks to my family for all their continued support over the years.

TABLE OF CONTENTS

	page
Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Figures	xv
List of Tables	xxiv
List of Schemes	xxv
Abbreviations	xxvi

CHAPTER ONE

OVARIAN PHYSIOLOGY, FERTILITY AND THE OVARIAN MONITOR

1.1 Introduction	1
1.2 The Fertile Menstrual Cycle	1
1.2.1 Initiation of Follicular Growth	1
1.2.2 The Follicular Phase	4
1.2.2.1 Follicular Estrogen Production	4
1.2.2.2 The Follicle Stimulating Hormone Threshold	7
1.2.3 Ovulation	8
1.2.4 The Luteal Phase	9
1.2.5 The Fertile Period	10
1.3 Natural Family Planning	12
1.3.1 Methods of Natural Family Planning	12
1.3.1.1 Calendar Method	12
1.3.1.2 Basal Body Temperature Method	12
1.3.1.3 Billings' Ovulation Method	13
1.3.1.4 Lactational Amenorrhoea Method (LAM)	15
1.3.2 Natural Family Planning Aids	15
1.3.2.1 Basal Body Temperature	16
1.3.2.2 Cervicovaginal Mucus Volume	16
1.3.2.3 Vaginal and Salivary Electrical Resistance	16
1.3.3 Natural Family Planning Aids and Hormone Measurement	16
1.3.3.1 Urinary Luteinising Hormone Home Tests	17
1.3.3.2 Urinary Follicular Stimulating Hormone Tests	17
1.3.3.3 Choice of Ovarian Steroid Metabolites as Markers of Fertility	18
1.3.3.4 The Persona Device	21
1.3.3.5 The Ovarian Monitor	22
1.3.3.6 Choice of Urine Collection Method for Hormonal Assays	23
1.3.3.7 Hormonal Definition of the Beginning of the Fertile Period using Measurements of Urinary Estrogen Metabolites	25

1.3.3.8	Hormonal Definition of the End of the Fertile Period using Measurements of Urinary Progesterone Metabolites	26
1.3.4	The Problems of Natural Family Planning	27
1.3.4.1	The Problem of Abstinence	27
1.3.4.2	The Ovarian Monitor and Abstinence	28
1.3.4.3	The Continuum	29
1.3.4.4	The Ovarian Monitor and Navigating through the Continuum	29
1.4	The Value of the Ovarian Monitor in the Treatment of the Subfertile Couple ..	32
1.5	The Application of the Ovarian Monitor in Reproductive Health	34
1.6	The Ovarian Monitor Homogeneous Immunoassay	35
1.6.1	The Ovarian Monitor System	35
1.6.2	The Estrone Glucuronide and Pregnanediol Glucuronide Assay Protocols ...	37
1.6.3	The Estrone Glucuronide and Pregnanediol Glucuronide Standard Curves ..	38
1.7	Aims of the Thesis	40

CHAPTER TWO

SYNTHESIS AND PURIFICATION OF STRUCTURALLY DEFINED HEN EGG WHITE LYSOZYME - CONJUGATES

2.1	Introduction	41
2.2	Materials and Methods	45
2.2.1	Apparatus	45
2.2.2	Reagents	46
2.2.3	Purification of Commercial Hen Egg White Lysozyme	47
2.2.4	Active Ester Conjugation of Hen Egg White Lysozyme with Estrone Glucuronide	47
2.2.5	Standard Analytical Mono-S Cation Exchange Chromatography	49
2.2.6	The Lysozyme Assay	49
2.2.6.1	Buffers	49
2.2.6.2	Lysozyme and Lysozyme Conjugate	49
2.2.6.3	Anti-Estrone Glucuronide Antiserum	50
2.2.6.4	Substrate	50
2.2.6.5	The Standard Activity Assay Protocol	50
2.2.6.6	Inhibition Studies	50
2.3	Results	51
2.3.1	Synthesis of 17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic Acid	51
2.3.1.1	Synthesis of Methyl 1-Bromo-1-deoxy-2,3,4-tri-O-acetyl-	

2.3.1.2	α -D-glucopyranuronate	51
2.3.1.2	Synthesis of Methyl [17-Oxoestra-1,3,5(10)-triene-3-yl-2',3',4'-tri-O-acetyl- β -D-glucopyranosid] Uronate	52
2.3.1.3	Preparation of 17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic Acid	54
2.3.2	Analysis of the Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Reaction Mixture	56
2.3.3	Purification of Hen Egg White Lysozyme-Estrone Glucuronide Conjugate in the Absence of Urea	58
2.3.3.1	Mono-S Cation Exchange Chromatography at pH 4.3	58
2.3.3.2	Mono-S Cation Exchange Chromatography at pH 9.5	59
2.3.4	Large Scale Purification of Hen Egg White Lysozyme Estrone Glucuronide Conjugates under Denaturing Conditions	62
2.3.4.1	Purification of Lysozyme Conjugates by S-Sepharose fast flow Chromatography	62
2.3.4.2	Purification of Lysozyme E1 and E3 Conjugates by Butyl Sepharose Chromatography	64
2.3.4.3	Analysis of Lysozyme E1 and E3 Conjugates by Electrospray Mass Spectrometry	68
2.3.4.4	Inhibition Studies of Lysozyme E1 and E3 Conjugates	68
2.4	Discussion	70
2.4.1	Synthesis of 17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic Acid	70
2.4.2	Synthesis of Hen Egg White Lysozyme-Estrone Glucuronide Conjugates and Analysis of the Conjugation Reaction Mixture	71
2.4.2.1	Reactivity of Lysine Residues Towards Acylation by Estrone Glucuronide	71
2.4.2.2	Effect of the Lysine Microenvironment on the Contribution to the Positive Electrostatic Field	74
2.4.2.3	Selection of Lysozyme-Estrone Glucuronide Conjugates for Further Study and the Effect of Substitution Level on the Specific Activity	77
2.4.2.4	Control of the Conjugation Reaction	79
2.4.3	Large Scale Purification of Hen Egg White Lysozyme Estrone Glucuronide Conjugates	80
2.4.3.1	Attempted Isolation and Purification of Lysozyme Conjugates E1 and E3 without Urea	80
2.4.3.2	The Purification of Lysozyme Conjugates using Urea	84
2.5	Conclusions	88

CHAPTER THREE

STRUCTURALLY DEFINED HEN EGG WHITE LYSOZYME-ESTRONE GLUCURONIDE CONJUGATES AS SIGNAL GENERATORS IN HOMOGENEOUS IMMUNOASSAYS FOR MEASUREMENT OF ESTRONE GLUCURONIDE

3.1	Introduction	89
3.1.1	The Substrate and Enzyme Activity Measurement	89
3.1.2	Hen Egg White Lysozyme Steroid Glucuronide Conjugates as Signal Generators	92
3.2	Materials and Methods	95
3.2.1	Apparatus	95
3.2.2	Preparation of Assay Reagents	95
3.2.2.1	Buffers	95
3.2.2.2	Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	95
3.2.2.3	Estrone Glucuronide Antisera	95
3.2.2.4	Estrone Glucuronide Standards	95
3.2.2.5	Blank Urine	96
3.2.2.6	Urine for determination of Estrone Glucuronide Excretion Rates throughout the Menstrual Cycle	97
3.2.2.7	Bacterial Substrate	97
3.2.3	Assays for Estrone Glucuronide using Pre-Coated Estrone Glucuronide Assay Tubes with Freeze-dried Components	97
3.2.4	Assays using Fresh Components Only	97
3.2.4.1	Standard Activity Assay	97
3.2.4.2	Inhibition Studies	98
3.2.4.3	Estrone Glucuronide Standard Curves	98
3.2.4.4	Assay for Estrone Glucuronide in Daily Urine Samples	98
3.2.5	Data Analysis	99
3.2.5.1	Conversion of Ovarian Monitor Transmission Data into Absorbance ...	99
3.2.5.2	Kinetic Analysis of the Rate Order of Clearing	99
3.2.5.3	Analysis of Antiserum Titration Curves	99
3.2.5.4	Fitting of the Estrone Glucuronide Standard Curves	100
3.2.5.5	Measurement of Urinary Estrone Glucuronide Excretion Rates using Estrone Glucuronide Standard Curves	100
3.2.5.6	Comparison of Estrone Glucuronide Cycle Excretion Rates throughout a Menstrual Cycle as measured by Different Methods	100
3.2.5.7	Standard Curve Simulations	101
3.3	Results	102
3.3.1	The Melbourne Estrone Glucuronide Standard Curves	102
3.3.2	Validation of Estrone Glucuronide Standards	103
3.3.3	Wallaceville Animal Research Anti-Estrone Glucuronide Antisera	105

3.3.4	Optimisation of the Buffer Ionic Strength	106
3.3.5	Relationship between the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Activities in Light Transmission and Absorbance ..	107
3.3.6	The Bleed Three Anti-Estrone Glucuronide Antisera and the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates	110
3.3.7	The E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates and the Anti-Estrone Glucuronide Antiserum 243 Bleed Four Titration Curves	112
3.3.8	Optimisation of the E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Standard Curve with Anti-Estrone Glucuronide Antiserum 243 Bleed Four	115
3.3.9	Optimisation of the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Standard Curve with the Bleed Four Anti-Estrone Glucuronide Antiserum 243	117
3.3.10	Further Comparison of the E1 and E3 Hen Egg White Lysozyme- Estrone Glucuronide Standard Curves by Changing Assay Conditions and Computer Simulations Studies	118
3.3.11	The Effect of Blank Urine on the E1 and E3 Hen Egg White Lysozyme- Estrone Glucuronide Conjugate Optimised Standard Curves	120
3.3.12	An Alternative Source of Blank Urine	122
3.3.13	The Use of the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate for the Determination of Urinary Estrone Glucuronide Excretion for a Complete Menstrual Cycle	123
3.4	Discussion	129
3.4.1	Determinants of the Ideal Ovarian Monitor Standard Curve	129
3.4.2	Stability of the Melbourne Standard Curves	131
3.4.3	Validation of the Estrone Glucuronide Standards	132
3.4.4	The Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera	133
3.4.5	Optimisation of the Buffer Ionic Strength	134
3.4.6	Measurement of the E1 and E3 Lysozyme Conjugate Activity	135
3.4.7	Affinities of E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for Anti-Estrone Glucuronide Antisera From Bleed Three	138
3.4.8	The Effect of Conjugate Structure and Antiserum Source on the Estrone Glucuronide Standard Curves	141
3.4.9	Reproducibility of the Estrone Glucuronide Standard Curves	143
3.4.10	The Effect of Blank Urine on the E1 and E3 Hen Egg White Lysozyme- Estrone Glucuronide Standard Curves	144
3.4.11	Application of the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Menstrual Cycle Urine Samples	146
3.5	Summary	148

CHAPTER FOUR

DEVELOPMENT OF A FAST HOMOGENEOUS ENZYME IMMUNOASSAY FOR ESTRONE GLUCURONIDE USING HUMAN LYSOZYME-ESTRONE GLUCURONIDE CONJUGATES

4.1	Introduction	150
4.1.1	Decreasing the Ovarian Monitor Assay Time	150
4.1.2	Lysozymes from Alternative Sources	151
4.1.3	Human Lysozyme	152
4.2	Materials and Methods	155
4.2.1	Apparatus	155
4.2.2	Reagents	155
4.2.3	General Methods	155
4.2.3.1	Estimation of Protein Content	155
4.2.3.2	Preparation of Human Lysozyme for Conjugation to Estrone Glucuronide	155
4.2.3.3	Conjugation of Human Lysozyme to Estrone Glucuronide	156
4.2.3.4	Preparation of the Human Lysozyme Conjugation Mixture for Analysis by Mono-S Fast Protein Liquid Chromatography	156
4.2.3.5	Reconjugation of the Human Lysozyme Conjugation Mixture	157
4.2.3.6	Separation of the Human Lysozyme Conjugation Mixture by Mono-S Cation Exchange Chromatography	157
4.2.3.7	Gel Electrophoresis	158
4.2.4	Preparation of Assay Reagents	158
4.2.4.1	Buffers	158
4.2.4.2	Substrate	159
4.2.4.3	Stock Enzyme Solutions	159
4.2.4.4	Anti-Estrone Glucuronide Antiserum	159
4.2.4.5	Estrone Glucuronide Standards	160
4.2.4.6	Blank Urine	160
4.2.4.7	Urine for Determination of Estrone Glucuronide Excretion Rates throughout the Menstrual Cycle	160
4.2.5	Assay Protocols	160
4.2.5.1	The General Activity Assay Protocol	160
4.2.5.2	Inhibition Studies	161
4.2.5.3	Kinetic Analysis of the Rate Order of Clearing	162
4.2.5.4	Human Lysozyme Conjugate Standard Curves	162
4.2.5.5	Human Lysozyme Conjugate Menstrual Cycle Data	162
4.2.5.6	Attempted Measurement of the Apparent Dissociation Constant of the Lysozyme Conjugate - Antibody Complex	163
4.2.5.7	Measurement of the Displacement (Off) Rate Constants for Lysozyme Conjugates from the Conjugate - Antibody Complexes	163
4.3	Results	165

4.3.1	Analysis of the Human Lysozyme Conjugation Mixtures by Mono-S Cation Exchange Chromatography	165
4.3.2	Physical Characterisation of the Major Human Lysozyme Conjugate Fractions	167
4.3.3	Optimisation of the Human Lysozyme Conjugate Activity Assay	170
4.3.3.1	The Clearing Curve and Selection of the Assay Method	170
4.3.3.2	Optimisation of Initial Rate Assay Conditions for Buffer Ionic Strength and pH	172
4.3.3.3	The Effect of Bovine Serum Albumin on Human Lysozyme Conjugate Activity and Stability	173
4.3.4	A Rapid Human Lysozyme HE2 Conjugate Based Estrone Glucuronide Immunoassay	176
4.3.5	A Rapid Human Lysozyme HE1 Conjugate Based Estrone Glucuronide Immunoassay	180
4.3.6	Comparison of the HE1 and HE2 Human Lysozyme Conjugates with HE5 ..	185
4.3.7	Lysozyme-Estrone Glucuronide Conjugate Titrations with Anti-Estrone Glucuronide Antibody 243-4	187
4.3.8	Displacement Rate Constants for Immune-Complexes with Antiserum 243-4 and Lysozyme Estrone Glucuronide Conjugates E1, HE1 and HE2	189
4.4	Discussion	190
4.4.1	The Insoluble Fraction of the Commercial Human Lysozyme Preparation ..	191
4.4.2	Conjugation of Human Lysozyme with Estrone Glucuronide	191
4.4.3	Possible Explanation for the Ease of Mono-Acylation of Human Lysozyme with the Estrone Glucuronide Active Ester Reagent	195
4.4.4	Kinetic Studies on Human Lysozyme Conjugates	199
4.4.4.1	Selection of the Assay Method	199
4.4.4.2	Optimisation of the Assay Conditions	201
4.4.5	Kinetic Characterisation of the Human Lysozyme Estrone Glucuronide Conjugates	202
4.4.6	Feasibility of using Lysozyme-Estrone Conjugates as Signal Generators in Homogeneous Enzyme Immunoassays for Estrone Glucuronide	205
4.4.7	A Comparison of the Different Kinetic Behaviour of the Human Lysozyme and Hen Egg White Lysozyme Conjugates in terms of their Dissociation Constants	209
4.5	Conclusions	210

CHAPTER FIVE

STUDIES TOWARDS DEVELOPMENT OF A HORSERADISH PEROXIDASE BASED ENZYME IMMUNOASSAY FOR ESTRONE GLUCURONIDE USING NOVEL HORSERADISH PEROXIDASE-ESTRONE GLUCURONIDE CONJUGATES

5.1	Introduction	216
5.1.1	Immunoassay Design	216
5.1.2	Horseradish Peroxidase Structure	211
5.1.3	Suitability of Horseradish Peroxidase C for Enzyme Immunoassay	212
5.1.4	Conjugation of Horseradish Peroxidase	213
5.1.5	Disadvantages of the Standard Conjugation Procedures	214
5.1.6	An Alternative Procedure for Conjugating Horseradish Peroxidase to Haptens	215
5.1.7	Advantages of using a Horseradish Peroxidase Hemin-Hapten Conjugate as a Signal Generator in Enzyme Immunoassay	219
5.1.8	Aims	221
5.2	Materials and Methods	222
5.2.1	Apparatus	222
5.2.2	Reagents	222
5.2.3	Important Horseradish Peroxidase Constants	223
5.2.4	Estimation of Protein Content by the Coomassie Blue Method	223
5.2.5	Preparation of Reagents for Standard Activity Assay for Horseradish Peroxidase	224
5.2.6	Standard Activity Assay for Horseradish Peroxidase	224
5.2.7	Preparation of ELISA Reagents	225
5.2.7.1	Horseradish Peroxidase - Estrone Glucuronide Active Ester Conjugate	225
5.2.7.2	Carbonate - Bicarbonate Coating Buffer (pH 9.6)	225
5.2.7.3	0.5% Gelatine Blocking Buffer (pH 9.6)	225
5.2.7.4	Citrate-Phosphate Substrate Buffer (pH 5.0)	226
5.2.7.5	Phosphate Washing Buffer (pH 7.4)	226
5.2.7.6	0.1% Gelatine Assay Buffer (pH 7.4)	226
5.2.7.7	<i>o</i> -Phenylenediamine Substrate Solution (pH 5)	226
5.2.7.8	Stopping Solution	226
5.2.7.9	Antibody Solution	226
5.2.8	The Standard ELISA Assay Protocol	227
5.2.8.1	Coating the Wells	227
5.2.8.2	Blocking the Wells	227
5.2.8.3	Washing the Wells	227
5.2.8.4	Addition of the Signal Generator and Hapten	227
5.2.8.5	The Activity Assay	228
5.2.8.6	General Notes on the ELISA Assay Methodology	228

5.2.9	Comparison of an Active Ester Horseradish Peroxidase Enzyme Linked Immunosorbent Assay (ELISA) for Estrone Glucuronide with Radioimmunoassay (RIA) and the Commercial Ovarian Monitor	229
5.2.10	Preparation of Apo Protein	229
5.2.10.1	Preparation of the Stock Horseradish Peroxidase Solution	230
5.2.10.2	Acidification	230
5.2.10.3	Methyl Ethyl Ketone Extraction	230
5.2.10.4	Removal of the Methyl Ethyl Ketone by Gel Filtration	230
5.2.11	Preparation of Hemin and Substituted Hemins for Reconstitution with Apo Horseradish Peroxidase	231
5.2.12	Titration of Apo Horseradish Peroxidase with Hemin and Modified Hemin	231
5.2.13	Removal of Unreacted Hemins from the Reconstituted Protein	231
5.2.14	Analysis of Hemin-Polyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPE1G) using a Reconstitution Assay Format with Apo Horseradish Peroxidase	232
5.2.15	Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Polyethyleneglycol-Estrone Glucuronide (HPE1G) (6 or 7) Mono-Conjugate by Solution Assay and Enzyme Linked Immunosorbent Assay (ELISA)	233
5.2.16	Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Polyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) by Enzyme Linked ImmunoSorbent Assay (ELISA)	234
5.2.17	Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) by the Ovarian Monitor	235
5.3	Results	237
5.3.1	Preparation of an Active Ester Horseradish Peroxidase Estrone Glucuronide Conjugate	237
5.3.2	Adaptation of an Active Ester Horseradish Peroxidase Enzyme Linked Immunosorbent Assay (ELISA) for Estrone Glucuronide	238
5.3.2.1	Effect of Assay Time on the Position and Amplitude of the ELISA Standard Curve	238
5.3.2.2	Effect of the Active Ester Horseradish Peroxidase Estrone Glucuronide Conjugate Concentration on the Position and Amplitude of the ELISA Standard Curve	239
5.3.2.3	Effect of the Concentration of the Anti-Estrone Glucuronide Antiserum on the Position and Amplitude of the ELISA Standard Curve	241
5.3.3	Comparison of an Active Ester Horseradish Peroxidase Enzyme Linked Immunosorbent Assay (ELISA) for Estrone Glucuronide with Radioimmunoassay (RIA) and the Ovarian Monitor	243
5.3.3.1	Cycle 002F-C2	243

5.3.3.2	Cycle 003J-C2	244
5.3.3.3	Cycle 009D-C2	245
5.3.3.4	Cycle 014X-C1	246
5.3.3.5	Cycle 014X-C2	247
5.3.3.6	Cycle 020K-C1	248
5.3.3.7	Cycle 021R-C2	249
5.3.3.8	Cycle 023B-C1	250
5.3.3.9	Cycle 023B-C2	251
5.3.3.10	Correlation Summary of Ovarian Monitor, ELISA and RIA Estrone Glucuronide Excretion Rate Data for World Health Organisation Trial Menstrual Cycles	252
5.3.4	Data from a Typical Apo Protein Preparation	254
5.3.5	Hemin and Substituted Hemin Titrations	255
5.3.5.1	Hemin Titration	255
5.3.5.2	Hemin-Lysine-Estrone Glucuronide (6 or 7) Mono-Conjugate (HLysE1G)	259
5.3.5.3	Hemin-Lysine-Pregnanediol Glucuronide (6 or 7) Mono-Conjugate (HLysPdG)	260
5.3.5.4	Hemin-Methionine (6 and 7) Di-Conjugate (HMet(di))	261
5.3.5.5	Hemin-Aminohexanoic Acid (6 or 7) Mono-Conjugate (HA)	261
5.3.5.6	Hemin-Aminohexanoic Acid (6 and 7) Di-Conjugate (HA(di))	262
5.3.5.7	Hemin-Aminohexanoate Methyl Ester (6 or 7) Mono-Conjugate (HAME)	263
5.3.5.8	Hemin-Hexylamine (6 or 7) Mono-Conjugate (HH)	264
5.3.5.9	Hemin-Phenylalanine Methyl Ester (6 or 7) Mono-Conjugate (HPheME)	266
5.3.5.10	Hemin-Methionine Methyl Ester (6 or 7) Mono-Conjugate (HMetME)	266
5.3.5.11	Hemin-Proline Methyl Ester (6 or 7) Mono-Conjugate (HProME)	267
5.3.5.12	Hemin-Diaminopolyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPE1G)	268
5.3.5.13	Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPAE1G)	269
5.3.5.14	Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6- Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPAAE1G)	270
5.3.6	Analysis of Hemin-Polyethyleneglycol-Estrone Glucuronide (6 or 7) Mono- Conjugate (HPE1G) using a Reconstitution Assay Format with Apo Horseradish Peroxidase	272
5.3.7	Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin- Polyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPE1G) by Solution Assay and Enzyme Linked ImmunoSorbent Assay (ELISA)	276
5.3.8	Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin- Polyethyleneglycol-6-Aminoheaxanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin- Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-	

	Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) by Enzyme Linked Immunosorbent Assay (ELISA)	277
5.3.9	Cross Reactivity of the Pre-Reconstituted Horseradish Peroxidase Hemin- Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate- Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) in the Ovarian Monitor	278
5.4	Discussion	282
5.4.1	Establishment of an ELISA Reference Assay for Estrone Glucuronide using Timed Urine Specimens	282
5.4.2	Reconstitution of Synthetic Hemins with Apo Horseradish Peroxidase	289
5.4.3	The Reconstitution Solution Assays	297
5.4.4	The ELISA Assays with the Reconstituted Enzymes	299

LIST OF FIGURES

FIGURE 1.1: The Five stages of Follicular Development	2
FIGURE 1.2: Schematic Diagram of the Two Cell Theory	5
FIGURE 1.3: Idealised Hormone Profile of Luteinising Hormone, Follicle Stimulating Hormone, Estradiol and Progesterone during the Human Menstrual Cycle	6
FIGURE 1.4: Structure of Estrone Glucuronide and Pregnanediol Glucuronide	21
FIGURE 1.5: Idealised Menstrual Cycle Profile for Urinary Estrone Glucuronide and Pregnanediol Glucuronide Excretion Rates	22
FIGURE 1.6: Urinary Excretion Rate Menstrual Cycle Profiles of Estrone Glucuronide and Pregnanediol Glucuronide for Early Morning Urine and Time Diluted Urine Samples	24
FIGURE 1.7: The Ovarian Monitor (Mark VI)	36
FIGURE 1.8: An Ovarian Monitor Assay Tube	36
FIGURE 1.9: Ovarian Monitor Estrone Glucuronide Standard Curve	39
FIGURE 2.1: Model of Hen Egg White Lysozyme's Tertiary Structure showing the Enzyme's Deep Enzymatic Cleft	41
FIGURE 2.2: Nuclear Magnetic Resonance Spectrum showing the Anomeric Proton and the Glucuronide Ring C5' Proton of the Acid Form of Estrone Glucuronide	55
FIGURE 2.3: Mono-S Profile of the Reaction Mixture of a Typical Hen Egg White Lysozyme-Estrone Glucuronide Active Ester Conjugation obtained under Standard Analytical Conditions	56
FIGURE 2.4: Mono-S Profiles of an Initial Active Ester Hen Egg White Lysozyme- Estrone Glucuronide Conjugation Mixture, and its Resulting Supernatant and Precipitate Fractions obtained under Standard Analytical Conditions	57
FIGURE 2.5: Mono-S Elution Profile obtained at pH 4.3 in the Absence of Urea for a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation	58
FIGURE 2.6: Mono-S Profile obtained under Standard Analytical Conditions of the Main Peak of a Mono-S pH 4.3 Non-Urea Based Conjugate Separation	58
FIGURE 2.7: Mono-S Profile obtained in the Absence of Urea at pH 9.5 of a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Mixture	59
FIGURE 2.8: Mono-S Profile obtained under Standard Analytical Conditions of the Main Peaks of a Mono-S pH 9.5 Non-Urea Based Separation	60
FIGURE 2.9: Mono-S Profile obtained in the Presence of Urea at pH 9.5 of a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Mixture	61
FIGURE 2.10: S-Sepharose fast flow Profile of a Hen Egg White Lysozyme- Estrone Glucuronide Conjugation Mixture	62
FIGURE 2.11: Overlay of the Elution Profiles of Hen Egg White Lysozyme- Estrone Glucuronide Conjugates, E1 and E3, after Repeat Chromatography through an S-Sepharose fast flow Column	63
FIGURE 2.12: Butyl Sepharose Elution Profile for E1 Hen Egg White Lysozyme- Estrone Glucuronide Conjugate after initial Purification by S-Sepharose fast flow Chromatography.	64
FIGURE 2.13: Butyl Sepharose Elution Profile for E3 Hen Egg White Lysozyme- Estrone Glucuronide Conjugate after initial Purification by S-Sepharose fast	

flow Chromatography.	65
FIGURE 2.14: Mono-S Profiles obtained under Standard Analytical Conditions of the Pooled Fractions of the Butyl Sepharose Purification of E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	66
FIGURE 2.15: Mono-S Profiles obtained under Standard Analytical Conditions of the Pooled Fractions of the Butyl Sepharose Purification of E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	67
FIGURE 2.16: Electrospray Mass Spectrometry Spectra for E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates after Final Purification by Butyl Sepharose Chromatography	68
FIGURE 2.17: Structural Model of Hen Egg White Lysozyme showing the Salt Bridges formed between Lysine 1 and Glutamic Acid 7, and Lysine 13 and the C-terminal Group of Leucine 129	73
FIGURE 2.18: Ball and Stick Model of the Urea Binding Site in Hen Egg White Lysozyme which is proximal to Lysine 116 obtained from Enzyme Crystals grown in High Concentration Urea Solution	75
FIGURE 2.19: The Surface Electrostatic Fields surrounding the most favoured Lysine Conjugation Sites on Hen Egg White Lysozyme	76
FIGURE 3.1: Hen Egg White Lysozyme Cleavage Site of the Polysaccharide Component of the <i>Micrococcus lysodeikticus</i> Cell Wall	89
FIGURE 3.2: Steric Occlusion of the Substrate Binding Site of Hen Egg White Lysozyme-Estrone Glucuronide E3 Conjugate by Antibody Binding to the Steroid Moiety	90
FIGURE 3.3: View of Hen Egg White Lysozyme from Opposite Sides showing the Positions of the Six Lysine Residues around the Substrate Binding Site	93
FIGURE 3.4: Variation in the Estrone Glucuronide Standard Curves for Pre-Coated Estrone Glucuronide Assay Tubes of the same Batch Number but different Box Lots	102
FIGURE 3.5: Reproducibility of Estrone Glucuronide Standard Curves for Pre-Coated Assay Tubes of Batch Number #14, Box Lot 25-43 over a Six Month Period	103
FIGURE 3.6: Day-to-Day Reproducibility of Estrone Glucuronide Standard Curves for Pre-Coated Assay Tubes of Batch Number #14, Box Lot 316-337)	103
FIGURE 3.7: Broad Titration Curves for the Third Bleeds of the Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera with Melbourne Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	105
FIGURE 3.8: Effect of Buffer Ionic Strength on the Specific Activity of E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	106
FIGURE 3.9: Relationship between Rate of Clearing in Transmission using a Twenty Minute End Point Assay and E1 and E3 Hen Egg White Lysozyme Estrone Glucuronide Conjugate Concentration	107
FIGURE 3.10: E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Clearing Curves as Change in Transmission in the Absence and Presence of Anti-Estrone Glucuronide Antiserum and High Levels of Estrone Glucuronide Standard	108

FIGURE 3.11: Fit of the E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Change in Absorbance Clearing Curve in the Absence and Presence of Anti-Estrone Glucuronide Antiserum and High Estrone Glucuronide Standard to a Second Order Rate of Clearing	108
FIGURE 3.12: Fit of the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Change in Absorbance Clearing Curve in the Absence and Presence of Anti-Estrone Glucuronide Antiserum and High Estrone Glucuronide Standard to a Second Order Rate of Clearing	109
FIGURE 3.13: Clearing Curve for a Pre-Coated Estrone Glucuronide Ovarian Monitor Assay Tube	109
FIGURE 3.14: Titration Curves for the Third Bleeds of the Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera with E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	110
FIGURE 3.15: Estrone Glucuronide Standard Curves for E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with the Third Bleeds of the Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera . . .	110
FIGURE 3.16: Optimised Estrone Glucuronide Standard Curves for E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-3	111
FIGURE 3.17: Broad Titration Curve for the Third Bleed of the Anti-Estrone Glucuronide Antiserum 243 with E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	112
FIGURE 3.18: Titration Curves for the Third and Fourth Bleeds of Anti-Estrone Glucuronide Antiserum 243 with E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate	113
FIGURE 3.19: Titration Curves for the Fourth Bleed of Anti-Estrone Glucuronide Antiserum 243 with E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates	113
FIGURE 3.20: Skeleton Estrone Glucuronide Standard Curves for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4	115
FIGURE 3.21: Optimised Estrone Glucuronide Standard Curve for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4	116
FIGURE 3.22: Day-to-Day Reproducibility of the Optimised Estrone Glucuronide Standard Curve for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4	116
FIGURE 3.23: The Optimised Estrone Glucuronide Standard Curves for E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates with Anti-Estrone Glucuronide Antiserum 243-4	117
FIGURE 3.24: The Effect of Performing the E1 Hen Egg White Lysozyme Estrone Glucuronide Standard Curve at the E3 Optimised Conditions and Optimisation of a Combined E1 and E3 Standard Curve	118
FIGURE 3.25: Computer Simulation of the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Standard Curves and the E1 Standard Curve performed at E3 Optimised Conjugate and Antiserum Conditions	119

FIGURE 3.26: The Optimised Estrone Glucuronide Standard Curves for the E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4 in the Presence and Absence of a Blank Urine Sample	120
FIGURE 3.27: The Optimised Estrone Glucuronide Standard Curves for the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4 in the Presence and Absence of a Blank Urine Sample	121
FIGURE 3.28: The Optimised Estrone Glucuronide Standard Curves for the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4 in the Presence and Absence of a Child's Blank Urine Sample Diluted using Two Methods	122
FIGURE 3.29: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle expressed as Twenty Minute Change in Transmission measured using the Pre-Coated Assay Tubes, and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays	123
FIGURE 3.30: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle expressed as Twenty Minute Change in Transmission measured using the Pre-Coated Assay Tubes, and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays and a Double Dilution over Days 15-18	125
FIGURE 3.31: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle measured using the Pre-Coated Assay Tubes, and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays and a Double Dilution over Days 15-18	126
FIGURE 3.32: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle measured using the Pre-Coated Assay Tubes, and the Optimised E1 Hen Egg White Lysozyme-Estrone Glucuronide Assays with and without blank urine and using a Double Dilution over Days 15-18	127
FIGURE 3.33: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle measured using the Pre-Coated Assay Tubes, and the Optimised E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays with and without Blank Urine and using a Double Dilution over Days 15-18	128
FIGURE 3.34: Deming Correlation between the Pre-Coated Ovarian Monitor Assay Tubes versus the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Urinary Estrone Glucuronide Excretion Rates throughout an Endocrinologically Normal Menstrual Cycle	128
FIGURE 3.35: Deming Correlation between the Pre-Coated Ovarian Monitor Assay Tubes versus the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Urinary Estrone Glucuronide Excretion Rates throughout an Endocrinologically Normal Menstrual Cycle ..	129
FIGURE 3.36: Model of E6 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate showing the Positions of the Lysine 33, 97 and 116 Estrone Glucuronide Moieties around the Substrate Binding Site	137
FIGURE 3.37: Model of Hen Egg White Lysozyme showing the Arginine Rich Area surrounding the Substrate Binding Site	137

FIGURE 4.1: Measurement of Lysozyme Lytic Activity by the End-Point Assay Method and the Initial Rate Method	161
FIGURE 4.2: Mono-S Profiles for an Active Ester Human Lysozyme Estrone Glucuronide Conjugation Mixture after Three Successive Exposures to the Active Ester Reagent	165
FIGURE 4.3: Electrospray Mass Spectrometry Spectra for Human Lysozyme, and Mono-S Chromatographic Conjugate Peaks HE1, HE2, HE3 and HE5	167
FIGURE 4.4: Polyacrylamide Gel Electrophoresis of Hen Egg White Lysozyme and Human Lysozyme and their Estrone Glucuronide Conjugates Purified by Mono-S Chromatography	169
FIGURE 4.5: Clearing Curve for the Human Lysozyme HE2 Conjugate at Variable Enzyme Concentrations	170
FIGURE 4.6: Linearisation of a Human Lysozyme HE2 Clearing Curve using Two Second Order Equations	171
FIGURE 4.7: Changes in the End-point Rates for the Human Lysozyme HE2 Conjugate with Enzyme Concentration and Time	171
FIGURE 4.8: Effect of Maximally Inhibiting Levels of the Anti-Estrone Glucuronide Antiserum 243-4 on the Human Lysozyme HE2 Conjugate Clearing Curve	172
FIGURE 4.9: Effect of pH and Ionic Strength of the Tris-Maleate Buffer on the Initial Rate of the Human Lysozyme HE2 Conjugate	173
FIGURE 4.10: Relationship between Initial Rates and the Human Lysozyme HE2 Conjugate Concentration	176
FIGURE 4.11: Relationship between the Second Order Rate Constant and the Human Lysozyme HE2 Conjugate Concentration for the First Phase of Clearing	176
FIGURE 4.12: Anti-Estrone Glucuronide Antiserum 243-4 Titration Curve for Human Lysozyme HE2 Conjugate	177
FIGURE 4.13: The Optimised Human Lysozyme HE2 Standard Curve obtained in the Presence of a Blank Urine Sample	177
FIGURE 4.14: Estrone Glucuronide Cycle Data from a Woman undergoing Clomiphene Treatment obtained using the Rapid Human Lysozyme HE2 Optimised Immunoassay Method expressed in Terms of Initial Rates and Estrone Glucuronide Excretion Rates after Conversion from a Blank Urine Standard Curve	178
FIGURE 4.15: Estrone Glucuronide (E1G) and Pregnanediol Glucuronide (PdG) Cycle Data from a Woman undergoing Clomiphene Treatment obtained using the Rapid Human Lysozyme HE2 Optimised Immunoassay Method (E1G) and the Current Ovarian Monitor Assay (E1G and PdG)	178
FIGURE 4.16: Correlation by Deming Regression of Estrone Glucuronide Cycle Data from a Woman undergoing Clomiphene Treatment obtained using the Rapid Human Lysozyme HE2 Optimised Immunoassay Method and the Current Ovarian Monitor Assay	179
FIGURE 4.17: Relationship between Initial Rates and the Human Lysozyme HE1 Conjugate Concentration	180
FIGURE 4.18: Anti-Estrone Glucuronide Antiserum (243-4) Titration Curve for Human Lysozyme HE1 Conjugate	180

FIGURE 4.19: The Optimisation of the Human Lysozyme HE1 Standard Curve for Anti-Estrone Glucuronide Antiserum 243-4	181
FIGURE 4.20: Day-to-Day Reproducibility of the Optimised Human Lysozyme HE1 Standard Curve	182
FIGURE 4.21: Effect of Blank Urine on the Optimised Human Lysozyme HE1 Standard Curve	182
FIGURE 4.22: A Comparison of the Estrone Glucuronide (E1G) Cycle Data from a Woman with an Endocrinologically Normal Cycle obtained using the Optimised Human Lysozyme HE1 Immunoassay Method after Conversion into E1G Excretion Rates by the Blank and Without Blank Urine Standard Curves	183
FIGURE 4.23: Estrone Glucuronide Cycle Data from a Woman with an Endocrinologically Normal Cycle obtained using the Human Lysozyme HE1 Optimised Immunoassay Method and the Current Ovarian Monitor Assay	184
FIGURE 4.24: Correlation by Deming Regression of Estrone Glucuronide Cycle Data from a Woman with an Endocrinologically Normal Cycle obtained using the Rapid Human Lysozyme HE1 Optimised Immunoassay Method and the Current Ovarian Monitor Assay	184
FIGURE 4.25: Effect of Changing the Amount of Anti-Estrone Glucuronide Antiserum 243-4 on the Human Lysozyme HE5 Standard Curve	185
FIGURE 4.26: The Optimised Human Lysozyme HE1, HE2 and HE5 Standard Curves obtained with Anti-Estrone Glucuronide Antiserum 243-4	186
FIGURE 4.27: Hen Egg White Lysozyme E3 Conjugate Titration of Anti-Estrone Glucuronide Antiserum 243-4	187
FIGURE 4.28: Human Lysozyme HE1 Conjugate Titration of Anti-Estrone Glucuronide Antiserum 243-4	187
FIGURE 4.29: Human Lysozyme HE2 Conjugate Titration of Anti-Estrone Glucuronide Antiserum 243-4	188
FIGURE 4.30: Measurement of the Displacement Rates for Hen Egg White Lysozyme E1, and Human Lysozyme HE1 and HE2 Estrone Glucuronide Conjugates from Anti-Estrone Glucuronide Antiserum (243-4) using an Excess of Estrone Glucuronide as a Displacing Agent	189
FIGURE 4.31: Structural Model of Amino Acid Backbone and the Lysine Residues of Hen Egg White Lysozyme and Human Lysozyme	190
FIGURE 4.32: Structural Model of Human Lysozyme showing the Exposure of Lysine 13	196
FIGURE 4.33: Structural Model of Hen Egg White Lysozyme showing the Exposure of Lysine 33	197
FIGURE 4.34: Structural Model of Human Lysozyme showing the Exposure of Lysine 33	197
FIGURE 4.35: Structure of Clomiphene and Estradiol	208
FIGURE 5.1: Hemin Chloride Structure Showing the Carbons Numbered by the Fischer System	217
FIGURE 5.2: Structural Model of Horseradish Peroxidase showing the Exposure of the Hemin through the Organic Substrate Channel	218

FIGURE 5.3: Structural Model of Horseradish Peroxidase showing the Exposure of the Hemin Propionate Groups	219
FIGURE 5.4: Active Ester Horseradish Peroxidase - Estrone Glucuronide Sephadex G-25 A ₂₇₈ and A ₄₀₄ Elution Profile	237
FIGURE 5.5: The Effect of Assay Time on the Position of the ELISA Estrone Glucuronide Standard Curve	238
FIGURE 5.6: The Effect of the Assay Time on the Position of the ELISA Standard Curve Expressed Relative to the Zero Standard Absorbance	239
FIGURE 5.7: The Effect of Conjugate Concentration on the Position and Amplitude of the ELISA Estrone Glucuronide Standard Curve	240
FIGURE 5.8: The Effect of Conjugate Concentration on the Position and Amplitude of the Estrone Glucuronide Standard Curve expressed Relative to the Zero Standard	240
FIGURE 5.9: The Effect of Conjugate Concentration on the Absorbance for an ELISA at Different Estrone Glucuronide Concentrations	241
FIGURE 5.10: The Effect of the Amount of Antiserum on the Position and Amplitude of the ELISA Estrone Glucuronide Standard Curve	242
FIGURE 5.11: The Effect of the Amount of Antiserum on the Position and Amplitude of the ELISA Estrone Glucuronide Standard Curve expressed Relative to the Zero Standard Absorbance	242
FIGURE 5.12: Estrone glucuronide Excretion Rates for Cycle 002F-C2 as measured by ELISA, RIA and the Ovarian Monitor	243
FIGURE 5.13: Least Squares Correlation of Estrone Glucuronide Excretion Rates for Cycle 002F-C2 for the ELISA Method with the RIA and Ovarian Monitor Methods	244
FIGURE 5.14: Estrone Glucuronide Excretion Rates for Cycle 003J-C2 as measured by ELISA, RIA and the Ovarian Monitor	244
FIGURE 5.15: Estrone Glucuronide Excretion Rates for Cycle 009D-C2 as measured by ELISA, RIA and the Ovarian Monitor	245
FIGURE 5.16: Estrone Glucuronide Excretion Rates for Cycle 014X-C1 as measured by ELISA, RIA and the Ovarian Monitor	246
FIGURE 5.17: Estrone Glucuronide Excretion Rates for Cycle 014X-C2 as measured by ELISA, RIA and the Ovarian Monitor	247
FIGURE 5.18: Estrone Glucuronide Excretion Rates for Cycle 020K-C1 as measured by ELISA, RIA and the Ovarian Monitor	248
FIGURE 5.19: Estrone Glucuronide Excretion Rates for Cycle 021R-C2 as measured by ELISA, RIA and the Ovarian Monitor	249
FIGURE 5.20: Estrone Glucuronide Excretion Rates for Cycle 023B-C1 as measured by ELISA, RIA and the Ovarian Monitor	250
FIGURE 5.21: Estrone Glucuronide Excretion Rates for Cycle 023B-C2 as measured by ELISA, RIA and the Ovarian Monitor	251
FIGURE 5.22: Comparison of the RIA and ELISA Data using the Deming and Least Squares Regression Methods	252
FIGURE 5.23: Comparison of the Ovarian Monitor and ELISA Data using the Least Squares and Deming Regression Methods	252
FIGURE 5.24: Absorbance Spectra for Native, Acidified and Apo Horseradish Peroxidase	254

FIGURE 5.25: Hemin Titration Curve obtained in the Presence and Absence of Apo Protein	256
FIGURE 5.26: The Absorbance Spectra of Native Horseradish Peroxidase and Hemin Chloride obtained at a Molar Concentration of 6.32 μ M	257
FIGURE 5.27: Hemin-Lysine-Estrone Glucuronide (6 or 7) Mono-Conjugate	259
FIGURE 5.28: Hemin-Lysine-E1G Titration Curve obtained in the Presence and Absence of Apo Protein	259
FIGURE 5.29: Hemin-Lysine-Pregnanediol Glucuronide (6 or 7) Mono-Conjugate ..	260
FIGURE 5.30: Hemin-Methionine (6 and 7) Di-Conjugate	261
FIGURE 5.31: Hemin-Aminohexanoic Acid (6 or 7) Mono-Conjugate	261
FIGURE 5.32: Hemin-Aminohexanoic Acid (6 and 7) Di-Conjugate	262
FIGURE 5.33: Absorption Spectra for Free Hemin, Mono-Substituted and Di-Substituted Hemin Aminohexanoic Acid	262
FIGURE 5.34: Absorption Spectra for Native Horseradish Peroxidase, and Horseradish Peroxidase Reconstituted with Hemin, and Hemin Mono-Substituted and Di-Substituted Aminohexanoic Acid	263
FIGURE 5.35: Hemin-Aminohexanoate Methyl Ester (6 or 7) Mono-Conjugate	263
FIGURE 5.36: Hemin-Hexylamine (6 or 7) Mono-Conjugate	264
FIGURE 5.37: Hemin, Hemin-Aminohexanoate Methyl Ester and Hemin-Hexylamine Titration Curves obtained in the Presence and Absence of Apo Protein	264
FIGURE 5.38: Absorption Spectra for Hemin, Hemin-Aminohexanoate Methyl Ester and Hemin-Hexylamine in the Free and Reconstituted Forms	265
FIGURE 5.39: Hemin-Phenylalanine Methyl Ester (6 or 7) Mono-Conjugate	266
FIGURE 5.40: Hemin-Methionine Methyl Ester (6 or 7) Mono-Conjugate	266
FIGURE 5.41: Hemin-Proline Methyl Ester (6 or 7) Mono-Conjugate	267
FIGURE 5.42: Hemin-Diaminopolyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate	268
FIGURE 5.43: Hemin and Hemin-Diaminopolyethyleneglycol Estrone Glucuronide Titration Curves obtained in the Presence and Absence of Apo Protein	268
FIGURE 5.44: Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate	269
FIGURE 5.45: Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate	270
FIGURE 5.46: Absorption Spectra for Free Hemin, HPE1G, HPAE1G and HPAAE1G	270
FIGURE 5.47: Absorption Spectra for Horseradish Peroxidase Reconstituted with Hemin, and HPE1G, HPAE1G and HPAAE1G after DE chromatography ...	271
FIGURE 5.48: Titration of Apo Protein (30 pmoles) and HPE1G (72 pmoles) with Monoclonal Anti-Estrone Glucuronide Antibody in a Reconstitution Format	274
FIGURE 5.49: Effect of Incubation Time on the Activity of an Apo Protein - HPE1G Reconstitution Assay	274
FIGURE 5.50: Effect of Incubation Time on the Activity of an Apo Protein - Hemin Reconstitution Assay	275
FIGURE 5.51: Titration of Apo Protein (30 pmoles) with HPE1G using a Reconstitution Assay Format and a 15 Minute Incubation Period	275

FIGURE 5.52: ELISA Plate Competition Assay between Active Ester
Horseradish Peroxidase-E1G and Reconstituted Horseradish Peroxidase-
Hemin, -HPAE1G and -HPAAE1G 277

FIGURE 5.53: Model of Antibody-Antigen Complex Formation for Horseradish
Peroxidase-Estrone Glucuronide Conjugate and Estrone Glucuronide at
Different Antibody Densities on the Solid Phase 284

FIGURE 5.54: Inter-assay Variation of the Nine Standard Curves used for the
Measurement of Estrone Glucuronide Excretion Rates from Nine Cycles
from the World Health Organisation on the Ovarian Monitor 285

FIGURE 5.55: Hydrogen Bonds of the Hemin 6 and 7 Propionate Groups to Amino
Acids Arginine 31, Serine 35, Serine 73 and Glutamine 176 of Horseradish
Peroxidase 291

FIGURE 5.56: Bis-Copper Lysine Complex 292

FIGURE 5.57: Steric Occlusion of the Substrate Binding Site of Horseradish
Peroxidase Hemin-Polyethyleneglycol-Estrone Glucuronide Conjugate by
Antibody Binding to the Steroid Moiety

LIST OF TABLES

TABLE 1.1: The Estrogens and their Principal Urinary Metabolites	20
TABLE 2.1: A Summary of the Performance of the Various Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Purification Schemes	69
TABLE 2.2: A Summary of the Characteristics of the Main Hen Egg White Lysozyme-Estrone Glucuronide Conjugates of the Active Ester and Mixed Anhydride Conjugation Reactions	72
TABLE 3.1: Dilution Series for Preparation of Estrone Glucuronide Standards	96
TABLE 3.2: Deming Correlation between the Pre-Coated Ovarian Monitor Assay Tubes and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Urinary Estrone Glucuronide Excretion Rates throughout an Endocrinologically Normal Menstrual Cycle	128
TABLE 4.1: Distribution of Human Lysozyme and the Various Lysozyme Conjugate Families expressed as a Percentage of the Total Protein for Three Different Levels of Exposure of the Reaction Mixture to the Active Ester Reagent	166
TABLE 4.2: The Effect of Bovine Serum Albumin in the Assay Buffer on Human Lysozyme HE2 Conjugate Activity	173
TABLE 4.3: The Effect of Bovine Serum Albumin (0.1%) on the Stability of Human Lysozyme HE2 Conjugate Stock Solutions	174
TABLE 5.1: Correlation Summary of the ELISA versus RIA and Ovarian Monitor Menstrual Cycle Data using the Least Squares Regression Method	253
TABLE 5.2: Comparison of the RZ and Specific Activity of Native Horseradish Peroxidase, with its Acidified and Apo Forms	255
TABLE 5.3: The Effect of the Assay Mixture Components on the Rate of the Peroxidase Assay	272
TABLE 5.4: Activation of Native Horseradish Peroxidase and Pre-Reconstituted Horseradish Peroxidase-HPE1G by Monoclonal and Polyclonal Anti-Estrone Glucuronide Antisera	276
TABLE 5.5: Cross reactivity of the Reconstituted Horseradish Peroxidase- Hemin, -HPAE1G, and -HPAAE1G Samples with the Ovarian Monitor Estrone Glucuronide Assay	279
TABLE 5.6: Peroxidase Activity of the Post Ovarian Monitor Horseradish Peroxidase-Hemin, -HPAE1G and -HPAAE1G Assay Mixtures as measured in Absorbance (490 nm) using a 150 Second <i>o</i> -Phenylenediamine End Point Assay	280
TABLE 5.7: The Reconstitution Characteristics of Thirteen Modified Hemin Compounds with Apo Horseradish Peroxidase	290

LIST OF SCHEMES

SCHEME 1.1: Biosynthetic Pathway of the Female Sex Steroids Estradiol and Progesterone 18

SCHEME 2.1: Synthesis of Hen Egg White Lysozyme - Estrone Glucuronide Conjugates by the Active Ester Method 48

SCHEME 2.2: Synthesis of the α -Bromo Sugar 51

SCHEME 2.3: Synthesis of Estrone Glucuronide from the α -Bromo Sugar and Estrone 53

SCHEME 2.4: Formation of the β -Glucuronide of Estrone via the 1,2-Dioxolenium Ion Intermediate 70

ABBREVIATIONS

ΔT	change in transmission
A_{278}	absorbance at 278 nm
A_{404}	absorbance at 404 nm
Ab	antibody
Abs	absorbance
Ag	antigen
AS	antiserum
BBT	basal body temperature
BSA	bovine serum albumin
C_b	conjugate bound
C_t	total conjugate
Cj	conjugate
CM	carboxymethyl cellulose
CV	co-efficient of variation
DCC	dicyclohexylcarbodiimide
DMF	dimethylformamide
DMSO	dimethylsulphoxide
DE	diethylaminoethyl resin
ELISA	enzyme linked immunosorbent assay
E1-E10	hen egg white lysozyme conjugates labelled according to elution order under standard conditions on the Mono-S column with 7 M urea buffers
E1G	estrone glucuronide (refers to acid form)
E1G-(H)	estrone glucuronide acid form
E1G-(Na)	estrone glucuronide sodium salt
eq	equilibrium
EC_{50}	analyte mid-point of a normalised standard curve
ESMS	electrospray mass spectroscopy
FAD	flavin adenine dinucleotide
FPLC	fast protein liquid chromatography
FSH	follicle stimulating hormone
HCG	human chorionic gonadotrophin
HE1-HE5	human lysozyme conjugates labelled according to elution order under standard conditions on the Mono-S column with 7 M urea buffers
HEWL	hen egg white lysozyme
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
HuL	human lysozyme
i.d.	internal diameter
IgG	immunoglobulin type G
IR	initial rate
K_a	equilibrium association constant
k_{cat}	enzyme turnover number
K_d	equilibrium dissociation constant
K_{d1}	equilibrium dissociation constant for E1G with anti-E1G antibody

K_{d2}	equilibrium dissociation constant for EIG-conjugate with anti-EIG antibody
K_m	Michaelis Mentis constant - measure of substrate affinity
k_{off}	dissociation rate constant
k_{on}	association rate constant
L	lysozyme
LAM	lactational amenorrhoea method
LH	luteinising hormone
MEK	methyl ethyl ketone (2-butanone)
<i>M. lysodeikticus</i>	<i>Micrococcus lysodeikticus</i> (bacteria particularly susceptible to lysis by lysozyme)
M_r	molecular weight
M.W	molecular weight
NADPH	Nicotinamide adenine dinucleotide phosphate
NFP	natural family planning
NHS	<i>N</i> -hydroxysuccinimide
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PEG	polyethyleneglycol
PdG	pregnanediol glucuronide
PGLIA	prosthetic group labelled immunoassay
pI	isoelectric point
r	correlation co-efficient
RIA	radioimmunoassay
RZ	Reinheits zahl (purity number)
SA	specific activity
std	standard
T	transmission
TLC	Thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
V_{max}	maximal enzymatic rate
WHO	World Health Organisation
H	hemin
HA	hemin-aminohexanoic acid mono-conjugate
HA(di)	hemin-aminohexanoic acid di-conjugate
HAME	hemin-aminohexanoate methyl ester mono-conjugate
HH	hemin-hexylamine mono-conjugate
HLysEIG	hemin-lysine-estrone glucuronide mono-conjugate
HLysPdG	hemin-lysine-pregnanediol glucuronide mono-conjugate
HMet(di)	hemin-methionine di-conjugate
HMetME	hemin-methionine methyl ester mono-conjugate
HPhME	hemin-phenylalanine methyl ester mono-conjugate
HProME	hemin-proline methyl ester mono-conjugate
HPEIG	hemin-diaminopolyethyleneglycol-estrone glucuronide mono-conjugate
HPAEIG	hemin-diaminopolyethyleneglycol-6-amino-hexanoate-estrone glucuronide mono-conjugate

HPAAE1G hemin-diaminopolyethyleneglycol-6-amino-hexanoate-6-aminohexanoate-estrone glucuronide mono-conjugate

Arg	arginine
Asp	aspartic acid
Gln	glutamine
Glu	glutamic acid
Gly	glycine
His	histidine
Leu	leucine
Lys	lysine
Phe	phenylalanine
Ser	serine
Thr	threonine
Trp	tryptophan
Tyr	tyrosine

CHAPTER ONE

OVARIAN PHYSIOLOGY, FERTILITY AND THE OVARIAN MONITOR

1.1 INTRODUCTION

Since time immemorial humankind has tried to control its fertility and often with only a minimal amount of knowledge. For example, it has been known since biblical times that a man can father children throughout all his adult life and that a woman's fertility is periodic with her ability to bear children disappearing completely in later years. However, it is only in the last century that the period of a woman's peak fertility has been correctly assigned to the middle of her menstrual cycle (Collins, 1981). Because men do not have natural periods of infertility, any attempt by a couple to regulate their fertility naturally, whether for the purposes of enhancing or reducing the chances of conception, can only be made through working with the woman's fertility cycle and thus requires an understanding of the underlying mechanisms of the menstrual cycle.

1.2 THE FERTILE MENSTRUAL CYCLE

In physiological terms, the human menstrual cycle can be considered as the periodic preparation in women for fertilisation and pregnancy. The classical textbook example of the menstrual cycle is 28 days between bleeds, but in reality it is far more variable than this and any length between 23 and 35 days is considered to be within the normal range (Burger, 1981). Although most women have their own characteristic cycle length, few women have completely regular cycles (Bonnar, 1994) with the length decreasing with age (Metcalf, 1988) and cycles being most variable during the periods of life associated with lower fertility i.e. immediately postpartum, and the years leading into the menopause and during adolescence.

1.2.1 Initiation of Follicular Growth

At the time of puberty, the ovaries contain a combined total of approximately 400,000 primordial follicles, each of which contain a single potentially fertilisable female germ cell (oocyte) (Scheele and Schoemaker, 1996). Over the course of a woman's reproductive life all of these primordial follicles are stimulated to further development (Scheele and Schoemaker, 1996). However, for the vast majority of follicles this is followed by atresia and only about 400 are destined to ovulate (i.e. be released from the ovary for fertilisation) before the primordial pool is emptied at the menopause (Hillier, 1994).

It is now known that it takes approximately 85 days from the initial recruitment of a primordial follicle until ovulation (Collins, 1985). Follicular development is classically

divided into five stages. The development from the primordial follicle to the pre-ovulatory follicle is shown in Figure 1.1.

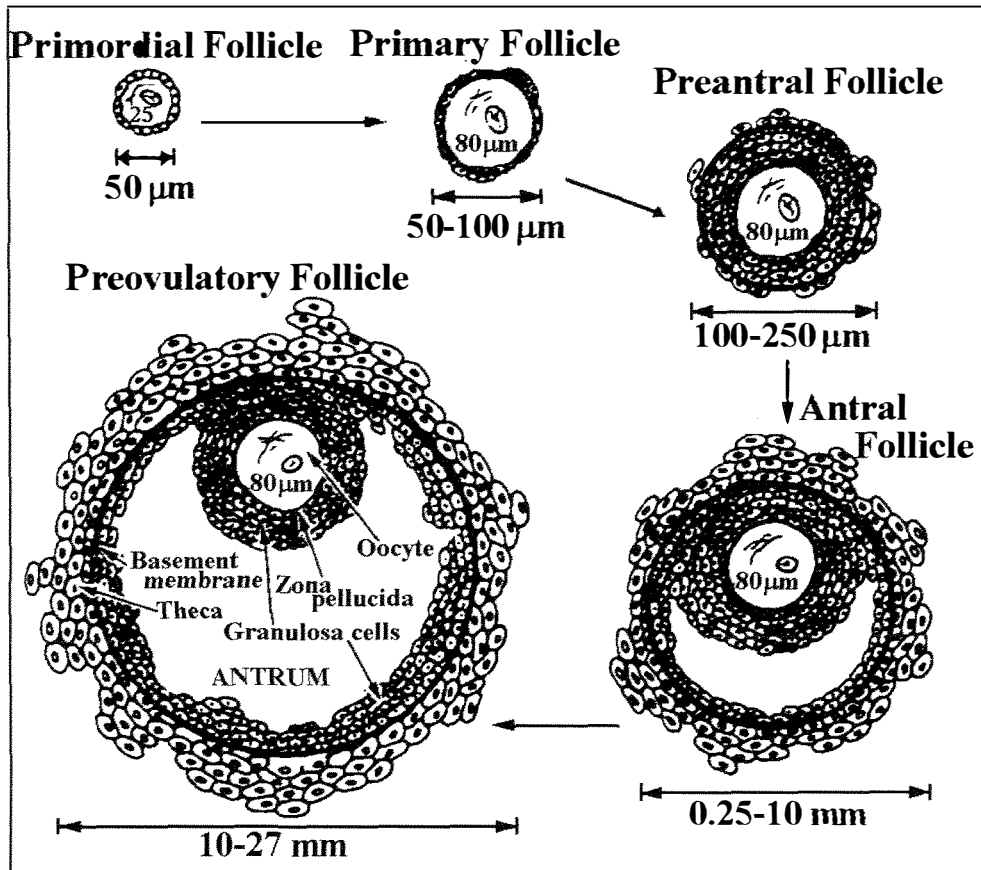


FIGURE 1.1: The Five stages of Follicular Development
modified from Fritz and Speroff, 1982

The primordial follicle is approximately $50\ \mu\text{m}$ in diameter and consists of an oocyte arrested in mid-meiosis and a surrounding single layer of granulosa cells separated from the ovarian stroma by a basement membrane (Fritz and Speroff, 1982; Hillier *et al.*, 1994a; Glasier *et al.*, 1989). Primordial follicles are recruited to further development every day from birth (Scheele and Schoemaker, 1996) with the number recruited decreasing with the size of the pool, and hence with age (Glasier *et al.*, 1989). Once growth of the primordial follicle is initiated, the follicle progresses to the primary stage which is characterised by an enlarged oocyte and differentiation of the pre-granulosa cell layer into a single layer of granulosa cells (Hadley, 1996a). At the end of the primary stage the follicle is approximately $100\ \mu\text{m}$ in diameter.

The next stage in the development of an ovulatory follicle, the preantral stage, covers the growth period from 100 to $250\ \mu\text{m}$. This stage is characterised by the development of a mucoid membrane (zona pellucida) around the oocyte from the granulosa layer, an increase in the number of granulosa cell layers, and the development of a thecal cell layer outside the basement membrane from the surrounding stroma (Fritz and Speroff, 1982; Glasier, *et al.*, 1989). It is during this period, that the follicle also develops an independent blood supply (Glasier, *et al.*, 1989). However, vascularisation is limited to the theca and no capillaries penetrate the granulosa layer (Hadley, 1996a).

In the follicle, the granulosa and thecal cells perform very different, although complementary functions and consequently are responsive to different hormonal cues. Thus while the granulosa cell membrane in the immature follicle contains follicle stimulating hormone (FSH) receptors only, the thecal cell membrane contains receptors only for luteinising hormone (LH) (Adashi, 1994). Both FSH and LH are anterior pituitary peptide hormones, and mediate their effects after receptor binding by activating gene expression via a cAMP/protein kinase A signalling pathway (Hillier *et al.*, 1994a). For normal folliculogenesis and steroidogenesis, both LH and FSH mediated processes are important. Only the process of recruitment from the primordial pool which occurs by a mechanism not yet understood, appears to be truly independent of pituitary control (Glasier *et al.*, 1989; Scheele and Schoemaker, 1996).

One of the most important functions of FSH is to stimulate granulosa cell mitosis (Scheele and Schoemaker, 1996) and hence growth of the follicle. Although follicular atresia can occur at any stage of follicular development regardless of the FSH status, atresia will always occur in the absence of FSH, at any stage post this early preantral stage. For example, *in vitro* studies on mouse follicles showed that the development of follicles to the final pre-ovulatory stage was absolutely dependent on FSH at all stages of development from the primary follicle and that the withdrawal of FSH at any stage resulted in atresia (Qvist *et al.*, 1990). Similar results were also obtained in *in vivo* primate studies on macaque monkeys (Zelevnik and Kubik, 1986).

At the basal serum levels of FSH present in a prepubescent girl, during pregnancy and during the fertile menstrual cycle FSH nadir, the levels of FSH are sufficient for the continued development of the preantral follicle into an antral follicle (Hillier, 1994). This stage of development is thought to begin approximately 70 days after the primordial follicle is first recruited for further development (Scheele and Schoemaker, 1996) and as the name suggests is associated with the development of an antrum. The antrum is formed from an increase in follicular fluid secreted by the granulosa cells which eventually coalesces to form one large fluid filled space. FSH is directly responsible for the follicular fluid production and the granulosa cell proliferation associated with this stage (Hillier, 1994).

All the stages of follicular development described so far occur throughout the cycle and are unaffected by the cyclic variations of the hormone concentrations, with only the development of follicles from past the early antral stage onwards being dependent on the hormonal changes associated with the menstrual cycle. Although antral follicles can develop at any time of the cycle (Baird, 1987), in the absence of increased FSH levels this is always followed by atresia. Only antral follicles which reach the early antral stage of development at the start of a new cycle when the FSH levels are just beginning to rise are saved (Hillier *et al.*, 1994a). The menstrual cycle is classically divided into two main phases separated by ovulation and menstruation; the follicular phase when a follicle is selected to become dominant and grows to the pre-ovulatory stage, and the luteal phase which encompasses the period from ovulation to conception or the next menstrual bleed.

1.2.2 The Follicular Phase

At the beginning of each new menstrual cycle the ovaries usually contain between ten and twenty antral follicles of 0.5-5.0 mm diameter available for 'recruitment' to pre-ovulatory development (Collins, 1989; Hillier *et al.*, 1994a). As all of the follicles of this cohort exist in a spectrum of maturational states, their ability to respond to the rising FSH levels also varies (Hillier *et al.*, 1994a). Although all follicles are stimulated to further development by FSH to a greater or lesser extent, there are usually only 4-6 follicles from the initial cohort which make it to the end of the antral stage (10 mm).

The minimum requirement for FSH above which further follicular development occurs, as judged by follicular estrogen production, is known as the FSH threshold (Brown, 1978). Although a sub-threshold level of FSH will not elicit an estrogen response, it does appear to condition the follicle which then responds more rapidly once the threshold is passed (Brown, 1978; Zeleznik and Kubik, 1986). The ovulatory follicle is the one that is most responsive to FSH (i.e. the follicle with the lowest FSH threshold) when the FSH rise begins and largely reflects this follicle's superior ability to produce estrogen in response to FSH stimulation (Hillier, 1994).

Endocrinologically the pre-ovulatory stage of development is characterised by the transition of a predominantly androgen producing follicle to a follicle in which most of the androgens are converted into estrogens. It is this transition of the follicle from an androgenic to an estrogenic environment which is absolutely critical for full pre-ovulatory development (McNatty, 1981).

Morphologically, this stage of development is also characterised by the resumption of meiosis by the oocyte, acquisition of lipid inclusions by the granulosa cells, and vacuolation and increased vascularisation of the thecal layer. The enlargement of the follicle associated with this stage is due to the enlargement of the granulosa cells, and an increase in the vacuolation of the thecal layer and follicular fluid volume (Fritz and Speroff, 1982).

Usually only one follicle develops to the fully ovulatory stage per cycle. It increases from a diameter of approximately 5 mm at the beginning of the cycle to approximately 10 mm in the first week with ovulation occurring approximately one week later (Hillier, 1994; Collins, 1985). By the time of ovulation the follicle is between 18-27 mm in diameter (Collins, 1985).

The rare occurrence of non-identical twins is due to more than one follicle responding optimally at the same time to the rising FSH levels at the beginning of the cycle and occurs in approximately one cycle in eighty (Brown, 1978).

1.2.2.1 Follicular Estrogen Production

Although the adrenal glands do produce androgens which can be peripherally aromatised into estrogens and thus are responsible for the baseline levels of estrogen observed throughout the early part of the cycle and in periods of infertility (Brown and Gronow, 1985), the vast majority of circulating estrogen found throughout most of the cycle is of

ovarian origin (Collins, 1989; Baird and Fraser, 1974). In the follicular phase of the cycle (i.e. the time between the first day of bleeding and ovulation), all ovarian estrogen is derived from maturing ovarian follicles.

In the ovarian follicle, the thecal cells are responsible for the production of androgens from progestagens, and the granulosa cells are responsible for the aromatisation of these androgens into estrogens. Thus for the follicular synthesis of estrogen, both the thecal and granulosa cells are required. This synthesis is largely controlled by the pituitary hormones. While LH stimulates androgen production by inducing thecal cell expression of the enzyme 17 α -hydroxylase/C₁₇₋₂₀-lyase which catalyses the conversion of progestagens into the corresponding androgens, FSH stimulates estrogen production by inducing granulosa cell expression of aromatase, the enzyme which catalyses the conversion of thecal androgens into estrogens (Hillier *et al.*, 1994a). This synthesis of estrogens by the granulosa cells from the thecal derived progestagens is referred to as the 'two cell' theory and is summarised in Figure 1.2.

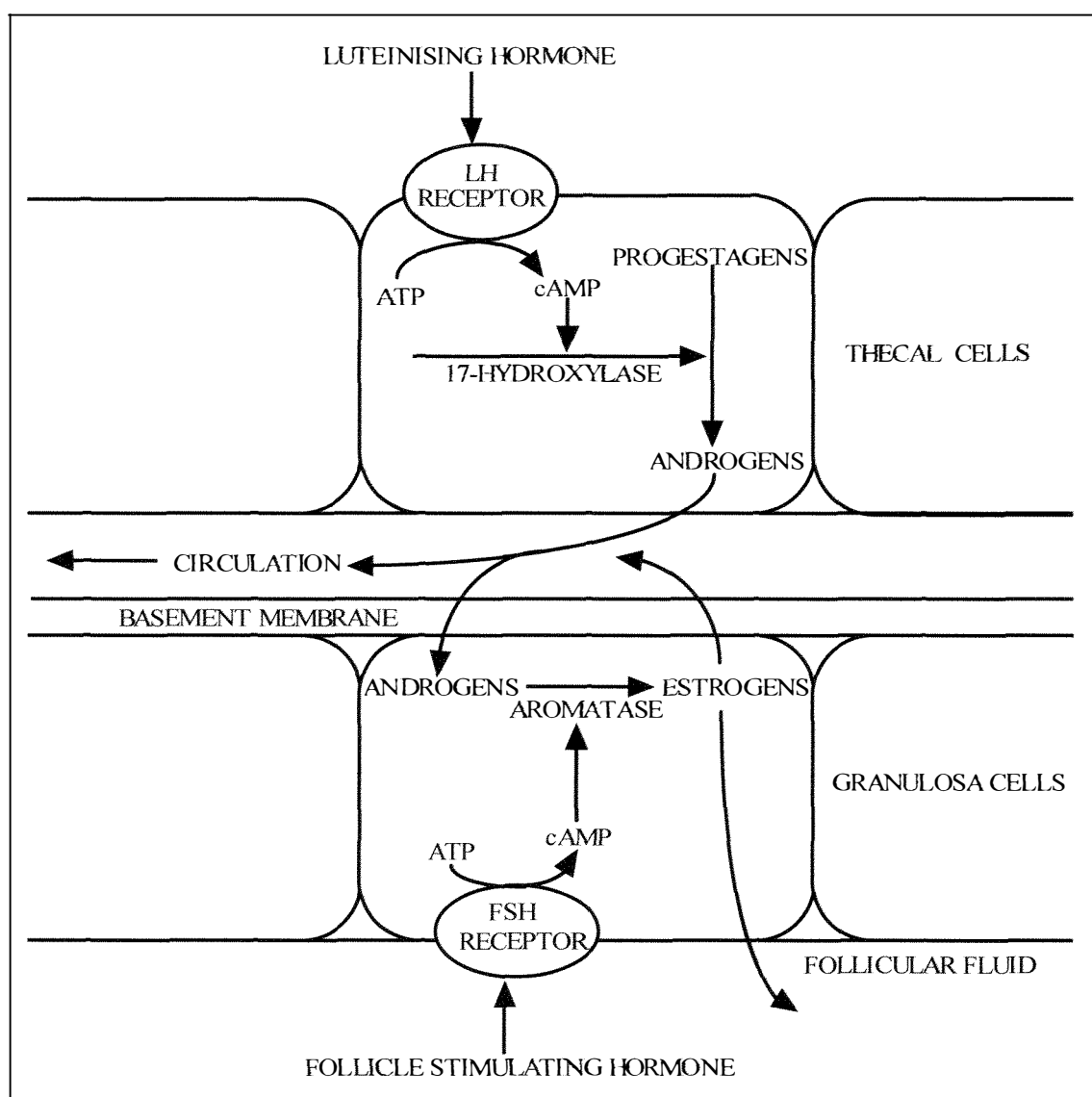


FIGURE 1.2: Schematic Diagram of the Two Cell Theory

modified from Adashi, 1994

Because granulosa cells are the only cells in the female body which express FSH receptors, FSH has no affect on the peripheral production of estrogen (Hillier *et al.*, 1994a). Thus, the first increase in circulating estrogen levels from baseline levels represents the acquisition of aromatase activity by the leading follicle. It is the first positive proof that a follicle has been selected for ovulation (Adashi, 1994) and represents the beginning of the fertile phase of the menstrual cycle.

A schematic diagram of the serum profile of the four main female sex hormones over the length of a menstrual cycle is shown in Figure 1.3.

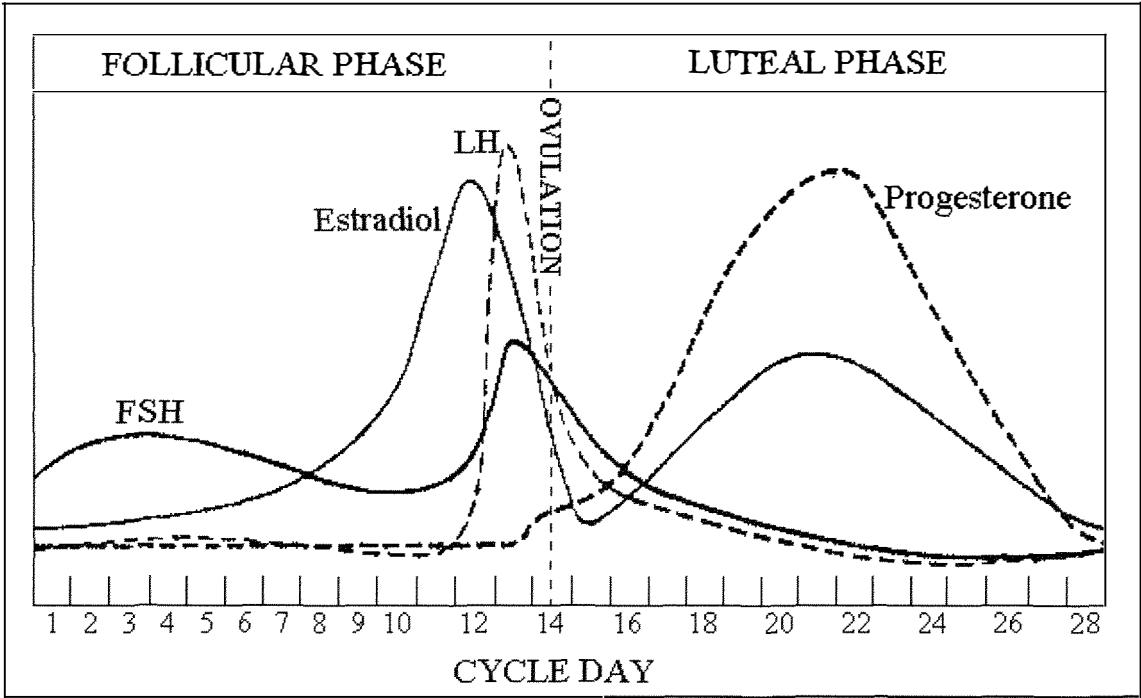


FIGURE 1.3: Idealised Hormone Profile of Luteinising Hormone, Follicle Stimulating Hormone, Estradiol and Progesterone during the Human Menstrual Cycle

Follicular estrogen production in response to FSH is limited by the follicle's FSH receptor content which is a product of the follicle's total number of granulosa cells and the density of the granulosa cell membrane receptors. FSH effectively sensitises the follicle to itself by up-regulating the FSH receptor expression (which increases the granulosa cell receptor density) and stimulating granulosa cell proliferation (which increases the total number of FSH responsive cells) (Adashi, 1994; Scheele and Schoemaker, 1996). Thus a sustained elevation of FSH is associated with increasing follicular sensitivity to FSH and a consequent increase in aromatase activity and estrogen production.

Estrogen itself also acts synergistically with FSH to promote follicular growth and increase FSH receptor formation (Fritz and Speroff, 1982) which would ultimately lead to the further amplification of aromatase transcription and activity. This ability of estrogens to augment the activity of the very enzyme responsible for their formation largely accounts for the exponential pre-ovulatory rise in the concentrations of circulating estrogens (Adashi, 1994).

Obviously this increase in estrogen output cannot be maintained without a corresponding increase in the thecal supply of androgen precursors. Although only LH acts directly on the thecal cell, the biosynthesis of thecal androgens is believed to be enhanced by both LH and FSH. With regards to LH, only a small sustained increase in the circulating concentrations of LH above its end of cycle nadir (see Figure 1.3), such as is present at the beginning of the cycle, is necessary to support androgen synthesis and continued follicular growth. The main means by which LH is thought to promote androgen biosynthesis is by activating the thecal transcription of 17α -hydroxylase/ c_{17-20} -lyase (Hillier *et al.*, 1994a). The FSH mediated process for the up-regulation of androgen production is less direct and is specific only to the pre-ovulatory follicle. In follicles of this advanced stage of maturity, FSH stimulates the development of LH receptors on the granulosa cells. Thus the granulosa cells of the mature follicle contain membrane receptors for both FSH and LH. As for the FSH receptor, binding to the LH receptor of the granulosa cell induces the transcription of aromatase. It also induces transcription of one of the subunits of the regulatory protein inhibin, a potent stimulator of lyase transcription in the thecal cell. Thus in the mature follicle, LH action on the granulosa cell leads to an increase in androgen production in the thecal cell (Hillier *et al.*, 1994a). Although the main method androgen production aids estrogen synthesis is by providing a source of aromatisable steroids, androgens also boost estrogen production by increasing aromatase transcription via a granulosa cell cytosol androgen receptor mechanism (Hillier *et al.*, 1994a).

In conclusion, the exponential increase in estrogen production associated with the development of the maturing ovarian follicle is the result of a battery of positive feedback mechanisms between FSH, LH, androgens and the estrogens themselves. Obviously with such a self-reinforcing system any follicle which is ideally placed in its stage of development to fully benefit from the increase in FSH levels at the exact time the FSH levels begin rising, will continue to consolidate their developmental lead (Baird, 1987).

1.2.2.2 *The Follicle Stimulating Hormone Threshold*

It is the leading follicle's developmental advantage, or more precisely its superior estrogen producing ability, which brings about the eventual demise of the other FSH responsive follicles. This is because high circulating levels of estrogens, such as are present in the blood when the leading follicle's estrogen production is entering its logarithmic stage, have a negative feedback effect on the release of FSH from the pituitary gland. While the leading follicle can survive this fall in FSH levels because of its lower FSH requirement, due to its greater FSH sensitivity and the unique ability of its granulosa cells to utilise an LH mediated induction of aromatase, the less advanced follicles are forced into atresia (Hillier *et al.*, 1994a) and no more are recruited from the antral pool.

The FSH level necessary to ensure only one follicle is selected for ovulation has a very narrow working range and is precisely controlled, with the usual range of operation of the FSH values above threshold during the normal cycle being estimated to be only 10%. This increase in FSH levels above threshold, although small is vital, as although threshold levels of the gonadotrophin are sufficient to stimulate ovarian estrogen production, they are not sufficient to boost it to pre-ovulatory levels. While the absolute FSH level between a sub-stimulating and a threshold dose varies from woman to woman, in a given individual

the difference could be as small as 20% (Baird, 1987). It is hypothesised that the episodic secretion of FSH prevents the FSH levels exceeding this range by allowing time for any responsive follicles to initiate estrogen synthesis and hence inhibit further FSH release (Brown, 1978). Although this negative feedback of estrogen on FSH secretion is critical for reducing the pool of pre-ovulatory follicles down to one follicle it can be easily over-ridden by gonadotrophin therapy. In fact, during the sixties and early seventies when gonadotrophin therapy for anovulatory women was still in its infancy, the high multiple pregnancy rate resulting from inappropriate hyperstimulation of the ovaries was a strong source of criticism of the technique (Brown, 1978). Today, with the greater control of FSH levels to within a small range of threshold values, this is rarely a problem (Scheele and Schoemaker, 1996). Although hyperstimulation of the ovaries is not a natural occurrence, artificial hyperstimulation is still a very useful technique for *in vitro* fertilisation programmes as it allows multiple oocytes at an advanced state of maturity to be collected at the one time (Aragona *et al.*, 1989).

In addition to these inhibitory effects against superovulation, follicular estrogen is also responsible for inducing the cascade of events which ultimately lead to ovulation and has other positive effects on fertility. At the level of the uterus, the increasing levels of circulating estrogen experienced over the follicular phase, stimulates the endometrial layer (lining of the uterus) to thicken and undergo vascularisation and glandular development (Cormack, 1984). This period of elevated estrogen levels unopposed by progesterone is also responsible for changing the composition of the cervical mucus to a form favourable to sperm survival (France, 1982; Bonnar, 1983; Katz *et al.*, 1997). With regards to ovulation the high circulating levels of estrogen present at the end of the follicular phase are responsible for inducing the mid-cycle LH surge necessary for ovulation (Fritz and Speroff, 1982). The variation in time taken to reach this estrogen threshold is now recognised as one of the main causes of cycle length variability between women. Changes in the actual length of the follicular phase (the period between menstruation and ovulation) are also responsible for the decrease in cycle length with age. This effect is such that the median duration of the menstrual cycle decreases from 28 days, five years after menarche, to 26 days in the 30 year old woman (Metcalf, 1988). The length of the follicular phase can also be increased dramatically if the leading follicle dies before it can induce the LH peak, in which case recruitment must start all over again, and may explain why menstruation is delayed in periods of high stress.

1.2.3 Ovulation

When the concentrations of circulating estrogens reach their upper physiological range there is an increase in the frequency and amplitude of the pulses of LH released from the pituitary gland (Collins, 1989). The LH surge typically lasts two days (Hoff *et al.*, 1983) and occurs concurrently with a surge in FSH which is significantly sharper and higher than the initial FSH peak associated with the first half of the follicular phase (see Figure 1.3). Twelve hours before the LH surge is initiated, a small but marked 24 hour rise in progesterone levels begins (Hoff *et al.*, 1983). Although estrogen is the main trigger for the pre-ovulatory LH surge, the small pre-ovulatory peak of progesterone is necessary for the normal mid-cycle gonadotrophin surges. Progesterone makes the LH surge earlier, higher and shorter in duration and appears to be essential for the induction of the second FSH peak

(Fritz and Speroff, 1982). These progesterone mediated effects are believed to be due to the high circulating estrogen levels present at mid-cycle stimulating the up-regulation of progesterone receptors in both the hypothalamus and anterior pituitary (Mahesh and Brann, 1998). In this model the increase in gonadotrophin release is due directly to progesterone action on the pituitary stimulating gonadotrophin secretion and inhibiting the degradation of gonadotrophin releasing hormone, and indirectly by progesterone and estrogen action on the hypothalamus increasing the secretion of gonadotrophin releasing hormone. The initial progesterone rise also appears to augment estrogen action, possibly by increasing the LH receptor content of the granulosa cell so that it is more sensitive to LH. The LH surge stimulates the resumption of meiosis in the oocyte, luteinises the thecal and granulosa cells so that the secretion of estrogens is reduced while that of progesterone can increase, and initiates the biochemical and mechanical processes which lead to ovulation (Collins, 1989).

The follicle reaches its greatest size just prior to ovulation and has a final diameter of 18-27 mm (Collins, 1989) with most of this final growth being associated with an increase in follicular fluid and elasticity of the follicle wall as opposed to an increase in granulosa cell numbers (Fritz and Speroff, 1982). Follicular rupture and release of the ovum (a mature female gamete) occurs on average 30 hours after the onset of the LH surge and 15 hours after the LH surge midpoint (Hoff *et al.*, 1983; Collins, 1991). The actual process of ovulation is not well understood and appears to be controlled by several mechanisms, of which most seem to be based around the proteolytic digestion of the follicular wall.

In the human, LH has been shown to stimulate the rupture of mature follicles and FSH has also been cited as a possible activator (Fritz and Speroff, 1982; Adashi, 1994). One of the ways that LH may mediate its effects is through prostaglandins. Prostaglandin synthesis has been shown to be essential for follicle rupture and LH is known to stimulate prostaglandin synthesis. It has been suggested that prostaglandins act by freeing lysosomal enzymes to digest the follicular wall. Another possible function for prostaglandins (and hence LH) is contraction of the smooth fibre in the thecal layer for the extrusion of the oocyte. Whereas LH appears to act through prostaglandins, FSH appears to act through plasminogen activator with the FSH dependent production of plasminogen activator by granulosa cells being particularly well documented. Plasminogen activator mediated conversion of the follicular fluid protein plasminogen to the proteolytic active enzyme plasmin may contribute to the digestion of the follicle wall. It may also act through the digestion of collagen, a major structural protein of the cell wall, as plasmin is a known activator of collagenase. It has also been suggested that it is the digestion of collagen and fibrin which is responsible for the increased distensibility of the follicle wall observed in the later stages of follicle growth. Finally FSH is also thought to act through plasminogen activator in the release of the oocyte and its associated cells from the internal granulosa layer allowing it to float free in the antral fluid just before follicle rupture.

1.2.4 The Luteal Phase

The phase following ovulation is known as the luteal phase and is characterised by the formation of the corpus luteum, an endocrine organ formed under the influence of LH from the remnants of the ovulated follicle. Luteal cells are derived mainly from the granulosa cells, and are much larger and have a much higher lipid content than their predecessors

(Fritz and Speroff, 1982). The accumulation of lipid is necessary for the high steroid production associated with this phase which is many times greater than the follicular phase requirements. A high lipid supply is ensured by the marked vascularisation of the corpus luteum which, unlike in the follicle of the follicular phase, penetrates the granulosa layer (Adashi, 1994). As the corpus luteum develops, its secretion of progesterone and estrogen increases. It is under the combined influence of these two hormones that the endometrial development converts from a growth phase to one of differentiation. The endometrium continues to thicken while the spiral arteries become increasingly prominent and the glands become secretory and more extensive in nature (Cormack, 1984). The combined action of estrogen and progesterone also inhibits the further release of LH and FSH from the pituitary gland.

Because the gonadotrophins are essential for the maintenance of the corpus luteum, and their release from the pituitary is inhibited by the hormones of the corpus luteum, its activity brings about its own regression. As the corpus luteum regresses, the steroid hormone secretion from the corpus luteum decreases until eventually the highly developed and vascularised endometrium can no longer be maintained. The spiral arteries constrict, followed by endometrial necrosis, sloughing off of the functional layer and bleeding (menstruation) (Cormack, 1984). Although the first day of bleeding is defined as the first day of the next follicular phase, the initiation of another wave of follicular growth begins in the late luteal phase when the inhibition of FSH release from the pituitary is first removed (Hadley, 1996a).

Unlike the follicular phase the luteal phase is very constant, lasting between 11-16 days. If the luteal phase is less than 11 days or is associated with a shallow progesterone peak, the progesterone output is inadequate for supporting implantation and the cycle is retrospectively diagnosed as having been infertile. A shortened luteal phase or inadequate luteal phase (i.e. one with low progesterone output) is commonly observed in cycles with low follicular FSH levels (Fritz and Speroff, 1982). Thus it has been postulated that the function of the second FSH peak is to induce the development of LH receptors on the granulosa cell, so that the cells are sufficiently sensitive to LH for optimal luteinisation and subsequent progesterone production. In the absence of this second FSH peak, low LH receptor levels on the granulosa cells will induce a sub-optimal response to LH and thus hinder luteinisation (Fritz and Speroff, 1982). In contrast a long luteal phase (> 16 days) is diagnostic of pregnancy as it shows that the maintenance of the corpus luteum has been taken over by the fetal gonadotrophin, human chorionic gonadotrophin (HCG) (Fritz and Speroff, 1982).

1.2.5 The Fertile Period

For the vast majority of women the most fertile time of life occurs between the ages of 20 and 35 years, during which period the ovaries are fully functional (Collins, 1989). Even at this period of peak fertility a woman is only fertile for a few days each cycle, with this length varying between women and even between consecutive cycles in the same woman. Strictly speaking the length of a woman's fertile period is really the product of her and her partner's fertility (Collins, 1989).

With regards to the woman's fertility, the ovum is viable for only a short period of time after ovulation with viability decreasing with the woman's age. This effect of aging is such that, the estimated mean fertilisable lifetime of the ovum for a forty year old woman is only 0.3 days. However, even in the years of optimal fertility this period is very short, with the mean period for ovum viability for a 25 year old woman being estimated at only 0.63 days (99% probability limit of 2 days). In contrast the mean lifetime of the sperm is much longer being estimated at 1.02 days with a 99% probability limit of 7 days (Royston, 1982; Collins, 1989). However even sperm viability times have a female component as their lifetimes are greatly affected by the conditions of the female genital tract which are in turn determined by hormonal factors. More specifically high levels of estrogen which are unopposed by progesterone change the nature of the cervical mucus into a form which produces an environment conducive to sperm survival. The presence of favourable conditions is extremely important as the fertilising capacity of the sperm is enhanced by time in the female genital tract. Thus the day of maximum fertility, which is defined as the day that an isolated act of intercourse is most likely to lead to conception, is not the day of ovulation as might be thought based on the ovum's short lifetime, but rather the preceding day. This extra time is necessary for sperm capacitation (dissolving of the sperm's acrosomal membrane), and is an essential prerequisite for the sperm to be able to penetrate the ovum. Although the length of the fertile period is highly variable depending on the combined fertility of the couple and the fertility of any given cycle most clinical trials usually assume it to be 6 days and base it on the LH peak (LH peak day -3 to LH peak day +2) (Adlercreutz, *et al.*, 1982; Collins, 1989).

1.3 NATURAL FAMILY PLANNING

Natural family planning (NFP) can be defined as the conscious avoidance (abstinence) or timing of intercourse by a couple during the fertile time of the woman's cycle to minimise or maximise the chances of conception. Although the people who use NFP for contraceptive purposes often do so because of their religious beliefs, NFP is increasingly becoming the method of choice for couples who want a chemical-, surgical- and invasive-free method. The desire for a more natural means of fertility regulation being sought by an increasing number of couples combined with today's impossible legal requirements for the design and introduction of new contraceptive methods provides a hitherto unknown opportunity for natural family planning (Djerassi, 1990; Vines, 1994).

1.3.1 Methods of Natural Family Planning

Several different natural family planning methods have evolved over the years for determining the natural period of fertility. Although the different methods rely on monitoring different cyclic markers, all these markers are reflections of the woman's current underlying ovarian hormonal status.

1.3.1.1 *Calendar Method*

The oldest and least reliable method of natural family planning is the calendar (rhythm) method (Ogino, 1930). This method is based on the recognition that the time interval between ovulation and the onset of the next bleed is relatively constant (12-16 days). By subtracting this value from a woman's average cycle length an estimate of the date of her next ovulation, and thus fertile period, can be made (Bonnar, 1994). Because the method relies on a relatively constant follicular phase, the method cannot be used by women with irregular cycles. Ogino recommended that the method not be used by women whose range of cycle length exceeds 10 days. The lack of universal application of this method is well illustrated by the data presented by Colombo (1989) who showed that when the rule was applied to a database of 2,355 sets of cycle data, 23% of women had cycle variabilities outside the method's recommended range. For women with more constant cycles, it was calculated that the method had a fertile length of 13 days.

Because the method is completely retrospective (i.e. it predicts the beginning and the end of the fertile period solely on the characteristics of the preceding cycles) and it has a high failure rate (18% pregnancy rate per year), the use of the method is discouraged by most natural family planning educators (France, 1988). Despite the limitations of the method, it is extremely simple to apply, and this is reflected in its popularity, with 85% of world NFP users incorporating a calendar method component into their method of choice (Bonnar, 1994).

1.3.1.2 *Basal Body Temperature Method*

In the combined calendar - basal body temperature method the beginning of the fertile phase is predicted according to the calendar method, and the end of the fertile phase is

determined by a sustained change in the basal body temperature (BBT) from the beginning of the cycle.

The general consensus is that when the vaginally or rectally measured waking temperature shifts to 0.2°C above the highest recorded temperature of the preceding six readings in a period of less than 48 hours and stays elevated for three days then the post-ovulatory infertile period has begun (Gross, 1989).

The change in BBT throughout the cycle is due to changes in the circulating levels of the thermogenic hormone, progesterone (France, 1982), with the two rising over the peri-ovulatory time and falling with the demise of the corpus luteum. However, the presence of a BBT shift cannot be taken as absolute proof of ovulation, as the lower concentrations of progesterone associated with a luteinised unruptured or regressing follicle is still often sufficient to result in a thermal shift (Gross, 1987). Furthermore, a shift is not always present with endocrinologically normal cycles, or the shift may be only slight. Difficulties in detecting the shift can be caused by illness, alcohol, stress, changes in waking time or anything that affects the body temperature (Gross, 1987; Bonnar, 1994; Gross, 1989).

The BBT method can also be used without the calendar method in which case intercourse is restricted only to the late safe days of the cycle. This method has an efficacy of 1.2 pregnancies per 100 women years compared to 5.0 for the combined calendar-BBT method (Marshall, 1968).

1.3.1.3 Billings' Ovulation Method

One of the more accepted methods of natural family planning, is based on the fact that the nature and volume of the cervicovaginal fluid (mucus) is determined by the ovarian hormone concentrations and thus change with the cycle (Odeblad, 1994a). In the Billings' Ovulation Method, the examination of this mucus at the level of the vulva is used to determine the beginning and end of fertility as well as the period of maximum fertility.

During the fertile menstrual cycle, menstruation is followed by a variable number of days of low estrogen production which are characterised by a pattern of unchanging dryness or a slight discharge, referred to in the Ovulation Method as the basic infertile pattern. The mucus produced over this period is slight and highly viscous and contains a complex network of glycoprotein fibrils (mucin) which forms an effective barrier to sperm penetration (France, 1982). The day this pattern changes represents the beginning of the potentially fertile phase and is correlated to an increase in estrogen production. Initially the mucus is thick and sticky, pasty yellow or white, but over the next few days it increases in volume and becomes slippery, clearer and stretchy, with the characteristics of raw egg white, and produces a characteristic wet, lubricative sensation (Gross, 1989). The low viscosity and high threadability (spinnbarkeit) is highly receptive to sperm. Under the influence of high concentrations of circulating estrogens, the glycoprotein fibrils in the mucus form a micellar structure orientated along the direction of the cervical canal. This structure aids in sperm migration directing them along channels between the micelles

towards the uterine cavity and into the crypts of the endocervix where reservoirs of sperm are formed and slowly released (Odeblad *et al.*, 1983; Odeblad, 1994a; France, 1982).

Because the slippery mucus is very favourable to sperm survival, its onset signals the beginning of the highly fertile period. The duration and onset of this high fertility mucus changes dramatically with age. In a post-pubertal girl, the slippery mucus appears early in the cycle and can last for more than a week. However, as a woman ages this mucus begins to appear later in the cycle and its duration decreases accordingly down to only one or two days by around age 35 (Odeblad, 1994b; Odeblad *et al.*, 1986). This decrease in days of slippery mucus is believed to be a major factor in decreasing fertility with age.

The last day of the slippery mucus, termed the peak day, is closely related to the day of ovulation and is thus considered to be the day of maximum fertility. Following ovulation, under the influence of progesterone the mucus becomes thick and sticky or dries up altogether (Gross, 1989). The end of fertility is defined as ending with the morning of the fourth day after the peak symptom.

The large and well documented World Health Organisation (WHO) trial on the Ovulation Method in 1976 showed that the average length of the fertile period for New Zealand trial women as calculated by the above rules was 10.8 days (WHO, 1983a). However, because the mucus symptoms can be masked by menstrual bleeding, seminal fluid and stress, for the avoidance of pregnancy abstinence is restricted to more than the fertile period. Thus, although the mucus defined the infertile period to an average length of 18 days, for the women in the New Zealand centre of the WHO trial who had an average cycle length of 29 days, when the additional guidelines were followed only an average of 12 days were available for intercourse.

A method based solely on the fertile characteristics of the mucus may seem vague as a contraceptive method, but its mode of action is quite similar to the mini-pill. In this progestagen only hormonal preparation, ovulation is only prevented in about half the cycles, and most of its contraceptive actions derive from its ability to maintain the exclusive secretion of infertile cervical mucus throughout the cycle (Baird and Glasier, 1993).

Studies have shown that the mini-pill is associated with a pregnancy rate of 3.0 pregnancies per 100 woman years (Baird and Glasier, 1993), and thus provides a similar level of protection as the Ovulation Method, which has a method failure rate of 2.8 when a couple correctly interprets the mucus pattern and abstains according to the rules (WHO, 1981). However, analysis of the same data by Trussell and Grummer-Strawn (1991) showed that for couples who deviated from the rules of the method, the pregnancy rate was extremely high at 84 pregnancies per 100 women years of experience, while breaking of the most serious rules of the method of no intercourse over the fertile period or during times of stress, resulted in a 27% risk of pregnancy per cycle. Considering that for young couples trying to conceive a child, the maximum live birth rate per menstrual cycle has been estimated as 21-28% (Royston, 1982), these results graphically illustrate how unforgiving the method is of imperfect use. This result may seem negative, but what the results really show is how good the method is at correctly defining the days of abstinence to include the entire fertile period, and how very fertile the fertile period is.

For an extra margin of security, the Ovulation Method can be combined with either the BBT or calendar method or both, in which case the method with the most conservative estimate for the beginning or end of fertility is used as the definitive marker. Thus combined methods are usually associated with a greater period of abstinence but a lower unplanned pregnancy rate. When the Ovulation Method is combined with the BBT and calendar method it is known as the symptothermal method. Studies have shown this method to be one of the most effective means of birth control by natural means, and to have a pregnancy rate of only 0.93 per 100 women years when the observations are interpreted correctly and the couples have abstained according to the rules (Rice *et al.*, 1981). The symptothermal method is the method most commonly endorsed by natural family planning educators including the Natural Family Planning Association of New Zealand.

1.3.1.4 *Lactational Amenorrhoea Method (LAM)*

It is well established that breastfeeding is associated with reduced fertility (Howie, 1991). Although breastfeeding is not usually used specifically as a means of birth control, on a global scale it is believed to prevent more births than most other methods of family planning (Gross, 1997).

In general terms the level of protection from pregnancy that breastfeeding provides decreases with time, and the sooner a baby receives its first breast feed, and the more suckling at the breast, then the later the return to fertility. This suppression of fertility, is such that a couple can rely on breastfeeding to provide natural protection against pregnancy for at least 6 months with a less than 2% chance of pregnancy, provided the baby is being fully, or almost fully breastfed and the mother has not spotted or recommenced menstruation (with the exception of any bleeding prior to the 57th postpartum day) (Gross, 1991). The results from one international study showed that the method could be applied for an average of 4.1 months, with only 16% of the new mothers being able to apply it to the whole six months (Kennedy *et al.*, 1991). However, the results from another study showed that when mothers were strongly committed to breastfeeding the method could be applied for the full six months for 81% of the women (Perez *et al.*, 1992).

In contrast, the confused mucus symptoms which are characteristic of the postpartum period and which are not always coupled to ovarian activity make application of the symptothermal method at this time difficult and unnecessarily restrictive in its abstinence requirements at a time of compromised fertility (Kennedy *et al.*, 1991; Flynn *et al.*, 1991; Gross, 1991). Thus the lactational amenorrhoea method provides a natural protection from pregnancy over the immediate postpartum period using guidelines which are both simple to apply and limited in their abstinence requirements.

1.3.2 Natural Family Planning Aids

A range of instruments or home tests based on detecting the changes which occur to various fertility markers throughout the menstrual cycle have been designed for the natural family planning market. One of the chief attractions of these devices is that they remove some of subjectiveness from the method.

1.3.2.1 *Basal Body Temperature*

For measurement of the BBT, the replacement of clinical thermometers with digital thermometers has reduced the recording time and made temperature readings much easier and more reliable (Flynn, 1989). The most sophisticated NFP thermometer currently on the market is the Bioself 110 Fertility Indicator which uses a combined BBT-calendar algorithm to determine the pre-ovulatory and post-ovulatory infertile days. Clinical trials have shown it to be an excellent refinement of the calendar/BBT method (Ismail *et al.*, 1989).

1.3.2.2 *Cervicovaginal Mucus Volume*

Another NFP aid currently on the market is a small disposable plastic vaginal aspirator (Rovumeter™) which measures the volume of cervicovaginal mucus that pools at the entrance to the cervix (Usala and Schumacher, 1983). As mentioned in Section 1.3.1.3, changes in the volume of the cervicovaginal mucus are believed to be largely under the influence of estradiol and parallel the first estrogen peak (Usala and Schumacher, 1983; Moghissi, 1980). Thus the volume of cervicovaginal mucus increases at the beginning of the fertile period to reach a peak one to two days before ovulation (Flynn *et al.*, 1988). After ovulation the volume drops abruptly and this fall is used to indicate the onset of the post-ovulatory infertile phase.

Although the results from the pilot study were encouraging (Usala and Schumacher, 1983), a larger more recent trial showed that for a significant proportion of women (21%) the device failed to detect all of the fertile period and it was concluded that the device was probably more useful as an aid for couples trying to achieve pregnancy than for those trying to avoid it (Flynn *et al.*, 1997).

1.3.2.3 *Vaginal and Salivary Electrical Resistance*

Another potentially useful natural family planning aid is the Cue™ Fertility Monitor which uses a vaginal and oral probe to measure changes in vaginal and salivary electrical resistance to predict ovulation and the beginning and end of the fertile period (Fernando *et al.*, 1988).

Once again, although the results from the developers of the method are very encouraging, independent studies on the method are less positive (Kesner *et al.*, 1992). However, although Freundl *et al.* also found the method unsatisfactory they did conclude that the potential of the method was not being fully realised and could be vastly improved by refining the monitor's basic algorithm (Freundl *et al.*, 1996).

1.3.3 **Natural Family Planning Aids and Hormone Measurement**

Most of the above methods of natural family planning rely on retrospective or indirect indicators of fertility i.e. although these markers may reflect the fertility status they are not important determinants of fertility in themselves. Thus they can easily be masked by other environmental influences. A woman's fertility is ultimately under hormonal control, and the above markers are really just symptoms of their actions. Thus a hormonal assay would

provide a more direct marker of fertility status. Although, serum hormone assays are the most common markers used in fertility clinics, because these assays require frequent blood sampling they can not be adapted readily for everyday use in the home. In contrast, the measurement of the excretion of the metabolites of these hormones into the urine can be. Apart from the fact that there is no blood letting, urine assays provide several other advantages over serum assays. Firstly, due to the short half-life of hormones in the peripheral circulation and the high ratio of blood volume to daily urinary output, hormones and their metabolites are present in much higher concentrations in urine, and thus are easier to measure. Although it may seem paradoxical, one of the biggest disadvantages of a serum hormone assay is that it measures the levels of circulating analyte at the exact time of sampling. Because all the principle ovarian and pituitary reproductive hormones (estrogens, progestagens, FSH and LH) have a pulsatile mode of secretion, this can cause considerable variations in blood levels throughout the day, making interpretation of the serum hormonal data particularly difficult. For example during the LH surge, the LH value in serum can vary by as much as three fold, while circulating levels of estradiol, the principal estrogen secreted by the ovary, can vary by as much as 50% within a space of 30 minutes (Baird, 1983). The use of urine samples avoids this problem because the peaks and troughs are integrated over the collection time.

1.3.3.1 Urinary Luteinising Hormone Home Tests

Because the LH peak is essentially an overshoot phenomenon it is not an ideal marker for impending ovulation. Similarly establishing a suitable threshold value for LH is difficult as there is considerable between subject variability (Jeffcoate, 1983). Although the changes in urinary LH excretion come far too late to be used to predict the beginning of the fertile period they are often used as markers for predicting the onset of the maximum fertile period in the home as well as at infertility clinics. Considering that the LH peak precedes ovulation by an average of only 15 hours (Collins, 1991), that there is a 6-7 hour delay in LH excretion (Martinez *et al.*, 1995) and that the maximum period of fertility is predicted to occur between 36 hours before and 12 hours after ovulation (Collins, 1989), the greatest disadvantage of using urinary LH as a fertility marker is that it often occurs too late to detect all of the period of peak fertility. Despite the lack of prospective evidence that LH kits have any positive effects on pregnancy rates (Agarwal and Hanney, 1994), their widespread use by physicians and the public continues.

1.3.3.2 Urinary Follicular Stimulating Hormone Tests

No home fertility tests based on FSH have been developed to date. The measurement of FSH in urine is much more difficult than for LH because unlike with LH where there is a low baseline and a very obvious surge, the FSH concentrations are changing throughout the follicular phase and at their mid-cycle peak they are only 20% of the LH peak values. Threshold limits are also particularly unsuitable for FSH assays as although the threshold levels of FSH required for inducing folliculogenesis are precise and practically constant for a given woman, it varies considerably between women (van Santbrink *et al.*, 1995). Thus it is possible for a woman with absent ovarian function to have an FSH production which is within the range for a normal population of fertile women, yet below her own threshold level (Brown, 1978).

Although some peripheral isomerisations and aromatisations of steroids does occur, for example in the liver and adipose tissue, in the human female, all steroids are ultimately of adrenal or ovarian origin (Baird, 1976). The conversion of the 27 carbon tetracyclic molecule, cholesterol, into pregnenolone, the 21 carbon precursor of all steroid synthesis, is catalysed by the desmolase system which uses a triad of cytochrome P₄₅₀ NADPH/O₂ dependent reactions to cleave a six carbon unit from the cholesterol side chain (Hadley 1996b; Stryer, 1981a).

Progesterone, the primary bioactive progestagen, is directly synthesised from pregnenolone by 3 β -hydroxy-dehydrogenase-isomerase which oxidises the secondary hydroxyl group at carbon 3 to a ketone and shifts the double bond from the 5 to the 4 position (Hadley, 1996b).

As can be seen from Scheme 1.1 the biosynthesis of the androgens is more complicated and can use either pregnenolone or progesterone as a precursor for the production of dehydroepiandrosterone and androstenedione respectively. Dehydroepiandrosterone is converted by an irreversible reaction into androstenedione, while androstenedione may be converted in a reversible reaction into testosterone by β reduction of the 17-keto functionality (Hadley, 1996b). The most important characteristic of the synthesis of the androgens from progestagen precursors is the removal of the two carbon side chain at carbon 17 by the P₄₅₀ enzyme, 17 α -hydroxylase/C₁₇₋₂₀-lyase to yield a 19 carbon steroid (Hadley, 1996b; Hillier *et al.*, 1994b).

Androstenedione and testosterone can be converted into their respective estrogens, estrone and estradiol by the P₄₅₀ NADPH/O₂ dependent aromatase enzymes (Hillier *et al.*, 1994a; Stryer, 1981a). These conversions are irreversible (Hillier *et al.*, 1994a) and require the reduction of the C₃ keto group, removal of the C₁₉ methyl group and aromatisation of the A ring (Stryer, 1981a). Although there is more androstenedione produced by the ovary than testosterone, the equilibrium of the 17 β -hydroxysteroid dehydrogenase catalysed reaction ensures that most of the resulting ovarian estrone is converted into estradiol (Hillier *et al.*, 1994a).

Estradiol is responsible for practically all of the estrogenic activity in the body (Burger, 1989). Thus it is estradiol which controls follicular development and oocyte maturation, stimulates the cervical mucus glands to secrete a fluid favourable to sperm survival and transport, and facilitates the mid-cycle surge of LH which precipitates ovulation. Estradiol is also involved in ovum transport and the proliferation of the endometrial layer in preparation for implantation.

To ensure the body's rapid response to changes in the levels of steroid production, circulating estradiol is rapidly converted into the less bioactive estrogens, estrone and estriol upon passage through the liver (Collins *et al.*, 1981) while the progestagen, progesterone, is similarly converted to less bioactive forms, including pregnanediol (Pd) (Cooke, 1976).

For excretion the lipid soluble steroids are conjugated most commonly to glucuronic acid, sulphuric acid or both (Collins *et al.*, 1981) which greatly enhances their water solubility.

Although the di-substituted conjugates are mainly excreted in the bile, the most common excretion route for the mono-substituted glucuronide conjugates is the urine (Collins, 1985). Estrone, estradiol, and estriol (see Table 1.1), and pregnanediol all appear in the urine as glucuronide conjugates.

TABLE 1.1: The Estrogens and their Principal Urinary Metabolites

Steroid	Conjugate (longhand)	(shorthand)
Estrone (E1)	Estrone-3-glucuronide	E1-3G (E1G)
	2-Hydroxyestrone-glucuronide	2-OH-E1G
Estradiol (E2)	Estradiol-17 β -3-glucuronide	E2-17 β -3G
Estriol (E3)	Estriol-16-glucuronide	E3-16G
	Estriol-3-glucuronide	E3-3G

There is a large range of values about the mean daily rate of urinary excretion for each of the three estrogens as measured individually by the split estrogen method (the separate measurement of estrone, estradiol and estriol), although the ratios between the various estrogens do tend to be relatively constant throughout the menstrual cycle for a given woman (Brown, 1955).

With regards to selecting a urinary marker for serum estradiol, the ideal conjugate should be excreted rapidly and in high concentrations into the urine, and show a large difference between the early follicular phase baseline and mid-cycle peak levels. Although E2-17 β -3G is the first metabolite to be excreted (Hobkirk and Nilsen, 1974), and has a steep rise from baseline values (Stanczyk *et al.*, 1980, Baker *et al.*, 1980), it is not a major urinary metabolite of estradiol (Musey *et al.*, 1972) and is only excreted at low concentrations. In contrast, the sum of the rates of excretion of the estriol glucuronides, E3-3G and E3-16G shows that they are present in large amounts. However, because their excretion is delayed by approximately 12 hours due to a complex enterohepatic circulation involving biliary excretion and reabsorption (Brown and Gronow, 1985), they are not ideal urinary markers for ovarian activity.

Next to E2-17 β -3G, estrone glucuronide (E1G) has the highest peak to baseline ratio (3.4 versus 2.8) and is found in the urine at 6.5 times higher concentration than E2-17 β -3G (Baker *et al.*, 1980). As well as being present in the urine in high quantities it is also usually excreted rapidly. Furthermore, estrone glucuronide levels appear to be directly correlated to circulating levels of estradiol (Munro *et al.*, 1991).

When it comes to the main bioactive progestagen, progesterone there are more than twenty metabolites, most of which are excreted via the bile into the gastrointestinal tract. Pregnanediol (5 β -pregnane-3 α -20 α -diol) is the major urinary metabolite of progesterone (Collins, 1989), and is mostly excreted in the urine as a pregnanediol glucuronide (PdG) conjugate. However, as a significant proportion of pregnenolone from the adrenal gland is also metabolised to pregnanediol, the pregnanediol glucuronide in the urine is not solely due to progesterone metabolism (Cooke, 1976). In addition to this, there are also significant amounts of ovarian progesterone produced in the first half of the cycle, thus the

urine always shows a baseline level of PdG. However, when it comes to the interpretation of PdG data for fertility monitoring this is not a cause for concern as the rise in PdG levels from baseline values due to increased circulating levels of progesterone in the luteal phase is very clear. Similarly, baseline levels of urinary estrogen metabolites which are not of ovarian origin are present throughout the cycle due to adrenal metabolism and the peripheral aromatisation of adrenal and ovarian androgens. However, once again the emergence of ovarian activity signalled by a rise above this peripheral activity is very clear, especially with estrone glucuronide.

Thus, in summary, PdG and E1G have become widely accepted as the most suitable urinary markers of ovarian progesterone and estradiol production respectively. The chemical structures of these two fertility markers are shown in Figure 1.4.

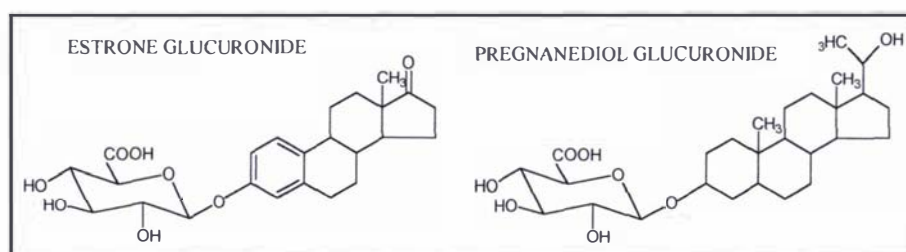


FIGURE 1.4: Structure of Estrone Glucuronide and Pregnanediol Glucuronide

1.3.3.4 The Persona Device

The first large scale commercialisation of a home hormonal test for defining the fertile period was released on the United Kingdom market in September, 1996 by Unipath (May, 1997). The Persona personal contraceptive system consists of a small battery held monitor which is used in combination with a series of disposable immunoassay test strips (May, 1996). This method uses early morning urine samples (first urinary void upon rising) to measure the E1G levels and define the LH peak (France, 1996), and is essentially a sophisticated calendar method based on the LH peak and the cycle length. The method uses the LH peak of preceding cycles to predict the beginning of the fertile period for the next cycle, and the end of fertility is calculated by counting forward from the current cycle's LH peak. The beginning of fertility is defined either according to the predictive LH algorithm or the current cycle's first rise in E1G levels, with the first E1G rise only being used if it predates the LH estimation (May, 1996).

The greatest attraction of the method is that it is very simple to apply and quick as it requires no interpretation from the woman (Kirkman, 1997) and an average of only 8 tests per month (May, 1997). On the first day of menstruation the woman presses the M button on the monitor, which then indicates day 1 of the cycle. Because the method is mainly used for the avoidance of pregnancy a red light is used to signal fertility, and a green light for infertility. A yellow light is also shown on days that testing is required, while an O is used to indicate the estimated day of ovulation (May, 1997).

The method incorporates an algorithm which relies on the data from the previous cycles to calculate when to commence sampling for the next cycle and also to help define the fertile

period. Thus for the first few months long periods of abstinence are still required. However, after this period for most women the system calculates a fertile period length of only 6-10 days per cycle (May, 1996). The chief disadvantage with the method is its pregnancy rate. According to field trials in Europe the method is associated with an effectiveness of only 94-95% compared to 98% for the symptothermal method (France, 1996; Freundl *et al.*, 1998), but it does have a much shorter requirement for abstinence. The method is also not suitable for women with irregular cycles or those approaching the menopause (Kirkman, 1997). Thus yet again a fertility detection method has been developed which only acknowledges the classical menstrual cycle.

1.3.3.5 The Ovarian Monitor

A reliable, much earlier, but less readily available natural family planning hormonal monitoring device is the Ovarian Monitor first developed by Professor Jim Brown in Melbourne in 1987 (Blackwell *et al.*, 1998; Brown *et al.*, 1988). The Ovarian Monitor utilises time diluted urine samples i.e. urine samples diluted to a constant urinary excretion rate ($150 \text{ mL } 24 \text{ hr}^{-1}$) and uses a visually determined increase in E1G levels above the follicular phase baseline as a marker for the onset of the fertile period. The day of the first urinary E1G peak and the following day are considered to be the days of maximum fertility, and an elevation in PdG levels past a threshold level of $6.3 \mu\text{mol PdG } 24 \text{ hr}^{-1}$ is used to mark the end of the fertile period (Brown *et al.*, 1991; Blackwell and Brown, 1992; Blackwell *et al.*, 1998). An idealised E1G and PdG hormone excretion profile showing the main fertility markers used by the Ovarian Monitor is illustrated in Figure 1.5.

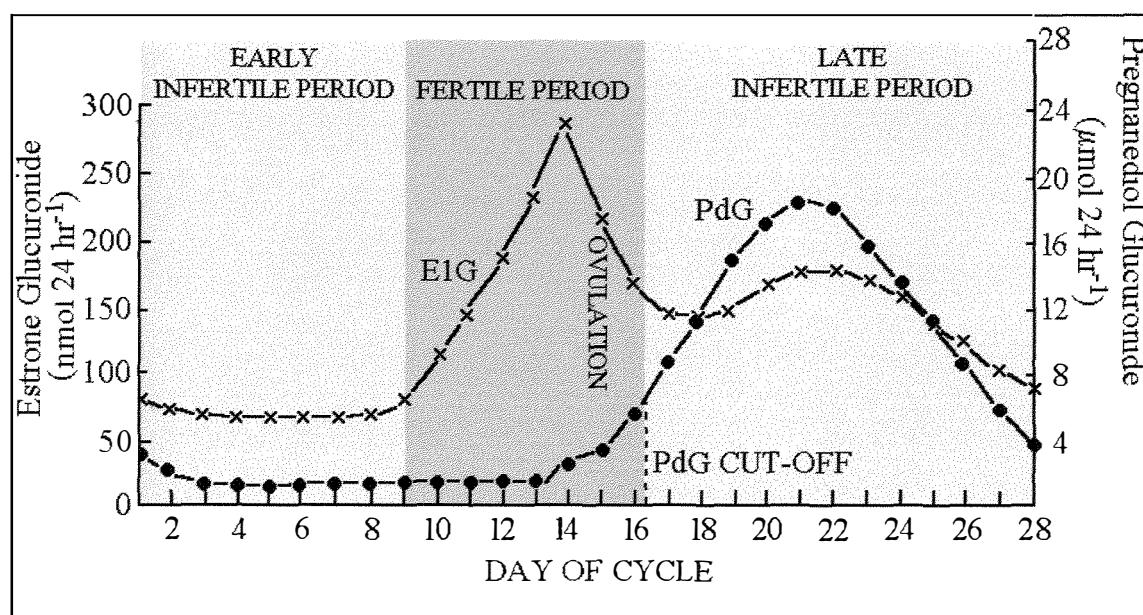


FIGURE 1.5: Idealised Menstrual Cycle Profile for Urinary Estrone Glucuronide and Pregnanediol Glucuronide Excretion Rates

The validity of the Ovarian Monitor method has been verified by numerous trials conducted by the developers of the Monitor (Brown *et al.*, 1991; Blackwell and Brown, 1992; Blackwell *et al.*, 1998) and more recently by the World Health Organisation (WHO, in preparation).

Apart from their method's of defining the fertile period, one of the most fundamental differences between the Ovarian Monitor and the Persona device is their method's of relaying the fertility status. Working on a simple red light - green light format, the Persona device allows no interpretation of the data by the user and shows no distinction between the gradients of fertility observed across a cycle or between cycles. In contrast the Ovarian Monitor provides the raw hormone data, and the training of the users to be able to interpret the hormonal pattern is an integral part of the method. Thus users are free to make their own informed decisions as to abstinence and family planning. Unlike the Persona device, the method provides information about the constantly changing fertility status as it unfolds across the cycle while careful attention to the patterns and absolute rates of excretion will enable the woman to determine the relative fertility of her cycles and provide a window to many aspects of her reproductive health.

1.3.3.6 Choice of Urine Collection Method for Hormonal Assays

Another difference between the Ovarian Monitor and the Persona device is their method of urine collection. Early morning urine samples and timed urine collections are usually looked upon as a sensible alternative to 24 hour samples as they require only a single urine collection. In contrast to the time diluted samples, when early morning urine samples are used no correction for variations in the rate of urine production is made. This is based on the premise that the rate of urine production is more constant over the night than during the day when daily changes in water intake, exercise and the environmental temperature can cause large diurnal variations. The main advantage of using early morning urines over timed collections is it simplifies the collection as no timing or dilution of the sample is required. In fact with the Persona personal contraceptive system no large scale collection of the sample is required at all; to collect the sample the test strip is simply placed in the early morning urine stream (May, 1997).

Although timed urine collections, as are required by the Ovarian Monitor system do require the collection of an entire void, they also give a more immediate reflection of the body's hormonal status as they can be collected over a period as short as 3 hours as opposed to an average closer to 8 or 9 hours which is required with the overnight collections. Timed collections also allow sampling to be taken at any time of day which can allow a more immediate correlation between the time of testing and intercourse in comparison to early morning urine samples in which case the mean of the collection period (e.g. 3 AM for a 11 PM - 7 AM collection) is usually quite removed from the next act of intercourse. However, the real advantage of timed samples is that they allow a more accurate measurement of the rate of hormone production to be made as it never assumes the rate of urine production to be constant. Thus, for example, the use of alcohol or aspirin, two substances well recognised for their diuretic properties, will not adversely affect the calculated hormone concentration on timed urine samples. Considering that alcohol plays an important, even if only occasional, role in most people's social lives and that the vast majority of it is consumed in the evening, lack of correction for rate of urine production of the hormonal markers with the use of overnight urine collections can have serious practical repercussions on fertility prediction.

In relative terms, to be able to utilise fully the information contained in the hormonal data it is necessary to distinguish between daily urine measurements that differ by no more than 40%. This is a stringent requirement which cannot be met if fluctuations in urine volume are occurring. A graphic example of the consequences of not correcting for urine volume is illustrated in Figure 1.6 which shows the estrone glucuronide and pregnanediol glucuronide cycle profile for a series of time diluted urines collected overnight and assayed for estrone glucuronide content using the Ovarian Monitor, and the profile which would have been observed for the same cycle if no correction had been made for changes in the rate of urine production.

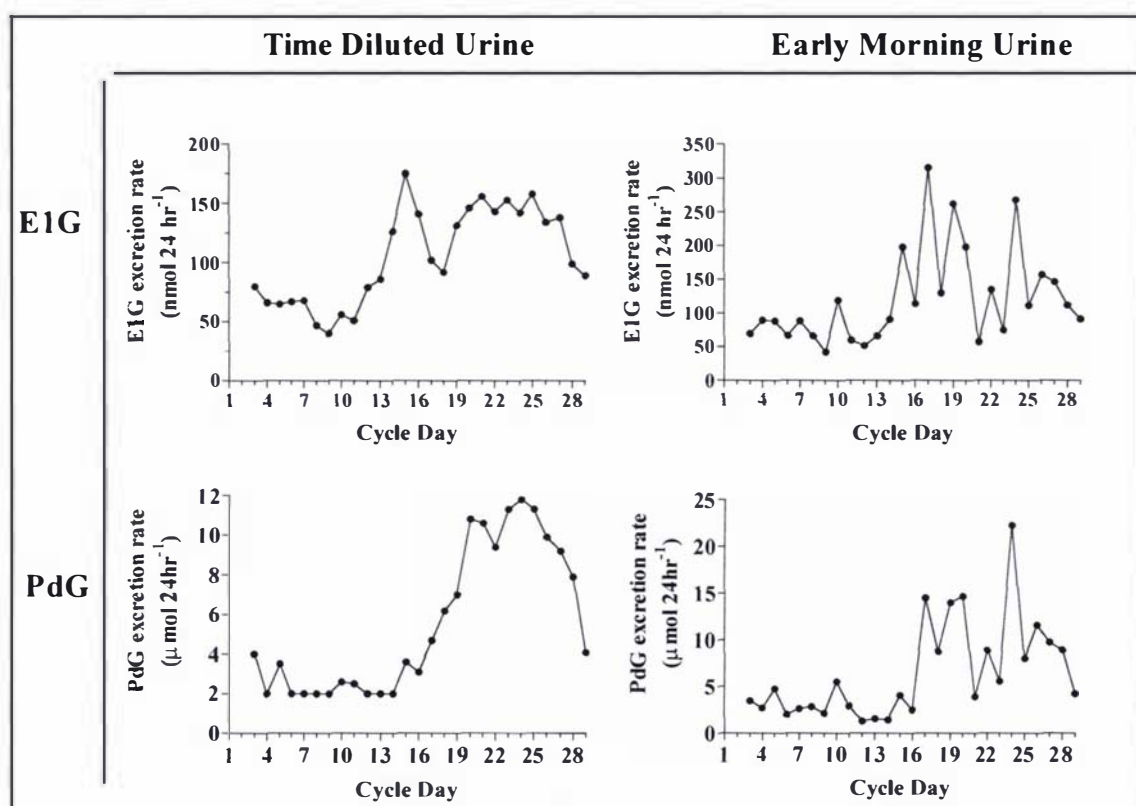


FIGURE 1.6: Urinary Excretion Rate Menstrual Cycle Profiles of Estrone Glucuronide and Pregnanediol Glucuronide for Early Morning Urine and Time Diluted Urine Samples

The similarity in the pattern of the E1G and PdG profiles for the timed urines with the idealised profile shown in Figure 1.5 is very clear, with all peaks and baseline periods being well defined. However, the distortion of the profiles calculated using the early morning urine data is so extreme that the profile is largely masked.

The difference in the willingness to use timed samples over early morning urine collections may explain the difference in opinion in the literature over the suitability of urinary ovarian steroid metabolites as markers for the beginning and end of the fertile period. Although most researchers concluded that urinary E1G and PdG concentrations were related to the beginning and end of the fertile period, this correlation was not considered to be sufficiently precise to be relied upon for contraceptive purposes (Adlercreutz, *et al.*, 1982; Cekan *et al.*, 1986; WHO, 1983b). However, it was also noted by many of the same workers that an E1G/PdG ratio or an E1G/creatinine and PdG/creatinine ratio may make more suitable

markers (Munro *et al.*, 1991; Adlercreutz *et al.*, 1982; Cekan *et al.*, 1986; WHO, 1983b; Collins *et al.*, 1981; Stanczyk *et al.*, 1980). In these cases the excretion of the metabolite of interest is corrected for changes in urine production by using the rate of excretion of another metabolite e.g. E1G excretion is corrected for by PdG levels. Although this is an improvement over the use of early morning samples, it must be remembered that it is only a relative correction. For example although creatinine is supposed to be excreted at a constant rate proportional to muscle mass and is commonly used to correct for the rate of urine production, its variation in rate of production in a single person is usually in the order of 20% (Paterson, 1967). While this is an improvement it will still obscure a 40% rise in E1G excretion rates which must be detected for the recognition of the start of the fertile period. Because the data generated from the Ovarian Monitor have shown that a rise in E1G levels above baseline and a threshold level of PdG make excellent markers for defining the fertile period (Blackwell and Brown, 1992; Blackwell *et al.*, 1998) and is in contrast to other research findings (Adlercreutz *et al.*, 1982; Cekan *et al.*, 1986), this strongly suggests that in an area as important as contraception, the extra accuracy provided by using timed urine samples, makes the inconvenience of their method of collection fully justified.

1.3.3.7 Hormonal Definition of the Beginning of the Fertile Period using Measurements of Urinary Estrogen Metabolites

The rate of excretion of urinary estrone glucuronide and granulosa cell number on the ovulatory follicle are linearly related from the time circulating FSH levels first stimulate estrogen synthesis in the follicle up until synthesis is inhibited by the high circulating estrogen feedback mechanism inhibiting further aromatase activation by FSH. Because the number of granulosa cells increases logarithmically relative to the size (diameter) (McNatty, 1981) of the follicle over this period, then the rate of estradiol synthesis (and estrone glucuronide excretion) also increases logarithmically with follicle growth. In other words, FSH stimulation causes a rapid increase in estradiol production by the leading follicles and provides a strong indication that the beginning of the active growth phase of a potentially ovulatory follicle has begun. Thus there is a sound physiological basis for using an increase in urinary E1G excretion above follicular phase baseline levels as a marker for the beginning of the fertile phase (Blackwell and Brown, 1992).

When time series analysis by Trigg's tracking signal, (Trigg, 1964) was applied to urinary total estrogen values from a database of 142 ovulatory cycles, a statistically significant increase in urinary total estrogens above baseline was detected for every cycle (Blackwell and Brown, 1992). The mean warning of ovulation given by these data using this first increase was 6.5 days (range 10.5-3.5 days) with 87% of cycles giving at least 6 days warning. Based on the fact that almost all pregnancies have resulted from sperm survivals of 3-4 days or less, ovulation would be predicted sufficiently in advance for contraceptive purposes for the vast majority of couples. Even in the 13% of cycles with a shorter warning, the increase in urinary estrogen levels from baseline was probably still far enough in advance of ovulation for pregnancy avoidance as sperm viability is highly dependent on the presence of sperm receptive mucus, which is in turn highly dependent on ovarian estradiol production. Thus the survival of sperm in the female reproductive tract before the estrogen rise and the sperm fertilising capacity would be severely compromised i.e. it is

unlikely that the conceptions resulting from long sperm survival times (> 6 days) would be associated with cycles with short estrogen rises.

1.3.3.8 *Hormonal Definition of the End of the Fertile Period using Measurements of Urinary Progesterone Metabolites*

Controversy reigns over whether a pregnanediol glucuronide (PdG) threshold marker or an increase in PdG levels from baseline would provide the most suitable marker for the end of the fertile period (Adlercreutz *et al.*, 1982; Cekan *et al.*, 1986). However, as discussed in Section 1.3.3.6 the suitability of an end of fertility marker largely depends on the quality of the data. For example a sensitive assay on time diluted urine samples will detect the small pre-ovulatory rise in PdG levels making an increase in PdG levels from baseline an unsuitable marker (Blackwell *et al.*, 1998; Cooke, 1993; Hoff *et al.*, 1983). Thus although the vast majority of the progesterone output during a menstrual cycle is restricted to the luteal phase, a sensitive PdG assay has more potential to be used as a marker for ovulation than for the end of fertility if first rises are being measured (see Figure 1.3). Because the pre-ovulatory progesterone surge is far smaller than the following luteal phase peak, the use of a threshold level of PdG makes a far more suitable end of fertility marker. However, to eliminate the high number of false positives (i.e. the number of premature predictions of the end of fertility) and false negatives (i.e. the number of excessively conservative estimates of the end of fertility) observed by Adlercreutz *et al.*, (1982) requires careful correction for the urinary excretion rate i.e. for a urinary PdG assay to provide maximum protection from pregnancy and minimum abstinence, the collection of time diluted urine samples is essential.

The suitability of a threshold level equivalent to an excretion rate of $6.3 \mu\text{mol PdG } 24 \text{ hr}^{-1}$ as is used by the Ovarian Monitor has been extensively checked against a 24 hour urinary Pd database of 180 cycles as well as the time diluted urinary PdG data of 107 cycles from a WHO trial on the Ovarian Monitor (Blackwell *et al.*, 1998; Cooke, 1993). With regards to the Pd data, this threshold level was reached at a mean of 3.01 days from the total estrogen peak day, with 96% of the cycles reaching it two or more days after this peak day. For the remaining 4% of cycles it was reached on the day of the estrogen peak. Similarly, for the PdG data which used the urinary LH peak as the mid-cycle marker, the threshold was reached on average 3.3 days after the LH peak day, with the threshold being exceeded for 91% of the cycles two or more days after the LH peak day. For the remaining cycles none reached it before the day of the LH peak day and only one exceeded the threshold value on the day of the peak. For both the Pd and PdG sets of data the threshold was equalled or exceeded in every cycle, and no cycle showed evidence of a subsequent ovulation until after the next bleed as judged by the hormone patterns and the length of the luteal phase. These results, combined with the fact that no pregnancies resulted from intercourse even in the cycles where the end of the fertile phase was indicated very close to the mid-cycle marker, suggest that the use of a threshold value equivalent to $6.3 \mu\text{mol PdG } 24 \text{ hr}^{-1}$ is valid and that the first rise in Pd or PdG levels may be more accurate markers of ovulation than either the estrogen or LH peaks (Blackwell *et al.*, 1998). This suggestion is supported by the results of another study which, found 33% of the LH peaks to occur after follicle rupture as proven by ultrasound (Adekunle *et al.*, 1988), although admittedly the sample size was small ($n=17$). Alternatively it may be that the PdG

threshold level is associated with infertility not because of its temporal distance from ovulation but because this concentration represents a circulating level of progesterone sufficient to cause an absolute bar to fertility. For example high circulating levels of progesterone are known to cause the production of infertile type mucus (Odeblad, 1994a; Odeblad *et al.*, 1983), while high levels of urinary Pd during the follicular phase of ovulatory cycles are known to be associated with infertility and high spontaneous abortion rates which can be reversed by using cortisone treatment to reduce the Pd levels back to normal levels (Brown, personal communication). Regardless of the reason for progesterone associated infertility, the fact that throughout an estimated total of 4,000 women years of experience, no pregnancies have resulted from the use of this threshold marker provides the strongest proof as to its validity for determining the end of the fertile period (Blackwell *et al.*, 1998).

1.3.4 The Problems of Natural Family Planning

In the broadest sense, family planning has two primary objectives. Firstly it is concerned with reducing the number of unwanted pregnancies and secondly with reducing the cases of infertility. With respect to natural family planning the areas which most need to be addressed to meet these demands are the problems of abstinence and the development of methods which are suitable for women whose fertility does not fit the classical menstrual cycle pattern.

1.3.4.1 *The Problem of Abstinence*

One of the biggest criticisms of the use of NFP for the avoidance of pregnancy is its requirement for abstinence. Part of this criticism stems from the fact that unplanned pregnancies resulting from method failure are more liable to be conceived from aging sperm or ova. However, the suggestions that these pregnancies are more liable to be associated with spontaneous abortions, pregnancy and birth complications, and low birth weight appear to be unsubstantiated (Bitto *et al.*, 1997; Mena *et al.*, 1997; Barbato *et al.*, 1997).

The real disadvantage with an abstinence method as might be predicted is the willpower required to abstain from intercourse, with the vast majority of unplanned pregnancies in NFP resulting from user failure as opposed to method failure. In fact the method failure rate of most NFP methods can be as low as other more accepted methods of contraception. However, the fact that NFP studies have shown that the vast majority of user failures are due to the purposeful breaking of a rule, not due to an inability to understand the rules, indicating that the problem is one of abstinence as opposed to the method itself (Trussell and Grummer-Strawn, 1991).

Compared to the theoretical length of the fertile period based on sperm and ovum survival times many of the methods of NFP are associated with excessive periods of abstinence. For example in a typical cycle the Ovulation Method requires abstinence for approximately half the cycle (Trussell and Grummer-Strawn, 1991), when the length of the fertile period is known to rarely exceed seven days. Likewise any method which is fully or partially based on the calendar method is associated with particularly high abstinence requirements for

women with irregular cycles. In fact when the rules of the most widespread calendar method, were applied to a large menstrual cycle database, it was found that 23% of the database's subjects had a cycle length variability too extreme to be able to apply the method (Colombo, 1989).

Although the methods may be extreme in their abstinence requirements they are still very good at defining the period of maximum fertility. For example in the WHO trial on the Ovulation Method all three cycles for which intercourse was recorded as having taken place on the peak day were associated with pregnancy. This suggests more emphasis needs to be placed on teaching couples to believe in the method and the relative chances of pregnancy they are exposing themselves to at different times of the cycle. It is interesting to note that the overall pearl index for natural family planning methods has decreased over the years to approximately one fourth of what it was at the time of the WHO trial on the Ovulation Method in the early eighties (Kambic, 1991; Guida *et al.*, 1997), and this is probably the result of better teaching as by and large the rules have not changed.

In summary, the greatest source of dissatisfaction with NFP methods is their long periods of abstinence and their high user failure rates which is largely due to the failure or inability of couples to comply with the long periods of abstinence; thus the two greatest drawbacks of the method are intrinsically linked. In fact it has been claimed that when a comparable period of abstinence is required between NFP methods the user failure rates are likely to be in the same range (Bonnar, 1994). Thus the greatest challenge facing NFP today is to be able to more accurately define the fertile period which would greatly improve the acceptability of the method by decreasing the period of abstinence and the overall failure rate at the same time.

1.3.4.2 *The Ovarian Monitor and Abstinence*

The Ovarian Monitor greatly reduces the period of abstinence of the symptothermal and Billings' Ovulation Methods. Because the hormones are not masked by menstruation, seminal fluid or stress, when the Ovarian Monitor is used intercourse is not restricted outside the calculated fertile period. Thus all the early infertile days as opposed to only alternative days, and all the days of bleeding and high stress are available for intercourse. The Ovarian Monitor not only limits abstinence to only the fertile period, it also calculates a substantially shorter fertile period than the symptothermal method. For example examination of the data from the Palmerston North centre of the WHO trial on the Ovarian Monitor showed that the Ovarian Monitor calculated a mean length of the fertile period of 7.6 days as opposed to the 9.4 days calculated by the symptothermal method (Cooke, 1993). Thus overall in comparison to the symptothermal method which requires a mean of 17 days of abstinence in a 29 day cycle when the extra rules are applied (see Section 1.3.1.3), the Ovarian Monitor requires closer to eight days. Because the Ovarian Monitor utilises timed urine samples, no excessive margins of security are required and all the estimated days of infertility can be used for intercourse with confidence.

1.3.4.3 *The Continuum*

The range of ovarian activities which may be experienced by a woman, from amenorrhoea to a fully fertile ovulatory cycle, is called the continuum (Brown, 1994). The progression up through the continuum is clearly observed from the year preceding menarche until approximately three years post-menarche, and also during the return to fertility postpartum, while regression back through the continuum is experienced as women approach the menopause. Other women included in the lower end of the continuum (i.e. with subfertile ovarian activity) include women with dysfunctional bleeding and women returning to fertility after oral contraceptive use. Deficient ovarian activity is also common in professional athletes, and in other women involved in circumstances which are associated with extreme physical or mental stress or weight loss. Even within the 20-40 year age group, the incidence of fully ovulatory cycles in unstressed women is only 90%.

The changes in a woman's position in the continuum from cycle to cycle are unpredictable; some stages in the continuum may be skipped, and a fully ovulatory cycle can occur at any time. Confusion in symptoms caused by the temporary passing through the infertile region of the continuum is one of the main causes of unplanned pregnancies in NFP.

The problem with most natural family planning methods is that they are primarily suited only to women from the top end of the continuum i.e. women with regular ovulatory cycles who exhibit the classical patterns of fertility. When these methods do take into account the 'other' women with irregular cycles, the guidelines are usually associated with long and excessive periods of abstinence over periods of life or conditions which are often in fact associated with low fertility.

1.3.4.4 *The Ovarian Monitor and Navigating through the Continuum*

Many of the women who use the Ovarian Monitor for pregnancy avoidance do so because of irregular cycling and the associated problems the more traditional methods of natural family planning produce for them. The proportion of this subgroup who have already experienced an unplanned pregnancy is particularly high (50%) (Brown *et al.*, 1991). For these women, the attraction of the Monitor is the security the method provides; the hormonal assays allow them to define precisely their periods of fertility and changing position within the continuum. The guidelines for the use of the Ovarian Monitor throughout the continuum are very straight forward. If the E1G levels rise a dominant follicle is present and fertility must be assumed. When the E1G levels fall, the cycle should be tested for ovulation with the PdG assay. Once the PdG threshold is reached infertility may be assumed with safety until the next menstrual bleed. The different positions within the continuum as determined by the Ovarian Monitor hormone assays are outlined below.

In the complete absence of ovarian activity, the E1G and PdG levels remain uniformly low and menstruation does not occur. Obviously this represents the lowest level of the continuum and is associated with absolute infertility. This situation may be permanent or temporary.

If E1G levels are observed to rise and fall between bleeding episodes but the PdG levels remain uniformly low, the cycle is anovulatory. Anovulatory cycles fall into two main categories. In the most common type, the E1G levels rise indicating that a follicle has developed, but the estrogen rise is insufficient to trigger the LH surge and the follicle dies by atresia, causing E1G levels to fall, and bleeding due to estrogen withdrawal. In other anovulatory cycles the E1G levels rise to reach a plateau and the values remain at this plateau for a variable period of time. The plateau levels of E1G are usually lower than for the usual pre-ovulatory E1G peak and bleeding eventually occurs as a breakthrough phenomenon i.e. the elevated levels of circulating estradiol over prolonged periods of time leads to extensive proliferation of the endometrial layer to such a level that it cannot be maintained.

The unruptured, luteinised follicle represents the next stage in the continuum. In these cycles the E1G levels rise and fall, but represent an estradiol level which is too low to induce a fully ovulatory LH peak. However it is sufficient to cause some LH mediated luteinisation of the follicle. Partial luteinisation results in a marginal elevation of PdG levels after the E1G fall, however their suboptimal levels provide proof that the follicle was never ovulatory. A cycle is defined as having a luteinised unruptured follicle if the PdG levels rise to between 4.5 to 6.3 $\mu\text{mol } 24 \text{ hour}^{-1}$ for two or more days.

The fact that all of the anovulatory conditions described above, may be followed immediately by a fertile ovulatory response with or without an intervening bleed, demonstrates some of the difficulties women face when moving within the continuum.

Cycles with short or deficient luteal phases represent the next step up in the continuum. A deficient luteal phase is one in which the PdG values rise to exceed 5 $\mu\text{mol } 24 \text{ hr}^{-1}$, but do not reach the ovulatory threshold of 9 $\mu\text{mol } 24 \text{ hr}^{-1}$, and a short luteal phase is one in which the PdG values exceed 9 $\mu\text{mol } 24 \text{ hr}^{-1}$ but the post-ovulatory phase lasts for ten days or less. Although both cycles are associated with a normal follicular phase, the cycles are infertile as their luteal phases are incapable of supporting pregnancy.

The fertile ovulatory cycle represents the highest level in the continuum. The fertile cycle is characterised by a well defined E1G peak followed by ovulation and a luteal phase which surpasses a PdG excretion rate of 9 $\mu\text{mol } 24 \text{ hr}^{-1}$ and lasts a minimum of 11 days. At these minimum levels conception rates are 25% per cycle. Higher E1G and PdG values (PdG $\geq 36 \mu\text{mol } 24 \text{ hr}^{-1}$) are more common and are associated with conception rates of 70% per cycle (Brown, 1994). The administration of estrogen analogues and gonadotrophins further elevates fertility and increases the possibility of multiple pregnancy by inducing superovulation. Such treatments have provided a 47% conception rate in patients with long-standing infertility (Brown, 1994).

The value of the Ovarian Monitor and the inadequacy of other natural family planning methods is well demonstrated with the passage through the continuum during the postpartum return to fertility. For example, the LAM guidelines only provide a maximum of six months protection, but since an Australian breastfeeding study showed that ovulation may be resumed in 12 weeks, the protection period this method provides can be quite short. Conversely for other women in the same study ovulation was delayed for up to two years

after delivery (Gross, 1991), in which case both LAM and the symptothermal method were grossly inadequate for these women's family planning needs. In comparison, the rules for using the Ovarian Monitor for detecting the return to fertility over this period of change are very straight forward. While the E1G levels are not rising, the E1G assay is measured twice a week for the first 6 months post partum, every third day for the next three months and then if the E1G levels are still not rising every second day until weaning. Interpretation of the data is essentially the same as for standard cycle monitoring - any rise in E1G levels above the woman's baseline which are not associated with elevated PdG levels must be considered as being potentially fertile.

1.4 THE VALUE OF THE OVARIAN MONITOR IN THE TREATMENT OF THE SUBFERTILE COUPLE

Problems of infertility are increasing due to both male and female factors. With regards to male infertility the average sperm count has dropped by 40% in the last 50 years and the fraction of abnormal sperm has increased (Carlsen *et al.*, 1992). These changes have largely been attributed to environmental factors; with exposure to pesticides and pollutants being believed to be the main causes (de Kretser, 1996; Feichtinger, 1991). In contrast, a lot of female infertility problems are due to older women trying to start a family when their own fertility is in decline. In such cases any other fertility problems of the couple are magnified by the woman's age. The other most common cause of female infertility is blocked fallopian tubes as the result of pelvic inflammatory disease; a silent symptom of untreated sexually transmitted diseases (STD's) (Morell, 1995).

Many subfertile couples have very little knowledge about their own fertility greatly limiting their chances of conception. The Ovarian Monitor can provide the first line of attack against infertility as it provides couples with knowledge about their fertility status and defines the period of maximum fertility so precisely. The Ovarian Monitor is more effective than the urinary LH home tests for optimising the timing of intercourse, as the E1G rise gives an advanced warning that the period of maximum fertility, i.e. the E1G peak day and the following day is approaching, whereas the urinary LH peak is often too late for all of the period of peak fertility to be detected (see Section 1.3.3.1).

Because the Ovarian Monitor measures metabolites from the two major ovarian steroids, it can be very useful in defining a woman's position within the continuum and any other hormone related fertility problems. Thus even if timed intercourse is not sufficient in itself to overcome a couple's fertility problems, the Ovarian Monitor can be used to provide information on what the next step in the treatment of infertility should be. For example, if the hormonal profile is normal then male infertility or pelvic inflammatory disease may be indicated. However, if the hormone levels are abnormally low then this could indicate gonadotrophin treatment as the next appropriate action.

Within the confines of an infertility clinic, the Ovarian Monitor has been used to advantage for hormonal monitoring in gonadotrophin releasing hormone therapy and gonadotrophin ovulation induction and in timing of ovum pick ups and implantations in *in vitro* fertilisation programmes (Thornton *et al.*, 1990; Brown *et al.*, 1988).

Finally, the Ovarian Monitor can be used to diagnose pregnancy, in which case both the PdG and E1G levels in the luteal phase continue to rise. This becomes obvious approximately 4-8 days after ovulation i.e. on an average of 6-10 days before the next period is due.

Another attraction of the Ovarian Monitor with regards to infertility treatments is that it is a much cheaper option than other laboratory assays, and in fact most of the time and labour requirements can be provided by the women themselves in their own home. This is of significant value as fertility treatments are expensive. Because the government does not consider fertility essential for health, state funding for fertility treatments often have a low

priority with the funding shortfall often being met by the couple. Home testing also greatly minimises the stress of fertility treatments by eliminating the need for daily monitoring at the clinic and allowing a more personal level of control and involvement. Considering the effect that stress has on fertility and that most couples find the period of intensive fertility treatment to be particularly stressful, self monitoring by the woman should be an attractive choice for both the couple and the clinician (Thornton *et al.*, 1990).

1.5 THE APPLICATION OF THE OVARIAN MONITOR IN REPRODUCTIVE HEALTH

The Ovarian Monitor should not be viewed so much as a method, but as an information provider. What a couple chooses to do with the information is up to them, but it does enable them to make informed decisions about fertility.

Thus, the Ovarian Monitor can be used as a natural aid by subfertile couples to maximise chances of conception, and as a natural contraceptive aid for those who are trying to avoid pregnancy. It can also be used to confirm pregnancy. Thus a woman can use it to answer all her most important reproductive questions, how do I avoid pregnancy, how do I achieve pregnancy, and am I pregnant? The information provided by the Ovarian Monitor can also be used by fertility services to aid in the diagnosis of the primary cause of subfertility, to assess the effectiveness of any hormonal treatments and to optimise the timing of any treatments.

Another potential use of the Ovarian Monitor is to optimise the timing of surgery for breast cancer in premenopausal women as studies have shown that surgery which is restricted to the luteal phase of the cycle is associated with an 84% chance of 10 years post-operative survival versus only 54% for women treated in the follicular phase (Badwe *et al.*, 1991).

The Ovarian Monitor also can provide a valuable adjunct for couples beginning natural family planning, as it demonstrates the cyclical nature of fertility very clearly, and the close correlation between the mucus symptoms and the hormones can be used to give the woman confidence in her own subjective ability to correctly interpret her mucus symptoms. Even people who use barrier methods would benefit from understanding the female cycle of fertility. Most barrier users would be interested to know that for most of the cycle the use of these methods is completely unnecessary, and that when a barrier method fails i.e. results in pregnancy, it is the result of intercourse from over only a small part of the cycle.

In conclusion the Ovarian Monitor is a very valuable device with numerous applications in reproductive health and which gives information about a woman's reproductive status which is unequalled by any other home method and is as accurate as that obtained in most clinical laboratories.

1.6 THE OVARIAN MONITOR HOMOGENEOUS IMMUNOASSAY

The homogeneous assay developed by Rubenstein in 1972 (Rubenstein *et al.*, 1972) was revolutionary as an immunoassay technique as, unlike the other immunoassays of the time, it required no purification, extraction, or separation of the free antigen from the antibody bound fraction, and thus gave the assay inherent simplicity and the potential to be utilised in home assays.

Rubenstein's work showed that the enzyme activity of a carboxymethylmorphine-lysozyme conjugate could be inhibited up to a maximum of 98% by the addition of anti-morphine antibodies, and that this inhibition was inversely proportional to the amount of free morphine in the system. In addition, because one molecule of free antigen is capable of freeing up one molecule of enzyme conjugate, which is in turn capable of catalysing the conversion of many molecules of substrate to product, this system shows intrinsic amplification.

In the method of Rubenstein, a lysozyme-hapten conjugate was made by combining hen egg white lysozyme (HEWL) with a mixed anhydride reagent derived from carboxymethylmorphine and isobutyl-chloroformate (Leute *et al.*, 1972), and the lysozyme activity of the immunoassay determined by changes in light transmission of a suspension of the bacterial substrate *Micrococcus lysodeikticus* (*M. lysodeikticus*).

This method forms the basis of the Ovarian Monitor assay where it has been adapted to measure urinary E1G and PdG (Brown *et al.*, 1989). The two immunoassay methods are essentially the same except that the morphine antibodies, conjugates and test samples have been replaced by steroid glucuronide equivalents.

The steroid glucuronide enzyme conjugates are prepared for use in the Ovarian Monitor by chemically synthesising the steroid glucuronides, conjugating them to HEWL by the mixed anhydride method (Rubenstein *et al.*, 1972; Leute *et al.*, 1972) and then purifying them by column chromatography.

The synthetic steroid glucuronides are also used to inoculate sheep for the generation of the assay antibodies. However, because the steroid glucuronide molecules are too small to be recognised as antigenic by the sheep's immune system they are first coupled to a protein carrier (also using the mixed anhydride method) before injection. A minimum of six months is required for the generation of high quality antibody. After bleeding, the blood is left to clot, with the resulting antiserum being of sufficient quality to be used directly in the assay without further purification (Brown *et al.*, 1988; Brown *et al.*, 1989).

1.6.1 The Ovarian Monitor System

The assay system described here has been developed and refined for over twenty years by Professor Jim Brown and co-workers in the Department of Obstetrics and Gynaecology at the University of Melbourne (Brown *et al.*, 1988; Brown *et al.*, 1989).



FIGURE 1.7: The Ovarian Monitor (Mark VI)

The Ovarian Monitor (Mark VI) unit is controlled by an automatic timer and a microchip, and consists of a cell holder housed in an electronically heated block with a 40°C thermostat, a 650 nm light source (light emitting diode) and a detector (see Figure 1.7).

Because separate assay tubes are required for the E1G and PdG assays and the timing requirements for the two assays are different, the Ovarian Monitor has been designed to differentiate electronically between the two tubes which are essentially the same except for their antibody specificity and the nature of their steroid glucuronide conjugates. In each tube, the *M. lysodeikticus* spot is freeze dried near the top of the tube, the conjugate spot approximately midway, and the buffer salts and the antibody spot together with a small glass mixing bead at the bottom. Upon the assay being initiated the instructions are

highlighted step-by-step, on the liquid crystal display according to the identity of the assay tube. A picture of the freeze dried (pre-coated) assay tube is shown in Figure 1.8.

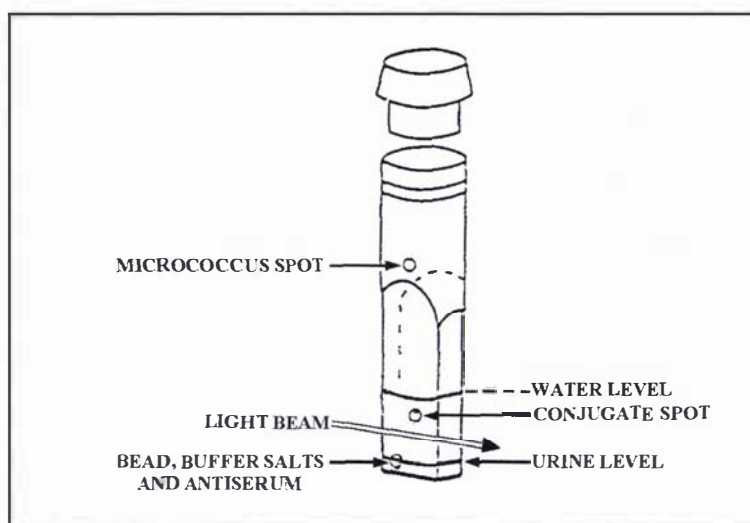


FIGURE 1.8: An Ovarian Monitor Assay Tube

The timed urine sample requires the collection of a single void (3-10 hours) into a jug calibrated in hours (150 mL hr⁻¹). The sample is then diluted by eye with tap water up to the nearest quarter hour according to the collection time.

1.6.2 The Estrone Glucuronide and Pregnanediol Glucuronide Assay Protocols

In practical terms, because the maximum rates of PdG excretion in a cycle are approximately 60 fold higher than the maximum E1G excretion rates, the incubation and reaction times for the PdG assay are much shorter than their E1G equivalents (Brown *et al.*, 1989). Unfortunately the high PdG levels in the urine means that the time diluted urine sample for PdG assays must be diluted an additional one in five times before assaying. This is done simply by mixing 200 μL of distilled water with 50 μL of the already time diluted urine sample and using 50 μL of this second dilution in the assay instead of 50 μL of the straight time diluted sample used in the E1G assay. Alternatively, 10 μL of the original time diluted urine sample could be diluted directly in the 50 μL syringe.

Step 1 *The Antigen-Antibody Reaction*

In the first step, 50 μL of the time diluted urine sample is carefully added by syringe to the bottom of an assay tube, placed inside the cell holder, and the tube is left to incubate for 5 minutes to allow time for the buffer salts and the antibody (Ab) dried at the bottom of the tube to dissolve. The end of this incubation period is indicated by a series of beeps at which point the tube is gently shaken horizontally for the count of twenty enabling the urinary steroid glucuronide, i.e. antigen (Ag), to mix freely with its antibody (see Equation 1.1). Binding of the steroid to the antibody is rapid and effectively irreversible within the assay time frame (Brown *et al.*, 1988).

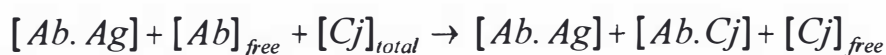


In summary, the level of antibody neutralisation is directly proportional to the E1G (or PdG) levels in the urine.

Step 2 *The Antibody E1G-HEWL Conjugate Reaction*

In the second step, the conjugate spot (Cj) is dissolved by the careful addition of 300 μL of distilled water by syringe, after which the tube is gently shaken horizontally for another count of twenty, returned to its cell holder and incubated for a further period (5 min, PdG; 10 min, E1G). This incubation is necessary to allow time for the reaction mixture to come to temperature in preparation for the activity assay in Step Three, and to allow any antibody remaining free after the first step to reach a new binding equilibrium with the conjugate in the presence of non-specific urinary components (see Equation 1.2). As before, this binding is irreversible within the assay time frame.

EQN. 1.2



In this step the level of antibody conjugate binding is directly proportional to the amount of antibody remaining free at the end of the first step. Thus, the level of free conjugate is directly proportional to the amount of the steroid glucuronide in the original urine sample.

Step 3 *The Micrococcus lysodeikticus Reaction*

The time to initiate the third step is signalled by a series of beeps. The assay tube is vigorously shaken vertically for a count of twenty to suspend uniformly the *M. lysodeikticus* cells, and then returned to the cell holder. Upon the addition of the cell to the cuvette holder, the Monitor recognises this as the beginning of the actual enzyme activity assay, and records the initial transmission value. In this final step the free conjugate remaining after Step Two, is finally given access to its substrate, *M. lysodeikticus*. As the conjugate proceeds to lyse the *M. lysodeikticus* cells by breaking the peptidoglycan bonds which form its walls, the initially turbid solution begins to clear as the cell wall collapses. The rate of this reaction is determined by measuring the amount of clearing of the solution over a set time period (PdG, 5 min; ELG, 20 min) i.e. the Monitor uses an end point assay as opposed to an initial rate assay. At the end of the assay period, the timer sounds and the difference in transmission between the two time points (ΔT) is left flashing permanently on the display.

Because only the free enzyme conjugate retains all of its enzyme activity, the rate of clearing is directly proportional to the amount of free conjugate remaining at the end of Step Two. In summary, when the concentration of urinary steroid glucuronides is low, most of the antibody remains free to bind and thus inactivate the enzyme conjugate resulting in low rates of clearing of the turbid bacterial suspension. However, as the concentration of urinary steroids increases, and thus becomes antibody bound, the proportion of antibody available to inactivate the enzyme conjugate decreases, resulting in an increased rate of clearing. Thus low levels of urinary steroid glucuronide are associated with only small changes in light transmission (ΔT), while high levels generate high ΔT values.

1.6.3 The Estrone Glucuronide and Pregnanediol Glucuronide Standard Curves

The standard curve for each batch of assay tubes fits into one of only a few limited patterns. As these standard curve profiles are programmed into the Monitor, and each batch of assay tubes is encoded with the standard curve family to which they belong, the Ovarian Monitor (Mark VI) can express the final reading as either 24 hour molar excretion rates or ΔT .

Conversion to 24 hour molar excretion rates is not necessary for home use and has only recently become available as an option with the development of the microchip Monitor. Monitoring of change in transmission levels is all that is necessary for general use because it is the pattern of hormone excretion which is important for the determination of fertility status. Even with regards to the PdG threshold, the molar excretion rate is not required, because each batch of PdG tubes is labelled with the ΔT 5 min⁻¹ equivalent to the threshold (6.3 μmol 24 hr⁻¹). Thus a woman just needs to check whether she has exceeded this equivalent ΔT value to know she has entered the late infertile days.

A typical example of an E1G standard curve relating the 24 hour molar excretion rates to ΔT per 20 minutes is illustrated in Figure 1.9 and shows the direct relationship between the rate of lysis and the urinary steroid levels.

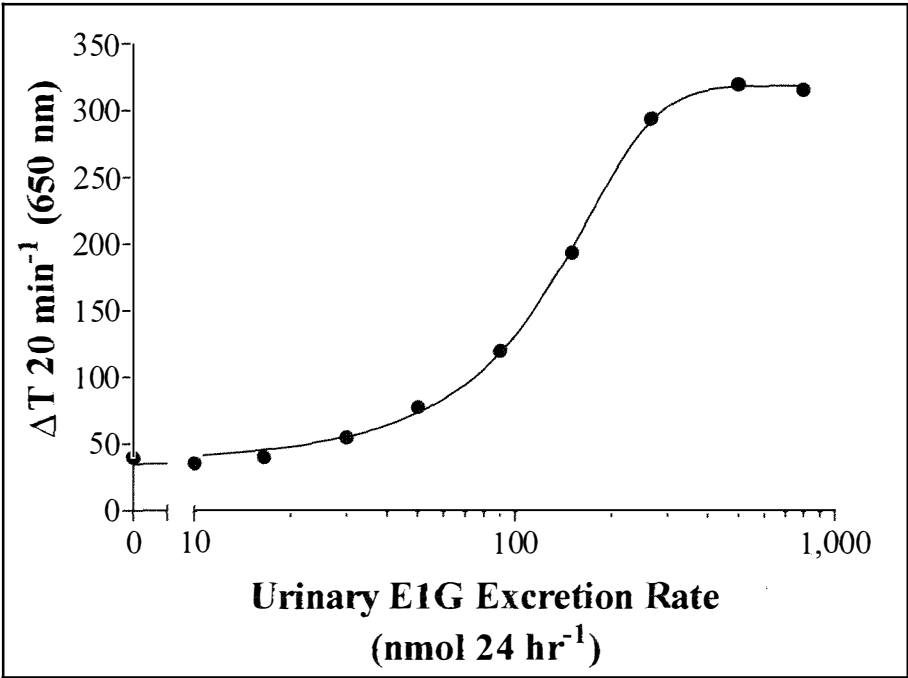


FIGURE 1.9: Ovarian Monitor Estrone Glucuronide Standard Curve

1.7 AIMS OF THE THESIS

The Ovarian Monitor System has already been fully validated by international natural family planning trials carried out by the World Health Organisation and Family Health International, by its long term independent use by Australian couples, and by its use as an aid in various fertility programmes at the Royal Women's Hospital in Melbourne. To date, over 400,000 EIG and 300,000 PdG assay tubes have been manufactured. The preparation of the biological components (steroid glucuronides, antibodies and enzyme conjugates), the freeze drying of the antibody, enzyme conjugates and substrate onto the assay tube wall, and the construction of the meters themselves are all currently performed in the laboratories of retired Professor Emeritus James Brown in Melbourne. Thus, the availability of the Monitor system is critically dependent on the continuing operation of the Melbourne group.

The various studies on the Ovarian Monitor as a natural family planning aid have shown great satisfaction with the quality of the data and the method. In fact the greatest source of dissatisfaction with the method was the time required to perform the EIG test.

Hence, the main aims of this thesis are to:

- establish the Ovarian Monitor system for the measurement of estrone glucuronide outside of Melbourne
- study the biological components of the Ovarian Monitor in order to gain a better understanding of the system
- attempt to improve the assay performance by increasing the sensitivity and decrease the reaction time for the EIG assay
- examine other assay systems which may be used to replace the Monitor with a rapid, accurate colour test for urinary EIG and PdG with the same precision as the current Ovarian Monitor

CHAPTER TWO

SYNTHESIS AND PURIFICATION OF STRUCTURALLY DEFINED HEN EGG WHITE LYSOZYME - ESTRONE GLUCURONIDE CONJUGATES

2.1 INTRODUCTION

In order to replicate the Ovarian Monitor immunoassay system for estrone glucuronide outside of Melbourne so that independent research can be performed, it is first necessary to prepare the assay biomaterials. These biomaterials must be then used to establish a standard curve with the required sensitivity and precision to measure menstrual cycle levels of estrone glucuronide.

This chapter is concerned with the development of procedures for the synthesis and purification of the hen egg white lysozyme - estrone glucuronide conjugates which can be used as signal generators (Paek *et al.*, 1993) in immunoassays for estrone glucuronide. A discussion on the synthesis and isolation of these conjugates requires a basic understanding of the hen egg white lysozyme structure.

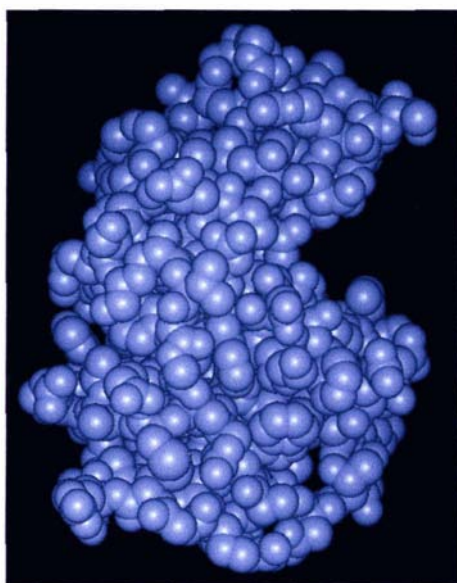


FIGURE 2.1: Model of Hen Egg White Lysozyme's Tertiary Structure showing the Enzyme's Deep Enzymatic Cleft

Hen Egg White Lysozyme (HEWL) is a relatively small ($M_r=14,300$), very basic protein with a roughly ellipsoidal shape ($45 \times 30 \times 30 \text{ \AA}$), and a well defined enzymatic cleft (see Figure 2.1) (Phillips, 1967). It is composed of a single polypeptide chain of 129 residues (Canfield, 1963), cross-linked by four disulphide bridges which are at least partly responsible for the unusually high stability of the enzyme. Lysozyme's high stability, combined with the large body of information already available on its structure and kinetics, and the fact that hen egg white is such a rich and ready source of the enzyme, has made hen egg white lysozyme a popular model protein.

Lysozyme's very basic nature ($pI=10.5$) (Verhamme *et al.*, 1988) is due to the high proportion of lysine and arginine residues in its structure. These positively charged amino acids are also essential for maintaining the kinetic integrity of the enzyme, with substrate binding being highly dependent upon the electrostatic attraction between the positively charged enzyme and the negatively charged surface of the *Micrococcus lysodeikticus* cell wall (Price and Pethig, 1986).

Although the positive charge contributed to hen egg white lysozyme by its six lysine residues is essential for activity, conjugation of lysozyme is also most simply achieved through the amino groups of these residues and the N-terminal amino group. In the standard conjugation protocol, lysine residues react with the acylating reagent to form stable amide linkages, but other amino acids can also react with the reagent. However as they form unstable ester or anhydride linkages which undergo hydrolysis during the subsequent purification procedures only the lysine acylations are of any significance in the synthesis of lysozyme conjugates.

Lysozyme contains lysine residues at positions 1, 13, 33, 96, 97 and 116 (Canfield, 1963).

The side chain amino group of all six lysine residues and the N-terminal amino group are all found near the surface of the molecule. Although it is theoretically possible to acylate all seven amino groups, because every acylation event results in the removal of a unit of positive charge, very high substitution levels result in the removal of all of the enzyme's specific activity (Habeeb and Atassi, 1971). Thus for maximum activity it is necessary to keep the average number of substitutions per lysozyme molecule low.

The Ovarian Monitor uses steroid glucuronide conjugates of hen egg white lysozyme as one of its main biomaterials, but few details of the methods of preparation and purification of the conjugates have been published (Brown *et al.*, 1988; Brown *et al.*, 1989).

All immunoassays need a binding component (usually an antibody) and a signal generator (often an enzyme conjugate) to indicate the concentration of occupied antibody binding sites and thus the concentration of analyte. Enzyme immunoassays can be divided broadly into homogeneous and heterogeneous assays. The chief advantage of a homogeneous assay is that it requires no separation step of the antibody bound conjugate fraction from the free enzyme fraction as it is the antibody binding itself which modulates the enzyme activity. Thus, whereas a heterogeneous assay requires several washing steps to ensure the complete separation of the unbound and bound signal generator fractions (which cannot be distinguished from each other), the homogeneous assay is much simpler as the hapten concentration can be measured by a simple measurement of the enzyme activity made directly on the complete immunoassay mixture (see Section 1.6). This direct measurement of the lytic activity makes the homogeneous assay an ideal candidate for conversion into a home assay format.

However, for simplicity in the preparation of an enzyme conjugate for immunoassay, the heterogeneous assay has the advantage. Conjugate prepared for a heterogeneous assay does not have to be separated from the unconjugated native enzyme as when the mixture is used in the assay any unconjugated material is removed during the washing steps anyway. In fact, most reported procedures for the preparation of conjugate for a heterogeneous assay (i.e. most immunoassays) simply process the resulting conjugation reaction mixture by dialysis, or a single gel filtration step, which only removes any unreacted haptens and coupling reagents. In contrast, because there is no washing step for the homogeneous assay, the purification from the reaction mixture of conjugate from the unconjugated material is much more involved and is an essential prerequisite for a successful assay.

The Ovarian Monitor assay can be considered as a classic example of a homogeneous assay and is based on the principle that the lytic activity of lysozyme-hapten conjugates are extensively inhibited (over 95%) by anti-hapten antibodies. In the presence of hapten this inhibition can be removed, with the level of reversal being determined by the free hapten concentration. The effect is absolute with very high hapten concentrations resulting in the complete removal of the inhibition. This graded reversal of the conjugate inhibition with increasing hapten concentration can be used to construct a standard curve from which the hapten concentrations can be determined in urine samples (see Section 1.6.3).

As this enzymatic method of measuring hapten levels requires no separation of the bound enzyme from the free fraction any unconjugated lysozyme left in the conjugate preparation will also show lytic activity. This background rate is constant for a given level of contamination, and unlike the lysozyme-conjugates, cannot be modulated by the presence of anti-hapten antibody. Because the ability of the assay to differentiate accurately between closely similar concentrations of haptens depends on a maximum difference between the minimum and maximum lysis rates, the presence of any uninhibitable lysozyme will reduce the assay discrimination by an amount determined by the level of the contamination (Daunert *et al.*, 1989). Thus, one of the most important considerations in the preparation of enzyme conjugate for use in a homogeneous immunoassay is the separation of the labelled enzyme from the native enzyme. Also, it is not clear to what extent the assay is compromised by using a mixture of conjugates. Thus, the purification method for the preparation of conjugates for immunoassay must ideally also be capable of separating the individual conjugate families as well.

To date there have been few reported purification procedures for the separation of lysozyme conjugates from free lysozyme.

In the original patent of Rubenstein *et al.* the unconjugated lysozyme fraction was separated from the conjugate fraction using repetitive dialysis (Rubenstein *et al.*, 1974). This method is based on the observation that in most conjugations a large amount of insoluble, mostly conjugated material is produced which can be resolubilised using solutions of high urea concentration.

In Rubenstein's method, this material was dissolved in 7 M urea and then the solution was dialysed exhaustively against water, to generate a new precipitate fraction and a supernatant. The resulting precipitate was then redissolved in 7 M urea and subjected to further dialysis. This redissolving and dialysing of successive precipitate fractions resulted in a series of supernatants containing decreasing amounts of lysozyme, and was continued until a supernatant was obtained which was highly inhibitable by anti-hapten antibodies. Although this procedure does eventually produce a conjugate fraction free of lysozyme, the yield is very low making the method unsuitable for the large scale preparation of conjugate required by the home Ovarian Monitor. In addition the final conjugate fraction is undefined and of variable composition. Thus the poor reproducibility of the method combined with the poor quality of the conjugates makes the method unsuitable for research purposes and alternative purification procedures are required.

One of the aims of the present work, therefore is to devise a separation scheme which is reproducible and delivers large amounts of pure, defined conjugates for use in immunoassays. For most enzymes this is a significant challenge since there is very little difference in physical characteristics between the conjugate and the free enzyme which makes separation by classical chromatographic procedures difficult. One potentially very powerful method for separating conjugates from unreacted enzyme and hapten is to use affinity chromatography. As only the conjugate contains the hapten, using an anti-hapten antibody immobilised to the resin, provides a very specific method of purifying the conjugate. For example, Paek *et al.* (1993) have successfully purified horseradish peroxidase-progesterone conjugates from the native enzyme using an immobilised anti-progesterone antibody as an affinity column. However not all purification schemes lend themselves to affinity chromatography. Firstly, for the simple elution of the conjugate a source of monoclonal antibody is required. Secondly, the binding energy between an antibody and its hapten is typically very high. Thus, although there are usually no problems associated with sample binding, the dissociation of a hapten conjugate (or hapten) from its resin-bound antibody cannot always be achieved within a practical time frame. Taking into account the fact that the rate of dissociation of hapten from its antibody is particularly slow in solid phase based systems (Nygren and Stenberg, 1989), the extensive screening necessary to find a suitable antibody, and the time required to determine a set of elution conditions necessary to overcome this naturally high avidity, the purification of conjugate by affinity chromatography was not regarded as a practical option in this work.

One feature of the lysozyme estrone- and pregnanediol-glucuronide conjugates which has the potential to be exploited in the design of conjugate purification schemes is the fact that every conjugation event removes one positive charge from the lysozyme molecule and replaces it with a highly hydrophobic residue. Analytical procedures based on these charge differences between the free lysozyme molecule and its conjugates are already part of the standard methodology utilised by the Ovarian Monitor Research Laboratory at Massey University (Cooke, 1993; Smales *et al.*, 1994).

In this chapter the development of a large scale simple two step purification protocol which utilises the differences in charge and hydrophobicity of the different conjugates and lysozyme is discussed. This procedure was used for the isolation of two of the main conjugate fractions from a conjugation reaction mixture in preparation for their employment as signal generators in immunoassays for estrone glucuronide which is described in Chapter Three.

2.2 MATERIALS AND METHODS

2.2.1 Apparatus

Nuclear magnetic resonance (NMR) spectral analyses were performed by the NMR Spectroscopy Service, Massey University, Palmerston North. All NMR spectra were performed on a JEOL GX-270 spectrometer using deuterated solvents and an operating frequency of 270 MHz. All spectra were referenced relative to the internal standard tetramethylsilane ($\delta = 0.0$ parts per million (ppm)). The data are reported as position (ppm), multiplicity (s = singlet, d = doublet, dd = double doublet, t = triplet, m = multiplet), relative integral, coupling constant (J Hz) and assignment.

Melting points were determined on a Bausch and Lomb hot plate melting point apparatus.

All protein chromatography was performed at room temperature on a Pharmacia Fast Protein Liquid Chromatography system (FPLC). This consisted of an LCC-500 liquid chromatography controller, two P-500 pumps, an MV-7 motor valve injector, a mixer, and a 280 nm single path UV-1 flow cell, the output of which was connected to a chart recorder.

All chromatographic separations were performed using Pharmacia resins. Small scale separations were performed using a prepacked Mono-S strong cation exchange column HR 5/5 (50 × 5 mm i.d.) and a prepacked Alkyl Superose hydrophobic interaction column HR 5/5 (50 × 5 mm i.d.). All large scale cation exchange separations were performed using either an S-Sepharose fast flow or CM-Sepharose fast flow resin packed into a Pharmacia HR 16/5 column (500 × 16 mm i.d.). Large scale hydrophobic interaction chromatography was performed using a Butyl Sepharose resin packed into a Pharmacia 16/15 column (150 × 16 mm i.d.).

All buffers and samples prepared for FPLC were prefiltered through 0.2 μm GVWP Millipore filters. Samples were injected into a loop on the motor valve using a syringe before being automatically loaded onto the column. All samples greater than 2 mL were loaded directly onto the column using a peristaltic pump. All large scale FPLC purifications had their A_{280} profiles determined manually on a Hewlett Packard diode array spectrophotometer by measuring the absorbance of the individual fractions at appropriate dilutions with the equilibrating buffer.

Hen egg white lysozyme-estrone glucuronide conjugates were concentrated using an Amicon Diaflow Apparatus fitted with a YM10 Amicon membrane (MW cut-off 10,000).

All electrospray mass spectral analyses were performed by Dr. C. M. Smales (University of Kent, Canterbury, United Kingdom). Samples were desalted and introduced into the electrospray source by reverse phase high pressure liquid chromatography (HPLC) using a Hewlett Packard 1100 series HPLC and a Jupiter C4 column (50 × 2 mm i.d.). Mass spectra were recorded in the positive ion mode on a Finnigan MAT LCQ ion trap mass spectrometer. The charge state ion distributions observed in the electrospray ionisation

spectra were deconvoluted using Finnigan Corps Bioexplore software (Version 1.0 for LCQ 1.2).

The Ovarian Monitor equipment including meter (Figure 1.7), empty assay tubes and multi-assay heating block were supplied by St Michael Natural Family Planning Services Pty, 2/380 Riversdale Road, East Hawthorn, Victoria, 3123, Australia. All syringes were manufactured by either the Hamilton Company (Reno, NV, USA) or Scientific Glass Engineering Pty Ltd (Melbourne, Australia). The 500 μ L substrate syringe was fitted with a stepper apparatus (also supplied by the Hamilton Company) which allowed the repetitive release of 10 μ L boluses of substrate from a single 500 μ L aliquot.

2.2.2 Reagents

Hen egg white lysozyme (grade VI, 3 times recrystallised, dialysed and lyophilised), the lyophilised *Micrococcus lysodeikticus*, 3-hydroxy-estra-1,3,5(10)-triene-17-one (estrone) and glucuronolactone were obtained from the Sigma Chemical Company (St. Louis, MO, USA). Acetic anhydride (analar), hydrogen bromide (45% w/v in glacial acetic acid) and cadmium carbonate were obtained from British Drug House Biochemicals Ltd (Poole, UK). The silica gel (60, F254) coated aluminum sheets for thin layer chromatography, and the dicyclohexylcarbodiimide, and deuterated (99%) dimethylsulphoxide (DMSO) were all obtained from Merck-Schuchardt (Darmstadt, Germany). The sodium (laboratory grade) was obtained from Hopkins and Williams (Essex, England), the perchloric acid (70% w/w; analar grade) from AJAX Chemicals (Auburn, NSW, Australia), and the deuterated (98.8%) chloroform from Aldrich Chemical Company (Milwaukee, USA). The XAD-2 resin was supplied by Serva (Heidelberg, Germany) and the *N*-hydroxysuccinimide from the United States Biochemical Corporation (Ohio, USA).

The dry distilled dimethylformamide was supplied by Dr. David Harding (Institute of Fundamental Sciences, Massey University, Palmerston North).

All polyclonal estrone glucuronide antisera were raised in sheep by Dr. Keith Henderson (AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand), using a thyroglobulin-estrone glucuronide conjugate prepared in our laboratory by the active ester method (Mattox *et al.*, 1969). The freeze-dried sample was dissolved in a 1:1.25 mixture of phosphate buffered saline and an oil adjuvant which was prepared using a 1:1:18 ratio of sorbiton triolate, tween 85 and macrol 52 mineral oil. The prepared antigen was administered subcutaneously using a total volume of 0.9 mL per sheep according to the following inoculation programme.

Day 1	Bleed 1 (10 mL, taken immediately prior to first immunisation)
	Primary immunisation (1.5 mg steroid glucuronide antigen)
3 Weeks	First booster immunisation (1 mg steroid glucuronide antigen)
5 Weeks	Bleed 2 (10 mL)
13 Months	Second booster immunisation (1 mg steroid glucuronide antigen)
13.5 Months	Third booster immunisation (1 mg steroid glucuronide antigen)
14 Months	Bleed 3 (50 mL)
20 Months	Fourth booster immunisation (1 mg steroid glucuronide antigen)
20.5 Months	Bleed 4 (500 mL)

All antisera based assays were performed using antisera from bleeds 3 and 4 only.

All other chemicals and reagents were of analytical grade or higher, and all water was of Milli-Q quality.

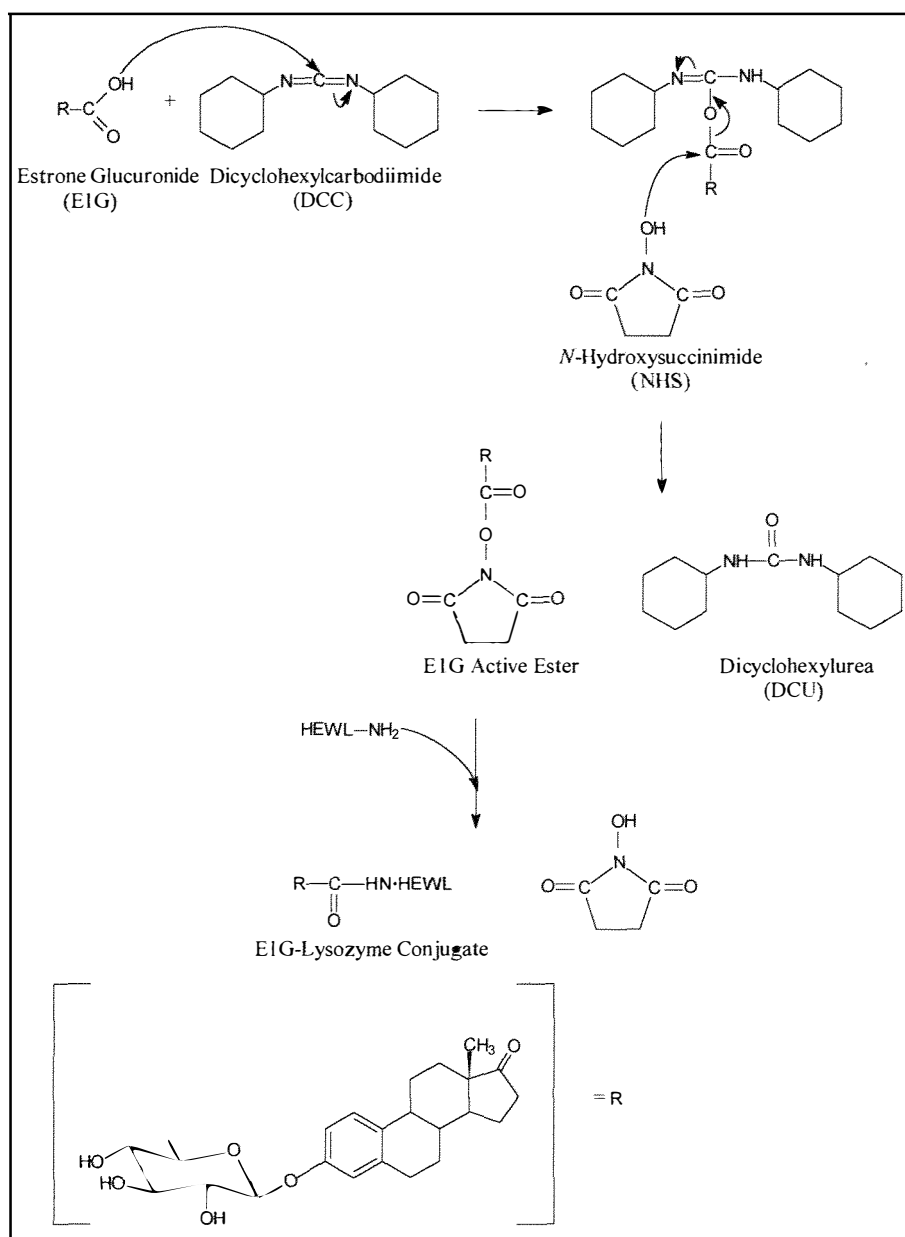
2.2.3 Purification of Commercial Hen Egg White Lysozyme

Before conjugation, commercially supplied hen egg white lysozyme was further purified by cation exchange chromatography on a large column packed with CM-Sepharose fast flow resin. Hen egg white lysozyme (2.99 g, 209 μmol) was dissolved in 40 mL of the equilibrating buffer (10 mM phosphate buffer, pH 6.0) and loaded, via a peristaltic pump, onto a pre-equilibrated column. Elution of the sample was effected using a sodium chloride gradient in 10 mM phosphate buffer (pH 6.0). A small impurity, thought to be deaminated lysozyme was removed from the sample as a small peak by running a 10 minute gradient from 0 to 0.15 M NaCl and holding at this salt concentration for 90 minutes. The main lysozyme peak was then eluted by using a gradient from 0.15-0.39 M NaCl over 500 minutes. Over the next 100 minutes, the salt concentration was increased to 0.6 M NaCl, using a linear gradient, and held until the absorbance had returned to baseline. The whole chromatographic procedure, including loading, was performed at a flow rate of 1 mL min⁻¹ with fractions collected every seven minutes. Due to the large size of the second peak (peak A₂₈₀ > 3.0 absorbance units), the absorbance profile had to be obtained manually. This showed it to consist of a single symmetrical peak. All fractions from the second peak that had an absorbance above 0.5 units at 280 nm were pooled and dialysed (3 changes $\times$ 20 L) against water, and then freeze dried before storage at -10°C.

2.2.4 Active Ester Conjugation of Hen Egg White Lysozyme with Estrone Glucuronide

Estrone glucuronide was conjugated to hen egg white lysozyme via its carboxylic acid group by activation with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS). An overview of the activation and conjugation reaction is shown in Scheme 2.1.

The formation of the active ester reagent was optimised by using a 50% molar excess of DCC and NHS relative to estrone glucuronide and a small total reaction volume (140 μL). Dry estrone glucuronide (4.6 mg, 10.4 μmoles) was weighed into a small dry glass vial, dissolved in dry dimethylformamide (DMF) (80 μL) and kept in a desiccator until required. Because of the difficulties in weighing out the small amounts of DCC (3.2 mg) and NHS (1.8 mg) required, stock solutions of DCC and NHS were prepared by weighing out 10-20 mg of the reagent and making up to the desired volume with dry DMF to give 520 mM solutions. The correct amount (15.6 μmoles) of DCC and NHS was then dispensed as 30 μL aliquots into the estrone glucuronide reaction vial. The resulting solution was mixed by inversion and left to equilibrate at room temperature for one hour in a desiccator.



SCHEME 2.1: Synthesis of Hen Egg White Lysozyme - Estrone Glucuronide Conjugates by the Active Ester Method

Estrone glucuronide was conjugated to hen egg white lysozyme routinely using a molar ratio of 1.5:1. Purified lysozyme (100 mg, 6.99 μ moles) was dissolved with stirring in a 1% sodium bicarbonate solution (4 mL) and then left to equilibrate to 4°C in preparation for the conjugation. The conjugation was performed with constant stirring by the dropwise addition of the active ester reaction mixture to the lysozyme solution over a period of 15 minutes and was associated with the formation of a fine white precipitate. The conjugation mixture was then left stirring for a further hour at 4°C, before being dialysed against water (3 changes $\times$ 2 L) to remove the sodium bicarbonate and any free estrone glucuronide or unreacted active ester reagent.

2.2.5 Standard Analytical Mono-S Cation Exchange Chromatography

Mono-S cation exchange chromatography was routinely used to analyse the initial HEWL-E1G conjugation reaction mixtures as well as assess the efficiency of any conjugate purification procedures.

All analyses on the conjugation reaction mixtures were performed on the dialysed sample. The thick milky precipitate which was associated with all conjugations was removed by centrifuging the dialysed reaction mixture for 15 minutes at speed 5 on a Gallenkamp Junior bench centrifuge. Although most analyses were performed on the supernatant some were performed on the total dialysed mixture. Both the total conjugation mixture and the supernatant were prepared for loading by preparing a 6:1 dilution of an aliquot of the sample with the equilibrating buffer (50 mM phosphate/7 M urea, pH 6.0). This method of sample preparation was also used for the post-purification samples, but due to their often comparatively dilute nature, they were generally prepared for analysis by dissolving urea directly into the dialysed sample (0.123 g of urea per 300 μ L).

After loading of the sample onto the column by injection, elution was effected using a linear sodium chloride gradient by using increasing quantities of the elution buffer (50 mM phosphate/7 M urea/1 M sodium chloride, pH 6.0). The standard chromatographic conditions were: flow rate 0.5 mL min⁻¹; programme - 0-5 minutes, 0 M NaCl; 5-45 minutes, 0-0.3 M NaCl; 45-50 minutes, 1 M NaCl). Sometimes a shorter, steeper gradient was used (5-35 minutes, 0-0.4 M NaCl) but all gradients are recorded in the figures of the FPLC profiles.

2.2.6 The Lysozyme Assay

To avoid cross contamination, separate syringes were used for the lysozyme, lysozyme conjugates, antiserum and substrate in the preparation of the reagents and in the assay.

2.2.6.1 Buffers

All tris-maleate buffers used in the assay were made by appropriate dilution of a 1 M stock buffer (pH 7.0) with water. The stock buffer was prepared by mixing maleic acid (7.25 g), tris (19.80 g), sodium chloride (12.75 g), tween 80 (20.0 mL) (prediluted 1/100 with water) and concentrated hydrochloric acid (2.8 mL) in water. The pH of the buffer was adjusted to 7.0 with concentrated hydrochloric acid and then made to a final volume of 375 mL.

2.2.6.2 Lysozyme and Lysozyme Conjugate

Enzyme was diluted fresh daily with 0.35 M tris-maleate buffer (pH 7.0) into a plastic vial. The high ionic strength of the buffer was necessary to prevent any non-specific binding of the conjugate to the walls of the dilution vessel, syringes and assay tubes, as well as to protect the enzyme's specific activity.

2.2.6.3 *Anti-Estrone Glucuronide Antiserum*

The polyclonal anti-estrone glucuronide antiserum (243/bleed 4) was diluted fresh daily with 75 mM tris-maleate buffer (pH 7.0) 1 part in 50 (20 μ L to 1,980 μ L buffer) into a small glass vial.

2.2.6.4 *Substrate*

The bacterial suspension of *Micrococcus lysodeikticus* (*M. lysodeikticus*) was prepared fresh daily. Lyophilised *M. lysodeikticus* (15 mg) was triturated with 2 mL of 75 mM tris-maleate buffer (pH 7.0) in a manual two piece glass homogeniser. When a visually uniform suspension was obtained, it was transferred to a small glass vial and sonicated using a Bandelin Sonorex RK100 sonicator for five minutes to ensure homogeneity. The sonicated suspension was kept on ice and resonicated every few hours.

2.2.6.5 *The Standard Activity Assay Protocol*

Aliquots (1-100 μ L) of the diluted enzyme were dispensed into empty plastic Ovarian Monitor assay tubes and a variable amount of 40 mM tris-maleate buffer added to give a combined total volume of 340 μ L per tube. The enzyme solution and buffer were preincubated for a minimum period of five minutes to a temperature of 40°C using the Ovarian Monitor for a single assay or a custom made heating block if there were multiple assays. The assay was initiated by the addition of a 10 μ L bolus of the bacterial suspension via a stepper syringe. The reaction mixture was quickly vortexed (2-3 seconds) and the assay tube placed in the Ovarian Monitor. The first transmission value was recorded upon shutting the lid, followed by the second transmission value five or twenty minutes later depending on the assay. The rate was recorded as change in transmission (ΔT) (650 nm). All assays were performed in duplicate or triplicate.

2.2.6.6 *Inhibition Studies*

For the inhibition studies, lysozyme conjugate was diluted such that a small aliquot (1-20 μ L) gave a change in transmission (ΔT) of approximately 350 over 20 minutes for the above standard activity assay.

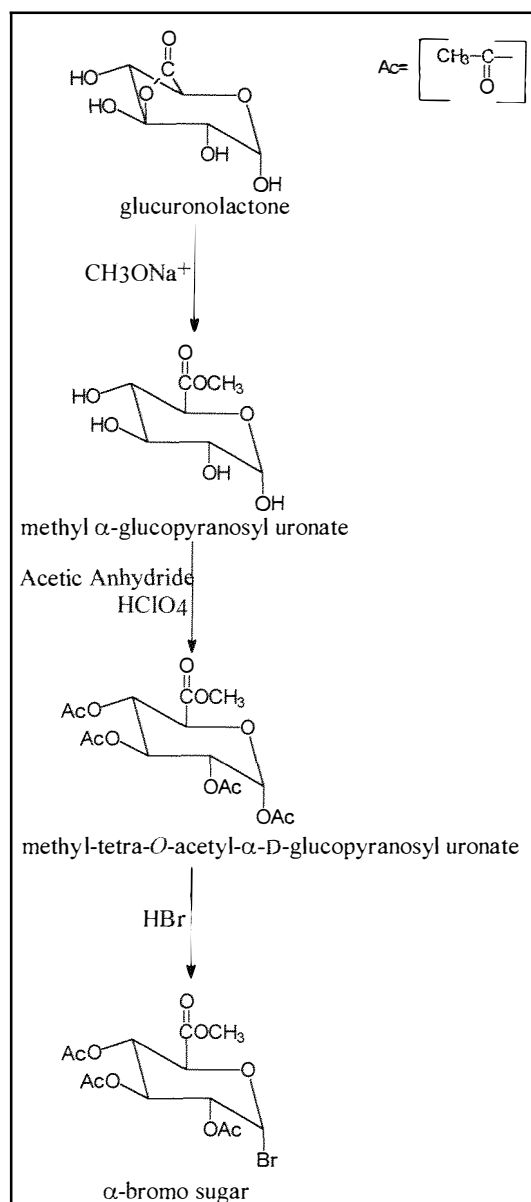
The diluted antiserum (1-50 μ L) and conjugate were added to opposite corners to the bottom of an empty Ovarian Monitor assay tube, the total volume in the assay tube made up to 100 μ L with 40 mM tris-maleate buffer (pH 7.0), and the resulting mixture shaken for a count of three. A further 240 μ L of 40 mM tris-maleate buffer was then added and the assay mixture left to incubate for 5 minutes at 40°C. The rest of the protocol was identical to the standard activity assay (Section 2.2.6.3). All assays were performed in duplicate or triplicate and all results were expressed relative to the control rate (i.e. rate of lysis without antiserum) and were corrected for the blank rate (i.e. spontaneous rate of lysis without enzyme). The reported percentage inhibition of a lysozyme conjugate or conjugate mixture refers to the maximal percentage inhibition which could be obtained, not to the percentage inhibition obtained at a given antiserum concentration.

2.3 RESULTS

2.3.1 Synthesis of 17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic Acid

17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic acid or estrone glucuronide (E1G) as it is more commonly known, will be referred to by its trivial name throughout this thesis.

2.3.1.1 Synthesis of Methyl 1-Bromo-1-deoxy-2,3,4-tri-*O*-acetyl- α -D-glucopyranuronate



SCHEME 2.2: Synthesis of the α -Bromo Sugar

The first step in the synthesis of estrone glucuronide is the synthesis of its sugar group in the form of an unstable, highly reactive bromo sugar. The α -bromo sugar (methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl- α -D-glucopyranuronate) was synthesised from glucuronolactone in a three step reaction (Bollenback *et al.*, 1955). An outline of the synthesis is shown in Scheme 2.2.

Glucuronolactone (35.2 g, 0.2 moles) was added gradually over approximately an hour with constant stirring, to a freshly prepared solution of sodium methoxide (0.47 g, 8.7 mmol) in methanol (200 mL), which was made by dissolving sodium (0.2 g, 8.7 mmol) in dry methanol (200 mL). The methanol was removed from the resulting yellow methyl glucuronate (methyl α -glucopyranosyl uronate) solution by rotary evaporation with the aid of a water bath (temperature $\leq 40^\circ\text{C}$) to yield a thick syrup.

The syrup was redissolved in acetic anhydride (136 mL) and a dropping funnel fitted with a drying tube was then used for the dropwise addition of a solution of perchloric acid (0.6 mL) in glacial acetic acid (20 mL) to the sugar. Due to the risk of the sugar decomposing at temperatures above 40°C and the highly exothermic nature of the reaction, the mixture was kept at 4°C , with constant stirring throughout the dropwise addition of the acid which took approximately an hour.

After the reaction mixture had stood overnight at room temperature, extra perchloric acid

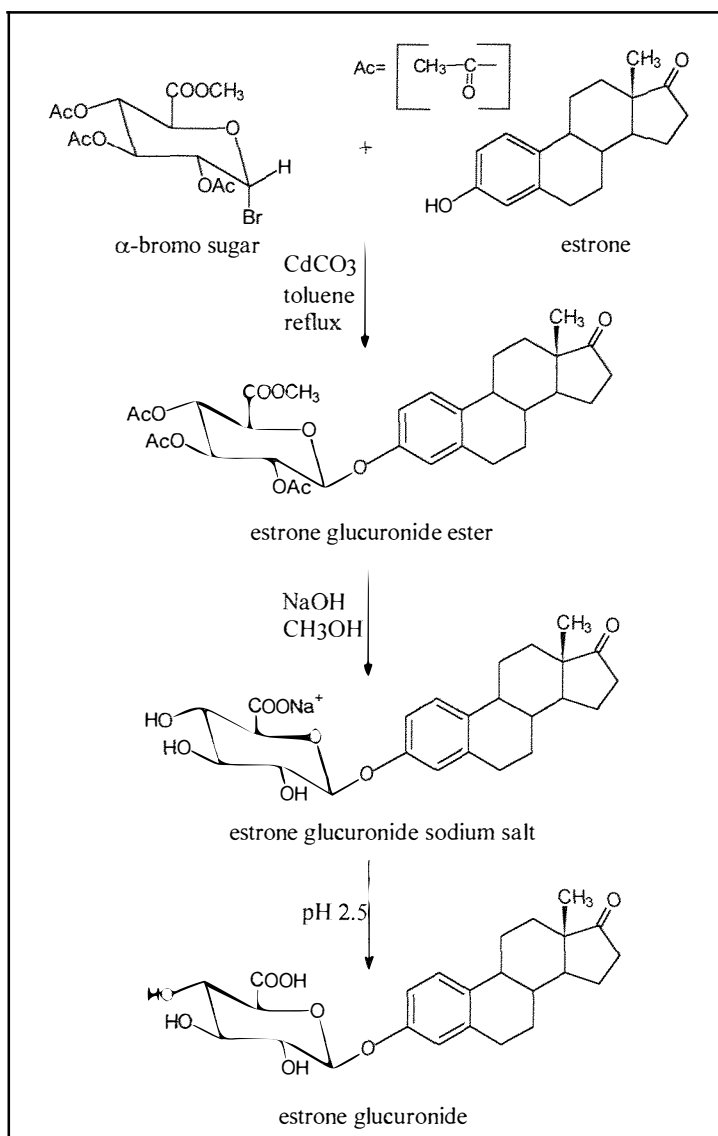
(0.2 mL) was added. The reaction mixture was then left overnight at -20°C . Crystals of the acetylated sugar (methyl tetra-*O*-acetyl- α -D-glucopyranuronate) were isolated by washing with diethylether and recrystallising from hot absolute ethanol. The recrystallisation mixture was left to cool to room temperature, then transferred to 4°C for 2 hours and finally left at -20°C for 48 hours. The resulting methyl tetra-*O*-acetyl- α -D-glucopyranuronate crystals were removed by filtration and then dried *in vacuo* to give (10.28 g, 27.3 mmol, 14% yield) of pure white crystalline solid that had a melting point of $179\text{--}181.5^{\circ}\text{C}$ (literature $176.5\text{--}178^{\circ}\text{C}$; Bollenback *et al.*, 1955).

Bromination of the sugar was initiated by dissolving all the acetylated sugar from the previous step (10.28 g, 27.3 mmol) in 30% hydrogen bromide (HBr) in acetic acid (41 mL) with the aid of gentle heating. The mixture was left to react overnight at 4°C after which the previously golden reaction mixture had turned brown. The acetic acid and unreacted HBr were removed by rotary evaporation to leave a dark syrup which was redissolved in chloroform (20 mL) and then washed with saturated sodium bicarbonate ($1 \times 30\text{ mL}$) followed by water ($1 \times 50\text{ mL}$). The bromo sugar solution was then dried over sodium sulphate and the chloroform removed by rotary evaporation to leave a dark wine coloured sugar syrup. The bromo sugar was recrystallised twice overnight at 4°C using cold absolute ethanol and the crystals combined and dried *in vacuo* to yield 6.93 g (17.5 mmol, 64% yield) of very crystalline dusky pink product (methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl- α -D-glucopyranuronate) which had a melting point of $105\text{--}106.5^{\circ}\text{C}$ (literature value $106\text{--}107^{\circ}\text{C}$; Bollenback *et al.*, 1955).

2.3.1.2 *Synthesis of Methyl [17-Oxoestra-1,3,5(10)-triene-3-yl-2',3',4'-tri-O-acetyl- β -D-glucopyranosid] Uronate*

Estrone glucuronide was synthesised in a three step process as shown in Scheme 2.3 (Conrow and Bernstein, 1971). In the first step, 3-hydroxy-estra-1,3,5(10)-triene-17-one, henceforth referred to by its trivial name estrone, was coupled to the bromo sugar, methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl- α -D-glucopyranuronate, using cadmium carbonate (CdCO_3) as a catalyst to produce the corresponding estrone glucuronide ester (methyl [17-oxoestra-1,3,5(10)-triene-3-yl-2',3',4'-tri-*O*-acetyl- β -D-glucopyranosid] uronate). The overall molar ratio of estrone:bromo sugar: CdCO_3 used in the synthesis was 1:3:3.

All toluene used in the preparation was freshly distilled and pre-dried over molecular sieves. The bromo sugar (6.9 g, 17 mmol) was weighed into a dry stoppered flask, dissolved in toluene (100 mL), and kept in the dark until required. Estrone (1.5 g, 5.6 mmol) and cadmium carbonate (2.9 g, 17 mmol) were weighed into a dry 500 mL two necked flask and toluene (120 mL) added. The solution was heated with stirring in a hot oil bath, and the toluene allowed to distil until the total volume was reduced by approximately two-thirds of the original. This volume was then kept constant by additions of the previously prepared bromo sugar - toluene solution via a dropping funnel over a period of approximately an hour during which time the reaction mixture changed from a clear solution to yellow and eventually pink. The reaction mixture was refluxed for a



SCHEME 2.3: Synthesis of Estrone Glucuronide from the α -Bromo Sugar and Estrone

further hour and the success of the reaction verified by thin layer chromatography (TLC). The TLC plate was run using standard estrone glucuronide ester as a control, acetone as the sample solvent, and a 1:1 mixture of hexane:ethyl acetate as the running solvent. The plate was stained by immersion in Kober Reagent (9 parts 95% ethanol + 1 part concentrated sulphuric acid) and developed for 10 minutes at 120°C.

The cadmium carbonate was removed by filtration and washed with distilled toluene. The filtrate was then rotary evaporated down to dryness, and the residual methyl [17-oxoestra-1,3,5(10)-triene-3-yl-2',3',4'-tri-*O*-acetyl- β -D-glucopyranosid] uronate (estrone glucuronide ester) redissolved in the minimum volume of acetone (70 mL) with gentle heating. The acetone content of the solution was then reduced to 20% by the addition of 280 mL of water and the mixture left at 4°C overnight. The product,

which precipitated out as a yellow fatty flocculent solid, was redissolved in a 1:1 mixture of dichloromethane:methanol (60 mL total). The solution was gently heated with stirring until the volume had decreased to just above the 30 mL mark. The flask was then sealed and the solution was left to crystallise at 4°C for 3 hours. The resulting crystals were isolated by filtration and washed with a minimal quantity of cold methanol. The mother liquor was left unsealed for 24 hours at room temperature after which time a second crop of crystals was isolated. In the final crystallisation the two crystal fractions were combined. The crystals were dried *in vacuo* to give 2.02 g (3.05 mmoles, 62% yield) of white crystalline solid (methyl [17-oxoestra-1,3,5(10)-triene-3-yl-2',3',4'-tri-*O*-acetyl- β -D-glucopyranosid] uronate). The purified crystals gave a single spot on TLC and had a melting point of 228-235°C (literature value 222-230°C; Conrow and Bernstein, 1971).

The structure of the product was confirmed by NMR spectroscopy in deuterated chloroform. The key spectral data for this analysis was: ^1H NMR (270 MHz; CDCl_3): δ 0.91 (s, 3H, 18- CH_3), 2.04 (s, 3H, COCH_3), 2.05 (s, 3H, COCH_3), 2.06 (s, 3H, COCH_3), 3.74 (s, 3H, OCH_3), 4.15 (m, 1H, $J = 9.51$ Hz, H-5'), 5.09 (d, 1H, $J = 7.33$ Hz, H-1'), 6.73 (d, 1H, $J = 2.19$ Hz, H-4), 6.77 (dd, 1H, $J = 8.43, 2.77$ Hz, H-1), 7.20 (d, 1H, $J = 8.43$ Hz, H-2).

2.3.1.3 Preparation of 17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic Acid

In the next step of the synthesis, the acetyl and methyl groups (C2', 3', 4' and 6') were hydrolysed using a slight excess of sodium hydroxide (i.e. a 2:3 molar ratio of hydrolysable groups:NaOH). Sodium hydroxide (8 mL, 2.58 M) in methanol was added dropwise with stirring to 2.02 g (3.05 mmoles) of the estrone glucuronide ester over 30 minutes. The mixture was then stirred for an additional 5 hours to allow the hydrolysis reaction to go to completion (as indicated by TLC). The reaction mixture was adjusted from pH 11.50 to 7.93 with glacial acetic acid, rotary evaporated to dryness, then redissolved in the minimal volume of methanol:water 3:2 and stored at 4°C. Crystals of sodium-[17-oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosid]uronate (E1G-(Na)), were collected by filtration 24 hours later, washed with methanol and dried to give 1.21 g (2.6 mmoles, 75% yield) of white crystalline solid. The E1G-(Na) was further purified by running it through an XAD-2 column (270 $\times$ 17 mm i.d.). The packed resin was prepared by sequential washing with 200 mL of water, methanol:water 50:50, methanol, methanol:water 50:50, and water again. The column was loaded with 0.2 g sample (0.43 mmoles) dissolved in 15 mL of water, and then washed with 90 mL water to remove any sodium acetate. The sample was eluted using a 50:50 mixture of methanol:water (200 mL), and the column regenerated with methanol (200 mL). The methanol and water were then removed from the purified product by rotary evaporation and freeze drying to give 0.177 g (0.378 mmoles, 89% yield) of sodium-[17-oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosid]uronate. The success of the purification of E1G-(Na) was verified by a single spot on TLC using the Kellie solvent system (ethyl acetate: absolute ethanol: acetic acid 3:1:1) and Kober reagent.

In the final step the sodium salt of estrone glucuronide was titrated into its acid form (E1G-(H)). The E1G sodium salt (0.177 g, 0.378 mmoles) was dissolved in a minimal volume (8.5 mL) of a 3:2 methanol:water mixture and titrated to pH 2.3 with 0.25 M HCl. The reaction mixture was then rotary evaporated until all of the methanol was removed. Methanol was then added dropwise (1.65 mL) until a thick white precipitate began to form. The solid product was isolated by filtration and dried *in vacuo* to give 0.125 g (0.281 mmoles, 74% yield) of 17-oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic acid, melting point 165-168°C (literature value 176.5-178°C, Elce *et al.*, 1967).

The structure of the E1G-(H) product was confirmed by NMR spectroscopy using deuterated dimethylsulphoxide (DMSO) as a solvent in which the compound was readily soluble. Due to the interference from the DMSO solvent in the NMR spectrum, only the part of the spectrum that displayed the anomeric proton and the C5' proton of the glucuronide ring is shown (see Figure 2.2).

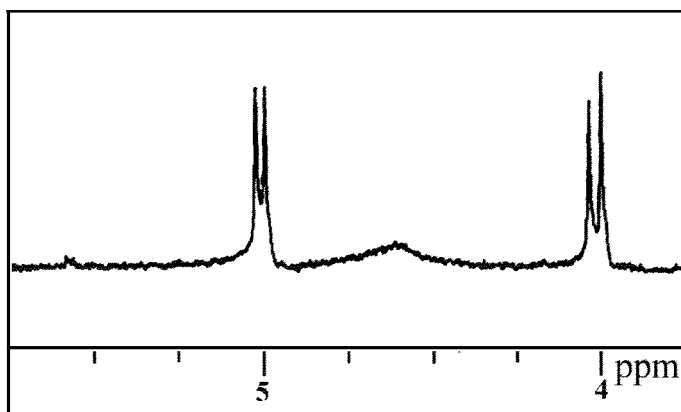


FIGURE 2.2: Nuclear Magnetic Resonance Spectrum showing the Anomeric Proton and the Glucuronide Ring C5' Proton of the Acid Form of Estrone Glucuronide

The key features of the NMR spectrum are summarised below.

¹H NMR (270 MHz; DMSO-d₆):
 δ 0.82 (s, 3H, 18-CH₃), 3.72 (d, 1H, $J = 8.79$ Hz, H-5'), 4.91 (d, 1H, $J = 7.96$ Hz, H-1' anomeric proton), 6.71 (s, 1H, H-4), 6.77 (d, 1H, $J = 8.43$ Hz, H-1), 7.17 (d, 1H, $J = 8.43$ Hz, H-2)

2.3.2 Analysis of the Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Reaction Mixture

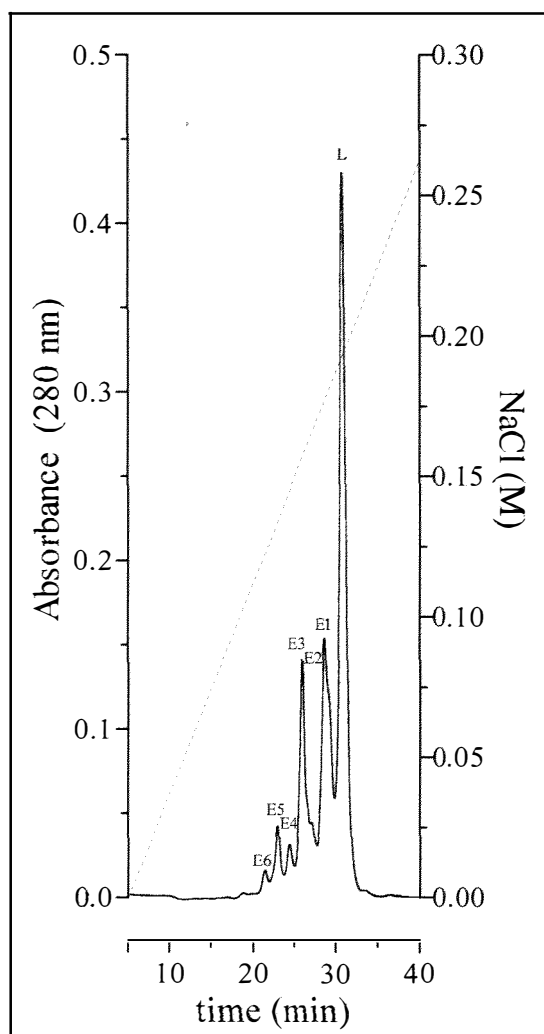


FIGURE 2.3: Mono-S Profile of the Reaction Mixture of a Typical Hen Egg White Lysozyme-Estrone Glucuronide Active Ester Conjugation obtained under Standard Analytical Conditions

Conjugation Conditions: Steroid:Enzyme 1.5:1

Throughout this work multiple hen egg white lysozyme - estrone glucuronide active ester conjugations were performed. Figure 2.3 shows a Mono-S trace from the reaction mixture of a typical 1.5:1 steroid:enzyme active ester conjugation. Under the standard conjugation conditions (Section 2.2.4), all of the 1.5:1 molar ratio conjugations gave the same seven chromatographically distinct peaks. The last peak eluted (labelled L) was unreacted lysozyme as determined by its identical retention time with that of a pure lysozyme control (profile not shown). As no other peaks were present in the chromatogram of the lysozyme control, all the earlier eluting peaks on the reaction mixture trace must be conjugate peaks and have been labelled E1-E6 accordingly with the conjugate peak closest to lysozyme being designated E1. As shown in Figure 2.3, the E2 peak sometimes was seen as a shoulder on the E3 peak as opposed to as a properly differentiated peak. Sometimes a shoulder could also be detected on the trailing edge of the E1 peak (also as shown in Figure 2.3), however this never differentiated itself into a fully resolved peak.

In the example conjugation, the ratio of unconjugated to conjugated lysozyme based on peak area was 1:1. The two largest conjugate fractions, E1 and E3, were present in 18% and 17% yield respectively and together comprised 68% of the total conjugate yield.

The relative peak heights were found to vary between conjugations under different reaction conditions such that coupling reactions which produced a higher overall conjugation ratio also gave a higher proportion of the early eluting peaks (E4-E6). In some conjugations, which gave a conjugation rate greater than 50%, up to three extra, very small peaks (E7-E9) eluted before the E6 peak. The height of these extra peaks (and the E4-E6 peaks) increased relative to the lysozyme (and E1-E3 peaks) as the ratio of steroid to enzyme was increased.

This can be seen in the Mono-S profile shown in Figure 2.4a which is from the total conjugation mixture of a 3:1 steroid to enzyme conjugation ratio.

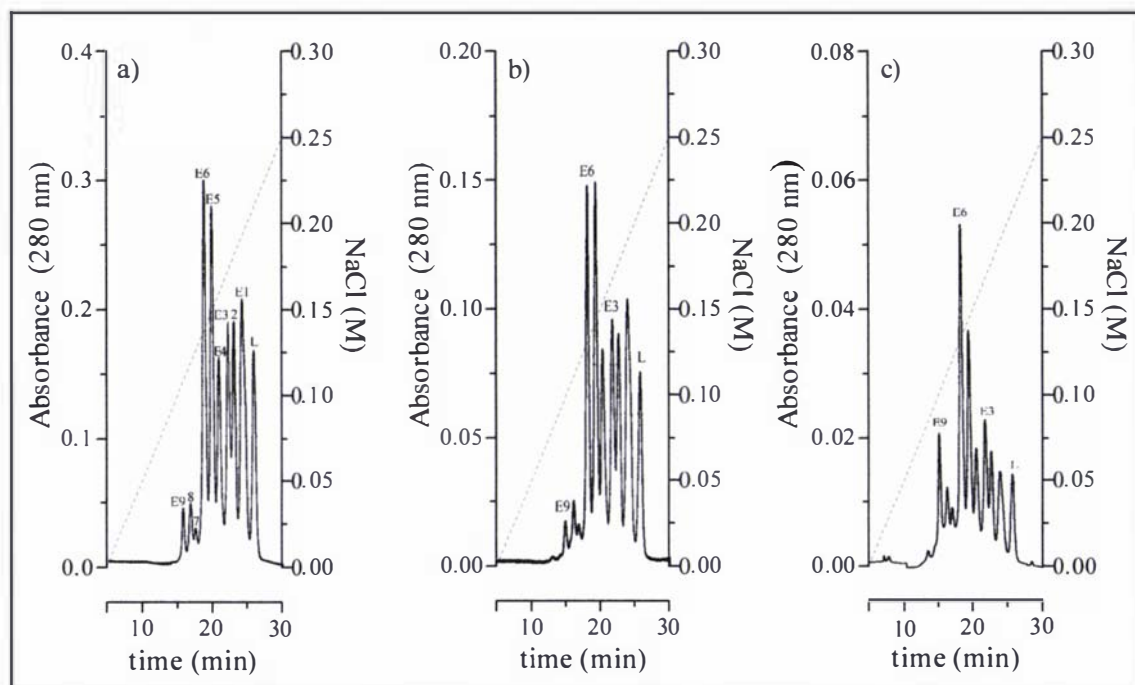


FIGURE 2.4: Mono-S Profiles of an Initial Active Ester Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Mixture, and its Resulting Supernatant and Precipitate Fractions obtained under Standard Analytical Conditions

Conjugation Conditions: Steroid:Enzyme 3:1

a) total reaction mixture; b) supernatant; c) precipitate

In this example, the lysozyme peak constituted only 10% of the total sample, and the two biggest conjugate peaks were now E5 and E6 which together made up 40% of the total conjugate yield compared with only 14% for a standard 1.5:1 conjugation.

There was a significant difference in the relative peak heights of the conjugates between the reaction mixture (Figure 2.4a) and the corresponding supernatant (Figure 2.4b) on the one hand and the redissolved precipitate (Figure 2.4c) on the other. Relative to the reaction mixture and the supernatant, which gave essentially identical analytical FPLC profiles, the precipitate sample (which was prepared by redissolving the precipitate in 7 M urea) had proportionally more of the early eluting peaks (E5 to E9).

2.3.3 Purification of Hen Egg White Lysozyme-Estrone Glucuronide Conjugate in the Absence of Urea

2.3.3.1 Mono-S Cation Exchange Chromatography at pH 4.3

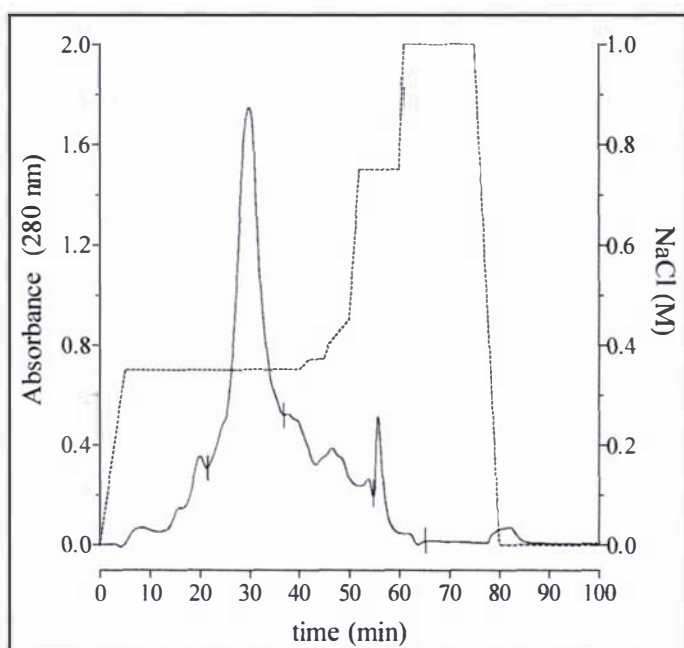


FIGURE 2.5: Mono-S Elution Profile obtained at pH 4.3 in the Absence of Urea for a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation

Aliquots of the dialysed supernatant of an active ester conjugation were injected onto a Mono-S column equilibrated with 50 mM phosphate buffer (pH 4.3). Elution of the sample was effected using an essentially two step sodium chloride gradient as recorded on the FPLC profile (see Figure 2.5). However, attempts to re-optimize the gradient to facilitate separation of the conjugates and lysozyme as reported previously (Smales *et al.*, 1994) were unsuccessful. Instead it resulted in the elution of one well defined peak followed by a broad, irregular band containing a small sharp peak at the end as the gradient was stepped to 0.75 M salt.

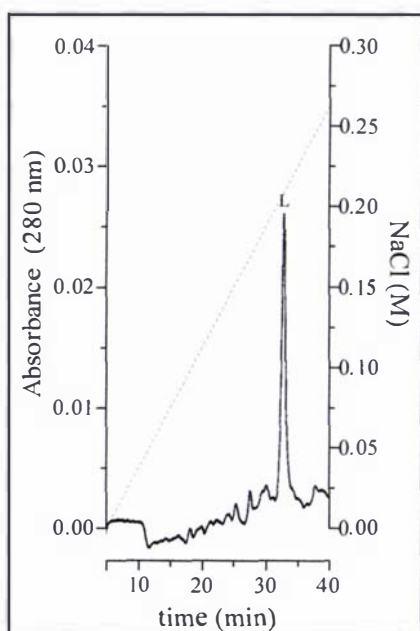


FIGURE 2.6: Mono-S Profile obtained under Standard Analytical Conditions of the Main Peak of a Mono-S pH 4.3 Non-Urea Based Conjugate Separation

Analysis of the fractions using standard denaturing conditions on the analytical Mono-S column (Cooke, 1993; Smales *et al.*, 1994) showed that the major peak consisted almost entirely of unreacted lysozyme and contained only trace amounts of the conjugate fractions E1-E6 (see Figure 2.6), and the late eluting fraction contained a mixture of conjugates and only trace amounts of lysozyme (figure not shown due to the low amplitude of the traces).

Thus at pH 4.3, the Mono-S column failed to separate the different conjugates from the reaction mixture into individual peaks. The method was also found to be only partially reproducible and although the mainly lysozyme fraction was always present as a well defined peak, the elution characteristics of the broad conjugate band were more variable.

2.3.3.2 Mono-S Cation Exchange Chromatography at pH 9.5

Attempts to purify the lysozyme conjugates in the absence of urea were also carried out at pH 9.5 on the Mono-S column using 50 mM glycine buffers (equilibrating buffer - 50 mM glycine, pH 9.5; eluting buffer - 50 mM glycine, 1 M sodium chloride, pH 9.5).

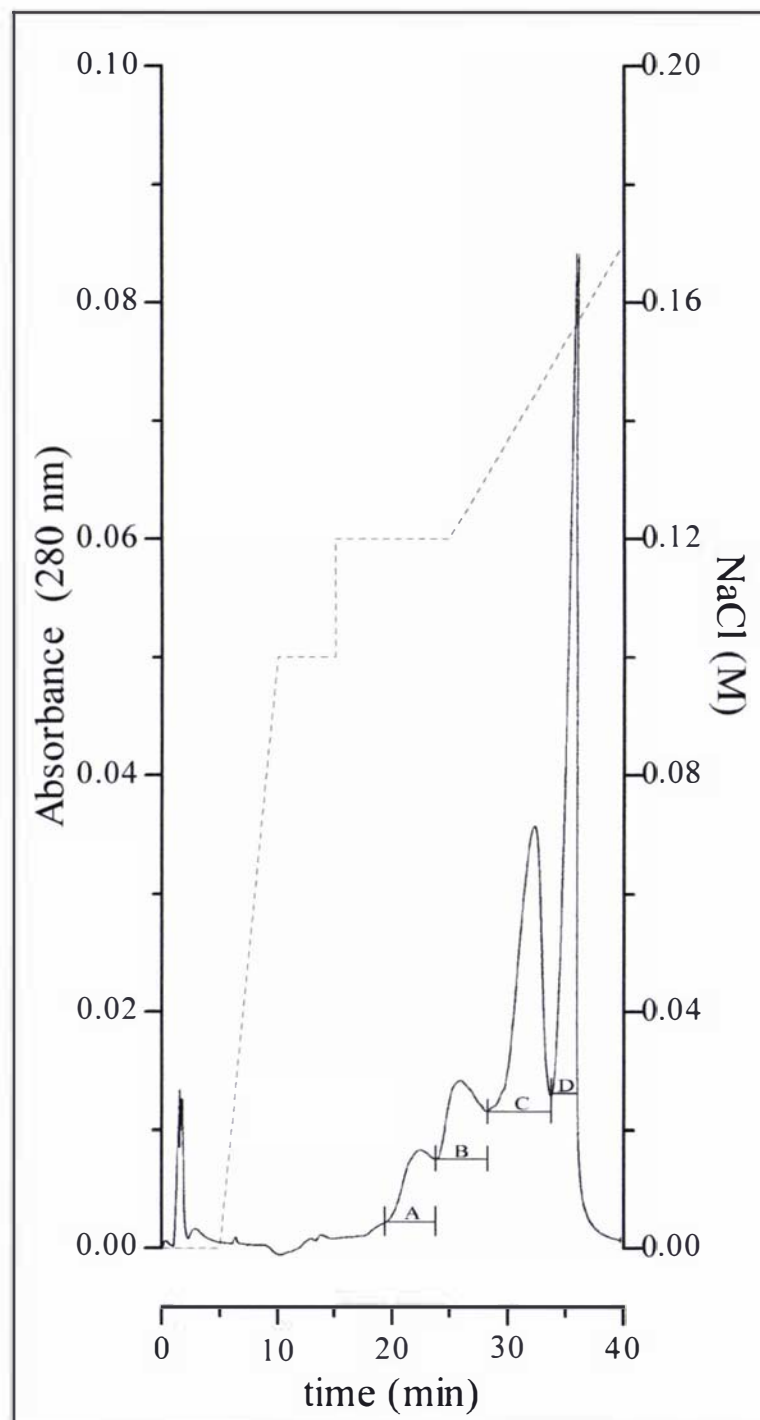


FIGURE 2.7: Mono-S Profile obtained in the Absence of Urea at pH 9.5 of a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Mixture

Dialysed supernatant from an active ester conjugation (prepared at a molar ratio of 1:1) was diluted one part in ten with equilibrating buffer, injected onto an equilibrated Mono-S column and eluted using a combination of step and linear sodium chloride gradients as recorded on the FPLC profile (Figure 2.7).

The profile consisted of one large well defined peak (D) preceded by three broader peaks; one moderately sized peak (C) and two smaller early eluting peaks (A and B).

The four different peaks were collected as indicated on Figure 2.7 and analysed using the standard 7 M urea (pH 6.0) conditions on the analytical Mono-S column (Cooke, 1993; Smales *et al.*, 1994).

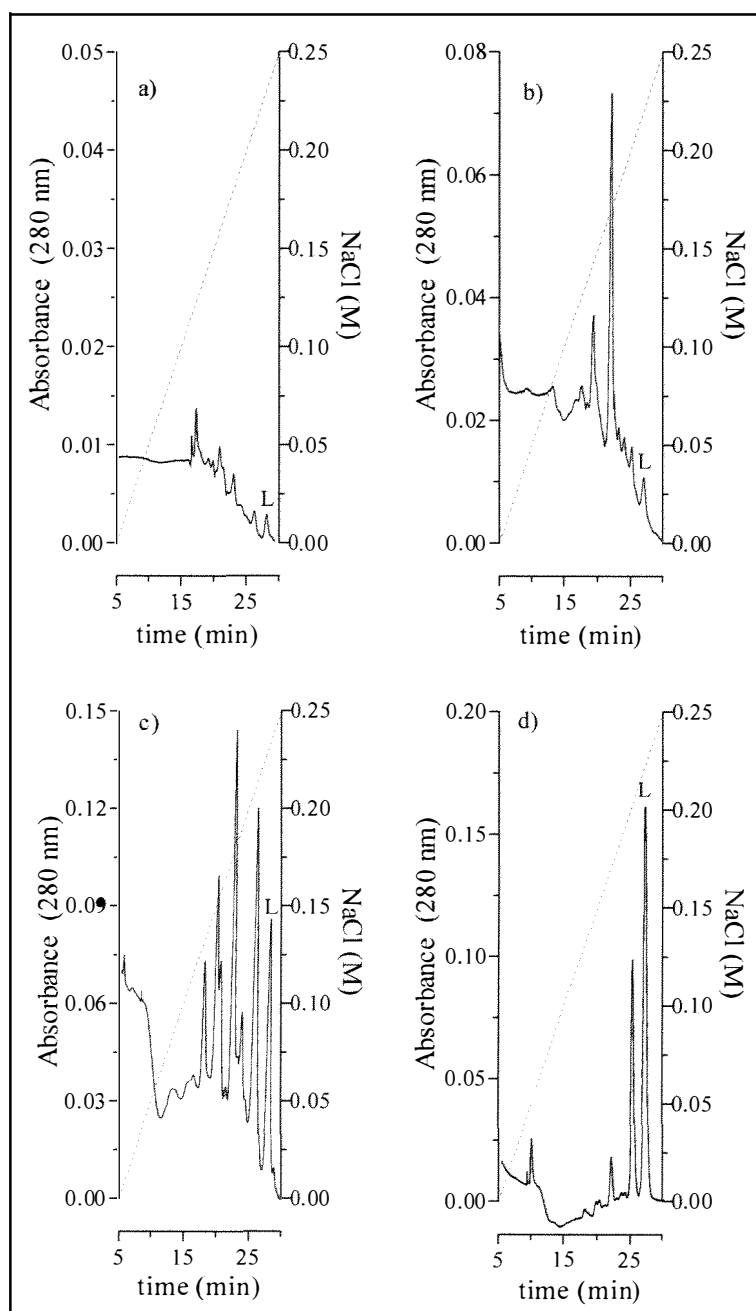


FIGURE 2.8: Mono-S Profile obtained under Standard Analytical Conditions of the Main Peaks of a Mono-S pH 9.5 Non-Urea Based Separation

a) peak A; b) peak B; c) peak C; d) peak D

This showed that all four peaks contained a mixture of lysozyme and conjugates with the three earlier eluting peaks (peaks A, B and C) consisting largely of conjugate (Figures 2.8a-c) and the late eluting peak (peak D) consisting mainly of lysozyme and E1 (Figure 2.8d). Although all of the earlier peaks contained some lysozyme, the level of lysozyme contamination was highest in peak C, the peak which eluted nearest to the large lysozyme peak. In addition to a large lysozyme component this peak also contained all six conjugates E1-E6 (see Figure 2.8c). Peak B also contained all of the conjugates, however the conjugate distribution was less even, and the largest conjugate components by far were conjugates E4 and E5 (Figure 2.8b). The FPLC profile for peak A was poor but showed a mixture of all of the conjugates as well as unreacted lysozyme in approximately equal proportions (Figure 2.8a). In this respect it was similar to peak C.

Thus, the conjugate distribution throughout the peaks of the pH 9.5 separation in the absence of urea was irregular compared with the progression observed at pH 6.0 using 7 M urea buffers (i.e. under the standard analytical conditions) and although the peaks still followed the same general elution order, they were less well resolved and contained a heterogeneous mixture of lysozyme and conjugates.

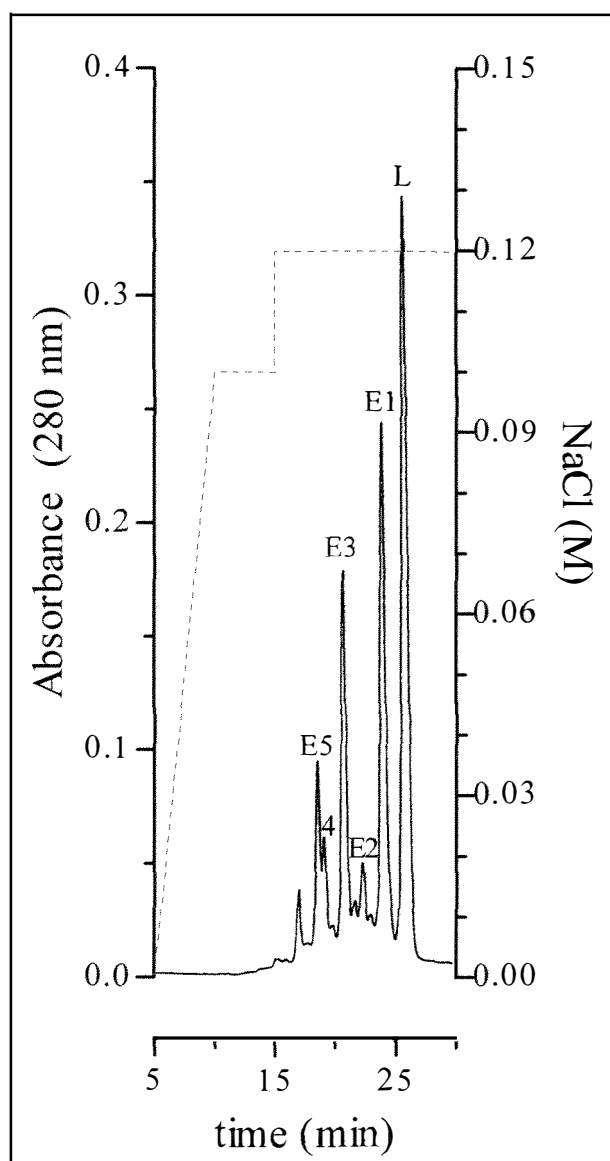


FIGURE 2.9: Mono-S Profile obtained in the Presence of Urea at pH 9.5 of a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Mixture

The sample was also run at pH 9.5 using 7 M urea buffers and a gradient optimised for maximum separation of the conjugate peaks to investigate the difference that high pH and 7 M urea made to the separation (see Figure 2.9). The resulting FPLC profile was very similar to that obtained under the standard analytical conditions (7.0 M urea, pH 6.0) (compare Figure 2.9 with Figure 2.3). Like the pH 6.0 separation, it consisted of six major, narrow, well resolved conjugate peaks which all eluted before the lysozyme peak. However, now E1 was a single sharp peak and a smaller peak (labelled E2) was well separated from E3, while the peaks labelled E4 and 5 now appeared as a split pair, presumably due to partial overlap.

2.3.4 Large Scale Purification of Hen Egg White Lysozyme Estrone Glucuronide Conjugates under Denaturing Conditions

Because of the failure of the Mono-S column to separate the individual conjugates from each other and from lysozyme in the absence of urea, it was decided to return to a urea based purification scheme. Although the Mono-S cation exchange column gave a very good separation of the different conjugates in the presence of urea, it is not commercially available as a large column and thus could not be used for the purification of the gram amounts of conjugate which needed to be processed. Thus, the purification procedure was scaled up by changing to an S-Sepharose fast flow cation exchange column.

The large scale separation and purification of the different lysozyme conjugates was always performed on the dialysed supernatant from the reaction mixture.

2.3.4.1 Purification of Lysozyme Conjugates by S-Sepharose fast flow Chromatography

The supernatant was prepared for loading onto a large S-Sepharose fast flow cation exchange column by the addition of urea (0.123 g per 300 μL of sample), and then sodium dihydrogen phosphate added to the new increased volume to give a 50 mM phosphate/7 M urea solution. After filtering the sample was loaded via a peristaltic pump onto the column which was pre-equilibrated with 50 mM phosphate buffer/7 M urea (pH 6.0) and elution effected using a long, linear sodium chloride gradient (0-0.21 M NaCl in 693 minutes).

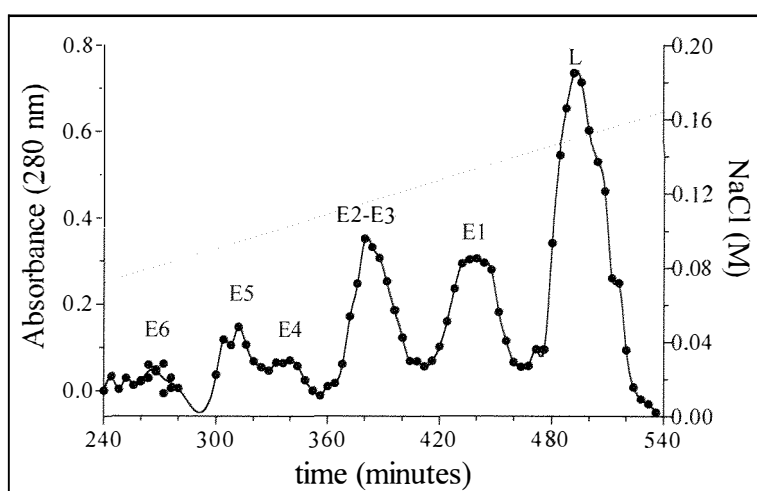


FIGURE 2.10: S-Sepharose fast flow Profile of a Hen Egg White Lysozyme-Estrone Glucuronide Conjugation Mixture

Conditions: flow rate - 1.5 mL min^{-1} ; fraction volume - 6 mL. Buffers and sample preparation as stated in the text.

Figure 2.10 shows the elution profile resulting from loading 35 mg of protein from the dialysed supernatant of a 1.5:1 estrone glucuronide:hen egg white lysozyme active ester conjugation onto the S-Sepharose fast flow column. The total protein recovery for this chromatographic step was 88%. The elution profile consisted of three large broad peaks preceded by three poorly resolved small peaks. Of the three large peaks, the last one was about twice the height of the other following peaks.

A comparison of this scaled up S-Sepharose fast flow profile with the Mono-S profile shown in Figure 2.3, which was obtained from the total reaction mixture from a similar

1.5:1 active ester conjugation, showed that the two urea based separations had similar FPLC profiles. If the E2 peak on the Mono-S trace is considered as part of the E3 peak on the S-Sepharose fast flow column then for both separations, six chromatographically distinct peaks were observed, and the relative retention times of the earlier eluting peaks compared with the last eluting peak (i.e. the lysozyme peak) were similar between the two separations. The only major visible difference between the two profiles was that the peaks from the S-Sepharose fast flow separation were broader, which is consistent with the heavier loading used and the column's greater size.

Chromatographic analysis of the S-Sepharose fast flow peak fractions on the analytical Mono-S column under standard denaturing conditions (profiles not shown) established that the two sets of peaks from the two cation exchange columns were chromatographically equivalent. Thus the peaks on the S-Sepharose fast flow profile have been labelled E1-E6 accordingly.

The peak fractions from the S-Sepharose fast flow column were pooled and depending on the resolution of the run, were either rechromatographed individually through the S-Sepharose fast flow column or passed directly through a Butyl Sepharose hydrophobic interaction column. In the S-Sepharose fast flow separation shown in Figure 2.10, where the absorbance returned close to baseline between peaks, the peak fractions were prepared for loading directly onto the Butyl Sepharose column.

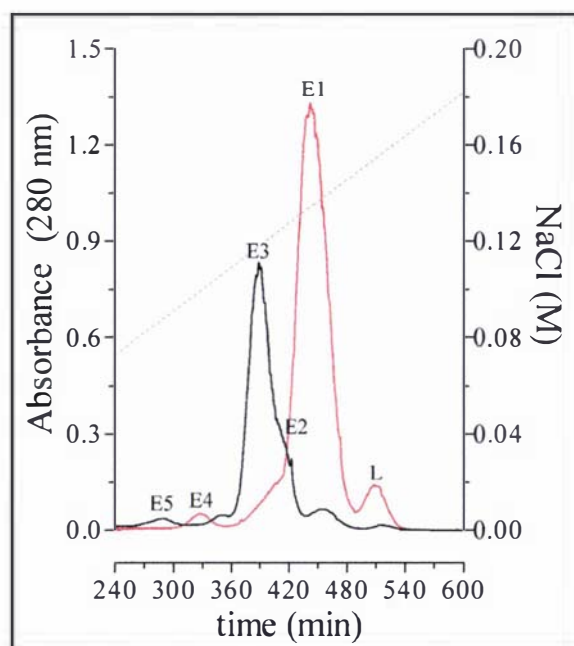


FIGURE 2.11: Overlay of the Elution Profiles of Hen Egg White Lysozyme-Estrone Glucuronide Conjugates, E1 and E3, after Repeat Chromatography through an S-Sepharose fast flow Column

Figure 2.11 shows an overlay of the elution profiles for E1 and E3 after repeat S-Sepharose fast flow chromatography where the peaks were not considered to be adequately resolved after the original separation. A comparison of the retention times of the fractions from the repeat S-Sepharose fast flow columns with the original S-Sepharose fast flow purification, showed that before loading for the second time the E1 sample was contaminated with only small amounts of lysozyme and E3, and a trace amount of E4. In contrast, the absorbance profile from the E3 purification showed E2 to have been a major contaminant which was not unexpected as the E2 peak was not always properly resolved from the E3 peak in the equivalent Mono-S profiles either. Small amounts of E1 were also present, but the E3 sample was essentially free of lysozyme contamination.

The pooled peak fractions from the repeat S-Sepharose fast flow columns were dialysed against water (3 changes $\times$ 20 L) and then concentrated to a final volume of 10-20 mL.

2.3.4.2 Purification of Lysozyme E1 and E3 Conjugates by Butyl Sepharose Chromatography

The final step in the purification of conjugates E1 and E3 involved a single passage through a hydrophobic interaction column. Ammonium sulphate and sodium dihydrogen phosphate were added to the concentrated samples from the S-Sepharose fast flow cation exchange column to give a 1.26 M ammonium sulphate/50 mM phosphate solution which was adjusted to pH 6.6 and filtered. The filtered sample was then loaded via a peristaltic pump, onto a Butyl Sepharose column which was pre-equilibrated with 1.26 M ammonium sulphate/50 mM phosphate buffer (pH 6.6). Elution of the sample from the column was effected using a linear negative ammonium sulphate gradient (1.26-0 M ammonium sulphate in 20 minutes). The profile obtained depended on the success of the separation of the conjugates achieved by the preceding S-Sepharose fast flow cation exchange column. The Butyl Sepharose E1 and E3 profiles shown (Figures 2.12 and 2.13 respectively) are from the pooled fractions of one of the less successful S-Sepharose fast flow separations, which provides a better example of the separation which could be effected by the Butyl Sepharose column.

In the example E1 purification, 31 mg of protein was loaded and generated an absorbance profile which consisted of two peaks; a symmetrical main peak which was preceded by a small plateau, and a later eluting, much smaller peak. The main peak constituted approximately 80% of the total recovered protein and the overall protein recovery of the column was 50%.

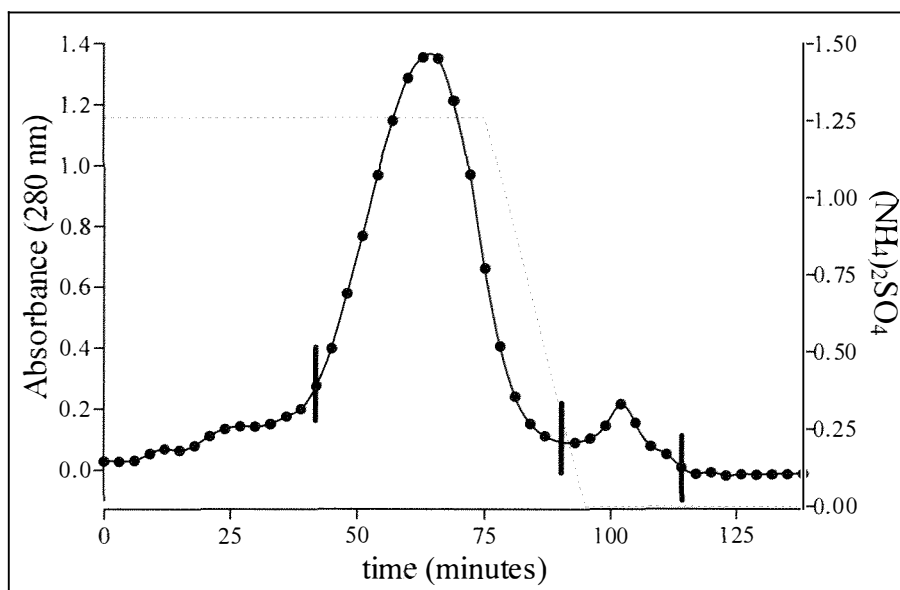


FIGURE 2.12: Butyl Sepharose Elution Profile for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate after initial Purification by S-Sepharose fast flow Chromatography

Conditions: flow rate - 1.5 mL min^{-1} ; fraction volume - 4.5 mL. Buffers and sample preparation as stated in the text.

The E3 purification profile shown in Figure 2.13 was obtained using a loading of 18 mg of protein. This showed the Butyl Sepharose profile for E3 to also consist of two peaks; a main peak which was preceded by a broad shoulder, and a later eluting medium sized, but sharp asymmetric peak. Due to the shoulder on the main peak the main peak fraction collection was over quite a restricted range and only represented 54% of the total recovered protein. The total protein recovery of the column was 76%.

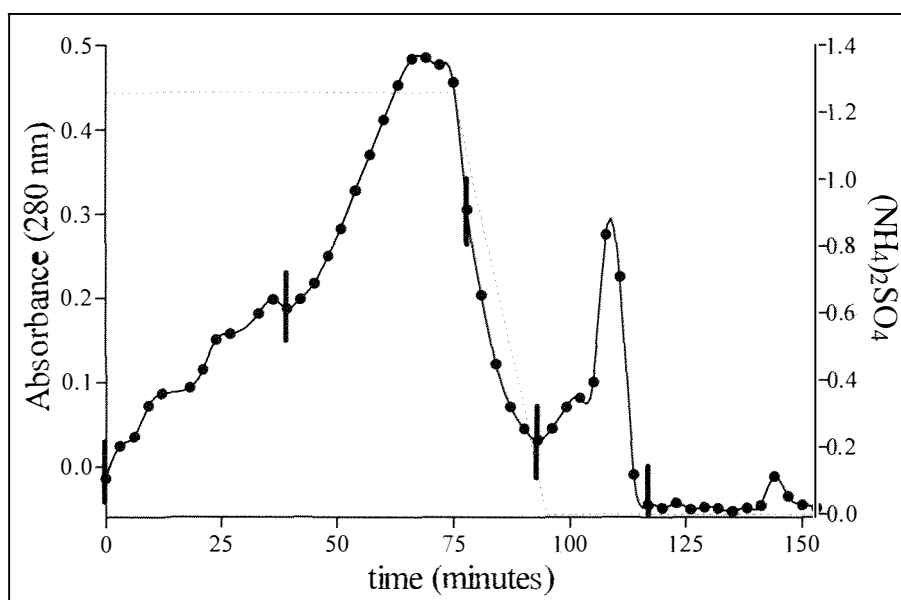


FIGURE 2.13: Butyl Sepharose Elution Profile for E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate after initial Purification by S-Sepharose fast flow Chromatography

Conditions: flow rate - 1.5 mL min^{-1} ; fraction volume - 4.5 mL. Buffers and sample preparation as stated in the text.

The fractions from the Butyl Sepharose separations were pooled as indicated on the absorbance profiles, dialysed against water (3 changes $\times$ 20 L) and concentrated by ultra filtration. The concentrated conjugates were then transferred into 0.35 M tris-maleate buffer (pH 7.0) by diluting the purified samples 1:1 with 0.7 M tris-maleate buffer before storage at -20°C . Under these conditions, after four years no decrease in the specific activity of the purified E1 and E3 conjugates was apparent.

The concentrated pooled fractions from the E1 and E3 Butyl Sepharose purifications were analysed by Mono-S chromatography under standard 7 M urea conditions (see Figures 2.14a-b for E1 and Figures 2.15a-d for E3).

With regards to the purification of E1, analysis by Mono-S chromatography showed the main peak from the Butyl Sepharose column to be essentially pure E1 conjugate containing only trace amounts of some of the earlier eluting conjugates (Figure 2.14a). In comparison, analysis of the small late eluting peak from the E1 separation showed that it was also mainly composed of E1 conjugate, but had a higher level of cross contamination with the other conjugates (Figure 2.14b). On the basis of relative retention times, the main contaminant was identified as E2 and composed 10% of the sample, but there were also trace amounts of some of the earlier eluting conjugates as well.

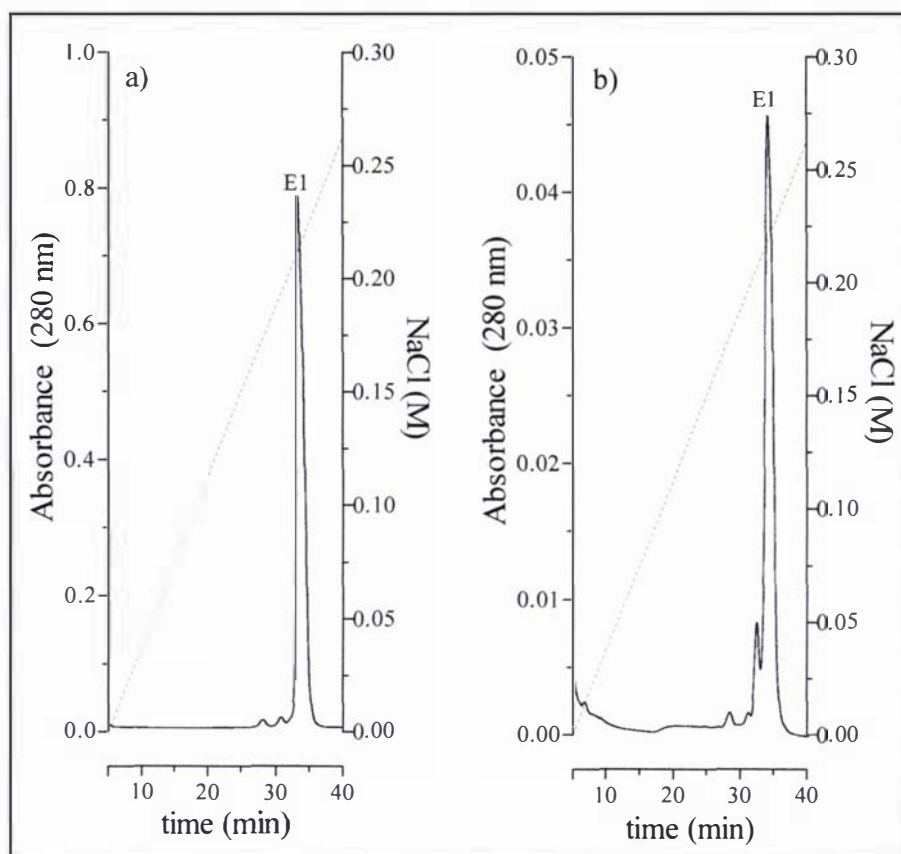


FIGURE 2.14: Mono-S Profiles obtained under Standard Analytical Conditions of the Pooled Fractions of the Butyl Sepharose Purification of E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

a) main peak: b) secondary late eluting peak

Four pooled fractions were analysed from the E3 separation on the Butyl Sepharose column. Consistent with the broad nature of the shoulder from the main peak, the pooled fractions from this portion of the profile formed the most heterogeneous mixture of all the samples analysed (see Mono-S profile in Figure 2.15a). It consisted mainly of E3 but also contained small to trace amounts of E1, E4, E5 and E6. It was also contaminated with approximately 10% lysozyme. The main peak from the Butyl Sepharose column was 100% pure E3 (Figure 2.15b); even when the sensitivity was increased and the E3 peak went heavily off scale (profile not shown) there was no evidence of any other peaks. The fractions collected between the main and secondary peak were also found to be essentially pure E3 (Figure 2.15c). Analysis of the secondary peak (Figure 2.15d) showed it to be a mixture of

70% E3 and 30% E4 which probably explains its curious asymmetric shape on the Butyl Sepharose column.

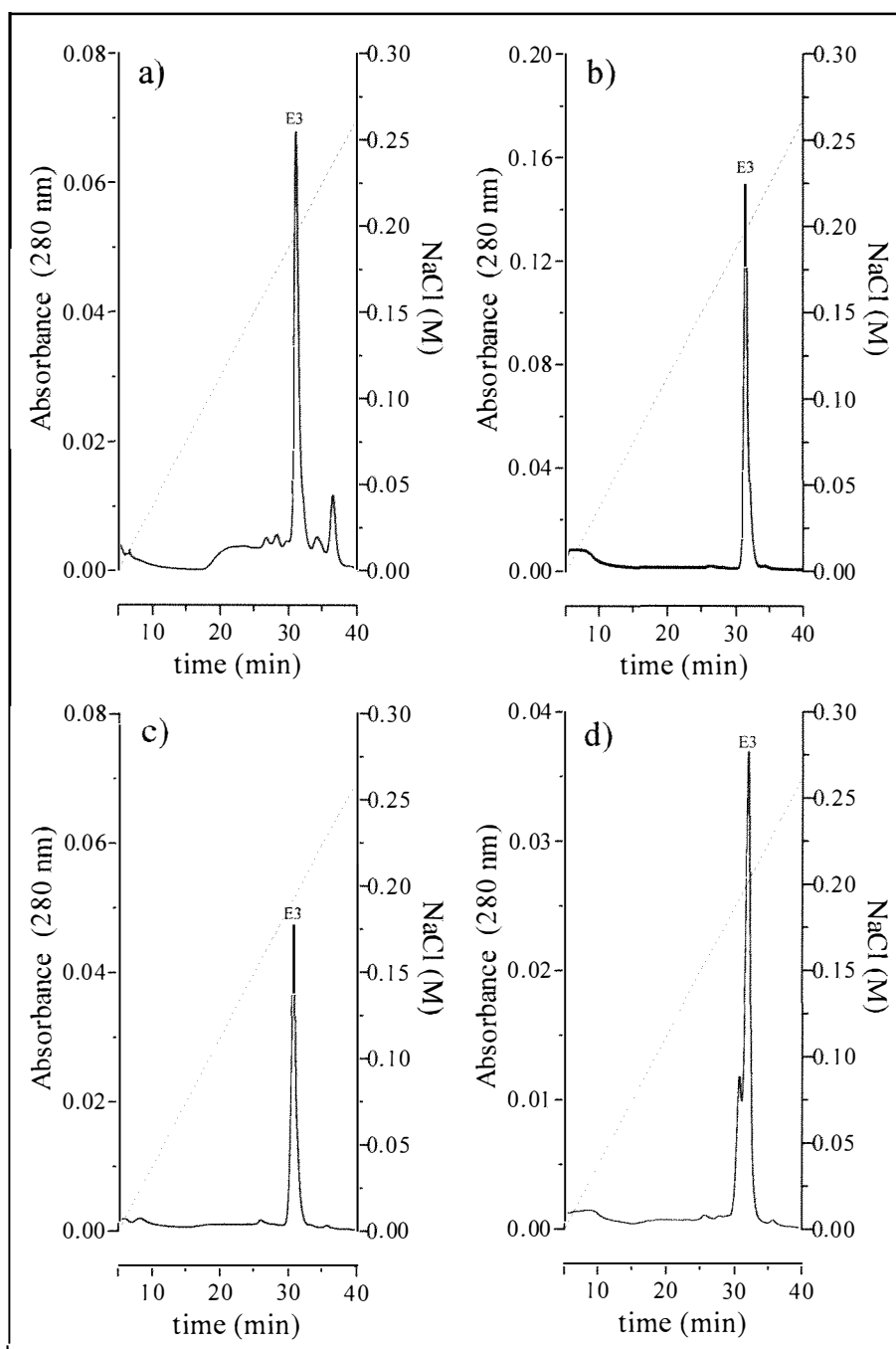


FIGURE 2.15: Mono-S Profiles obtained under Standard Analytical Conditions of the Pooled Fractions of the Butyl Sepharose Purification of E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

a) early eluting shoulder of main peak; b) main peak; c) late eluting edge of main peak; d) small late eluting asymmetric peak

2.3.4.3 Analysis of Lysozyme E1 and E3 Conjugates by Electrospray Mass Spectrometry

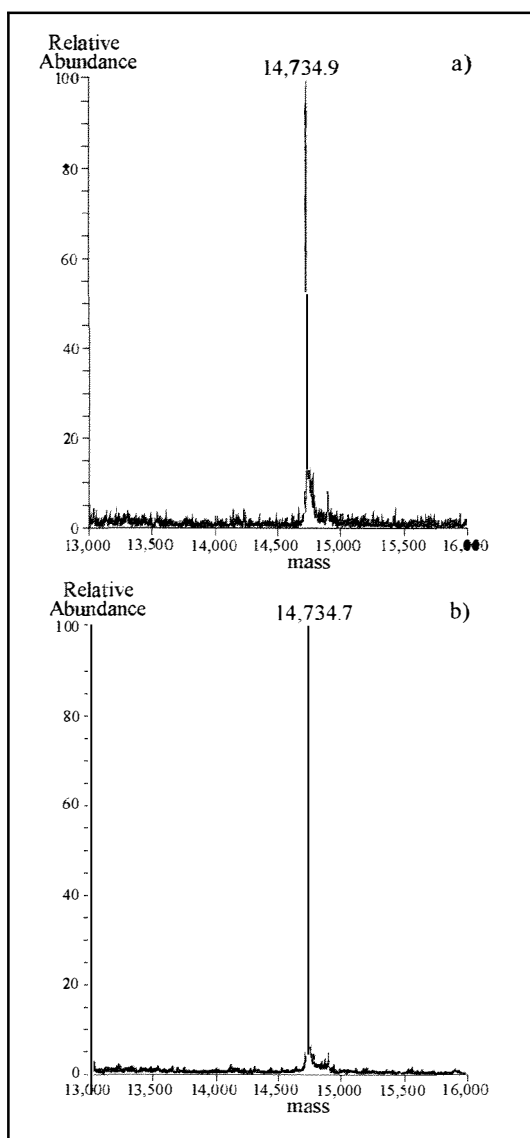


FIGURE 2.16: Electrospray Mass Spectrometry Spectra for E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates after Final Purification by Butyl Sepharose Chromatography

a) E1; b) E3

The purified E1 and E3 conjugate fractions after the Butyl Sepharose purification step were analysed by electrospray mass spectrometry (ESMS). Figures 2.16a and 2.16b show the mass spectra obtained for the purified E1 and E3 conjugate samples used in the kinetic studies reported in Chapter Three. In both cases the principal mass peak of the spectrum had a calculated mass of 14,735.

As the molecular weight for native hen egg white lysozyme is 14,305.5, and acylation with estrone glucuronide increases the mass by 428.5 mass units, these spectra provide conclusive evidence that the purified E1 and E3 samples are both mono-substituted lysozyme conjugates. The spectra also confirmed the lack of contamination of the samples with more highly substituted conjugates which would have increased the mass by 428.5 units per additional estrone glucuronide molecule. The absence of any peak at around 14,305 confirmed that the samples were not contaminated with lysozyme. The results also showed no evidence that the exposure of the samples to urea caused any obvious carbamylation of the final product which would have increased the mass of any protein present by 42 mass units. In both cases an unidentified very small impurity at a mass of 14,897 was observed.

2.3.4.4 Inhibition Studies of Lysozyme E1 and E3 Conjugates

After purification on the S-Sepharose fast flow column the degree of inhibition of the conjugates was low and passage through the Butyl Sepharose column was essential for the production of highly inhibitable conjugate. For example, for the E1 Butyl Sepharose profile shown in Figure 2.12 the percentage inhibition of the sample was 65% before the column and 96% for the main peak after the column. A similar result was observed for the

equivalent E3 purification (see Figure 2.13) where the inhibition increased from 59% to 99%. For both the E1 and the E3 purifications this change in the percentage inhibition was more than could be attributed to contamination of the sample (before the Butyl Sepharose column) with lysozyme as analysis of all the components across the Butyl Sepharose profiles by Mono-S chromatography showed there to be very little if any lysozyme present.

The low level of conjugate inhibition after the S-Sepharose fast flow column was also found for the Mono-S based separations as well.

A summary of the recoveries and conjugate characteristics obtained by the various purification schemes on the S-Sepharose, Butyl Sepharose and Mono-S columns is given in Table 2.1.

TABLE 2.1: A Summary of the Performance of the Various Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Purification Schemes

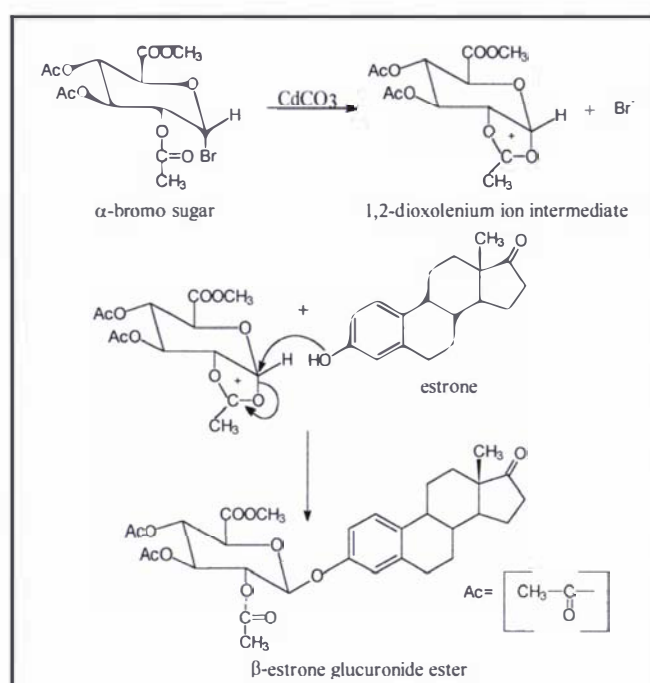
Column and Conditions	Total Protein Recovery (%)	Specific Activity (ΔT 20 min ⁻¹ pmol ⁻¹)	Specific Activity in Presence of anti-E1G Antiserum	Inhibition (%)
S-Sepharose ff (total)	88	-	-	-
S-Sepharose ff (E1)	-	44	15	65
S-Sepharose ff (E3)	-	49	20	59
Butyl Sepharose (E1)	50	45	0.8	96
Butyl Sepharose (E3)	76	50	0.5	99
Mono-S (E3) 7 M urea, pH 6.0	80	50	38	76
Mono-S non-urea, pH 4.3	-	poor separation from lysozyme fraction		
Mono-S (fraction B) non-urea, pH 9.5	-	48	29	60

2.4 DISCUSSION

2.4.1 Synthesis of 17-Oxoestra-1,3,5(10)-triene-3-yl- β -D-glucopyranosiduronic Acid

Because immunoassays are highly hapten specific, it is essential that the purity of the final synthetic product is high (i.e. $\geq 99\%$) and that it has the correct β stereochemistry (i.e. that it is the same as the naturally occurring estrone glucuronide which is excreted in urine).

Before the bromo sugar can be coupled to estrone it is activated with CdCO_3 (a halophilic reagent) in a one to one molar ratio with the sugar to form a 1,2-dioxolenium ion intermediate (Banoub and Bundle, 1979) that can react with the steroid. This coupling



SCHEME 2.4: Formation of the β -Glucuronide of Estrone via the 1,2-Dioxolenium Ion Intermediate

occurs in a stereospecific manner (Scheme 2.4) as a result of neighbouring group participation by the 2-acetoxy group to give the 1,2-dioxolenium ion. Hence, in the product forming step the alcohol partner (estrone in this case) is restricted to attack from the β -face giving the required β -glucuronide isomer. The reaction was driven in the forward direction by using an excess of the bromo sugar, which ensured that most of the steroid was reacted and hence simplified the purification procedure for the steroid-sugar derivative.

The synthesis was confirmed at each stage by both NMR spectral analysis and melting points. The melting point of the acid form of estrone glucuronide (E1G-(H)) was found to

be consistently low relative to the literature value (by approximately 10°C in this example). However, as the final product was found to be immunologically reactive with anti-estrone glucuronide antiserum, and its NMR spectrum was consistent with the known estrone glucuronide structure this was considered as adequate proof of the final product's structural integrity.

More specifically, at each stage in the synthesis, the large coupling constants (7-9 Hz) for the anomeric proton (C1') and the C5' proton (see Figure 2.2) were consistent with a trans configuration of the C1' and C2' protons, and the C4' and C5' protons of the sugar ring. This showed that the sugar ring was in the chair configuration, and more importantly that the steroid group was attached in the β position as required. This was a particularly important feature of the E1G-(H) NMR spectrum as it confirmed that no racemisation of the sugar

ring had taken place during the exposure of the E1G-sodium salt to the acidic conditions necessary for the formation of E1G-(H).

2.4.2 Synthesis of Hen Egg White Lysozyme-Estrone Glucuronide Conjugates and Analysis of the Conjugation Reaction Mixture

For the successful conjugation of estrone glucuronide to hen egg white lysozyme, the steroid glucuronide molecule must first be activated. For all lysozyme conjugates previously and currently employed in the pre-coated Ovarian Monitor assay tubes, the steroid glucuronide has been activated using the mixed anhydride method (Erlanger *et al.*, 1957). However, success with the mixed anhydride method is critically dependent on the strict maintenance of an anhydrous environment, and good temperature control, with different stages of the reaction having to be performed at -10, 4 and 10°C respectively. For research purposes, the active ester method (Mattox *et al.*, 1969) has also been utilised for reaction of the steroid glucuronide with lysozyme. Because the active ester method can be performed at room temperature and its requirements for an anhydrous environment are much less stringent than the mixed anhydride method, it is quicker, less technically demanding, and more reliable. The active ester method is also associated with a greater acylating ability than the mixed anhydride method (Mattox *et al.*, 1969), and thus for the same ratio of steroid to enzyme, produces a higher proportion of conjugate (Cooke, 1993; Smales, *et al.*, 1994). For all of these reasons, the active ester method has largely superseded the mixed anhydride method as the standard conjugation procedure in our research laboratory.

2.4.2.1 Reactivity of Lysine Residues Towards Acylation by Estrone Glucuronide

Because it is possible for a lysozyme molecule to be conjugated at multiple sites, the reaction mixture from a lysozyme conjugation is consequently very complex, containing a diverse range of lysozyme conjugates that vary in terms of both the number and position of lysine residues acylated. For example, when Lee *et al.* (1975) acylated lysozyme with a succinyl group, a range of six different di- to penta- substituted lysozyme products were produced which covered all seven conjugation sites.

With regards to a lysozyme - steroid glucuronide conjugation, an indication of the range of conjugate products formed can be obtained by analysing a small aliquot of the reaction mixture by Mono-S cation exchange chromatography used in combination with 7 M urea buffers (Cooke, 1993; Smales *et al.*, 1994). Depending on the initial conjugation conditions, such an analysis of an active ester reaction mixture results in the elution of up to ten peaks (as shown in Figure 2.4).

In the conjugation of lysozyme with estrone glucuronide, each conjugation event replaces a positively charged lysine residue with an uncharged lysine steroid moiety and hence reduces lysozyme's overall positive charge by one unit. Thus, if the separation on the cation exchange column (Figure 2.3) was based solely on simple charge differences, then lysozyme and its conjugates would elute in strict order of their levels of substitution with each peak representing a different level of substitution. However, as there are only seven possible conjugation sites on lysozyme this would give a maximum of seven conjugate

peaks. The fact that it is possible to get up to ten conjugate peaks, shows that at least some of the substitution levels are represented by more than one chromatographic peak.

For a separation based on cation exchange, the most highly substituted conjugates should elute first, followed by the less highly substituted conjugates and finally by unreacted lysozyme. With regards to unreacted lysozyme, a comparison of retention times of lysozyme only controls with conjugation mixture runs showed that lysozyme, which retains all of its positive charges, always eluted last as predicted. The elution order of the remaining conjugate peaks is not so simply defined and requires the conjugates of the different peaks to be structurally characterised.

Recently in a parallel study (Smales *et al.*, 1999), the six main conjugate peaks (E1-E6) from the active ester reaction mixture and the main conjugate peak (E3) from the mixed anhydride reaction mixture have been identified by using a combination of tryptic digestion, amino acid sequencing and electrospray mass spectrometry. Only one conjugate family was analysed for the mixed anhydride reaction (E3) because although other conjugate peaks were present, they were not produced in sufficient quantities for analysis. A summary of the results for the peaks which were analysed is given in Table 2.2.

TABLE 2.2: A Summary of the Characteristics of the Main Hen Egg White Lysozyme-Estrone Glucuronide Conjugates of the Active Ester and Mixed Anhydride Conjugation Reactions

Lysozyme-E1G Conjugate	No. of E1G molecules per lysozyme molecule	Lysine Residue Acylated	Specific Activity Relative to Lysozyme
lysozyme	0		100
<u>Active Ester</u>			
E1	1	97/116	95
E2	2	97+116	88
E3	1	33	94
E4	2	33+116	61
E5	2	33+97	58
E6	3	33+97+116	42
<u>Mixed Anhydride</u>			
E3	1	33	99

Modified from Smales, 1997

Characterisation of the E3 peak from the mixed anhydride and the active ester conjugations showed that the two peaks were identical both consisting of a single mono-substituted lysozyme - estrone glucuronide conjugate acylated at the same position (lysine 33).

Examination of the active ester results shows that, with the exception of the E1 peak, all the other chromatographic peaks consist of a single conjugate which is uniform with respect to the number and position of the acylations. In contrast, the E1 peak consists of two mono-substituted conjugates; a lysine 97 conjugate and a lysine 116 conjugate. This microheterogeneity is consistent with the slight shoulder which could sometimes be

resolved on the trailing edge of the E1 peak (see Figure 2.3). Electrospray mass spectral analysis of the tryptic digest of the E1 peak showed it to consist of 60% of lysine 97 conjugate and 40% of lysine 116 conjugate. This suggests that the later eluting shoulder is due to the lysine 116 conjugate.

The structural identification of the active ester conjugates also showed that under the near stoichiometric ratios of active ester to lysozyme (1.5:1) only three of lysozyme's six lysine residues (33, 97 and 116) are favoured for conjugation. Because the early eluting conjugate peaks (E1-E6) included all seven possible mono-, di- and tri-substituted conjugate combinations for the three lysine residues, at least one other lysine residue must sometimes be conjugated to produce the very small, very early eluting peaks (E7-E9) which were observed with conjugation mixtures resulting from higher active ester:lysozyme ratios.

An indication of the order of relative reactivity of the different lysine residues can be obtained by comparing the sum of the peak heights for the different conjugate peaks containing the same acylated lysine residue with the sum of the peak heights for the other acylated residues. For example the sum of the peak heights for conjugates with an acylated lysine 97 residue is equal to the sum of the peak heights of E1, E2, E5 and E6, while the sum of the peak heights for conjugates with an acylated lysine 33 residue is equal to the sum of the peak heights of E3, E4, E5 and E6.

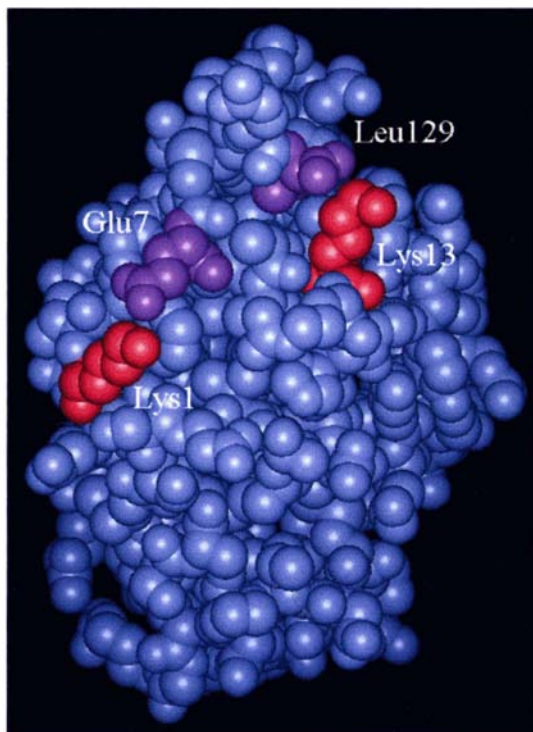


FIGURE 2.17: Structural Model of Hen Egg White Lysozyme showing the Salt Bridges formed between Lysine 1 and Glutamic Acid 7, and Lysine 13 and the C-terminal Group of Leucine 129

When the Mono-S profile of a 1.5:1 steroid glucuronide:lysozyme conjugation mixture (Figure 2.3) was examined using this analysis the lysine reactivity order was $97 > 33 > 116$ which is in good agreement with their calculated ratios of solvent exposure of 1.0, 0.9 and 0.5 respectively (Gerken *et al.*, 1982; Imoto *et al.*, 1972; Lee and Richards, 1971) obtained from the crystal structure. The slightly higher reactivity of the lysine 97 residue deduced from the present work is probably also partially attributable to the complete lack of interactions of its side chain with the rest of the molecule which is in contrast to all the other lysine side chains and the N-terminal amino group. While the side chains of both lysine 33 and 116 were involved in single hydrogen bonds (with asparagine 37 and 106 respectively) and were still readily conjugated, the acylation of the other lysine residues was probably energetically unfavourable due to the nature of their intramolecular interactions. Both the side chain amino groups of lysine residues 1

and 13, which have calculated solvent exposure ratios of 0.6 and 0.4 respectively, are involved in strong salt bridges to glutamine 7 and the terminal carboxyl group of

leucine 129 respectively (see Figure 2.17), while the side chain amino group of lysine 96, which has a solvent exposure ratio of 0.5, is involved in two hydrogen bonds with residues histidine 15 and asparagine 93 (Gerken *et al.*, 1982). Thus, in spite of the intermediate levels of exposure of these residues, their strong interactions with the rest of the protein probably protected them from acylation in any significant amounts at the near stoichiometric concentrations of steroid glucuronide to lysozyme used in this work. In contrast, the N-terminal amino group of lysine 1 has a very low level of solvent exposure (0.1) and it was probably this as opposed to its single hydrogen bond to threonine 40 which protected it from acylation.

Thus, it appears that it is the degree of exposure of the amino group to the solvent in combination with the strength of its interactions with the rest of the protein which determines its level of reactivity towards acylation.

2.4.2.2 *Effect of the Lysine Microenvironment on the Contribution to the Positive Electrostatic Field*

The degree of exposure of the different amino groups to the solvent did not explain the separation order of the lysozyme conjugates on the Mono-S cation exchange column. With the exception of the di-substituted E2 peak, which eluted later than the other di-substituted conjugates, E4 and E5, and also the mono-substituted conjugate, E3, the conjugates eluted in order of their overall positive charges based on their substitution levels although conjugates with the same charge did not necessarily elute as a single peak. These results were similar to the separation order observed for cytochrome c - carboxydinitrophenyl lysine conjugates as reported by Brautigan *et al.* (1978) who also used a salt gradient in conjunction with a cation exchange column for the separation of the different fractions. Under their reported conditions the multi-, di- and mono-substituted conjugates all eluted as distinct groups with retention times which decreased in strict order with their substitution levels. However, all the peaks making up each of these groups contained a range of structural isomers (i.e. conjugates with the same level of substitution, but at different sites) and had to be rechromatographed under a variety of conditions to yield homogeneous conjugates. The failure of the first ion exchange column to produce a single isomer per peak, unlike with the lysozyme conjugates, probably partly reflects the fact that nineteen lysine residues are available for conjugation in cytochrome c compared to only six in lysozyme.

It is interesting to note that for both lysozyme and cytochrome c, conjugates with the same level of substitution separated into several peaks upon cation exchange chromatography. This suggests that although the separation of conjugates in ion exchange chromatography primarily depends upon the overall net charge of the protein for the general separation of the mono-, di- and tri- substituted conjugates from each other, it is not the only factor influencing the separation as conjugates with the same net charge also have different retention times. For example, if the lysozyme-estrone glucuronide conjugates eluted in strict order of their calculated net charges (i.e. degree of substitution) on the Mono-S column the expected order of elution would have been: tri-substituted > di-substituted > mono-substituted > lysozyme, or E6 > E5=E4=E2 > E3=E1 (Lys 116 conjugate)=E1 (Lys 97 conjugate) > lysozyme.

Comparison of the relative orders of elution of the lysozyme mono-substituted conjugates with each other and the di-substituted conjugates with each other showed that conjugates which were substituted at lysine 116 always eluted later, while conjugates which were substituted at lysine 33 always eluted earlier. Thus there was a definite pattern in the order of elution in 7 M urea of the conjugates according to the acylation position which was: Lys 33 > Lys 97 > Lys 116.

Part of this difference may be a consequence of using 7 M urea for the separation. Although the interaction between urea and the protein is poorly understood, compared with other enzymes, lysozyme is known to be particularly resistant to denaturation by urea and the evidence from many studies indicates that the effect of urea on the tertiary structure of lysozyme is minimal (Barnes *et al.*, 1972; Hamaguchi, 1958; Hamaguchi and Rokkaku, 1960). Recently, the tertiary structure of hen egg white lysozyme crystals grown in urea has been determined (Pike and Acharya, 1994). This structure shows nine urea binding sites, including one where urea hydrogen bonds to lysine 116 (see Figure 2.18).

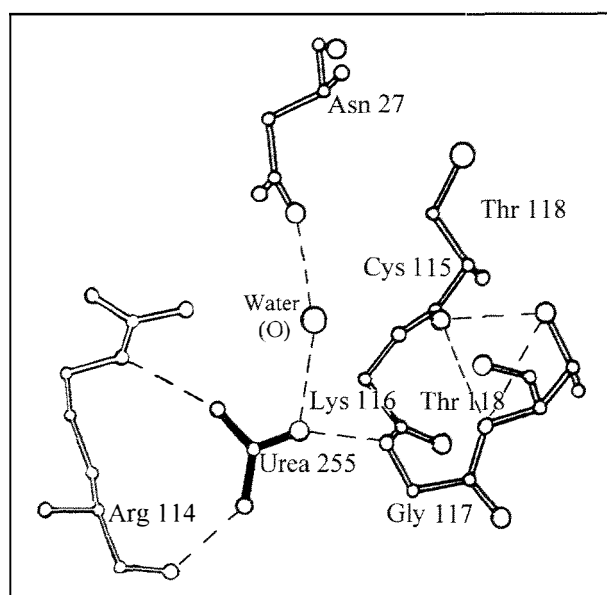


FIGURE 2.18: Ball and Stick Model of the Urea Binding Site in Hen Egg White Lysozyme which is proximal to Lysine 116 obtained from Enzyme Crystals grown in High Concentration Urea Solution

Hydrogen bonds shown as dashed lines

Modified from Pike and Acharya, 1994

The close proximity of the urea molecule to the 116 residue could affect the retention time of the conjugates by partially masking the positive charge of this residue and hence reducing its interaction with the cation exchange column. However, this would not affect the retention time for any conjugate substituted at lysine residue 116 (i.e. E2, E4 and E6 and the lysine 116 conjugate of the E1 peak), as the positive charge contribution from this residue has already been removed by acylation. However, this effect could theoretically reduce the retention times for lysozyme and all other conjugates (i.e. E3, E5 and the Lys 97 conjugate of the E1 peak) making them elute ahead of their equivalent lysine 116 substituted conjugate isomers. Thus, the contribution of lysine 116 to the positive electrostatic field may be underestimated in 7 M urea solutions.

Further, evidence that this is the case has been presented recently by Smales *et al.* (1998) who showed that the separation of lysozyme-E1G conjugates obtained by polyacrylamide gel electrophoresis (PAGE) in the absence of urea resulted in a migration order much closer to that predicted by simple net charge effects.

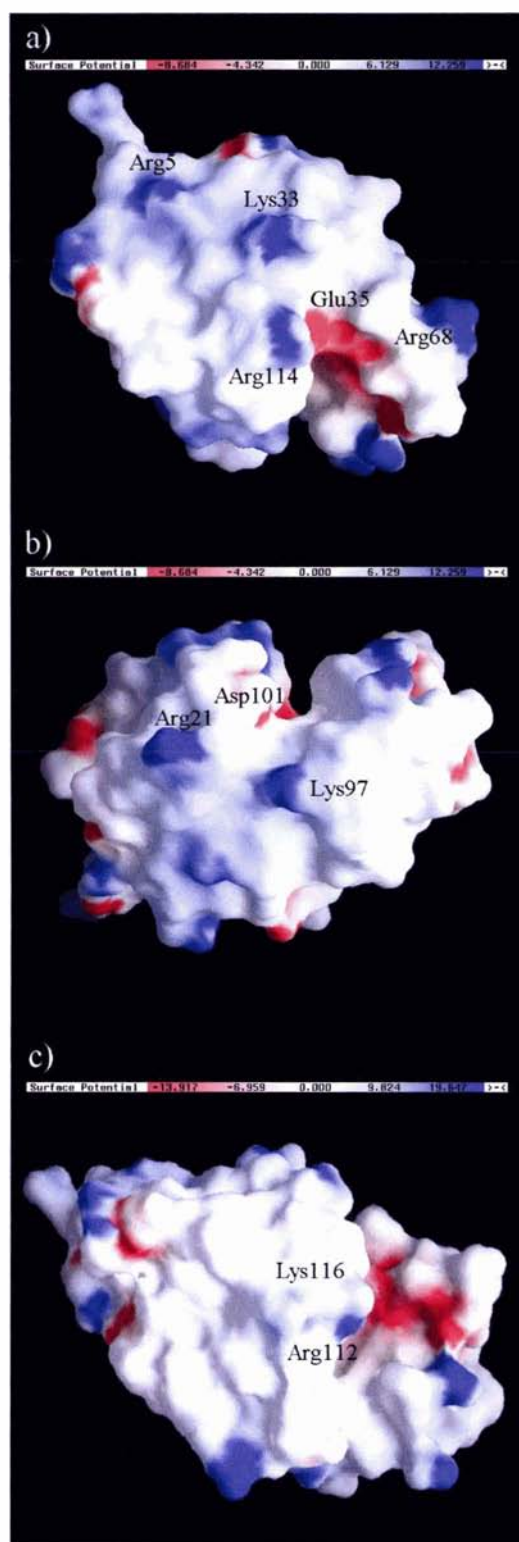


FIGURE 2.19: The Surface Electrostatic Fields surrounding the most favoured Lysine Conjugation Sites on Hen Egg White Lysozyme

a) Lysine 33; b) Lysine 97, and c) Lysine 116

Red - negative charge, blue - positive charge

If this partial masking of charge by urea together with the number of unconjugated lysine residues is taken into account when predicting the elution order from the column, it would be as follows: $E6 > E5 > E4=E2 > E3=E1(\text{Lys } 97) > E1(\text{Lys } 116) > \text{lysozyme}$, which is much closer to the observed order of elution.

The discrepancies between this predicted order, where the contribution to the electrostatic field by lysine 97 equals that of lysine 33, and the actual order, where lysine 33 appears to be more highly charged, is probably the result of the asymmetric distribution of charge on the molecule surface and its consequent effects on binding. Sites of high electrostatic potential on the surface of the protein can help orient its approach to a charged support (Kopaciewicz *et al.*, 1983), with the interaction between the resin and protein at multiple sites depending on the relative positions of charges within the domains of interaction (Brautigan *et al.*, 1978). This means that the reduction in charge of a highly positive region of lysozyme by conjugation of a lysine residue with estrone glucuronide, will affect the binding more than the removal of a positive charge from a less concentrated area of charge.

Figure 2.19 shows the electrostatic charge distribution around the three most favoured lysine conjugation sites as visualised using the program GRASP on a Silicon graphics workstation. This clearly showed the charge microenvironment around the three positions to be different. Lysine 33 (Figure 2.19a) due to its proximity to arginines 5, 68 and 114 is close to many positively charged areas of the protein but is not near any negative charges with the closest being glutamic acid 35 in the active site at 13 Å distant. In contrast, in the vicinity of lysine 97 there was a negative patch of charge (see Figure 2.19b) from the carboxyl group of aspartic acid 101 which sits 4.9 Å from the lysine ε-amino group. Although this negative patch of charge is too distant to constitute a salt

bridge it could well reduce the magnitude of the overall positive charge and interact unfavourably with the negative charges on the cation exchange matrix. Taken together, these two factors could account for the fact that lysine 33 is a more significant contributor to the positive electrostatic field than lysines 97 or 116 at pH 6.0.

Lysine 116 is only partly exposed (Figure 2.19c) and it is therefore not surprising that it produces the weakest electrostatic field, particularly when the fact that it is also shielded by a urea molecule is taken into account. Thus the order of conjugate elution from a cation exchange column appears to be determined by a combination of the local differences between charge distributions on the different conjugates as well as their overall net charge i.e. their level of substitution.

2.4.2.3 *Selection of Lysozyme-Estrone Glucuronide Conjugates for Further Study and the Effect of Substitution Level on the Specific Activity*

The specific activity of the lysozyme conjugates was also affected by their substitution level with the mono-, di-, and tri-substituted conjugates having an average specific activity relative to native lysozyme of 95 (E1 and E3), 69 (E2, E4 and E5) and 42% (E6) respectively. These results are consistent with the findings of Lee *et al.* (1975) who also found that the specific activity of conjugates decreased with the level of substitution. However, in the case of Lee *et al.* the change in specific activity was more dramatic than here with their di-substituted succinate conjugates having a specific activity of only 10% of the native rate, and all higher substituted conjugates having no detectable activity. Although no mono-substituted succinate conjugates were produced to allow comparison of their specific activities with those obtained with the mono-substituted estrone glucuronide conjugates, it is clear that the conjugation of lysozyme with succinate has the greater effect on lysozyme activity.

It is well known that the electrostatic attraction between the normally very basic lysozyme molecule and its negatively charged bacterial substrate is important for binding (Price and Pethig, 1986). The fact that in an estrone glucuronide conjugation a positive charge is only neutralised, while in a succinyl conjugation a positive charge is replaced with a negative one is probably responsible for the observed difference between the two sets of conjugate specific activities. Further evidence that it is charge effects which are responsible for the different relative specific activities observed between acylating agents is provided by Habeeb and Atassi (1971). These authors showed that lysozyme could be substituted at five amino groups without any loss in enzyme activity if the positive charge on the lysine residue was replaced by a positively charged guanidine molecule. Thus the effect on the specific activity of the introduction of a neutral estrone glucuronide group is intermediate between the addition of a positive charge and a negative charge. Of course the more charges which are neutralised the greater the effect on the specific activity.

The lower specific activity associated with the more highly substituted conjugates (E2, and E4-E6) makes them less suitable for use in the Ovarian Monitor based enzyme immunoassay as they decrease the sensitivity and precision of the assay (Daunert *et al.*, 1989). For example, if the enzyme conjugate concentration in an assay is increased to offset a low specific activity, the amount of antibody used in the assay must also be

increased to ensure that the rate of lysis for the zero standard point remains low. This combined increase of the conjugate and antibody concentrations would be expected to make the assay less sensitive to low levels of hormone. Alternatively, if an enzyme conjugate is used without correction for a low specific activity and the corresponding compensatory increase in antibody concentration, the rates of lysis at all steroid concentrations would be reduced, making the assay less precise.

The multi-substituted conjugates are also less suitable for use in immunoassays because of their intrinsically higher antibody binding capacity (Daunert *et al.*, 1989). For example, at any given concentration of conjugate, a di-substituted conjugate has the potential to bind twice as much antibody for the same amount of inhibition as a mono-substituted conjugate. This also makes the assay inherently less sensitive.

Because the multi-substituted conjugates, E2, E4, E5 and E6, have a higher number of hydrophobic groups than lysozyme and the mono-substituted conjugates, E1 and E3, they are also less soluble. This is most clearly illustrated by comparing the 7 M urea Mono-S profile of a pre-centrifugation conjugation reaction mixture with the Mono-S profiles of the supernatant and precipitate from the same conjugation (Figures 2.4b and c). These profiles showed that relative to the original reaction mixture and the supernatant, the precipitate had a much higher proportion of the early eluting (i.e. more highly substituted) conjugate peaks. The fact that the reaction mixture and the supernatant gave such similar profiles most likely reflects the fact that only a small proportion of the total sample is removed with the precipitate. This finding, combined with the high proportion of highly substituted conjugates in the precipitate fraction indicated that the losses in yield of immunoassay quality conjugate from the total conjugation mixture to the precipitate were not significant.

These profiles also provide an illustration of the serial dialysis method (Rubenstein *et al.*, 1974) and its disadvantages. If the dialysis performed here on a 3:1 steroid:enzyme active ester reaction mixture had been used for the purification of conjugate, at the end of the first step most of the conjugate would have been lost to the supernatant, while the level of highly substituted conjugates would have increased, especially the E7-E9 conjugates which were proportionally 4 fold higher in the precipitate fraction than the supernatant. Despite the increasing amounts of the highly substituted conjugates, the purity of the precipitate fraction from lysozyme was also low with the sample still being contaminated with relatively high levels of lysozyme (6%) compared to the total reaction mixture (10%).

Although the E2, E4, E5 and E6 conjugates were present in high yield as these multi-substituted conjugates had low solubilities and specific activities and an excessively high antibody binding capacity, they are not suitable for use as signal generators in the Ovarian Monitor immunoassay, and only the mono-substituted conjugates, E1 and E3, were investigated in detail.

The two mono-substituted conjugates which make up the E1 peak, i.e. the lysine 116 and lysine 97 conjugates, always co-eluted under all chromatographic conditions investigated. In contrast, isolation and recovery of these conjugates could be achieved by gel electrophoresis, but only on an analytical scale (Smales *et al.*, 1998). Thus, for simplicity the two E1 conjugates were studied as a mixture as without a simple means of purifying

them in preparative amounts they could never be used individually in the pre-coated Ovarian Monitor assay tubes. The only other mono-substituted conjugate to be produced in any quantity was the lysine 33 conjugate, the sole constituent of the E3 peak, which was also purified on a large scale and investigated in detail.

2.4.2.4 *Control of the Conjugation Reaction*

The proportions and range of the conjugate products changed according to the conjugation method used. For example at near stoichiometric ratios of steroid to enzyme, the most predominant conjugate species for the mixed anhydride method was E3, while for the active ester method the major conjugate products were E1 and E3. However the steroid:activating agent ratio, their absolute concentrations in the activating vessel, and the steroid:enzyme ratio were also important determinants. For example if the amount of activating agent was increased, or the reaction volume in the activating vessel decreased while keeping the amounts of steroid and activating agent the same, the overall conjugation rate, the percentage of the highly substituted conjugates produced and the total range of conjugate products were all increased. The same effect was observed when the steroid:enzyme ratio was increased. For example when using a 3:1, as opposed to a 1.5:1, ratio for the active ester conjugation of estrone glucuronide to lysozyme, although the total amount of conjugate increased, it was at the expense of the mono-substituted E1 and E3 yield. Thus, although the choice of conjugation method and manipulation of the reaction conditions did allow some degree of control over the distribution of conjugate in the final product, any optimisation of the reaction conditions for the increased yield of mono-substituted conjugates resulted in a reaction mixture where unreacted lysozyme made up a significant proportion of the total product.

In summary, a successful lysozyme conjugation resulted in a reaction mixture that contained a mixture of unreacted steroid, lysozyme, and a range of lysozyme conjugate products of which the E1 and E3 conjugates were the dominant forms.

2.4.3 Large Scale Purification of Hen Egg White Lysozyme Estrone Glucuronide Conjugates

Although no details have been published, currently all conjugate used in the pre-coated Ovarian Monitor assay tubes is made by the mixed anhydride method and purified from native (i.e. unreacted) lysozyme by cation exchange chromatography without urea (Brown, personal communication). In this method, aliquots of the reaction mixture are passed through a CM-Sepharose column equilibrated with 10 mM phosphate buffer (pH 6.0) and the column eluted with a sodium chloride gradient. This results in the elution of a single broad peak, the trailing edge of which contains increasing levels of conjugate. Fractions from the trailing edge must then be rechromatographed several times to remove all contamination of the sample by free lysozyme before it can be utilised in the Ovarian Monitor.

Analysis by Mono-S cation exchange chromatography in 7 M urea (Cooke, 1993; Smales *et al.*, 1994) of estrone glucuronide conjugate, prepared and purified by the above procedure, has shown it to consist mainly of the E3 family, with smaller amounts of E1 and E2. This is consistent with the distribution of conjugate in the mixed anhydride reaction mixture where E3 is shown to dominate (Cooke, 1993; Smales *et al.*, 1994).

Lysozyme conjugate purified by the Melbourne large scale CM-Sepharose procedure has a percentage inhibition equal to, or greater than, 95% in the presence of excess hapten specific antibody, and a high specific activity. However, the method is also only partially reproducible, as although the conjugate and free lysozyme fractions usually elute as a single peak, the separation can vary, and sometimes the conjugate and lysozyme peaks are partially resolved. Not only is this procedure limited in terms of resolution and reproducibility, but also in terms of scale. The soft nature of the CM-Sepharose resin means that it is easily compacted under pressure which limits the size of the column which can be used and consequently the amount of conjugate which can be purified in a single chromatographic run. Thus, although conjugate of high quality is produced, alternative purification schemes which allow the large scale purification of individual conjugates are still required.

2.4.3.1 *Attempted Isolation and Purification of Lysozyme Conjugates E1 and E3 without Urea*

Because the Mono-S cation exchange column gives excellent separation of the different conjugates in the presence of 7 M urea initial attempts to purify the conjugates in the absence of urea were also performed using Mono-S chromatography with the aim of scaling up to the larger cation exchange S-Sepharose fast flow column. Previous work (Cooke, 1993) has shown that the elution order for lysozyme and its conjugates in non-urea based purification schemes on the Mono-S column is highly pH dependent. The conjugate fraction elutes last at low pH, first at high pH, and co-elutes with lysozyme at pH 6.0. This behaviour at pH 6.0 is also consistent with the elution behaviour observed in the current preparation of conjugate for the Ovarian Monitor which is also performed using cation exchange chromatography at pH 6.0, but with a CM-Sepharose resin.

This result at pH 6.0 on the Mono-S and CM-Sepharose columns in the absence of denaturing buffers is in complete contrast to the results obtained on the Mono-S column at pH 6.0 in the presence of 7 M urea where the conjugate elutes in discrete peaks well separated from the lysozyme peak (see Figure 2.3) and the entire separation can be explained by simple charge effects (see Section 2.4.2.2). Clearly, the separations observed in the non-urea based Mono-S purification schemes must be determined, at least in part, by other factors. In this work, attempts to purify conjugate without urea on the Mono-S column were made at pH 4.3 and 9.5. The basis for the two separations were clearly different as using the optimised pH 4.3 gradient, the bulk of the lysozyme fraction eluted first (see Figures 2.5 and 2.6), while with the optimised pH 9.5 gradient, lysozyme eluted last (Figures 2.7 and 2.8d) in common with its elution position under the standard 7 M urea, pH 6.0 conditions.

Using the gradients reported in this thesis, at pH 4.3, a salt concentration of 0.35 M sodium chloride was required to effect elution of the main lysozyme peak, while at pH 9.5 a much narrower peak was obtained with only 0.2 M salt. This is in agreement with the findings of Gupta *et al.* (1983) who have also observed that a decrease in pH is associated with a broadening of the lysozyme peak and an increase in the salt concentration required for its elution. The change to lysozyme retention with pH is not unexpected since the net positive charge on lysozyme will increase with decreasing pH. Thus as the pH is decreased a progressively higher salt concentration will be required to overcome the stronger electrostatic attraction between the sample and negatively charged cation exchange resin. This difference in the salt concentration required for the elution of the lysozyme peak at high and low pH values may also explain the difference in the elution order of the main conjugate peaks relative to the lysozyme peak as observed in this work.

At high salt concentrations protein solubility is decreased, and protein interactions with other protein molecules or other more hydrophobic compounds are favoured. Thus, any separation procedure which requires a high salt concentration for elution, will also be associated with an increase in interactions between any hydrophobic regions of the sample and any hydrophobic regions of the resin matrix. This means that a resin which is not customarily used for hydrophobic interaction chromatography may begin to display some hydrophobic properties under these conditions. For example, a Pharmacia Mono-S column with its sulpho groups is a strong cation exchanger but its matrix is based on cross-linked polystyrene beads (Henry, 1990) which could theoretically contribute some hydrophobic characteristics to the resin.

This cation exchange and hydrophobic partitioning with changing salt concentration has already been demonstrated on a size exclusion column for lysozyme which showed the protein retention time to decrease with increasing salt and then increase again i.e. the retention times were minimal at intermediate salt concentrations when the partitioning between both the organic and charged portions of the resin was minimal (Pfannkoch and Regnier, 1980).

In the case of the pH 4.3 separation (see Figure 2.5), the higher salt concentrations required for the elution of lysozyme and its conjugates due to their increased charge at this low pH enhances any hydrophobic interactions between the sample and the column. Because

of the conjugate was distributed across three peaks. However, none of these three conjugate peaks were homogeneous (see Figure 2.8a-c), and some conjugate was also present in the lysozyme peak. A possible reason for the failure of these two procedures to produce a single homogeneous conjugate peak may be due to intermolecular associations between lysozyme and the conjugates, or different conjugates with each other as it is well known that at high protein concentrations lysozyme will form complexes with itself (Hampe *et al.* 1982).

With respect to the low pH separation, the high salt concentration required for the elution of the conjugates may also cause a salting out effect increasing the hydrophobic attraction between the conjugates. However, at pH 9.5, the repulsive forces between lysozyme and its conjugates would be decreased reducing the positive charges on both the lysozyme and conjugate molecules. Thus while at low pH the main conjugate band was contaminated only with other conjugates (i.e. other molecules with hydrophobic patches) at high pH all the conjugate peaks were contaminated with lysozyme as well due to an increase in electrostatic interactions. Association complexes form more readily in conditions of high concentration thus it is also possible that their formation during ion exchange chromatography may be encouraged as the samples become more concentrated on the column. As denaturing mobile phases are known to allow the separation of associating systems (Hancock and Sparrow, 1984), this effect may also explain the excellent separation of lysozyme and its conjugates achieved with the 7 M urea buffers systems which showed no cross contamination of the different conjugate families with lysozyme.

In contrast to the results from the Mono-S column, analysis of the conjugation reaction mixture by acid PAGE, which separates protein on the basis of size and charge, did not show any evidence for the formation of association complexes (Smales *et al.*, 1998). However, because the gel separation is performed in the absence of salt, any hydrophobic interactions are minimal. Similarly, the low pH which would make a very basic protein even more positively charged than normal ensures maximum repulsion between molecules. Thus, given that both the inter and intra electrostatic and hydrophobic attraction between lysozyme and conjugate are minimal under these conditions, the fact that the results from the gel electrophoresis did not show any evidence of association complexes, is not unexpected.

Finally, the lack of a second discrete peak of conjugate at pH 4.3 (Figure 2.6) was disappointing since earlier work (Cooke, 1993; Smales *et al.*, 1994), had shown that a separation was possible in the absence of urea. However, during this thesis the separation always gave only a broad band richer in a mixture of E1-E6 than the main peak, but no discrete second peak, and the profile was always similar to that shown in Figure 2.6. The reason for this difference is not obvious but given the hydrophobic character of the Mono-S column it is possible that during the long term heavy use of the column, its hydrophobic character has changed. It is conceivable that repeated exposure to steroid-lysozyme conjugates has coated the hydrophobic patches of the column with a layer of hydrophilic protein. Regardless of the reason for this change in column behaviour, the fact that the separation could not be repeated meant that further attempts at purification at pH 4.3 using the Mono-S column were abandoned. Although the work presented in this section provided some interesting insights into the basis of this separation on the Mono-S column under a

steroids are highly non-polar, any such hydrophobic interactions will be much more pronounced with the steroid conjugates than with lysozyme itself. Thus as the salt concentration is increased, the conjugates may become adsorbed more tightly to the column and their elution delayed relative to lysozyme despite the lower overall charge on the conjugates. A separation based on the above interactions would be expected to give an elution profile similar to the one observed (see Figure 2.5). Thus the retention time on the Mono-S column for steroid conjugates in the presence of high salt appears to be dependent upon the sum of two opposing forces. Only when the increased hydrophobic interactions are cancelled out by the displacing effect of the increased number of ions in solution will elution occur.

Spiking the buffers with 2-propanol has been used previously to check for hydrophobicity effects of the Mono-S column (Kopaciewicz *et al.*, 1983) while lysozyme and cytochrome c have both been previously used for determining gross column hydrophobicity (Pfannkoch and Regnier, 1980). When Kopaciewicz *et al.* compared the retention time for both lysozyme and cytochrome c on the Mono-S column at pH 4.5, 5.5 and 6.5, the retention times were found to be largely unaffected by the presence of 2-propanol. Thus, the authors concluded that the Mono-S column had little if any hydrophobicity. We also found that with our salt gradient optimised for low pH, that 2-propanol had little effect on the elution time for lysozyme (Smales *et al.*, 1994). However, under these conditions the elution time for the conjugates decreased causing them to co-elute with the lysozyme peak. Thus, it appears that the Mono-S column does have some underlying hydrophobic character, but it requires localised regions of very high hydrophobicity on the sample and a high ionic buffer strength for it to be displayed.

At pH 9.5 it would be expected that the hydrophobic interactions would be of minor importance because the lysozyme and conjugates are not so highly charged and hence required a lower salt concentration for their elution. Thus at high pH any hydrophobic interactions would be minimised and the order of elution of lysozyme and its conjugates would largely be determined by their relative charges with the most highly charged fractions eluting last as observed experimentally (see Figure 2.8a-d).

The fact that the order of elution of the conjugate peaks at pH 9.5 in the absence of urea was broadly similar to the order of elution observed at pH 6.0 with the standard 7 M urea separations, confirms that electrostatic effects predominate under both conditions. Certainly the ability of urea to diminish hydrophobic forces is very well established (Warren, 1970; Berthou and Jollès, 1973; Pike and Acharya, 1994), thus it is highly probable that the improvement in the separation of the conjugates due to the presence of urea is the result of a reduction in the hydrophobic interactions between the matrix and the sample. This factor would also help to explain why at pH 6.0 in the absence of urea, the conjugate and lysozyme fractions co-elute, while at the same pH in the presence of urea the conjugate fraction eluted before lysozyme.

Although hydrophobic interactions can explain the difference in the elution order observed for the pH 4.3 and 9.5 separations in the absence of urea, it is less clear why the two conditions gave different resolutions for the conjugate fractions. In the pH 4.3 separation, the conjugates (E1-E6) eluted as a single, broad, asymmetric band while at pH 9.5 the bulk

variety of conditions, neither the pH 4.3 and 9.5 buffer systems were successful in producing pure E1 and E3 samples free from contamination by lysozyme or other conjugates. Because one of the aims of this work was to separate mono-substituted conjugates from each other to enable them to be compared, the use of the Mono-S column without urea was abandoned as a practical method for providing this separation.

2.4.3.2 *The Purification of Lysozyme Conjugates using Urea*

Urea is now used routinely in our laboratory on an analytical scale for the analysis of conjugation reaction mixtures and assessment of purification procedures by cation exchange chromatography (Cooke, 1993; Smales *et al.*, 1994) and gives an excellent separation of conjugates from free lysozyme with high reproducibility (Figure 2.3). However, because of urea's well known ability to denature proteins, concerns about incorrect refolding has meant that the use of urea in purification schemes has generally been avoided.

The effect urea has on the separation of a conjugation reaction mixture on the Mono-S column is considerable. Unlike the separations performed in the absence of a denaturing agent, 7 M urea based separations were largely pH independent. In fact the 7 M urea pH 6.0 and 9.5 separations were similar, in the sense that both consisted of a series of six moderate to large earlier eluting conjugate peaks followed by a lysozyme peak. Because the relative peak heights were similar between the two different pH conditions, this suggests that the peak contents were also the same. Thus, the peaks from the pH 9.5 separation are also likely to be pure, in stark contrast to the non-urea separations. Finally, in comparison with the broad peaks characteristic of the non-urea separations, all analyses of the reaction mixture performed in the presence of 7 M urea resulted in the elution of peaks which were well resolved and very sharp. The sum of these differences between the two sets of conditions add up to show the superior separation which can be offered by a urea based purification scheme.

Most of urea's effects on the separation can be explained in terms of its water structure breaking properties which act to decrease surface tension and hence weaken any hydrophobic interactions (Warren and Gordon, 1970; Pike and Acharya, 1994; Berthou and Jollès, 1973; Henry, 1990).

For example, at a concentration of 7 M urea any hydrophobic interactions between the sample and the column or the sample with itself are clearly drastically reduced and the separation is determined mainly by electrostatic interactions.

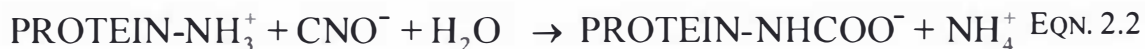
Secondly, the purity of the peaks, as discussed in Section 2.4.3.1, is probably due to the high urea concentration preventing the formation of any protein-protein complexes and thus allowing the unmodified lysozyme molecules and the conjugates to elute as single entities (Hancock and Sparrow, 1984).

Finally, compared to the non-urea separations, the elution of lysozyme and its conjugates were effected at a much lower salt concentration when urea buffers were used. This is consistent with previous observations where it has been reported that urea reduces the

retention time for the isocratic elution of lysozyme from a cation exchange column (Parente and Wetlaufer, 1984). This effect of urea is attributed to its ability to increase the dielectric constant of the buffer (Parente and Wetlaufer, 1984). As any increase in the dielectric constant reduces the attractive forces between the protein and the resin (Kopaciewicz *et al.*, 1983), then the salt concentration required for elution is also reduced.

Thus all the observed effects of urea on the separation can be explained without requiring any large structural changes to the enzyme. This is in agreement with other lysozyme studies (Warren and Gordon, 1970; Parente and Wetlaufer, 1984; Hamaguchi and Rokkaku, 1960) including analysis of the lysozyme-urea crystal structure (Pike and Acharya, 1994), which all showed urea to cause minimal changes to the structural integrity of the protein. Hence, it appears that concerns about the permanent denaturation of the lysozyme conjugates from the use of urea based purification schemes may be unwarranted.

Although the above findings suggest that urea causes no serious changes to the tertiary structure of lysozyme, fear of denaturation is not the only perceived hazard of using urea in an enzyme preparation. Its use is also avoided because of the potential exposure of the protein to cyanates (Equation 2.1) which can cause carbamylation of the amide groups (Equation 2.2).



As can be seen by the above equation, each event of carbamylation replaces a positive charge with a negative one, causing the net positive charge on a protein to decrease by two. Since the net charge of a protein changes with carbamylation, its elution characteristics on an ion exchange column will be modified.

• Obviously such an event would have serious repercussions on the purification of lysozyme conjugates for immunoassay. If lysozyme is carbamylated at one site, the unconjugated enzyme would have the same overall charge as a di-substituted lysozyme conjugate, and thus may have the same elution characteristics on cation exchange as the di-substituted conjugates resulting in their co-elution.

Carbamylation of a single lysine residue has little affect on the enzyme activity so a conjugate sample contaminated with carbamylated lysozyme would have good specific activity (i.e. rate per unit of protein), but the apparent inhibition would be reduced by the same percentage as the level of contamination. Due to the difficulties in separating carbamylated lysozyme from conjugate, it is important that carbamylation is avoided at all costs in the production of conjugate for use in the Ovarian Monitor.

Because carbamylation adds to the molecular weight of a protein by 43 atomic units, contamination of a lysozyme or lysozyme conjugate sample with carbamylated protein can be detected easily by electrospray mass spectrometry. In the work presented in this thesis, no peaks due to carbamylation of the protein were found (see Figures 2.16a and b) if the

buffers were made up fresh and kept cold, and the sample was dialysed immediately out of the urea buffer after elution.

Although it was not the initial intention, for the isolation of conjugates in high yield it was found that the use of urea could not be avoided and the final purification procedure involved an initial urea based cation exchange step followed by hydrophobic interaction chromatography without urea. However, the fact that highly inhibitable conjugates with high specific activity and no carbamylation were isolated using this procedure, provides strong evidence that the urea used in the purification did not cause any permanent changes to the structural and immunological integrity of the conjugates and in fact a preparation of E3, purified in this manner has remained stable and immunologically active for over three years.

The first step of the purification scheme was essentially a scaled up version of the standard analytical method, with both methods using a strong cation exchanger in conjunction with 7 M urea buffers and a linear salt gradient to effect elution. Under the reported conditions both the Mono-S and S-Sepharose fast flow columns gave essentially identical profiles but the resolution of the peaks on the S-Sepharose fast flow column was lower as must be expected for a much larger column. Although the final purified product was highly inhibitable, the conjugate purified at the end of this first step was not. This poor inhibition was also a characteristic of conjugates purified with and without urea using the Mono-S column. In contrast, conjugates purified on the cation exchange CM-Sepharose column without urea were highly inhibitable (>95%) without requiring passage through another column (Brown, personal communication). Because the CM-Sepharose column could be used to produce heterogeneous mixtures of good quality conjugate, whereas the separations performed in both the presence and absence of urea on the Mono-S column resulted in poor quality conjugate, this suggests that it is not the use of cation exchange chromatography or urea *per se* which is responsible for the low inhibitions observed. The fact that the S-Sepharose fast flow and the Mono-S resins are strong cation exchangers compared to the much weaker CM-Sepharose resin may be responsible for the differences in the quality of the conjugate produced on the three columns. One possibility is that the strength of the electrostatic interaction with the strong cation exchange columns induces small conformational changes which affects the antibody binding. For example, if the binding constant with the antibody was lowered, this could lead to a faster dissociation rate for the antibody from the conjugate. If this was the case, then rapid equilibration during the twenty minute assay time could favour the formation of the free conjugate. Thus, the rate of lysis would increase during the assay time accounting for the apparent low degree of inhibition.

Interestingly, this low inhibition returned by the conjugates after S-Sepharose fast flow or Mono-S cation exchange chromatography was readily reversed upon their passage through a Butyl Sepharose or Alkyl Sepharose hydrophobic interaction column. Thus if the reason for the low inhibition after the cation exchange step is due to a conformational modification, then it follows that the passage of the conjugate through a hydrophobic interaction column reverts it back to its native state. Although the mechanism for this cannot be determined from this work, it is possible that the binding of the hydrophobic steroid groups to the resin, allows the protein to rearrange itself into its native form.

The excellent separation of conjugate with highly inhibitory properties obtained by the Butyl Sepharose column suggests that this procedure could be used for a one step purification. However, previous experience with the Alkyl Superose column precludes the use of a hydrophobic interaction column for the purification of conjugate directly from the conjugation reaction mixture. When the Alkyl Superose column has been used to purify conjugates contaminated with large amounts of unreacted lysozyme, elution of a large proportion of the lysozyme fraction is retarded and co-elutes with the conjugate.

This effect where the predominant species co-elutes with the other components of the sample was also observed on the Butyl Sepharose column in the purification of both the E1 and E3 partially purified samples. Although in both cases the main peak was pure for the conjugate of interest, it also dominated the composition of all the other fractions in the profile as well. For example in the E1 purification (Figures 2.12 and 2.14a and b), although the primary contaminant was the di-substituted E2 conjugate which eluted later than the main E1 peak as expected for a compound with a greater hydrophobicity than E1, some E1 always co-eluted with it. Likewise, in the E3 purification (Figures 2.13 and 2.15a-d), where the chief contaminants were lysozyme and the di-substituted E4 conjugate, lysozyme eluted before the main E3 peak and the E4 conjugate eluted after the E3 peak as predicted by their relative hydrophobicities. However, both the main lysozyme and E4 fractions were still dominated by E3. Thus, it appears to be impossible to separate the different lysozyme steroid conjugates from each other and lysozyme in a single chromatographic step while maintaining the inhibitory integrity of the conjugates. Possibly if the lysozyme contamination could be reduced to less than about 20% then a hydrophobic interaction column could be used to give a mono-substituted highly inhibitable conjugate in a single chromatographic step. However, given the effect that increasing the conjugation rate has on the yield of mono-substituted conjugates, this seems unlikely.

Hence, the two step isolation and purification procedure reported in this chapter, is currently the best method for the large scale purification of mono-substituted hen egg white lysozyme-estrone glucuronide conjugates, free from lysozyme contamination.

2.5 CONCLUSIONS

Based on the work presented in this chapter, there is no evidence that the use of urea for the purification of lysozyme conjugates has any irreversible detrimental effects upon lysozyme or its conjugates. Given the superior separation achieved by urea based cation exchange chromatography over non-urea based separations the use of urea forms an essential part of the first step in the purification of high quality conjugate in high yield. Although the S-Sepharose fast flow cation exchange column produces conjugate which is only poorly inhibited, this low inhibition is readily reversed in a single step by passing the conjugate through a Butyl Sepharose hydrophobic interaction column. Analysis of the thus purified conjugate by electrospray mass spectrometry and 7 M urea Mono-S chromatography confirmed that individual conjugates produced by this method had a very high level of purity (>99%).

Thus, high quality E1 and E3 conjugate were purified from a conjugation reaction mixture in good yield by a simple two step chromatographic procedure. This allowed the two main conjugates from an active ester conjugation to be characterised and compared for their suitability as a signal generator in the Ovarian Monitor immunoassay system as described in Chapter Three.

CHAPTER THREE

STRUCTURALLY DEFINED HEN EGG WHITE LYSOZYME - ESTRONE GLUCURONIDE CONJUGATES AS SIGNAL GENERATORS IN HOMOGENEOUS ENZYME IMMUNOASSAYS FOR MEASUREMENT OF ESTRONE GLUCURONIDE

3.1 INTRODUCTION

The key components of the Ovarian Monitor assay include the lysozyme-steroid glucuronide conjugates as signal generators, the anti-steroid glucuronide antibodies, the *Micrococcus lysodeikticus* substrate, and the steroid glucuronide standards necessary for the construction of the standard curves. In this chapter the assay characteristics of these biomaterials have been analysed and in particular, the effect of the structurally isomeric lysozyme-estrone glucuronide conjugates E1 and E3 on the estrone glucuronide assay and the measurement of urinary estrone glucuronide concentrations has been studied.

3.1.1 The Substrate and Enzyme Activity Measurement

The Ovarian Monitor lytic assay is based on the hydrolytic activity of hen egg white lysozyme against the cell wall of the gram positive lyophilised bacterium, *M. lysodeikticus*. More specifically the enzyme cleaves the C1-C4 glycosidic bond of the *N*-acetyl glucosamine (NAG) and *N*-acetyl muramic acid (NAM) repeating units of the polysaccharide component of the cell wall (Stryer, 1981b). The site of cleavage is shown in Figure 3.1.

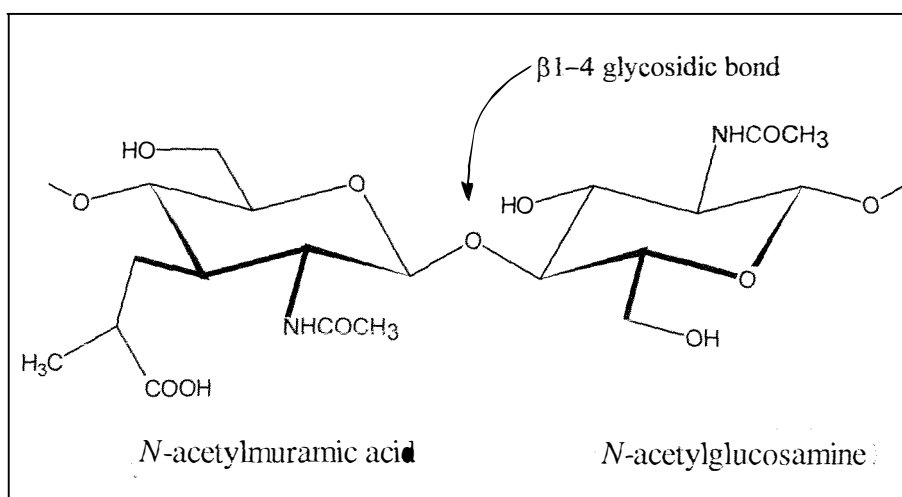


FIGURE 3.1: Hen Egg White Lysozyme Cleavage Site of the Polysaccharide Component of the *Micrococcus lysodeikticus* Cell Wall

The carbohydrate chains are cross-linked into a three-dimensional lattice by small negatively charged peptides between the lactic acid groups of some of the *N*-acetyl muramic acid residues (Matsumura and Kirsch, 1996). The cross-linking peptides confer a high negative charge to the cell wall while the high proportion of basic residues on the lysozyme molecule makes the enzyme highly positively charged. The importance of the electrostatic attraction in the lytic reaction, is well illustrated by the reduction in lysozyme's specific activity which occurs at high levels of acylation of the six surface lysine residues with neutral or negatively charged groups (Habeeb and Atassi, 1971).

The standard assay for lysozyme activity is unusual compared with most enzyme assays as it measures changes in the number of whole cells as opposed to changes in the concentration of a small defined chromophoric molecule. When lysozyme's direct catalytic activity (i.e. the hydrolysis of glycosidic bonds) is compared with the catalytic activity of other enzymes, it scores relatively poorly (Gorin *et al.*, 1971). However, when whole cells are used as the substrate, lysozyme generates a very large change in the apparent absorbance for a given enzyme concentration. This is because in the lytic assay the observed absorbance is due to the physical obstruction of the light path by the intact cell wall. As the enzymatic cleavage reaction between lysozyme and the cell wall substrate proceeds, the walls of the numerous *M. lysodeikticus* cells are weakened and ultimately collapse from the internal osmotic pressure, resulting in a very large change in absorbance for a relatively small number of catalytic events.

The cell lysis based assay is not only far more sensitive than the hydrolytic assay using chromophoric substrates, it is essential for the performance of the lysozyme homogeneous immunoassay developed by Rubenstein *et al.* (1972) and utilised by the Ovarian Monitor (Brown *et al.*, 1988, Brown *et al.*, 1989). This assay is dependent upon the steric exclusion of a macromolecular substrate upon antibody binding extensively inhibiting (over 90%) the activity of a lysozyme-hapten conjugate.

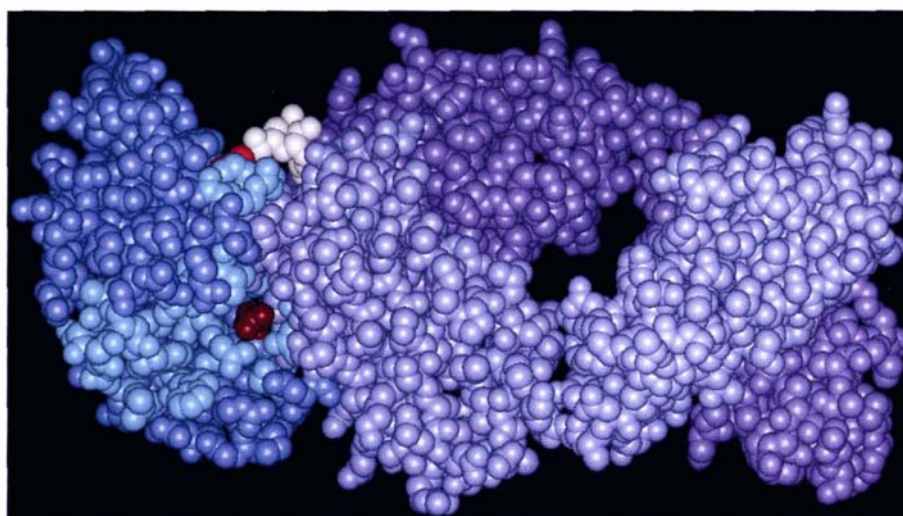


FIGURE 3.2: Steric Occlusion of the Substrate Binding Site of Hen Egg White Lysozyme-Estrone Glucuronide E3 Conjugate by Antibody Binding to the Steroid Moiety

Enzyme: general protein - medium blue, binding site cleft - pale blue, Asp 52 active site residue - brown, Lysine 33 - red; Estrone glucuronide - white; Antibody: heavy chain, purple, light chain - lilac

Figure 3.2 shows a model of an antibody binding to the steroid moiety of hen egg white lysozyme E3 conjugate (Dr. C. M. Smales, University of Kent, Canterbury, United Kingdom). As a crystal structure for anti-estrone glucuronide antibody was not available this model was generated using the co-ordinates from the Fab portion of a progesterone monoclonal antibody structure. Thus, the glucuronide ring is still visible as the model antibody lacked an antigenic determinant for it. However despite the poor complementarity between estrone glucuronide and the anti-progesterone antibody, the model still clearly illustrates the steric occlusion by antibody of the six sugar substrate binding site of the enzyme.

Figure 3.2 also clearly shows the extensive nature of the substrate binding site. Steric inhibition of enzyme activity by indirect antibody binding as is observed with lysozyme is unusual and inhibition of the lytic assay really reflects steric inhibition of the access of the intact cell wall to the binding site as opposed to just the enzyme's six unit substrate requirements. This steric inhibition of lysozyme activity by indirect antibody binding is probably also partly due to the disproportionately large size of the substrate binding site on a very small enzyme.

Despite the very obvious advantage of an assay which shows intrinsic amplification, measuring lysozyme activity indirectly, in common with many indirect assays, does have some added complications. In the case of the lysozyme assay, catalytic activity and cell lysis and thus consequently the rate of absorbance change do not necessarily proceed in parallel. For example in hypertonic solutions, lysozyme activity and cell lysis can be completely uncoupled (Colobert and Lenior, 1957) as the cell is no longer dependent upon the cell wall for its mechanical support. However, this obviously requires very specific reaction conditions.

Of more relevance, is the fact that lysozyme activity is not specific to the whole cell. Since lysozyme can attack the intact cell wall as well as the resulting cell wall fragments, cell lysis and collapse do not necessarily result in a decrease in the amount of substrate available to the enzyme (Gorin *et al.*, 1971). Thus although the enzyme's actual catalytic activity may not be affected with time by the removal of the intact cells, as the level of remaining intact cells is decreased with time so is the apparent rate of absorbance change. This means that although the initial rates of both the hydrolytic cleavage of the NAM-NAG polymer and the indirect cell lytic assays may be linear, a lytic end point assay which measures changes in absorbance between two time intervals, will show more curvature with time. This also means that in an end point assay, the whole cell wall assay will show less percentage changes in rate at the higher enzyme concentrations than at lower ones. Because of the non-linear relationship between transmission and absorbance, this effect of increasing curvature with increasing enzyme concentration is magnified when the end point rates are measured in transmission.

These differences are of particular significance to the Ovarian Monitor system, where, in the interests of cost effectiveness which is a major consideration of a home assay system, the rate is measured as a change in transmission end point assay.

3.1.2 Hen Egg White Lysozyme Steroid Glucuronide Conjugates as Signal Generators

The Ovarian Monitor assay is critically dependent upon the lysozyme conjugate (i.e. signal generator) and the steroid glucuronide antibody (signal mediator). Ideally the enzyme conjugate should have the following characteristics:

- i) a high specific activity i.e. the conjugation of hapten to the enzyme should have a minimal effect on the enzyme's activity,
- ii) an activity which is highly inhibited (minimum 95%) when mixed with saturating levels of anti-hapten antibody to ensure a low background lytic activity,
- iii) contain only one hapten molecule per enzyme molecule to ensure maximal assay sensitivity i.e. every instance of binding of antibody to the enzyme should result in a reduction in enzymic activity, and
- iv) have its long-term stability unaffected by conjugation.

The ideal antiserum for a homogeneous immunoassay should complement these features. Thus it should:

- i) be capable of causing high inhibition (at least 95%) of the enzyme conjugate activity.
- ii) require low volumes of serum to achieve maximal inhibition (i.e. have a high titre),
- iii) have a high specificity towards the hapten of interest and a low level of non-specific binding, and
- iv) have a rapid association rate and slow dissociation rate of binding i.e. a low dissociation constant.

For an assay based on the steric inhibition of the enzyme activity by anti-hapten antibody binding, it follows that the sites of hapten conjugation on the enzyme must be located close to the active site region. For lysozyme, only three of its six lysine residues are readily conjugated to estrone glucuronide by the active ester and mixed anhydride reactions. However, all three positions (lysine's 33, 97 and 116) are located close to lysozyme's active site.

The distribution of lysine residues around the binding site of hen egg white lysozyme is shown in Figures 3.3a and b. Lysine 116 is positioned under the mouth of the active site cleft, lysine residues 96 and 97 to one corner of the mouth and lysine 33 on the other. In comparison, lysine residues 1 and 13 are positioned well away from the cleft on the opposite side of the molecule.

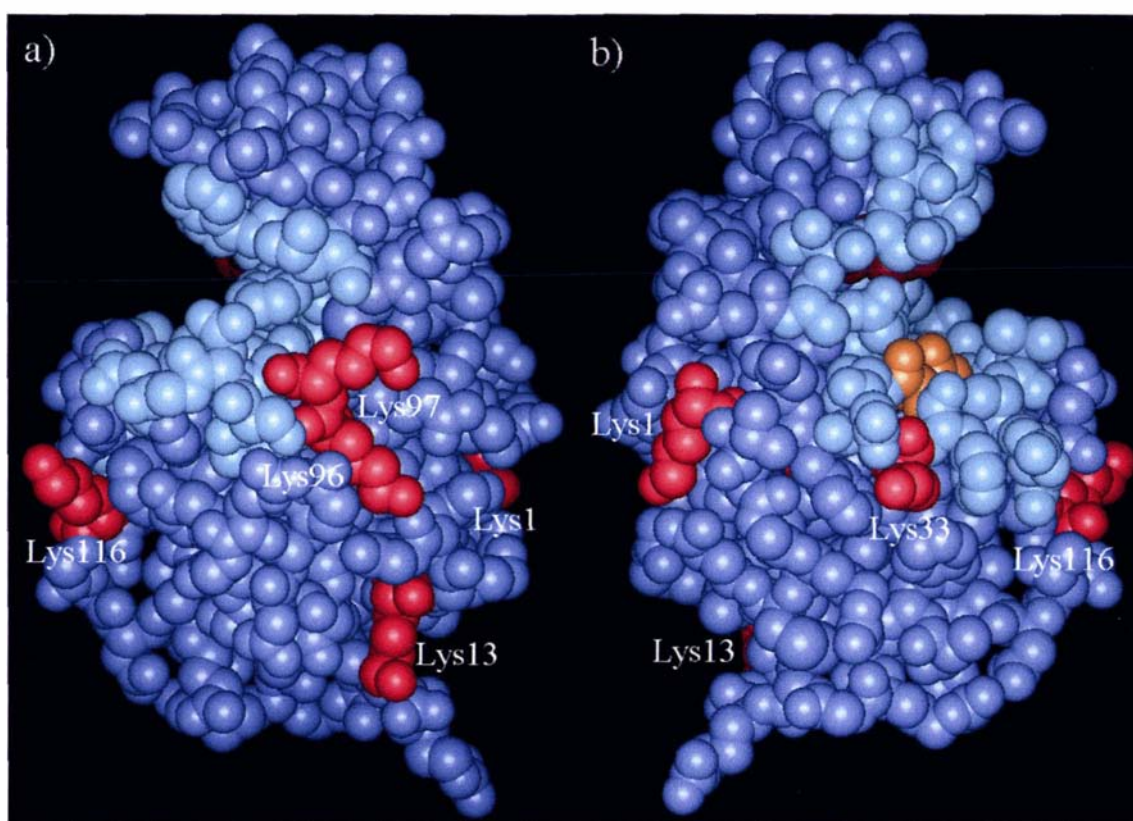


FIGURE 3.3: View of Hen Egg White Lysozyme from Opposite Sides showing the Positions of the Six Lysine Residues around the Substrate Binding Site

General protein - medium blue, Binding site cleft - pale blue, Glu 35 active site residue - orange, Asp 52 active site residue - brown, Lysines - red

The two predominant conjugate fractions (E1 and E3) from the active ester conjugation mixture were both considered suitable for investigation as immunoassay signal generators as they were mono-substituted and showed very little decrease in their specific activities relative to the native enzyme.

Although the E3 conjugate fraction was substituted solely at lysine 33 the E1 sample consisted of a 60:40 mixture of lysine 97 and 116 mono-substituted conjugates. No column procedure was found which separated the two E1 conjugates, and because they could not be readily separated on a large scale, it is unlikely that it will ever become feasible to use either of the two E1 conjugates separately in the Ovarian Monitor assay. Thus the two E1 conjugates were analysed together as the 60:40 mixture, and the terminology E1 should be understood to refer to a mixture of two mono-substituted conjugates of known structure and composition.

Despite the dual nature of the E1 conjugate, the purification of the E1 and E3 conjugates still represents the first time that enzyme conjugates which are of a known and uniform structural composition have been available for individual use and characterisation in an enzyme immunoassay system. This provides a unique opportunity for examining the

behaviour and relative performance of different structurally defined enzyme conjugates in an enzyme immunoassay format.

Thus, the two main aims of the work presented in this chapter were to:

- establish the Ovarian Monitor assay in our laboratories with the new biomaterials
- compare the performance of the structurally isomeric, characterised mono-substituted conjugate E1 and E3 for their relative potential as signal generators capable of replacement for the conjugation mixture currently utilised in the Ovarian Monitor

3.2 MATERIALS AND METHODS

3.2.1 Apparatus

The Ovarian Monitor equipment, including the pre-coated E1G assay tubes (Figure 1.8), was supplied as described in Section 2.2.1, as were the assay syringes.

All curve fitting was performed using the PRISM data analysis and graphing program (Graphpad™).

3.2.2 Preparation of Assay Reagents

All assay reagents were prepared with water of Milli-Q quality. To avoid cross contamination, separate syringes were used always for the urine samples, estrone glucuronide standards, lysozyme, lysozyme conjugates, antiserum and substrate in the preparation of the assay reagents and in the assay.

3.2.2.1 Buffers

The tris-maleate buffers (pH 7.0) were prepared as described in Section 2.2.6.1.

3.2.2.2 *Hen Egg White Lysozyme-Estrone Glucuronide Conjugate*

The lysozyme conjugate used in the wet chemistry antiserum titrations described in Section 3.3.3 which were performed using the same lysozyme conjugate as used in the pre-coated E1G assay tubes, was a gift from Professor Emeritus James Brown in Melbourne. All other assays were performed using either the E1 conjugate or the E3 conjugate prepared in Section 2.3.4. The enzyme was diluted fresh daily as described in Section 2.2.6.2.

3.2.2.3 *Estrone Glucuronide Antisera*

All polyclonal estrone glucuronide antisera were raised in sheep by Dr. Keith Henderson (AgResearch, Wallaceville Animal Research Centre, Upper Hutt). The protocol including the inoculation and bleeding program is described in Section 2.2.2.

All antiserum based assays were performed using antisera from bleeds 3 and 4 only, which were obtained at 14 and 20.5 months respectively. The antisera were diluted fresh daily as described in Section 2.2.6.3. Although the antisera were generally used diluted, all antiserum volumes used in the assays are reported in terms of the equivalent volume of undiluted antiserum.

3.2.2.4 *Estrone Glucuronide Standards*

All references to estrone glucuronide refer to the acid form which was synthesised according to procedures described in Section 2.3.1. Before weighing, the estrone glucuronide was redried under high vacuum for a minimum period of two hours, and

transferred to a desiccator with an oven-dried weighing boat. The desiccator was then transferred to a humidity-controlled balance room and left to equilibrate to the new room temperature. All weight measurements of estrone glucuronide were made to the nearest 0.01 mg using a five figure analytical balance.

Estrone glucuronide (3.11 mg) was weighed into a glass weighing boat and washed quantitatively into a 50 mL volumetric flask with 10 mM tris-maleate buffer (pH 7.0). The solution was then made to the mark with more buffer to give a stock solution equivalent to a urinary excretion rate of 500,000 nmol E1G 24 hr⁻¹ (see Section 1.3.3.5). All E1G standards were prepared by serial dilution from this stock solution using 10 mM tris-maleate buffer as the diluent according to Table 3.1. The dilutions were performed using two sets of glass syringes; one set was reserved for use with E1G solutions only and the other for dispensing the tris-maleate buffer. To minimise any systematic dilution errors of the standards, the E1G syringes were never rinsed with water or buffer throughout the dilution series. After each standard in the dilution series had been prepared, the E1G syringe was then rinsed a minimum of 5 times with the new standard. Only then was an aliquot from the new standard drawn for the next standard in the dilution series. Thus, carry over from the high to the low standards was minimised.

TABLE 3.1: *Dilution Series for Preparation of Estrone Glucuronide Standards*

Standard nmol E1G 24 hr ⁻¹	Volume (standard) μL (nmol E1G 24 hr ⁻¹)	Volume (buffer) μL
50,000	200 (500,000)	1,800
5,000	200 (50,000)	1,800
2,000	1,000 (5,000)	1,500
800	1,000 (2,000)	1,500
267	600 (800)	1,200
150	1,126 (267)	874
90	1,200 (150)	800
50	1,000 (90)	800
30	1,200 (50)	800
16.5	1,100 (30)	900
10	800 (16.5)	520
2.8	336 (10)	864

3.2.2.5 *Blank Urine*

Two different sources of urine were used as a blank urine throughout the course of this work. The blank urine from an ovariectomised woman was supplied by The Royal Woman's Hospital, Melbourne, Australia, and was diluted to an excretion rate equivalent to 150 mL 24 hr⁻¹. The other sample was provided by a young girl (aged 24 months). This

sample was diluted using two methods; in one method the sample was diluted to the standard urinary excretion rate of 150 mL hr^{-1} and in the other method the conductivity of the urine was measured and the sample diluted to the same conductivity as the 10 mM tris-maleate buffer. The source of urine and method of dilution has been reported in the Results Section of all blank urine experiments.

3.2.2.6 *Urine for determination of Estrone Glucuronide Excretion Rates throughout the Menstrual Cycle*

All estrone glucuronide cycle profiles were obtained using time diluted urines (150 mL hr^{-1}) collected by a 26 year old woman over cycle days 2-29 of a 29 day cycle.

3.2.2.7 *Bacterial Substrate*

The *M. lysodeikticus* substrate was prepared fresh daily as described in Section 2.2.6.4.

3.2.3 Assays for Estrone Glucuronide using Pre-Coated Estrone Glucuronide Assay Tubes with Freeze-dried Components

Single assays were performed according to the instructions given in Section 1.6.2. In standard curves prepared using these tubes, the $50 \mu\text{L}$ urine sample was replaced with $50 \mu\text{L}$ of the appropriate estrone glucuronide standard solution. When more than one sample needed to be assayed, a custom made heating block was utilised to incubate the samples at 40°C between transmission readings. All assays were generally performed in duplicate.

The fitting of the standard curves is described in Section 3.2.5.4, and the conversion of transmission data into estrone glucuronide excretion rates for urine samples is described in Section 3.2.5.5.

3.2.4 Assays using Fresh Components Only

The conjugate, and conjugate concentration, antiserum number and bleed, and the equivalent volume of undiluted antiserum used in each assay, is recorded in the Results Section for each individual experiment. The $500 \mu\text{L}$ substrate syringe was fitted with a stepper apparatus which allowed the repetitive release of $10 \mu\text{L}$ boluses of substrate from a single $500 \mu\text{L}$ aliquot. All assays were generally performed in duplicate or triplicate.

3.2.4.1 *Standard Activity Assay*

The standard activity assay with enzyme conjugate only was measured using a five or twenty minute end point assay and was performed as described in Section 2.2.6.5.

The clearing curve data presented in Section 3.3.5, were collected over twenty minutes. Transmission values were recorded at 0 time, then every 20 seconds for the first two minutes, every 30 seconds for the 3rd-5th minute, and then every minute until the end of the collection time.

3.2.4.2 *Inhibition Studies*

The inhibition studies were performed using a five or twenty minute end point assay. The assay protocol was essentially as described in Section 2.2.6.6.

Results were either expressed as a percentage relative to the control rate (i.e. rate without antiserum) or as the rate of transmission change, and were not corrected for the blank rate (i.e. spontaneous lysis rate without enzyme present) unless specified.

Fitting and data analysis of the titration curves is described in Section 3.2.5.3.

3.2.4.3 *Estrone Glucuronide Standard Curves*

For the estrone glucuronide standard curves, both the antiserum and conjugate stock solutions were diluted such that the required amount would be added in a volume between 1-10 μL . The assay buffer, 40 mM tris-maleate (pH 7.0), was the same as that used for the standard activity and inhibition assays.

The following general protocol was used in the production of the standard curves. Estrone glucuronide standard (50 μL) and diluted antiserum (1-10 μL) were dispensed into diagonally opposed corners of an empty assay tube, and the immune reaction was initiated by vortexing the assay tube for a count of three. After the mixture had been incubated for 5 minutes at 40°C, the diluted conjugate stock solution was added and the contents of the assay tube vortexed again. Assay buffer was then added to bring the total volume in the assay tube to 340 μL , and the resulting reaction mixture remixed and left to incubate for 10 minutes at 40°C. The assay was initiated as reported for the standard activity assay (Section 2.2.6.5).

Assays in the presence of a urinary control were performed using the general protocol described above except that an aliquot of blank urine (50 μL) was added with the estrone glucuronide standards. The final reaction volume was maintained at 350 μL by reducing the volume of assay buffer.

The fitting of the standard curves is described in Section 3.2.5.4.

The clearing curves in Section 3.3.5 which were obtained using conjugate, antiserum and estrone glucuronide standard, were performed using the standard curve protocol described here. The conjugate only controls for these experiments were also performed using this standard protocol, but with the following modifications. The estrone glucuronide standard was replaced with 50 μL of 10 mM tris-maleate buffer, and the volume of assay buffer increased to compensate for the omission of the antiserum.

3.2.4.4 *Assay for Estrone Glucuronide in Daily Urine Samples*

Urine samples were assayed for estrone glucuronide levels using the same general assay protocol as was used in the generation of the standard curves. The only difference was that the estrone glucuronide standards were replaced with a 50 μL aliquot of time diluted urine.

Conversion of the transmission data into estrone glucuronide excretion rates is described in Section 3.2.5.5.

3.2.5 Data Analysis

3.2.5.1 Conversion of Ovarian Monitor Transmission Data into Absorbance

Ovarian Monitor transmission data was converted into absorbance using Equation 3.1:

$$Abs_{650} = \log \left(\frac{T_{650}^{buffer}}{T_{650}^{sample}} \right) \quad \text{EQN. 3.1}$$

3.2.5.2 Kinetic Analysis of the Rate Order of Clearing

The second order rate equation is defined in Equation 3.2 (McKenzie and White, 1986) where S_0 and S_t are the concentrations of substrate at time 0 and time t respectively and k_2' is the second order rate constant.

$$\frac{[S_0]}{[S_t]} = k_2' S_0 \cdot t + 1 \quad \text{EQN. 3.2}$$

This equation has the form of a straight line ($y=mx+c$) where the gradient (m) equals $k_2' S_0$ and the y intercept (c) equals 1. As the absorbance of an *M. lysodeikticus* suspension at 650 nm is directly proportional to its concentration (Mörsky, 1982), the concentration terms can be substituted for their equivalent absorbance values. The modified equation is given in Equation 3.3.

$$\frac{(Abs_{650}^0)}{(Abs_{650}^t)} = k_2' \cdot Abs_{650}^0 \cdot t + 1 \quad \text{EQN. 3.3}$$

Thus the clearing curve data of a second order reaction can be linearised by plotting the initial absorbance divided by the absorbance at time t against time and the second order rate constant calculated by dividing the gradient of the resulting plot by the initial absorbance.

The fit of the transmission clearing curve data to a second order reaction was shown by converting the transmission data into absorbance using Equation 3.1 and then plotting the data as change in absorbance against time with the line predicted by a second order rate of clearing.

3.2.5.3 Analysis of Antiserum Titration Curves

All titration curves were fitted using a sigmoidal dose response curve with a variable slope (see Equation 3.5) using a data analysis and graphics programme (PRISM). The fit of the titration curve data was used to calculate the inhibition midpoint of the different antisera.

The antiserum midpoint is defined as the antiserum volume required for 50% inhibition of conjugate activity for a given conjugate concentration and was calculated by extrapolation from the change in transmission midpoint of the fitted titration curve according to Equation 3.4.

$$\Delta T_{midpoint} = \left(\frac{(highest \Delta T - lowest \Delta T)}{2} \right) + lowest \Delta T \quad \text{EQN. 3.4}$$

3.2.5.4 *Fitting of the Estrone Glucuronide Standard Curves*

Standard curves were fitted to a sigmoidal dose response curve with a variable slope using Equation 3.5.

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogEC_{50} - LogX) \cdot Hill\ Slope}} \quad \text{EQN. 3.5}$$

In this equation, X represents the concentration of EIG in terms of urinary excretion rates, Y the rate of the assay, and Bottom and Top the rate at the bottom and top plateau of the standard curve. EC₅₀ is the X value halfway between the bottom and top plateaus and thus is equal to the EIG excretion rate that gives a response halfway between the minimum and maximum rates. The Hill slope variable controls the gradient of the curve. If the Hill slope is equal to 1, then the equation generates the standard dose-response curve and the response shifts from 10-90% of the maximal rate over two log units. A Hill slope less than 1 results in a shallower standard curve and a Hill slope greater than 1 results in a steeper standard curve.

3.2.5.5 *Measurement of Urinary Estrone Glucuronide Excretion Rates using Estrone Glucuronide Standard Curves*

A standard curve performed under identical conditions to the assay for unknowns was used to convert the transmission data into urinary estrone glucuronide excretion rates. This reading of the unknowns off the standard curve was performed using the fitted standard curve calculation of unknowns option on the PRISM data analysis and graphing program.

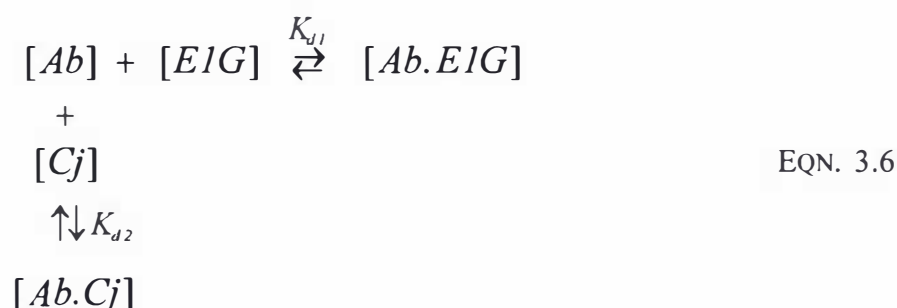
3.2.5.6 *Comparison of Estrone Glucuronide Cycle Excretion Rates throughout a Menstrual Cycle as measured by Different Methods*

The EIG excretion rate data calculated for the same cycle using different methods were compared using the Deming Regression Model (Deming, 1943; Martin, 2000) as measured by the Best Fit program written by Mr. Andrew Rowsall of the Professorial Unit, Royal Women's Hospital, Melbourne. This model is similar to the standard linear regression model except that it places an equal weighting on the two sets of data i.e. it recognises that variations between the two sets of data may be due to inaccuracies in both sets of data, not just the Y data set.

3.2.5.7 Standard Curve Simulations

Standard curve simulations were performed using the EIA computer program written originally by Dr. Alistair McGibbon (Dairy Research Institute, Palmerston North) and Mr. Bryce Cummock (Cummock, 1998) and converted into a user friendly windows version by Mr. Bob Lambourne (Lambourne Computing, Palmerston North). Variables in the program included dissociation constants for the antibody and hapten (K_{d1}), and antibody and hapten conjugate (K_{d2}), and antibody binding site and hapten conjugate concentrations and the hapten concentration range.

From these variables the program calculates the concentration of bound conjugate from the two competing equilibria shown in Equation 3.6.



3.2.5.8 Error Bars

All graphical data for which error bars are displayed are for one standard deviation from the mean ($n=2$).

3.3 RESULTS

3.3.1 The Melbourne Estrone Glucuronide Standard Curves

Melbourne pre-coated assay tubes of the same batch number contain hen egg white lysozyme-estrone glucuronide conjugate produced from a given conjugation and purified in the same chromatographic run, and antiserum produced by a given sheep and obtained from the same bleed. Assay tubes from the same batch but with different box lot numbers contain the same conjugate and antiserum but are produced on different days.

Figure 3.4 shows the standard curves obtained in Melbourne using the pre-coated Ovarian Monitor assay tubes of the same batch number (#14) but different box lots (25-43 versus 316-337) at the time of production.

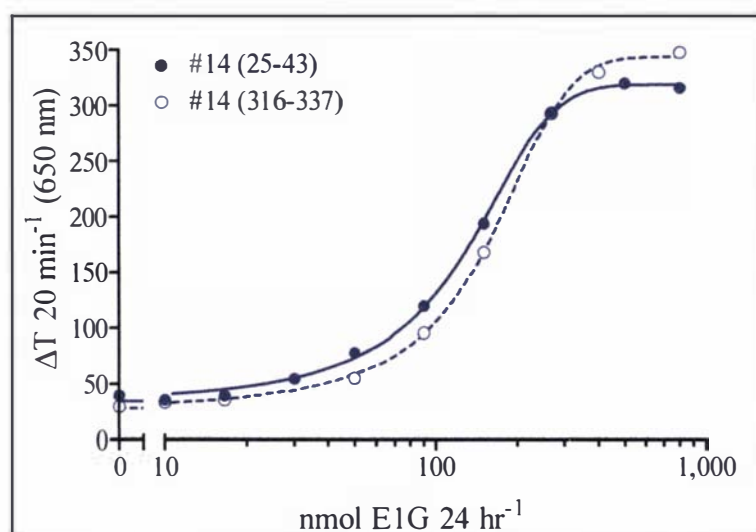


FIGURE 3.4: Variation in the Estrone Glucuronide Standard Curves for Pre-Coated Estrone Glucuronide Assay Tubes of the same Batch Number but different Box Lots

Although the standard curves were obviously similar they were not identical. Using the fit of the standard curve to a sigmoidal dose response curve, the curve obtained with the 25-43 box lot of assay tubes had its first visible rise above the zero standard value occurring at 35 nmol E1G 24 hr⁻¹ and a mid-point of 120 nmol E1G 24 hr⁻¹ while the standard curve obtained with the 316-337 box lot had its first rise at 28 nmol E1G 24 hr⁻¹ and a mid-point of 150 nmol E1G 24 hr⁻¹.

3.3.2 Validation of Estrone Glucuronide Standards

To assess accurately the effect of any assay modifications encountered in this work resulting from structural differences between E1 and E3, the concentration and integrity of the Palmerston North estrone glucuronide standards had to be properly validated. The simplest method by which to achieve this was to compare the standard curve provided from Melbourne which was generated using the validated Melbourne standards and a given batch and lot number of pre-coated Ovarian Monitor assay tubes with the corresponding curve generated using the new standards and pre-coated assay tubes, of the same batch and lot number. In the absence of this check, it is difficult to determine whether any deviations from the Melbourne standard curves shown by the alternative assays constructed with the new biomaterials prepared during this thesis are real and not just due to a difference in the quality of the estrone glucuronide standards.

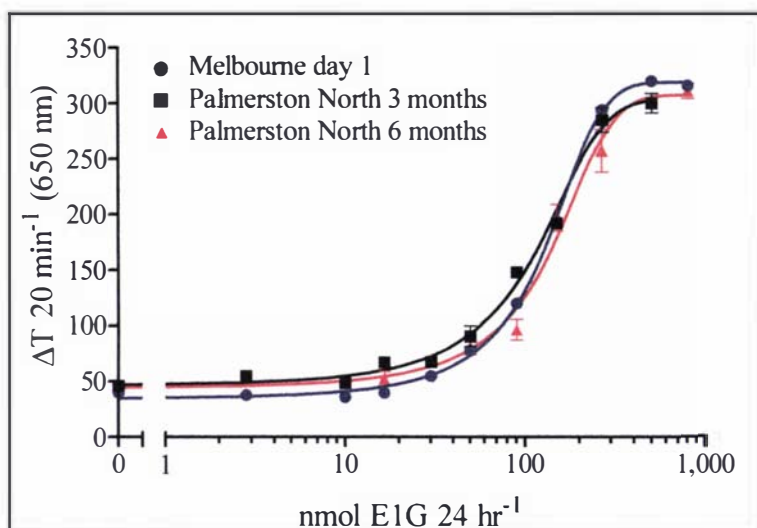


FIGURE 3.5: Reproducibility of Estrone Glucuronide Standard Curves for Pre-Coated Assay Tubes of Batch Number #14, Box Lot 25-43 over a Six Month Period

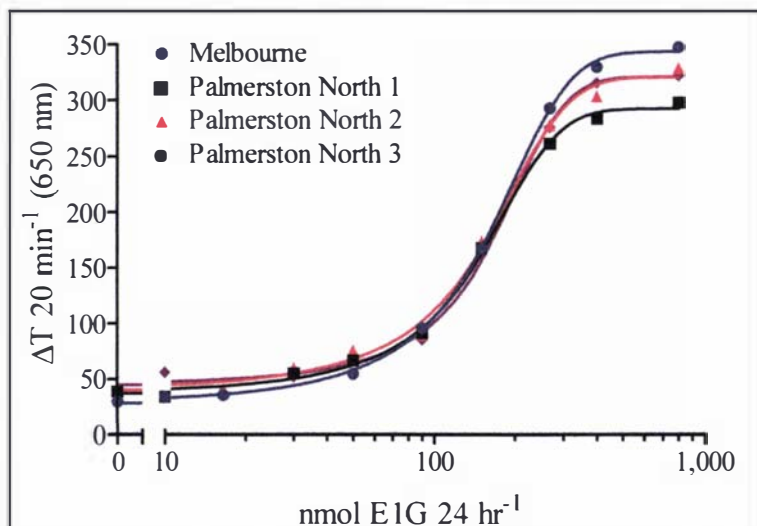


FIGURE 3.6: Day-to-Day Reproducibility of Estrone Glucuronide Standard Curves for Pre-Coated Assay Tubes of Batch Number #14, Box Lot 316-337)

Figures 3.5 and 3.6 show the reproducibility of the standard curves for batch #14, box lots 25-43 and 316-337 respectively for the pre-coated estrone glucuronide assay tubes. The standard curves supplied with each box lot of assay tubes (i.e. the curves obtained in Melbourne with the Melbourne standards) are outlined in blue, while the other sets of data show the standard curves obtained with the same box lot of tubes on different days using the estrone glucuronide standards prepared in Section 3.2.2.4. The data presented show that the reproducibility of the standard curves was excellent for the two different box lots.

For box lot 316-337, the fits of the four standard curves almost superimposed on each other from the low to middle E1G concentration

range. Only when the E1G concentration reached an excretion rate equivalent to about 267 nmol E1G 24 hr⁻¹ did the four standard curves begin to deviate from each other, with the degree of deviation increasing with the estrone glucuronide concentration. In comparison there was less overall variation in the shapes of the standard curves observed with the box lot 25-43 E1G assay tubes although the agreement at the lower end of the concentration range was not as close as that observed for the standard curves generated with the box lot 316-337 assay tubes.

The three standard curves presented in Figure 3.5 were obtained over a 6 month period. The Melbourne standard curve was performed at the time the assay tubes were made and the two Palmerston North standard curves generated with the pre-coated assay tubes were obtained 3 and 6 months after the tubes were first prepared. These data show that within a six month time scale the position and shape of the standard curves for a given batch of tubes was stable irrespective of the source of the estrone glucuronide used in the standards.

The standard curves obtained with the box lot 25-43 assay tubes can be considered as typical of the standard curves obtained with the pre-coated tubes and shows the characteristic sigmoidal dose response curve typical of a competitive binding assay. The curves were anchored at a lysis rate of approximately 40 ΔT 20 min⁻¹. This baseline rate (i.e. the rate of lysis for the zero standard) was maintained over an E1G concentration range equivalent to urinary excretion rates of 0-10 nmol E1G 24 hr⁻¹ with the individual fitted curves showing an increase from baseline rates at excretion rates of 20-30 nmol E1G 24 hr⁻¹ or less. However, taking into account the variation in the data, the first significant rise above baseline which could be determined visually and unambiguously from the raw data occurred later at an excretion rate of 50 nmol 24 hr⁻¹. This E1G excretion rate signalled the beginning of the steepest and thus most sensitive region of the standard curve.

Increases in lysis rates with increasing amounts of estrone glucuronide were most apparent at urinary excretion rates between 50 to 267 nmol E1G 24 hr⁻¹ which is equivalent to a urinary concentration of 13.9 to 74.2 nM. Over this range, the relationship between the end point lysis rate (ΔT 20 min⁻¹) and the estrone glucuronide excretion rates was virtually linear. This linear E1G concentration range corresponded to a lysis rate over 20 minutes (ΔT) of between 80-275 units. Thus, if a time diluted urine sample analysed using an assay tube from this batch and box lot recorded a 20 minute ΔT greater than 275 units, it would have to be re-assayed after further dilution before its estrone glucuronide concentration could be quantified accurately. However, these assay tubes could be used to determine directly and accurately the estrone glucuronide concentration for all urinary samples which had an E1G excretion rate between 50 and 267 nmol 24 hr⁻¹.

Changes in rates between estrone glucuronide levels of 267 and 500 nmol E1G 24 hr⁻¹ were small and there was no detectable increase in rates at estrone glucuronide concentrations equivalent to 500 nmol E1G 24 hr⁻¹ and higher. This corresponded to a maximal recorded rate of 325 ΔT 20 min⁻¹. As the blank rate was 40 ΔT 20 min⁻¹ this in turn corresponded to a maximum apparent inhibition for the assay of 88%.

3.3.3 Wallaceville Animal Research Anti-Estrone Glucuronide Antisera

To justify the use of the alternative antisera raised by the Wallaceville Animal Research Centre, their ability to inhibit the Melbourne lysozyme-estrone glucuronide conjugate had to be confirmed. Figure 3.7 shows the broad partial titration curves obtained for the third bleeds of all four Wallaceville estrone glucuronide antisera using the Melbourne lysozyme-estrone glucuronide conjugate.

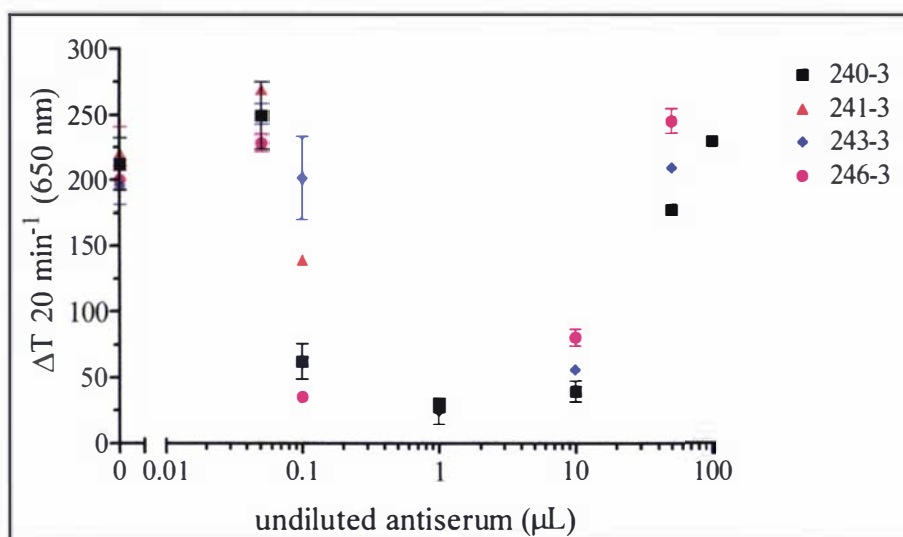


FIGURE 3.7: Broad Titration Curves for the Third Bleeds of the Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera with Melbourne Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

All four sets of data showed very similar profiles. As the concentration of antiserum was increased the activity decreased to a minimum of approximately 10% of the conjugate only lysis rate (i.e. 90% inhibition) and then increased again. At very high antiserum levels the lysis rates increased back to the level of the original conjugate only rates, while at very low levels, the antisera appeared to have a slight activating effect on the conjugate activity.

3.3.4 Optimisation of the Buffer Ionic Strength

Figure 3.8 shows the bell shaped relationship between the enzymic activity of the E3 conjugate at pH 7.0 and the total ionic strength of the buffer.

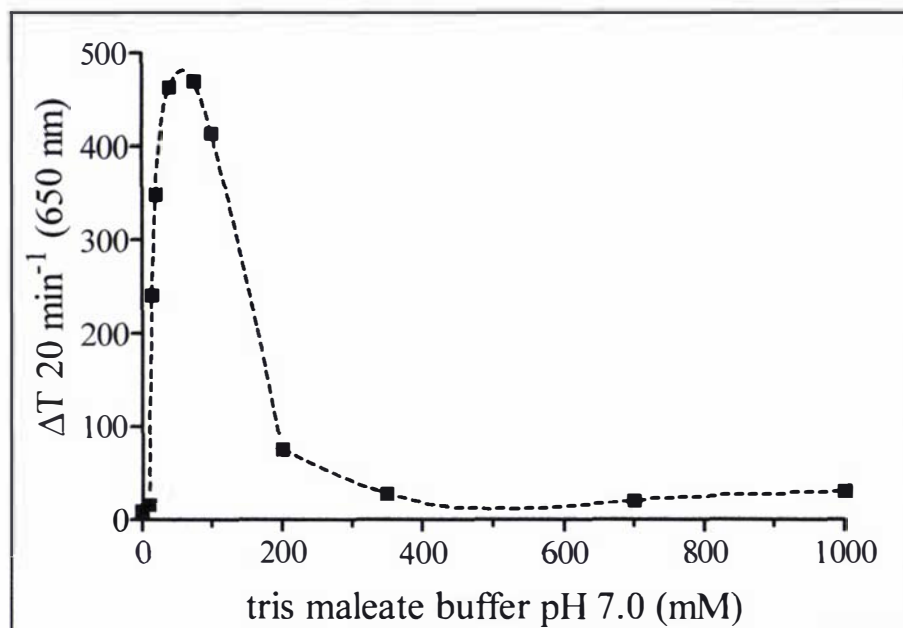


FIGURE 3.8: Effect of Buffer Ionic Strength on the Specific Activity of E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

Conditions: E3 in Assay Mixture - 30.3 nM

In terms of the tris-maleate buffer concentration, the lowest lytic activities were observed below 10 mM tris-maleate and above 350 mM, while the maximal enzymic activity was observed between a buffer ionic strength of 40 and 75 mM tris-maleate.

3.3.5 Relationship between the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Activities in Light Transmission and Absorbance

Figure 3.9 shows the total change in units of transmission at 20 minutes as measured by the Ovarian Monitor (650 nm), for a standard *M. lysodeikticus* substrate solution (214 mg L⁻¹), over a range of conjugate concentrations for E1 and E3 and using a reaction mixture at the optimum ionic strength.

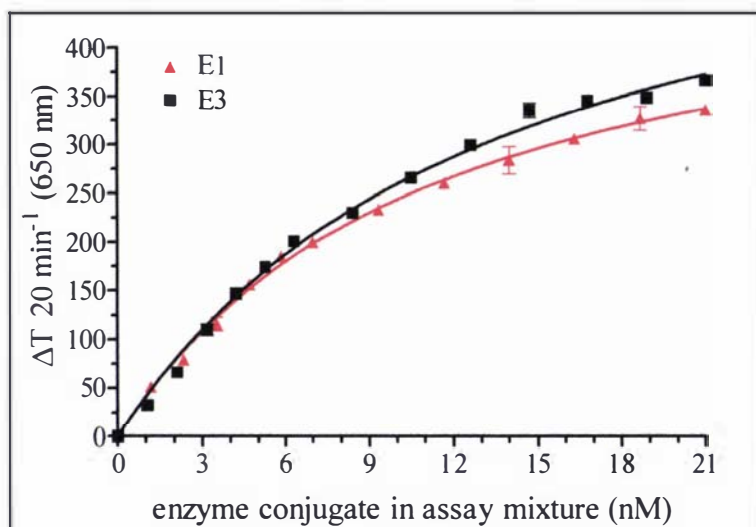


FIGURE 3.9: Relationship between Rate of Clearing in Transmission using a Twenty Minute End Point Assay and E1 and E3 Hen Egg White Lysozyme Estrone Glucuronide Conjugate Concentration

The fact that the two sets of data were never linear but fitted equally well to a hyperbolic equation ($r=0.994$ and 0.995 respectively) showed that the relationship between the conjugate concentration and the rate as measured under the above conditions was essentially the same for the E1 and E3 conjugates. The magnitude of the specific activity was also very similar for the two conjugates. For example, at a conjugate concentration of 21 nM in

the assay (i.e. the highest conjugate concentration on the graphs in Figure 3.9) both E1 and E3 had a twenty minute ΔT of approximately 350 units, although the specific activity of the E3 conjugate appeared to be the highest of the two.

The transmission clearing curves (i.e. change in transmission versus time plots) for E1 and E3 at this conjugate concentration are shown in Figure 3.10 (see page 108).

As might be predicted from the results above the actual clearing curves were also essentially identical for the two conjugates. Visual examination of the clearing curves showed them to consist of two phases. At the conjugate reaction mixture concentration of 21 nM, the length of the first phase was only 4.5 minutes, with the slope of the first phase being steeper than the second by approximately a factor of three.

Figure 3.10 also shows the same E1 and E3 transmission clearing curves obtained in the presence of a slight excess of anti-estrone glucuronide antiserum over the conjugate concentration and 50 μ L of a high estrone glucuronide standard (2,000 nmol E1G 24 hr⁻¹). These conditions provide a more accurate representation of the shape of the clearing curve which would be observed if these conjugates were utilised in the Ovarian Monitor.

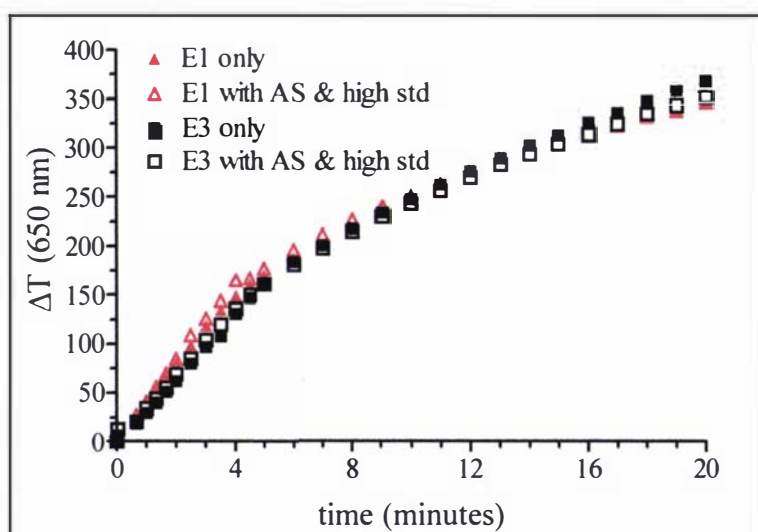


FIGURE 3.10: E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Clearing Curves as Change in Transmission in the Absence and Presence of Anti-Estrone Glucuronide Antiserum and High Levels of Estrone Glucuronide Standard

Conditions in Assay Mixture: E1 - 21 nM enzyme, 0.085 μ L antiserum; E3- 21 nM enzyme, 0.062 μ L antiserum; Antiserum (AS) - 243-4; High Standard (std) - 2,000 nmol E1G 24 hr⁻¹

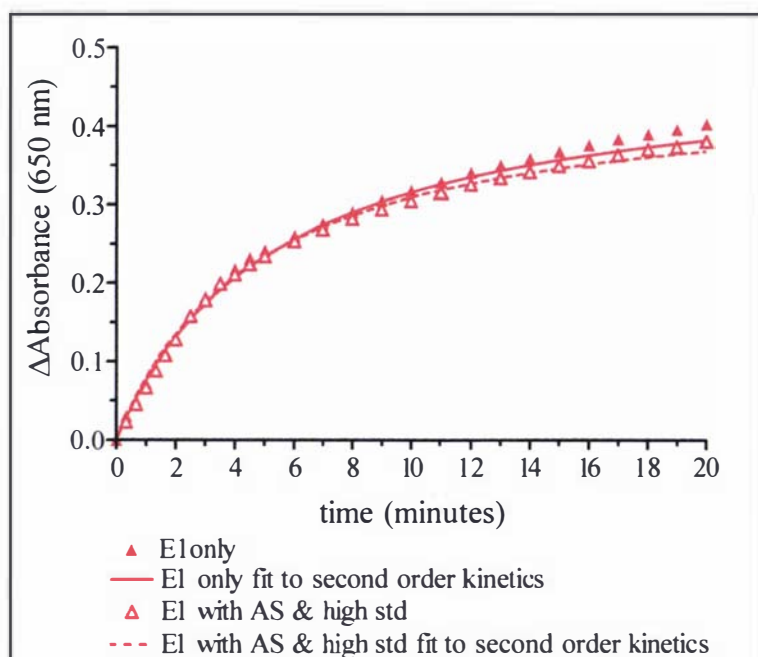


FIGURE 3.11: Fit of the E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Change in Absorbance Clearing Curve in the Absence and Presence of Anti-Estrone Glucuronide Antiserum and High Estrone Glucuronide Standard to a Second Order Rate of Clearing

Conditions: see Figure 3.10

Neither the E1, nor the E3 clearing curves showed any detectable differences between the shape, or specific activity, of their conjugate only curves compared with their equivalent curves obtained in the presence of antiserum and high levels of estrone glucuronide.

As the relationship between absorbance and the concentration of intact *M. lysodeikticus* cells is linear, most kinetic studies of lysozyme are performed in absorbance. The Ovarian Monitor transmission data can be converted into absorbance using Equation 3.1 (see Section 3.2.5.1).

Figure 3.11 (this page) and Figure 3.12 (page 109) show the transmission data from the clearing curves presented in Figure 3.10 after conversion into absorbance change.

By using units which are linearly related to substrate concentration, a relative measure of the rate constant of the lytic reaction could be calculated as outlined in Section 3.2.5.2 (Equation 3.3), which allowed the raw absorbance data to be fitted to a second order equation. As indicated by the broken lines in Figures 3.11

and 3.12, the clearing curves obtained in the presence of E1 and E3, and the equivalent antiserum/estrone glucuronide curves were all closely fitted by a second order rate equation (see Section 3.2.5.2).

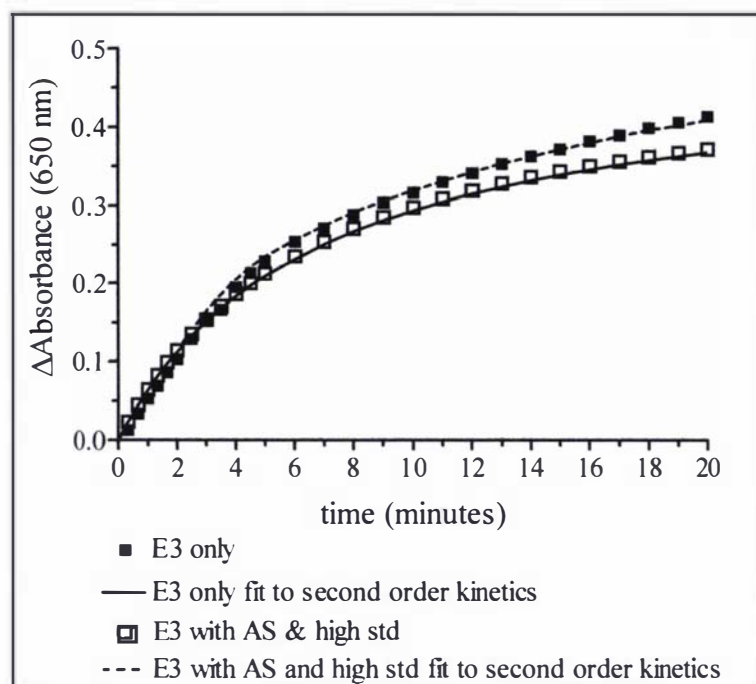


FIGURE 3.12: Fit of the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Change in Absorbance Clearing Curve in the Absence and Presence of Anti-Estrone Glucuronide Antiserum and High Estrone Glucuronide Standard to a Second Order Rate of Clearing

Conditions: see Figure 3.10

Transmission data for the clearing curve for the pre-coated estrone glucuronide assay tubes was also collected and is shown in Figure 3.13. However as this curve showed a very obvious lag phase it was not converted into absorbance values as it could not be fitted to a second order reaction.

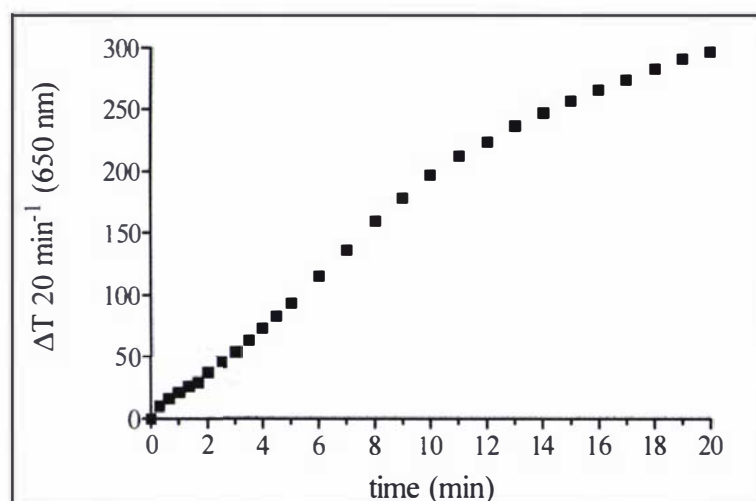


FIGURE 3.13: Clearing Curve for a Pre-Coated Estrone Glucuronide Ovarian Monitor Assay Tube

3.3.6 The Bleed Three Anti-Estrone Glucuronide Antisera and the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates

Figure 3.14 shows the titration curves for the four anti-estrone glucuronide antisera obtained from the third bleeds using the Ovarian Monitor end point assay.

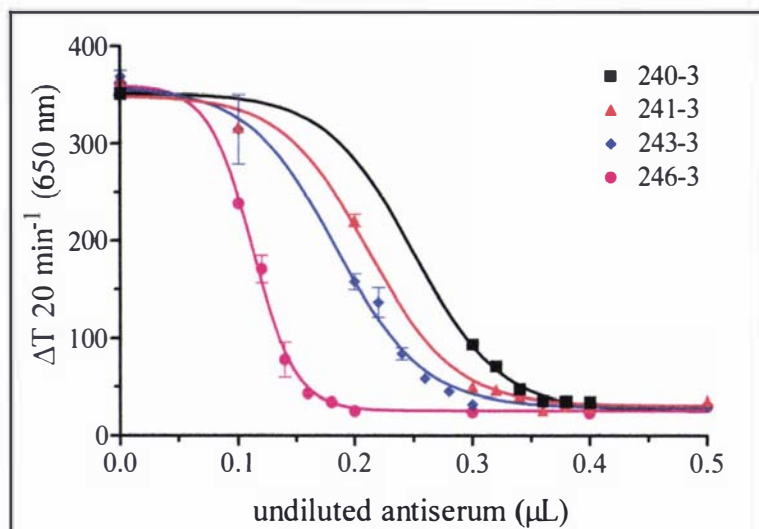


FIGURE 3.14: Titration Curves for the Third Bleeds of the Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera with E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

Conditions in Assay Mixture: E3 - 16.8 nM

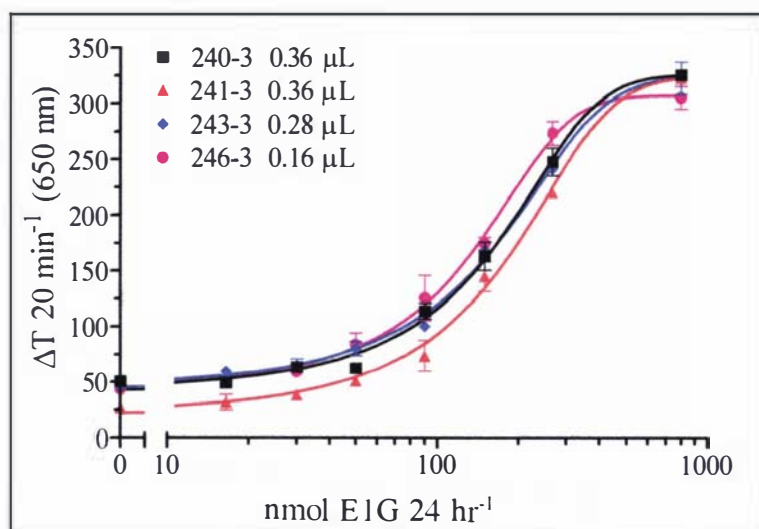


FIGURE 3.15: Estrone Glucuronide Standard Curves for E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with the Third Bleeds of the Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera

Conditions in Assay Mixture: E3 - 16.8 nM; refer to key for volumes of antiserum

All four antisera were successfully titrated against a constant concentration of E3 (16.8 nM). Although no difference was observed in the maximal inhibition of the E3 conjugate which could be achieved by the different antisera, they varied considerably in the titre required to achieve this level of inhibition. The inhibition midpoint, i.e. the antiserum volume in the assay which was halfway between the conjugate only rate and the maximally recorded inhibited rate, was estimated at 0.250, 0.212, 0.184 and 0.113 μL of neat antiserum, for antisera 240-3, 241-3, 243-3 and 246-3 respectively. These volumes corresponded to titres (i.e. ratio of mid-point volume of antiserum to the total volume of assay mixture) of 1:1400, 1:1650, 1:1902 and 1:3097 respectively. Thus the titre of antiserum 246-3 was significantly higher than that observed for the other antisera, with the antiserum with the titre, of the next highest titrating antiserum, 243-3, having a titre more than 60% lower.

Figure 3.15 shows the optimised standard curves for the four different anti-E1G antisera of the third bleed with the E3 conjugate.

An acceptable standard curve was obtained with all four antisera. However, based on the data presented in Figure 3.15, antiserum 241-3 produced a standard curve which had a significantly lower baseline than the other three antisera (a zero standard value of 25 as opposed to 45 ΔT 20 min⁻¹), and a mid-point which further up the concentration axis making it less sensitive to changes in estrone glucuronide excretion rates over the critical range. Although antiserum 246-3 appeared to be the most sensitive producing a standard curve which was slightly further to the left on the concentration axis than the others and which had a steeper slope its discrimination (i.e. difference between its minimum and maximal response) was slightly less. Antiserum 243-3 and 240-3 were essentially identical. However as the titre of 243-3 was slightly higher, antiserum 243-3 was chosen as the most suitable antiserum for immunoassay and all further studies were performed with this antiserum.

Figure 3.16 shows the optimised standard curve for the E1 and E3 conjugates with the third bleed of the anti-estrone glucuronide 243 antiserum.

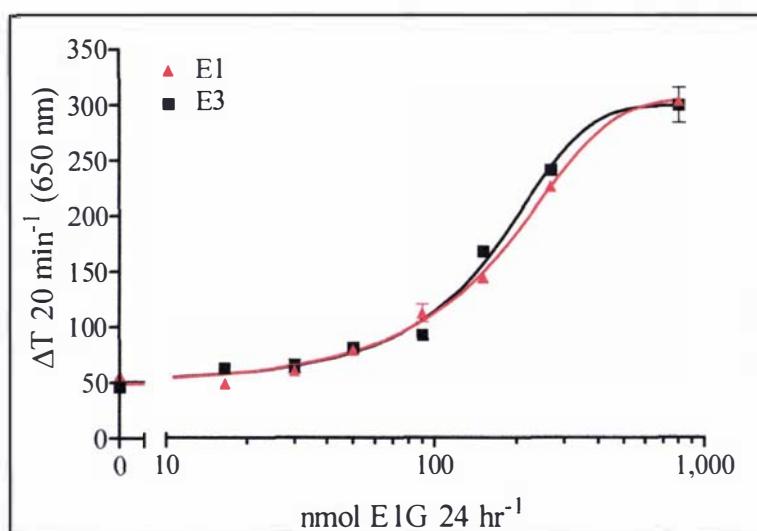


FIGURE 3.16: Optimised Estrone Glucuronide Standard Curves for E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-3

Conditions in Assay Mixture: E1 - 11.7 nM enzyme, 0.3 μ L antiserum;
E3 - 16.8 nM enzyme, 0.28 μ L antiserum

All points on the standard curves were performed in triplicate, and as indicated by the error bars, the within assay precision for both conjugate standard curves was very high. The position and shape of the two optimised standard curves for the conjugates were essentially identical. The optimised E1 standard curve was generated using 11.7 nM of conjugate and 0.3 μ L of undiluted antiserum, in comparison to the E3 standard curve which was produced using 16.8 nM of conjugate and 0.28 μ L of undiluted

antiserum per assay. Thus although the concentration of conjugate was quite different between the two assays, the amount of antiserum was similar. The relative ratio of conjugate concentration to antiserum volume between the E3 and E1 optimised standard curves was 1.0:1.55 i.e. relative to the E3 assay, the E1 assay required 55% more antiserum for a given amount of conjugate to produce a standard curve in the right position for fertility monitoring.

3.3.7 The E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates and the Anti-Estrone Glucuronide Antiserum 243 Bleed Four Titration Curves

Figure 3.17 shows the broad partial titration curve obtained when antiserum 243-4 was titrated against a constant concentration of E1 lysozyme conjugate.

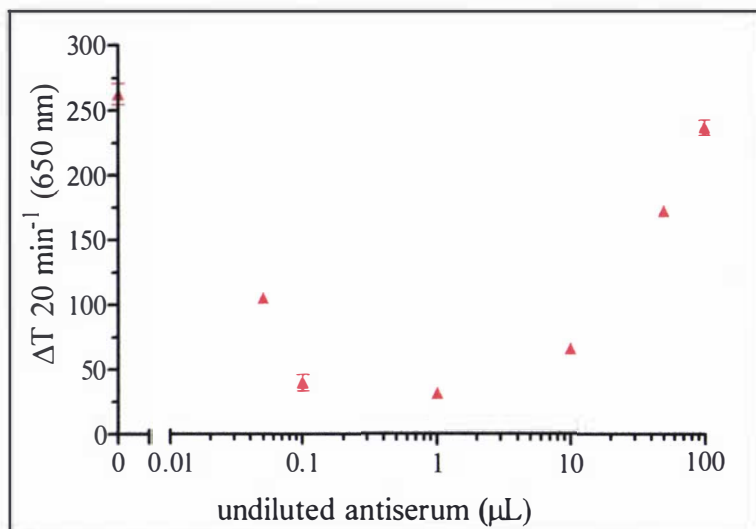


FIGURE 3.17: Broad Titration Curve for the Third Bleed of the Anti-Estrone Glucuronide Antiserum 243 with E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

Conditions in Assay Mixture: 11.7 nM enzyme

This showed a similar profile to that obtained when the third bleed of the same antiserum was titrated against a constant concentration of the Melbourne conjugate (see Figure 3.7).

The most significant difference between the two profiles was that in the 243-4 titration the percentage inhibition increased at a lower volume of the neat antiserum. For the 243-4 titration, the lysis rates decreased with increases in antiserum volume to a

minimum equivalent to approximately 12% of the conjugate only control rate before increasing again at higher antiserum concentrations. At very high levels of antiserum (100 μL undiluted per reaction mixture) the rates were again back to the level of the conjugate only control.

The minimum lysis rate occurred at an antiserum concentration equivalent to 0.1 μL of neat antiserum. Taking into account the fact that the spontaneous rate of lysis (i.e. the rate of lysis in an assay without lysozyme or lysozyme conjugate) was approximately 12 transmission units per 20 minutes, the true inhibited rate would be closer to 7% of the conjugate only rate (i.e. 93% inhibition).

Figure 3.18 (page 113) shows a comparison of the titration curves generated over the critical range when antiserum 243 of the third and fourth bleeds was titrated against a constant concentration of E1 lysozyme conjugate (11.7 nM).

Although the fourth bleed titration was only performed in a skeleton, (i.e. partial), format, it was obvious that much lower volumes of antiserum were required to produce the same level of inhibition for the fourth bleed preparation than for the third. The inhibition mid-point was 0.22 μL of neat antiserum for the third bleed antiserum (titre 1:1590) compared to only 0.03 μL for the fourth bleed antiserum (titre 1:11,667). At maximally inhibiting levels of antiserum both conjugates had a base rate of 25 ΔT 20 min⁻¹. The two

titration curves presented in Figure 3.18 were both performed with the latest antiserum available at that time. Thus the bleed four titration curve was performed significantly later than the bleed three titration.

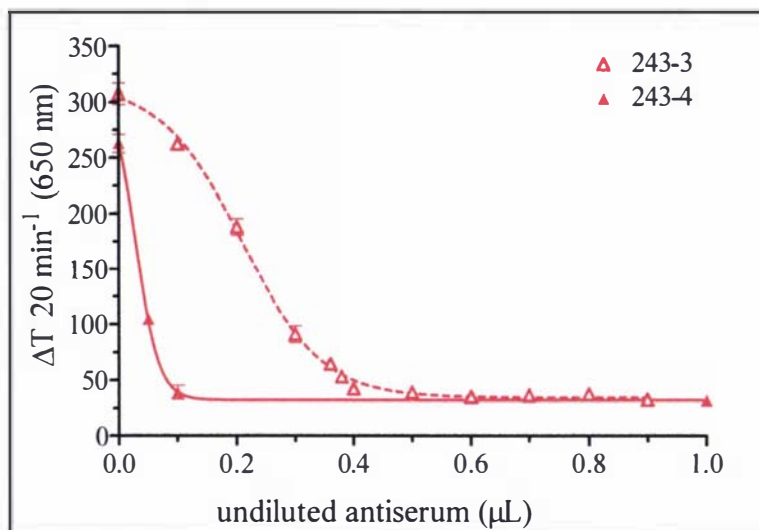


FIGURE 3.18: Titration Curves for the Third and Fourth Bleeds of Anti-Estrone Glucuronide Antiserum 243 with E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate

Conditions in Assay Mixture: E1 - 11.7 nM

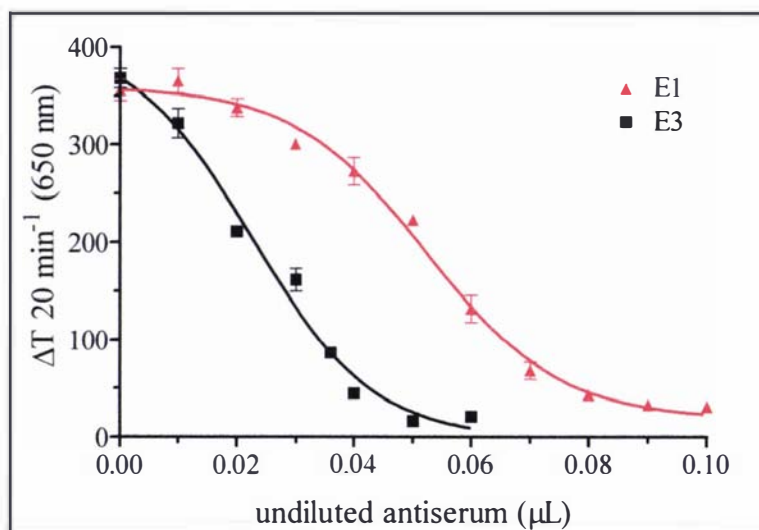


FIGURE 3.19: Titration Curves for the Fourth Bleed of Anti-Estrone Glucuronide Antiserum 243 with E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates

Conditions in Assay Mixture: E1 - 21 nM; E3 - 19 nM

Although the final rate of lysis was the same for the two antisera, these values actually represent different maximal levels of inhibition since the control rates for the two titration curves were different. Thus the maximum inhibition of the lysis rates for the third and fourth bleeds were 88% and 90% respectively. If the spontaneous rate of lysis was taken into account these levels of inhibition increased to 92% and 93% respectively.

Figure 3.19 shows the comparison of the antiserum titration curves for the fourth bleed of antiserum 243 for both the E1 and the E3 conjugates.

Both conjugates had a control rate i.e. conjugate only rate of approximately 350 transmission units per 20 minutes. Although the enzyme concentration required to achieve this rate was very similar for the two conjugates (E1, 21 nM; E3, 19 nM), the E1 conjugate required approximately twice as much antiserum to achieve the same level of inhibition as the E3.

The two titration curves showed the characteristic sigmoidal inhibition pattern when measured with the end point assay with an apparent lag at the beginning of the titration curve followed by a rapid increase in the inhibition and an extended tail as the inhibitions began to approach their maximum levels. The shape of the two curves was essentially identical except that the E1 conjugate had an extended lag phase relative to the E3 inhibition curve. At the intermediate and high levels of inhibition, the two curves had the same slope i.e. for the same absolute increase in the antiserum concentrations, the level of inhibition increased in parallel.

3.3.8 Optimisation of the E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Standard Curve with Anti-Estrone Glucuronide Antiserum 243 Bleed Four

The most rapid method of determining the optimum standard curve conditions for a given antiserum and conjugate combination is to first optimise the conjugate concentration and then use the titration curve to estimate the approximate antiserum concentration required for inhibition. This value can then be used to determine a range of antiserum concentrations to test in a skeleton standard curve format. Skeleton standard curves i.e. standard curves constructed with just a few key estrone glucuronide concentrations, were used to screen rapidly a range of antiserum concentrations at a given conjugate concentration for their potential to generate a standard curve with the desired shape and sensitivity limits.

Figure 3.20 shows the skeleton curves for the E1 conjugate (21 nM) at nine different concentrations of antiserum 243-4.

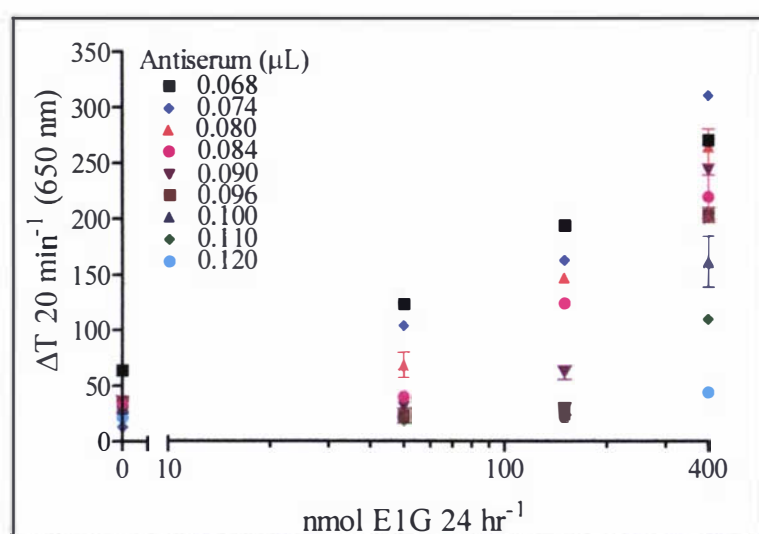


FIGURE 3.20: Skeleton Estrone Glucuronide Standard Curves for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: E1 - 21 nM, refer to key for volumes of antiserum

the equivalent of 0.084 μL of undiluted antiserum and higher, the lysis rate was still maximally inhibited at this E1G concentration and thus resulted in a standard curve which was too insensitive to be utilised for fertility assays. In contrast, at antiserum concentrations equivalent to 0.074 μL of undiluted antiserum and lower, the baseline became elevated and the shape of the standard curve was too shallow for the accurate determination of the urinary estrone glucuronide concentrations over the critical physiological range.

These sets of data clearly showed how small changes in the antiserum concentration had large effects on the position and shape of the standard curve. Of the nine antiserum concentrations tested the data obtained with 0.08 μL of undiluted sample provided the most promising skeleton standard curve generating a curve with a low baseline, but with significant increases in rate above the baseline being observed at an E1G concentration equivalent to an excretion rate of 50 $\text{nmol } 24 \text{ hr}^{-1}$. At

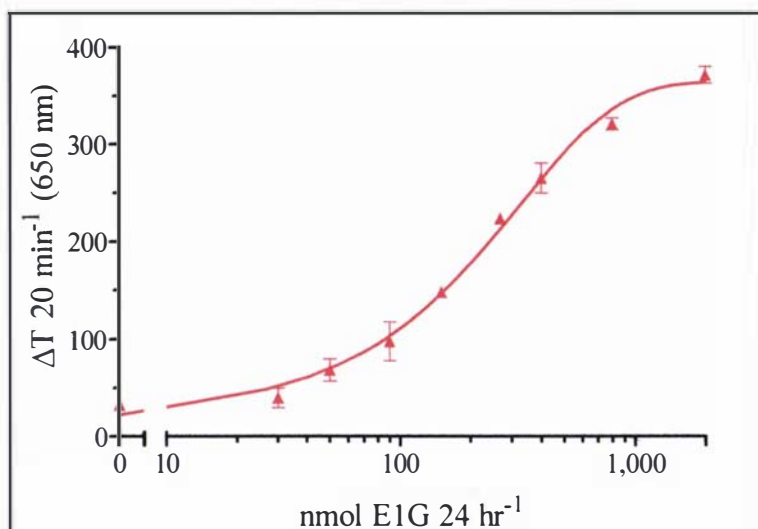


FIGURE 3.21: Optimised Estrone Glucuronide Standard Curve for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: E1 - 21 nM enzyme, 0.08 μ L antiserum

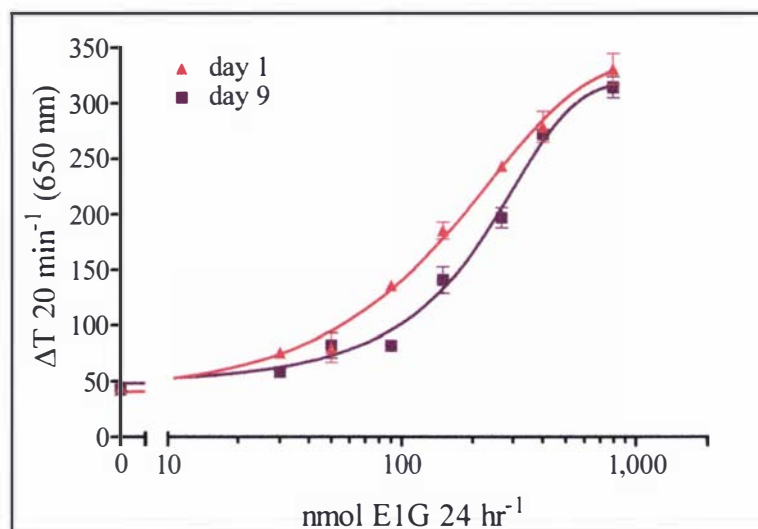


FIGURE 3.22: Day-to-Day Reproducibility of the Optimised Estrone Glucuronide Standard Curve for E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: E1 - 21 nM enzyme, 0.08 μ L antiserum

Based on the estimated optimal E1 and antiserum 243-4 combined conditions i.e. 21 nM conjugate and 0.08 μ L undiluted antiserum per assay mixture, a full standard curve was generated. This standard curve (see Figure 3.21) showed all the desired features required for the determination of unknown urinary E1G excretion rates such as experienced throughout the natural menstrual cycle. The baseline was low, the sensitivity of the assay was such that increases in E1G excretion rates to 50 nmol 24 hr⁻¹ or higher above baseline levels were clearly detectable, and the assay was maximally sensitive over the critical physiological range 50-400 nmol E1G 24 hr⁻¹.

Figure 3.22 illustrates the day-to-day variation in lysis rates as observed for the optimised E1 conjugate standard curve with the 243-4 antiserum.

These two standard curves were performed nine days apart and were produced with fresh batches of substrate, and fresh dilutions of conjugate and antiserum from the stock preparations. Although the two curves were clearly similar some movement in the position and shape of the standard curve had occurred.

3.3.9 Optimisation of the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Standard Curve with the Bleed Four Anti-Estrone Glucuronide Antiserum 243

The conjugate concentration was titrated down in a series of conjugate only assays so that in twenty minutes the change in transmission units was equal to approximately 350 units. The antiserum 243-4 standard curve with the E3 conjugate was then optimised using the same method described for the E1 conjugate. The final E3 optimised standard curve had a conjugate concentration of 19 nM and an antiserum concentration equivalent to 0.048 μL of undiluted antiserum per assay. The full standard curve for E3 under these optimised conditions is shown in Figure 3.23 along with the E1 optimised standard curve which was generated on the same day.

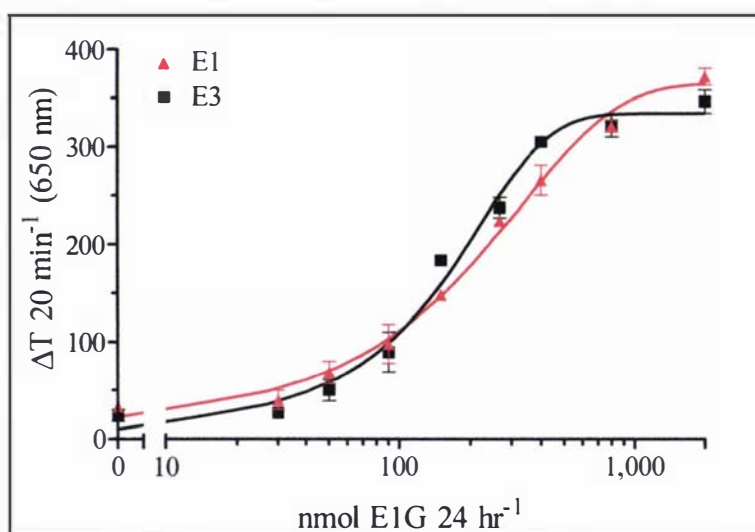


FIGURE 3.23: The Optimised Estrone Glucuronide Standard Curves for E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates with Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: E1 - 21 nM enzyme, 0.08 μL antiserum; E3 - 19 nM enzyme, 0.048 μL antiserum

more antiserum (0.080 μL versus 0.048 μL). The relative ratio of the conjugate concentration to antiserum volume for the E1 and E3 optimised standard curves was 1.0:1.51 i.e. relative to the E3 assay, the E1 assay required 51% more antiserum for a given amount of conjugate to produce a standard curve in approximately the same position.

Although the position and shape of the two standard curves were both considered to be appropriate for the determination of urinary E1G excretion rates, the two curves were not identical. The E3 standard curve appeared to be marginally superior having a lower baseline, and a more rapid increase in lysis rates with increasing E1G excretion rates resulting in a steeper curve. Thus for approximately the same amount of conjugate (21 nM versus 19 nM) the E1 assay required much

3.3.10 Further Comparison of the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Standard Curves by Changing Assay Conditions and Computer Simulations Studies

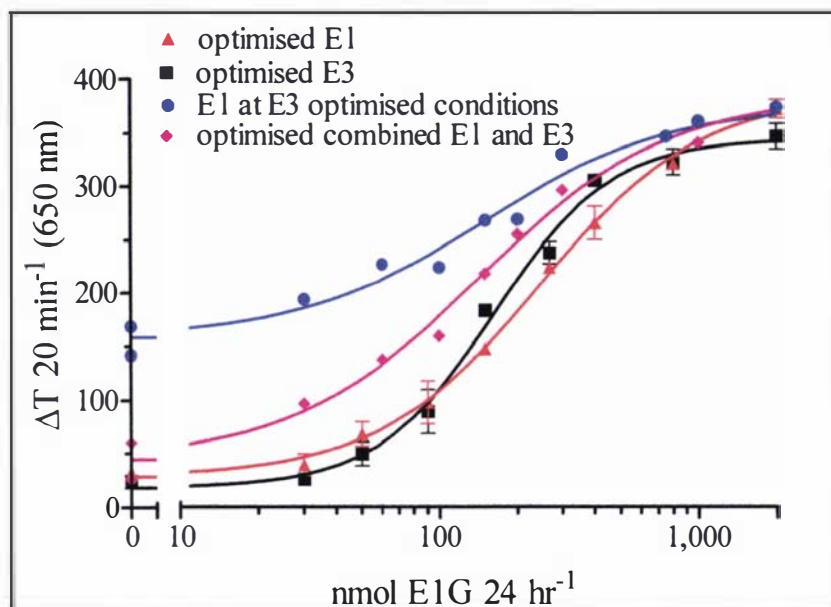


Figure 3.24: The Effect of Performing the E1 Hen Egg White Lysozyme Estrone Glucuronide Standard Curve at the E3 Optimised Conditions and Optimisation of a Combined E1 and E3 Standard Curve

Conditions in Assay Mixture: Optimised E1 - 21 nM enzyme, 0.08 μ L antiserum; Optimised E3 - 19 nM enzyme, 0.048 μ L antiserum; Optimised E1 and E3 - 14 nM E1, 7 nM E3, 0.06 mL antiserum; Antiserum - Anti-Estrone Glucuronide 243-4

For approximately the same amount of conjugate, E3 required significantly less 243-4 anti-estrone glucuronide antiserum than E1 (0.048 μ L vs 0.080 μ L). Figure 3.24 shows the optimised E1 and E3 standard curves, and the E1 standard curve performed at the E3 optimised conjugate and antiserum conditions i.e. 19 nM conjugate and 0.048 μ L antiserum. The large decrease in the volume of antiserum from the E1 optimised conditions resulted in a large shift in the standard curve's baseline with the rate of lysis for the zero standard increasing from approximately 30 ΔT 20 min⁻¹ to 160 ΔT 20 min⁻¹.

Figure 3.24 also shows a standard curve obtained using a mixture of E1 and E3 after re-optimisation of the conjugate and antiserum conditions. The final optimised conditions in the assay mixture were 14 nM E1, 7 nM E3 (i.e. 21 nM total conjugate), and 0.06 μ L of antiserum. This resulted in a standard curve with a blank rate of lysis of 44 ΔT 20 min⁻¹, which was only marginally higher than the optimised E1 and E3 standard curves. However, the curve was much more sensitive to changes in estrone glucuronide concentrations over the early part of the standard curve, with the first clear increase in baseline rates occurring at an excretion rate equivalent to 20 nmol E1G 24 hr⁻¹ as opposed to 50 nmol E1G 24 hr⁻¹ as was obtained with the E1 and E3 optimised curves. The difference between the blank (zero standard) and maximum rates of lysis was 340 ΔT 20 min⁻¹. Thus the assay discrimination of the estrone glucuronide concentrations

over the working range of the combined E1 and E3 standard curve was very similar to the E1 and E3 optimised standard curves.

The E1 and E3 standard curves were modelled using the EIA program. Standard curves were simulated by entering values for the antibody binding site and hapten conjugate concentration in the assay, and the dissociation constant (K_{d1}) of the antiserum with the unconjugated hormone (K_{d1}) and the antiserum with the hapten conjugate (K_{d2}), which allowed the level of bound conjugate to be calculated for a range of estrone glucuronide concentrations. The results of these simulations are shown in Figure 3.25.

A good approximation of the experimentally determined E3 standard curve was made by using a K_{d1} and K_{d2} value of 0.1 and 0.4 nM respectively, a conjugate concentration of 19 nM and a slight excess of antibody binding sites (20 nM). The E1 standard curve performed at the E3 optimised conditions was modelled by increasing the conjugate dissociation constant to 3 nM. Like the experimental E1 data this resulted in a standard curve with an elevated baseline and a lower E1G working range. The E1 standard curve was brought back to the optimised E3 position by increasing the concentration of antiserum to 29 nM.

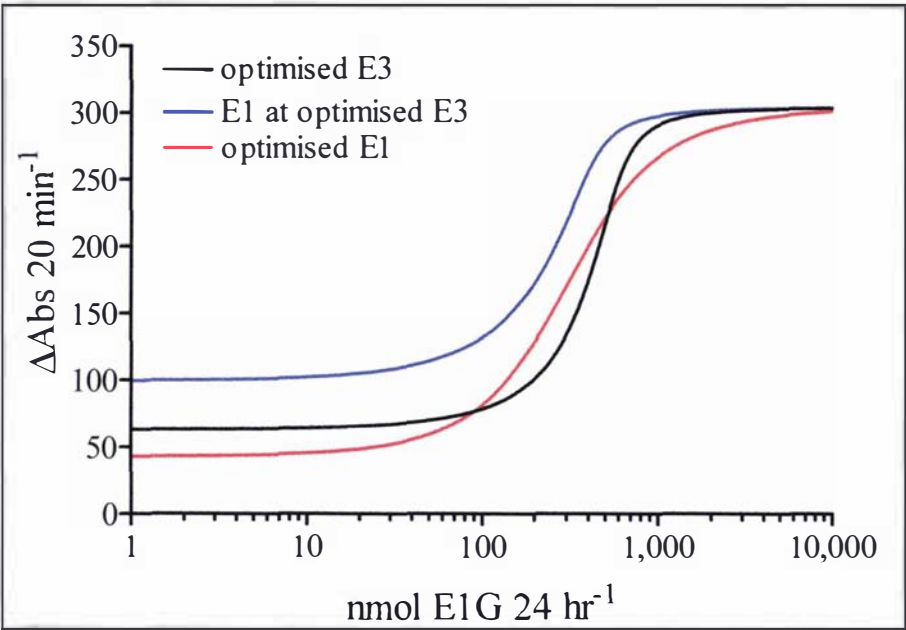


FIGURE 3.25: Computer Simulation of the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Standard Curves and the E1 Standard Curve performed at E3 Optimised Conjugate and Antiserum Conditions

Conditions	Optimised E1	Optimised E3	E1 at E3 Optimised
K_{d1} (nM)	0.1	0.1	0.1
K_{d2} (nM)	3.0	0.4	3.0
Conjugate (nM)	19.0	19.0	19.0
Antibody sites (nM)	29.0	20.0	20.0

3.3.11 The Effect of Blank Urine on the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate Optimised Standard Curves

The function of the blank urine in the EIG assay is to correct the standard curve for any non-specific urinary matrix effects which are not due to the presence of urinary EIG but may affect the urinary EIG assay. Figure 3.26 shows the effect of addition of a blank urine sample obtained from an ovariectomised woman on the shape and position of the optimised standard curve obtained with the E1 conjugate and the 243-4 antiserum.

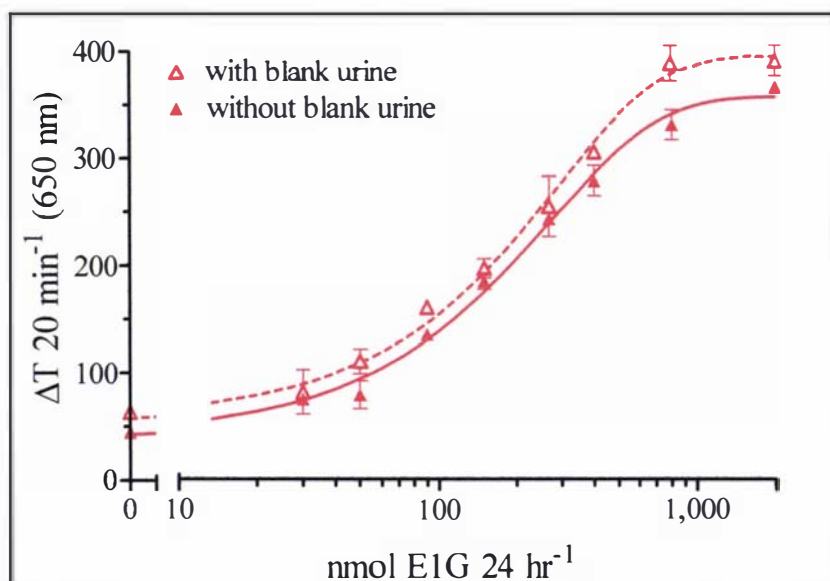


FIGURE 3.26: The Optimised Estrone Glucuronide Standard Curves for the E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4 in the Presence and Absence of a Blank Urine Sample

Conditions in Assay Mixture: E1 - 21 nM enzyme, 0.08 μ L antiserum; blank urine - from an ovariectomised woman

The two curves were performed on the same day and thus were produced using the same batch of substrate and the same dilutions of conjugate and antiserum. The only obvious difference between the shapes and positions of the two standard curves was that the rates measured in the presence of the blank urine were consistently and slightly elevated relative to the control rates.

Figure 3.27 (page 121) shows the effect of the same blank urine on the optimised standard curve obtained with the E3 conjugate and the 243-4 antiserum. The standard curves outlined with solid lines and markers were produced in the absence of a blank urine sample. These curves were obtained on three separate days which covered a total period of nine days. Thus each of these curves was produced using a fresh batch of substrate and with fresh dilutions of the antiserum and the conjugate.

The position of the standard curves was very stable showing little day-to-day variation. The blank urine standard curve was produced using the same blank urine as that used in

Figure 3.26 with the E1 conjugate, and was produced on the same day as the control standard curve outlined in blue. Its effect on the shape of the standard curve was much more dramatic with the E3 sample. Compared to the control standard curve, the baseline of the E3 blank standard curve was greatly elevated and the whole curve was much shallower.

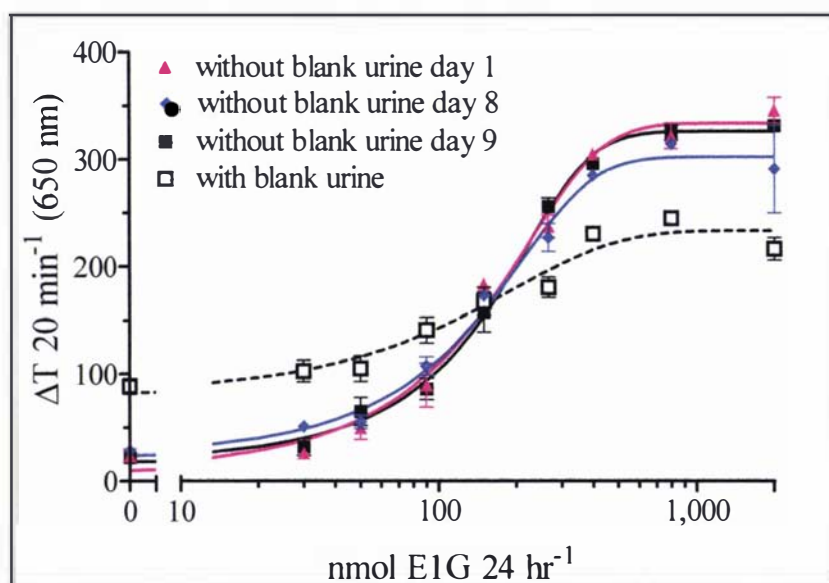


FIGURE 3.27: The Optimised Estrone Glucuronide Standard Curves for the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4 in the Presence and Absence of a Blank Urine Sample

Conditions in Assay Mixture: E3 - 19 nM enzyme, 0.048 μ L antiserum; blank urine - from an ovariectomised woman

Although the shape of the standard curve was greatly affected by the blank urine, the standard curve's position appeared to be stable. Both curves had a mid-point (point of inflection) equivalent to approximately 150 nmol E1G 24 hr⁻¹. In contrast the baseline changed from the relatively low level of 30 ΔT 20 min⁻¹ to 85 ΔT 20 min⁻¹ and the maximum rate was reduced from 319 ΔT 20 min⁻¹ down to only 230 ΔT 20 min⁻¹.

3.3.12 An Alternative Source of Blank Urine

The blank urine standard curve data presented in the previous Section were obtained using a urine sample from an ovariectomised woman. This sample was supplied by the Royal Woman's Hospital in Melbourne and is no longer available to us. Thus the ability of a urine collected from a young girl (aged 24 months) to replace this sample was tested. Usually a blank urine is diluted to a constant urine excretion rate of 150 mL hr^{-1} of collection i.e. the same rate as for the daily urine samples collected by the Ovarian Monitor clients. The average molar strength of a thus diluted urine sample is approximately 10 mM . Two different methods of diluting the child's urine were examined. In one method the sample was diluted to the standard urinary excretion rate of 150 mL hr^{-1} and in the other method the conductivity of the urine was measured and the sample diluted to the same conductivity as the 10 mM tris-maleate buffer.

Figure 3.28 shows the optimised E3 conjugate/antiserum 243-4 standard curves obtained under the standard conditions and with the two diluted child urine samples. All three standard curves were produced on the same day. Compared to the control standard curve, the two blank urine samples did not affect the position or shape of the standard curve.

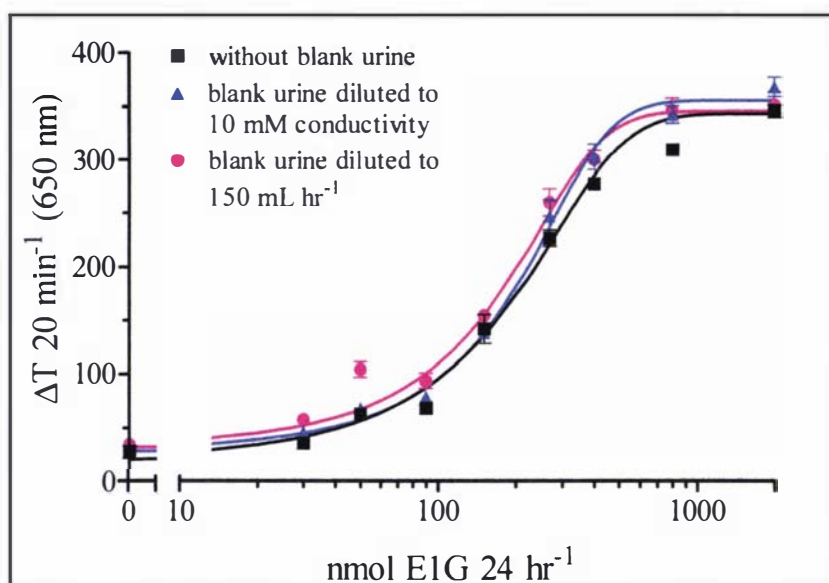


FIGURE 3.28: The Optimised Estrone Glucuronide Standard Curves for the E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate with Anti-Estrone Glucuronide Antiserum 243-4 in the Presence and Absence of a Child's Blank Urine Sample Diluted using Two Methods

Conditions in Assay Mixture: E3 - 19 nM enzyme, $0.048 \text{ }\mu\text{L}$ antiserum; blank urine - from a 24 month female child, see key for dilutions

3.3.13 The Use of the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate for the Determination of Urinary Estrone Glucuronide Excretion for a Complete Menstrual Cycle

Figure 3.29 shows the daily urinary $\Delta T \ 20 \text{ min}^{-1}$ data for a complete menstrual cycle measured using different estrone glucuronide assay procedures.

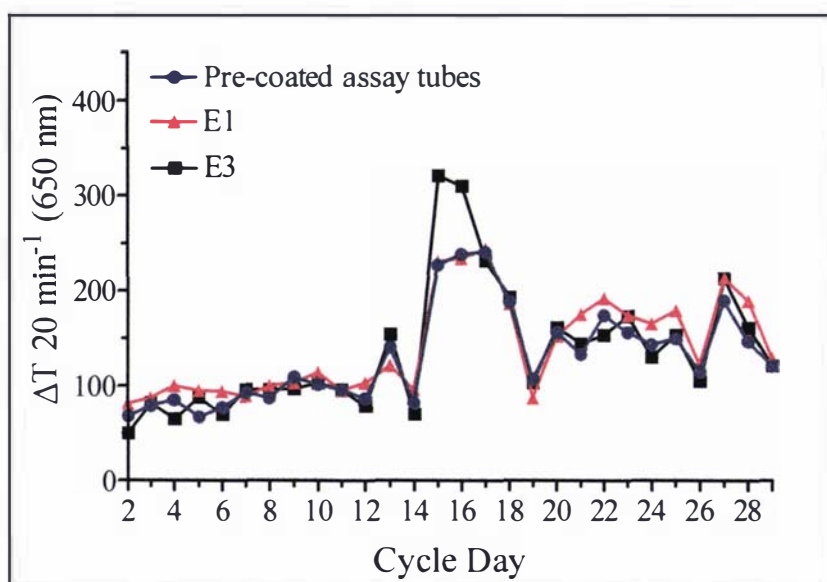


FIGURE 3.29: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle expressed as Twenty Minute Change in Transmission measured using the Pre-Coated Assay Tubes, and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays

Pre-coated Assay Tubes: #14 (429-435)

Conditions of Wet Chemistry Assay Mixtures: E1 - 21 nM enzyme, 0.08 μL antiserum; E3 - 19 nM enzyme, 0.048 μL antiserum; antiserum - 243-4

Data were collected using the pre-coated Ovarian Monitor assay tubes (batch 14, lot 429-435); and also using the wet chemistry laboratory assay with the E1 and E3 conjugates and the fourth bleed of antiserum 243. The E1 assays were performed using 21 nM of conjugate in the assay mixture and the equivalent of 0.08 μL of the undiluted antiserum, and the E3 assays were performed using 19 nM of conjugate in the assay mixture and the equivalent of 0.048 μL of the undiluted antiserum. The wet chemistry assays were all performed on the same day and each urine sample was only assayed once with each conjugate. The pre-coated assay was performed in duplicate with each sample being assayed once on two separate days. The pre-coated assay tube data presented in Figures 3.29-3.33 represents the average of these two sets of results.

The profile of the daily transmission data showed very good agreement between the different methods. A fairly flat baseline was observed between days 2-12 of the cycle, followed by a short peak on day 13 and another dip back to baseline levels again. The major peak occurred between days 15 and 18. The agreement between the data using the E1 conjugate and the pre-coated assay was very high over these four days. In both assays

the highest rates were recorded over days 15 to 18 and resulted in two essentially identical flat topped peaks with an average twenty minute ΔT of 236 units. Significantly higher rates (320 average ΔT 20 min⁻¹) were recorded for the data obtained with the E3 conjugate over days 15 and 16 resulting in two peak days, as opposed to a three day plateau, after which the rates again decreased. From day 17 onwards the agreement between the rates observed with the three assays was again very high. The rates fell to a minimum on day 19, and then increased to give a broad shallow peak between days 20 and 25 which ended in another small fall on day 26 followed by a second low peak. The rates of this second rise peaked on day 27 and then fell for two consecutive days.

At the very high urinary E1G excretion rates experienced over the peak period, the lysis rates were elevated into the top region of the standard curve and thus needed to be reassayed at a higher dilution. For home monitoring this is always by a factor of two. If the woman is recording her daily data chart in transmission units, the ΔT 20 min⁻¹ for the two-fold diluted urine sample is simply doubled and then plotted on her chart. However, if the woman is charting her cycle as nmol E1G 24 hr⁻¹, then the observed rate of lysis for the doubly diluted sample is used to measure the equivalent nmol E1G 24 hr⁻¹ from the standard curve and then this value is multiplied by two to give the concentration of E1G for the original time diluted sample. The cycle illustrated in Figure 3.29 is unusual in that there were several days with an almost equal exceptionally high urinary E1G output. Thus the cycle days with these four highest rates of lysis (15-18) were repeated at twice their normal dilution under all three different protocols and the resulting data replotted as described above (Figure 3.30).

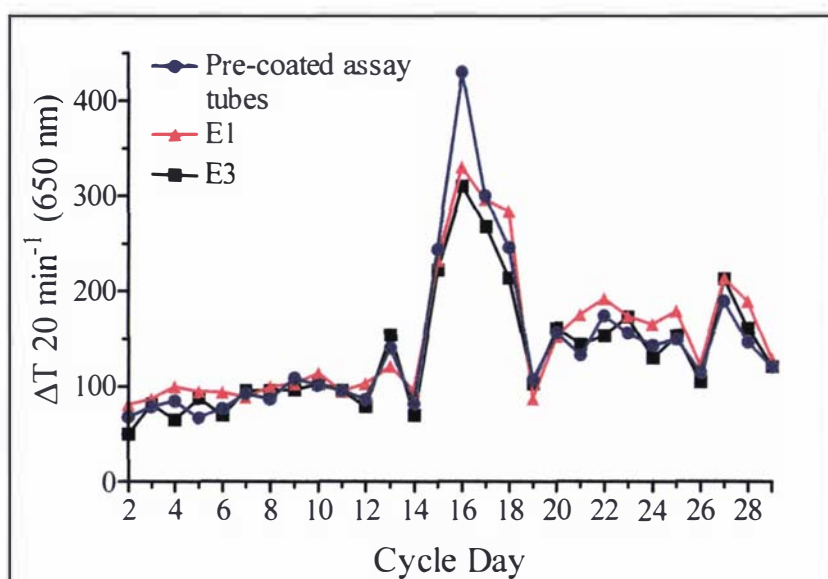


FIGURE 3.30: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle expressed as Twenty Minute Change in Transmission measured using the Pre-Coated Assay Tubes, and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays and a Double Dilution over Days 15-18

Pre-coated Assay Tubes: #14 (429-435)

Conditions of Wet Chemistry Assay Mixtures: E1 - 21 nM enzyme, 0.08 μ L antiserum; E3 - 19 nM enzyme, 0.048 μ L antiserum; antiserum - 243-4

The pattern of rising and falling rates of estrone glucuronide excretion over the peak period observed between the different protocols was more consistent for the doubly-diluted urine samples. The peak was also better resolved using the repeat values. In contrast to the original data where the peak was observed as a 2-3 day plateau and in which no actual peak day could be identified, the repeat analyses allowed it to be identified unambiguously by all three methods as occurring on day 16 of the cycle.

Figure 3.31 shows the same data after conversion into E1G excretion rates using the standard curve appropriate to each method.

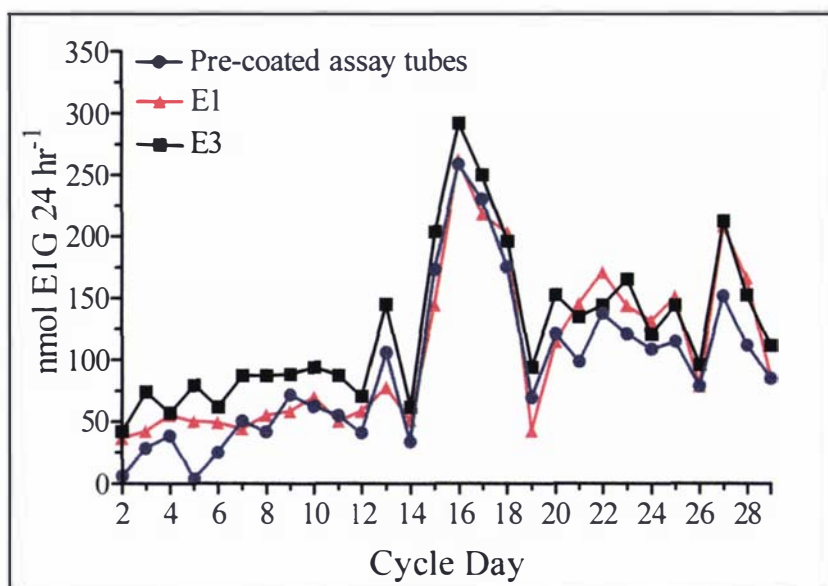


FIGURE 3.31: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle measured using the Pre-Coated Assay Tubes, and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays and a Double Dilution over Days 15-18

Pre-coated Assay Tubes: #14 (429-435)

Conditions of Wet Chemistry Assay Mixtures: E1 - 21 nM enzyme, 0.08 μ L antiserum; E3 - 19 nM enzyme, 0.048 μ L antiserum; antiserum - 243-4

Although the two sets of daily profiles must obviously increase and fall together, the magnitude of these rises and falls does change due to the logarithmic relationship between the rates of lysis and the hormone concentrations. Interestingly at low to intermediate E1G excretion rates (days 2-12), the agreement between the different methods was higher when the data were charted as enzymatic rates (ΔT) as opposed to E1G excretion rates. In contrast, over the major peak period the correlation between the different methods appeared to be higher when the data were expressed in terms of the E1G excretion rates. Expressing the results in terms of E1G excretion rates also helped to define the major E1G peak (i.e. between days 14 and 19) by making it more symmetrical. This was especially the case with the data obtained with the E1 conjugate and the pre-coated tube data. In addition when the data were plotted in terms of the E1G excretion rates the secondary peak which appeared as a very minor change in the baseline, just prior to the major peak,

became much more defined. This effect was particularly apparent for the data obtained with the E3 conjugate and the pre-coated assay tubes.

Figure 3.32 shows the effect of using the blank urine E1 standard curve as opposed to the control E1 standard curve for calculating the apparent estrone glucuronide excretion rates.

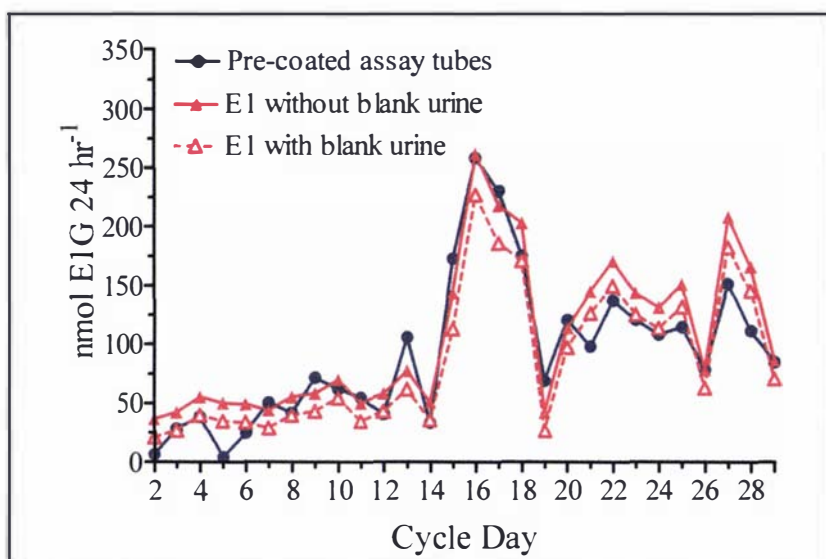


FIGURE 3.32: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle measured using the Pre-Coated Assay Tubes, and the Optimised E1 Hen Egg White Lysozyme-Estrone Glucuronide Assays with and without blank urine and using a Double Dilution over Days 15-18

Pre-coated Assay Tubes: #14 (429-435)

Conditions of Wet Chemistry Assay Mixtures: E1 - 21 nM enzyme, 0.08 μ L antiserum; blank urine - from an ovariectomised woman

The major effect of using the blank standard curve was to reduce the apparent magnitude of the estrone glucuronide excretion rates. This effect although significant was not dramatic with the peak estrone glucuronide value being reduced from a maximum of approximately 300 nmol E1G 24 hr⁻¹ to approximately 230 nmol E1G 24 hr⁻¹.

Figure 3.33 (page 127) is equivalent to Figure 3.32 except that the E1 conjugate assay data was replaced with the E3 conjugate data.

The effect of using the blank urine standard curve to calculate the E1G excretion rates was similar to that observed for the E1 assay, except that the baseline changes were more pronounced. Thus the magnitude of the E1G excretion rates appeared to be quite heavily depressed on days 2 to 12 by using the E3 blank urine standard curves. Because of the high baseline of the blank urine standard curve for the E3 conjugate (Figure 3.27), in the E3 assay the E1G excretion rate could not be measured for the daily urine samples with very low E1G concentrations. Cycle days 2-4, 6, 12 and 14 all gave a ΔT 20 min⁻¹ rate too low to be read from the E3 blank urine standard curve. These days are recorded on the graph as having an E1G excretion rate of 0 nmol E1G 24 hr⁻¹. In contrast, at high rates of urinary

E1G excretion, the E3 blank urine standard curve had an opposite effect on the estimation of the E1G excretion rates. Compared to the equivalent cycle data obtained without blank urine in the generation of the standard curve and the pre-coated assay tube standard curve, when the E3 blank urine standard curve was used to calculate the rate of urinary excretion for cycle day 28, a very high E1G excretion rate of 340 nmol 24 hr⁻¹ was estimated which was in excess of that recorded for the main peak. Paradoxically, there was little effect of reading from the blank urine curve for the main E1G peak day of day 16 (280 versus 260 nmol 24 hr⁻¹).

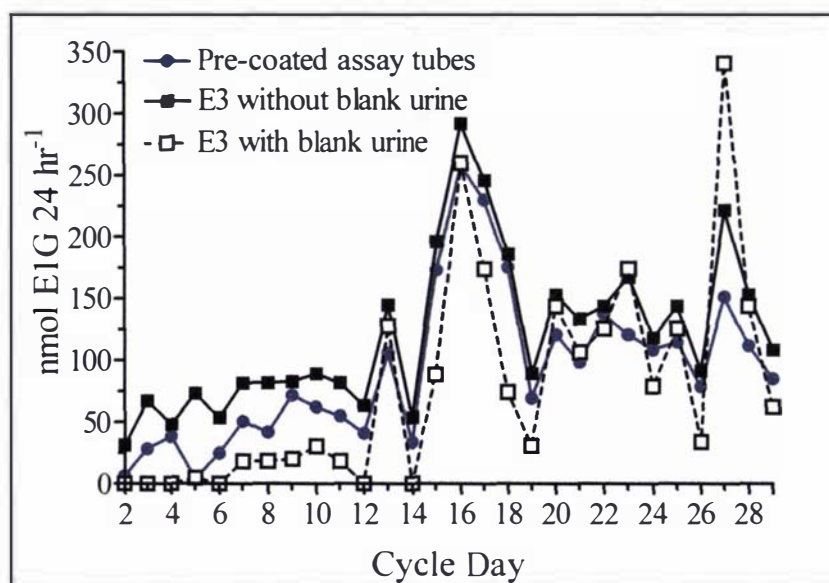


FIGURE 3.33: Estrone Glucuronide Excretion Profile for a Normal Menstrual Cycle measured using the Pre-Coated Assay Tubes, and the Optimised E3 Hen Egg White Lysozyme-Estrone Glucuronide Assays with and without Blank Urine and using a Double Dilution over Days 15-18

Pre-coated Assay Tubes: #14 (429-435)

Conditions of Wet Chemistry Assay Mixtures: E3 - 19 nM enzyme, 0.048 μ L antiserum; blank urine - from an ovariectomised woman

The cycle data obtained by the three assay methods, pre-coated assay tubes, and the optimised E1 and E3 assays, were compared by Deming Regression. This showed that the correlation between all three methods was high ($r \geq 0.93$) but there was some variation in the Y-intercept with both the E1 and E3 assays measuring higher excretion rates than the Ovarian Monitor assay, and the E3 assay measuring consistently higher excretion rates than the E1 assay. However, the gradients of the correlations were all very close to 1 thus, the three methods were measuring the same absolute changes in concentration. The correlation lines between the three methods are shown in Figures 3.4 and 3.5 and a summary of the correlation data is given in Table 3.2.

TABLE 3.2: Deming Correlation between the Pre-Coated Ovarian Monitor Assay Tubes and the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Urinary Estrone Glucuronide Excretion Rates throughout an Endocrinologically Normal Menstrual Cycle

Correlation	Slope	Y-Intercept (nmol E1G 24 hr ⁻¹)	Correlation Co-efficient (r)
Pre-coated versus E1	1.023	10.9	0.930
Pre-coated versus E3	0.968	35.4	0.974
E3 versus E1	1.070	-35.4	0.935

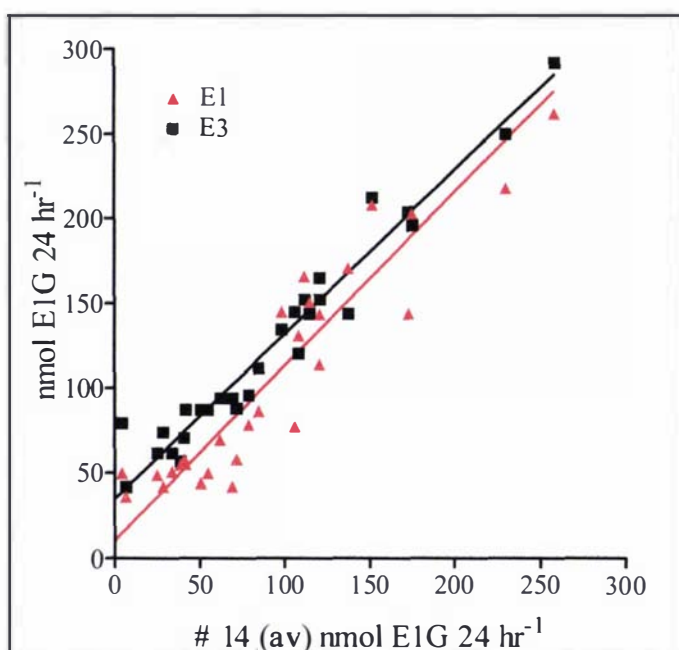


FIGURE 3.34: Deming Correlation between the Pre-Coated Ovarian Monitor Assay Tubes versus the Optimised E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Urinary Estrone Glucuronide Excretion Rates throughout an Endocrinologically Normal Menstrual Cycle

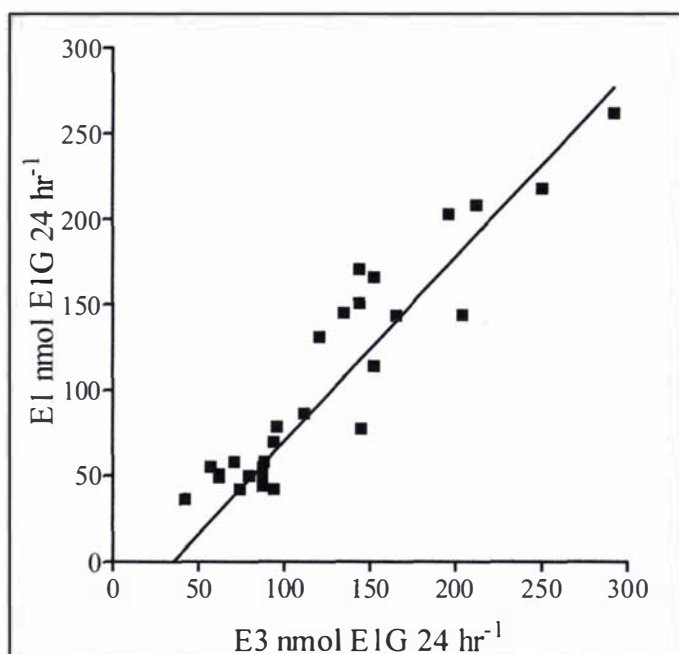


FIGURE 3.35: Deming Correlation between the Optimised E1 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate versus the Optimised E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate for the Measurement of Urinary Estrone Glucuronide Excretion Rates throughout an Endocrinologically Normal Menstrual Cycle

3.4 DISCUSSION

3.4.1 Determinants of the Ideal Ovarian Monitor Standard Curve

From a theoretical point of view the shape of an estrone glucuronide standard curve and its position on the concentration axis is determined by several factors. Critical parameters which are easily controlled include the concentration of the antiserum and lysozyme-E1G conjugate in the assay and their relative concentrations. Other important determinants of the shape and position of the standard curve include the structure and specific activity of the conjugate, the nature of the antibody and any antibody related matrix effects, and the dissociation constants for the antibody.E1G (K_{d1}) and antibody.E1G-conjugate (K_{d2}) immune complexes (Daunert *et al.*, 1989).

In this work, new antibodies, conjugates and standards have had to be prepared. The main aims of the work presented in this chapter were to determine whether the Melbourne home estrone glucuronide assay can be reproduced in terms of speed, accuracy and sensitivity using these new biomaterials and whether there are any advantages to be gained by using a defined estrone glucuronide conjugate as the signal generator.

This is the first time that structurally defined enzyme conjugates have been available for study. Without the crystal structure of the lysozyme conjugates the exact orientation of the substituted groups cannot be definitively determined. However, as the conjugation sites

are known and the orientation of the estrone glucuronide moiety relative to the protein surface is partially fixed by the β 1-6 linkage of the protein and steroid to the glucuronide ring, it can be modelled. A model of the E6 conjugate which is acylated to estrone glucuronide at all three of the E1 and E3 acylation sites (i.e. lysine residues 33, 97 and 116) is shown in Figure 3.36.

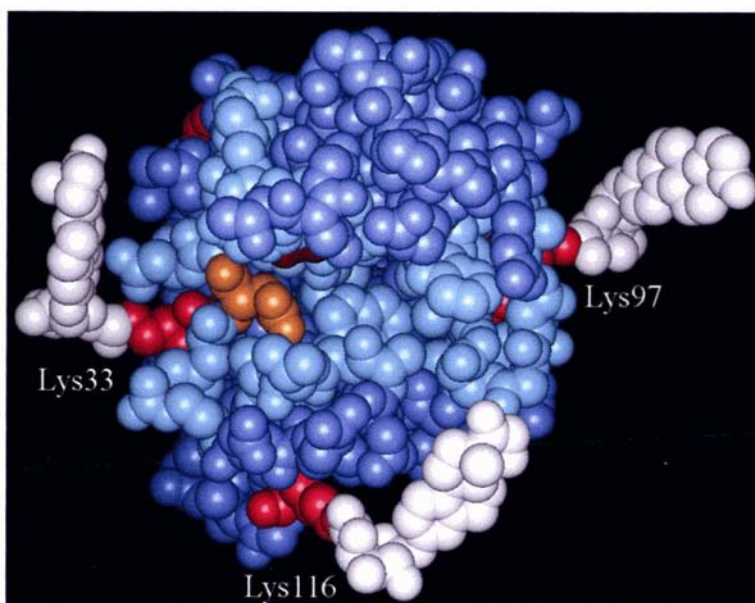


Figure 3.36: Model of E6 Hen Egg White Lysozyme-Estrone Glucuronide Conjugate showing the Positions of the Lysine 33, 97 and 116 Estrone Glucuronide Moieties around the Substrate Binding Site

General protein - medium blue, Binding site cleft - pale blue, Glu 35 active site residue - orange, Asp 52 active site residue - brown, Lysines - red, Estrone glucuronide - white

One of the obvious features of the structure is that the almost planar steroid moieties are more or less parallel to the lysozyme surface. Although there is no three dimensional structure

available for an anti glucuronide antibody it is known that the binding sites for small haptens generally lie in a cleft in the centre of the antibody binding site (Vargas-Madrado *et al.*, 1995). Hence it is interesting that the antibodies can bind so well to the lysozyme-E1G conjugates given that the plane of the steroid is so close to the lysozyme surface. Hence, it is probable that there will be significant contacts between the antibody and the enzyme surfaces.

The conjugation site determines not only the distance of the estrone glucuronide moieties to the binding cleft, but also the electrostatic and hydrophobic environments surrounding the steroid substituents as well. Since binding proteins such as antibodies are highly selective, these different microenvironments and accessibility of the steroid ligand can impair, or promote, their binding affinity. Such changes would be expected to alter the immunochemical behaviour of the conjugates with a given antibody and hence the immunoassay characteristics.

The gold standard for the recognition of any beneficial or detrimental changes to the assay has to be the standard curves produced by the current antibodies, conjugate and standards used in the Melbourne pre-coated assay tubes. This assay has been accredited by the Australian National Association of Technical Authorities for use in normal cycles and gonadotrophin induction programmes at the Royal Woman's Hospital, and the Mercy Hospital in Melbourne.

The key features of an estrone glucuronide standard curve suitable for the measurement of urinary estrone glucuronide excretion rates during the menstrual cycle are:

- i) the anchor point of the zero standard. That is the rate of lysis (in units of ΔT 20 min⁻¹) for the zero standard on any new standard curve should be either the same as, or less than, that observed for the zero standard on the Melbourne standard curve,
- ii) the E1G excretion rates for which the rate of lysis is first clearly observed by visual examination to be higher than that measured for the zero standard. This concentration (which determines the assay sensitivity) should be at the same, or lower, concentration than that which is currently found with the Melbourne biomaterials, i.e. 50 nmol E1G 24 hr⁻¹,
- iii) the mid-point of the curve i.e. the point of inflection. This represents the most sensitive portion of the standard curve and should be close to 140 nmoles E1G 24 hr⁻¹, and
- iv) the maximum workable range of the standard curve i.e. the E1G concentration equivalent to the upper end of the standard curve before the rates begins to level off dramatically. This should be no less than 267 nmol E1G 24 hr⁻¹.

As illustrated in Figure 3.4, the standard curves obtained with the pre-coated Ovarian Monitor assay tubes vary between box lots. This is because the standard curve is very sensitive to the ratio of conjugate to antibody, the amounts of both varying during

manufacture due to losses from the drying process. To deal with this variation a standard curve which is specific to the batch and lot number is included with every order of assay tubes. Variations in the position and shape of the standard curve as observed in Figure 3.4 although significant, are not sufficient to compromise the precision of the home assay as the changes in the urinary excretion rates which signify changes in fertility are much greater than the experimental errors introduced from this source.

The normal ranges for estrone glucuronide used in Melbourne as part of the certification procedure are: a mean follicular phase baseline of $65 \text{ nmol } 24 \text{ hr}^{-1}$ (range for 10th to 90th percentiles of 35-115) and a mean pre-ovulatory estrone glucuronide peak value of $315 \text{ nmol } 24 \text{ hr}^{-1}$ (range for 10th to 90th percentiles of 225-465). Figure 3.4 illustrates two typical standard curve profiles for two different batches of pre-coated estrone glucuronide assay tubes. The region of maximum sensitivity on these standard curves ($50\text{-}267 \text{ nmol E1G } 24 \text{ hr}^{-1}$) lay almost in the middle of the normal ranges. The lower limit of the standard curves is just sufficient to provide an accurate marker for the beginning of the fertile period as rates of urinary estrone glucuronide excretion of less than $50 \text{ nmol } 24 \text{ hr}^{-1}$ are almost exclusively the result of the peripheral aromatisation of androgens derived from the adrenal gland. Hence only when the urinary estrone glucuronide excretion rates exceed this baseline level is there an estrone glucuronide component attributable to ovarian activity. The lower these baseline levels of estrone glucuronide are, the earlier the first rise above these baseline levels can be detected, and hence the beginning of potential fertility. However, current experience suggests that for home monitoring a urinary excretion rate equivalent to $50 \text{ nmol E1G } 24 \text{ hr}^{-1}$ makes an appropriate limit for the assay sensitivity.

Although the region of maximum sensitivity of the standard curve covers the early period of ovarian estrogen production, the upper operational limit of the standard curve (for example $267 \text{ nmol E1G } 24 \text{ hr}^{-1}$ for the standard curves in Figure 3.14) may be exceeded by some women at the mid-cycle peak. To enable the rate of urinary estrone glucuronide excretion to be determined accurately, the Ovarian Monitor protocol recommends that these urine samples be reassayed after further dilution. However, it should also be noted that the assay is optimised to detect first rises and for many uses the actual value of the estrone glucuronide excretion rate on the peak day is not vital to a correct interpretation of the fertility patterns. Hence, in practise the extra dilution step is rarely necessary as the most important information about the fertility status has already been determined by daily plotting of the $\Delta T \text{ } 20 \text{ min}^{-1}$ values. In other words, for home monitoring of estrone glucuronide as a fertility indicator it is the pattern which is important for the user, not the absolute values. However, for clinical use conversion of the raw data into actual urinary excretion rates is often required.

3.4.2 Stability of the Melbourne Standard Curves

The pre-coated assay tubes are usually considered to be viable for up to 9 months after production, with a conservative expiry date of 6 months issued with every order. Figure 3.6 showed that the day-to-day variation in the shape of the standard curve for the same batch of assay tubes was limited to the upper end of the curve and thus occurred

largely outside the normal physiological range for estrone glucuronide excretion which is observed throughout the menstrual cycle. The fact that for a given batch of pre-coated assay tubes the actual position of the standard curve did not change over this six month period illustrates the exceptional stability of the standard curve and the robustness of the assay once the assay components have been dried.

3.4.3 Validation of the Estrone Glucuronide Standards

The concentration, purity and structural integrity of the synthetic estrone glucuronide and pregnanediol glucuronide standards used to obtain the Melbourne standard curves have already been fully validated experimentally using naturally occurring estrone glucuronide and pregnanediol glucuronide, and radio labelled estrone glucuronide and pregnanediol glucuronide as controls. The naturally occurring estrone glucuronide and pregnanediol glucuronide used in the original tests were isolated from pregnancy urines (Brown, personal communication). The purification of these steroid glucuronides from urine is time consuming, unpleasant and low yielding, requiring approximately 80 litres of pregnancy urine to produce 400 and 600 mg of estrone glucuronide and pregnanediol glucuronide respectively. The radio labelled steroid glucuronides were prepared by conjugating tritium labelled estrone and pregnanediol enzymatically to glucuronic acid with the enzyme, uridine diphosphate glucuronate glucuronyl transferase. The catalytic activity of this enzyme, as its name implies, is specific to the synthesis of β -orientated sugar conjugates only. This is the same stereochemistry of the sugar as in the steroid glucuronides excreted in human urine.

Although the integrity of the estrone glucuronide and pregnanediol glucuronide produced by the enzyme is not questioned, in common with the isolation of steroid glucuronides from urine, the low yield means it cannot be employed to supply estrone glucuronide and pregnanediol glucuronide for all the potential commercial and research applications of the Ovarian Monitor. Thus, all the estrone glucuronide and pregnanediol glucuronide used to make the standards, the lysozyme conjugates, and the protein conjugates required for estrone glucuronide and pregnanediol glucuronide antibody production, is chemically synthesised.

As the specificity of the transferase ensures the structural integrity of the enzymatically synthesised steroid glucuronides, the tritiated form of these compounds make ideal tracers for examining the chromatographic behaviour of estrone glucuronide and pregnanediol glucuronide i.e. the target compounds of the organic synthesis. Chromatographic experiments have shown that urinary estrone glucuronide and pregnanediol glucuronide, and their equivalent tritiated enzymatically synthesised forms, co-elute with the Melbourne chemically synthesised steroid glucuronides under a wide range of chromatographic conditions. All experimental tests confirmed the purity and structural integrity of the chemically synthesised steroid glucuronides as well as the concentrations of the standard solutions made from them. This comprehensive validation of the Melbourne standards means that the pre-coated Ovarian Monitor assay tubes can be used with confidence for determining the concentrations of unknown estrone glucuronide or pregnanediol glucuronide solutions, provided that the correct standard curve for the assay tube batch

number is always used. It also means that the pre-coated assay tubes may be used in combination with their corresponding standard curves to assess the accuracy of any other set of standards of unknown quality, for example as in the present work.

The estrone glucuronide standards prepared according to Section 3.2.2.4 in this thesis generated standard curves which were identical within experimental error over the critical concentration range with the Melbourne standard curves supplied with the pre-coated assay tubes (Figures 3.5 and 3.6). These results can be considered as conclusive proof that the estrone glucuronide sample synthesised in Chapter Two from which the Palmerston North standards were prepared was of the correct stereochemistry and was pure and that the preparation of the estrone glucuronide stock solution and the subsequent dilution series used to make the standards were accurately performed. Thus the estrone glucuronide standards prepared in this work can be used with confidence to examine the properties of the anti-estrone glucuronide antisera and lysozyme-estrone glucuronide conjugates and to determine their effect on the Ovarian Monitor estrone glucuronide assay.

3.4.4 The Wallaceville Animal Research Centre Anti-Estrone Glucuronide Antisera

The characteristics of the estrone glucuronide antiserum is one of the main uncertain variables in the establishment of a reproducible immunoassay for estrone glucuronide. The pre-coated tubes supplied by Melbourne use a polyclonal antiserum raised in sheep, and the finite supplies of this serum limit the general applicability of the test. There is no guarantee that a similar antiserum can be produced, hence, the necessity of raising new sheep polyclonal anti-E1G antisera to establish an independent estrone glucuronide assay in this work provides a test of the sensitivity of the assay to the source of the polyclonal antiserum. The few points in the titration curve and the broad range of antiserum volumes (0.05-100 μ L) examined in the partial titration of the Wallaceville sheep antisera using the Melbourne conjugate only allowed a rough overall titration profile to be determined. However, all four anti-E1G antisera caused high inhibition of conjugate activity, and showed, for the antiserum volumes tested, their maximum inhibition at a concentration equivalent to 1 μ L of the neat antiserum per assay tube. However, because the next lowest and highest antiserum concentrations differed in concentration by a factor of 10, neither the maximal percentage inhibition nor the lowest antiserum concentration required for maximum inhibition could be determined from such a titration (Figure 3.7).

The fact that all four new sheep antisera resulted in a high level of inhibition of the Melbourne conjugate suggested that they could all be used to test the suitability of the defined lysozyme-E1G conjugates for use in the Ovarian Monitor assays and in the development of alternative assay protocols.

The observation that very high volumes of antiserum resulted in a loss of inhibition is not easily explained. One possible explanation is that the antibody molecules form complexes with either other antibody molecules or other antiserum components at high concentration which prevents their interaction with the lysozyme conjugates. Although the explanation for this apparently contrary inhibitory response can only be speculative, there are important practical ramifications for such a phenomenon. Obviously, using a single very high

antiserum concentration to estimate the maximal inhibitory action of an antiserum against a new conjugate solution is not valid and will almost certainly result in very low estimates for the inhibition and possible rejection of a highly inhibiting antiserum or highly inhibitable lysozyme conjugate. Thus, the antiserum must always be titrated down to its maximal inhibitory level.

3.4.5 Optimisation of the Buffer Ionic Strength

Before the new antisera could be tested in a standard curve format it was necessary to optimise the assay performance of the E1 and E3 signal generators.

The action of native lysozyme against its bacterial substrate, the *M. lysodeikticus* cell wall, and the effect of changes in the pH and ionic strength of the reaction buffer on this activity is well established (Maurel and Douzou, 1976; Price and Pethig, 1986). As the buffer concentration increases from a low to a high ionic strength at a single pH value, the lytic activity of lysozyme increases and then falls resulting in a bell shaped activity curve similar to that shown in Figure 3.8. For native lysozyme at pH 7.0, the optimum ionic strength is about 60-80 mM (Maurel and Douzou, 1976). As the pH is decreased the activity curve broadens and the optimum salt concentration shifts to higher ionic strengths.

The fact that the lytic activity is highly dependent upon the basic nature of lysozyme and the ionic strength of the reaction buffer in a complex manner suggests that the optimum ionic strength may be modified by the conjugation and subsequent neutralisation of some of the positively charged lysine residues. Furthermore the magnitude of this effect may be dependent upon the site of conjugation, with conjugation to the lysine residues closest to the active site having the potential for the greatest detrimental effect on the activity.

A charge based model to explain the shape of these curves has been developed by Maurel and Douzou (1976) and further validated and refined by Price and Pethig (1986). The *M. lysodeikticus* bacterial cell wall is a very negatively charged entity having a constant net negative surface charge of approximately $1.5 \mu\text{C cm}^2$ for the pH range of 4-8 (Price and Pethig, 1986). In the model of Price and Pethig, the pH and ionic strength of the buffer are thought to affect lytic activity by modifying the electrostatic attraction between the positively charged enzyme and the negatively charged bacterial cell wall. At ionic strengths above the optimal range for lytic activity, the electrostatic potential in the immediate environment of the cell wall and the enzyme is lowered due to an increase in the ionic interactions of the enzyme and its substrate with the buffer salts. Paradoxically, the decrease in the rates of cell lysis at low ionic strengths is also postulated to be due to the charges on the cell wall and lysozyme molecule becoming inadequately masked by the counter ions.

The enzyme and the cell wall will always be highly positively and negatively charged respectively over the middle pH range regardless of the ionic strength. However over this range they both still possess some local heterogeneity of the negative and positive charges respectively. The low lytic activity observed at low ionic strength is postulated therefore to be due to the lack of counter ions to mask this heterogeneity. More specifically, this fall

in lytic activity is thought to be due to the poor shielding of the negatively charged catalytic Asp 52 residue by other ions in solution causing an electrostatic repulsion between the enzyme and the cell wall which in turn hinders the formation of the catalytically productive enzyme-substrate complexes.

The results displayed in Figure 3.8 show that changes in ionic strength have an effect on the activity profile of E3 similar to those observed for native lysozyme by Maurel and Douzou (1976). The tris-maleate buffer concentration range for optimum E3 activity at pH 7.0 was narrow, with the maximum activity being observed at a buffer concentration between 40-75 mM, which was slightly lower than for native lysozyme. This result carries with it the implication that more highly substituted lysozyme conjugates might have an even lower ionic strength optimum at pH 7.0. Thus, it is feasible that the lower specific activities reported for E2, E4, E5 and E6 (see Table 2.2) might be due in part to inhibition of their lytic activities by an inappropriately high ionic strength and that optimising the buffer medium may increase the activities of these higher substituted E1G-lysozyme conjugates.

In the Ovarian Monitor assay, the constituents were diluted with tris-maleate buffer of different molarities. For example the estrone glucuronide standards, antiserum and conjugate were diluted with 10, 75 and 350 mM tris-maleate buffer respectively. The volumes of the different diluted constituents also varied during the optimisation and characterisation of the assay with the difference being made up with 40 mM general reaction buffer which was added to give a total reaction volume before the addition of substrate of 340 μ L. Despite these variations, the final molar concentration of the reaction mixture never exceeded the optimum concentration range of 40-75 mM. Similar results were obtained for the E1 conjugate. Thus over the course of this work, all studies on the Ovarian Monitor assay were performed using this optimal buffer concentration for the mono-substituted conjugates.

3.4.6 Measurement of the E1 and E3 Lysozyme Conjugate Activity

Before constructing the standard curves it is advisable to determine the dependence of the enzymic activity on the concentration of the enzyme conjugates since this defines the shape of the standard curve and its working range. As shown in Figure 3.9, the relationship between the observed rate of lysis and enzyme concentration was essentially the same for the E1 and E3 conjugates. Although this relationship was non-linear, it could be used to convert end point assay lytic rates into concentrations of conjugate.

The degree of curvature observed largely depends upon the units used to express the enzyme activity. As discussed in the Introduction (Section 3.1.1), the Ovarian Monitor measures lytic activity as a change in transmission units (Figure 3.9) which is not linearly related to the concentration of intact cells. This curvature is exaggerated at high enzyme concentration and with increasing assay times. This is because as the lytic assay proceeds the proportion of the substrate available to the enzyme as an intact cell decreases and the enzyme's activity becomes increasingly directed towards reducing the lysed cell wall remnants into smaller fragments which cannot be observed spectrophotometrically.

A measure of the extent of lysis can be obtained by comparing the transmission value at the end of the assay time with the transmission value for an equivalent buffer only control. For example, in the Ovarian Monitor the transmission of a buffer only reaction mixture is approximately 650 units before the addition of the bacterial substrate and is approximately 240 units at the initiation of the reaction. The change in transmission of a 21 nM conjugate reaction mixture over 20 minutes is approximately 350 units. Thus, at this conjugate concentration the transmission level at the end of the assay period is already at 590 units, which is starting to approach the transmission level of the completely cleared reaction mixture.

Hence, the results are inherently more accurate at low conjugate concentrations which is important for detecting the first rise in estrone glucuronide levels. In effect this assay truncates the top end of the standard curve so that very different estrone glucuronide excretion rates give the same 20 minute ΔT (see Figure 3.4) and is the reason why urine samples which give rates outside the linear region of the standard curve are repeated using a further 1 in 2 dilution.

McKenzie and White (1986) showed that the lytic reaction catalysed by lysozyme followed a second order rate of clearing and that the clearing curve data could be linearised. The E1 and E3 clearing curve data could also be linearised by a second order rate equation by first converting the transmission clearing curve data into absorbance using Equation 3.1, and then linearising the data using the second order rate equation given in Equation 3.3. The gradient and intercept of the line was then measured by least squares analysis and the parameters used to generate the second order clearing curves shown in Figures 3.11 and 3.12 for E1 and E3. The fact that all the clearing curves including those performed in the presence of antiserum and estrone glucuronide could be fitted successfully to a second order equation (Figures 3.11 and 3.12), shows that cell lysis by both E1 and E3 is still essentially a second order reaction.

It may be possible to shorten the assay by measuring the rates in terms of the rate constant as opposed to an end point assay. However, for this to be utilised by the Ovarian Monitor, the Monitor would have to be fitted with a silicon chip encoded with several formulae and algorithms. This would include a program for converting the transmission data into absorbance and an algorithm for determining when sufficient data points had been collected over a time period which represented a significant change in the total transmission to enable the data to be accurately linearised with respect to time. This time period would depend upon the active enzyme concentration, and thus would be longer at the lower hormone concentrations. Not only would the Monitor have to also recognise when this time period was reached but it would also have to then linearise the data, extrapolate the line, calculate the line's gradient and hence the rate constant of the reaction, and finally convert this value into a urinary hormone excretion rate using an appropriate standard curve.

However, when this procedure was applied to the pre-coated Melbourne assay tubes it was discovered that the shape of the clearing curve was significantly altered by the drying procedure used to prepare the tubes (see Figure 3.13). Instead of the second order clearing curve seen with all solutions of lysozyme, E1 or E3, the plot of transmission versus assay

time had an initial lag phase followed by an almost second order clearing curve as normal. The reason for the change is unknown but presumably relates to the drying of the assay components onto the wall of the assay tube. A possible reason could be that the biomaterials in the tube need to become rehydrated before behaviour similar to that observed with fresh solutions is obtained. Whatever the reason the use of a second order algorithm to shorten the assay time for estrone glucuronide cannot be applied directly to the present pre-coated assay tubes. It might be possible to measure initial rates for the pre-coated tubes from the initial linear region of their clearing curves and thus obtain a workable standard curve. However, this illustrates the difficulty in working with fresh solutions and extrapolating from their behaviour to predict what happens with actual dried pre-coated assay tubes.

In hindsight it may be profitable to study methods for retaining the integrity of the shape of the clearing curve after drying since this would allow the application of the second order curve fitting procedures discussed above to reduce the assay time. However, at present, assaying for hormone concentration as a change in transmission over a fixed time period remains the best option and has the added advantage that it requires less complicated instrumentation and is thus cheaper - an important consideration for a home test.

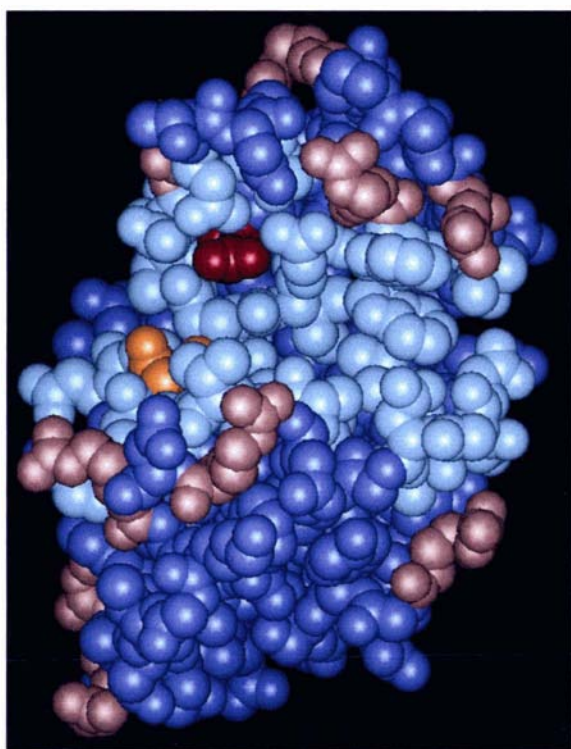


FIGURE 3.37: Model of Hen Egg White Lysozyme showing the Arginine Rich Area surrounding the Substrate Binding Site

General protein - medium blue, Binding site cleft - pale blue, Glu 35 active site residue - orange, Asp 52 active site residue - brown, Arginines - tan

Probably one of the most obvious methods for shortening the assay time is to use an enzyme with a higher specific activity. However, as the differences in specific activity between the E1 and E3 conjugates were minimal this showed that in terms of the potential minimum assay time there was little advantage in one conjugate over the other.

Given that E1 and E3 have different structures and that the positive charges are involved in the primary interaction between lysozymes and the negatively charged bacterial cell wall (Price and Pethig, 1986) it might have been anticipated that there would have been differences in the relationship between lysis rate and enzyme concentration. Probably the most important area of charge for substrate binding and catalysis on the lysozyme molecule is around the binding site cleft. As shown in Figure 3.37 both sides of the binding site cleft are flanked by a line of positively charged arginine residues.

The fact that the E1 (lysines 33 and 97 modified) and E3 (lysine 33 modified) conjugates had very similar specific activities probably reflects the much greater importance of the arginine rich area in binding to the cell wall than the single lysine residues. Modification of a single lysine residue therefore either has no effect on the binding between the lysozyme surface and the bacterial cell wall, or the same amount of binding energy is lost in each case. Clearly, there can be no significant steric effects either since the calibration plots for E1 and E3 are similar to those previously obtained with native lysozyme (Cooke, 1993).

3.4.7 Affinities of E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for Anti-Estrone Glucuronide Antisera From Bleed Three

The sensitivity of the assay depends on the combined affinities of the signal generators for the antibody in the anti-E1G antisera.

The rate of conjugate-antibody binding for a given conjugate-antibody system is determined by the association rate constant (k_{on}) and the concentration of free antibody and conjugate as shown in Equation 3.7 where $[Ab]_t$ is the concentration of free antibody at time t and $[Cj]_t$ is the concentration of free conjugate at time t :

$$rate = k_{on}[Cj]_t[Ab]_t \quad \text{EQN. 3.7}$$

On the other hand, the rate of dissociation of this immune complex is determined by its concentration and dissociation rate constant (k_{off}) as reported in Equation 3.8 where $[Cj.Ab]_t$ is the concentration of conjugate-antibody complex present at time t :

$$rate = k_{off}[Cj.Ab]_t \quad \text{EQN. 3.8}$$

Equilibrium is reached when the rates of conjugate and antibody association and dissociation are equal as shown in Equation 3.9:

$$k_{on}[Cj]_{eq}[Ab]_{eq} = k_{off}[Cj.Ab]_{eq} \quad \text{EQN. 3.9}$$

The position of the equilibrium reflects the affinity of the antibody for the conjugate and the starting concentrations of the antibody and conjugate. As shown in Equation 3.10, the equilibrium association constant, K_a (otherwise known as the affinity constant), is equal to the ratio of k_{on} to k_{off} :

$$K_a = \frac{k_{on}}{k_{off}} = \frac{[Cj.Ab]_{eq}}{[Cj]_{eq} \cdot [Ab]_{eq}} \quad \text{EQN. 3.10}$$

while the equilibrium dissociation constant, K_d , is the inverse of the equilibrium association constant as shown in Equation 3.11:

$$K_d = \frac{k_{off}}{k_{on}} = \frac{[Cj]_{eq} \cdot [Ab]_{eq}}{[Cj \cdot Ab]_{eq}} = \frac{1}{K_a} \quad \text{EQN. 3.11}$$

The two equilibrium constants are measures of the affinity of a given antibody for a given hapten (or in this case enzyme-hapten conjugate) but for the purposes of discussion here only the dissociation constant of the immune complexes will be used as the measure of affinity.

For a given conjugate, the shape of the antiserum titration curve is determined by the characteristics of the different antisera with different affinity constants and antibody concentrations. A difference in antiserum titre (see Figure 3.14) can be due to a difference in the dissociation constant of the different antisera for the lysozyme-ELG conjugate i.e. K_d , a difference in the concentration of the antibody in the antiserum, or due to a combination of the two factors.

The antiserum titration curves for the four different antisera of the third bleeds indicated that they could all inhibit the E3 conjugate activity to a similar level (>92% without subtraction of the blank rate), but that the volume (i.e. μL of undiluted antiserum) required to achieve this level of inhibition varied substantially between the antisera produced by the different sheep. All four antisera were tested using the same conjugate and at the same conjugate concentration, thus any difference in the titres was due solely to differences in the properties of the antisera. The antiserum with the highest titre (246) had the steepest titration curve, indicating that it contained either antibodies at a higher concentration or with lower K_d values, than the antisera produced by the other sheep.

When the fourth bleed antisera became available it was necessary to recheck some of the parameters. However when these were tested over a wide range of antiserum concentrations, they still showed the characteristic trough effect (Figure 3.17) as was observed with the third bleed antisera. Thus the decreases in lytic inhibition observed at high antiserum concentrations were not removed by using antiserum from the later bleed. The most notable feature was that less antiserum was required now to achieve the same level of inhibition for any given antiserum. This implies that the antibody concentration of the antiserum or its affinity had increased. The titration end point for antiserum 243 was calculated from the intersection point of the tangent to the steepest part of the antiserum titration curve with the baseline and was 0.08 μL of neat antiserum for the fourth bleed compared with only 0.35 μL for the third bleed. Thus the avidity of antiserum 243 had improved by a factor of 4.4 with the extra incubation time in the sheep. The time difference between bleeds three and four was a period of 6.5 months and included one extra booster shot. Thus, these results clearly show the importance of time and repetitive challenges for the greatest activation of the immune response and the consequent generation of high titre antisera (Tijssen, 1985a).

The fourth bleed titration curves for the E1 and E3 conjugates shown in Figure 3.19 were performed using the same antiserum (243-4) thus the differences in the titration curves cannot be attributable to differences in the concentration of antibody binding sites and must be due to differences in the affinities of the E1 and E3 conjugates for the E1G antibodies in the serum. The antiserum titration curve for the E1 conjugate was shallower and the end point required a much higher volume of the neat antiserum than did the E3 titration curve showing that the E1 conjugate had a higher mean K_d value.

The calibration curves relating the rate of the end point assay to conjugate concentration (Figure 3.9) could be used to calculate the concentration of the free conjugate at each point on the titration curve assuming that the antibody-bound conjugate had a negligible lysis rate ($<5\%$ of the control). Hence, the ratio of the bound conjugate fraction over the free (C_B/C_F) could be calculated and used to construct a Scatchard plot (C_B/C_F versus C_B). However, this resulted in a non-linear plot (data not shown). The lack of linearity must be due to the polyclonal nature of the antiserum since the conjugates were mono-substituted and hence should give linear Scatchard plots if the antiserum contained a single population of antibodies with the same binding site affinity. Because the Scatchard plot was curved, it could not be used to calculate the K_d or antiserum binding site concentration.

Small differences in free conjugate concentration caused by the addition of small concentrations of antiserum showed little difference in lysis rates due to the hyperbolic shape of the conjugate concentration calibration curves (Figure 3.9). Thus the lag phases evident in the titration curves (see Figures 3.18-3.19) are partially an artefact of the poor discrimination of the end point assay procedure at high conjugate concentrations (Section 3.4.6). This effect was more pronounced for the E1 conjugate which had the lower affinity. In fact the relative length of the lag phases was a crude measure of the relative antibody affinities. As E1 had the longer lag phase, this indicates that more antiserum must be added to bind the E1 conjugate for the lysis rate to be reduced significantly below that of the control. Hence, on this basis the K_d value for E1 was approximately three times higher than that for E3.

The reason for the differences between the K_d values for E1 and E3 must lie in the interaction between the protein and the total antibody binding surface and the local environment surrounding the estrone glucuronide moiety. The most likely explanation is that in the immune complex for E3 (E3.Ab) there are more favourable interactions between the antibody binding surface (including the E1G binding site) and the contact region of lysozyme with the surface than there are for the E1.Ab complexes. If the contact regions are in the vicinity of the acylated lysines then from the tertiary structure of E3 (Figure 2.19a) it would appear that the favoured microenvironment for tight binding is a basic and non-polar one. However, this explanation must remain speculative since the orientation of the estrone glucuronide moiety relative to the lysozyme surface and the actual area of protein-protein contact in the immune complex are unknown. For example, in the model constructed between E3 and the progesterone Fab fragment, the points of maximum contact between the proteins are seen to be removed from the estrone glucuronide acylation site. However, for the E1G antibodies it is known that the glucuronide moiety is recognised as otherwise the cross-reactivity with estrone would be much higher than 1.9% (Binnie, 1999). Hence the area of contact between the total

antibody binding site and the lysozyme surface is likely to be nearer the acylated lysine position than shown in Figure 3.36.

3.4.8 The Effect of Conjugate Structure and Antiserum Source on the Estrone Glucuronide Standard Curves

The fact that E3 has a lower K_d value than E1 with antiserum 243-4 suggests that a more sensitive immunoassay might result by using E3 as the signal generator. This is because less free antiserum will be required to maximally inhibit E3 in the absence of any steroid glucuronide. Thus, since the E1G is added first in the assay less of it will be required to bind to the antibody before the subsequent binding of E3 is affected thus resulting in an earlier rise in the rate of lysis.

The skeleton standard curves performed with antiserum 243-4 and the E1 conjugate (see Figure 3.20) clearly demonstrated how sensitive the standard curve was to small changes in the amount of antiserum. At an E1G concentration equivalent to a urinary excretion rate of $50 \text{ nmol } 24 \text{ hr}^{-1}$, an increase from the baseline rate was virtually undetectable using $0.084 \mu\text{L}$ of the undiluted antiserum. However, when the level of antiserum in the assay was decreased to $0.08 \mu\text{L}$ (i.e. $\approx 5\%$) the increase from the baseline became very obvious with the rate of lysis being more than double the $0.084 \mu\text{L}$ value.

The series of skeleton standard curves depicted in Figure 3.20 also clearly illustrates the difficulties with choosing an antiserum concentration high enough to maintain a low baseline rate of lysis while at the same time ensuring that the antiserum concentration is kept low enough that there is an early increase in lysis rates from baseline values with increasing E1G concentration i.e. there is a trade off between maintaining a low background and keeping the assay sensitivity.

When the same concentration of conjugate and antiserum ($0.048 \mu\text{L}$) were used for E1 and E3 as signal generators (Figure 3.24), E3 gave a standard curve similar to the Melbourne control. Although the mid-points of the two standard curves were very similar with E1 having a mid-point at $149 \text{ nmol E1G } 24 \text{ hr}^{-1}$ compared with $162 \text{ nmol E1G } 24 \text{ hr}^{-1}$ for E3, the assay discrimination for the E1 standard curve was approximately reduced by a third due to its high baseline rate of lysis ($160 \text{ nmol E1G } 24 \text{ hr}^{-1}$) making it unsuitable for fertility detection. Nevertheless, despite its apparently higher K_d value, the E1 conjugate - antiserum 243-4 combination could be used also to obtain a quality standard curve which was almost indistinguishable experimentally from that generated with the E3 conjugate (Figure 3.23) by increasing the amount of antiserum used to $0.08 \mu\text{L}$. Under these conditions, the resulting standard curve met all the criteria set for an estrone glucuronide fertility assay (Section 3.4.1). The baseline rate of lysis was low, the increase from this baseline was clearly apparent at an estrone glucuronide concentration equivalent to $50 \text{ nmol E1G } 24 \text{ hr}^{-1}$, the assay was maximally sensitive over the critical physiological range ($50\text{-}400 \text{ nmol E1G } 24 \text{ hr}^{-1}$), and the within run assay variability was low.

At first sight the fact that standard curves could be obtained using E1 and E3 which were so similar by using different amounts of antiserum is surprising given the presumed

differences in their K_d values. E1 required more antiserum to anchor the zero standard point than did E3 as expected from the respective titration curves. This might have been expected to give rise to a less sensitive standard curve using the E1 conjugate as signal generator. The effect of varying the concentrations of antiserum, conjugates and using conjugate with differing K_d 's was studied by simulation using the EIA program. As shown above in Equation 3.6 (page 101), the dynamics of the binding of the antibody with the conjugate and hapten (i.e. as occurs in the urinary E1G assay or standard curve), are complicated as there are two association and dissociation equilibria to be considered. Also, in reality, the system is not at equilibrium and assay and incubation times need to be controlled to obtain reproducible data. Since the urine, (or standard), is added first (in Step 1) this reaction is probably at equilibrium. However, when the conjugate is added and binds rapidly to a proportion of the free antibody binding sites, equilibrium is probably not established although since it is known that the dissociation rates are slow ($1 \times 10^{-3} \text{ s}^{-1}$) not much equilibration occurs during the performance of the assay. Hence the equilibrium model is a reasonable approximation to the experimental situation for the polyclonal antisera.

K_{d2} can be measured experimentally by titration of the hapten-conjugate against a fixed amount of antiserum (Rubenstein *et al.*, 1972). However, when this method was applied to the E3/antiserum 243-4 system, the conjugate titration curve consisted of two essentially linear phases with little or no transition point between them (see Figure 4.27). Thus K_{d2} could not be measured directly for this system. However, this did provide evidence of tight binding. Further evidence that the dissociation constants were low came from the simulation studies. A reasonable approximation of the E1 and E3 standard curves was obtained by setting dissociation constants of 0.1 nM for the antiserum with the unconjugated hormone, and 3.0 nM and 0.4 nM for the E1 and E3 conjugates respectively. However, if the curves were simulated using high K_d values the curve became significantly less sensitive than those obtained experimentally.

Figure 3.25 shows three standard curves as modelled by the EIA program. The line corresponding to the E3 standard curve was simulated using the above K_{d1} and K_{d2} values and antibody binding site and lysozyme conjugate concentrations of 20 nM and 19 nM respectively. The same standard curve was recalculated using the same antiserum and conjugate concentrations, but increasing the conjugate dissociation constant K_d by a factor of 7.5 to 3 nM. As shown in Figure 3.25, the program predicted that this increase in the K_{d2} value for the conjugate, would cause the baseline rate of lysis to become elevated and the whole curve to shift to the left as observed experimentally when E1 was substituted for E3 as the signal generator with no change in the antiserum concentration (Figure 3.24). If the antiserum concentration was then incrementally increased the simulated E1 standard curve slowly moved back towards the simulated E3 standard curve. When the antiserum concentration was increased to 29 nM although the new curve was not identical with the original simulated E3 standard curve the two would not be distinguishable experimentally. Thus, the increase in K_d (as encountered in changing from E3 as signal generator to E1) was almost exactly countered by increasing the antibody concentration.

Despite differences in titres of the different sheep anti-E1G antisera (Figure 3.14), the antiserum and E3 conjugate concentrations could be optimised for all third bleed antisera

to produce workable standard curves for the Ovarian Monitor (Figure 3.15), although the standard curve for antiserum 241 was slightly less sensitive to changes in hormone concentration (Figure 3.15). However, even this standard curve could probably have also been made to superimpose on the others by using a slight decrease in the antiserum concentration since the usual effect of decreasing the antiserum concentration is to shift the curve further to the left and increase the baseline rate. Thus for the antisera from the different sheep adjustment of the ratio of antibody:conjugate concentrations could be used to produce experimentally identical standard curves as well.

The major conclusion drawn from the simulations and the experimental data is that all of the antisera can be used to produce workable standard curves according to the criteria outlined in Section 3.4.1. The increase in K_d (as for the E1 conjugate mixture for example) is offset by increasing the antiserum concentration. The two trends, one towards higher baseline lysis rates and a lower EC_{50} caused by the increase in the K_d value, and the other towards lower baseline lysis rates and a higher EC_{50} value caused by increasing the antiserum concentration, almost exactly compensate each other. The model also indicated that this compensatory effect only operated over a limited range of increased K_d values. When the increases in K_d in the model were an order of magnitude greater, it was never possible to shift the computer generated standard curves back to coincide with the original by adding extra antiserum. Thus the increase in K_d for E1 compared with E3 must be small irrespective of the individual sheep the antibody was obtained from.

In a parallel study with conjugate E5, where lysines 33 and 97 are both acylated with E1G, a similar standard curve was again produced although the amount of antiserum had to be increased again over that used for E1. Thus, experimentally it appears that indeed any hen egg white lysozyme-E1G conjugate can be made to give a workable standard curve. Equally, as seen with the third bleed antibodies, any of them could be used to generate an acceptable standard curve showing the generality of this compensation effect for the sheep anti-E1G antisera.

3.4.9 Reproducibility of the Estrone Glucuronide Standard Curves

The wet chemistry standard curve day-to-day reproducibility using the E1 conjugate as the signal generator was noticeably lower than the day-to-day standard curve reproducibility observed for the pre-coated assay tubes of a given batch and lot number (compare Figure 3.22 with Figure's 3.5 and 3.6). This result was not surprising as the assay tubes from the same batch and lot are produced on the same day and thus are prepared using not only antiserum from the same sheep and bleed as well as conjugate from the same stock solution, but also conjugate and antiserum from the same dilution mixture, and substrate from the same preparation. In contrast, the day-to-day reproducibility of the wet chemistry standard curves is limited by the accuracy of the syringes used to dispense the conjugate, antiserum, and diluting buffer. Because the conjugate and antiserum samples were diluted fresh each day, although they were recorded as being diluted using the same dilution factor, slight variations in the actual concentration of the diluted samples may occur due to dispensing inaccuracies. The effect of slight variations in the preparation of the antiserum and conjugate solutions is well illustrated for antiserum 243-4 in Figure 3.20. The

antiserum aliquots of some of these standard curves varied by only 0.04 μL , but the effect of these volume differences on the position and shape of the standard curve were significant. These results show how only small inaccuracies in dispensing can lead to day-to-day variations in the standard curve. Although theoretically these dilution errors could be removed by using the same diluted antiserum and lysozyme conjugate sample from day-to-day, at very low concentrations, proteins can become partially denatured, and a significant proportion of the sample can absorb onto the walls of the storage vessel. In our experience this is particularly a problem with lysozyme stored in glass and antiserum stored in plastic.

Another major source of variation in the day-to-day reproducibility of the standard curve is the substrate suspension. The suspension cannot be stored overnight as some spontaneous lysis occurs with time which causes a decrease in the initial substrate concentration. The substrate suspension can also not be frozen for storage as the formation of ice crystals will also result in cell lysis. Thus the substrate suspension was prepared fresh daily. However, the substrate suspension has also been reported as being difficult to prepare consistently and is one of the reasons that lysozyme activity is now commonly studied using different non-cellular substrates even though they are associated with a much smaller absorbance change per unit of enzyme activity. However, these alternative substrates cannot be used in the Ovarian Monitor as a substitute for the *M. lysodeiktiticus* as the method is dependent upon using a very large substrate that is capable of being blocked from the enzyme conjugate's active site upon antibody binding (Figure 3.2).

In summary, the difficulties in setting up a standard curve with the wet chemistry which is reproducible from day-to-day are removed once the assay components are dried provided a standard curve is supplied with each batch of tubes. This is a very important feature of the Ovarian Monitor as the method would not be viable as a home assay if every day the woman had to repeat the standard curve to obtain the urinary hormone output for that day.

3.4.10 The Effect of Blank Urine on the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Standard Curves

The accuracy of the data produced by the immunoassays depends, in part, on the specificity of the anti-E1G antisera and the similarity of the urine matrix to the medium used to generate the standard curve. The detailed specificity of the antisera were not examined as part of this thesis but other work has demonstrated that cross reactivities with antiserum 243-4 with other related steroids, including estrone sulphate, estrone, estradiol and pregnanediol glucuronide, is low (Binnie, 1999). Figure 3.26 demonstrated the effect of the addition of a urine sample from an ovariectomised woman, to the E1 standard curve assay mixture. Although this urine sample did not contain any significant amounts of estrone glucuronide, it still caused the standard curve to become uniformly lifted by approximately 12 transmission units between an estrone glucuronide excretion rate equivalent to 0-400 nmol E1G 24 hr^{-1} , with the effect being slightly more pronounced at E1G concentrations equivalent to an excretion rate of 800 nmol 24 hr^{-1} and higher. Thus the conjugate rate of lysis was affected by the presence of some urinary factor which was not estrone glucuronide. Because all urine samples probably contain these interfering

substances, for any estrone glucuronide concentration, the conjugate rates of lysis for the urinary assay will be higher relative to the standard curve. Thus, to correct for these factors the standard curve needs to be performed in the presence of an estrone glucuronide free urine. The reason for this blank urine effect is not known, but the results are consistent with the presence of an antibody binding compound. The most likely candidates for this non-specific binding would be other steroid glucuronides or possibly even just other glucuronide conjugates, or estrogen based sulphate conjugates of which there are relatively large amounts in urine.

The effect of the blank urine on the E3 standard curve was unexpected (see Figure 3.27). Why the blank urine had such a dramatic effect on the E3 conjugate system compared to the E1 system is not known. However, the blank urine sample was very old (1989) and may have deteriorated with time. This combined with the fact that there was very little of the sample left meant that another blank urine needed to be sourced.

The replacement sample was from a very young girl (24 months) and thus was much more readily obtained than a sample from an ovariectomised woman, who is usually on some form of hormonal supplementation. Although the usual method of diluting a urine sample is to dilute it to a constant excretion rate of 150 mL hr^{-1} , in the case of a young child this may not be valid due to her much lower body weight i.e. rate of metabolite and salt excretion into the urine, is probably much lower. Thus, in an attempt to correct for this, an aliquot of the urine sample was also diluted to the same average ionic strength as a woman's time diluted urine. This is equivalent to 10 mM tris-maleate buffer i.e. the same concentration as the buffer used to dilute the estrone glucuronide standards.

The stock urine needed to be diluted by a factor of approximately 2.7 for the time diluted sample and by a factor of 5.7 for the sample to be diluted to a constant ionic strength. These results are the opposite to what would be expected if the child had a lower rate of salt excretion than an adult.

A comparison of the effect of diluting the child's urine to an excretion rate equivalent to 150 mL hr^{-1} versus diluting it according to its ionic strength was shown in Figure 3.28. The E3 conjugate optimised standard curve was repeated on the same day without urine and with the 150 mL hr^{-1} and the ionic strength adjusted (10 mM) child's blank urine samples. The effect of the two diluted samples on the position of the standard curve were similar and slight. In both cases the standard curve was slightly elevated. Thus regardless of the blank urine concentration, the effect on the standard curve was small which implies that the child's urine contained few cross-reacting substances. The difference between the blank urine effect with the ovariectomised woman's sample versus the child's urine, combined with the difficulty Melbourne has found in replacing their current blank urine sample, shows the difficulty in obtaining a consistent blank urine supply and the individual variability which exists. Hence, the data reported in this thesis were collected from standard curves without using blank urine samples with the standards. However, the error produced by this omission is likely to be small.

In conclusion, the variability in the effect of the blank urine suggests that the level of urinary based interference in the assay may vary between individuals preventing the

universal application of a single blank urine sample. Based on these results the necessity and feasibility of using a blank urine must be questioned.

3.4.11 Application of the E1 and E3 Hen Egg White Lysozyme-Estrone Glucuronide Conjugates for the Measurement of Menstrual Cycle Urine Samples

The ultimate test of any assay system using any signal generator to construct a standard curve must be in the quality of the data produced from its application to clinical samples. One of the difficulties with immunoassays is that it is never possible to assume that a response in the assay is caused necessarily by the analyte in question. In the present case, the validation of E1 and E3 as signal generators was performed by comparing the data obtained using these conjugates with the data provided by the pre-coated Ovarian Monitor tube assay for daily time-diluted urine samples collected over a menstrual cycle.

Although the general pattern of daily estrone glucuronide excretion for a cycle could be followed using the Ovarian Monitor transmission data directly as is done by individuals at home, using a standard curve to convert the transmission data into nmol E1G 24 hr⁻¹ allowed the pattern of estrone glucuronide excretion to be more clearly delineated. Likewise repeating the assay for urines which gave a high ΔT in 20 minutes after diluting an extra 1 in 2, allowed the first estrogen peak to be defined more clearly. Most notably in the example cycle shown (see Figure 3.30), using this method allowed the peak day to be unambiguously assigned to day 16 of the cycle. Visual inspection of the cycle profiles obtained with the E1, E3 and the pre-coated tube conjugate mixtures, after conversion into estrone glucuronide excretion rates using their respective standard curves, clearly showed an excellent agreement in the absolute values for the different conjugate based assays. The hormone excretion rates calculated for each cycle day obtained with the different conjugates were correlated individually against each other using the Deming regression model (Deming, 1943; Martin, 2000). These correlations showed that the three assays gave the same values for the daily estrone glucuronide excretion rates (slope ≈ 1) within experimental error (Table 3.2). However the data generated using E3 as the signal generator contained a larger urine bias than either the E1 assay or the pre-coated tubes. Thus when the assay data were presented in terms of estrone glucuronide excretion rates, the E1 assay appeared to be slightly superior compared to E3 due to its lower baseline (Figure 3.31). Conversely, when the data were presented in terms of transmission change the data generated with the E3 assay showed a more clearly defined main estrogen peak (Figure 3.29) due to a large difference between the peak value and baseline rates.

In conclusion, with respect to the Ovarian Monitor assay, there appeared to be very little advantage in purifying the different mono-substituted conjugates from each other. This was shown clearly by the close agreement between the estrone glucuronide excretion rates irrespective of whether E1, E3 or the Melbourne conjugate was used as signal generator in the assay. Surprisingly, there was no significant advantage in assay sensitivity in using E3 as the signal generator despite its lower K_d value. At a K_d value of 0.4 nM for the conjugate (as used in the computer simulations) only a slight excess of antibody was required to give maximum binding of the conjugate at zero standard concentrations. Thus

to increase the assay sensitivity the amount of excess antibody cannot be reduced significantly.

Computer simulations showed that the assay sensitivity can be increased by increasing the specific activity of the signal generator so that the concentrations of all the assay components can be reduced. This cannot be done using any of the purified hen egg white lysozyme-estrone glucuronide conjugates, thus this system may already be at the limits of its sensitivity.

The final question to be addressed is whether it is necessary to separate E1 and E3 from the active ester conjugation mixtures. On the basis of the above results this is questionable as long as the highly substituted conjugates and native lysozyme are removed during the purification.

For the combined E1/E3 experiment a 2:1 molar ratio of E1 and E3 was used to give a ΔT 20 min⁻¹ of 350 units. Under these conditions with the 243-4 anti-estrone glucuronide antiserum, an acceptable standard curve was obtained using an antiserum volume intermediate between that used for the pure E3 and the pure E1 optimised standard curves. This was consistent with the greater volume of antiserum required to anchor the lysis rate for the zero standard due to the presence of E1. The interesting result was that an apparently more sensitive curve was obtained. The zero standard lysis rate was anchored at $\approx 44 \Delta T$ 20 min⁻¹, the rise from the baseline lysis rates occurred at 20 nmol E1G 24 hr⁻¹ and the mid-point of the curve was about 140 nmol E1G 24 hr⁻¹. Thus, these results suggest that a mixture of E1 and E3 may be superior to either of the pure conjugates as a signal generator. However, these studies were only preliminary, and the performance of this combined conjugate system still remains to be tested under clinical conditions. Full testing of the combined conjugate hypothesis requires experimentation with the other antibodies (which may have different ratios of K_{d1} and K_{d2}) and with different ratios of E1 and E3. As the combined conjugate conditions have not been optimised it may be possible that a standard curve superior even to the one presented in Figure 3.24 could be obtained.

3.5 SUMMARY

The Ovarian Monitor system measures urinary estrone and pregnanediol glucuronide within the range experienced throughout a woman's menstrual cycle. The daily measurement of the rate of excretion of these two compounds into the urine provides a comprehensive up-to-date commentary on the fertility status which is unequalled by any other home method. Any attempt to further the understanding of the underlying dynamics of the assay requires the assay to be dissected into its component parts. The production of the biomaterials required for this system and their assembly into an assay tube format has previously been the exclusive domain of an Australian enterprise. The work presented in this thesis represents the first complete reproduction of the Ovarian Monitor system outside of Australia. Although the assay has only been reproduced here in a wet chemistry format, this is the first time that all the biomaterials required for the assay have been produced independently of Melbourne. This required the synthesis of estrone glucuronide, lysozyme- and thyroglobulin-estrone glucuronide conjugations, and the generation of new polyclonal estrone glucuronide antisera.

Like most immunoassay systems, the Ovarian Monitor utilises a heterogeneous mixture of protein conjugates. Because it relies on the specific lytic activity of the lysozyme conjugates, and this is reduced at high levels of hapten substitution, unlike in most other immunoassay conjugations the ratio of activated hapten to enzyme is always kept low. Thus although for most immunoassays the conjugation mixture can be used without further purification in the Ovarian Monitor system it is necessary to separate the lysozyme conjugate from the unreacted enzyme. In the work presented in the previous chapter a new method for purifying the conjugate was developed. Using this method the different conjugates could not only be separated from lysozyme but also from each other. Thus for the first time, the individual immunoassay characteristics of the different lysozyme estrone glucuronides conjugates produced in the conjugation reaction could be examined. Because the structure of these different conjugates is also known their behaviour in the immunoassay could be related back to any structural differences between the conjugates. This is the first time that the individual protein conjugates used in any immunoassay system have been isolated and characterised for their individual immunoassay properties.

In the work presented in this chapter, the standard curves generated with the new biomaterials enabled the dependence of the standard curves and the antibody reactions on the structure of the conjugate to be investigated. These standard curves were used to measure the EIG excretion rate from time diluted urine samples and gave results comparable to those achieved with the pre-coated assay tubes of the Ovarian Monitor system. Thus the integrity of the new biomaterials and the independent duplication of the Ovarian Monitor was fully validated.

In summary, the results showed that:

- the organically synthesised estrone glucuronide was of the correct stereochemistry and pure.

- inoculation of sheep with thyroglobulin-estrone glucuronide conjugates was a reliable method for obtaining estrone glucuronide antisera of a quality suitable for use in immunoassay, as all four sheep inoculated with the conjugate produced a workable antiserum, and furthermore that the best antisera were obtained several years after the first inoculation and after several booster injections.
- there were definite differences in the antibody affinities for the different conjugates. However, there was also a compensation between the increased amount of antiserum required for the weaker binding conjugates and the amount of free E1G required to cause a decrease in inhibition of the conjugates' lytic activity.
- despite differences in affinity and concentration all lysozyme - estrone glucuronide conjugates and antisera in the present work could be made to give a similar standard curve by careful adjustment of the conjugate:antiserum ratio. All these standard curves could then be used successfully for the determination of the concentration of urinary estrone glucuronide.
- when used on the same urine samples all curves gave essentially identical results with the pre-coated Ovarian Monitor assay tubes with most differences being accounted for by the intrinsic variation of the assay.
- there was no practical advantage in separating out the individual hen egg white lysozyme-estrone glucuronide conjugates. In fact a combination of E3 and E1 appeared to be the best signal generator. Hence, the active ester conjugation method together with the general purification scheme developed in this work to remove E4-E6 and lysozyme represents the simplest and most reproducible procedure for producing high quality conjugate for use in homogeneous enzyme immunoassays.

Thus overall the results in this chapter have proven the Ovarian Monitor system represents a very robust and practical immunoassay method for home use.

CHAPTER FOUR

DEVELOPMENT OF A FAST HOMOGENEOUS ENZYME IMMUNOASSAY FOR ESTRONE GLUCURONIDE USING HUMAN LYSOZYME - ESTRONE GLUCURONIDE CONJUGATES

4.1 INTRODUCTION

4.1.1 Decreasing the Ovarian Monitor Assay Time

Feedback from participants in field trials of the Ovarian Monitor have consistently shown that users found it to be a very useful adjunct for natural family planning, with the assay being easy to perform and the resulting data simple to interpret and apply. Although the quality of the data was never questioned and the validity of the method was substantiated, the consensus from these trials was that a reduction in the 20 minute assay time of the estrone glucuronide assay would be a valuable improvement (Cavero, 1995; WHO, in preparation b).

In theory, there are several methods by which the assay time can be reduced.

One potentially very simple way to reduce the assay time, given the high thermal stability of hen egg white lysozyme (HEWL), is to increase the assay temperature. A useful and oft quoted rule of thumb states that a 10°C increase in temperature will result in an approximate doubling of the rate of reaction. Given that hen egg white lysozyme is usually considered to be stable to a temperature of 77°C (Imoto *et al.*, 1972), the time of the current estrone glucuronide assay could be reduced by half to 10 minutes by increasing the assay temperature to 50°C, or possibly reduced to even 5 minutes by increasing the temperature to 60°C. Although attempts to decrease the assay time by this method do show the expected increase in enzyme activity, the increased temperature also has the side effect of weakening the immune reaction resulting in a dramatically less sensitive standard curve (Brown, personal communication). Thus the thermodynamics of the Ovarian Monitor assay means that increasing the current assay temperature from 40°C is not a viable method for decreasing the assay time.

Another obvious method by which the assay time could be reduced would be to replace the current HEWL conjugate mixture, which contains conjugates with a range of specific activities, with a pure lysozyme conjugate of higher specific activity, or with a lysozyme conjugate whose activity is more sensitive to inhibition by an E1G specific antibody. Although increasing levels of acylation decreases the specific activity of lysozyme, the specific activities of neither E1 nor E3, the main mono-substituted conjugates resulting from an active ester conjugation, were sufficiently higher than the current mixture to decrease the Ovarian Monitor assay time. Interestingly, the results presented in Chapter Three suggested that even though the optimised standard curves obtained with E1

and E3 were identical, a mixture of the two could result in a more sensitive assay. However, even this difference would not be sufficient for a reduction in assay time.

Although there appears to be no simple method for decreasing the assay time using an active ester derived HEWL conjugate or mixture of conjugates as the signal generator, this does not preclude the possibility of replacing the current HEWL conjugate with an enzyme conjugate based on a lysozyme molecule derived from an alternative source.

4.1.2 Lysozymes from Alternative Sources

Lysozyme is widely distributed throughout nature with avian egg white being a particularly ubiquitous and rich source of the enzyme. In addition to avian sources, lysozyme is also found in mammals, reptiles, insects, plants, fungi and bacteria. In the human, lysozyme is found in various tissues and fluids with the best sources being tears, followed by pus, nasal mucus, stomach juice and milk. The exact physiological significance of lysozyme is not well defined. However, based on its distribution patterns and its ability to digest the cell wall of certain bacteria, it is generally believed to have a protective, or bacteriostatic, role. For example, the high levels of lysozyme observed in egg white have been hypothesised to help protect the developing chick embryo from infection until its immune system matures (Jollès and Jollès, 1984).

Lysozyme is found in many different forms which can be grouped into distinct families based on their structural and catalytic similarities, with some species possessing lysozymes from more than one family. All the avian egg white lysozymes so far examined can be divided into two major categories. Those which are structurally analogous to hen egg white lysozyme, for example turkey egg white, are commonly referred to as chicken or c type lysozymes while lysozymes which are structurally analogous to goose egg white are referred to as g type enzymes. All the mammalian lysozymes so far examined, including human, are of the c type, while the distribution of the type g isoforms seems to be restricted to members of the avian class only. T4 lysozyme, another well characterised enzyme, has a structure distinct from the two avian types so far discovered (Jollès and Jollès, 1984).

Human lysozyme, and the lysozyme's from T4 bacteriophage, turkey egg white and goose egg white, are all reported in the literature as having a specific activity higher than that observed for HEWL. For example, when *Escherichia coli* (*E. coli*) is used as a substrate, T4 lysozyme is particularly active having a reported specific activity 250 times greater than HEWL. However, as *E. coli* is an opportunistic human pathogen the use of a T4 lysozyme based immunoassay with *E. coli* is not a viable option for a home test kit. When *Micrococcus lysodeikticus* (*M. lysodeikticus*), the standard lysozyme and Ovarian Monitor substrate, is assayed with T4 lysozyme, the enzyme has a more modest increase in specific activity of only 6.3 relative to HEWL (Tsugita *et al.*, 1968). This compares favourably with the rates observed for the turkey egg white, goose egg white and human lysozymes with *M. lysodeikticus* as the substrate. Turkey and goose egg white have specific activities relative to HEWL of 1.5 and 3.0 respectively (La Rue and Speck, 1970; Canfield and McMurray, 1967) while the rates reported for human lysozyme are more variable. The relative specific activity of human lysozyme has been found to be highly dependent on the

reaction conditions and is generally reported as being between 3 and 5 (Mörsky, 1983; Mulvey *et al.*, 1973).

The greater reported specific activity of the above four lysozymes relative to HEWL suggests that the Ovarian Monitor assay time could be reduced possibly by using a conjugate of one of these alternative enzymes as a replacement for the current HEWL conjugates. Although the literature rates reported for the different enzymes are expressed relative to HEWL, as they were all obtained under different assay conditions, they are not directly comparable. Thus all five lysozymes were reassayed at 450 nm under $V_{\max}$ conditions using a lysis assay based on the initial rate of clearing of a turbid *M. lysodeikticus* suspension. Under these conditions, the lysozymes isolated from T4 bacteriophage, turkey egg white, and human milk had specific activities relative to HEWL of 5.8, 1.8, and 3.3 respectively which were in close agreement with the relative initial rate values reported in the literature.

As both human lysozyme and HEWL belonged to the c type class, it may be expected that their kinetic behaviour would also be similar allowing a more straightforward substitution in the Ovarian Monitor. Compared to turkey egg white lysozyme which was also from the same class, the greater specific activity of the human lysozyme suggested that the human enzyme would be the most effective enzyme for reducing the assay time.

4.1.3 Human Lysozyme

Human lysozyme is distributed throughout the body, and sequencing studies have shown no difference in the amino acid sequence between the enzymes isolated from different tissues and body fluids (Fett *et al.*, 1985). Crystallographic studies have also determined the tertiary structure of human lysozyme (Artymiuk and Blake, 1981). Apart from showing a very strong overall homology with HEWL these studies strongly implicated the same amino acids as the main catalytic residues of the human isoform. Whereas HEWL utilises residues, aspartic acid 52 and glutamic acid 35, human lysozyme utilises residues aspartic acid 53 and glutamic acid 35. This change in sequence position of the aspartic acid catalytic residue is due to a difference in sequence alignment as opposed to a true change in the geometry of the active site region. Human lysozyme contains an extra amino acid at position 48 which occurs in one of the external loop regions and has little effect on the rest of the enzyme's tertiary structure. Despite the fact that no other insertions or deletions occur throughout the sequence relative to HEWL, the overall sequence homology between the two enzymes is not particularly high. Human lysozyme consists of a single polypeptide chain of 130 residues and contains (including the insertion) 52 point mutations relative to HEWL (Jollès and Jollès, 1984). Thus there is only 60% sequence homology between the two enzymes. Of the 51 substitutions, 27 occur at external positions with most of the remaining 24 surface and internal substitutions representing conservative changes (Mulvey *et al.*, 1973). This finding is consistent with the close homology observed between the two enzyme's tertiary structures.

In common with most proteins the highly charged residues are largely restricted to the outside of the molecule for the two enzymes. HEWL contains a total of nine acidic residues (7 Asp, 2 Glu) and eighteen basic residues (6 Lys, 11 Arg, 1 His) compared to

human lysozyme which contains two more acidic residues (8 Asp, 3 Glu) and two more basic residues (5 Lys, 14 Arg, 1 His) (Jollès and Jollès, 1984). Thus the net difference in charged residues is effectively the same with both enzymes having a large excess of basic residues. This is reflected in their isoelectric points which are reported as 10.5 in both cases (Fett *et al.*, 1985; Verhamme *et al.*, 1988).

Although HEWL has been conjugated many times using several different methods and many different ligands, to date no conjugation of human lysozyme has been reported. Despite the overall similarity in the tertiary structures of the two enzymes, the difference in the number and position of the lysine residues present in native human lysozyme compared to HEWL may have important effects on its conjugation to estrone glucuronide.

In HEWL, lysine residues are located at positions 1, 13, 33, 96, 97 and 116 along the polypeptide chain, with positions 33, 97 and 116 being especially favoured for conjugation to estrone glucuronide (Smales *et al.*, 1999). In human lysozyme, lysine residues are located at positions 1, 13, 33, 69 and 97, with positions 69 and 97 being equivalent to positions 68 and 96 in HEWL due to the insertion of the extra amino acid at position 48 (Jollès and Jollès, 1984). Thus, not only does human lysozyme have one less lysine residue than HEWL (five as opposed to six), and thus one less potential conjugation site, it also lacks an equivalent residue to lysine's 97 and 116 in HEWL i.e. it only contains the equivalent of one of the three favoured conjugation sites of HEWL.

These differences could favour the conjugation of HEWL over the human isoform. The ability to predict the effect or ease of conjugation of human lysozyme from the HEWL conjugation pattern is complicated by the large number of differences in the exterior of the two proteins. A large percentage of the total number of substitutions occur externally and these represent the least conservative substitutions. Thus one of the chief determinants of conjugation has been dramatically altered - the microenvironment of the target lysine residues. Similarly two of the chief determinants of the lysozyme conjugation mixture's chromatographic behaviour have been altered - the probable conjugation sites, and the surface distribution of charge.

Finally, as human lysozyme has not been conjugated before, the effect of conjugation on the enzyme's kinetics has never been determined. More specifically, the ability of an anti-hapten antibody to inhibit a human lysozyme-hapten conjugate, and the effect of the physical characteristics of this interaction on the estrone glucuronide standard curve, are not known.

This chapter is concerned with an investigation of the feasibility of developing a rapid human lysozyme based estrone glucuronide home assay.

The aims are:

- to examine and optimise the human lysozyme - estrone glucuronide conjugation and purification conditions

- to analyse the kinetics of the purified human lysozyme conjugates and compare their behaviour with the HEWL-E1G conjugates
- to develop an alternative assay procedure and assess the assay under clinical conditions

4.2 MATERIALS AND METHODS

4.2.1 Apparatus

All column chromatography of human lysozyme and the human lysozyme conjugates was performed on a Pharmacia Fast Protein Liquid Chromatography (FPLC) system (see Section 2.2.1) using a Pharmacia Mono-S cation exchange column HR 5/5 (50 mm × 5 mm i.d.). All buffers and samples prepared for FPLC were prefiltered through 0.2 µm GVWP Millipore filters. Samples were injected into a loop on the motor valve using a syringe before being automatically loaded onto the column.

All manually obtained absorbances were measured on a Hewlett Packard diode array spectrophotometer using a quartz cuvette.

All mass spectral analyses were performed by Dr. C. M. Smales (University of Kent, Canterbury, United Kingdom) as described in Section 2.2.1.

Gel electrophoresis was performed using a Protean II xi system from Bio-Rad Laboratories (Richmond, CA, USA). Gels were dried for permanent storage on a gel vacuum dryer from Bio-Rad Laboratories.

All Ovarian Monitor equipment including the pre-coated estrone glucuronide assay tubes, was supplied as described in Section 2.2.1 as were the assay syringes.

All curve fitting was performed using the PRISM data analysis and graphing program (Graphpad™).

4.2.2 Reagents

Estrone glucuronide was synthesised according to the method outlined in Section 2.3.1 and all reagents for making the active ester reagent were as reported in Section 2.2.2. The bovine serum albumin (BSA), cytochrome c (bovine heart) and human milk lysozyme were obtained from the Sigma Chemical Company (St Louis, MO, USA). All other reagents and chemicals were of analytical grade or higher, and the water was of Milli-Q quality.

4.2.3 General Methods

4.2.3.1 *Estimation of Protein Content*

The spectrophotometer was blanked against the appropriate buffer or water depending on the sample and the concentration of human lysozyme in the unknown samples was calculated using the $A_{280}^{1\%}$ human lysozyme value of 25.65 (Mörsky, 1983).

4.2.3.2 *Preparation of Human Lysozyme for Conjugation to Estrone Glucuronide*

According to the information provided with the commercial samples of human lysozyme, each vial contained approximately 5% human lysozyme by weight (1.3 mg), and 95%

buffer salts (sodium phosphate and sodium chloride). In preparation for the conjugation, the contents of 14 vials of human lysozyme with an estimated total enzyme content of 18.2 mg were combined and dialysed. To ensure the complete removal of the enzyme from the vials without excessive dilution of the sample, the vial contents were removed by serial transfer. This was achieved by dissolving the contents of the first vial in a small aliquot of water (1 mL), and then using the washings from this vial to dissolve the contents of the next vial. This sequence was repeated in series until all the vials were washed, with the washings from the final vial being transferred to a dialysis sac. The vials were then all rinsed with a second aliquot of water (1 mL) using the same washing sequence. After the washings from the final vial were transferred, the last vial was then rinsed with a small final amount of water (approximately 200 μ L). This attempt to dissolve the contents of the vials was not completely successful as a fraction of the sample remained in suspension as a white powder.

The dissolved sample was dialysed against water (2 changes $\times$ 2 L) over a period of 24 hours, followed by dialysis against a 1% sodium bicarbonate (NaHCO_3) solution (2 L) in preparation for the conjugation reaction. Dialysis against the sodium bicarbonate solution failed to dissolve the white precipitate which was finally removed by centrifugation. The final sample was estimated to have a volume of 3.6 mL and calculated to have a concentration of 1.8 mg mL⁻¹ i.e. the total protein content was estimated to be 6.6 mg, which represented only a 36% recovery from the calculated original 18.2 mg of enzyme.

4.2.3.3 *Conjugation of Human Lysozyme to Estrone Glucuronide*

Human lysozyme was conjugated to estrone glucuronide using the active ester method (Scheme 2.1, page 48). The estrone glucuronide active ester reagent was prepared using the same protocol, concentrations and volumes used previously in its preparation for the HEWL conjugation (see Section 2.2.4). The human lysozyme conjugation was performed at 4°C with constant stirring by the dropwise addition of the active ester reagent (30 μ L) to the enzyme (6.6 mg) NaHCO_3 solution (3.6 mL) using a dry pasteur pipette. As usual the addition of the active ester reagent was associated with the formation of a white precipitate. Upon the complete addition of the reagent the reaction mixture was left for an hour at 4°C before dialysis into water (see Section 4.2.3.4).

4.2.3.4 *Preparation of the Human Lysozyme Conjugation Mixture for Analysis by Mono-S Fast Protein Liquid Chromatography*

Usually a lysozyme conjugation mixture can be analysed directly by 7 M urea Mono-S column chromatography by mixing a fraction of the reaction mixture (1 part in 6) with the starting buffer and thus diluting out the sodium bicarbonate. However in the case of the human lysozyme conjugation with its low protein concentration this procedure would have made the sample too dilute to have enabled a quality FPLC absorbance profile to be obtained. Instead the sample was transferred to a dialysis sac, using an additional 0.4 mL of water to rinse the conjugation vessel, and the sodium bicarbonate content of the reaction mixture reduced by dialysis against water (2 L) for 1 hour. An aliquot of this sample was

then prepared for loading onto the column by adding urea at the rate of 0.123 g per 300 μL of sample, and filtering.

4.2.3.5 *Reconjugation of the Human Lysozyme Conjugation Mixture*

After analysis of a sample of the initial conjugation mixture by Mono-S chromatography (see Section 4.2.3.6), the reaction mixture was reconjugated. This involved adding 0.038 g sodium bicarbonate to the remaining 3.8 mL of sample to make a 1% bicarbonate solution, adding a further 30 μL of the original active ester reagent, and then leaving the reaction mixture for 10 minutes with stirring at 4°C. The reconjugated sample was then dialysed back into water as before resulting in a 4.1 mL solution which was analysed as for the first conjugation by Mono-S chromatography.

The sample was then conjugated a third time. Thus, a further 0.04 g of sodium bicarbonate and 30 μL of active ester reagent were added to the remaining 4 mL of sample. The resulting reaction mixture was left stirring at 4°C for 30 minutes before being dialysed back into water as previously to give a final volume of 4.3 mL. All kinetic work was performed using conjugates purified from this final conjugation mixture.

4.2.3.6 *Separation of the Human Lysozyme Conjugation Mixture by Mono-S Cation Exchange Chromatography*

As for the analytical chromatography, samples prepared for a preparative scale separation were transferred into 7 M urea by the addition of 0.123 g of urea per 300 μL of sample.

All analytical and preparative chromatographic separations were performed on a Mono-S cation exchange column using 50 mM phosphate/7 M urea buffers (pH 6.0) and a flow rate of 0.5 mL min⁻¹. Elution was effected using the following sodium chloride gradient: 0 M NaCl, 5 minutes; 0-0.3 M NaCl, 40 minutes; 0.3-1.0 M NaCl, 5 minutes; 1 M NaCl, 10 minutes.

Loadings for the analytical and preparative runs are reported in the figure legends of the individual traces. The absorbances recorded on these traces were low relative to the true protein concentration. This was because of concern from the technical officer in charge of the FPLC apparatus that crystals formed from the high concentrations of urea used in these separations would scratch the UV cell. Consequently all 7 M urea chromatographic work was performed using an old cracked cell which reduced the apparent absorbance of the eluent and the resolution of the profile.

A total of three preparative runs was required to process all the sample from the final conjugation mixture. The individual peaks from these runs were collected directly into plastic 1.5 mL snap-cap vials which had the centre cut out of their lids. Each vial with the lid still ajar was then covered with a piece of dialysis tubing cut open into a single layer, and the lid closed. The samples were then dialysed by placing the vials upside down in a container of water (24 hours $\times$ 3 changes $\times$ 2 L). The vials were sealed by replacing the dialysis tubing with a double thickness of parafilm before storing upright at -20°C.

4.2.3.7 Gel Electrophoresis

Human lysozyme conjugates were analysed by non-denaturing acid-polyacrylamide gel electrophoresis (acid-PAGE) using large (160 × 200 mm), continuous 15% acrylamide slab gels. To enable the positively charged conjugates to be separated, in contrast to the standard gel electrophoresis methodology, the separation was performed at pH 4.08 with the polarity of the electrodes reversed (Smales *et al.*, 1998).

Glycine buffer (pH 4.08), which was used to prepare the gels and also as a running buffer, was prepared fresh daily from glycine (28.1 g), glacial acetic acid (3.06 mL) and water to give a final buffer volume of 1 L. A 40% stock acrylamide solution was prepared by dissolving acrylamide (97.4 g) and *N, N* methylene bis-acrylamide (2.6 g) in water (250 mL) and a 15% acrylamide solution was prepared by mixing 7.5 mL of the glycine buffer with 12.5 mL of the 40% acrylamide solution. The resulting solution was degassed for 10 minutes with stirring using a water vacuum pump. After polymerisation of the gel matrix was initiated by the simultaneous addition of 30 µL of *N, N, N', N'*-tetramethylethylenediamine and 200 µL of a 10% solution of ammonium persulphate in glycine buffer, the gels were quickly poured and then left to set overnight. The gels were pre-run for 3.5 hours at constant voltage (200 volts) to remove any persulphate contaminants. The buffer in the chambers (i.e. glycine buffer) was then changed and the samples loaded including a cytochrome c marker. The samples were prepared for loading by mixing 150 µL of sample with 50 µL of sample buffer (glycine buffer with 20% glycerol).

Electrophoresis was performed using a constant voltage (200 volts) and with the electrodes reversed so that the current ran from the anode in the upper chamber to the cathode in the lower chamber. When the cytochrome c marker reached the bottom of the gel, the current was stopped. Gels were stained with a 0.1% Coomassie blue R-250 solution (40% methanol, 10% acetic acid and 50% water), destained using the same solvent mixture without the dye (3 changes), and then dried for one hour at 70°C using a gel vacuum drier.

4.2.4 Preparation of Assay Reagents

All assay reagents were prepared with water of Milli-Q quality. To avoid cross contamination, separate syringes were used always for the urine samples, estrone glucuronide standards, lysozyme, lysozyme conjugates, antiserum and substrate in the preparation of the assay reagents and in the assay.

4.2.4.1 Buffers

All buffers were prepared from a standard 1 M tris-maleate stock buffer (pH 7.0) (see Section 2.2.6.1). The pH 6.0 and pH 6.2 stock buffers (1 M) were prepared by titrating the pH of the standard stock buffer down with 1 M hydrochloric acid. All other tris-maleate buffers were made by dilution of the appropriate 1 M stock solution with water.

The substrate suspension, estrone glucuronide standards, and antiserum and stock enzyme solutions were prepared using tris-maleate buffers of the same ionic strength as was used

for the HEWL conjugate work reported in Chapters Two and Three. Thus the stock enzyme solution was prepared in 0.35 M buffer, the substrate suspension and antiserum dilutions were prepared in 75 mM buffer, and the estrone glucuronide standards were prepared in 10 mM buffer. All early kinetic studies (Section 4.3.3.1) and the study of the effect of albumin on enzyme stability (Section 4.3.3.3) were performed using 40 mM tris-maleate assay buffer. The study on the effect of albumin on enzyme activity (Section 4.3.3.3) and all later studies (Section 4.3.4-4.3.9) were performed using 55 mM assay buffer.

Unless reported otherwise, all individual assays were performed using stock enzyme, antiserum, substrate, assay and estrone glucuronide standard buffers of the same pH. All early kinetic studies (Section 4.3.3.1) were performed using pH 7.0 buffers and all the studies reported in Section 4.3.3.3 and onwards were performed using pH 6.2 buffers.

Albumin containing buffers were also prepared. Because of the small weights involved, the bovine serum albumin (BSA) used in the preparation of these buffers, was weighed out on a four figure balance. The buffers were then prepared by dissolving the BSA directly into tris-maleate buffer of the appropriate ionic strength and pH. A 0.1% albumin stock enzyme buffer (0.35 M, pH 7.0) and a 0.01% albumin assay buffer (55 mM, pH 6.2) were prepared. These buffers were used for the studies reported in Section 4.3.3.3 only.

4.2.4.2 *Substrate*

Apart from the differences in buffer pH reported above, the preparation of the *M. lysodeikticus* substrate was identical to that described in Section 2.2.6.4.

4.2.4.3 *Stock Enzyme Solutions*

As only small quantities of enzyme were available, the human lysozyme conjugates could not be diluted fresh each day. Typically 500-1,000 μL of enzyme stock was prepared at a time by dilution into plastic vials, and refrozen at the end of each day. Fresh dilutions were only prepared when there was insufficient stock solution left to complete a full days work. Stock solutions were diluted from the dialysed samples so that the required rate could be achieved using 1-10 μL of the stock enzyme solution in the assay. Apart from some of the early work (see Section 4.2.4.1), all stock solutions were prepared using 0.35 M tris-maleate buffers (pH 6.2).

Stock enzyme solutions of HEWL conjugate were prepared as described in Section 2.2.6.2.

4.2.4.4 *Anti-Estrone Glucuronide Antiserum*

All antisera based activity assays were performed using antiserum 243/bleed 4 (see Section 2.2.2). Again, apart from some of the initial early work (see Section 4.2.4.1), all antiserum solutions were prepared using 75 mM tris-maleate buffer (pH 6.2). The antiserum was diluted fresh each day 1 part in 50 (20 μL to 1,980 μL buffer) into a small glass vial. At this dilution, the aliquot of the antiserum solution added to the assay could

usually be maintained within the volume limits of 1-10 μL . All antiserum volumes used in the assays are reported in terms of the equivalent volume of undiluted antiserum.

4.2.4.5 *Estrone Glucuronide Standards*

Estrone glucuronide standards were prepared using the same protocol outlined in Section 3.2.2.4 except that they were diluted with pH 6.2 tris-maleate buffer (10 mM).

4.2.4.6 *Blank Urine*

The urine from a 24 month female child was diluted to the same conductivity as the 10 mM tris-maleate buffer.

4.2.4.7 *Urine for Determination of Estrone Glucuronide Excretion Rates throughout the Menstrual Cycle*

All endocrinologically normal estrone glucuronide cycle profiles were obtained using time diluted urines (150 mL hr^{-1}) collected by a 26 year old woman over cycle days 2-34 of a 34 day cycle.

Time-diluted urines collected daily over cycle days 2-28 of an artificially controlled cycle for a 33 year old woman undergoing clomiphene treatment for idiopathic infertility were also assayed for estrone glucuronide.

4.2.5 Assay Protocols

The conjugate, and conjugate concentration, and the volumes of equivalent undiluted antiserum used in each assay, is recorded in the Results Section for each individual experiment. Depending on time constraints and the quality of the data, assays were performed in duplicate, triplicate or as single values.

4.2.5.1 *The General Activity Assay Protocol*

Apart from changes in the data collection method and the addition of the enzyme after equilibration of the assay buffer, the human lysozyme conjugates were assayed for activity using essentially the same protocol outlined in Section 2.2.6.5 for the HEWL conjugates. Modifications to the buffer for the various assay components were as noted in Section 4.2.4.

Data were collected typically as 3, 5 or 20 minute clearing curves. Transmission values were recorded at 0 time, then every 10 seconds for the first minute, every 20 seconds for the second minute, every 30 seconds for the 3rd-5th minute and then at every minute until the end of the collection time. Generally data were collected only over the full twenty minute period for kinetic analysis of the clearing curve or the end-point assay method. End-point rates were calculated, for the same clearing curve, at 1, 2, 5, 10 and 20 minutes and were expressed in terms of the transmission change calculated from the transmission at zero time. Apart from for the comparison of the end-point assay method with the initial

rate assay the initial rate data were collected from 3 or 5 minute clearing curves only. Initial rates were calculated using the initial linear portion of the clearing curve, or in the case of clearing curves which were never truly linear, by using a tangent to the earliest part of the curve.

Figure 4.1 shows an example of the measurement of the enzyme activity by the initial rate and end-point assay methods.

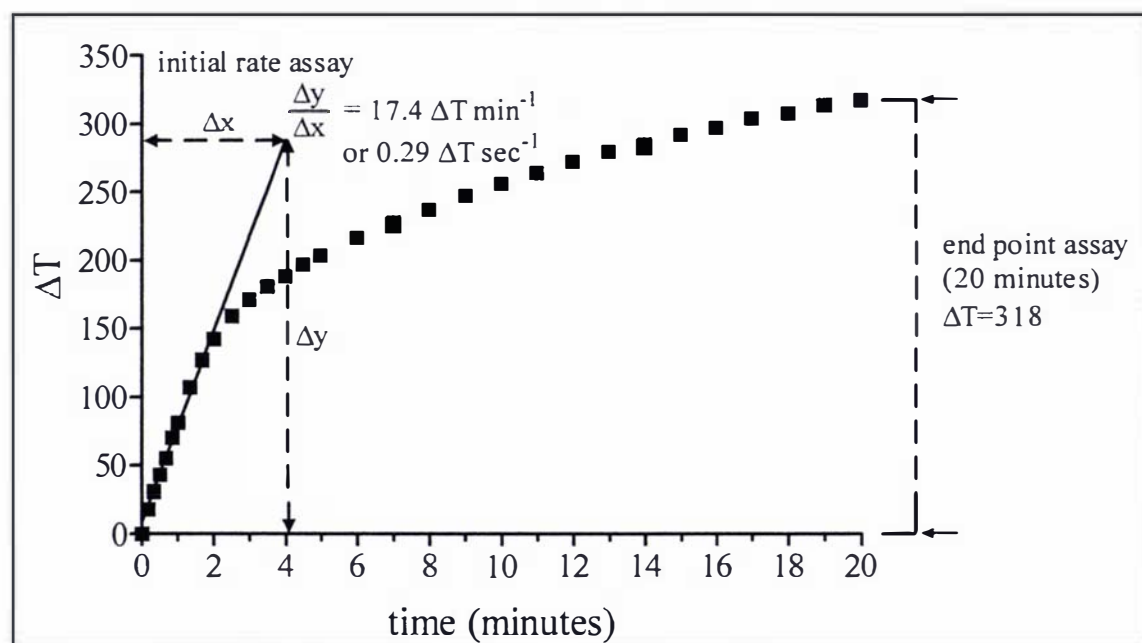


FIGURE 4.1: Measurement of Lysozyme Lytic Activity by the End-Point Assay Method and the Initial Rate Method

For many of the clearing curves the first data point (i.e. 0 time) was inconsistent with the rest of the curve and the initial rates for most curves were calculated according to the initial slope between 10 and 60 seconds. All initial rate data were expressed in terms of change in Ovarian Monitor transmission units per second.

4.2.5.2 Inhibition Studies

Inhibition assays were performed using essentially the same method as for the standard human lysozyme conjugate assay. Assay buffer was added to an empty assay tube, which was then left to equilibrate to 40°C in the heating block. The volume of assay buffer initially added to the tube was such that the final assay volume, i.e. after the addition of all the components, was 350 μL . Following a minimum equilibration period of 5 minutes, an aliquot of dilute antiserum solution (1-10 μL) was added, and the contents of the assay tube vortexed for a count of three. The diluted conjugate stock solution (1-10 μL) was then added and the contents of the assay tube mixed as before. The mixture was then incubated for a minimum period of 2 minutes, to ensure the temperature of the assay mixture was back at 40°C.

The assay was initiated by the addition of a bolus of the *M. lysodeikticus* suspension (10 μL) via a 500 μL stepper syringe. The final assay mixture was then quickly vortexed and the first transmission value recorded (time 0). Data collection and rate calculation were as for the standard human lysozyme conjugate assay (see Section 4.2.5.1). Rates were reported either directly as an initial rate or relative to the conjugate only rate (percentage inhibition). No subtraction of the blank rate (rate of lysis in the absence of enzyme), was used in the calculation of the inhibition values unless specified.

4.2.5.3 *Kinetic Analysis of the Rate Order of Clearing*

Values for the relative rate constants for the human lysozyme conjugate data at different enzyme concentrations were obtained by first converting the Ovarian Monitor transmission clearing curve data into absorbance using Equation 3.1, linearising the data by the second order rate equation (Equation 3.3) and then calculating the relative second order rate constant from the gradient as described in Section 3.2.5.2.

4.2.5.4 *Human Lysozyme Conjugate Standard Curves*

All components of the standard curves were prepared in pH 6.2 buffers. The assay buffer was 55 mM tris-maleate (pH 6.2). Both the antiserum and conjugate stock solutions were diluted such that the required amount would be added in a volume between 1-10 μL .

The following general protocol was used in the production of the standard curves. Estrone glucuronide standard (50 μL) and diluted antiserum (1-10 μL) were dispensed into diagonally opposed corners of an empty assay tube. The immune reaction was initiated by vortexing the assay tube for a count of three. After the mixture had been incubated for 2 minutes at 40°C, the diluted conjugate stock solution was added and the contents of the assay tube vortexed again. Assay buffer was then added to bring the total volume in the assay tube to 340 μL , and the resulting reaction mixture revortexed and left to incubate for 10 minutes at 40°C. The assay was initiated as reported for the inhibition studies. Data were collected over three minutes with transmission values recorded every 10 seconds for the first minute, every 20 seconds for the second minute and every 30 seconds for the last minute. All rates were calculated as initial rates ($\Delta T \text{ sec}^{-1}$) as described for the standard human lysozyme conjugate assay (Section 4.2.5.1).

Assays performed in the presence of a urinary control used the general protocol described above except that an aliquot of blank urine (50 μL) was added with the estrone glucuronide standards. The final reaction volume was maintained at 350 μL by reducing the volume of assay buffer.

All standard curves were fitted to a sigmoidal dose response curve with a variable slope using Equation 3.5 (PRISM) as described in Section 3.2.5.4.

4.2.5.5 *Human Lysozyme Conjugate Menstrual Cycle Data*

Urines were assayed for estrone glucuronide levels using the same general assay protocol as was used in the generation of the standard curves. The only difference was that the

estrone glucuronide standards were replaced with a 50 μL aliquot of a time diluted urine sample (150 mL hr^{-1}). A standard curve performed under identical conditions was used to convert the initial rate data into urinary estrone glucuronide excretion rates. This reading of the unknowns from the standard curve was performed using the fitted standard curve calculation of unknowns option on the PRISM data analysis and graphing program.

The E1G excretion rate data calculated for the same cycle using different methods were compared using the Deming Regression Model (Deming, 1943; Martin, 2000) as described in Section 3.2.5.6.

4.2.5.6 *Attempted Measurement of the Apparent Dissociation Constant of the Lysozyme Conjugate - Antibody Complex*

This involved the titration of varying amounts of lysozyme-estrone glucuronide conjugate against a constant amount of anti-E1G antiserum 243-4. The amount of antiserum in the assay was the same as that used for a given conjugate's optimised standard curve.

Assay buffer and a constant volume of diluted antiserum solution were mixed together in a series of empty assay tubes. This was followed by the addition of a variable and increasing volume of conjugate (0-10 μL). The volume of assay buffer added at the beginning of the protocol was chosen to give a final assay volume of 350 μL after the addition of all assay components. After the addition of the conjugate solution, each assay tube was mixed by vortexing and stoppered. The assay mixtures were then incubated at 40°C for a minimum period of 30 minutes.

The assay was initiated, the data collected and the initial rate data calculated as previously described for the standard human lysozyme conjugate assay (Section 4.2.5.1).

Control conjugate only rates were also performed covering the same range of conjugate concentrations as was used for the conjugate with antiserum rates. These rates were performed using essentially the same protocol as that described for the conjugate with antiserum rates but without the addition of the antiserum.

Assays for the human lysozyme conjugates and HEWL conjugates were performed using pH 6.2 and pH 7.0 buffers respectively.

4.2.5.7 *Measurement of the Displacement (Off) Rate Constants for Lysozyme Conjugates from the Conjugate - Antibody Complexes*

Displacement (off) rates for HEWL E1, and the human lysozyme HE1 and HE2 conjugates from antiserum 243-4 were measured using an excess of estrone glucuronide as the displacing reagent. These assays were performed using the same concentrations of antiserum and conjugate as were used to obtain the conjugate in question's optimised standard curve. The diluted stock solutions for both the antiserum and conjugate were prepared so that the required amount of reagent could be dispensed using a single aliquot for each with a volume between 1-10 μL . The diluted conjugate stock solution and diluted antiserum were dispensed into diagonally opposed corners of a series of empty assay tubes.

Assay buffer (55 mM tris-maleate, pH 6.2) was then added to bring the total volume in each assay tube up to 290 μL , and the subsequent immune complex reaction mixture vortexed for a count of three. After the mixture had been incubated for 5 minutes at 40°C, the displacement of the lysozyme conjugate from the antibody was initiated by the addition of a 50 μL aliquot of a concentrated E1G standard stock solution (139 μM ; 500,000 nmol E1G 24 hr^{-1}). Thus the concentration of non-lysozyme associated E1G in the reaction mixture was 20 μM . As the concentration of lysozyme estrone glucuronide conjugate used in this series of experiments ranged from 13 to 23 nM, relative to the lysozyme conjugate, the non-conjugated E1G fraction was present in an approximately 1,000 fold excess.

Upon the addition of the estrone glucuronide solution, the reaction mixtures were incubated at 40°C for a range of time intervals. Typical incubation times for the collection of a data set for a single set of conditions were 0, 1, 2, 5, 10, 20 30, 45, 60, 80, 100, 120, 150 and 180 minutes. The incubation was ended and the conjugate activity assay initiated by the addition a 10 μL bolus of a standard *M. lysodeikticus* suspension. Data were collected over three minutes and the initial rates calculated as previously described for the standard human lysozyme conjugate assay (Section 4.2.5.1).

The initial rate data were plotted against time and fitted by a one phase exponential equation using the non-linear regression option of the PRISM data analysis and graphics program. Obvious outliers were manually excluded from the fit.

4.2.5.8

Error Bars

All graphical data for which error bars are displayed are for one standard deviation from the mean ($n=2$).

4.3 RESULTS

4.3.1 Analysis of the Human Lysozyme Conjugation Mixtures by Mono-S Cation Exchange Chromatography

Figure 4.2a-c shows the 7 M urea Mono-S profiles for the same human lysozyme reaction mixture after each of three successive exposures of the enzyme to the same amount of the E1G active ester reagent.

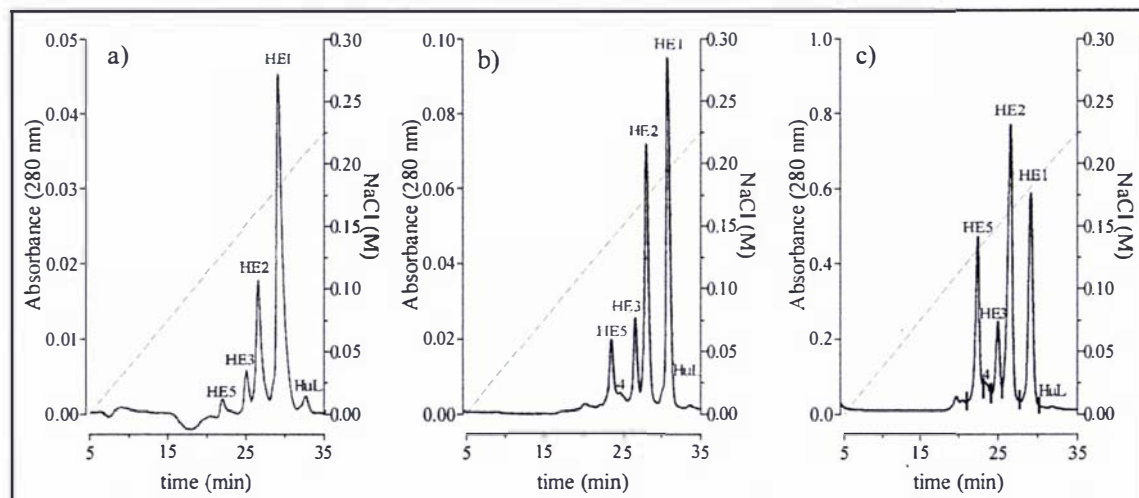


FIGURE 4.2: Mono-S Profiles for an Active Ester Human Lysozyme Estrone Glucuronide Conjugation Mixture after Three Successive Exposures to the Active Ester Reagent

a) initial conjugation mixture - loading 100 μ L, b) conjugation mixture from second exposure - loading 75 μ L, c) final conjugation mixture with a preparative scale loading - 900 μ L

See Section 4.2.3.4 and 4.2.3.6 for sample preparation and column conditions

A control run of a human lysozyme only preparation (profile not shown) showed that the unconjugated enzyme eluted in the same position as the very small latest eluting peak of the conjugation mixtures. Thus this peak has been labelled HuL for unconjugated human lysozyme on the conjugation mixture profiles. The remaining peaks have been numbered using the same system as was used for the HEWL conjugation profiles, but with the addition of an H prefix to distinguish them from the HEWL conjugates. Thus, all peaks are labelled according to their elution position relative to the free human lysozyme peak, with the conjugate peak closest to this peak being designated HE1.

The three FPLC profiles clearly showed a shift in the distribution of the protein between the conjugate peaks and the free lysozyme peak which changed according to the level of exposure of the conjugation mixture to the E1G active ester reagent. As the level of exposure increased, there was a shift from the later eluting peaks (free lysozyme, HE1, HE2 and HE3) to the earlier eluting peaks (HE4-HE6). Table 4.1 shows the percentage distribution of protein amongst the different peaks as calculated from the peak area data for the three consecutive conjugation mixtures.

TABLE 4.1: Distribution of Human Lysozyme and the Various Lysozyme Conjugate Families expressed as a Percentage of the Total Protein for Three Different Levels of Exposure of the Reaction Mixture to the Active Ester Reagent

	total number of exposures to the active ester reagent		
	1	2	3
Lysozyme	3.9	0.9	0.6
HE1	59.6	42.5	24.2
HE2	23.7	32.3	34.9
HE3	8.3	11.5	11.3
HE4	1.3	2.7	4.1
HE5	3.2	8.8	22.7
HE6	negligible	1.3	2.2
% total conjugation	96.1	99.1	99.4

The rearrangement of the conjugate distribution is most dramatically illustrated by the changing proportions of the HE1 peak. In the initial conjugation mixture, this peak constituted nearly 60% of the total protein. However after the conjugation mixture had been re-exposed to the active ester reagent an additional two more times, the contribution of the HE1 peak to the total protein yield was reduced to only 24.2%.

It was this third and final conjugation mixture which was used for all preparative chromatography. Figure 4.2c shows an example of the fractionation and collection of the conjugate peaks from one of the preparative runs. Only the conjugate peaks corresponding to HE1, HE2, HE3 and HE5 were collected for further characterisation. The small conjugate HE4 peak appeared as a shoulder on peak HE5 and due to the small scale of the purification could not be separated in amounts sufficient for further study.

4.3.2 Physical Characterisation of the Major Human Lysozyme Conjugate Fractions

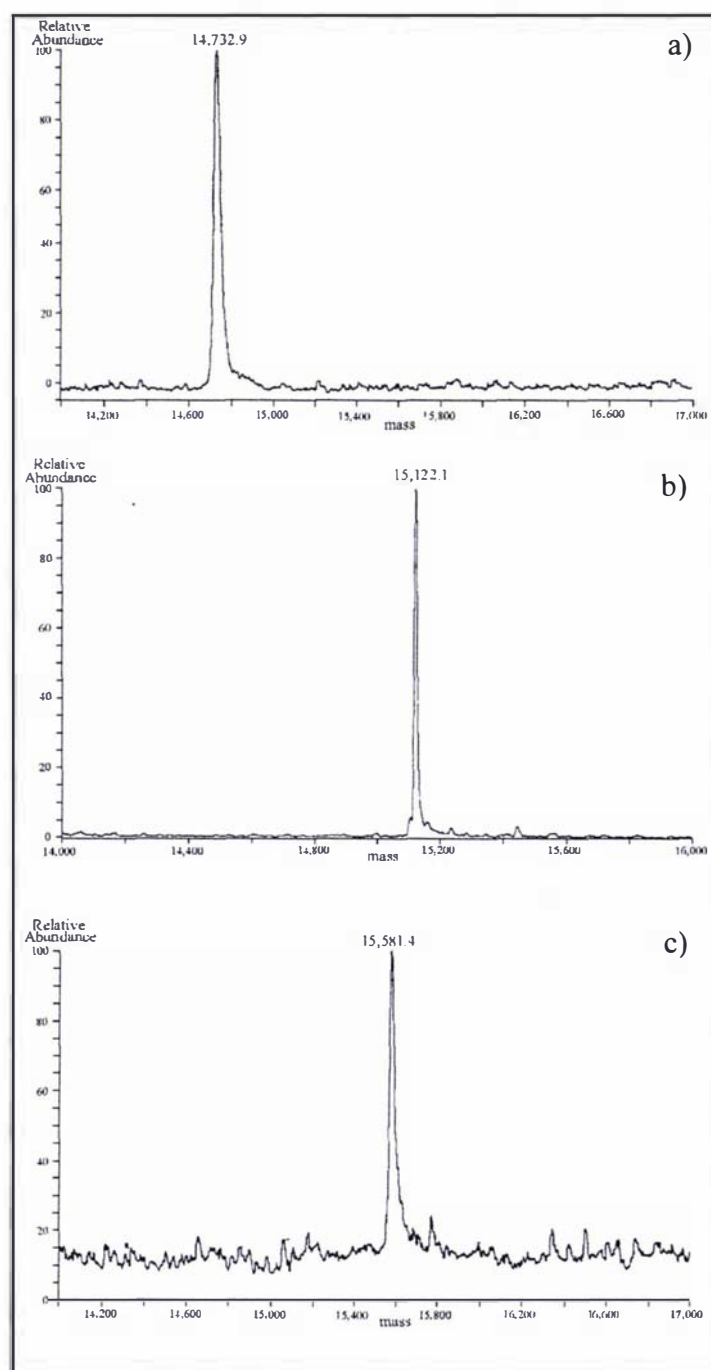


FIGURE 4.3: Electrospray Mass Spectrometry Spectra for Human Lysozyme, and Mono-S Chromatographic Conjugate Peaks HE1, HE2, HE3 and HE5

a) Human Lysozyme; b) HE1; c) HE2; d) HE3 and e) HE5

Continued page 168

Each of the major fractions separated from the final conjugation mixture (HE1, HE2, HE3 and HE5) were analysed for purity and degree of substitution by electrospray mass spectrometry (ESMS) (see Figures 4.3b-e). A commercial human lysozyme sample was also analysed by ESMS as a control (Figure 4.3a) and consisted of a single mass peak at 14,732.9 Daltons. The ESMS profile of the HE1 fraction (Figure 4.3b) also consisted of a single mass peak, having a molecular weight of 15,122.1 Daltons which was 389.2 Daltons higher than the native human lysozyme peak.

The major HE2 peak component had a mass of 15,581.4 Daltons which was 848.5 Daltons greater than the human lysozyme mass and 459 mass units greater than that measured for the HE1 fraction. Although the ESMS profile of the HE2 fraction was dominated by a single peak, there were also some minor components which constituted less than 5% of the total. The minor components were not dominated by any one form.

Of the human lysozyme conjugate fractions analysed by ESMS, fraction HE3 was the most heterogeneous. However, once again the profile was dominated by a single mass peak.

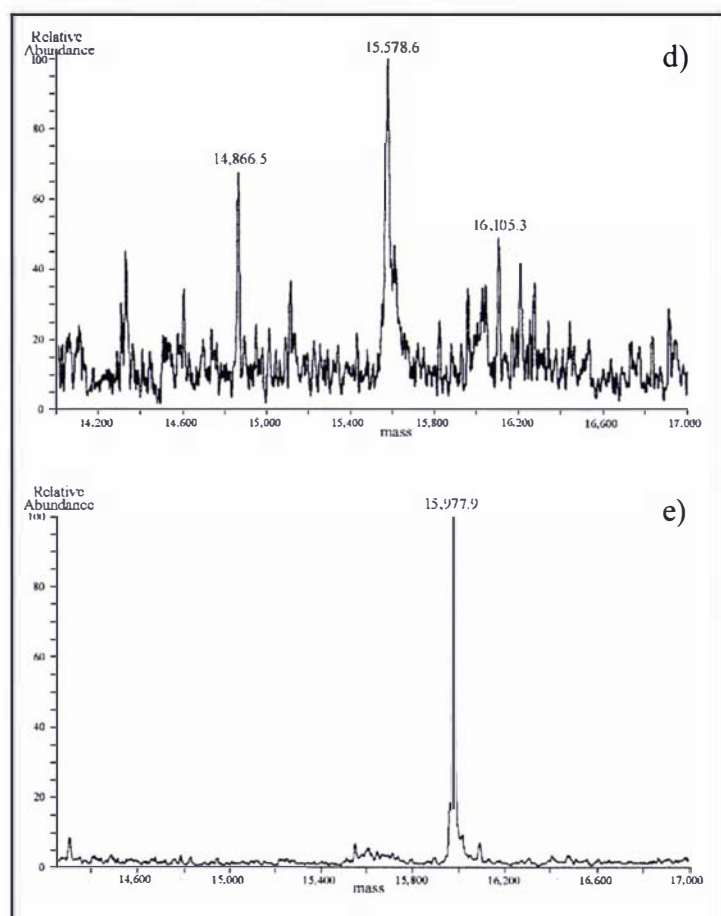


FIGURE 4.3 (continued)

d) HE3 and e) HE5

This peak had a molecular weight of 15,578.6 Daltons which was similar to the mass obtained for the main HE2 conjugate peak, suggesting that the major components of the HE2 and HE3 conjugate peaks are structural isomers.

The HE5 sample was the earliest eluting fraction from the Mono-S separation which was analysed by ESMS. The analysis showed that the HE5 conjugate peak was homogeneous, consisting of a single mass peak of 15,977.9 Daltons. This was 1,245.0 and 855.8 Daltons higher than that measured for the native enzyme and the HE1 conjugate fraction respectively, and an average of 397.9 units higher than that measured for the main components of the HE2 and HE3 conjugate peaks.

The major peaks from the final conjugation mixture (HE1, HE2 and HE5) were also analysed by non-denaturing acid gel electrophoresis (see Figure 4.4) and their migration distances compared with those of the corresponding HEWL E1-E6 active ester conjugates, and the native hen egg white and human lysozymes. Each of the human lysozyme conjugate fractions and the commercial human lysozyme sample migrated as a single band which was in contrast to the separation pattern of the purified HEWL conjugate fractions in which one of the major conjugate peaks collected from the Mono-S column (E1) separated into two bands upon electrophoresis.

The order of the electrophoretic migration and chromatographic elution times of the human lysozyme samples was also different from the HEWL samples. For HEWL and its estrone glucuronide active ester conjugates the migration order was HEWL>E1(lys97)>E3>E1(lys116)>E5>E2>E4>E6 while for the four human lysozyme samples analysed by gel electrophoresis the migration order was HuL>HE1>HE2>HE5. Thus the relative order of the migration distances on the gel and the chromatographic elution times from the cation exchange column separation were, at least for the four fractions analysed, the same for the human lysozyme samples in contrast to the behaviour of the HEWL conjugates (Section 2.4.2.2).

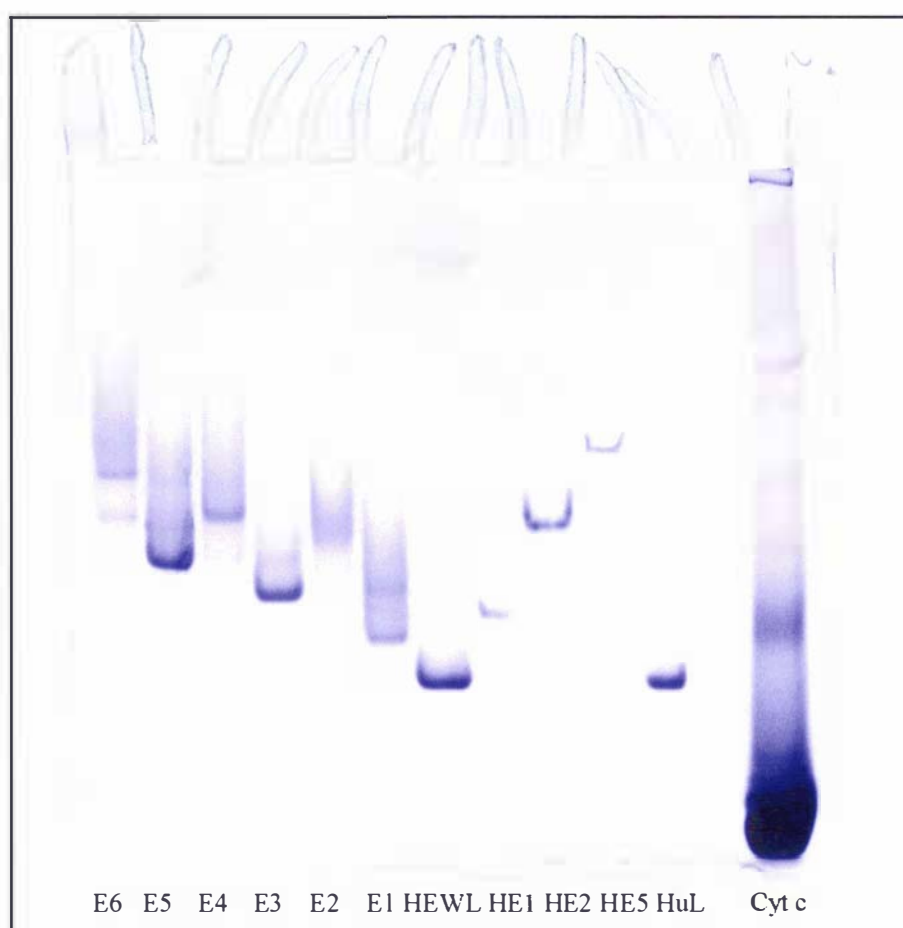


FIGURE 4.4: Polyacrylamide Gel Electrophoresis of Hen Egg White Lysozyme and Human Lysozyme and their Estrone Glucuronide Conjugates Purified by Mono-S Chromatography

Although there was some discrepancy in the relative order of electrophoretic migration for the HEWL versus the human lysozyme samples, in both cases, the native lysozyme migrated the furthest relative to the conjugated fractions which was also consistent with the relatively long column retention time for the two unconjugated enzymes. No difference in the migration distance was observed between the native human lysozyme and HEWL samples. This was in contrast to the electrophoretic behaviour of the conjugated samples, where upon electrophoresis each human lysozyme conjugate peak collected from the Mono-S column migrated a lesser distance compared with its HEWL counterpart.

4.3.3 Optimisation of the Human Lysozyme Conjugate Activity Assay

4.3.3.1 The Clearing Curve and Selection of the Assay Method

Figure 4.5 shows the change in transmission (ΔT) plotted as a function of time of the *M. lysodeikticus* clearing curves as measured on the Ovarian Monitor for a range of HE2 human lysozyme concentrations.

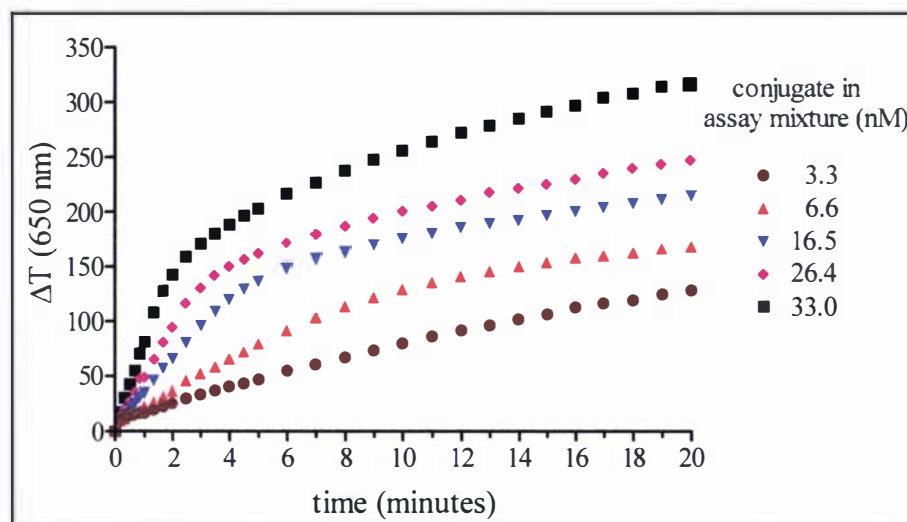


FIGURE 4.5: Clearing Curve for the Human Lysozyme HE2 Conjugate at Variable Enzyme Concentrations

These curves showed the same bi-phasic pattern of clearing as was observed for the native human enzyme (Cooke, 1993) with the degree of curvature increasing with an increase in the conjugate concentration. The rate of clearing of the first phase showed a clear dependence on the enzyme concentration, but in the second phase the clearing appeared to be almost independent.

It has previously been shown (Cooke, 1993) that the clearing of a *M. lysodeikticus* suspension by human lysozyme can be fitted with a reasonable level of accuracy to two consecutive second order processes. The order of clearing of the human lysozyme conjugate was analysed, by converting the Ovarian Monitor transmission data into absorbance by Equation 3.1, and then fitting it by the second order rate equation (Equation 3.3). Figure 4.6 (page 171) shows the human lysozyme conjugate HE2 clearing curve absorbance data from a high enzyme concentration reaction mixture (33 nmol L^{-1}) after the transformation. The second order plot (A_{650}^0/A_{650}^t versus time) clearly consisted of two consecutive linear phases, with the first phase (0-2.5 minutes) being considerably steeper than the second phase (2.5-20 minutes).

A relative measure of the rate constants for the two phases could be made by dividing the slope of the gradients with the starting absorbance.

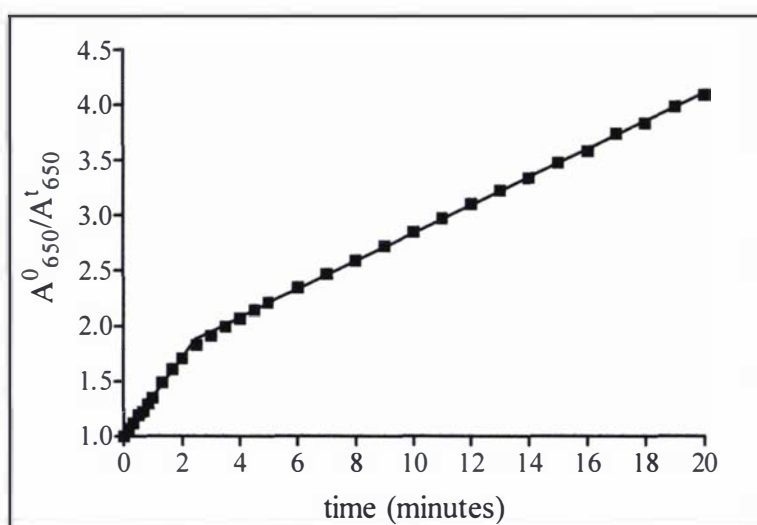


FIGURE 4.6: Linearisation of a Human Lysozyme HE2 Clearing Curve using Two Second Order Equations

Conditions in Assay Mixture: 33 nM enzyme

Thus, at a conjugate concentration of 33 nM in the assay mixture the apparent second order rate constants for the fast and slow phases were 0.758 min^{-1} and 0.269 min^{-1} respectively i.e. the second order rate constant was a factor of 2.8 fold higher for the first phase relative to the second phase. However this ratio was reduced at lower concentrations of enzyme as indicated by the clearing curves for different enzyme concentrations (Figure 4.5).

The corresponding end-point assay rates shown in Figure 4.7 were derived from the average of two sets of clearing curves; the clearing curves shown in Figure 4.5 and their duplicate assays (not shown).

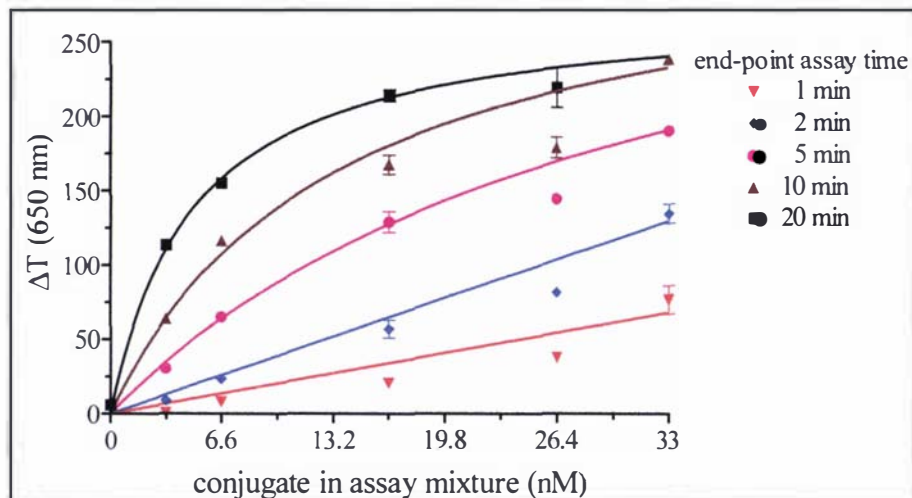


FIGURE 4.7: Changes in the End-point Rates for the Human Lysozyme HE2 Conjugate with Enzyme Concentration and Time

This plot of the end-point rate versus conjugate concentration, clearly illustrates how the selection of the end-point assay time affected the shape and magnitude of the apparent rate-dose response curve. When an end-point assay time of one minute was used, the ΔT values increased linearly with the conjugate concentration. However, as the end-point time was increased, the measured end-point rate became less linear with increases in conjugate concentration. This levelling off of the rate with the assay time and increasing conjugate concentration was such that at the end of twenty minutes, even at the highest conjugate concentration of 33 nmoles L^{-1} of assay mixture, the change in transmission was only

310 units. This was very low and compared unfavourably with the current Ovarian Monitor assay where only approximately 20 nmol L⁻¹ of HEWL conjugate were required to produce a twenty minute change of 350 transmission units.

Compared to the end-point assay, measuring the human lysozyme lytic activity by an initial rate assay was found to be a practical alternative. Although the total transmission change used to calculate these rates was small and only represented a small proportion of the total clearing curve, the relationship between the initial rates and conjugate concentration was linear over the concentration range tested (see Figures 4.10 and 4.17). Based on these results, the initial rate data provided the best method for determining the concentration of active enzyme conjugate. Therefore all other conjugate rates reported in this chapter, including those performed in the presence of antiserum, are derived from initial rate data unless otherwise specified.

The method of measuring the enzyme activity also had a significant effect on the apparent inhibition of its lytic activity by anti-E1G antibody.

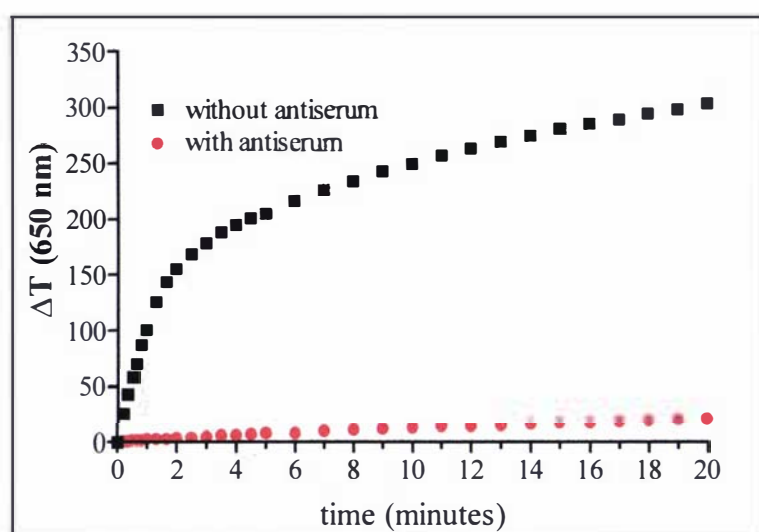


FIGURE 4.8: Effect of Maximally Inhibiting Levels of the Anti-Estrone Glucuronide Antiserum 243-4 on the Human Lysozyme HE2 Conjugate Clearing Curve

Conditions in Assay Mixture: 33 nM enzyme; 0.2 μL antiserum

Figure 4.8 shows the twenty minute clearing curve catalysed by the HE2 human lysozyme conjugate fraction in the presence, and absence, of maximally inhibiting levels of anti-estrone glucuronide antiserum.

When the level of inhibition was calculated based on the final change in transmission at twenty minutes, it was 93%, but when it was calculated based on the initial rate of clearing of the two curves the inhibition was increased to 98%.

4.3.3.2 Optimisation of Initial Rate Assay Conditions for Buffer Ionic Strength and pH

After establishing that an initial rate determination was the method of choice for measuring conjugate lytic activity and that the conjugate was of a high quality i.e. highly inhibitable, the conjugate assay conditions were optimised for pH and ionic strength.

The effect of buffer pH on conjugate activity was measured over a range of ionic strengths and showed the conjugate activity to be highly dependent upon the ionic strength of the

tris-maleate buffer resulting in the typical bell shaped activity curve for all three pH values examined (Figure 4.9).

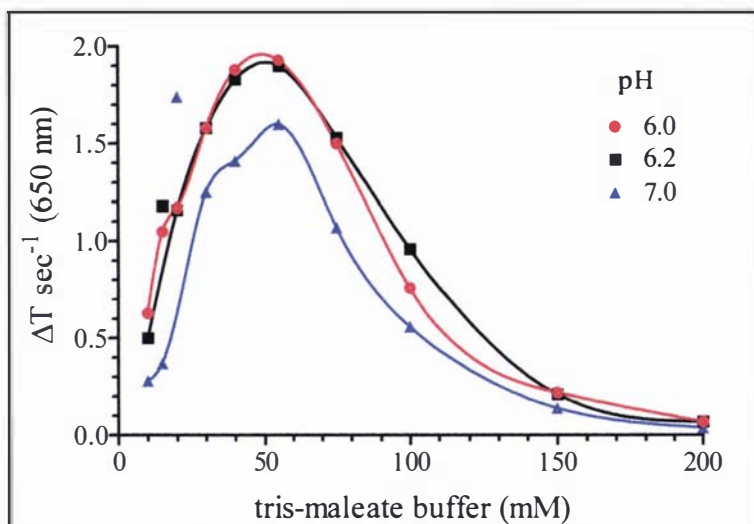


FIGURE 4.9: Effect of pH and Ionic Strength of the Tris-Maleate Buffer on the Initial Rate of the Human Lysozyme HE2 Conjugate

Apart from a difference in amplitude, the three activity curves were essentially the same. The curves were all clearly skewed towards higher ionic strengths and had a maximal lytic activity between 40-55 mM tris-maleate. With regards to buffer pH, the assays performed using the pH 7.0 buffers generated significantly lower rates than the pH 6.2 and pH 6.0 assays. On the basis of the above results all further

human lysozyme conjugate kinetic work reported in this chapter was performed using 55 mM tris-maleate buffer (pH 6.2) as the assay medium.

4.3.3.3 The Effect of Bovine Serum Albumin on Human Lysozyme Conjugate Activity and Stability

Native human lysozyme assays performed in the presence of albumin have been reported to have a higher lytic activity (Mörsky, 1983). In order to examine the effect of bovine serum albumin (BSA) on the activity of the human lysozyme HE2 conjugate, the initial rate of clearing by the conjugate was measured with and without the addition of BSA (0.1 g L^{-1}) in the assay buffer. The data are summarised in Table 4.2.

TABLE 4.2: The Effect of Bovine Serum Albumin in the Assay Buffer on Human Lysozyme HE2 Conjugate Activity

without albumin in assay buffer				with albumin in assay buffer			
$\bar{x}$ IR* ($\Delta T \text{ sec}^{-1}$)	n	SD [†] (n-1)	CV [§] (%)	$\bar{x}$ IR* ($\Delta T \text{ sec}^{-1}$)	n	SD [†] (n-1)	CV [§] (%)
1.86	3	0.138	7.4	1.75	3	0.172	9.8
1.63	3	0.023	1.4	1.92	3	0.050	2.6
1.63	3	0.051	3.1	1.52	3	0.055	3.6

$\bar{x}$ IR* - mean initial rate; SD[†] - standard deviation; CV[§] - co-efficient of variation.

Each of the six sets of data in the table corresponds to a triplicate set of rates obtained with one of six fresh dilutions of the conjugate made with albumin free stock buffer. Fresh dilutions of the conjugate were used for each set of rates to minimise any problems caused

by time dependent enzyme denaturation, hence none of the with and without albumin assays were performed using the same conjugate dilution. To minimise any differences in the observed rates due to dilution errors in the preparation of the enzyme stock solutions, both sets of conditions were repeated using three enzyme dilutions. Thus a total of nine assays were performed for each condition.

The initial rates measured for the assays performed in the presence of the 0.01% albumin assay buffer were associated with an average of 25% more variation than the data collected using albumin free assay buffer. Comparison of the initial rate data measured in the presence and absence of albumin in the assay buffer by a one-tailed P test generated a P value of 0.878 for the averages of the three data sets.

Human lysozyme is also reported to be less stable than HEWL (Mörsky, 1983; Selsted and Martinez, 1980). Since BSA has been reported to stabilise native human lysozyme, the effect of the addition of BSA to the stability of a human lysozyme conjugate stock solution was investigated. In this experiment, the activity of an aliquot of an HE2 conjugate stock solution prepared in the absence and presence of albumin (0.1% w/v) was measured immediately after the stock solution had been prepared and again after the same stock solution had been stored at 4°C for 14 days. The enzyme stock solutions (1.156 μ M) were all prepared in 0.35 M tris-maleate buffer (pH 7.0) and the activity assays were all performed using albumin free assay buffer and a final enzyme concentration of 33 nM in the assay mixture. The results of the activity assays are summarised in Table 4.3.

TABLE 4.3: The Effect of Bovine Serum Albumin (0.1%) on the Stability of Human Lysozyme HE2 Conjugate Stock Solutions

storage conditions	freshly diluted enzyme				after storage for 14 days at 4°C				decrease in activity after storage (%)
	$\bar{x}$ IR* (ΔT sec ⁻¹)	n	SD [‡] (n-1)	CV [§] (%)	$\bar{x}$ IR* (ΔT sec ⁻¹)	n	SD [‡] (n-1)	CV [§] (%)	
without BSA	1.92	3	0.079	4.1	1.58	2	0.007	0.4	18
with BSA	1.96	3	0.220	11.2	1.52	2	0.028	1.9	23

$\bar{x}$ IR* - mean initial rate; SD[‡] - standard deviation; CV[§] - co-efficient of variation.

The data presented in this table gives an indication of the range of values obtained under a single condition. The co-efficient of variation for the initial rate data ranged from 0.4% on day 14 to 11.2% on day 1. This range was typical of what was experienced in the collection of the human lysozyme conjugate initial rate data. Sometimes the rates were very reproducible, while at other times they were far more variable, with the reproducibility seeming to vary from day-to-day. The number of replicates used to obtain a data set usually reflected the variability of the day. For example, in the data presented in Table 4.3, the experiment was performed in triplicate on day 1, but for day 14 a duplicate assay was found to be sufficient. The variation in the data was higher for the assays performed using the conjugate from the albumin-containing stock solution. With the freshly diluted stock solutions and the 14 day old samples, the co-efficient of variation

was 2.7 and 4.8 fold higher respectively for the assays performed using conjugate from the stock solution with albumin relative to the albumin free stock. Within the limits of the experimental error of the experiment, the mean initial rates measured for conjugates obtained from the albumin and albumin free enzyme stock solutions were the same for both the freshly diluted and 14 day old samples.

On the basis of the data presented in the above two tables, all further work reported in this chapter was performed using assay and enzyme stock buffers prepared in the absence of albumin.

4.3.4 A Rapid Human Lysozyme HE2 Conjugate Based Estrone Glucuronide Immunoassay

After the Ovarian Monitor buffer conditions had been optimised for the human lysozyme conjugates, the potential of the human lysozyme HE2 conjugate for use in a rapid immunoassay system for E1G was investigated.

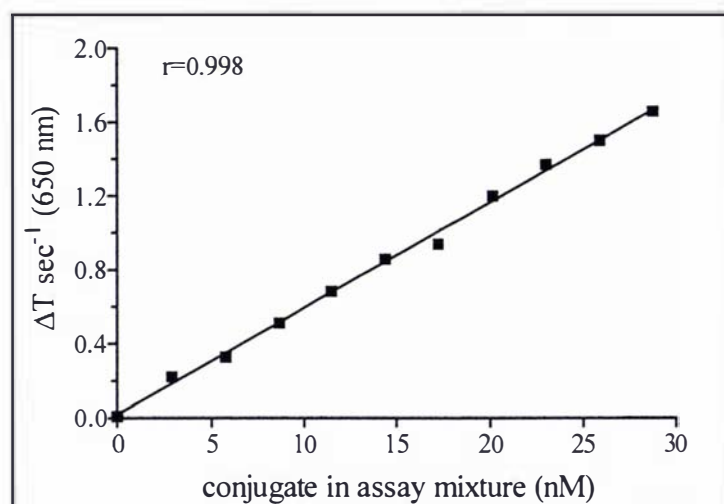


FIGURE 4.10: Relationship between Initial Rates and the Human Lysozyme HE2 Conjugate Concentration

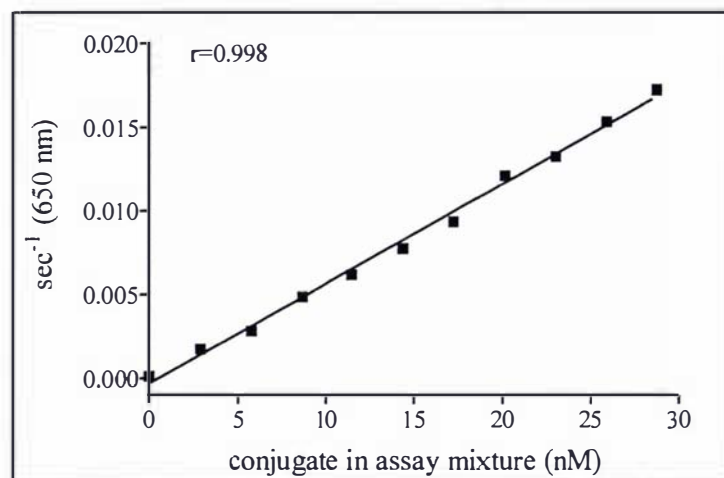


FIGURE 4.11: Relationship between the Second Order Rate Constant and the Human Lysozyme HE2 Conjugate Concentration for the First Phase of Clearing

Figures 4.10 and 4.11 show the increase in the initial rates ($\Delta T \text{ sec}^{-1}$) and the second order rate constant (1st phase) respectively with enzyme concentration over a conjugate concentration range in the assay mixture of 0-28.8 nM. The relationships were clearly linear over this range of concentrations and when the data were fitted to a straight line using a least squares regression program (PRISM), the correlation co-efficient (r) was 0.998 for both sets of data.

Since the concentration of substrate used in these assays was 214 mg L^{-1} and the K_m for human lysozyme at an enzyme concentration in the assay mixture of 7 nM has been calculated as 39 mg mL^{-1} , (Cooke, 1993) it can be assumed that these rates were obtained under saturating substrate conditions. Thus the gradient of initial rate plot ($0.057 \Delta T \text{ sec}^{-1} \text{ nmoles L}^{-1}$) equals k_{cat} for the human lysozyme HE2 conjugate.

Because at a conjugate concentration of 28.8 nM in the assay mixture the conjugate only rate was high and the relationship between enzyme concentration and the initial rate of clearing was still linear, this concentration allowed for the maximum discrimination between different levels of antibody-mediated conjugate inhibition.

Figure 4.12 shows a titration curve obtained when this concentration of human lysozyme HE2 conjugate was titrated against increasing aliquots of the anti-E1G antiserum 243-4.

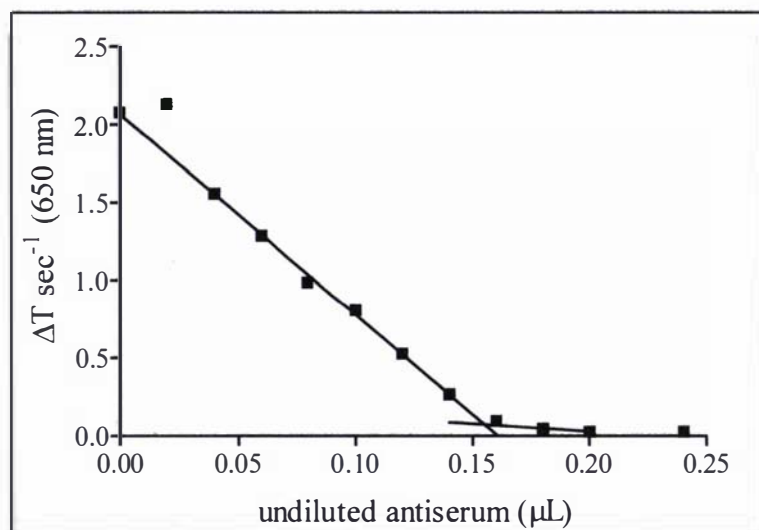


FIGURE 4.12: Anti-Estrone Glucuronide Antiserum 243-4 Titration Curve for Human Lysozyme HE2 Conjugate

Conditions in Assay Mixture: 28.8 nM enzyme

The antiserum titration curve consisted of a steep initial linear phase followed by a second much shallower linear phase. The transition between the phases (i.e. the intersection point between the two extrapolated fitted lines) occurred at an antiserum concentration equivalent to 0.155 μL of undiluted antiserum 243-4 per assay mixture. At this point the lytic activity of HE2 was inhibited by 96.5%.

Based on the above results, a conjugate concentration of 28.8 nM and the equivalent of 0.155 μL of undiluted antiserum, was used as a starting point in the optimisation of the HE2 human lysozyme standard curve. The final optimised assay conditions were 23 nM HE2 human lysozyme conjugate and 0.09 μL of undiluted antiserum 243-4.

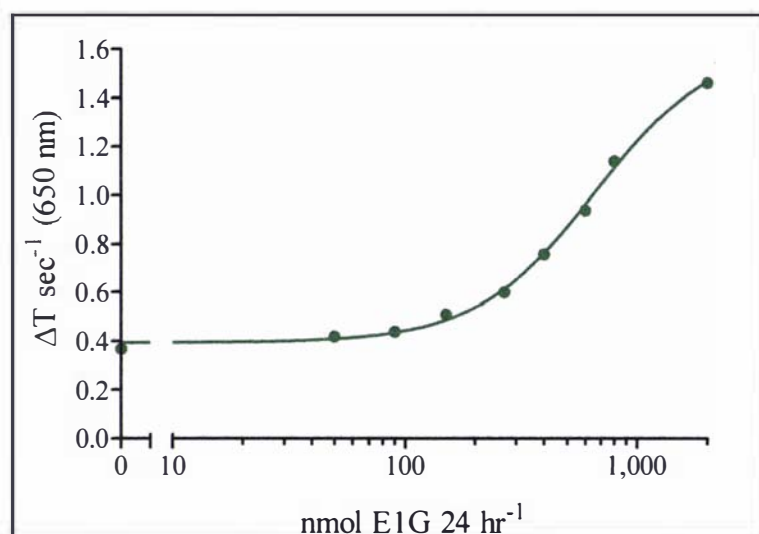


FIGURE 4.13: The Optimised Human Lysozyme HE2 Standard Curve obtained in the Presence of a Blank Urine Sample

Conditions in Assay Mixture: 23 nM enzyme; 0.09 μL antiserum; antiserum - anti-estrone glucuronide 243-4

Figure 4.13 shows the final optimised standard curve performed in the presence of the child's blank urine and using only a single lytic assay at each E1G concentration.

By visual inspection, the standard curve had a sensitivity of about 150 nmol E1G 24 hr⁻¹ and an optimum working range of 200-2,000 nmoles of E1G 24 hr⁻¹.

Figure 4.14 shows the daily E1G profile for a woman undergoing clomiphene treatment for unexplained (idiopathic) infertility as measured by the rapid HE2 human lysozyme conjugate system.

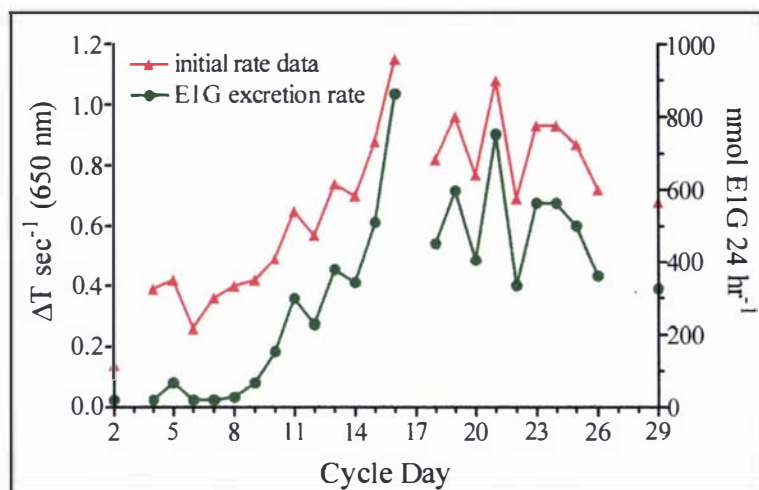


FIGURE 4.14: Estrone Glucuronide Cycle Data from a Woman undergoing Clomiphene Treatment obtained using the Rapid Human Lysozyme HE2 Optimised Immunoassay Method expressed in Terms of Initial Rates and Estrone Glucuronide Excretion Rates after Conversion from a Blank Urine Standard Curve

Conditions in Assay Mixture: 23 nM enzyme; 0.09 μ L antiserum; anti-estrone glucuronide antiserum 243-4
Standard Curve: Figure 4.13

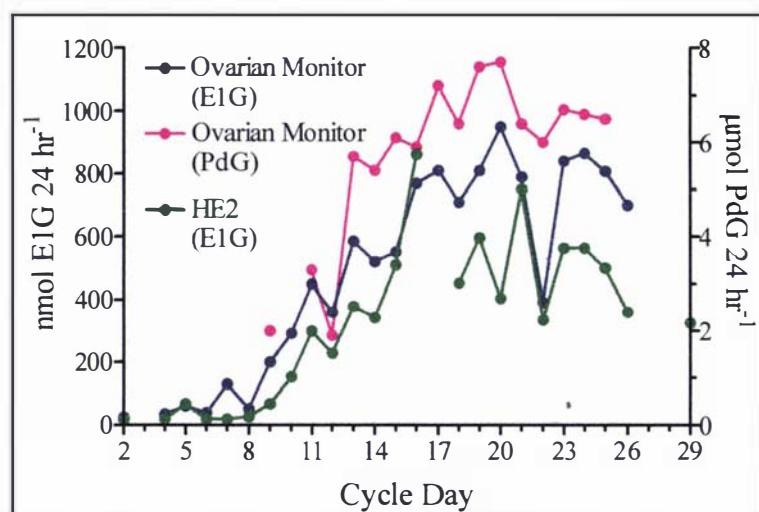


FIGURE 4.15: Estrone Glucuronide (E1G) and Pregnanediol Glucuronide (PdG) Cycle Data from a Woman undergoing Clomiphene Treatment obtained using the Rapid Human Lysozyme HE2 Optimised Immunoassay Method (E1G) and the Current Ovarian Monitor Assay (E1G and PdG)

Pre-coated Assay Tubes: E1G - #14 (80-85); PdG - #16 (224-229)

Wet Chemistry Assay Conditions and Standard Curve: see Figure 4.14

The data were expressed in terms of the initial rates of clearing and as urinary excretion rates after conversion of the initial rate data using the initial rate standard curve in Figure 4.13. Unfortunately, no urine samples were available from the patient for days 3, 17, 27 and 28 of the cycle, thus the complete urinary excretion profile could not be obtained. However, based on the data which were available, the first (pre-ovulatory) E1G peak occurred on day 11 of the cycle (299 nmoles E1G 24 hr⁻¹) and the highest E1G excretion rate was recorded on cycle day 16 (863 nmoles E1G 24 hr⁻¹). The E1G values remained above 400 nmoles E1G 24 hr⁻¹ for the remainder of the cycle until just before the next menstrual bleed.

Figure 4.15 shows the daily urinary E1G excretion rates for the same clomiphene treatment cycle as measured by the alternative HE2 human lysozyme conjugate system in the laboratory and as measured by the Ovarian Monitor at home by the client with the pre-coated assay tubes.

Although a urinary E1G excretion rate is recorded for day 17 in the woman's home data, the client failed to provide a urine sample to the laboratory for this cycle day, thus there is a break in the urinary E1G excretion rate data for the alternative human lysozyme assay on this day. Apart from an obvious discrepancy at day 20, the two sets of data rose and fell in parallel for the duration of the cycle.

The urinary pregnanediol glucuronide (PdG) excretion rates as measured by the Ovarian Monitor assay at home by the client with the PdG pre-coated tubes are also recorded on Figure 4.15. Using the standard Ovarian Monitor PdG threshold value of $6.3 \mu\text{mol } 24 \text{ hr}^{-1}$, the post-ovulatory infertile days began on day 13.

Figure 4.16 shows the correlation of the two sets of E1G excretion rates as measured by the two methods using the Deming Regression Model (Deming, 1943; Martin, 2000).

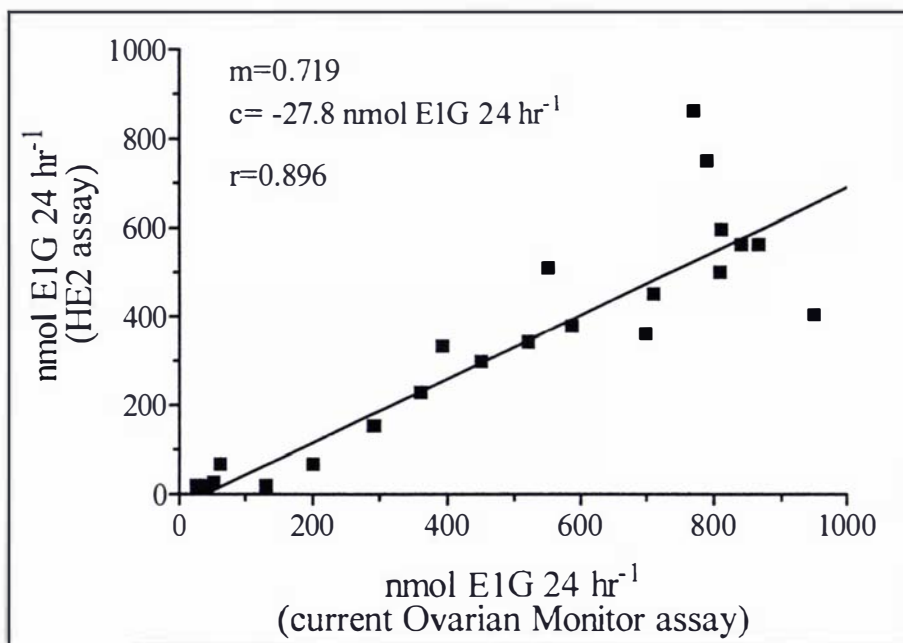


FIGURE 4.16: Correlation by Deming Regression of Estrone Glucuronide Cycle Data from a Woman undergoing Clomiphene Treatment obtained using the Rapid Human Lysozyme HE2 Optimised Immunoassay Method and the Current Ovarian Monitor Assay

Conditions of wet chemistry and pre-coated assay tubes as stated in Figures 4.14 and 4.15

The correlation co-efficient was 0.896, and if the day 20 outlier was excluded this was increased to 0.927. The line had a gradient of 0.72 and a Y intercept of $-28 \text{ nmol E1G } 24 \text{ hr}^{-1}$.

4.3.5 A Rapid Human Lysozyme HE1 Conjugate Based Estrone Glucuronide Immunoassay

Under the optimised assay conditions described in Section 4.3.3, the human lysozyme HE2 conjugate had a specific activity of $0.165 \Delta T \text{ sec}^{-1} \text{ pmol}^{-1}$, while the HE1 conjugate had a specific activity of $0.372 \Delta T \text{ sec}^{-1} \text{ pmol}^{-1}$. Thus its specific activity was more than double that of the HE2 conjugate. As one method of increasing an immunoassay's sensitivity is to use a smaller amount of an enzyme with a higher specific activity, the human lysozyme-E1 G conjugate immunoassay was also optimised using the HE1 conjugate as the signal generator.

Figure 4.17 shows the increase in the initial rates ($\Delta T \text{ sec}^{-1}$) with increasing conjugate concentration over the range of 0-13.6 nM.

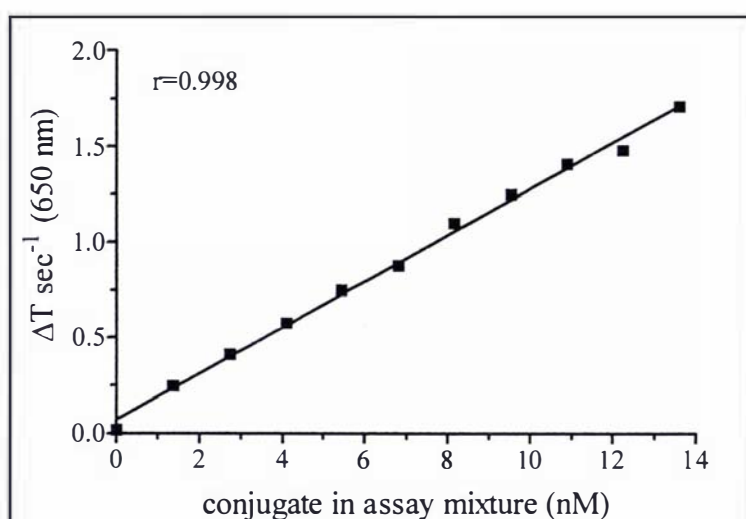


FIGURE 4.17: Relationship between Initial Rates and the Human Lysozyme HE1 Conjugate Concentration

This data set was obtained without any repeat assays. As was observed for the HE2 human lysozyme-E1 G conjugate, the relationship between the initial rates and the conjugate concentration was clearly linear over this range ($r=0.998$). Thus k_{cat} could also be calculated for the HE1 data ($0.12 \Delta T \text{ sec}^{-1} \text{ nmoles L}^{-1}$).

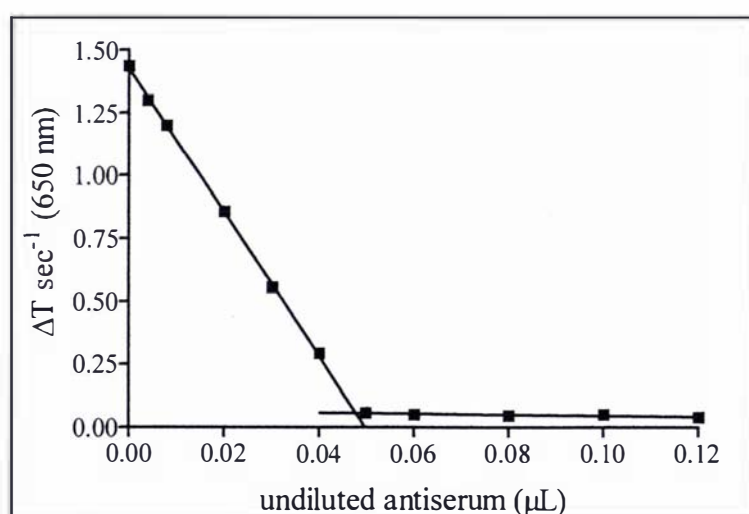


FIGURE 4.18: Anti-Estrone Glucuronide Antiserum (243-4) Titration Curve for Human Lysozyme HE1 Conjugate

Conditions in Assay Mixture: 12.2 nM enzyme

Figure 4.18 shows a titration curve of antiserum 243-4 against a constant concentration of human lysozyme HE1 conjugate (12.2 nM). As for the conjugate concentration versus initial rate plot, the overall titration profile was very similar to the antiserum titration curve obtained with the HE2 conjugate, with only the absolute values varying. Thus the antiserum titration curve of the HE1 conjugate also consisted of two linear phases with the

transition point between the two phases being sharply defined. Over the steep initial phase the initial rate decreased from 1.43 to 0.05 $\Delta T \text{ min}^{-1}$. Thus at the end-point (which occurred at an antiserum concentration equivalent to 0.048 μL of undiluted antiserum), the conjugate activity was inhibited by 96.5%.

Based on the results of the antiserum titration curve, a conjugate concentration of 12.2 nM and the equivalent of 0.050 μL of undiluted antiserum were used as a starting point in the optimisation of the HE1 human lysozyme standard curve. These conditions resulted in a standard curve which was too high up the concentration axis. The standard method for decreasing the working range of an immunoassay is to decrease the concentration of antiserum. However, this had little effect on the standard curve's position on the concentration axis and only increased the baseline lytic activity of the standard curve thus decreasing the assay discrimination.

Using the same rationale, a small increase in antiserum concentration may act to decrease the baseline even further without significantly increasing the optimum concentration range of the assay. The results of this experiment are shown in Figure 4.19.

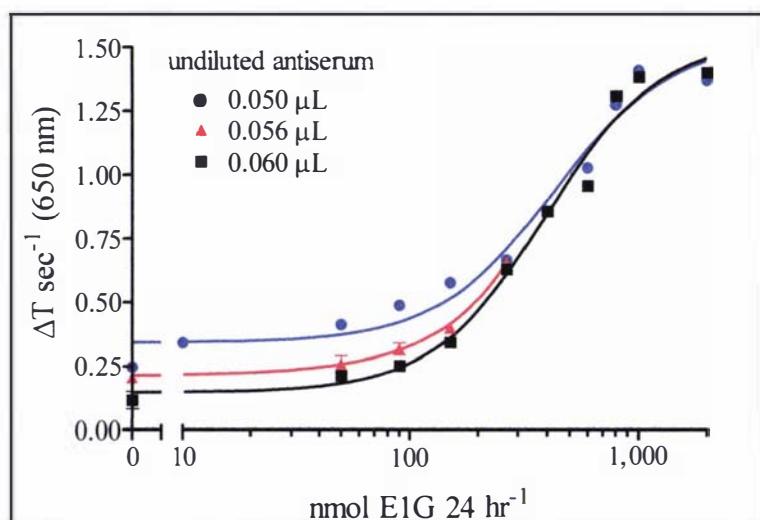


FIGURE 4.19: The Optimisation of the Human Lysozyme HE1 Standard Curve for Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: 12.2 nM enzyme; see key for antiserum conditions

All the standard curves outlined in Figure 4.19 were produced with the same concentration of HE1 conjugate (12.2 nM) but with different amounts of antiserum 243-4 (0.050, 0.056 and 0.060 μL). Within the limits of experimental error, increases in the amount of antiserum over this range had little discernable effect on the horizontal position of the standard curve, but were associated with a significant baseline decrease.

The baseline initial rates for the three standard curves were 0.34, 0.21 and 0.15 $\Delta T \text{ sec}^{-1}$ for the equivalent of 0.050, 0.056 and 0.060 μL of undiluted antiserum increasing the extent of inhibition at the baseline from 77% to 90%. On the basis of these results, the final optimised standard curve conditions for the HE1 human lysozyme conjugate were 12.2 nM enzyme with 0.060 μL of undiluted antiserum.

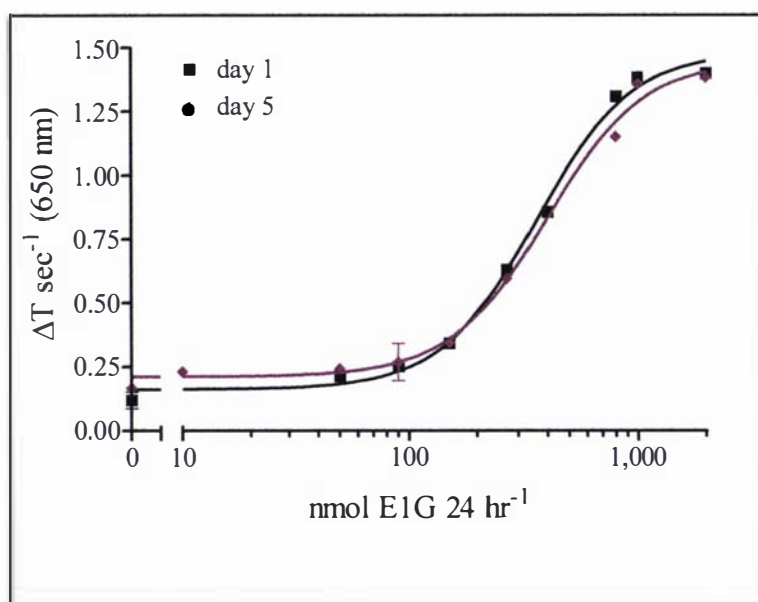


FIGURE 4.20: Day-to-Day Reproducibility of the Optimised Human Lysozyme HE1 Standard Curve

Conditions in Assay Mixture: 12.2 nM enzyme; 0.06 μ L antiserum; anti-estrone glucuronide antiserum 243-4

Figure 4.20 shows the HE1 standard curve as obtained under these optimal conditions, but on different days and showed that the day-to-day reproducibility of the position of the standard curve was extremely high.

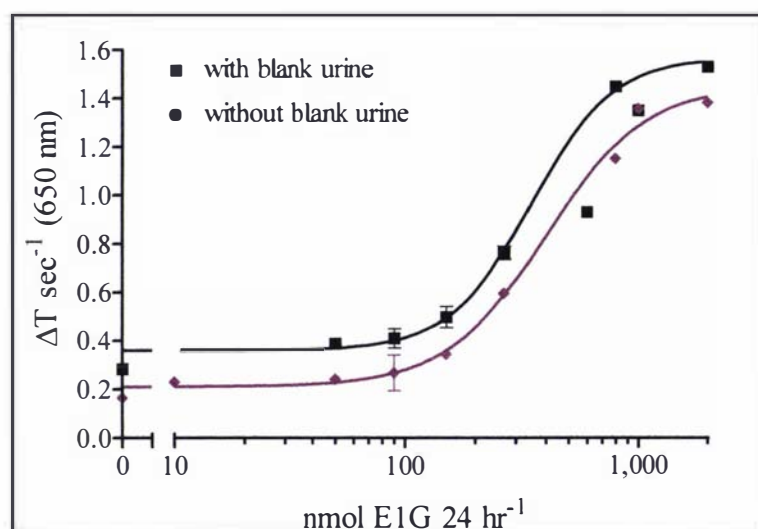


FIGURE 4.21: Effect of Blank Urine on the Optimised Human Lysozyme HE1 Standard Curve

Conditions in Assay Mixture: see Figure 4.20

Figure 4.21 shows the optimised HE1 standard curve obtained on the same day in the presence and absence of a blank urine sample (see Section 4.2.5.4). Based on the fit of the standard curves to a sigmoidal dose response curve, the standard curve obtained in the absence of a blank urine sample had a baseline rate of 0.21 $\Delta T \text{ sec}^{-1}$ and a mid-point value (EC_{50}) of 400 nmol E1G 24 hr⁻¹ with a rise from the zero standard value occurring at an excretion rate of 150

nmol E1G 24 hr⁻¹. In comparison, the standard curve obtained in the presence of the blank urine sample was of the same sensitivity (i.e. $\approx 150 \text{ nmol E1G 24 hr}^{-1}$), but had a baseline rate of 0.36 $\Delta T \text{ sec}^{-1}$ and a mid-point value of 346 nmol E1G 24 hr⁻¹.

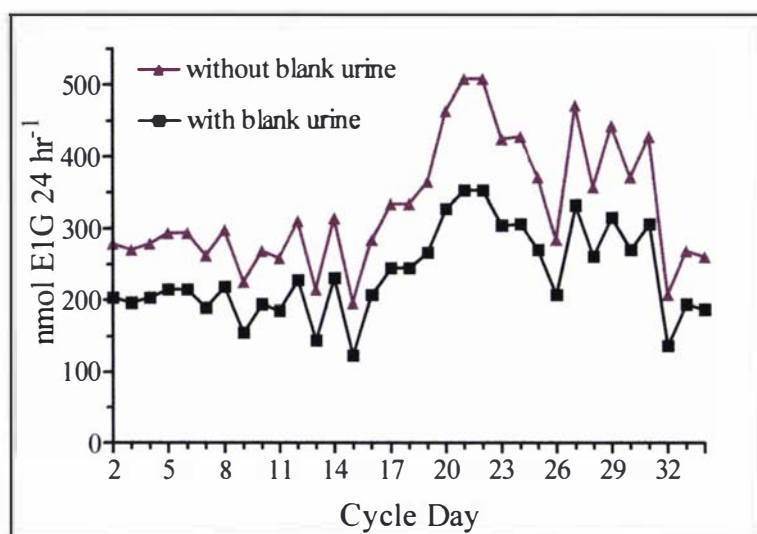


FIGURE 4.22: A Comparison of the Estrone Glucuronide (E1G) Cycle Data from a Woman with an Endocrinologically Normal Cycle obtained using the Optimised Human Lysozyme HE1 Immunoassay Method after Conversion into E1G Excretion Rates by the Blank and Without Blank Urine Standard Curves

Conditions in Assay Mixture: 12.2 nM enzyme; 0.06 μ L antiserum; anti-estrone glucuronide antiserum 243-4

two sets of data rose and fell in parallel, with the urinary E1G excretion rates calculated using the standard curve obtained in the presence of blank urine being reduced by approximately one-third. For example, the day of maximum E1G excretion (day 17) was calculated to have a urinary E1G excretion rate of 334 nmol 24 hr⁻¹ when the buffer control standard curve was used to convert the initial rate data, compared to only 245 nmol E1G 24 hr⁻¹ when the blank urine standard curve was used. This shows the extent that the selection of the standard curve can have on the apparent urinary E1G excretion rates.

The ultimate test for an alternative E1G immunoassay system requires that the measurement of the urinary E1G levels for a menstrual cycle by the new and the current method be compared. Figure 4.23 (page 184) shows the urinary E1G excretion rates for the same complete menstrual cycle as measured by the Ovarian Monitor system with the pre-coated assay tubes and the HEWL conjugate mixture, and as measured by the new HE1 human lysozyme conjugate rapid assay system.

Both sets of data were converted into urinary E1G excretion rates using standard curves obtained in the presence of a blank urine sample. Although relative to the home Monitor assay, the HE1 human lysozyme conjugate system consistently over-estimated the E1G excretion rate, the two methods generated profiles which showed essentially the same E1G excretion pattern. Both sets of data showed the classical menstrual cycle estrogen profile; a period of low estrogen levels at the beginning of the cycle followed by two peaks and ending with a drop back to pre-peak E1G levels immediately before the next menstruation. The two sets of data would have estimated the key estrogen markers of fertility as

Figure 4.22 shows the difference that the addition of a blank urine sample to the standard curve assay mixture made to the apparent urinary E1G excretion rates for an endocrinologically normal menstrual cycle measured by the optimised rapid HE1 human lysozyme immunoassay.

The two E1G cycle profiles were derived from the same initial rate data and were converted into urinary E1G excretion rates using the buffer control and blank urine standard curves presented in Figures 4.20 and 4.21 respectively. The

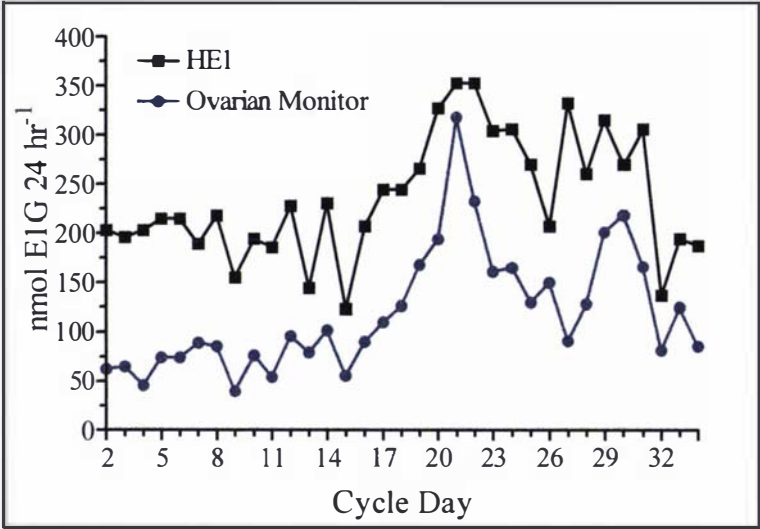


FIGURE 4.23: Estrone Glucuronide Cycle Data from a Woman with an Endocrinologically Normal Cycle obtained using the Human Lysozyme HE1 Optimised Immunoassay Method and the Current Ovarian Monitor Assay

Pre-coated E1G Assay Tubes: #14 (80-85)

Wet Chemistry Assay Conditions: see Figure 4.22; initial rate data converted using a blank standard curve

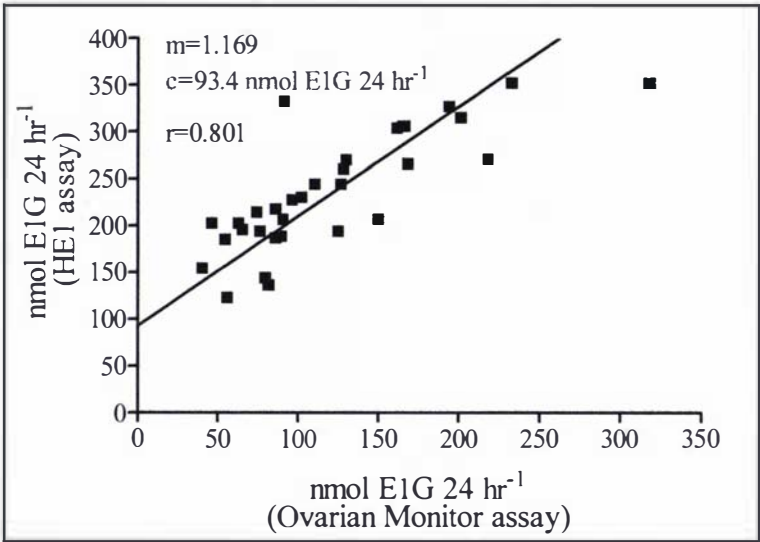


FIGURE 4.24: Correlation by Deming Regression of Estrone Glucuronide Cycle Data from a Woman with an Endocrinologically Normal Cycle obtained using the Rapid Human Lysozyme HE1 Optimised Immunoassay Method and the Current Ovarian Monitor Assay

Conditions as stated in Figures 4.22 and 4.23

occurring on the same days. The day of the first rise in E1G excretion rates from baseline levels occurred on day 17 of the cycle, and the first estrogen peak on day 21. The biggest discrepancy in the data was recorded on day 27 in the second half of the cycle which was depicted as the nadir between the two estrogen peaks for the Monitor assay and as an early high of the second peak for the human lysozyme E1G assay.

Figure 4.24 shows the correlation between the data from the two methods ($r=0.801$) using the Deming Regression Model as described in Section 3.2.5.6.

The line had a gradient of 1.17, and a Y intercept of 93 nmol E1G 24 hr⁻¹.

4.3.6 Comparison of the HE1 and HE2 Human Lysozyme Conjugates with HE5

Under the optimised assay conditions described in Section 4.3.3, the HE1, HE2 and HE5 conjugate fractions had specific activities of 0.372, 0.165 and 0.086 $\Delta T \text{ sec}^{-1} \text{ pmol}^{-1}$ respectively. Thus the specific activity of the HE5 fraction was approximately half that of the HE2 conjugate fraction which in turn had approximately half the specific activity of the HE1 fraction.

Figure 4.25 shows an attempt to optimise the standard curve using the HE5 conjugate as a signal generator and anti-E1G antiserum 243-4 for measurement of urinary E1G excretion rates over the normal physiological range.

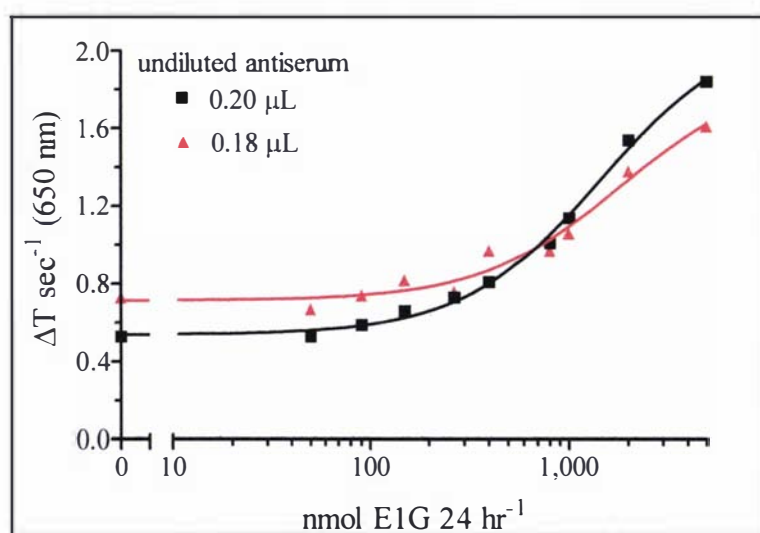


FIGURE 4.25: Effect of Changing the Amount of Anti-Estrone Glucuronide Antiserum 243-4 on the Human Lysozyme HE5 Standard Curve

Conditions in Assay Mixture: 98.3 nM enzyme

Although under these conditions the standard curve showed good discrimination between the minimum and maximal initial rates, as predicted from the low specific activity of the HE5 conjugate, the position of the standard curve along the concentration axis made it completely unsuitable for use in a home fertility assay. A reduction in the volume of antiserum in the assay to 0.18 μL per assay mixture resulted in a lifting of the baseline (0.715 $\Delta T \text{ sec}^{-1}$), reduction of the maximal rate (1.87 $\Delta T \text{ sec}^{-1}$) and an increase in the standard curve E1G excretion rate mid-point (1791 nmol 24 hr⁻¹) without a significant increase in sensitivity.

Thus, the final optimised conditions for the three fast assays using HE1, HE2 and HE5 human lysozyme conjugates as signal generators were 12.2, 28.8 and 98.3 nM of conjugate with 0.06, 0.11 and 0.20 μL of undiluted antiserum 243-4 respectively.

To achieve an acceptable initial rate of lysis the amount of the HE5 conjugate in the standard curve assay mixture had to be increased to 98 nM. The optimised HE5 standard curve was obtained with the equivalent of 0.2 μL of undiluted antiserum and had a baseline rate of 0.54 $\Delta T \text{ sec}^{-1}$, a maximal rate of 2.12 $\Delta T \text{ sec}^{-1}$, a mid-point equivalent to an E1G excretion rate of 1431 nmol 24 hr⁻¹, and a sensitivity of about 300 nmol E1G 24 hr⁻¹. Although under these

Figure 4.26 shows a comparison of the optimised standard curves all obtained in the absence of a blank urine sample.

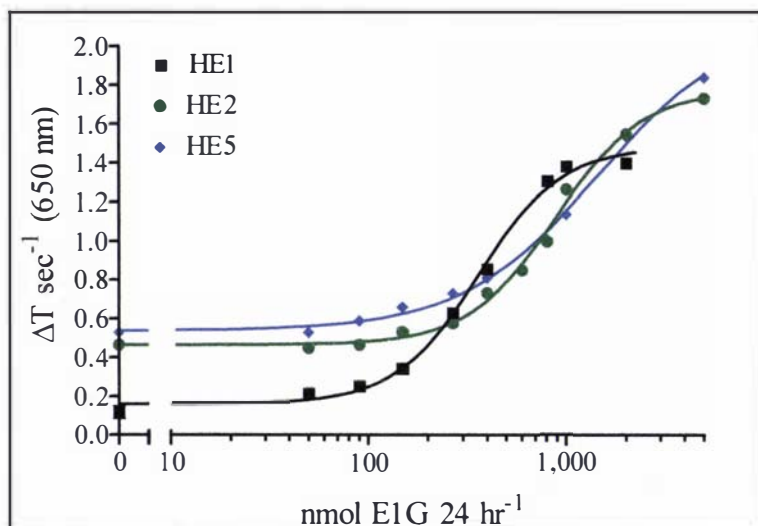


FIGURE 4.26: The Optimised Human Lysozyme HE1, HE2 and HE5 Standard Curves obtained with Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: HE1 - 12.2 nM enzyme, 0.06 μ L antiserum; HE2 - 28.8 nM enzyme, 0.11 μ L antiserum; HE5 - 98.3 nM enzyme, 0.20 μ L antiserum; anti-estrone glucuronide antiserum 243-4

For the HE1, HE2 and HE5 standard curves, the baseline initial rates increased with decreasing specific activity of the signal generator as did the mid-points of the standard curves, while the assay sensitivities decreased. For the HE1, HE2 and HE5 standard curves, the baseline initial rates were 0.16, 0.47, and 0.54 $\Delta T \text{ sec}^{-1}$, the first rises occurred at 150, 300 and 300 nmol E1G 24 hr⁻¹ and the standard curve mid-points were 358, 884, and 1431 nmol E1G 24 hr⁻¹ respectively.

4.3.7 Lysozyme-Estrone Glucuronide Conjugate Titrations with Anti-Estrone Glucuronide Antibody 243-4

Conjugate titrations in which increasing concentrations of HEWL-E1G and human lysozyme-E1G were added to fixed amounts of the anti-E1G antibody 243-4 were undertaken in an attempt to calculate the dissociation constants for the immune complexes.

Figure 4.27 shows the results of an initial rate titration with 0.048 μL of antiserum 243-4 and with increasing amounts of HEWL conjugate E3.

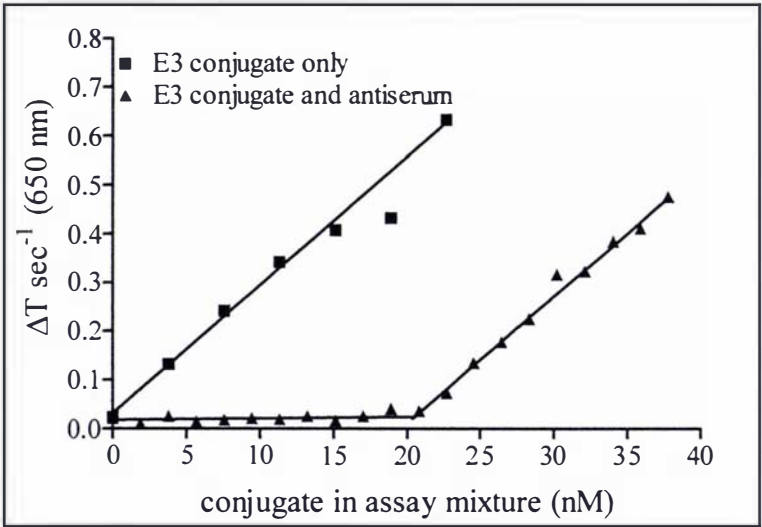


FIGURE 4.27: Hen Egg White Lysozyme E3 Conjugate Titration of Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: 0.048 μL antiserum; assay buffer - 55 mM tris-maleate buffer (pH 6.2)

As the amount of E3 added to the titration vessel increased from 0-20 nM there was only a very small linear increase in the initial rate of lysis. However, once the amount of conjugate exceeded 20 nM there was an abrupt linear increase in the initial rate of lysis which was parallel to the control line obtained in the absence of antibody. The intersection point (at 20.5 nM E3 conjugate for 0.048 μL of undiluted antiserum) corresponds to an apparent antiserum binding site concentration of 150 μM .

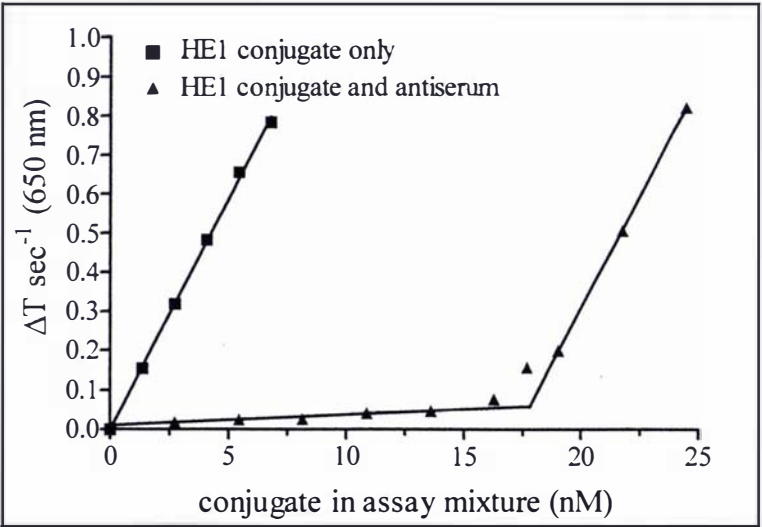


FIGURE 4.28: Human Lysozyme HE1 Conjugate Titration of Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: 0.06 μL antiserum; assay buffer - 55 mM tris-maleate buffer (pH 6.2)

When the experiment was repeated using HE1 a similar titration curve (Figure 4.28) was obtained consisting of two intersecting straight lines with the steep line being parallel to the control, conjugate only titration line. From the known concentration of conjugate in the titration at the intersection point (17.5 μM) the concentration of antibody binding sites was

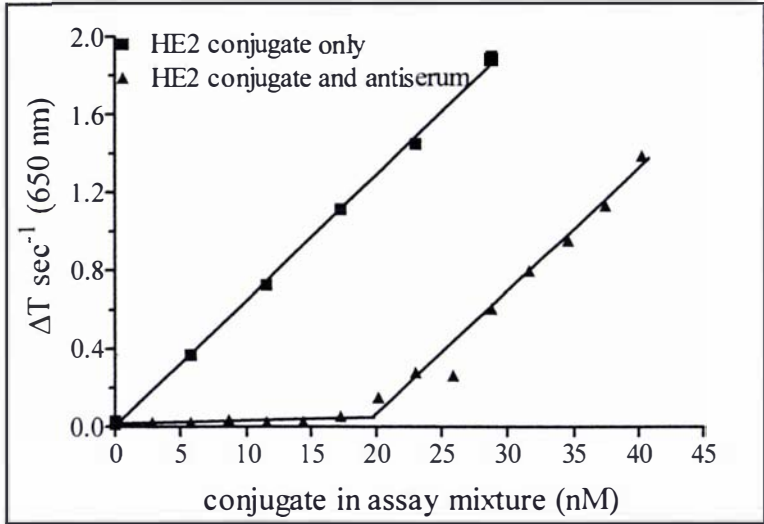


FIGURE 4.29: Human Lysozyme HE2 Conjugate Titration of Anti-Estrone Glucuronide Antiserum 243-4

Conditions in Assay Mixture: 0.09 μL antiserum; assay buffer - 55 mM tris-maleate buffer (pH 6.2)

calculated as 105 μM . For the HE2 conjugate a similar titration curve was obtained (Figure 4.29) from which an apparent antibody binding site concentration of 78 μM was calculated from the intersection point. For neither of the human lysozyme conjugates or the HEWL E3 conjugate could a dissociation constant (K_d) be calculated from the data.

4.3.9 Displacement Rate Constants for Immune-Complexes with Antiserum 243-4 and Lysozyme Estrone Glucuronide Conjugates E1, HE1 and HE2

Displacement rate constants for the HEWL E1 conjugate and the human lysozyme HE1 and HE2 conjugates with antiserum 243-4 were measured using the antiserum and conjugate conditions of their optimised standard curves, and pH 6.2, 55 mM tris-maleate as the assay buffer. The antiserum and conjugate were premixed in assay cuvettes for 5 minutes before the addition of an excess amount of E1G at time 0 as described in Section 4.2.5.7. All titrations resulted in an exponential increase in the rate of lysis with time as shown in Figure 4.30.

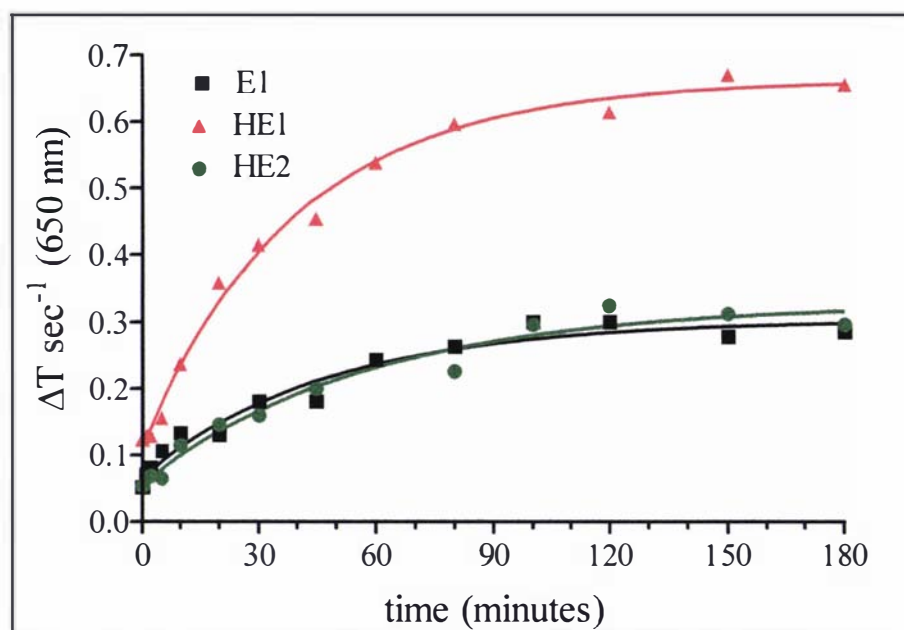


FIGURE 4.30: Measurement of the Displacement Rates for Hen Egg White Lysozyme E1, and Human Lysozyme HE1 and HE2 Estrone Glucuronide Conjugates from Anti-Estrone Glucuronide Antiserum (243-4) using an Excess of Estrone Glucuronide as a Displacing Agent

Conditions in Assay Mixture: E1 - 21 nM enzyme, 0.08 mL antiserum; HE1 - 12.2 nM enzyme, 0.06 mL antiserum; HE2 - 23 nM enzyme, 0.09 mL antiserum

The fit of the HEWL displacement curve (conjugate, 21 nM; antiserum, 0.08 μ L) gave a displacement rate constant (k_{off}) of 0.067 min^{-1} and had an amplitude of $0.24 \Delta T \text{ sec}^{-1}$. Since the control rate was $0.46 \Delta T \text{ sec}^{-1}$ this corresponded to 51% of the total free conjugate at the end of the displacement experiment.

For the HE1 (12.2 nM) - antiserum (0.06 μ L) system, the displacement gave a first order rate constant (k_{off}) of 0.11 min^{-1} with an amplitude of $0.55 \Delta T \text{ sec}^{-1}$. For HE1 this was 43% of the available free conjugate displaced. For HE2 (23 nM) pre-incubated with 0.09 μ L of antiserum 243-4 the displacement experiment yielded a first order rate constant (k_{off}) of 0.057 min^{-1} and an apparent maximum free conjugate rate of $0.27 \Delta T \text{ sec}^{-1}$. The control rate was $0.99 \Delta T \text{ sec}^{-1}$ which corresponded to a displacement of 27% of the initial conjugate.

4.4 DISCUSSION

As outlined in the introduction to this chapter the tertiary structure of human lysozyme is nearly identical with that of HEWL and the two enzymes catalyse the same hydrolytic reaction by very similar mechanisms.

The similarity of their structures is well illustrated in Figure 4.31 which shows the amino acid backbone of the two molecules superimposed using a ribbon display, and the positioning of the lysine residues.

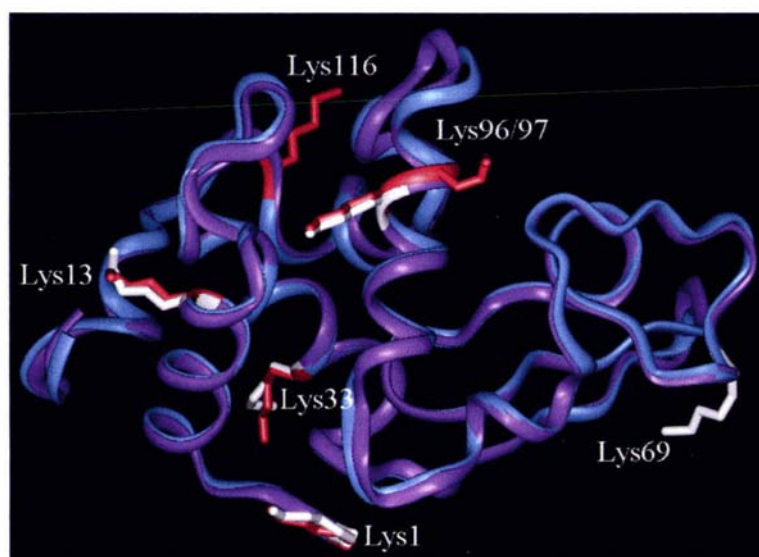


FIGURE 4.31: Structural Model of Amino Acid Backbone and the Lysine Residues of Hen Egg White Lysozyme and Human Lysozyme

Hen Egg White Lysozyme: backbone - blue, lysine - red
Human Lysozyme: backbone - purple, lysine - white

The major variations in their structures occurs in the 46-49/50 (HEWL/HuL) external loop region shown in the background of the right hand side of the molecule which contains the extra amino acid in human lysozyme (Gly 48), and the 100/101-105/106 external loop region shown in the central upper background of the molecule. Apart from these differences, the main chains are almost perfectly aligned with the overall root mean square difference for the α -carbon positions of the crystal

structures for the two enzymes being only 0.74 Å (Artymiuk and Blake, 1981). Based on the similarities of their crystal structures and electrostatic fields it could be expected that they will behave similarly under the same conditions and that the protocols already determined for HEWL can also be applied to the human enzyme and give similar results. The clear conclusions from the experiments reported in this chapter were that despite the strong structural homology between the two enzymes their physical and chemical behaviours are very different, and that the outcomes observed with one enzyme cannot be directly extrapolated to the other. This of course increases the difficulty and amount of work which must be carried out even when trying to replace a signal generator such as the HEWL-E1G conjugates by an apparently similar but more active human lysozyme-E1G conjugate. In the discussion which follows the very significant differences between HEWL and human lysozyme should be noted.

4.4.1 The Insoluble Fraction of the Commercial Human Lysozyme Preparation

The cost of the human lysozyme sample (\$4,000 NZ, 50 mg) used in this work and its reputed instability (Mörsky, 1983), necessitated that steps be taken to ensure that it was used efficiently. Hence, the presence of an insoluble fraction left in the commercial vials of human lysozyme was of considerable concern.

This insoluble fraction from the human lysozyme vials was never characterised, but considering the lower than expected absorbance of the soluble fraction based on the stated weight of the enzyme in each vial, it probably also was lysozyme. One explanation is that the precipitate results from the formation of insoluble multi-enzyme complexes of human lysozyme. Protein precipitates are formed from large protein aggregates and the fact that HEWL readily forms multi-enzyme complexes has long been known (Hampe and Tondo, 1982), while the possibility that human lysozyme forms such associations has also been implied (Fett *et al.*, 1985).

As urea is known to minimise inter-protein interactions (Section 2.4.3.1), conjugation of the combined soluble and insoluble fractions in urea could provide a means of dissolving the putative complexes and hence utilising the entire sample in a single step. Although the effects of urea on the active ester conjugation reaction are unknown, the lack of major structural changes for HEWL in urea implies that the conjugation pattern would be unchanged.

4.4.2 Conjugation of Human Lysozyme with Estrone Glucuronide

The reported instability of human lysozyme (Mörsky, 1983) had some important implications on the experimental methodology which was applied to the human lysozyme studies, with all possible precautions being taken to avoid leaving the enzyme for long periods of time unfrozen or in contact with possible denaturing reagents. For this reason the initial conjugation of human lysozyme and subsequent analysis on a Mono-S column in 7 M urea (as reported in Section 4.2.3.3-4.2.3.6) were planned to be carried out as rapidly as possible.

If it is assumed, based on the strong structural homology of the two enzymes, that their chemical reactivities were also similar then it would be expected that a large amount of unconjugated human lysozyme would be left (40-70%), if the enzyme was conjugated using the same enzyme and active ester reagent concentrations as used in the standard HEWL conjugation reaction.

Although the molar ratio of the human enzyme to the EIG active ester reagent was similar for the two enzyme conjugations the actual enzyme and reagent concentrations were very different. This was because to maintain the enzyme concentration of the human lysozyme conjugation mixture at the same concentration as that used in the standard HEWL conjugation protocol, the volume of the reaction mixture would have had to have been reduced to an impractical level due to the much smaller amount of human lysozyme available for conjugation (≈ 7 mg versus 100 mg). In the human lysozyme reaction

mixture, the concentration of protein and active ester reagent were reduced by approximately a factor of 15 and 5 respectively, relative to the concentrations used in the HEWL conjugation. According to the law of mass action the rate of complex formation is proportional to the product of the reactant concentrations (Kurstak, 1985). Thus, if HEWL and human lysozyme were equally reactive to the estrone glucuronide active ester reagent, the rate of human lysozyme conjugate formation and hence the initial yield of product would have been expected to be decreased by a factor of approximately 75 fold. This, combined with the fact that human lysozyme had one less potential conjugation site (i.e. 5 lysine residues as opposed to 6), and had no equivalent lysine residue for two of the three favoured conjugation sites of the hen enzyme (i.e. lysine's 97 and 116) meant that a much lower conjugation level was expected for the human enzyme. Thus, the experiment was designed so that an analytical FPLC column (Cooke, 1993; Smales *et al.*, 1994) could be run immediately after the addition of the reagent, and if a large amount of unreacted human lysozyme still remained a second or third aliquot of the active ester reagent could be added to maximise the conjugate yield. The underlying principle was that all manipulations should be done as rapidly as possible, with a minimum of steps, lest the reported instability of the human enzyme left a conjugated but inactive enzyme.

Analysis of the first E1G active ester conjugation reaction with human lysozyme by Mono-S chromatography appeared to conform to all expectations. The elution profile was similar to that obtained for an active ester HEWL conjugation mixture separated under identical conditions (compare Figure 2.3 with Figure 4.2a). Assuming that the large late eluting peak in the human lysozyme profile was therefore also unconjugated lysozyme it appeared that the human lysozyme conjugation had resulted in a similar distribution of conjugates to the HEWL preparation, except with a proportionally much larger amount of unreacted lysozyme as expected on the basis of the very much lower reagent concentrations.

Thus after analysis, the reaction mixture was acylated immediately by the active ester reagent for a second time to improve the yield of conjugate since it was considered necessary to maximise the conjugate yield as rapidly as possible. Analysis of the resulting mixture by Mono-S chromatography showed that the large late eluting peak was indeed significantly reduced in magnitude (Figure 4.2b), but it was still larger than any of the other peaks on the profile. Thus to reduce the concentration of what was considered to be unreacted lysozyme even further, the reaction mixture was acylated for a third and final time. Analysis of the resulting reaction mixture showed for the first time a Mono-S profile in which this large late eluting peak was no longer the highest and this was judged sufficient to allow purification according to the scheme developed in Chapter Two.

As discussed in Chapter Two, removal of unreacted lysozyme is a major pre-requisite in the purification of HEWL-E1G conjugates. Previous experience with HEWL indicated that reducing this level by increasing the extent of conjugation was the easiest way of simplifying the purification as long as not too much poly-acylated enzyme was produced. The Mono-S traces from the active ester conjugation of the human enzyme (Figures 4.2a-c) were superficially completely in accord with those previously found with increasing ratios of the active ester reagent and HEWL (see Figures 2.3 and 2.4a). Clearly, a comparison of the retention times with that of pure human lysozyme would have revealed the mistake

but the control wasn't run at the time due to fears about instability outlined above, the cost of the sample and the erroneous assumption that human lysozyme would behave the same as HEWL. However, it should be pointed out that the retention time of the human lysozyme was only 7% less than that of HEWL, thus the profile in Figure 4.2a was visually similar to those encountered previously with HEWL.

Based on the considerations of the principles of cation exchange chromatography, if the latest eluting peak was indeed unreacted human lysozyme, then the peak immediately preceding it, should have consisted of mono-substituted conjugate. Because the most lowly substituted conjugates are generally considered to be the most appropriate for immunoassay (Paek *et al.*, 1993), all preliminary kinetic work was performed on this second to last large eluting peak subsequently labelled as HE2.

However, when a method for gel electrophoretic analysis of lysozyme conjugates was developed (Smales *et al.*, 1998) and easy access to electrospray mass spectrometric (ESMS) analysis became available, the initial assignment of the peaks from the human lysozyme conjugation mixture profile was proven to be incorrect.

Estrone glucuronide has a molecular weight of 446.5 Daltons and in the conjugation reaction, the condensation of an estrone glucuronide molecule with a lysine residue results in the release of a single water molecule. Thus, the molecular weight of a lysozyme molecule should increase by increments of 428.5 Daltons for every site conjugated. The degree of substitution (acylation) of each fraction separated from the reaction mixture by the Mono-S column (Figure 4.2c) was assigned by comparison of the actual masses with that predicted by the addition of units of 428.5 to the mass of the commercial human lysozyme peak of 14,732.9. For example, fraction HE1, which consisted of a single main peak, was recognised as a pure mono-acylated isoform of human lysozyme since the experimental mass was close to 15,161.4. Although, the actual mass was 39.3 units less than that calculated for the attachment of a single E1G moiety, this difference is unlikely to arise from any change to the E1G structure. If this were the case, recognition by the anti-E1G antibody would be affected since the antibody is highly specific (Binnie, 1999). This was clearly not the case since all the evidence points to very tight binding between the human lysozyme-E1G conjugates and the antibodies from the anti-E1G 243-4 antiserum (see Figures 4.12 and 4.18).

Similarly, the main components of fractions HE2 and HE3 were clearly pure di-substituted human lysozyme-E1G conjugates since within experimental error their masses corresponded to the base human lysozyme peak plus two E1G moieties (i.e. 2×428.5). Hence the predominant conjugate forms in the HE2 and HE3 fractions must be structural isomers of each other. The other fraction, HE5, was most likely a pure tri-substituted human lysozyme-E1G conjugate since the experimentally determined mass was equivalent to two E1G moieties greater than the mass of HE1. Again one of the E1G structures has apparently contributed only 389 Daltons as was the case for HE1. This difference is most likely due to modification of a protein side chain on storage of the conjugate since, as discussed above, the immune reactivity of all of the conjugates with the anti-E1G antiserum was extremely high. The nature of this modification is unknown as is the reason why it occurs for HE1 and HE5 but apparently not for HE2 and HE3.

The major surprise from these results was the discovery that the peak which is labelled correctly as HE2 in the Mono-S traces (Figure 4.2a-c) was in fact a di-substituted conjugate and HE1, originally assumed to be unreacted human lysozyme, was a mono-substituted human lysozyme-E1G conjugate. This reassignment of the final large eluting peak (HE1) from the Mono-S column as a pure mono-substituted conjugate was confirmed when acid-PAGE analysis also became available (Smales *et al.*, 1998). When this fraction was compared with the commercial human lysozyme sample, they were clearly different (Figure 4.4) with HE1 eluting as a single band on the gel with an R_f value relative to the native human lysozyme control of only 0.92.

After the peaks had been correctly assigned, the conjugation yield for the first conjugation mixture increased from an apparent 37% to a surprising 96%. The fact that in the first conjugation attempt, close to a 100% conjugation rate of the human lysozyme was achieved was completely unexpected, especially considering the low concentration of the reactants.

For the first conjugation mixture (Figure 4.2a), the mono-substituted fraction (HE1) composed 60% of the total yield. This very high proportion of the mono-substituted conjugate was also unexpected as it has previously been the experience that as the level of unreacted protein in a conjugation reaction decreases, the levels of multi-substituted conjugates increase in such a manner that the mono-substituted conjugate never dominates the reaction mixture. For example in the conjugation of enzyme to immunoglobulin G (IgG), Kurstak (1985) found that irrespective of the molar ratio of the two reactants, not more than about 37% of the IgG could be conjugated with a single enzyme molecule. This was in accord with our extensive experience with the active ester conjugation of HEWL in which the level of mono-substituted conjugate was never observed to exceed 30%. Any attempts to increase the percentage of mono-substituted HEWL conjugates by changing the reaction conditions so that conjugation would be favoured, only resulted in an increase in the level of the more highly substituted conjugates at the expense of the mono-substituted and native lysozyme molecules.

Thus in comparison to HEWL, human lysozyme was an unexpectedly ideal conjugation candidate for immunoassay. It can be mono-acylated very readily with a minimum of reagents, and by careful control of the reaction conditions it will produce a large quantity of presumably pure, highly inhibitable, mono-substituted conjugate with a minimum of purification.

Compared to HEWL, the chromatographic purification of the human lysozyme conjugates of immunoassay quality (i.e. highly inhibitable) was very straight forward. Because conjugation of the human lysozyme was only performed on a small scale, all cation exchange chromatography could be performed on the small (50 × 5 mm) Mono-S column. The separation obtained on this column was very satisfactory compared to the performance of the large S-Sepharose fast flow column which was used for the large scale purification of hen egg white lysozyme conjugates (Figure 2.10) and sometimes required two passes of the material through the column for proper separation of the conjugate peaks due to its lower resolving power (see Section 2.3.4.1).

The value of 98% for the inhibition of the human lysozyme HE2 fraction measured by initial rates was obtained after a single pass through the Mono-S column. This was much higher than that recorded for any of the HEWL conjugates collected after a single pass through either the S-Sepharose fast flow or Mono-S cation exchange columns which usually had an inhibition of closer to 70% and had to be passed through a hydrophobic interaction column to become highly inhibitable (see Chapter Two). Thus, the chromatographic preparation of conjugates of immunoassay quality was much simpler for human lysozyme.

Why passage through a hydrophobic interaction column was necessary to produce highly inhibitable HEWL conjugates but not for human lysozyme conjugates is not known. In Chapter Two it was suggested that the poor inhibition was due to contact denaturation of the highly charged lysozyme conjugate molecules during binding to the strong cation exchange column. In most cases, after denaturation a protein will refold spontaneously into its original conformation. This suggests that all the information necessary to specify the tertiary structure of a protein is contained within the amino acid sequence itself. One of the major driving forces of this renaturation is provided by the side chains of the hydrophobic amino acids. Because of the poor water solubility of these groups, the most energetically favourable arrangement of the amino acid chain is one in which the contact of these groups with water is minimised. Thus the hydrophobic side chains tend to be pushed together to form the interior of the molecule. With regards to the HEWL conjugates it was suggested (Section 2.4.3.2) that after the contact denaturation, the hydrophobic nature (and perhaps bulk) of the extra steroid moiety (or moieties) prevented the conjugate from refolding back into its native lysozyme conformation and that this misalignment was only reversed upon exposure to the hydrophobic resin. Although the reason why the combination of cation exchange chromatography and urea denaturation produced highly inhibitable conjugate with the human enzyme and only poorly inhibitable conjugate with the hen egg white enzyme is not known, the difference must be derived from some of the 52 sequence differences between the two enzymes. If nothing else this anomaly has furnished us with yet another example of the highly divergent behaviour found between these two supposedly highly conserved and similar enzymes.

4.4.3 Possible Explanation for the Ease of Mono-Acylation of Human Lysozyme with the Estrone Glucuronide Active Ester Reagent

Because the gross topological features of the two proteins are almost identical, the higher reactivity of human lysozyme must be found in the lysine microenvironments. In the absence of detailed information regarding the actual sites of acylation of the conjugates any explanation of the unexpected ease of reaction of the human enzyme with the estrone glucuronide active ester reagent must be speculative. However, a comparison of the structures of HEWL and human lysozyme does allow some rationalisation of the most likely candidates for ready conjugation in the human lysozyme molecule.

From Figure 4.31 the relative positions of the different lysine residues on the two molecules are easily observed. For the HEWL lysine residues for which equivalent lysine residues are present in the human enzyme (i.e. residues 1, 13, 33 and 96 (=97 in human)),

the crystal structure shows the orientation of the 96/97 residues to be particularly close, while the 33 residues show some major differences in the positioning of the side chain.

With regards to lysine's 1 and 13, peptide analysis of the HEWL estrone glucuronide conjugates (Smales *et al.*, 1999) always showed peptides arising from tryptic cleavage next to these residues. The lability of these two sites to cleavage provides strong evidence that they were never conjugated. In Section 2.4.2.1, both residues were postulated to be protected from acylation (at least under the near stoichiometric ratios of acylating reagent used in these conjugations) due to their involvement in salt bridges (see Figure 2.17). Examination of these equivalent residues in the human lysozyme crystal structure suggests

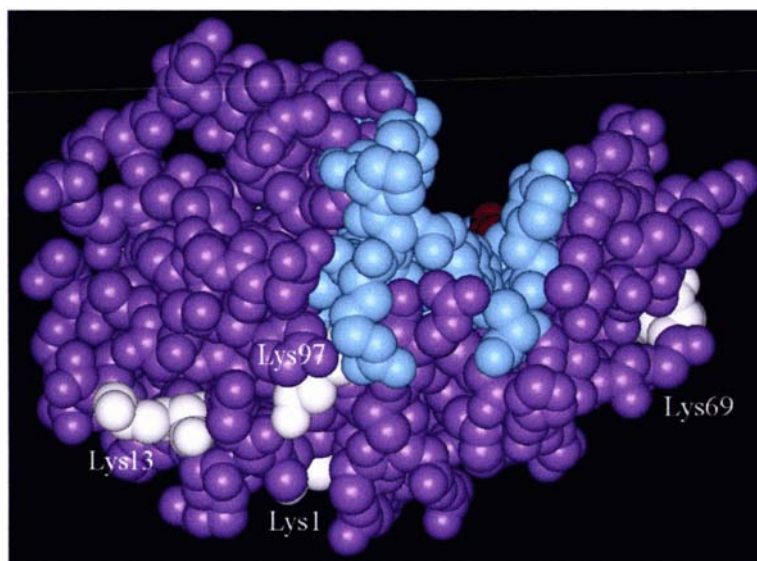


FIGURE 4.32: Structural Model of Human Lysozyme showing the Exposure of Lysine 13

General protein - purple; binding site cleft - pale blue; Asp 53 active site residue - brown; lysines - white

that the ϵ -amino group of lysine 1 is also involved in a salt bridge with glutamine 7 while the α -amino group of this residue is involved in two hydrogen bonds (with Thr 40 and Asp 87), as opposed to one in HEWL (Thr 40). Thus neither of the lysine 1 amino groups appeared likely to be more susceptible to acylation in the human enzyme. However, compared to HEWL, the crystal structure of human lysozyme showed lysine 13 to be extremely exposed and there was no evidence of a salt bridge or any hydrogen bonds (Figure 4.32).

Lysine 33 which is the most easily acylated lysine in the hen enzyme also appears to be more exposed in the human isoform. Figures 4.33 and 4.34 (page 197) show the position of these residues with the surrounding amino acids highlighted, for the hen and human enzymes respectively. Whereas in HEWL, the side chain of lysine 33 is partially occluded and bonded by a strong hydrogen bond to asparagine 37, in the human enzyme the side chain of the asparagine is replaced by a single hydrogen from glycine 37 leaving the access to the terminal nitrogen more open to attack by the acylating agent.

The side chain of lysine residue 96 in HEWL is involved in two hydrogen bonds and there is no evidence of acylation at this site for the enzyme under standard active ester conditions. The equivalent residue in the human enzyme, lysine 97, was only involved in a single hydrogen bond with valine 93 and did not appear to be more exposed, thus

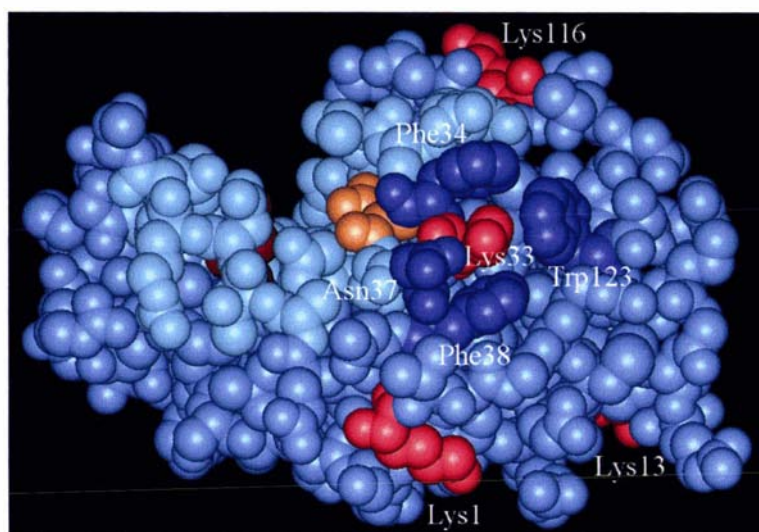


FIGURE 4.33: Structural Model of Hen Egg White Lysozyme showing the Exposure of Lysine 33

General protein - medium blue; binding site cleft - pale blue; Glu 35 active site residue - orange; Asp 52 active site residue - brown; lysines - red; Phe 34, Asn 37, Phe 38, Trp 123 - dark blue

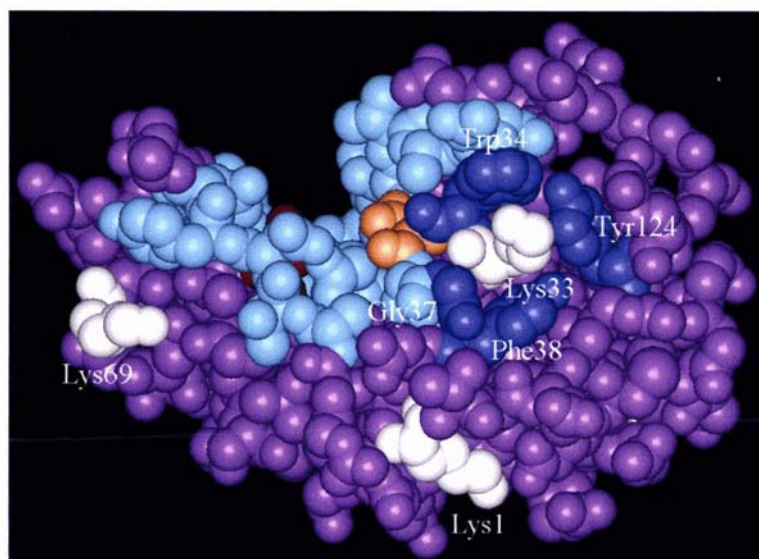


FIGURE 4.34: Structural Model of Human Lysozyme showing the Exposure of Lysine 33

General protein - purple; binding site cleft - pale blue; Glu 35 active site residue - orange; Asp 53 active site residue - brown; lysines - white; Trp 34, Gly 37, Phe 38, Tyr 124 - dark blue

although theoretically it may have been sometimes conjugated in the human enzyme it was probably not one of the most preferred conjugation sites.

Although the human enzyme lacked equivalent residues for two of hen egg white lysozyme's three favoured conjugation sites, the extra site it contained at position 69 appeared to be particularly exposed. When the exposure of the side chain at this position to the solvent was calculated using the Connolly's surface rolling ball analysis (probe radius = $1.45 \text{ \AA}$, $\approx \text{H}_2\text{O}$), the exposure of this residue was almost twice as high as that calculated for position 33 on the same enzyme. Considering that position 33 is the favoured acylation site on HEWL, and that the side chain of this residue is more exposed in human lysozyme, this strongly suggests that lysine 69 will be particularly susceptible to acylation. The side chains of lysine 69 or 13 were calculated to have a similarly high exposure which was in excess of that calculated for the other

three lysine residues, and neither were involved in strong interactions with the rest of the protein. Thus, it is highly likely that these two residues represented the most favoured conjugation sites on the human enzyme, possibly followed by position 33. The fact that mono-substituted conjugates so strongly dominated the human lysozyme conjugation mixture suggests that acylation at one of these sites was particularly favoured. However, with only structural information available on the unmodified enzymes, the true order of

reactivity of residues 69 or 13, or indeed the relative reactivities of any of the lysine residues to acylation by estrone glucuronide, cannot be known.

If the separation of the human lysozyme conjugates from each other and the unreacted native enzyme by both acid-PAGE and Mono-S chromatography is based solely on the overall charge on the protein, then the conjugates should elute and migrate strictly in order of their substitution level. The migration distances of the human lysozyme conjugates under acid-PAGE were broadly similar to those of their equivalent HEWL conjugates with the same level of substitution (see Figure 4.4). In particular, the migration distance of the unconjugated human and hen egg white lysozyme samples appeared to be identical within the bounds of the band resolution which is consistent with them having the same isoelectric point (10.5) (Fett *et al.*, 1985; Verhamme *et al.*, 1988) and a similar overall net surface charge.

The mono-substituted human lysozyme conjugate band (HE1) ran between the bands of the mono-substituted HEWL E1 (Lys116) and the mono-substituted HEWL E3 and E1 (Lys 97) bands (Figure 4.4) indicating that the electrostatic field of the human conjugate must be intermediate between those of the HEWL mono-substituted conjugates. The migration distance of the di-substituted human lysozyme conjugate band (HE2) ran in line with the HEWL di-substituted E2 and E4 bands while the migration distance of the human lysozyme tri-substituted conjugate (HE5), although slightly retarded, most closely mapped the HEWL E6 tri-substituted conjugate. Thus, the migratory behaviour of human lysozyme and its conjugates under acidic electrophoretic conditions was very similar to HEWL for the same level of substitution.

An important difference between the chromatographic behaviour of the two sets of conjugates was that in the separation of the human lysozyme conjugates there was only a single peak and band for the mono-substituted conjugate, compared to two peaks and a shoulder on the Mono-S column and a total of three bands on the gel for the mono-substituted fractions of HEWL. This lack of behavioural heterogeneity of the human mono-substituted fraction compared to HEWL suggests that in the human lysozyme conjugation the mono-substituted fraction is composed of only a single isoform compared to three in HEWL.

In fact the shift in the distribution of protein across the conjugate peaks in the Mono-S profiles of the three consecutive conjugations is completely consistent with a very strong hierarchical order of preferential acylation sites which would explain the existence of only a single mono-substituted isoform. If the first site was much more easily acylated than any other, almost all lysozyme molecules would be acylated at this site first before any other site, resulting in a single mono-substituted peak. This would also explain why there were only two as opposed to three di-substituted conjugate peaks, when mass spectral analysis of the HE5 peak proved there to be three conjugation sites. If the first site was extremely reactive, this would mean that there would be no di-substituted conjugate peak for a molecule which was di-substituted at the two less favoured sites. The fact that there was a large difference in the magnitude of the peak heights of the two di-substituted conjugate peaks (Figure 4.2a-c) also suggests that there was a large gradient in the relative reactivity between the second and third favoured conjugation sites as well.

In summary, on the basis of both the Mono-S and the electrophoretic separations, and the ESMS analyses, there was no evidence of heterogeneity for the purified mono- di- and tri-substituted HE1, HE2 and HE5 conjugate samples respectively which were utilised in the kinetic studies of this thesis.

4.4.4 Kinetic Studies on Human Lysozyme Conjugates

4.4.4.1 Selection of the Assay Method

An important consideration in the design of any enzyme immunoassay is the method of assaying for the enzyme activity. For the enzymes most commonly used in immunoassay, for example, horseradish peroxidase and alkaline phosphatase, there are a variety of synthetic substrates which can be used to provide a sensitive and rapid assay. These assays all provide a direct method of measuring the catalytic activity based on a change in the concentration of a chromophoric substrate.

Although, the lysozyme homogeneous immunoassay is restricted to an indirect lytic assay, there are also several different methods available for measuring this activity. In most lysozyme assays reported in the literature, lytic activity is measured using an initial rate of clearing which requires a series of data points to be collected over a period of time near the start of the lysis reaction. In contrast the end-point assay (see Figure 4.1) is essentially a two point assay, which makes it very simple to apply and renders any automation of the method correspondingly basic in its instrumentation. Apart from the more demanding requirements of the initial rate assay for accurate data collection, this method is also complicated by the calculation of the initial rate gradient. In addition to the time consuming plotting and calculation of the slope, the rates are dependent upon the ability and discrimination of the user to determine the correct tangent. With high quality data this is less of a problem. However, in the case of a turbidity assay, interpretation of the data is often complicated by fluctuating absorbance values which distort the early portion of a clearing curve of a recently dispersed suspension. This effect was apparent in the raw data used in the calculation of the initial rates presented in this work, with most initial rates being calculated by ignoring the first 10 seconds of the data collection.

Automation of an initial rate assay must take the above factors into account, and requires more sophisticated (and expensive) instrumentation and data processing capability than that required for an equivalent end-point assay. Despite these disadvantages, for the human lysozyme conjugates, the initial rate assay was the more practical alternative due to the bi-phasic nature of the human lysozyme clearing curve.

Since the second clearing phase was much less dependent on the conjugate concentration than the first phase, the degree of bi-phasicity increased with the conjugate concentration (Figure 4.5), resulting in a hyperbolic end-point rate versus enzyme concentration plot (Figure 4.7). The bi-phasic nature of the clearing curve also meant that the degree of non-linearity in this dose-response curve increased with the end-point time. Consequently as the length of the assay time increased, there was less discrimination at the upper concentration range compared to the lower concentration range. Thus the end-point assay

sensitivity changed dramatically with the human lysozyme E1G-conjugate concentration and the assay time making it completely unsuitable for immunoassays.

This factor also effectively precludes the use of the turkey, goose and phage T4 enzymes as signal generators in an end-point immunoassay as well. The *M. lysodeikticus* clearing curves obtained with these alternative enzymes were also bi-phasic with the enzymes associated with the fastest initial rates producing the clearing curves with the greatest bi-phasicity. When these other lysozymes were assayed using the same enzyme concentration as was required to give a transmission change equivalent to 350 units per 20 minutes for HEWL, they all required longer than 20 minutes to reach the same total transmission change (Cooke, 1993). Thus it appears unlikely that the Ovarian Monitor assay time for E1G can be shortened simply by replacing the current HEWL-E1G conjugate with any E1G-conjugate derived from one of these other enzymes and using the current end-point assay method.

Since the HEWL clearing curve consists of a single second order clearing process, this still leaves open the possibility of using a HEWL mutant to achieve the same end. Although most enzyme mutants are associated with a reduction in enzyme activity relative to the native form, several HEWL mutants have been reported which exhibit a higher specific activity. The initial rate of clearing by these mutant enzymes ranged from a factor of 1.33 to 3 fold greater than the wild type (Kumagai *et al.*, 1987; Kumagai and Miura, 1989; Kumagai *et al.*, 1992). However, in the only literature example which included the clearing curve for a mutant with a faster initial rate, the curve was decidedly more bi-phasic than the curve of the native enzyme (Kumagai *et al.*, 1987). Considering that there is a positive correlation between increasing initial rate and the degree of bi-phasicity of the clearing curves obtained with the lysozymes from the five different sources (Cooke, 1993), it is quite likely that the clearing curves generated by the other mutants which were not reported were similarly affected. Thus, the viability of conjugating a mutant lysozyme to estrone glucuronide and using the resulting conjugate as a direct substitute in the Ovarian Monitor end-point assay is also doubtful.

Compared to the end-point assay, for the same human lysozyme conjugate concentration range the initial rate data increased linearly with the conjugate concentration (see Figures 4.10 and 4.17). Thus the sensitivity of the initial rate assay was unaffected by the conjugate concentration. Furthermore, due to the high degree of linearity in the initial rate versus conjugate concentration plot, the assay could be used to provide an extremely accurate and reliable measure of the conjugate concentration (HE2, $r=0.998$; HE1, $r=0.998$). This linearity of the rate response with increasing enzyme concentration is a decided advantage in an immunoassay system, as it means that a simple measurement of the lytic rate is directly proportional to the amount of free conjugate present in the system unlike in an end-point assay (see Figure 4.5).

The other major advantage in the initial rate assay was the shorter collection time of the assay. When only initial rate data were needed, the standard collection period for the transmission data was 3 or 5 minutes. In fact, for all but the lowest lytic rates, the departure of the change in transmission values from a straight line with time occurred almost immediately after the first minute with most of the initial rates being calculated by

fitting only the first 10-60 seconds of the clearing curve. Although when performed manually the initial rate method was very labour intensive with each assay requiring a series of transmission values to be recorded and plotted before the initial gradient could be calculated, if automated the assay has the potential to provide a very rapid (1-2 minutes) measurement of urinary estrone glucuronide concentrations.

Alternatively, due to the excellent fit of the clearing curve data obtained with the human lysozyme conjugates to two second order processes, the data could be linearised by the second order rate equation, and the gradient of the resulting plot used as a measure of the enzyme concentration. This has the advantage that it utilises a greater proportion of the clearing curve than the initial rate assay, but has none of the inherent inaccuracy of the end-point assay. However, in reality it fitted little more of the clearing curve due to the change over to the second phase of second order clearing, and based on the excellent correlation of the conjugate concentration with the initial rate data, the gain in data quality obtained from this extra manipulation does not seem warranted. Thus the goal of providing a new signal generator using human lysozyme-E1G conjugates was achieved using initial rates.

4.4.4.2 *Optimisation of the Assay Conditions*

The excellent fit of the human lysozyme conjugate data to two second order processes showed that there was no obvious difference between the clearing kinetics of the HE2 human lysozyme conjugate and the native enzyme. However, modification of the enzyme structure by acylation, may still change the enzyme's optimal assay conditions, while reports of human lysozyme's instability were also of concern. Thus, before the human lysozyme conjugates could be assessed as signal generators for use in immunoassays for urinary E1G concentrations the assay conditions had to be optimised and the question of potential conjugate instability addressed. The ionic strength and pH optimum of the assay buffer for human lysozyme have been determined previously with phosphate buffers to be 55 mM and pH 6.2 (Mörsky, 1983). Neither the acylation of the enzyme nor a change in the buffer agent to the tris-maleate system used in the Ovarian Monitor had any effect on these parameters in the human lysozyme assay for the HE2 human lysozyme estrone glucuronide conjugate. As these buffer effects were examined using a di-substituted conjugate, which was structurally less analogous to the native enzyme than the equivalent mono-substituted conjugate, HE1, these findings probably also apply to the HE1 conjugate. Thus, the similarity in the enzyme activity profiles observed using the tris-maleate buffer system compared with the phosphate buffer system, justified the continuation of the use of tris-maleate buffer as the main Ovarian Monitor buffer for this alternative lysozyme signal generator system.

In contrast to the above findings, the results observed with the addition of albumin to the assay buffers and stock enzyme solutions were at variance with the literature data. Mörsky (1983) reported that the addition of human albumin to the phosphate assay buffer increased the enzymic activity of human lysozyme by a factor of 1.3. However, there was no evidence for bovine serum albumin increasing the reaction rate of the human lysozyme conjugate activity with the tris-maleate buffer system used in the present work. It is possible that the difference between the results reported in the literature and the results

reported here could be due to a species specific interaction between the albumin and lysozyme molecules with the human lysozyme activity being best activated by human albumin rather than BSA. However, this is not supported by the work of Maeda *et al.*, (1980) which showed by the more primitive lysoplate method, that the addition of BSA to the medium resulted in an increase in the apparent lytic activity of human lysozyme by a factor of 1.4.

Likewise the finding by Mörsky that the addition of albumin increased the stability of human lysozyme was not substantiated with the human lysozyme-E1G conjugates. In the work presented by Mörsky, human lysozyme stock solutions prepared in the absence of albumin were found to show a distinct decrease in activity after storage for 7 days at 4°C while albumin containing stock solutions (0.1%) were found to be completely stable for a month when stored at 4°C. However, given the experimental error of the experiments, the differences in conjugate activity measured after 14 days at 4°C for the two sets of conditions in the present work were not statistically significant (Table 4.3). Thus the storage of the human lysozyme conjugate stock solution in a 0.1% BSA solution had no obvious beneficial stabilising effect on the enzyme's stability. The difference between the present results and the literature data may simply reflect the fact that some of the positive charges are removed in the human lysozyme-E1G conjugates. Since the interaction between lysozyme and BSA is electrostatic the lower charge of the human lysozyme conjugates may compromise the formation of the lysozyme.BSA complexes.

4.4.5 Kinetic Characterisation of the Human Lysozyme Estrone Glucuronide Conjugates

The fact that the initial rate data collected over a range of conjugate concentrations for the HE1 and HE2 conjugates were obtained without any repeat assays, combined with the very low deviation of the individual data points from a straight line, clearly illustrated the reliability and quality of the data which could be obtained by the rapid initial rate method with a human lysozyme conjugate.

The only significant difference between HE1 and HE2 as signal generators was in the gradient of their concentration versus lytic activity plots (Figures 4.10 and 4.17) with the di-substituted conjugate having a specific activity half as high as the mono-substituted conjugate. The tri-substituted HE5 conjugate also had half the activity of the HE2, thus although only single samples of a mono-, di- and tri-substituted conjugate were analysed in this work, the results indicated a strong inverse relationship between the degree of acylation by E1G and the specific activity with the specific activity being approximately halved by each additional estrone glucuronide substituent added.

As one of the main methods suggested for increasing the sensitivity and speed of an immunoassay is to use a signal generator with a higher specific activity, these results indicate that the tri-substituted conjugate would be a particularly unsuitable replacement for the current Ovarian Monitor HEWL conjugate, and most comparative work was therefore performed on the mono- and di-substituted conjugate fractions, HE1 and HE2.

The human lysozyme conjugate clearing curve very clearly fitted to two second order rates of clearing, with the time of transition from the first to the second phase decreasing and the ratio of the relative gradients of the two phases increasing with an increase in the concentration of free enzyme (see Figures 4.5 and 4.6). Thus the method for assaying for activity had a significant effect on the apparent inhibition, with the percentage inhibition for the human lysozyme conjugates calculated using an end-point assay being significantly lower than that calculated for the initial rate assay. Because the correlation between enzyme concentration and activity using the end-point assay was also not linear for the HEWL conjugate system (Figure 3.9), the inhibitions reported in Chapter's Two and Three would also be slightly increased if an initial rate assay had been used although not as dramatically as with the human conjugate samples.

The relationship between the concentration of antiserum 243-4 and the decrease in the initial rate of clearing was linear throughout the titration for both the HE1 and HE2 conjugates (see Figure 4.12 and 4.18) which showed that the ability of this antiserum to bind the free conjugate did not decrease as the antiserum concentration approached the equivalence point. These results imply therefore that the binding between the anti-E1G 243-4 antiserum and both the HE1 and HE2 conjugates was very tight. This conclusion was confirmed by their conjugate titration curves (Figures 4.28 and 4.29) which also consisted of two straight lines, in each case, rather than a binding curve which could be analysed to give the K_d values for the conjugate-antiserum 243-4 system. The E3 conjugate titration curve also consisted of two straight lines (Figure 4.27). Thus both the hen and the human lysozyme conjugates bound to the anti-E1G antiserum with high affinity.

The binding of the antiserum to the human lysozyme conjugates appeared to be so tight that the relationship between the amount of antiserum added and the amount of free conjugate was effectively stoichiometric. The end-point of the antiserum and conjugate titration curves for the mono-substituted HE1 conjugate-antiserum system was therefore used to calculate the antibody binding site concentration for the 243-4 antiserum. For a stoichiometric mixture of the mono-substituted conjugate and the antiserum to produce a high level of inhibition of the conjugate's lytic activity, all E1G acylation sites must be capable of binding to the antibody, and all instances of binding between these groups with the antibody must result in a high level of conjugate inhibition irrespective of the acylation position. In such a tight binding conjugate-antiserum system the end-point of the titration curve will be reached when the molar ratio of antibody to conjugate is 1:1. In the 243-4 antiserum titration for HE1, the end-point occurred at 0.048 μL of undiluted antiserum per assay mixture (Figure 4.18) i.e. 0.048 μL of undiluted antiserum was needed to bind 4.28 picomoles of conjugate (where 4.28 picomoles of conjugate is the amount of conjugate in 350 μL of a 12.2 nM conjugate reaction mixture). Based on this information the antiserum E1G binding site concentration was 89 $\mu\text{mol L}^{-1}$ of undiluted antiserum. This compares favourably with the value of 105 $\mu\text{mol L}^{-1}$ determined by the corresponding HE1 conjugate titration.

For a di-substituted conjugate-antiserum system such as in the HE2 system, the calculation of the E1G binding site concentration of the antiserum is more complicated as there are several possible mechanisms by which a high level of conjugate inhibition can be achieved. However, the molar ratio of conjugate to antiserum at the end-point can be determined.

In the titration of the HE2 system, which was performed using 10.01 pmoles of conjugate per assay mixture (i.e. 28.8 nM), the end-point occurred at 0.155 μL of undiluted antiserum per assay tube. Thus the ratio of pmoles of conjugate per μL of undiluted antiserum at the end-point for the di-substituted HE2 conjugate system was apparently equal to 65 $\mu\text{mol L}^{-1}$ of undiluted antiserum. However, the actual concentration of antibody binding sites in the 243-4 antiserum must be the same for the antiserum titrations of the HE1 and HE2 conjugates. Thus the apparently lower value given by the antiserum titration of the HE2 conjugate must be due to the extra antibody binding site (i.e. E1G moiety) on the conjugate. On this basis the molar ratio of conjugate to antiserum for the HE2 conjugate-antiserum 243-4 system at the equivalence point was calculated to equal 1:1.37 (89/65). Thus HE2 requires 37% more antiserum to reach the same inhibition end-point.

A high level of inhibition of the lytic activity may be achieved for HE2 if both steroid moieties on the conjugate are capable of binding to the antiserum, with binding at either site causing inhibition. In this case a high level of inhibition would be achieved regardless of whether binding occurred at either one of the sites or at both sites simultaneously. A high level of inhibition would also be obtained if only one site was capable of binding the antibody, and this binding caused inhibition. The only situation which can be dismissed on the basis of the experimental evidence, is if only one E1G binding site was associated with inhibition, but both E1G sites were capable of binding to the antibody, and binding at the non-inhibitable site prevented binding at the inhibitable binding site. In this case the maximal level of inhibition in the antiserum titration would never exceed 50%. The apparent binding site concentration for the antiserum calculated according to the equivalence point of the di-substituted HE2 conjugate antiserum titration curve will depend upon the which of the above scenarios is correct for the HE2 antiserum 243-4 system.

If a lysozyme-E1G conjugate had two inhibitable binding sites (A and B) of equally high affinity which could bind antibody simultaneously, then statistically at an equimolar ratio of conjugate and antibody only 50% of the total conjugate E1G binding sites would be occupied. In other words for a di-substituted conjugate population, 50% of the total A binding sites and 50% of the total B binding sites would be occupied. If this binding was randomly distributed, then 25% of the conjugate molecules would have no antibody bound at either site, 25% would have antibody bound at site A only, 25% would have antibody bound at site B only and 25% would have antibody bound at both sites. Thus at a 1:1 molar ratio, only 75% of the conjugate population would be antibody bound and hence have their activity inhibited while the remaining 25% would be free and uninhibited. At the actual equivalence point for this di-substituted conjugate system, every conjugate molecule would have to have at least one antibody molecule bound. Thus, again from statistical considerations 33% of the conjugate molecules would have antibody bound at site A only, 33% would have antibody bound at site B only and 33% of the conjugate would have antibody molecules bound at both sites. Hence, a minimum 33% molar excess of antibody relative to the conjugate would be required for maximal inhibition of a di-substituted conjugate with two binding sites of the same high affinity. In other words the equivalence point would be reached when the molar ratio of conjugate to antibody binding sites was 1:1.33, which is very close to the experimentally determined value of 1.37. From these results it can be concluded that the di-substituted HE2 conjugate binds anti-E1G antibody at both E1G sites with high affinity and simultaneously, with binding at either site being

sufficient to cause almost complete high inhibition of the HE2 conjugate activity. Thus the titration curves of the HE1 and HE2 conjugates provided a very clear example of how an increase in the level of substitution increases the amount of conjugate (or hapten) required to neutralise the antiserum.

The fact that two antibody molecules could bind simultaneously to the HE2 molecule without steric hindrance supports the identity of the acylation sites proposed in Section 4.4.3 based on the surface accessibility of the different lysines. Simultaneous binding of the antibody to a di-substituted HE2 molecule could only occur if the two acylation sites were well separated on the molecule. As shown in Figure 4.32, these residues, lysine 13 and 69, are on opposite sides of the enzymatic cleft and are separated by a distance equal to more than half the length of the molecule.

4.4.6 Feasibility of using Lysozyme-Estrone Conjugates as Signal Generators in Homogeneous Enzyme Immunoassays for Estrone Glucuronide

When examining the feasibility of any new signal generator or method for immunoassay one of the first things which must be established is its ability to produce a working standard curve and its performance under clinical conditions. Standard curves for E1G were obtained for all the human lysozyme conjugates (HE1, HE2 and HE5) using a rapid assay (1-2 minutes), although HE1 was the most sensitive signal generator (Figure 4.26). The performance of the new assays was investigated by using them to measure the E1G concentration of daily time diluted urine samples collected over the duration of a cycle from a normally cycling woman and a woman with an artificially stimulated cycle, and comparing the results with the corresponding current Ovarian Monitor assay.

Based on the HE1 conjugate concentration curve (Figure 4.28) and its antiserum titration curve (Figure 4.18), HE1 might have been expected to give a similar or superior standard curve to those generated with the HEWL E1 and E3 conjugates (Figure 3.23). The very high degree of inhibition (98%) obtained with the HE1 conjugate should slightly increase the assay discrimination (maximum rate - minimum rate), and the linear relationship between HE1's lytic activity and enzyme concentration, meant that a greater proportion of the HE1 standard curve should be available to measure the rate of E1G excretion before the urine sample needed to be further diluted and re-assayed compared to the HE WL signal generators and the end-point assay. The assay sensitivity might have also been expected to be improved somewhat as the amount of antiserum required for complete inhibition was less than that required for E1 and E3. However, unfortunately and unexpectedly for the measurement of urinary E1G, the standard curve generated with HE1 conjugate was worse than that obtained with the current HEWL based Ovarian Monitor assay, although the effect of greater linearity did allow concentrations of up to 800 nmoles E1G 24 hr⁻¹ to be read directly off the standard curve.

The greatest challenge in developing an alternative rapid estrone glucuronide assay suitable for home monitoring is to produce one which has the sensitivity and accuracy required to measure the range of E1G excretion rates experienced over a natural menstrual cycle. For

natural family planning purposes, the assay must be capable of detecting the first increase in E1G excretion rates above pre-follicular phase selection levels.

The minimum sensitivity limit for the Ovarian Monitor is defined from the standard curve as occurring at the first unambiguous rise in conjugate lytic activity above baseline levels (or 2 standard deviations above the zero standard). For the optimised HE1 standard curve this occurred at an E1G excretion rate of about 100 nmoles 24 hr^{-1} which was almost twice that obtained using E3 as the signal generator (compare Figure 4.20 with Figure 3.23). Given that the sensitivity of the E3 assay is considered only just sufficient for application with the normal menstrual cycle, this meant that the HE1 assay had approximately half the required sensitivity, making it unsuitable for use in a home assay.

When converted into concentration units the sensitivity limit using HE1 as the signal generator was about 27.8 nmoles of E1G L^{-1} of time diluted urine sample or 1.39 pmoles of E1G per assay tube. The HE1 standard curve was performed using 4.28 pmoles of conjugate and an E1G binding site concentration of 5.34 pmoles for the antiserum in the assay tube. Thus assuming tight binding for the HE1 conjugate to the antiserum as indicated by the antiserum titration curve (Figure 4.18), there was an excess of 1.06 pmoles of binding sites available in an assay mixture without any unconjugated E1G present. If it is assumed that the free E1G binds to the antiserum with equal affinity as the HE1 conjugate, then at the sensitivity limit of the assay (i.e. 1.39 pmoles), there was a slight molar excess of conjugate of approximately 0.33 pmoles (1.39 pmoles - 1.06 pmoles) or 8% of the total conjugate free at this point. Thus the position of the observed first rise in the lysis rates was consistent with the underlying assumption of near stoichiometric binding of human lysozyme HE1 conjugate and anti-E1G antibody.

This relative insensitivity of the standard curve generated with HE1 was probably due to the very tight binding of HE1 to antiserum 243-4. This was supported by simulation studies which showed that the sensitivity of the standard curve decreased with a decrease in the dissociation constant (K_{d2}) for the antibody with the hapten-conjugate.

If every pmole of antibody left free after the addition of the urinary or standard E1G, bound a picomole of conjugate, more E1G would be required to observe the first increase in free conjugate, compared to an assay where the antiserum binding was not so tight. Clearly, the HE1 conjugate bound more tightly than E3 did to the same antiserum since in the latter case there was more unbound conjugate present in the assay for the same amount of total E1G and a similar amount of antiserum (i.e. the sensitivity was 50 nmoles 24 hr^{-1} instead of 100 nmoles 24 hr^{-1}).

The effect the relative lack of sensitivity of the HE1 standard curve had on the measurement of urinary E1G excretion rates was examined using a blank urine in the standard curve assay mixture. The blank urine caused an elevation in the baseline lysis rates of the standard curve and the mid-point was shifted to a slightly lower concentration range. These results were consistent with non-specific binding between the antiserum and some non E1G component of the urine which decreases the amount of antiserum available for binding to the conjugate at all E1G concentrations. The fact that the blank urine standard curve was associated with a baseline shift, suggested that the amount of antiserum

which was effectively removed from the assay by the non-specific binding was sufficient to convert the immunoassay from an assay performed with a slight molar excess of antiserum, to an assay performed with a slight molar excess of conjugate.

An increase in baseline is not desirable for any immunoassay. However, for the blank urine standard curve this disadvantage was partly offset by the shifting of the position of the E1G standard curve closer to the normal physiological range of urinary E1G excretion rates. Although in the menstrual cycle shown in Figure 4.22 the follicular phase baseline data appeared to be more clearly defined for the profile obtained using the blank urine standard curve and the E1G peaks appeared better defined in the non-blank urine profile this was really an illusion of scaling since if each data set was plotted on its own optimum scale the two profiles almost completely superimposed. Thus for the range of E1G excretion rates experienced throughout this sample cycle, the difference that a blank urine standard curve made to the calculated E1G concentrations was minimal.

The fast E1G assay using HE1 as the signal generator gave unacceptable hormonal data even though its correlation with the normal menstrual cycle Ovarian Monitor assay data was high. According to the regression line (Figure 4.24) the mean differences between the E1G values were 17% larger for the human lysozyme immunoassay. This is acceptable but the mean baseline values from the human lysozyme data were nearly 100 nmol E1G 24 hr⁻¹ higher than for the Ovarian Monitor assay, being close to 150-175 nmol E1G 24 hr⁻¹ for this cycle. This is clearly unacceptable for general use in normal menstrual cycles. A good indication of the lower sensitivity of the fast HE1 human lysozyme E1G assay is given by the lower discrimination between the peak and baseline values which was 1.75 (350/200) compared with 4.3 (325/75) for the Ovarian Monitor assay. In this cycle this was definitely due to the higher baseline data for the human lysozyme assay since the excretion rates on the mid-cycle E1G peak day were similar (350 nmoles 24 hr⁻¹ compared with 325 nmoles 24 hr⁻¹). Thus, the fast E1G assay using HE1 as the signal generator, although meeting the criterion of speed, failed to meet the important criteria of sensitivity and a low urinary baseline excretion rate.

In a home monitoring method, fertility status may be defined by the pattern of E1G excretion rates, not their absolute rates of excretion although these are more accurate. For example, in Figure 4.23 the day of the first significant rise above baseline which represents the beginning of the fertile period can be recognised easily with the fast HE1 assay data as can the day of the estrogen peak which is used to define the day of peak fertility. Thus, it would be possible to use the human lysozyme conjugate HE1 as a signal generator in a crude home assay for fertility monitoring. However, any further work involved in validating such an assay was not considered justified here since the reduction in assay time was not sufficient to outweigh the loss in sensitivity.

With regards to the development of an alternative home E1G monitoring system for everyday use, the work reported in Section 4.3.4 has shown that the HE2 human lysozyme conjugate was also unsuitable as a signal generator for use in monitoring the normal menstrual cycle, since it was even less sensitive over the critical concentration range. The quality of the initial rate data for the HE2 standard curve was clearly exceptional, following a sigmoidal dose response pattern with a minimum level of scatter, but the

optimum working range of the optimised standard curve (200-2,000 nmoles of E1G 24 hr⁻¹) was high compared to the normal range of urinary E1G excretion values experienced throughout a menstrual cycle (35-465 nmoles 24 hr⁻¹). Thus, despite the high quality of the immunoassay data, the working range of the HE2 immunoassay made it unsuitable for everyday cycle monitoring.

However, not all urinary E1G excretion rates fall within the above defined normal limits. In pregnancy, in the presence of some forms of hormonal supplementation and under certain pathological conditions, for example ovarian cysts, these limits are exceeded and it is possible that the HE2 and HE1 assays might be used in these cases.

One of the hormonal preparations commonly used in the treatment of female infertility is clomiphene, an estrogen analogue (Figure 4.35), which acts to enhance estrogen production by competitively binding to the hypothalamic estradiol receptors in the brain and thus negating the usual negative feedback mechanism for estrogen production by an antagonistic action (Khatkhatay *et al.*, 1990; Hadley, 1996a).

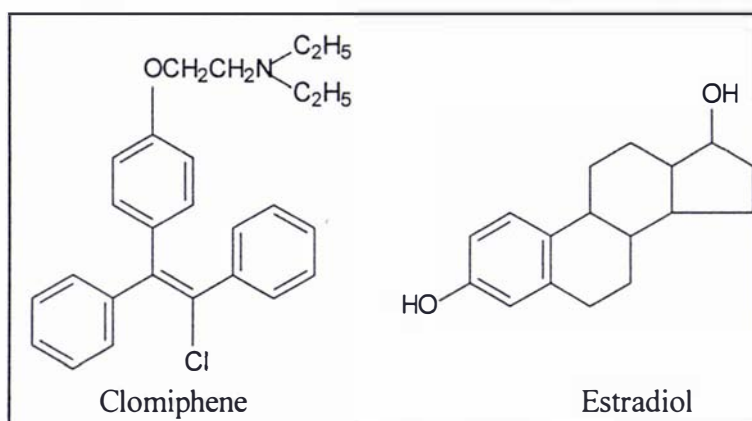


FIGURE 4.35: Structure of Clomiphene and Estradiol

The correlation obtained for the E1G cycle data of a woman undergoing clomiphene treatment as measured by the HE2 assay and by the client using the Ovarian Monitor was high showing that the new assay was accurately detecting the daily changes in the urinary E1G excretion rates. The follicular phase baseline data were similar to those determined with the Ovarian Monitor assay however, the absolute values of the E1G excretion rates were only 72% of the Ovarian Monitor values (Figure 4.15). Nevertheless, the fast E1G assay would have guided the woman successfully through her treatment cycle.

One of the main problems of infertility treatment with estrogen analogues is that although they stimulate ovarian activity, very high levels of estradiol have a negative effect on the fertile characteristics of the cervical mucus making it hostile to sperm survival. The extreme E1G excretion rates in Figure 4.15, clearly demonstrate this ovarian hyperstimulation.

The period of maximum fertility occurs over the pre-ovulatory peak day and the following day. In the normal cycle the first E1G peak is usually the largest but in this cycle it would not have been recognised as the pre-ovulatory peak if it were not for the concomitant rise

in PdG (Figure 4.15). This rise clearly identified the first (relatively small) E1G peak on day 11 as the pre-ovulatory one. Counting from this E1G peak as day zero gave a luteal phase length of 18 days which was longer than that usually encountered in the normal menstrual cycle (12-16 days). This was however typical of the increased length of the luteal phase encountered in clomiphene stimulated cycles. In general the more nearly that a stimulated ovarian cycle follows the normal pattern the more likely it is that pregnancy will result. It is interesting to note that this woman never conceived.

Clearly, this fast assay using HE2 as the signal generator (and presumably using HE1) could be used in the monitoring of stimulated cycles. However, again the performance of the assay was not judged to be superior to the Ovarian Monitor assay and hence the extra work which would need to be done to convert the laboratory assay into a fast home assay and to carry out its validation according to standard procedures was not warranted.

4.4.7 A Comparison of the Different Kinetic Behaviour of the Human Lysozyme and Hen Egg White Lysozyme Conjugates in terms of their Dissociation Constants

As the only difference between the mono-substituted hen and human lysozyme conjugate assay systems was in the interactions between the conjugates and the antibodies contained in the antiserum, the lower sensitivity obtained using the human lysozyme-E1G conjugates as signal generators must be found in the microenvironments of the acylated residues.

The apparent decrease in the K_d values for HE1 and HE2 compared to E3 could result from either a larger k_{on} , a lower k_{off} , or both. Since there appeared to be little difference in the k_{off} values for the three conjugates, at least for the antibody population present in the experiment, it seems likely that the difference is in the association step. As a result of the sequence differences between human lysozyme and HEWL, lysine residues 13 and 33 are more exposed in the human enzyme, while lysine residue 69 (which is not present in HEWL) is one of the most exposed residues in human lysozyme and more exposed than any lysine residue present in HEWL (see Section 4.4.3). The high exposure of these residues in human lysozyme could conceivably give rise to faster rates of binding between the E1G conjugates and the anti-E1G antibodies which would account for the apparently lower K_d values and the lack of sensitivity of the standard curves generated with the human lysozyme conjugates. Although a higher exposure of the steroid moieties, may result in a faster rate of association, it would have little effect on the rates of dissociation. Thus the rate constant, k_{off} , would be similar between the two protein as observed experimentally.

4.5 CONCLUSIONS

The results of this chapter highlighted the differences between human lysozyme and HEWL as opposed to their similarities and showed that the so called conservative changes in amino acid sequence between the two enzymes have very far reaching effects. This suggests a call for caution in fields such as structural genomics where the function of unknown proteins expressed from the genome of species are determined by a comparison of tertiary structures and sequence homology. For example, one structural element which has come to be recognised as a defining characteristic of dehydrogenases, the Rossman fold (Rossman *et al.*, 1974), is not present in aldehyde dehydrogenase, one of the most studied enzymes of this group.

The different behaviour and characteristics of the human enzyme and its conjugates compared to the equivalent HEWL isoforms became a recurring theme for this chapter. These differences include:

- i) their relative ease of conjugation. Human lysozyme was much more easily conjugated than HEWL, and analysis of the crystal structure suggested that the most favoured conjugation sites were probably also different between the two enzymes.
- ii) their purification protocols. Unlike with HEWL, with the human enzyme, highly inhibitable conjugate could be obtained in a single step without hydrophobic interaction chromatography.
- iii) their kinetics. Human lysozyme and its conjugates had a much more bi-phasic clearing curve with the *M. lysodeikticus* substrate than the equivalent HEWL isoforms, and also displayed much tighter antibody binding.
- iv) their immunoassay characteristics. Only the mono-substituted HEWL conjugates could be used in an immunoassay for clearly defining the E1G menstrual cycle profile.

The poor sensitivity of the immunoassays obtained with the human lysozyme conjugates shows that an increase in specific activity of the signal generator does not necessarily result in a more sensitive assay. Although within the same system there was indeed a relationship between the signal generator, specific activity and the assay sensitivity (for example see Figure 4.26) when different systems (HEWL versus human lysozyme) are involved this cannot be assumed. The poor sensitivity of the assays using HE1 and HE2 as signal generators meant that they could not be utilised in a fast assay for cycle monitoring of urinary estrone glucuronide. Although the results obtained with the twenty minute HEWL conjugate are adequate for home monitoring, based on the results obtained with the human enzyme it does not appear that a faster assay can be obtained by using a different lysozyme, and that if the assay time is to be reduced a completely different assay system will be required.

CHAPTER FIVE

STUDIES TOWARDS DEVELOPMENT OF A HORSERADISH PEROXIDASE BASED ENZYME IMMUNOASSAY FOR ESTRONE GLUCURONIDE USING NOVEL HORSERADISH PEROXIDASE - ESTRONE GLUCURONIDE CONJUGATES

5.1 INTRODUCTION

5.1.1 Immunoassay Design

Immunoassays exploit the interaction between an antibody and its antigen to provide a very powerful, versatile and sensitive analytical tool which can be used to measure a vast array of compounds of biological interest ranging from high molecular weight proteins to low molecular weight drugs and hormones. In a laboratory setting a wide range of detection labels are available for use with immunoassays including radioisotopes, and chromophoric, chemiluminescent and fluorescent compounds (Gosling, 1990). In a home assay the choice of label is much more restricted by safety issues and instrumentation, with the majority of immunoassays being based on a chromophoric reagent. Popular chromophores include colloidal gold, latex beads and the enzymatic products generated from synthetic substrates. Immunoassays based on enzyme activity as a signal generator are generally more sensitive than the latex bead and colloidal gold methods. Ideally, an immunoassay enzyme should contain reactive groups suitable for conjugation and its catalytic activity should be easily detectable. It should also be readily and cheaply available, and stable (Gosling, 1990). The most commonly used enzyme labels are horseradish peroxidase, alkaline phosphatase, glucose oxidase and β -galactosidase, of which horseradish peroxidase is particularly versatile.

5.1.2 Horseradish Peroxidase Structure

Although a large number of horseradish peroxidase isoforms have been reported, these can be divided broadly into three groups according to their electrophoretic behaviour. Isozyme's belonging to group A are acidic, those belonging to groups B and C are neutral or slightly basic, and isozymes belonging to groups D and E are strongly basic (Fujiyama *et al.*, 1988). Of the isoperoxidases of horseradish root, the type C peroxidases dominate quantitatively (Welinder, 1979) with almost all horseradish peroxidase studies and horseradish peroxidase enzyme immunoassays being based on this isoform (Dunford, 1991; Ryan *et al.*, 1994).

In common with the other horseradish peroxidase isozymes, the type C isozymes are moderately small glycoproteins composed of a single polypeptide chain cross-linked by four disulphide bonds, and contain a ferric hemin prosthetic group (Welinder, 1985). Like peroxidases from the A and B families, they also contain two calcium ions per molecule

of enzyme (Welinder, 1985). Their true distinguishing feature is their moderately high isoelectric point which is usually about nine or slightly higher (Welinder, 1979). The horseradish peroxidase C isoforms undergo considerable post translational modification including N-linked glycosylation (Kelly *et al.*, 1996), and cleavage at the N and C termini (Fujiyama *et al.*, 1988), followed by conversion of the N-terminal residue into a pyrrolidonecarboxyl group (Welinder, 1979).

Heterogeneity in the C isoforms comes from variations in the amino acid sequence (Fujiyama *et al.*, 1988) and in the glycosylation pattern (Kelly *et al.*, 1996). Homology in the amino acid sequence between the C variants is between 91-94% compared to a minimum of at least 70% between the C isoforms and isoforms from the other divisions (Fujiyama *et al.*, 1988). The amino acids of the C isoforms involved in catalysis and binding are all highly conserved as are the eight cysteine residues involved in the four di-sulphide bonds (Fujiyama *et al.*, 1988). Although genetic analysis suggests that all C isoforms consist of a single polypeptide chain of 308 residues and a C-terminal serine residue, amino acid analysis shows this C-terminal serine residue to be particularly labile (Welinder, 1979) and that more than half of the horseradish peroxidase C molecules are without this residue (Kelly *et al.*, 1996). Genetic analysis has also shown the number of potential N-glycosylation sites to vary from 6-8 between the different isoforms (Fujiyama *et al.*, 1998) while mass spectral analysis shows that, within a given amino acid variant, there is also extensive variation in the number of sites actually glycosylated and also in the composition of the sugar chains attached to these sites (Kelly *et al.*, 1996).

Most commercial samples of horseradish peroxidase C consist of a mix of isoforms (Dunford, 1991; Kelly *et al.*, 1996), thus it is difficult to give a precise estimate of the molecular weight. The amino acid component of the most prevalent amino acid isoform, which has the C-terminal serine residue intact, has a molecular weight of 33,890 Daltons (Welinder, 1979). Taking the known variations in the carbohydrate content of this isoform into account, which have been estimated to compose 25-30% of the weight of the native enzyme, the enzyme's total molecular weight, including the hemin group (550.5 Daltons) and two calcium ions ($40 \text{ Daltons} \times 2$), is estimated to be between 41,000 and 44,000 Daltons (Kelly *et al.*, 1996). Although the heterogeneity of the commercial preparations (and most laboratory preparations) makes a complete structural characterisation of these samples impossible it is generally believed to be of little kinetic importance (Dunford, 1991).

In this chapter all unspecified references to horseradish peroxidase will refer to the type C variant.

5.1.3 Suitability of Horseradish Peroxidase C for Enzyme Immunoassay

One of the characteristics of horseradish peroxidase C which make it a particularly versatile immunoassay signal generator is its stability under a wide range of assay conditions. The enzyme is tolerant of a variety of buffer agents, and is stable over a wide range of pH values (5-10) and ionic strengths. It also has high inherent thermostability, being stable for long periods of time at room temperature and having a minimum short term upper tolerance limit of 80°C (Ryan *et al.*, 1994; Dunford, 1991).

Another of the advantages of horseradish peroxidase C is the wide range of substrates available for assaying its activity. In the cell, horseradish peroxidase utilises H_2O_2 to catalyse the oxidation of a wide variety of organic and inorganic substrates (Dunford, 1997). Because the specificity of horseradish peroxidase for the second molecule of hydrogen peroxide is low, there are many indicator molecules which can be used as electron donors to monitor peroxidase activity (Ryan *et al.*, 1994; Dunford, 1991). Substrates that are involved in chromogenic, fluorescent, chemiluminescent, electrochemical and hydroxylation reactions are all used as a measure of horseradish peroxidase activity (Ryan *et al.*, 1994). Of these, chromogenic assays are the most popular requiring only the most standard pieces of laboratory equipment for their quantification. Chromogenic assays are also most readily adapted into a home assay. Chromogenic substrates include *o*-phenylenediamine (OPD), 2,2'-azino-di-[3-ethyl-benzothiazoline-(6)-sulfonic acid] (ABTS), 3,3',5,5'-tetramethylbenzidine (TMB), diaminobenzidine (DAB), 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH), *o*-dianisidine and guaiacol (Ryan *et al.*, 1994; Dunford, 1991). One of the chief disadvantages of the chromogenic assays is that many of the substrates and their oxidation products are carcinogenic or mutagenic including ABTS, DAB, OPD and *o*-dianisidine. In contrast guaiacol, MBTH and TMB are not carcinogenic (Ryan *et al.*, 1994) and can be used safely in a home assay, although the smell of guaiacol can be unpleasant. Another common complication, of the chromogenic assays is that many of them cannot be easily quantified. This is because, upon oxidation many of them, for example guaiacol, produce a mixture of unknown oxidation products so that absolute molar absorptivities cannot be defined (Dunford, 1991). However, in a home assay, a relative assay is usually all that is required. Although most enzyme immunoassays can be detected via chromogenic substrates, compared to other commonly used enzyme labels, horseradish peroxidase is particularly sensitive. For example it is reported as being 40 and 400 times more sensitive than standard β -galactosidase and alkaline phosphatase assays respectively (Gosling, 1990).

Other properties of horseradish peroxidase which make it particularly suitable for enzyme immunoassay include the fact that it is available in a form pure from non-peroxidase contaminants and that it is relatively cheap. Also, there are few horseradish peroxidase inhibitors and most of these are not usually present in blood or urine (Ryan *et al.*, 1994), the most commonly tested body fluids.

5.1.4 Conjugation of Horseradish Peroxidase

In all enzyme immunoassay systems, the enzyme must be present as a conjugate. Most commonly it is conjugated to the anti-hapten antibody or to the hapten itself. The simplest method of horseradish peroxidase conjugate production involves using the same methods as are used in the production of the lysozyme conjugates for the Ovarian Monitor i.e. using either the mixed anhydride or active ester methods (Erlanger *et al.*, 1957; Mattox *et al.*, 1969) to conjugate directly onto a free amino group. The number of potential conjugation sites for this method can thus usually be considered to be equal to the number of lysine residues plus one for the N-terminal amino group. However, due to the post translational carboxylation of the N-terminus, only the number of lysine residues needs to be included in this calculation for the horseradish peroxidase C isozymes. Examination of the nucleotide sequences from horseradish peroxidase C cDNA clones has shown that the

number of lysine residues varies between different horseradish peroxidase C isozymes (Fujiyama *et al.*, 1988). For example, the C1, C2 and C3 cDNA clones reported in the work of Fujiyama contained a codon sequence for 6, 9 and 7 lysine residues respectively. The amino acid sequence predicted by the C1 cDNA corresponds to the horseradish peroxidase C sequence determined by Welinder (1979) for what is generally considered to be the most prevalent horseradish peroxidase C isoform. Thus, despite most horseradish peroxidase C preparations consisting of a mix of isoforms, in most conjugations the total number of potential conjugation sites is usually quoted as six (Ryan *et al.*, 1994) which is the same as the number of lysine residues in hen egg white lysozyme. In common with lysozyme, not all the lysine groups are readily conjugated under mild non-denaturing conditions (Paek *et al.*, 1993). These differences in the reactivity of the different lysine residues are believed to be determined also by differences in their local environment. Local factors which could affect the sites and rates of conjugation for the different isozymes include their degree of exposure and their intramolecular interactions with the rest of the protein (see Sections 2.4.2.1 and 4.4.3) and their proximity to glycosylation sites.

In most studies on commercial horseradish peroxidase C preparations there are only two lysine residues which can be readily conjugated, giving a total of only three possible conjugate variants i.e. two mono-substituted conjugates and a di-substituted conjugate. This low number of conjugate variants enables the isolation of pure conjugates of known substitution level. In fact for most other immunoassay enzymes conjugated by this procedure, this is impossible due to their high total lysine content. For example there are more than 13 lysine residues available for reaction on a glucose oxidase molecule and a maximum of 116 on β -galactosidase (Paek *et al.*, 1993).

Another method by which horseradish peroxidase C conjugates can be produced is via the enzyme's carbohydrate moieties using the periodate method (Tijssen, 1985b). Like the conjugation of glucose oxidase and β -galactosidase via the lysine residues, due to the high sugar content of the horseradish peroxidase glycoprotein this method also has the potential to produce a large number of variants (Tijssen and Kurstak, 1984).

5.1.5 Disadvantages of the Standard Conjugation Procedures

The lysozyme conjugate utilised in the Ovarian Monitor homogeneous immunoassay is unusual in that it can be purified to homogeneity from other conjugates and the unreacted enzyme in the conjugation reaction mixture using simple ion exchange and hydrophobic interaction chromatography (Section 2.3.4). For horseradish peroxidase C the high number of structural isomers present in the native enzyme preparations, makes any attempt to isolate a pure conjugate from a horseradish peroxidase conjugation mixture by standard chromatographic techniques very difficult if not impossible.

Usually, the separation of a protein-hapten conjugate from unreacted protein requires immunoabsorption affinity chromatography using anti-hapten antibody chemically bound to a solid matrix (Paek *et al.*, 1993). Although affinity chromatography can provide a very specific and powerful means of purification it is not always practical due to problems of the conjugate binding irreversibly to the solid phase, and the low capacity of the column.

One approach of beating the elution problem is to use an affinity column in which the antibody to the hapten of interest is replaced by an antibody to a structurally related but non-identical form of the hapten. Using this system, the enzyme-hapten is easily eluted from the column using the heterologous hapten as the eluting agent (Exley and Abuknesha, 1977). The disadvantage with this method is the time required to screen for a suitable eluting hapten and generate the antibody, a procedure which must be repeated for every new hapten.

Another common method of dealing with the removal of unreacted protein which bypasses the problem entirely is to perform the conjugation reaction at such a high ratio of activated hapten to protein that no unconjugated protein remains. However this approach suffers from the disadvantage that it produces a high level of multiply substituted conjugates which will decrease the assay sensitivity (Hosoda *et al.*, 1985; Paek *et al.*, 1993; Exley and Abuknesha, 1977). Alternatively in many solid phase assays, any unreacted protein will be removed in the washing step (Henderson *et al.*, 1995). However this method is unsuitable for immunoassays which require no physical separation of the bound and free fractions, for example as in homogeneous assays.

Because of the problems outlined above in separating a defined singly substituted conjugate, most enzyme immunoassay systems utilise a complex undefined conjugate mixture. Apart from providing a more sensitive assay due to the removal of multiply substituted conjugates and minimising the background due to contamination with unconjugated protein, working with defined mono-substituted conjugates can provide some additional benefits. Not only does it allow the kinetics of the assay to be more fully characterised, but as not all isomers of a given conjugate have identical properties, by judicious screening a conjugate of superior affinity may be selected (Paek *et al.*, 1993).

5.1.6 An Alternative Procedure for Conjugating Horseradish Peroxidase to Haptens

Of the three major structural elements of horseradish peroxidase; the amino acid residues, the carbohydrate groups and the heme prosthetic group, only the amino acid residues and the carbohydrate groups have been used previously in the preparation of enzyme-hapten conjugates for use as immunoassay signal generators.

In the work presented in this chapter, the feasibility of using a horseradish peroxidase-hapten conjugate linked through the heme prosthetic group as an immunoassay enzyme hapten conjugate is examined for the first time.

The idea of using a prosthetic group conjugate in an enzyme immunoassay is not new, and was first patented in 1979 (Hornby and Morris). This patent used as its example a Flavin Adenine Dinucleotide (FAD)-glucose oxidase conjugate assay which was used to measure *N*-2',4'-dinitrophenyl-6-aminocaproate, immunoglobulin G (IgG), theophylline, and thyroxine. The main disadvantage with their chosen method was that the synthetic route used to prepare the FAD-conjugates was elaborate and resulted in low yields (less than 1% from the starting reagents). However, the reconstitution results obtained with the FAD-hapten and the glucose oxidase apo enzyme were very encouraging. Although the reconstitution of the glucose oxidase with the modified FAD was slower than that observed

for FAD itself, its natural prosthetic group, it was sufficient to allow the development of a new homogeneous immunoassay system (Morris *et al.*, 1981) which has been exploited commercially.

As discussed in Section 2.1, all homogeneous enzyme immunoassays are characterised by the binding of antibody to a hapten conjugate directly modulating the enzyme's activity. The advantage with these systems is that they do not require the separation of bound from free fractions as the level of hapten in the sample directly determines the enzyme activity. Modulation of the activity can be in the form of enzyme activation or enzyme inhibition, and can be initiated by conformational changes or steric constraints brought about by antibody binding (Blake and Gould, 1984). The now classical example of a homogeneous assay is the one initially developed by Rubenstein *et al.* (1972) and upon which the current Ovarian Monitor assay is based. In this assay the direct binding of antibody to a lysozyme-hapten conjugate physically restricts access of the substrate to the active site (see Figure 3.2). As the concentration of sample hapten in the assay is increased, the proportion of antibody bound to the enzyme-hapten conjugate decreases, and the enzyme activity increases (see Section 1.6.2-1.6.3). However all homogeneous immunoassays do not involve direct conjugation to the enzyme, and homogeneous assays based on cofactor-, prosthetic group-, substrate- and inhibitor-hapten conjugates have all been shown to be viable (Blake and Gould, 1984). The work of Hornby and Morris represents the first prosthetic group labelled immunoassay (PGLIA).

Their PGLIA glucose oxidase assay for the cardiac drug, theophylline has been reported in detail (Morris *et al.*, 1981). The assay works on the principle that enzyme activity is controlled by antibody binding to the FAD-hapten conjugate preventing reconstitution of the apo glucose oxidase enzyme into its active form, with the extent of antibody binding to the FAD-hapten conjugate itself being determined by the level of unconjugated theophylline in the sample. This assay had a lower sensitivity limit of approximately 25 nmoles mL⁻¹ of serum which although adequate for the administrative control of theophylline patients is approximately 2,000 times higher than that required for the Ovarian Monitor E1G assay (0.012 nmoles mL⁻¹ of time diluted urine).

In a review of the time, it was suggested that the prosthetic group labelled system was a superior method to the direct enzyme conjugate systems (Blake and Gould, 1984). Firstly it allows a wider range of reaction conditions to be used in the synthesis of the hapten conjugate, as a prosthetic group is generally more stable than the average enzyme, and secondly, because of their smaller size and different structure, the products of reactions performed on these molecules should generally be more specific than those involving enzymes which usually have several comparable functional groups, many of which are likely to react.

However, it must be borne in mind that the chemistry for the modification of a prosthetic group is rarely as straightforward as that required in a simple enzyme conjugation with most procedures requiring a multi-step synthesis. Modification of only a single functional group on a prosthetic group is not necessarily simple either.

For example, hemin (see Figure 5.1 for structure and Fischer numbering system), possesses two propionate groups (positions 6 and 7), two vinyl groups (positions 2 and 4) and four methyl groups (positions 1, 3, 5 and 8), in addition to four meso carbons (labelled α , β , γ , δ).

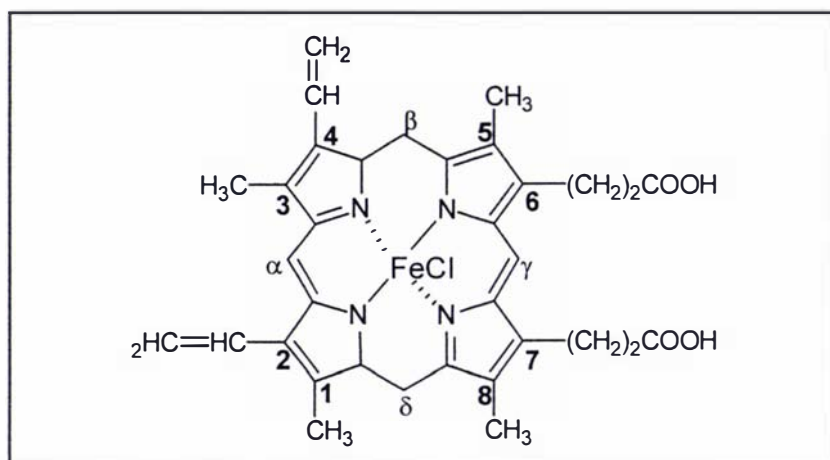


FIGURE 5.1: Hemin Chloride Structure Showing the Carbons Numbered by the Fischer System

Although in theory any of these functional groups can be modified most chemical modification procedures are unable to select between the different positions of a given functional group. In fact one novel method for selectively modifying a single methyl group involved modifying the hemin on the intact protein, where access of the modifying reagent to the substrate channel restricted the reaction to a single position (Harris *et al.*, 1993). Selective modification of the carboxyl groups on the propionate side chains is also a challenge due to their similar reactivity, and most reports of modifications in these positions have involved the conversion of both residues (DiNello and Dolphin, 1981; Harris *et al.*, 1993). Furthermore, although there are several examples in the literature of modifications to the hemin of horseradish peroxidase for kinetic studies (Harris *et al.*, 1993; Fernandez *et al.*, 1993; DiNello and Dolphin, 1981), to date there have been no reports of the linkage of hemin with a hapten and its subsequent reconstitution back into the protein. This includes the owners of the now outdated PGLIA patent, who although claiming the use of an horseradish peroxidase-hemin-hapten conjugate in a PGLIA format as their own intellectual property have not performed any experiments to prove its validity, including the synthesis of the hemin-hapten. Since then, nobody has taken on the synthetic challenge, and the ability of such a conjugate to perform in a PGLIA system or even as a general enzyme label remains unknown.

Any attempt to plan strategies for modifying the hemin into a form which can be reconstituted into the horseradish peroxidase apo protein requires a structural approach. The major consideration in the reconstitution of a hemin-hapten is steric, with the best position for the molecular linker between the hapten and the hemin being the one that minimises steric contacts with the protein.

Figures 5.2 and 5.3 show the native hemin in its binding pocket of horseradish peroxidase.

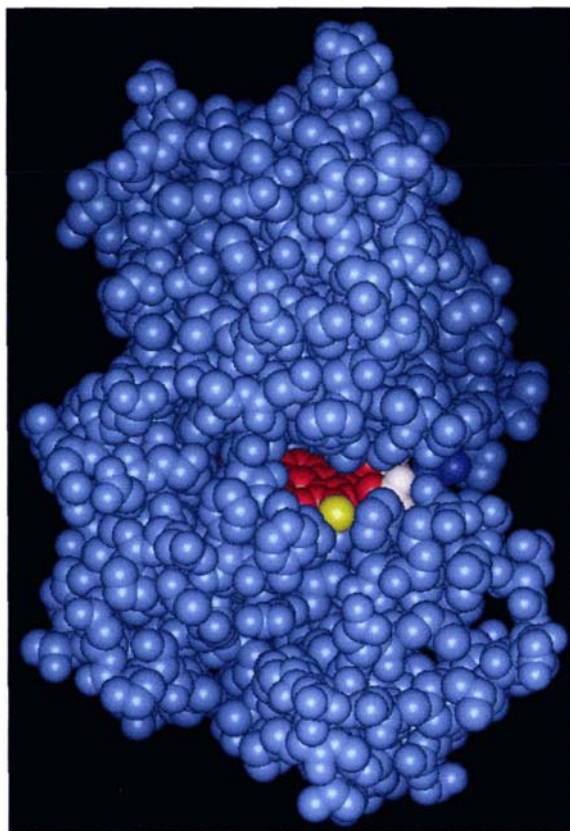


FIGURE 5.2: Structural Model of Horseradish Peroxidase showing the Exposure of the Hemin through the Organic Substrate Channel

Protein - Blue (Arg 31 - Dark Blue), Hemin - Red (7-Propionate Side Chain- White, 8-Methyl Position - Yellow)

In the model of the holo enzyme structure, parts of the hemin are still clearly visible from the protein surface.

Based on exposure of the hemin, the possible sites for modification are limited to the 8-methyl and the δ meso positions on the hemin edge which are exposed through the organic substrate channel, and the propionate side chain at position 7 which is exposed to the surface through the inorganic substrate channel.

Attaching a linker at the 8-methyl position is not a feasible option since selective modification at this residue requires it to be first converted into its hydroxymethyl derivative which can only be achieved by using either total synthesis or by a low yielding protein modification method where access of the modifying reagent to the holo-protein's substrate channel restricts the reaction to the 8-position (Snow and Smith, 1989). Furthermore, as the 8-methyl - δ meso side of the hemin edge is believed to be involved in electron transfer from the organic substrate, a large scale substitution at either of these positions may result in an inactive

reconstituted enzyme. Equally likely, enzyme prepared from one of these hemin derivatives may be inhibited by the linker chain passing through the substrate channel and thus preventing the access of bulky aromatic substrate molecules to the binding site.

In comparison the 7-propionate side chain seems to a much better candidate for modification. It is positioned close to the organic substrate channel (as shown in Figure 5.3), but does not have direct access to it. Thus conjugation at this position with an organic linker-steroid conjugate is unlikely to result in the direct occlusion of the substrate channel. Another advantage of using the 7-propionate group for modification, is that the propionate groups are not believed to be directly involved in the peroxidative mechanism. However, this is not to say that modification of these groups is not associated with a loss of peroxidase activity. Spectral and kinetic studies on the modification of these groups, has suggested rather that their role is one of hemin orientation, and that the correct orientation of the hemin in its binding pocket is an important requirement for catalytic activity (Adak and Banerjee, 1998). In fact the effect of modification of the propionate groups on enzyme activity can be very large, with most two site modifications resulting in

a complete loss of activity, and modification at a single site resulting in a partial loss (Adak and Banerjee, 1998; Fernandez *et al.*, 1993; Tamura *et al.*, 1972).

The model of horseradish peroxidase in Figure 5.3 shows the exposure of the two propionate groups to the surface.

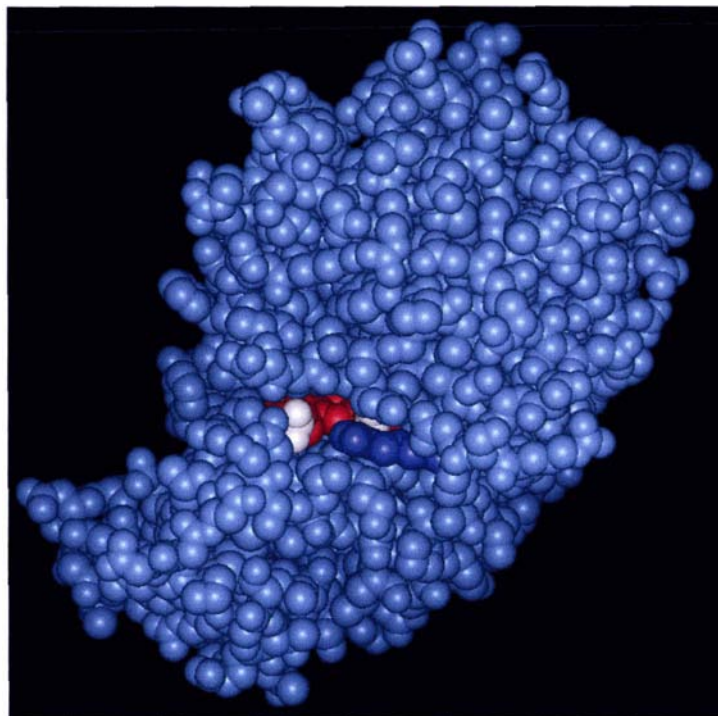


FIGURE 5.3: Structural Model of Horseradish Peroxidase showing the Exposure of the Hemin Propionate Groups

Protein - Blue (Arg 31 - Dark Blue), Hemin - Red (Propionate Side Chains- White - Position 6 on right, Position 7 on left)

This shows very clearly that the 7-propionate group is quite open to the protein surface, and that the 6-propionate group is almost completely occluded by arginine 31. Thus whereas it appears that modifications to the propionate group at the 6 position may not be favoured for reconstitution, if a 7-mono substituted hemin derivative can be made, it should have a good chance of binding to the apo horseradish peroxidase without major disruption of the tertiary structure.

The final advantage with using a propionate group for attaching the hapten is that the terminal carboxyl groups are also the most chemically reactive groups on the hemin

molecule. However, as the propionate groups have similar reactivities, limiting modifications to a single site is difficult to control. To date, only very simple selective modifications of these propionate groups have been reported (Tamura *et al.*, 1972; Adak and Banerjee, 1998). The organic chemists of our research group have recently developed a procedure to limit large scale modifications of the propionate side chains to a single site. As one of the advantages of using a prosthetic group conjugate is to limit the number of hapten molecules per enzyme for greater immunoassay sensitivity, and the conjugation of a minimal number of sites on the hemin is vital for functional integrity of the molecule and to minimise any steric constraints, the ability to be able to produce a mixture of 6 and 7 mono-substituted propionate groups is an important development.

5.1.7 Advantages of using a Horseradish Peroxidase Hemin-Hapten Conjugate as a Signal Generator in Enzyme Immunoassay

According to a review on steroid enzyme immunoassays (Pratt, 1978), sensitivity is principally limited by bridge recognition of the hapten conjugate, and by using a

multivalent hapten-enzyme conjugate. In bridge recognition, the antibody recognises the chemical structure linking the hapten to the enzyme because it is similar to the bridge used in the synthesis of the protein-hapten immunogen used to generate the antibody in the first place (Danilova, 1994). For example in the Ovarian Monitor assay, enzyme conjugates and the protein-hapten immunogen conjugates are both conjugated via the lysine residues. Thus it is possible for the antibody to have a greater affinity for the enzyme-hapten than the free hapten as the enzyme-hapten is more analogous to the protein-hapten used to generate the anti-hapten antibody than to the hapten itself. Linkage of the hapten via the hemin provides an opportunity to use a completely different linker to that usually used with proteins which effectively minimises all bridge recognition problems. Secondly the problem of a multivalent hapten-enzyme conjugate does not arise as in the hemin-E1G conjugate there is only one linkage site.

The use of a horseradish peroxidase hemin substituted conjugate also has the potential to simplify greatly the purification of a singly substituted hapten conjugate, as reconstituted enzyme can be separated from the apo form by passage through a carboxymethyl cellulose column (Tamura *et al.*, 1972). This gives a procedure which is universally applicable unlike the current affinity chromatography methods which require a separate affinity column to be prepared for every hapten.

Finally, one of the biggest disadvantages of the current lysozyme assay is its dependence on a turbidimetric assay which restricts the method to a liquid assay format. Converting to a chromogenic horseradish peroxidase assay as the enzyme signal generator removes this restriction and enables more user friendly solid phase formats to be devised. Although, numerous immunoassays for steroids and their metabolic products exist, most of these are for use in a clinical laboratory and are designed for the clinician as opposed to the individual at home. For a home test, simplicity and speed are of paramount importance, and the public perception of home testing, especially for contraceptive purposes where daily monitoring can be required, demands a test-strip format.

Thus, apart from providing a structurally characterised and defined conjugate, the advantages of using a horseradish peroxidase hemin-hapten conjugate as a signal generator are the potential for simplification of conjugate purification, increased immunoassay sensitivity and dry chemistry assay formats.

In order to compare the performance of the new horseradish peroxidase estrone glucuronide conjugates as signal generators, a urinary estrone glucuronide immunoassay using a horseradish peroxidase-estrone glucuronide active ester was established based on the method described by Henderson *et al.* (1995).

Converting to the horseradish peroxidase enzyme immunoassay of Henderson from the current hen egg white lysozyme Ovarian Monitor immunoassay as the standard laboratory reference assay offers a number of advantages. Because the assay of Henderson is an ELISA plate assay, it is much more easily automated, and has potential for a much higher throughput than the current Ovarian Monitor assay. Other potential advantages are a decrease in background interference from non-specific binding due to the low sample volumes required and the removal of weak binding interactions by the washing steps.

5.1.9 Aims

In summary the major aims of the work presented in this chapter were:

- to explore the feasibility of using a hapten linked hemin molecule as a defined singly substituted hapten conjugate of horseradish peroxidase for use in enzyme immunoassay
- to compare the urinary estrone glucuronide cycle data collected using the horseradish peroxidase ELISA plate method of Henderson with the results obtained from the Ovarian Monitor and a World Health Organisation reference radioimmunoassay using the same cycles as a reference against which the performance of assays using new signal generators can be compared

5.2 MATERIALS AND METHODS

5.2.1 Apparatus

Centricon (2 mL) and Macrosep (20 mL) centrifugal concentrators, both with a 10,000 molecular weight cut-off, were supplied by Amicon (Beverly, MA, USA) and Filtron Technology Corporation (Northborough, MA, USA) respectively. All manually obtained absorbances including kinetic assay rates were measured on a diode array spectrophotometer (Hewlett Packard). A quartz cuvette was used for all measurements for protein and hemin content and plastic 3 mL cuvettes for all kinetic assays. All ELISA plate absorbance readings were measured using an 8 channel single-beam microplate absorption photometer (Anthos Labtec Instruments, Salzberg, Austria). Polystyrene 96 well ELISA microtitre plates (Maxisorp) were supplied by Nalge Nunc International (Kamstrup, Roskilde, Denmark). The Ovarian Monitor equipment including pre-coated E1G assay tubes was supplied as described in Section 2.2.1 as were the assay syringes.

All curve fitting was performed using the PRISM data analysis and graphing program (Graphpad™).

5.2.2 Reagents

Estrone glucuronide was synthesised according to the method outlined in Section 2.4.1 and all reagents for making the active ester reagent were as reported in Section 2.2.2. The horseradish peroxidase (grade VI) and hemin chloride were obtained from the Sigma Chemical Company (St Louis, MO, USA) and the gelatine and *o*-dianisidine from British Drug House Biochemicals (Poole, UK). The *o*-phenylenediamine was obtained from Merck-Schuchardt (Darmstadt, Germany), the hydrogen peroxide (30% w/v) from AJAX (Auburn, NSW, Australia), and the resins, diethylaminoethyl cellulose (DE 52) and carboxymethyl cellulose from Whatman (Kent, UK) and Pharmacia (Uppsala, Sweden) respectively. The sephadex G-25 resin used for the purification of active ester protein conjugate (Section 5.2.7.1), the PD10 sephadex G-25M gel filtration column (60 × 14 mm i.d.) used in the preparation of apo protein (Section 5.2.10), and the Coomassie blue G250 were also supplied by Pharmacia. All modified hemins were synthesised by Zhandong Zhao and Dr. Yinqi Wu, and the structures confirmed by thin layer chromatography and electrospray mass spectrometry (Zhao, 1998; Wu, 1996).

Most antibody based assays were performed using unpurified anti-estrone glucuronide antiserum 243-4 (Section 2.2.2), although for the solution reconstitution assays several other antibody preparations were also used. These included a purified form of antiserum 243-4, an anti-pregnanediol glucuronide antiserum (ET2-4), and a monoclonal anti-estrone glucuronide antibody. The purified form of the anti-estrone glucuronide antiserum 243-4 was prepared by Mr Bryce Cummock (1998) using ammonium sulphate precipitation (Harlow and Lane, 1988). All references to antiserum 243-4 refer to the unpurified form, unless specified otherwise. The anti-pregnanediol glucuronide antiserum was generated using exactly the same methodology, and immunisation and bleeding schedule, as was used to produce antiserum 243-4 except that thyroglobulin-pregnanediol glucuronide was used as the immunogen. The monoclonal anti-estrone glucuronide antibody was generated from

Balb/c mice using standard methodology (Harlow and Lane, 1988) and was provided by Dr. Keith Henderson (AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand).

All other reagents and chemicals were of analytical grade or higher, and the water was of Milli-Q quality.

5.2.3 Important Horseradish Peroxidase Constants

The reinheits zahl (RZ) number is often used as a measure of purity and integrity for the horseradish peroxidase enzyme (Tijssen, 1985c) and was used in this work as a measure of hemin (and modified hemin) binding. It is usually calculated by expressing the absorbance obtained for a horseradish peroxidase solution at 403 nm, over the absorbance obtained at 278 nm (Tamura *et al.*, 1972). The RZ was calculated from the absorbance at 404 nm as opposed to 403 nm as the recording spectrophotometer only had a 2 nm slit width. The absorbance peak at around 404 nm is due to hemin bound at the hemin binding site, and the absorbance peak around 278 nm is the standard ultra-violet protein peak. The concentrations of horseradish peroxidase apo protein and holo enzyme were calculated using a molar absorption co-efficient of 20,000 at 278 nm and 107,000 at 404 nm respectively (Tamura *et al.*, 1972).

In the work presented in this chapter, two synthetic enzyme substrates have been utilised - *o*-dianisidine for the standard horseradish peroxidase assay and *o*-phenylenediamine for the enzyme linked immunosorbent assay (ELISA) studies. The absorption products of the peroxidase reaction with its synthetic substrates, including *o*-dianisidine and *o*-phenylenediamine, are ill-defined and depend on the reaction conditions (Claiborne and Fridovich, 1979; Tijssen, 1985c; Dunford, 1991) making it difficult to assign absorption co-efficients for the resulting product mixture. Thus peroxidase activity is usually reported in the arbitrary units of absorbance change as opposed to the concentration of product, and is the method which has been utilised here.

5.2.4 Estimation of Protein Content by the Coomassie Blue Method

Horseradish peroxidase solutions of unknown concentration were assayed for protein content using native horseradish peroxidase as the standard curve protein, and standard Coomassie reagent (Kruger, 1994). The assay was performed on a microscale using ELISA plates and an ELISA plate reader, and all dilutions of the standards and samples were prepared using phosphate buffer (5 mM, pH 6.5) as the diluent. The top standard ($\approx 6 \mu\text{M}$) was prepared by diluting a previously prepared stock horseradish peroxidase solution of known concentration (see Section 5.2.10.1) by a factor of 25. All other standards were prepared by direct dilution of this top standard directly on the plate to generate a six point standard curve. The samples were also generally diluted directly on the plate. The final well volume for all standards and unknowns was 20 μL , and all assays were performed in duplicate. After the diluting buffer had been added to the wells, the wells were mixed using a multi-channel pipette and the protein assay initiated by the addition of 200 μL of Coomassie reagent. The wells were then mixed as before and the plate left to incubate for 10 minutes at room temperature before reading at 595 nm.

The standard curve data were fitted using a one site binding hyperbola on the data analysis and graphics program PRISM and the protein content (μM) determined using the 'calculation of unknowns' option.

5.2.5 Preparation of Reagents for Standard Activity Assay for Horseradish Peroxidase

Citrate buffer (0.1 M, pH 5.3) was prepared by dissolving disodium citrate (10.3 g) and trisodium citrate (14.4 g) in water (≈ 900 mL) and adjusting the pH to 5.3. The buffer was then transferred to a 1 L volumetric flask and made up to volume with water.

Hydrogen peroxide solution was prepared by adding 1 mL of a commercial hydrogen peroxide solution (30% w/v) in a 100 mL volumetric flask and making up to volume with water.

An *o*-dianisidine solution was prepared by weighing approximately 8 mg of *o*-dianisidine accurately on a 5 figure balance and then dissolving it in the appropriate amount of methanol to produce a 10 mg mL^{-1} solution. The solution was freshly sonicated daily for 5 minutes before use.

The enzyme was diluted fresh daily with 5 mM phosphate buffer (pH 6.5) by dilution series.

5.2.6 Standard Activity Assay for Horseradish Peroxidase

Apart from the ELISA plate assays, all assays for peroxidase activity were measured using the assay protocol described below which utilises *o*-dianisidine as the hydrogen donor.

All *o*-dianisidine activity assays were performed at 40°C and measured at 460 nm using a diode array spectrophotometer. The temperature of the cuvette was kept constant throughout the assay by means of a flow cell holder connected to a circulating water bath. For the best temperature control, the stock bottle of assay buffer was kept in the circulating water bath and the addition of all assay components was made directly into the cuvette which was kept in the cell holder of the machine. A glass plunger was used to mix the assay mixture which allowed the cuvette to stay in the cell holder throughout the procedure.

The spectrophotometer was blanked against a solution of citrate buffer (1.9 mL), *o*-dianisidine (20 μL) and enzyme (20 μL), and the assay initiated by the addition of hydrogen peroxide (20 μL). Data were typically collected for 1 minute and all rates were expressed as initial rates.

5.2.7 Preparation of ELISA Reagents

5.2.7.1 *Horseradish Peroxidase - Estrone Glucuronide Active Ester Conjugate*

Active ester horseradish peroxidase - estrone glucuronide conjugate was prepared by standard conjugation procedures (Section 2.2.4), using a molar ratio of enzyme to steroid of 1:78. A solution of horseradish peroxidase (0.25 mM) was prepared for conjugation by dissolving 21.2 mg of the enzyme (77% purity, i.e. 0.38 μ moles) in 2 mL of sodium bicarbonate solution (0.13 M). The active ester reagent was prepared by adding aliquots (50 μ L) of stock solutions (0.602 M, 30.1 μ moles) of estrone glucuronide, *N*-hydroxysuccinimide and dicyclocarbodiimide dissolved in dimethyl formamide together. After 90 minutes the active ester reagent was added dropwise over 5 minutes with stirring to the horseradish peroxidase - sodium bicarbonate solution (4°C) using a dry pasteur pipette. The active ester reagent vial was rinsed with a 0.5 mL aliquot of sodium bicarbonate solution and the washings transferred to the conjugation reaction mixture. The reaction mixture was left gently stirring at room temperature. After 2 hours the reaction mixture was transferred to a dialysis sac. A further 0.5 mL of bicarbonate solution was used to rinse the rest of the sample from the conjugation vial into the dialysis sac. The reaction was then dialysed against water (3 $\times$ 2 L) over a period of 24 hours. After dialysis the sample was transferred to two small plastic test-tubes, using a further 0.5 mL aliquot of water to rinse the dialysis sac into the test-tubes. The test tubes were then spun at speed 5 on a Gallenkamp bench centrifuge for 20 minutes. Upon centrifugation the sample separated into a clear supernatant and a small powdery precipitate at the bottom of the test tube with a red-black spot. The supernatant was then passed through a sephadex G-25 gel filtration column (235 mm $\times$ 22 mm i.d.) pre-equilibrated with *N*'-2-hydroxyethylpiperazine-*N*'-ethanesulphonic acid (HEPES) buffer (0.154 M, pH 7.0). An estimated 4.2 mL of sample was loaded. The sample was clearly observed as a wide red band. Collection of the eluent (1.5 mL fractions) began when the band approached the bottom of the column. A total of 15 fractions was collected. Based on the A_{404} profile shown in Figure 5.4 (page 237), the first 7 fractions were combined and concentrated using a 10 K Macrosep centrifugation cup (6,500 rpm, 90 minutes).

The concentrated conjugate was assayed for activity using the standard horseradish peroxidase *o*-dianisidine assay (see Section 5.2.6).

5.2.7.2 *Carbonate - Bicarbonate Coating Buffer (pH 9.6)*

Coating buffer was prepared fresh daily by dissolving sodium carbonate (0.318 g) and sodium bicarbonate (0.586 g) in water to give a total volume of 200 mL. No adjustment of pH was usually necessary.

5.2.7.3 *0.5% Gelatine Blocking Buffer (pH 9.6)*

Blocking buffer was prepared fresh daily by dissolving gelatine (0.5 g) in water (approximately 80 mL) over gentle heat. Upon cooling the solution was transferred to a 100 mL volumetric flask and made up to volume.

5.2.7.4 Citrate-Phosphate Substrate Buffer (pH 5.0)

Substrate buffer was prepared fresh daily by dissolving citric acid (0.511 g) and anhydrous disodium phosphate (0.730 g) in water and making up to 100 mL in a volumetric flask. No adjustment of pH was usually necessary.

5.2.7.5 Phosphate Washing Buffer (pH 7.4)

Washing buffer was prepared by dissolving potassium dihydrogen phosphate (0.4 g), disodium hydrogen phosphate (5.8 g), and sodium chloride (15 g) in water (approximately 1.8 L) with Tween 20 (4 mL, 25% aqueous solution). The pH was adjusted to 7.4, the contents transferred to a 2 L volumetric flask and the buffer made up to volume with water. Fresh washing buffer was prepared every 2-3 weeks and stored at 4°C.

5.2.7.6 0.1% Gelatine Assay Buffer (pH 7.4)

Gelatine assay buffer was prepared fresh daily by dissolving gelatine (0.1 g) in washing buffer (90 mL) with the aid of gentle heat. Upon cooling it was transferred to a volumetric flask (100 mL) and made up to volume with more washing buffer. No adjustment of pH was usually necessary.

5.2.7.7 *o*-Phenylenediamine Substrate Solution (pH 5)

Substrate solution was prepared by dissolving *o*-phenylenediamine (40 mg) in substrate buffer (approximately 90 mL), transferring to a volumetric flask (100 mL) and then making to volume with further substrate buffer. The *o*-phenylenediamine solution was prepared approximately 5 minutes before required and stored wrapped in aluminium foil in a closed cupboard. Immediately before use hydrogen peroxide (30% commercial solution, 40 µL) was added and the resulting substrate solution mixed by inversion.

5.2.7.8 Stopping Solution

A 2.5 M sulphuric acid solution was used as a stopping solution and was made by adding concentrated sulphuric acid (98% purity, 13.5 mL) to water (86.5 mL). The stopping solution was stored at room temperature and was only prepared when the previous batch was finished.

5.2.7.9 Antibody Solution

Anti-estrone glucuronide antiserum (243-4) was diluted by dilution series using coating buffer as the diluent. In the initial dilution 20 µL of stock antiserum was diluted 1 in 50 with 980 µL of buffer. Apart from this initial dilution, no individual dilution step greater than 20, or antiserum aliquot smaller than 0.5 mL, was used for the rest of the dilution series. To avoid problems of the antiserum binding to glass, only plastic vessels were used for the dilution series.

5.2.8 The Standard ELISA Assay Protocol

The assay protocol was performed over three days.

DAY ONE

5.2.8.1 *Coating the Wells*

Diluted antiserum (100 μL) was added to all wells on the plate, apart from the four reserved for the blank and non specific binding assays which had coating buffer only (100 μL) added to them instead. After all wells were filled, the plate was lightly tapped to remove any air bubbles adhering to the sides of the wells. The plates were covered with aluminium foil and left at 4°C overnight.

DAY TWO

5.2.8.2 *Blocking the Wells*

The excess coating buffer was removed by inverting the plates and shaking. The final droplets were removed by tapping the plate firmly upside down on a double layer of paper towels. Immediately afterwards freshly prepared blocking buffer (250 μL) was added to all the wells.

5.2.8.3 *Washing the Wells*

After thirty minutes the plates were emptied as before and the plates washed with washing buffer. This involved filling all the wells to capacity with washing buffer, leaving for 3 minutes and then emptying. This sequence was repeated three times.

5.2.8.4 *Addition of the Signal Generator and Hapten*

After the plates had been filled and emptied with the washing buffer for the final time, assay buffer (50 μL) was immediately added to all the wells to stop the plates from drying out. The horseradish peroxidase - estrone glucuronide active ester conjugate, estrone glucuronide standards and urine samples were freshly diluted from the stock preparations on the day using the assay buffer. In this definition estrone glucuronide stock refers to the standard estrone glucuronide dilution series (Section 3.2.2.4) and the urinary stock refers to either early morning or time diluted (150 mL hr^{-1}) urine samples. Due to the expense of the enzyme, the first dilution was performed using only 2 μL of conjugate with 198 μL of buffer (1 in 50 dilution). Apart from this first step, no individual dilution step greater than 20 or conjugate aliquot smaller than 50 μL was used. Because during the time span of this work, the amounts of the enzyme conjugate, standards and samples in the assay varied, the exact concentrations and equivalent volumes for each experiment are referenced in the text and figure legends of the Results Section.

For competitive binding between the signal generator (i.e. enzyme conjugate) and the free hapten (estrone glucuronide standard or urinary sample) for the antiserum binding sites, the two had to be added simultaneously. Initially this was performed by pre-mixing the conjugate (300 μL) and standard or urine sample (150 μL) first in a 1.5 mL plastic vial and then adding an aliquot (150 μL) of the mixture to the well using a standard mechanical laboratory pipette. However upon purchase of a dual dispensing autopipette the pre-mixing step was no longer necessary and the diluted conjugate (100 μL) and hapten (50 μL) could be added directly.

As opposed to the conjugate - hapten mixture the blank wells contained assay buffer only (150 μL) and the non specific binding wells the signal generator only (100 μL and 50 μL assay buffer). To remove any evaporative effects, all otherwise empty wells were filled with assay buffer (150 μL). After all the wells had been filled, the well contents were mixed using a 200 μL 8-channel multi-pipette by aspirating the well contents up and down once using a fresh tip for each well. The plates were then covered with aluminium foil and stored at 4°C overnight, although occasionally a 2 hour incubation at room temperature was used instead.

DAY THREE

5.2.8.5 *The Activity Assay*

The plates were removed and the wells emptied and washed three times with washing buffer as described for the washing step on Day Two. Immediately after the plates had been washed and emptied for the third time, substrate was added to all the wells (100 μL), the plates covered with aluminium foil and placed in a closed cupboard at room temperature. The plates were checked intermittently for colour development.

Depending on the level of colour development, the reaction was stopped between 15 minutes and 1 hour by the addition of the stopping solution (50 μL) using the same well order of addition as was used for the substrate addition. The plate was read at 490 nm on an ELISA plate reader.

5.2.8.6 *General Notes on the ELISA Assay Methodology*

All assays including the blank and non-specific binding assays were performed at least in duplicate. In order to minimise any evaporative effects, all wells which were not being used were always treated the same as the assay wells. For example during the coating step, any otherwise empty wells were filled with coating buffer (100 μL) and during the signal generator - hapten step all otherwise empty wells were filled with assay buffer (200 μL). All empty wells were also washed and had the substrate and stopping solutions added to them the same as for the assay wells.

5.2.8.7 *Error Bars*

Apart from the data presented in Figure 5.54, all graphical data for which error bars are displayed are for one standard deviation from the mean ($n=2$).

5.2.9 Comparison of an Active Ester Horseradish Peroxidase Enzyme Linked Immunosorbent Assay (ELISA) for Estrone Glucuronide with Radioimmunoassay (RIA) and the Commercial Ovarian Monitor

Urine samples collected as part of the World Health Organisation (WHO) trial on the Ovarian Monitor were assayed for estrone glucuronide concentration using the active ester horseradish peroxidase ELISA assay and compared with the results obtained independently by the WHO reference laboratory in London using both their reference radioimmunoassay for E1G and the Ovarian Monitor. During the trial, time diluted urines were collected daily by the women participants over six consecutive menstrual cycles. Urinary samples from a total of nine cycles collected by seven women were analysed by the ELISA assay as a measure of the validity and accuracy of the optimised assay.

All the urines for a given cycle were assayed on a single ELISA plate. These assays were performed using a 20,000 fold dilution of anti-estrone glucuronide antiserum (243-4), a conjugate concentration of 51 fmole well⁻¹, and the equivalent of 5 μ L of time diluted urine per well. Each plate also contained a standard curve from which the cycle absorbance data were converted into estrone glucuronide excretion rates. The standard curve was performed under identical conditions except that the urine sample was replaced with 5 μ L of estrone glucuronide standard. The standard curve covered the range of 5-2,000 nmol E1G 24 hr⁻¹ (i.e. 6.9-278 fmole well⁻¹).

The colour assay was stopped at either 15, 30 or 45 minutes, depending upon when the zero standard wells of the standard curve were determined by eye to have reached a high level of colour development. All urinary samples and standards were assayed in duplicate and the cycles are referred to using the WHO numbering system, where the subject code is used together with the cycle number for that subject.

All standard curves were fitted to a sigmoidal dose response curve with a variable slope using Equation 3.5 from the data analysis and graphics program, PRISM. The reading of the unknowns from the standard curve was performed using the fitted standard curve calculation of unknowns option on the same program.

The correlation between the E1G excretion rate data calculated for the same cycle by the different assay methods was performed using the least squares regression method. A summary of the combined cycle data obtained by the different methods was correlated using both the least squares regression method and the Deming Regression Method (see Section 3.2.5.6). The method of analysis did not affect the correlation co-efficients.

5.2.10 Preparation of Apo Protein

Apo protein was prepared several times throughout the work presented in this thesis. The general protocol is given below with the specific details of one of these preparations reported in Section 5.3.4 of the Results.

5.2.10.1 *Preparation of the Stock Horseradish Peroxidase Solution*

A concentrated horseradish peroxidase stock solution was prepared by dissolving approximately 12 mg of commercial horseradish peroxidase powder in phosphate buffer (5 mM, pH 6.5) to give a 9.01 mg mL⁻¹ solution by weight. A low ionic strength buffer was used as this allowed the pH to be more readily altered during the acidification process.

5.2.10.2 *Acidification*

An aliquot of the stock enzyme (0.8 mL) was transferred into a small capped glass vial (2 mL capacity) with a small magnetic stirrer bar. Acidification was performed by the addition of hydrochloric acid (1 M) at room temperature with constant stirring and pH monitoring using a micro pH probe. The acid was added in 10 µL aliquots until a pH value of approximately 2 was reached. After acidification a 60 µL aliquot was removed for absorbance readings, Coomassie protein assays and activity assays.

5.2.10.3 *Methyl Ethyl Ketone Extraction*

For an efficient extraction it was necessary that the protein and solvent were kept cold and that the procedure was performed as rapidly as possible. The acidified horseradish peroxidase was placed at -20°C until just frozen. It was then warmed by hand until just thawed and transferred to an ice bath. The 2-butanone (methyl ethyl ketone, MEK) which was stored permanently at -20°C in preparation for the extraction, was also transferred to an ice bath. The hemin extraction was performed by the addition of 0.8 mL of the ice-cold MEK to the acidified horseradish peroxidase, and shaking vigorously. The phase separation was aided by keeping the vial contents cold by repeatedly dipping the vial in and out of a -40°C methanol bath. The mixture quickly separated into two phases - a top pink solvent phase and a bottom clear aqueous layer. The MEK was carefully siphoned off with a pasteur pipette and a further 0.8 mL of MEK added and the extraction repeated. The extraction was usually performed a total of three times. After the final extraction the volume of aqueous sample was estimated to the nearest 50 µL.

5.2.10.4 *Removal of the Methyl Ethyl Ketone by Gel Filtration*

A PD10 Sephadex G-25M gel filtration column (50 × 5 mm i.d.) was used to remove any MEK remaining with the aqueous apo horseradish peroxidase layer.

The column was pre-equilibrated with 30 mL of tris buffer (50 mM, pH 8.0). Before loading the buffer was run down to just above the level of the top frit. The sample was applied to the top of the column and carefully run in with the collection of the eluent into a 5 mL measuring cylinder beginning at the same time. When the sample had reached the level of the top frit, the column flow was stopped, the column topped with tris buffer washings from the sample vial and the washings run in. This process was repeated several times until a total of 2.5 mL of sample and buffer had been applied. When all the sample and sample washings had been applied and run into the column, the column reservoir above the frit was filled with tris buffer. After 2.5 mL of eluent had been collected the measuring cylinder was replaced with a small glass vial (3 mL capacity). Collection was

stopped when 3 mL of eluent had been collected (using an identical vial containing 3 mL of water to estimate the volume). The next drop of eluent was collected into a spotting plate containing an approximately equal volume of Brady's reagent, the standard chemical reagent for testing for ketones. A lack of precipitate in the spotting plate was taken as proof that the collected eluent was not contaminated with any methyl ethyl ketone.

The 3 mL of eluent (apo protein) was assayed for protein content using the micro Coomassie method (Section 5.2.4) and activity using the standard horseradish peroxidase *o*-dianisidine assay (Section 5.2.6). The RZ value was also recorded. The apo protein was stored at -20°C and was replaced about once a month.

5.2.11 Preparation of Hemin and Substituted Hemins for Reconstitution with Apo Horseradish Peroxidase

The preparation of hemin and the various substituted hemins is described with their structures in the Results Section.

5.2.12 Titration of Apo Horseradish Peroxidase with Hemin and Modified Hemin

Titration was carried out at room temperature in a 1 mL quartz cuvette. Tris buffer (50 mM, pH 8.0) was used to dilute the apo protein in the cuvette so that the initial protein concentration was 4.07 μ M and the initial volume (i.e. before the addition of hemin or modified hemin) was 1 mL. The resulting solution was used to blank the spectrophotometer at 404 nm. Hemin or modified hemin was then added in 1-5 μ L aliquots. After each addition, the contents were mixed by drawing the contents up and down a siliconised pasteur pipette several times, and the absorbance measured. The titration was usually stopped when the absorbance reached about 1.0. A full wavelength scan was then run before removing the cuvette, and the sample transferred to a small glass vial.

For every apo protein titration a control titration was also carried out. These titrations were identical to the sample titrations except that they were performed in the absence of protein. For every substituted hemin titration performed an unsubstituted hemin titration was also performed on the same day under identical conditions using the same batch of apo protein as a control.

After titration, the reconstituted mixtures were assayed for enzyme activity. In order to compare the rates with the native enzyme, freshly diluted enzyme from a native horseradish peroxidase stock solution was also assayed for peroxidase activity on the same day. This stock solution was the same stock solution that the apo protein for the hemin and substituted hemin titrations was prepared from.

5.2.13 Removal of Unreacted Hemins from the Reconstituted Protein

Unreacted hemins were removed from the reconstituted enzyme mixtures by passing the mixtures through a small bed of DE 52 resin (DiNello and Dolphin, 1981).

Tris buffer (50 mM, pH 8.0) was added to some dry DE resin to form a thick slurry and the pH of the slurry adjusted back to 8.0. The slurry was then poured into small plastic columns (8 mm diameter) fitted with a bottom glass frit, and allowed to settle. The volume of slurry was adjusted to form a 3 mm resin bed and the excess buffer removed. The titrated protein was then mixed with 0.4 mL of resin slurry. This was then applied to the column, and the column allowed to drain directly into a 2 mL Centricon device (10,000 molecular weight cut-off). The sample vial was rinsed with two 0.4 mL aliquots of buffer, and the washings applied to the column. The last of the eluent was forced into the Centricon device by compressed air. The sample was concentrated using a speed of 6,500 rpm typically to a volume of approximately 200 μ L. After all the sample had been concentrated, either a pre-weighed empty Ovarian Monitor assay vial or a collection vessel supplied with the Centricon apparatus was fitted over the top end of the device and the sample transferred according to the instructions supplied with the Centricon (1,000 rpm, 2 minutes). The volume of concentrated sample was estimated by weighing the collection vessel after the transfer and subtracting the weight of the empty vial.

After the samples had been concentrated their RZ values were recorded, and they were assayed for protein content and enzyme activity. Measurement of the protein content and enzyme activity of the reconstituted fractions allowed both the percentage reconstitution for each titration and the specific activity of the reconstituted fraction to be calculated. Native horseradish peroxidase was assayed for activity on the same day as the reconstituted samples and was also used as the standard protein in the Coomassie protein assay. In both cases, the native sample was freshly diluted from the same stock solution which was used in the apo protein preparation from which the reconstituted enzyme was generated from.

5.2.14 Analysis of Hemin-Polyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPE1G) using a Reconstitution Assay Format with Apo Horseradish Peroxidase

Described below is a general reconstitution assay protocol performed in the absence of free estrone glucuronide.

Antiserum and phosphate buffer (5 mM, pH 6.5) were added to diagonally opposed corners of a 3 mL plastic cuvette. In order to prevent the cuvette from being destroyed by the HPE1G solvent, the hemin compound was added directly to the phosphate buffer. The volume of phosphate buffer was adjusted so that the total volume of hemin compound (1-10 μ L), antiserum (1-10 μ L) and phosphate buffer was equal to 100 μ L. Mixing of the small volume was aided by the addition of a glass bead. After shaking for a count of three, the mixture was incubated for 5 minutes at 40°C. Dilute apo protein (20 μ L) was then added and the mixture shaken and incubated for a second time (5 minutes, 40°C). This was followed by the addition of citrate buffer (1.8 mL, 40°C) and the *o*-dianisidine substrate (20 μ L, 10 mg mL⁻¹). The initiation of the assay, data collection and calculation of enzyme activity was the same as described for the specific activity assay (Section 5.2.6).

The reconstitutions performed in the presence of free estrone glucuronide were carried out using the same general protocol as above with the following modifications. The hemin compound of the first step was replaced with estrone glucuronide standard (50 μ L). After

the buffer, antiserum and estrone glucuronide standard were mixed with the glass bead, the HPE1G was added and the sample mixed and left to incubate as for the non-estrone glucuronide protocol. The volume of buffer was adjusted so that the final volume of buffer, standard, antiserum and HPE1G at the end of this step was 100 μ L. The rest of this protocol was identical to the non-estrone glucuronide protocol.

Assays which were missing some or all the above biochemical reagents or used substitute reagents were also performed. The only difference in these protocols was the omission (or substitution) of the reagent (or reagents) and the adjustment of the volume of phosphate buffer, so that the final volume of the reagents and buffer before the addition of the citrate buffer was maintained at 100 μ L.

In later work the general reconstitution assay was performed with the following modifications. The phosphate buffer used directly in the assay and also for the dilution of the samples was replaced with tris buffer (50 mM, pH 8.0), the first 5 minute incubation at 40°C was removed, and the second 5 minute incubation was reduced to 1 minute and performed at room temperature. Prior to the addition of the apo protein, a further 100 μ L of buffer was added. Thus the final volume of tris buffer, hemin compound, antiserum and apo protein was 200 μ L as opposed to 100 μ L. The volume of citrate buffer was adjusted accordingly. This modified method was used for the time dependent analysis of the reconstitution assay, the HPE1G, hemin and antiserum titration curves, and also for the monoclonal based inhibition assays which were performed with a hemin control.

5.2.15 Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Polyethyleneglycol-Estrone Glucuronide (HPE1G) (6 or 7) Mono-Conjugate by Solution Assay and Enzyme Linked Immunosorbent Assay (ELISA)

The effect of anti-estrone glucuronide polyclonal antiserum and monoclonal antibody on the peroxidase activity of the post DE reconstitution mixture of a HPE1G titration mixture was analysed using the following modification of the standard peroxidase activity assay (Section 5.2.6).

The peroxidase sample (20 μ L) was mixed with undiluted anti-estrone glucuronide polyclonal antiserum (unpurified, 243-4) or undiluted anti-estrone glucuronide monoclonal antibody (4 μ L) in a total volume of 200 μ L (tris buffer; 50 mM, pH 7.0) using a glass bead. The sample mixture was then incubated at 40°C for 1 minute, before the addition of 1.7 mL of 40°C citrate buffer (0.1 M, pH 5.3). The rest of the assay was the same as the standard peroxidase assay.

The assay was repeated without antiserum as a control and also with native horseradish peroxidase as a replacement for the reconstituted enzyme. The protein and enzyme content of the final assay mixture for the native horseradish peroxidase control was 0.149 pmoles while the protein content (as determined by Coomassie) and enzyme content (as determined by the A_{404}) of the reconstituted assay mixtures were 1.2 picomoles and 0.22 picomoles respectively.

The post DE reconstitution mixture of a HPE1G titration mixture was also analysed by a modification of the standard ELISA assay. In this modified method the horseradish peroxidase active ester estrone glucuronide conjugate was replaced with the post DE HPE1G reconstitution mixture. This analysis was performed using two dilutions of the 243-4 anti-estrone glucuronide antiserum (1 in 3,500 and 1 in 1,000) and four dilutions of the reconstituted sample. The reconstituted sample was applied at enzyme concentrations equivalent to 440, 660, 881 and 1,101 fmole well⁻¹. These concentrations are based on the estimated concentration of reconstituted protein in the sample, not total protein.

As a control, wells containing the active ester horseradish peroxidase - estrone glucuronide conjugate, as opposed to the reconstituted samples, were assayed at the same time using the same antiserum dilutions, and two enzyme concentrations. The active ester enzyme conjugate concentrations were 140 and 255 fmole well⁻¹. Thus the 140 and 255 fmole well⁻¹ control samples had enzyme contents which were from 1.7-4.3 and 3.1-7.9 fold greater respectively than the enzyme content of the reconstitution sample wells.

5.2.16 Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Polyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) by Enzyme Linked ImmunoSorbent Assay (ELISA)

The post DE reconstitution titration mixtures of the polyethyleneglycol based hemin estrone glucuronide compounds, HPAE1G and HPAAE1G, were analysed by ELISA using two different variations of the standard ELISA method. The conditions for the standard ELISA were a 20,000 fold dilution of anti-estrone glucuronide antiserum 243-4 and a horseradish peroxidase active ester estrone glucuronide conjugate concentration of 51 fmole well⁻¹.

In the first method, a non-competitive ELISA assay, the horseradish peroxidase active ester estrone glucuronide conjugate was replaced by the reconstituted hemin, HPAE1G and HPAAE1G horseradish peroxidase samples using two different protein concentrations. The two protein concentrations (255 and 1,020 fmole well⁻¹) were based on the calculated total protein in the sample, not the calculated level of reconstituted protein and were a factor of 5 and 20 fold higher than the horseradish peroxidase concentrations used in the standard active ester conjugate ELISA assay. The active ester conjugate at standard concentrations (51 fmole well⁻¹) was also assayed at the same time as a control.

The second method utilised a competitive ELISA format. In this method, a competition ELISA between the standard ELISA tracer, i.e. the active ester horseradish peroxidase estrone glucuronide conjugate, and the reconstituted enzymes, HRP-HPAE1G and HRP-HPAAE1G, for the anti-estrone glucuronide antibody was setup. This was performed using essentially the same assay as was used to produce the estrone glucuronide standard curve in the standard assay except that the free estrone glucuronide was replaced with the reconstituted enzymes.

The competition assays were performed using a constant concentration of the active ester conjugate (51 fmole well⁻¹) and antiserum (20,000 fold dilution). Competition between the active ester conjugate and the reconstituted horseradish peroxidase samples was set up using increasing molar ratios of the competitor (i.e. reconstituted protein) to the active ester conjugate across the plate by varying the concentration of the reconstituted samples. Molar ratios of 0 (no reconstituted protein), 0.125, 0.25, 0.50, 0.75, 1.0, 2.0, 3.0 and 4.0 were used. These ratios were calculated based on the concentration of reconstituted protein in the samples, not on the concentration of total protein.

This experiment was performed using horseradish peroxidase reconstituted from hemin as a control and an active ester estrone glucuronide conjugate as the signal generator control series. The reconstituted horseradish peroxidase hemin control series was performed under identical conditions to the competition assays described above except that the samples of horseradish peroxidase reconstituted with hemin-estrone glucuronide were replaced with the reconstituted horseradish peroxidase hemin samples. The active ester estrone glucuronide control series was performed without a competing ligand and was set up using a decreasing concentration of conjugate across the plate. The conjugate concentrations for this control series were 51.0, 46.4, 40.8, 33.7, 29.1, 25.5, 16.8, 12.8 and 10.2 fmole well⁻¹.

5.2.17 Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) by the Ovarian Monitor

In the Ovarian Monitor analysis of the pre-reconstituted horseradish peroxidase-HPAE1G and -HPAAE1G samples, the free estrone glucuronide in the standard Ovarian Monitor assay was replaced with the reconstituted samples as a source of estrone glucuronide. Apart from this substitution, the protocol for these assays was identical to the standard Ovarian Monitor procedure. The reconstituted enzymes were assayed using both the pre-coated estrone glucuronide assay tubes (Batch 24, lot 107-109) and a fresh reagent format (wet chemistry). The wet chemistry assays were performed using conditions optimised with the lysozyme-estrone glucuronide E3 conjugate (7.35 pmoles per assay) and anti-estrone glucuronide antiserum 243-4 ($\approx 0.058 \mu\text{L}$ undiluted per assay).

The hemin-E1G content of the post DE apo protein-reconstituted enzyme mixtures was estimated from the A_{404} values. These values were used to calculate the dilution factor necessary to dilute the conjugated hemin samples to an estimated hemin-E1G concentration of 0.074 and 0.222 nmol E1G mL⁻¹. These estrone glucuronide concentrations are equivalent to the estrone glucuronide concentration of a time diluted urine sample with an excretion rate of 267 and 800 nmol E1G 24 hr⁻¹ respectively and represent intermediate and high values on an estrone glucuronide standard curve optimised to measure the range of urinary estrone glucuronide excretion rates observed in the menstrual cycle. As well as the reconstituted horseradish peroxidase hemin-E1G samples, controls in the form of the equivalent estrone glucuronide standards and the horseradish peroxidase samples reconstituted with hemin were also assayed in the Ovarian Monitor.

Reaction mixtures without antiserum for the three reconstituted samples at the two concentrations were also assayed using the wet chemistry format.

A sample of DE eluent from a standard estrone glucuronide preparation was also prepared for analysis. Standard estrone glucuronide ($20,000 \text{ nmol E1G } 24 \text{ hr}^{-1}$) was added to some dry DE resin to form a thick slurry, left to stand for 10 minutes with intermittent mixing and then spun at speed 5 on a Gallenkamp bench centrifuge for 5 minutes. The eluent was then assayed for the presence of estrone glucuronide using the pre-coated Ovarian Monitor assay tubes and standard assay.

All assays were performed in duplicate.

The post lysis reaction mixtures from the Ovarian Monitor wet chemistry assays for the three reconstituted samples were assayed for peroxidase activity. These assays were performed on both duplicates of the with, and without, antiserum mixtures at both reconstituted enzyme concentrations. As the original assays were performed in duplicate, and each of these post lysis assay mixtures was assayed in duplicate, there was a total of four peroxidase assays for each condition.

The peroxidase assay was initiated by the addition of $150 \mu\text{L}$ of freshly prepared *o*-phenylenediamine substrate solution (Section 5.2.7.7) to $3.5 \mu\text{L}$ of the post lysis Ovarian Monitor sample in a 2 mL plastic cuvette. The peroxidase activity assay was stopped after 150 seconds by the addition of $50 \mu\text{L}$ of stopping solution (Section 5.2.7.8). The reaction mixture was then diluted by the addition of 1 mL of water, mixed by inversion and the absorbance of the resulting solution read at 490 nm on a Hewlett Packard Diode Array Spectrophotometer. The machine was blanked using a peroxidase assay mixture prepared from $3.5 \mu\text{L}$ of the Ovarian Monitor assay buffer (40 mM tris maleate).

5.3 RESULTS

5.3.1 Preparation of an Active Ester Horseradish Peroxidase Estrone Glucuronide Conjugate

Figure 5.4 shows the gel filtration elution profile of an active ester horseradish peroxidase - estrone glucuronide conjugate preparation.

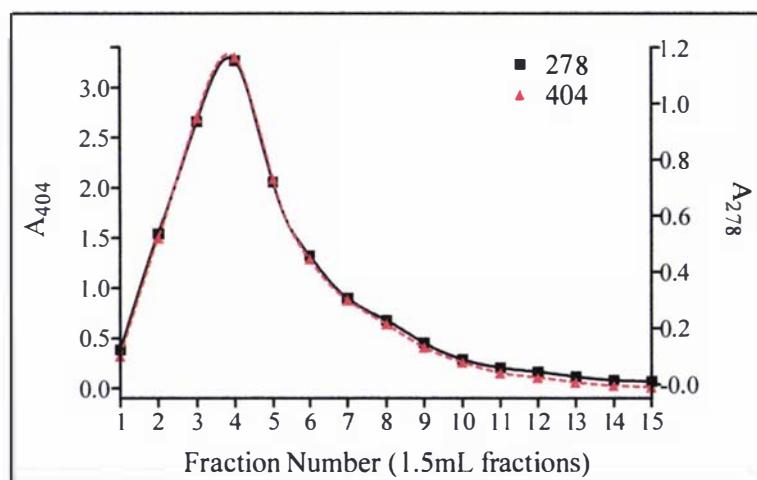


FIGURE 5.4: Active Ester Horseradish Peroxidase - Estrone Glucuronide Sephadex G-25 A_{278} and A_{404} Elution Profile

This profile was characterised by a steep leading edge and long tail which is typical of the protein elution pattern from a gel filtration column. The fact that the 278 nm and 404 nm absorption profiles were perfectly aligned with a constant RZ of 2.84 suggests that the sample was pure horseradish peroxidase protein and that little hemin had been lost during the synthesis and subsequent

processing of the active ester conjugate. Most of the protein eluted in the first eight fractions, and only the fractions with the highest absorbance (fractions 1-7) were combined and concentrated. After concentrating, the conjugate sample had an estimated concentration of 225 μM .

When assayed for activity, the active ester enzyme conjugate had an estimated peroxidase specific activity of $1,668 (\pm 30) \Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ enzyme compared to $1,651 (\pm 8) \Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ enzyme for the native enzyme. Thus it can be concluded that at a molar ratio of enzyme:steroid of 1:79, conjugation of the enzyme horseradish peroxidase with estrone glucuronide had no detrimental effect on the enzyme activity.

5.3.2 Adaptation of an Active Ester Horseradish Peroxidase Enzyme Linked Immunosorbent Assay (ELISA) for Estrone Glucuronide

5.3.2.1 Effect of Assay Time on the Position and Amplitude of the ELISA Standard Curve

The enzyme reaction of the ELISA assay for estrone glucuronide was terminated by the addition of stopping solution (2.5 M sulphuric acid) when the colour of the most highly developed wells on the plate was judged by eye to be of a moderately high to high intensity. The reaction was stopped using this arbitrary method as opposed to a constant time as the rate of colour development varied daily even when performed under apparently identical conditions. The source of this variation was never defined.

The use of this arbitrary method was justified by the following single plate experiment which used time as a variable. In this experiment the plate was blocked with a 10,000 fold dilution of antiserum (243-4), and the experiment was performed using the equivalent of 5 μL of estrone glucuronide (10-2,000 nmol E1G 24 hr^{-1}) and 25.5 fmole of conjugate per well. Each row of the plate represented a single standard curve, and each column a single estrone glucuronide concentration. The activity assay of the first standard curve (i.e. row) was stopped at 5 minutes and the activity assays of the remaining standard curves were stopped using a series of 5 minute intervals between rows.

Figure 5.5 illustrates the effect of colour development time on the estrone glucuronide standard curve as measured by the ELISA plate method and clearly shows the absorbance at all estrone glucuronide concentrations to increase with the assay time.

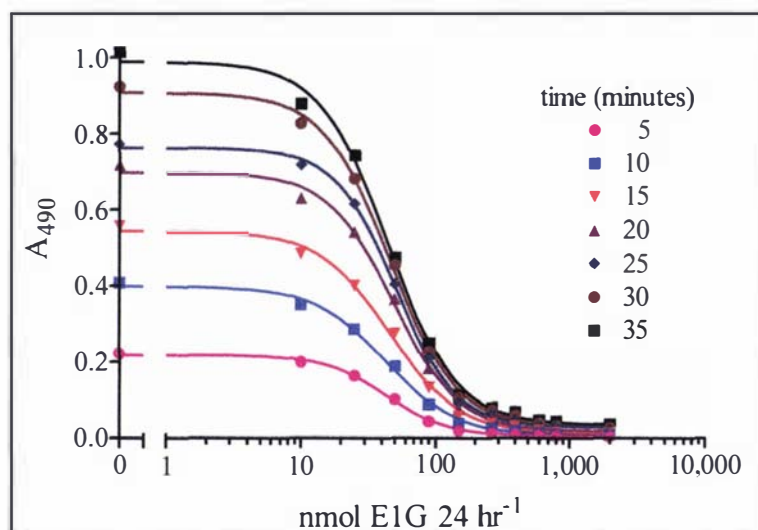


FIGURE 5.5: The Effect of Assay Time on the Position of the ELISA Estrone Glucuronide Standard Curve

Unlike all other ELISA plate data presented in this thesis which were derived from duplicate assays, the data for the standard curves presented in Figure 5.5 were obtained using a single data point at each estrone glucuronide concentration. However, the assay variation for this experiment was such that the shape and position of the standard curve could be obtained with a reasonable level of approximation for all time intervals.

Figure 5.6 shows the same data as in Figure 5.5 but expressed as Abs/Abs^0 i.e. the absorbance at any concentration of standard divided by the absorbance for the zero standard.

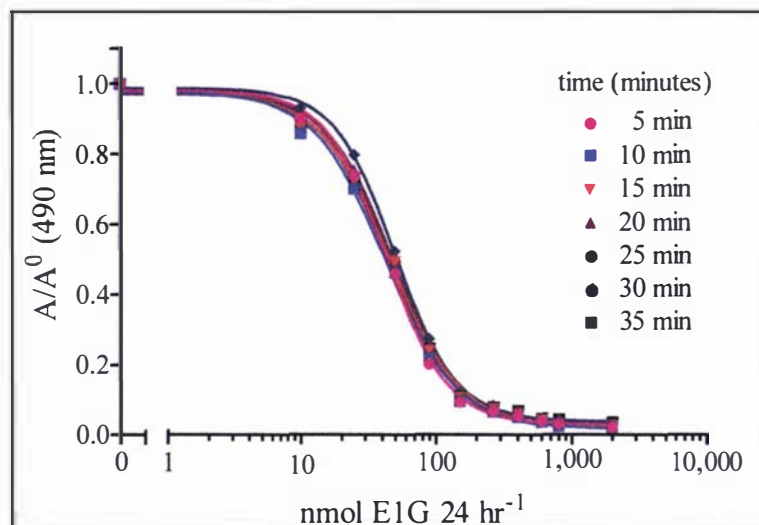


FIGURE 5.6: The Effect of the Assay Time on the Position of the ELISA Standard Curve Expressed Relative to the Zero Standard Absorbance

This resulted in the superimposition of all the standard curves. Thus the difference in the standard curves produced by stopping the enzyme reaction at different time intervals was one of amplitude alone.

5.3.2.2 Effect of the Active Ester Horseradish Peroxidase Estrone Glucuronide Conjugate Concentration on the Position and Amplitude of the ELISA Standard Curve

The effect of the conjugate concentration on the ELISA plate assay performance was examined by comparing the position of the standard curve obtained with four different conjugate concentrations.

In this experiment the plate was blocked using a 20,000 fold dilution of antiserum (243-4), and an equivalent of 5 μ L of estrone glucuronide standards (10-2,000 nmol E1G 24 hr⁻¹) was added to each well (13.8-278 fmoles). The lowest conjugate concentration examined was 12.75 fmole well⁻¹. The other conjugate concentrations were multiples of 2, 4 and 8 times this value (i.e. 25.5, 51 and 102 fmole well⁻¹). All estrone glucuronide concentrations on the four standard curves were assayed in duplicate, and the enzyme assay was stopped at 30 minutes.

The data for the four resulting standard curves are shown in Figure 5.7.

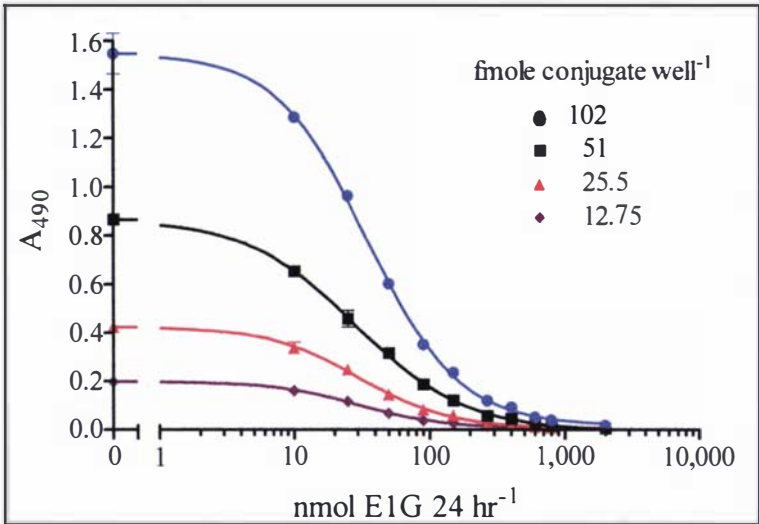


FIGURE 5.7: The Effect of Conjugate Concentration on the Position and Amplitude of the ELISA Estrone Glucuronide Standard Curve

The absorbance data displayed in this graph were obtained when the enzyme assay was stopped in all the wells based on the blank estrone glucuronide wells of the 102 fmole well⁻¹ conjugate concentration series reaching a high level of colour development. This graph showed that under these conditions, the degree of colour development obtained with each standard curve was directly proportional with the conjugate concentration. This relationship was most clearly illustrated by the absorbances of the maximally absorbing wells of each standard curve (i.e. the zero standard blank hapten wells). At the highest conjugate concentration (102 fmole well⁻¹), the zero standard had an absorbance (to the nearest 0.1 absorbance unit) of 1.6. At half this conjugate concentration (51 fmole well⁻¹) the maximum absorbance was approximately halved (0.8 absorbance units). This pattern was repeated for the 25.5 and 12.75 fmole standard curves which had maximum absorbances of 0.4 and 0.2 units respectively.

The true effect of a reduction in the conjugate concentration on the position of the standard

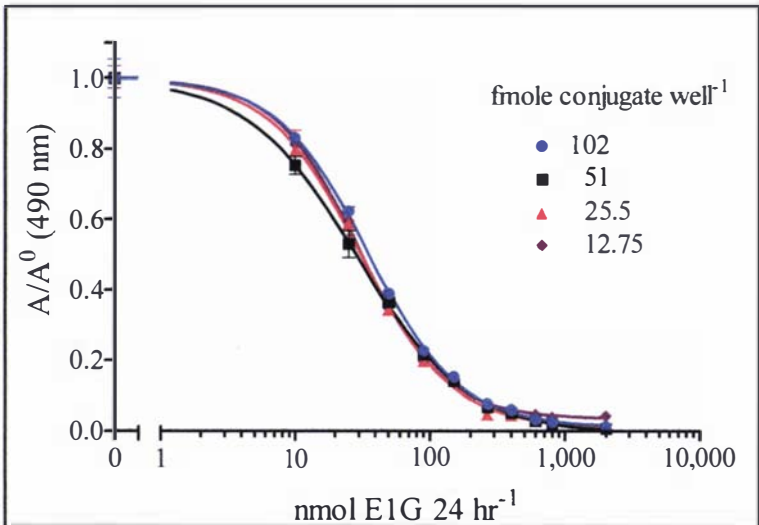


FIGURE 5.8: The Effect of Conjugate Concentration on the Position and Amplitude of the Estrone Glucuronide Standard Curve expressed Relative to the Zero Standard

This relationship was most clearly illustrated by the absorbances of the maximally absorbing wells of each standard curve (i.e. the zero standard blank hapten wells). At the highest conjugate concentration (102 fmole well⁻¹), the zero standard had an absorbance (to the nearest 0.1 absorbance unit) of 1.6. At half this conjugate concentration (51 fmole well⁻¹) the maximum absorbance was approximately halved (0.8 absorbance units). This pattern was repeated for the 25.5 and 12.75 fmole standard curves which had maximum absorbances of 0.4 and 0.2 units respectively.

curve is most clearly illustrated in Figure 5.8 where the absorbance data has been expressed again as A/A^0 . Within experimental error, scaling resulted in the superimposition of all the standard curves. Thus the position of the standard curve was not affected by the conjugate concentration, and differences in the standard curves produced with different conjugate concentrations but the same assay reaction time were of amplitude alone.

The graph in Figure 5.9 was generated using the same data as was used in Figures 5.7 and 5.8.

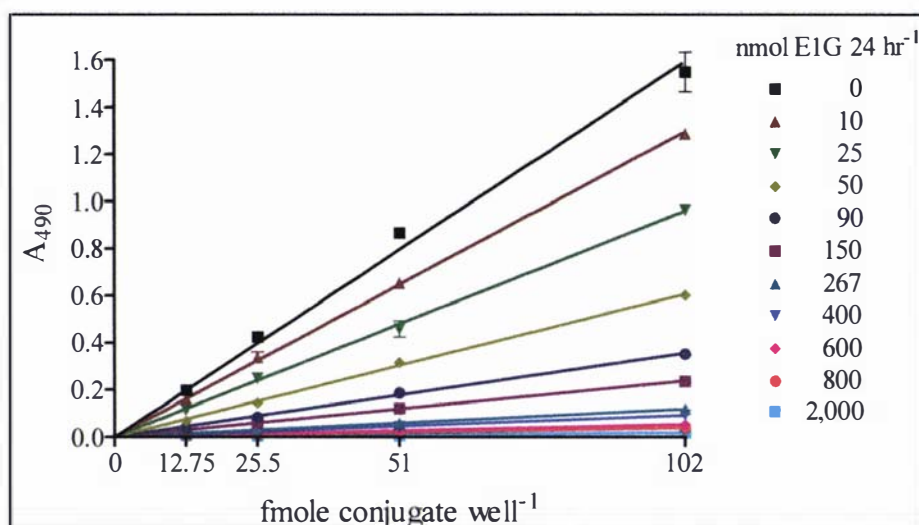


FIGURE 5.9: The Effect of Conjugate Concentration on the Absorbance for an ELISA at Different Estrone Glucuronide Concentrations

In this graph the conjugate concentration was plotted against the absorbance for each estrone glucuronide concentration. This graph clearly showed a linear relationship between the concentration of the conjugate signal generator and the level of colour development for all estrone glucuronide concentrations, with the level of the response decreasing with an increase in hormone concentration.

5.3.2.3 Effect of the Concentration of the Anti-Estrone Glucuronide Antiserum on the Position and Amplitude of the ELISA Standard Curve

The effect of the antiserum concentration on the ELISA plate assay performance was examined by comparing the position and amplitude of the standard curve obtained using four different dilutions (10,000, 20,000, 40,000 and 80,000) of anti-estrone glucuronide antiserum 243-4 to coat the wells.

In this experiment a conjugate concentration of 51 fmole well⁻¹, and 5 μ L of estrone glucuronide standards (5-2,000 nmol E1G 24 hr⁻¹) per well (which is equivalent to a range of 6.9-278 fmole E1G) were used. The activity assay was stopped when the blank estrone glucuronide wells of the highest antiserum concentration (10,000 fold dilution) were determined by eye to have reached a high level of colour development. This occurred at a reaction time of 30 minutes. All assays were performed in duplicate.

The data for the four resulting standard curves are shown in Figure 5.10.

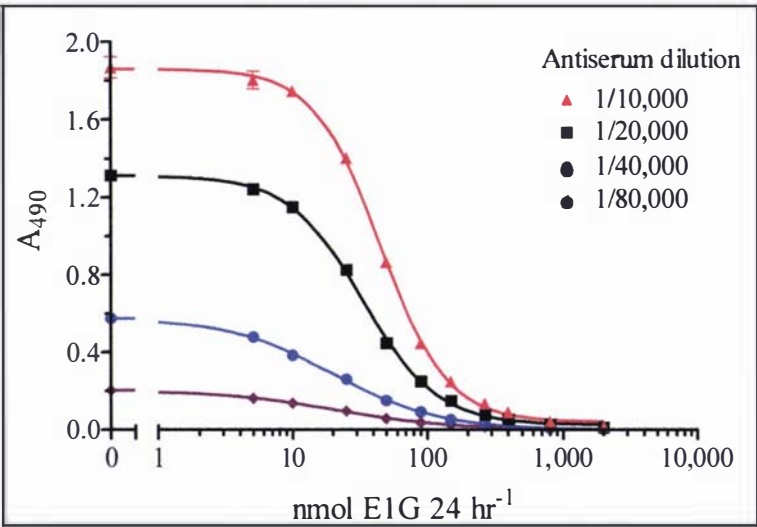


FIGURE 5.10: The Effect of the Amount of Antiserum on the Position and Amplitude of the ELISA Estrone Glucuronide Standard Curve

This graph shows that when the assay was stopped using the above criteria, the amplitude of the standard curves increased with the concentration of the antiserum.

Figure 5.11 shows the same absorbance data expressed as A/A^0 .

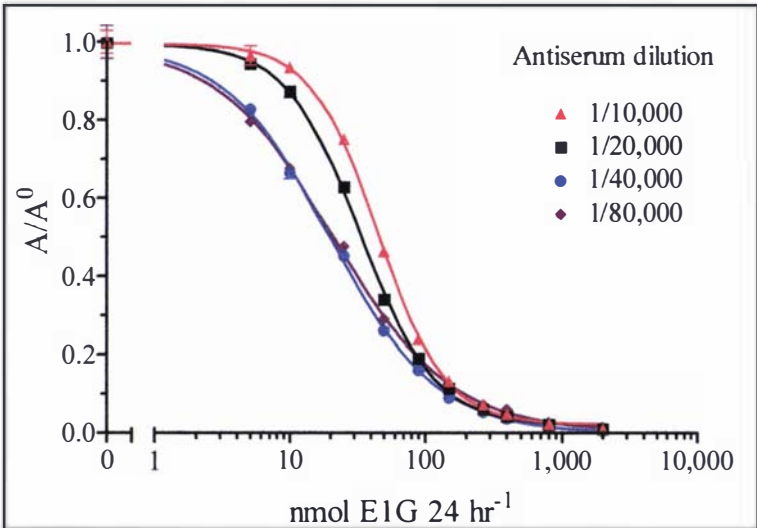


FIGURE 5.11: The Effect of the Amount of Antiserum on the Position and Amplitude of the ELISA Estrone Glucuronide Standard Curve expressed Relative to the Zero Standard Absorbance

Unlike when the conjugate concentration and the reaction time were varied, the position of the standard curve was shifted by changing the antiserum concentration.

The standard curves obtained with the two most diluted antiserum samples (40,000 and 80,000 dilution), appeared to be identical. As the antiserum concentration increased from these levels to a 20,000 fold dilution, and then to 10,000, the mid-point of the standard

curve (EC_{50}) was shifted progressively to a higher estrone glucuronide concentration range and the assay became increasingly less sensitive to low concentrations of the hormone. The standard curve mid-points were 46, 33, 20 and 21 nmol E1G 24 hr⁻¹ for the 10,000, 20,000, 40,000 and 80,000 fold antiserum dilutions respectively. Despite this shift in the standard curve the position of the standard curve at the higher end of the concentration range (150-2,000 nmol E1G 24 hr⁻¹) was largely unaffected by the antiserum concentration over the 10,000-80,000 fold dilution range.

5.3.3 Comparison of an Active Ester Horseradish Peroxidase Enzyme Linked Immunosorbent Assay (ELISA) for Estrone Glucuronide with Radioimmunoassay (RIA) and the Ovarian Monitor

5.3.3.1 Cycle 002F-C2

Figure 5.12 shows the daily urinary estrone glucuronide excretion rates as measured by RIA, the Ovarian Monitor and the ELISA method for cycle 002F-C2 of the WHO trial. The E1G excretion rate for day 5 as measured by the RIA method was not provided and the urine sample for day 1 was no longer available at the time of the ELISA assay.

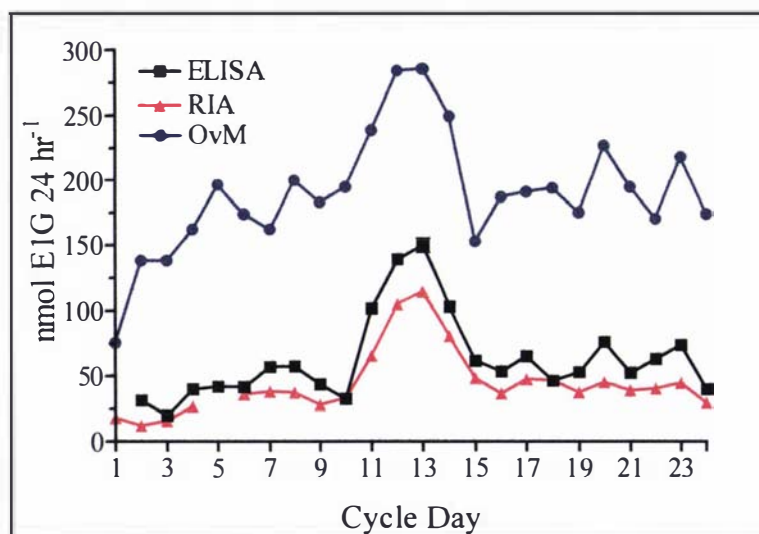


FIGURE 5.12: Estrone glucuronide Excretion Rates for Cycle 002F-C2 as measured by ELISA, RIA and the Ovarian Monitor

The general pattern of estrone glucuronide excretion as measured by the three methods was clearly similar and could be divided broadly into three phases - an initial baseline period, a large peak, and a second broad peak which was best described as an elongated elevation of the baseline. The three methods were in complete agreement as to the duration of the three phases and the peak day. The initial baseline period

covered the first 10 days of the cycle, and the large estrone glucuronide peak covered days 11 to 14 with the estrone glucuronide levels reaching a maximum for the cycle on day 13. The second estrogen peak covered the remaining 10 days of the cycle (days 15 to 24).

The agreement between the ELISA and the RIA data was particularly high, with the two sets of data rising and falling in strict unison over the length of the cycle. The correlation co-efficient (r) for the two sets of data was 0.969. Although not as high, the correlation between the WHO Ovarian Monitor data and the ELISA data was also very strong ($r=0.902$) showing a similar pattern of two small early peaks and three small late peaks relative to the main estrogen peak.

Visually, the most striking difference between the three profiles was in their position along the estrone glucuronide excretion rate axis. The difference in the excretion levels recorded for the Ovarian Monitor data were an average of 126 nmol 24 hr⁻¹ higher than those recorded for the ELISA assay, but the slope of the correlation (1.1) was very close to 1 (see Figure 5.13 and Table 5.1) showing that the two methods were measuring the same absolute changes in excretion rates. In turn, the excretion rates recorded for the ELISA assay were positively displaced relative to the RIA data. However, this effect was much more apparent at the higher excretion rates reflecting a difference in the gradient (0.738)

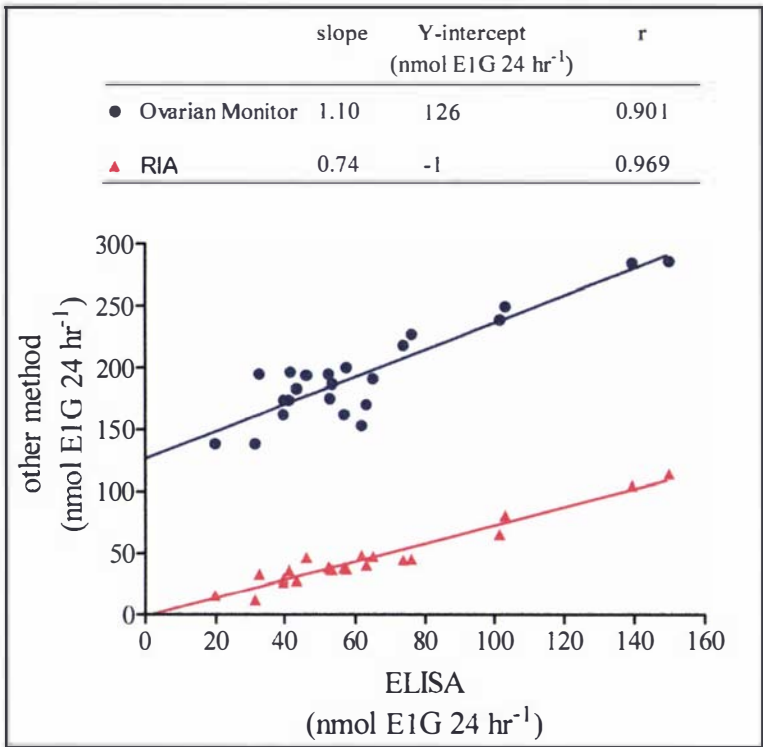


FIGURE 5.13: Least Squares Correlation of Estrone Glucuronide Excretion Rates for Cycle 002F-C2 for the ELISA Method with the RIA and Ovarian Monitor Methods

rates for the RIA, ELISA and Ovarian Monitor data were 156, 150 and 160 nmol E1G 24 hr⁻¹ respectively.

5.3.3.2 Cycle 003J-C2

For cycle 003J-C2 (Figure 5.14), the RIA data profile appears as a series of four lines since the RIA estrone glucuronide excretion rates for cycle days 4 and 5, 10 and 11, and day 25 were not provided.

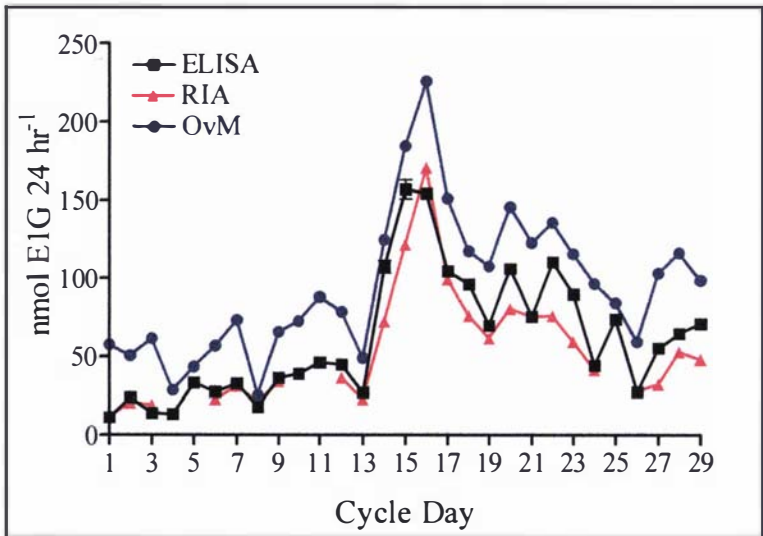


FIGURE 5.14: Estrone Glucuronide Excretion Rates for Cycle 003J-C2 as measured by ELISA, RIA and the Ovarian Monitor

of the ELISA-RIA correlation as opposed to the y-intercept. This vertical displacement of the profiles resulted in large discrepancies in the absolute excretion rates between the different methods. For example for the peak day, the rate of estrone glucuronide excretion was recorded as 115, 150 and 286 nmol E1G 24 hr⁻¹ for the RIA, ELISA and Ovarian Monitor data respectively. However, if the difference in intercepts between the ELISA and the Ovarian Monitor data, and the difference in slopes between the ELISA and the RIA data are taken into account, the peak excretion

As observed for cycle 002F-C2, the general pattern of estrone glucuronide excretion rates was very similar for the three methods, with all showing an initial baseline period of quiescent ovarian activity (days 1-13) followed by a mid-cycle estrone glucuronide peak (days 14-18) and an extended second less defined peak (days 19-29). The mid-cycle peak was defined unambiguously by

the RIA and the WHO Ovarian Monitor data as occurring on cycle day 16, while the ELISA data showed it as a two day peak (days 15-16).

The minor peaks and troughs in the estrone glucuronide excretion rates as measured by the ELISA method were largely reproduced by the RIA and Ovarian Monitor data. This was reflected by the high correlation co-efficients which were 0.945 and 0.949 for the ELISA-RIA, the ELISA-Ovarian Monitor pairing respectively.

The positive displacement of the Ovarian Monitor excretion rate data for this cycle relative to the ELISA data although clearly apparent was fairly low compared to cycle 002F-C2 at approximately 30 nmol E1G 24 hr⁻¹, and the slope for the ELISA-Ovarian Monitor correlation was again close to 1 (1.08) showing that the two methods were measuring the same absolute changes in E1G excretion rates. The excretion rates measured using the ELISA method were higher than the RIA data especially at the higher excretion rates (ELISA versus RIA slope=0.833), but due to the missing RIA values and the variation in the results this could not be absolutely defined. In terms of the peak day as defined by the Ovarian Monitor and RIA data (i.e. day 16), the estrone glucuronide excretion rates were 154, 171 and 226 nmol E1G 24 hr⁻¹ for the ELISA, RIA and Ovarian Monitor data respectively.

5.3.3.3 Cycle 009D-C2

Figure 5.15 shows the estrone glucuronide excretion rates for cycle 009D-C2 as measured by the three different immunoassay methods. The excretion rates as measured by the RIA method for cycle day 23 and as measured by the Ovarian Monitor (WHO) for days 23 and 24 were not available.

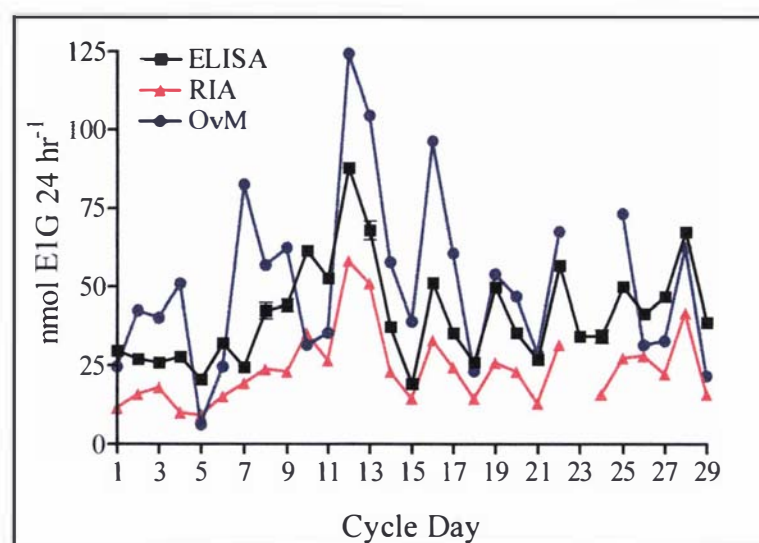


FIGURE 5.15: Estrone Glucuronide Excretion Rates for Cycle 009D-C2 as measured by ELISA, RIA and the Ovarian Monitor

Based on the normal cycle profile of a low initial baseline, a mid-cycle peak and a second broad, less well defined estrone glucuronide peak, the ELISA data generated the most acceptable cycle profile. According to the results obtained with the ELISA method, the initial baseline period covered the first seven days of the cycle and was associated with an excretion rate of about 25 nmol E1G 24 hr⁻¹. In the second phase of the cycle, the estrogen levels

slowly hunted upwards from days 8 to 11 to reach a maximum excretion rate of 88 nmol E1G 24 hr⁻¹ on day 12 and then fell to reach their post-ovulatory nadir (19 nmol E1G 24 hr⁻¹) on cycle day 15. The excretion rates increased on day 16 to reach

a value of 52 nmol E1G 24 hr⁻¹ and then fluctuated around this level for the rest of the cycle. This pattern of estrone glucuronide excretion was very closely followed by the RIA data, but the absolute values were about 30% lower (ELISA versus RIA slope=0.674).

Although the Ovarian Monitor data followed the same general excretion pattern as the RIA and ELISA data it was characterised by a more extreme profile in terms of the peaks and nadirs of E1G excretion. Large discrepancies in the Ovarian Monitor data relative to the ELISA and RIA data were also evident in the pattern of excretion in the immediate pre-ovulatory peak period. Thus whereas the correlation co-efficient was 0.944 for the comparison of the excretion rates measured by the ELISA and RIA methods, this was reduced to 0.645 for the ELISA versus WHO data.

However, in all methods the pattern of excretion over the main ovulatory peak (days 11-15) was highly reproducible, and the only difference was one of total amplitude. The estrone glucuronide excretion rate of the peak day which was the same for all methods (day 12) was calculated as 58, 88 and 124 nmol E1G 24 hr⁻¹ for the RIA, ELISA and WHO Ovarian Monitor data respectively.

5.3.3.4 Cycle 014X-C1

Figure 5.16 shows the daily estrone glucuronide excretion rates for cycle 014X-C1. The Ovarian Monitor data shown in Figure 5.16 were obtained after the World Health Organisation trial had been completed, by independent laboratory analysis as part of the work of this thesis. However at the time of analysis, the urine sample for day 20 was no longer available. Estrone glucuronide excretion rates as measured by the RIA method were also not available for cycle days 3 and 4, and 21.

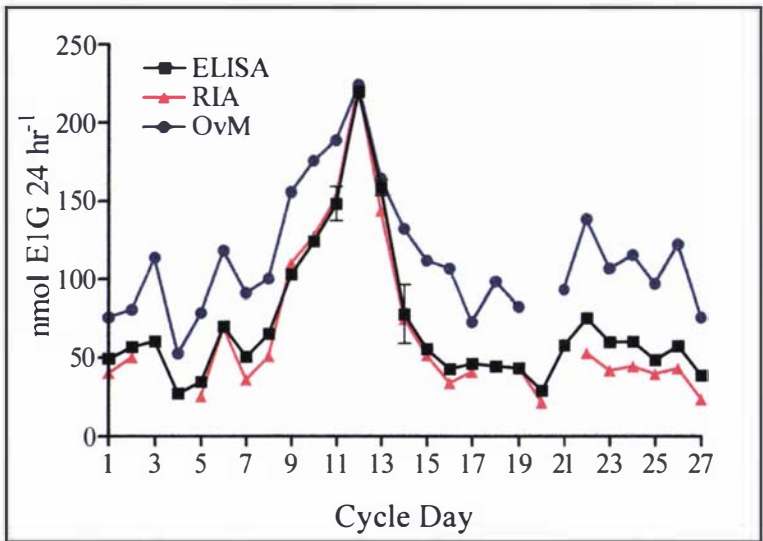


FIGURE 5.16: Estrone Glucuronide Excretion Rates for Cycle 014X-C1 as measured by ELISA, RIA and the Ovarian Monitor

The agreement between the estrone glucuronide excretion rate profile for the ELISA and RIA data for cycle 014X-C1 was clearly exceptional with the two methods resulting in essentially identical profiles (slope=1.07). This was reflected in the very high correlation co-efficient (0.990) observed for the two sets of data. The only real difference between them was a slight displacement of the RIA data downwards relative to the ELISA data

at the lower excretion rates (Y-intercept = -13 nmol E1G 24 hr⁻¹). However the superimposition of the main estrogen peak itself was almost perfect.

The Ovarian Monitor data showed a similar pattern of estrogen excretion as the ELISA and RIA data, with the excretion rates measured by the three methods rising and falling together. Differences between the profiles arose from some of the peaks and troughs measured by the Ovarian Monitor method being smaller or larger than those observed by the RIA and ELISA methods. However, these discrepancies were relatively minor, and the overall correlation co-efficient between the cycle data for the Ovarian Monitor and ELISA was 0.932. Once again, positive displacement of the Ovarian Monitor data on the concentration axis relative to the ELISA and RIA data was apparent. This was particularly obvious in the lower ranges which were an average of 54 nmol E1G 24 hr⁻¹ higher than the values recorded by the ELISA method.

All methods resulted in a very well defined ovulatory peak and agreed on the length of the initial baseline period (days 1-9), the ovulatory estrone glucuronide peak (days 10-14) and the post-ovulatory phase (days 15-27). The day of peak estrogen excretion (day 12) and its excretion rate (≈ 225 nmol E1G 24 hr⁻¹) determined by the three methods were also the same.

5.3.3.5 Cycle 014X-C2

Menstrual cycles 014X-C1 and 014X-C2 are from the same woman with cycle 014X-C2 immediately following cycle 014X-C1. As for cycle 014X-C1, the Ovarian Monitor data presented in this thesis for cycle 014X-C2 were collected by independent analysis after the World Health Organisation trial had been completed. The daily urinary estrone glucuronide excretion rates as measured by RIA, ELISA and the Ovarian Monitor method for this cycle are shown in Figure 5.17.

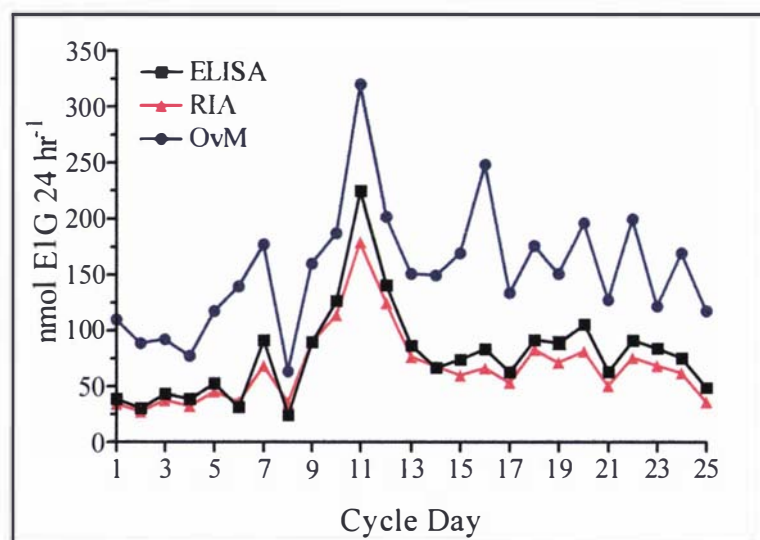


FIGURE 5.17: Estrone Glucuronide Excretion Rates for Cycle 014X-C2 as measured by ELISA, RIA and the Ovarian Monitor

As for the previous cycle, the agreement between the ELISA and RIA data was striking ($r = 0.983$) with the Ovarian Monitor data also having the same general pattern of estrogen excretion and all three methods showing a clear delineation between the three phases of estrogen excretion. As usual there was, relative to the ELISA method, a slight displacement in the excretion rate profile downwards for the RIA data (slope=0.79) and a

much larger displacement upwards for the Ovarian Monitor data (Y-intercept = 64 nmol E1G 24 hr⁻¹). The Ovarian Monitor data also showed the common feature of having exaggerated spikes and troughs relative to the ELISA and RIA data making the

excretion profile for this method more difficult to interpret and decreasing the correlation co-efficient between the Ovarian Monitor and the ELISA excretion data (0.879).

Despite the differences in the profiles, the three methods agreed on the lengths of the three phases (days 1-8, initial baseline phase; days 9-14, main estrogen peak; days 15-25, the post-ovulatory estrogen peak) and the day of peak estrogen excretion (day 11).

The rates of estrone glucuronide excretion as measured by the ELISA method for this cycle generated a profile with a classical menstrual cycle pattern. The baseline was low and stable, the main ovulatory peak was steep and clearly defined, and the post-ovulatory estrogen peak was more accurately described as a moderate elevation of the baseline as opposed to as a properly defined peak. In absolute terms (based on the ELISA results), the baseline levels fluctuated around 40 nmol E1G 24 hr⁻¹, the peak day had an excretion rate equivalent to 225 nmol E1G 24 hr⁻¹ and the excretion rate of the post-ovulatory phase fluctuated around 75 nmol E1G 24 hr⁻¹.

Although the similarity between this cycle, and the preceding cycle from the same woman (014X-C1) may not be readily apparent due to the different scales used on the concentration axes, a more careful comparison shows that they had similar rates and patterns of estrone glucuronide excretion, including the same peak excretion rate (225 nmol E1G 24 hr⁻¹). No other two cycles of the nine cycles examined in this study were so obviously alike.

5.3.3.6 Cycle 020K-C1

Figure 5.18 shows the daily estrone glucuronide excretion rates measured for cycle 020K-C1. RIA data were not available for cycle days 17 and 18.

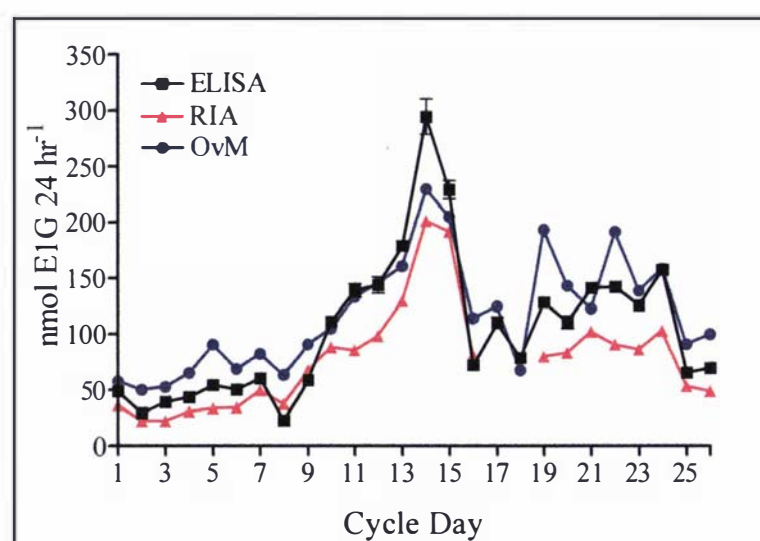


FIGURE 5.18: Estrone Glucuronide Excretion Rates for Cycle 020K-C1 as measured by ELISA, RIA and the Ovarian Monitor

The general estrone glucuronide excretion pattern as measured by the three different methods were again similar. The features described below were common to all three profiles. A low estrogen excretion rate in the initial baseline period (days 1-8), was followed by a long slow increase in excretion rates to reach a maximum on day 14. The ovulatory peak phase (days 9-16) ended in a dramatic fall on day 16 from which the estrogen levels recovered to

form a moderately sized ill defined peak until two days from the end of the cycle when the excretion rates fell again before the next menstrual bleed.

Once again the correlation between the ELISA and Ovarian Monitor data ($r = 0.916$) was poorer than that observed between the ELISA and RIA data ($r = 0.966$), and there was a distinct bias in the excretion rates measured by RIA and the Ovarian Monitor. Relative to the ELISA data, the RIA data showed a smaller change in absolute excretion rates (slope = 0.68). The Ovarian Monitor data also showed a smaller change in absolute excretion rates (slope = 0.72), in addition to its usual positive displacement which was for this cycle 43 nmol E1G 24 hr⁻¹. Hence, over the period of very high estrogen excretion rates the ELISA method recorded values higher than that measured by the Ovarian Monitor. Over this period the peak excretion, as measured by the ELISA method was 300 nmol E1G 24 hr⁻¹ which was higher than the excretion rates recorded by this method for any of the other eight cycles.

5.3.3.7 Cycle 021R-C2

For Figure 5.19 of cycle 021R-C2 of the World Health Organisation trial, the Ovarian Monitor data were obtained independently as part of this work, and were not provided by the World Health Organisation. No value was provided for cycle day 19 by the RIA method.

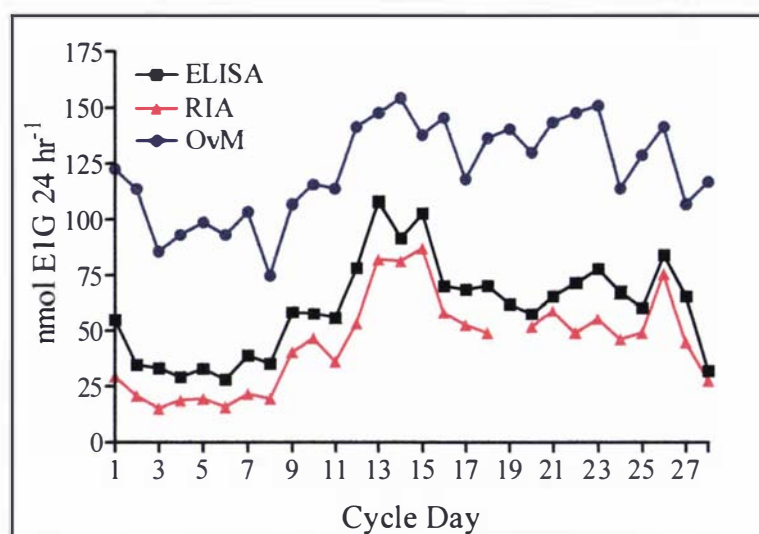


FIGURE 5.19: Estrone Glucuronide Excretion Rates for Cycle 021R-C2 as measured by ELISA, RIA and the Ovarian Monitor

The pattern of estrone glucuronide excretion rates as measured by the ELISA method was closely followed by the RIA data ($r = 0.961$), although once again the absolute values recorded for the RIA data were lower than those obtained by the ELISA method. This showed in the correlation between methods as a negative intercept and a low slope (-11 nmol E1G 24 hr⁻¹, 0.915 gradient).

Both the ELISA and RIA profiles began with a gentle fall in estrone glucuronide levels into a steady baseline (days 1-8) followed by a slow biphasic rise into a split peak (peak days 13 and 15). The estrogen excretion rates then fell (day 16) and stabilised at an intermediate level until the last few days of the cycle when they fell back to the levels observed at the beginning of the cycle. Apart from the splitting of the main estrogen peak, one of the major distinguishing characteristics of this cycle was its particularly low estrogen output. According to the ELISA method the rate of estrogen excretion never exceeded 110 nmol E1G 24 hr⁻¹.

The Ovarian Monitor profile was much more difficult to interpret, and although the data generally rose and fell with the ELISA data ($r = 0.806$), there were large discrepancies in the relative sizes of these changes, with the Ovarian Monitor data exaggerating many of the smaller peaks and troughs. Interpretation of the data was also further complicated by the poor discrimination between the ovulatory and post-ovulatory peaks which were of a similar amplitude. The Ovarian Monitor profile for this cycle also showed a high vertical displacement which had an average value of $74 \text{ nmol E1G } 24 \text{ hr}^{-1}$.

5.3.3.8 Cycle 023B-C1

Figure 5.20 shows the estrone glucuronide excretion rates for cycle 023B-C1. The RIA and Ovarian Monitor data were provided by the World Health Organisation. Excretion rates for cycle days 8 and 25, as measured by the RIA and Ovarian Monitor method respectively, were not available.

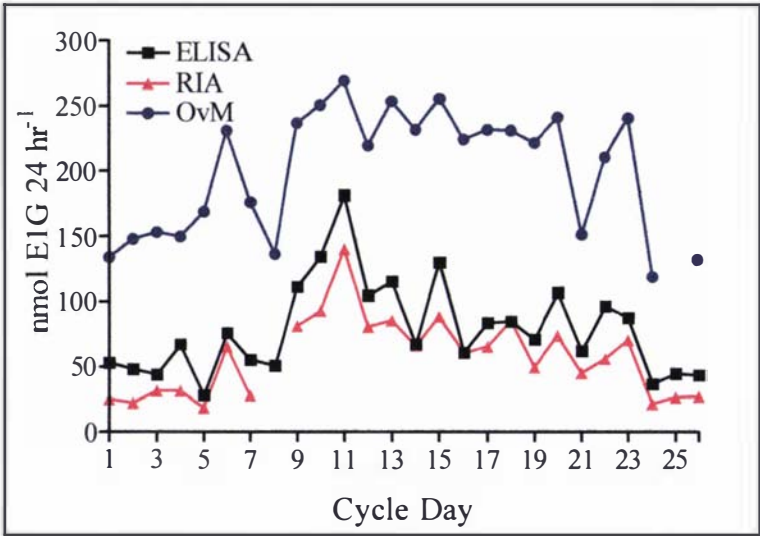


FIGURE 5.20: Estrone Glucuronide Excretion Rates for Cycle 023B-C1 as measured by ELISA, RIA and the Ovarian Monitor

Once again the excretion profile as measured by the RIA method ran parallel to the ELISA generated data ($r = 0.940$), with the excretion rates recorded by the ELISA method showing the characteristic vertical displacement from the RIA data being on average only 78% of the ELISA values. The Ovarian Monitor profile showed many similarities with the Ovarian Monitor profile from cycle 021R-C2. Relative to the ELISA data, minor peaks appeared as

spikes (e.g. day 6) and shallow dips as deep troughs (days 8 and 21), the ovulatory and post-ovulatory estrogen peaks were not clearly distinguished, and there was a large shift in baseline. From the correlation line the average bias in the results was $114 \text{ nmol E1G } 24 \text{ hr}^{-1}$.

The following description of this cycle's estrone glucuronide excretion pattern, is based on the ELISA results, the only method for which a complete set of values was available. The excretion rates of the initial baseline period (cycle days 1-8) fluctuated around $50 \text{ nmol E1G } 24 \text{ hr}^{-1}$ compared to the ovulatory peak (days 9-13) which reached a maximum of $170 \text{ nmol E1G } 24 \text{ hr}^{-1}$ (day 11), and the post-ovulatory peak (days 12-24) which had an average excretion rate of $80 \text{ nmol E1G } 24 \text{ hr}^{-1}$. This cycle also showed a return to a baseline level of excretion at the end of the cycle (days 24-26).

5.3.3.9 Cycle 023B-C2

Menstrual cycles 023B-C1 and 023B-C2 were from the same woman, with cycle 023B-C2 (Figure 5.21) following directly after cycle 023B-C1. As part of the World Health Organisation trial on the Ovarian Monitor all trial centres first measured the daily estrone glucuronide and pregnanediol glucuronide excretion rates for each cycle collected in their centre in the laboratory by the Ovarian Monitor method before sending them to the main trial centre in London where the cycles were re-analysed by the Ovarian Monitor and RIA methods. The Ovarian Monitor excretion rates presented in Figure 5.21 were obtained in the laboratory of the Palmerston North Centre. Estrone glucuronide excretion rates for cycle day 10 as measured by the RIA method were not available.

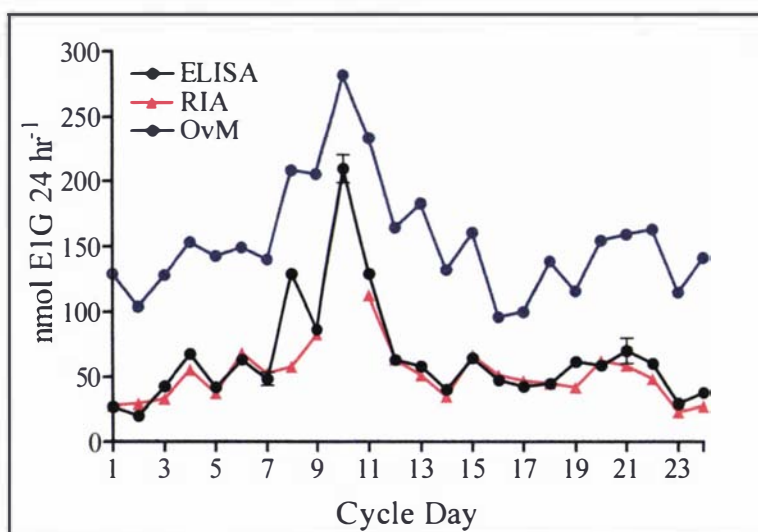


FIGURE 5.21: Estrone Glucuronide Excretion Rates for Cycle 023B-C2 as measured by ELISA, RIA and the Ovarian Monitor

A day by day analysis of this cycle shows that the estrone glucuronide excretion rates measured by the three immunoassay methods rose and fell together. The only obvious exception was day 8, which was observed as a spike for the ELISA data, a shoulder for the Ovarian Monitor data and a nadir for the RIA data. The disagreement between the ELISA and RIA data was particularly obvious. If day 8 was included in a

comparison between the two methods the correlation co-efficient (r) was 0.811, and if excluded this increased to 0.940. The correlation co-efficient between the Ovarian Monitor and ELISA data was 0.908.

Of the nine cycles analysed this cycle was the only cycle for which there was no obvious visual displacement between excretion rates of the RIA and ELISA data. However the Ovarian Monitor excretion data showed its usual positive bias relative to the ELISA data which in this case was 92 nmol E1G 24 hr⁻¹.

The following description of the cycle is based on the ELISA data. This cycle was particularly short (24 days) and although the baseline period could be strictly defined as ending on cycle day 8, the rate of estrogen excretion appeared to be increasing as early as day 3. For the first two days of the cycle the rate of estrone glucuronide excretion was particularly low (25 nmol E1G 24 hr⁻¹) and then fluctuated around 50 nmol E1G 24 hr⁻¹ for the rest of the baseline period. The main estrogen peak occurred between cycle days 9 and 13 peaking on day 10 with an excretion rate of 200 nmol E1G 24 hr⁻¹. The main peak was clearly defined from the baseline and post-ovulatory phases. In fact the rates of excretion of the post-ovulatory phase estrogen peak were very similar to those observed during the

later part of the baseline phase. At the very end of the cycle (days 23-24) the estrogen levels fell back to the levels observed during the first days of the cycle.

5.3.3.10 Correlation Summary of Ovarian Monitor, ELISA and RIA Estrone Glucuronide Excretion Rate Data for World Health Organisation Trial Menstrual Cycles

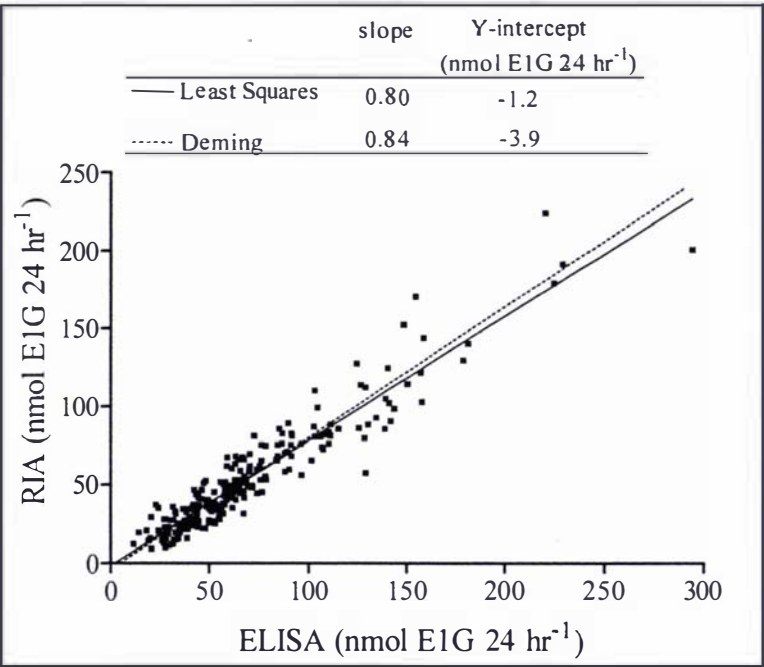


FIGURE 5.22: Comparison of the RIA and ELISA Data using the Deming and Least Squares Regression Methods

When the RIA and ELISA data were pooled, the overall correlations shown in Figure 5.22 were obtained. The two sets of data showed an excellent correlation using both the Deming and least squares regression models with a correlation co-efficient of 0.94, and gave similar slopes and intercepts. The intercept obtained was negligible, and the RIA values were on average between 80% (least squares) and 84% (Deming) of the ELISA values.

Correlation of the combined Ovarian Monitor and ELISA data by the two regression methods is shown in Figure 5.23.

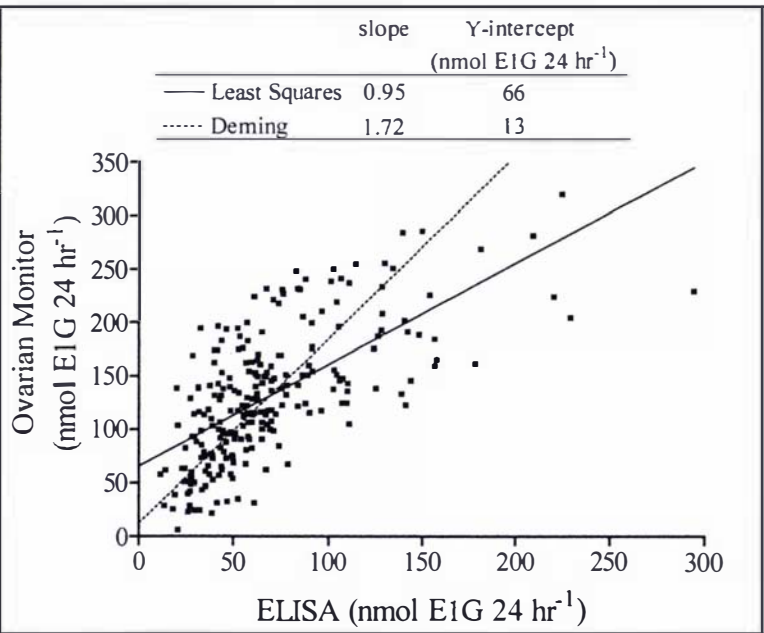


FIGURE 5.23: Comparison of the Ovarian Monitor and ELISA Data using the Least Squares and Deming Regression Methods

The correlation was markedly lower for the ELISA method with the Ovarian Monitor having a correlation co-efficient of 0.659. The method of analysis had a large affect on the parameters of the correlation line. A linear least squares analysis gave a line with an intercept of 66 nmol E1G 24 hr⁻¹ and a slope of 0.95 compared to the Deming's method which gave a line with an intercept of only

13 nmol E1G 24 hr⁻¹ and a slope of 1.72.

TABLE 5.1: Correlation Summary of the ELISA versus RIA and Ovarian Monitor Menstrual Cycle Data using the Least Squares Regression Method

Cycle	R*	N†	RIA		R*	N†	Ovarian Monitor	
			slope	intercept§			slope	intercept§
002F-C2	0.969	22	0.738±0.042	-1.21±3.01	0.901	23	1.098±0.116	126.40± 8.14
003J-C2	0.945	24	0.833±0.062	0.44±4.77	0.949	29	1.076±0.069	28.67± 5.02
009D-C2	0.940	28	0.674±0.049	-3.93±2.16	0.645	27	1.068±0.258	6.75±11.32
014X-C1	0.990	23	1.073±0.034	-13.03±2.95	0.932	26	0.841±0.067	53.71± 5.63
014X-C2	0.983	25	0.790±0.031	5.39±2.70	0.879	25	1.154±0.130	63.78±11.53
020K-C1	0.966	24	0.677±0.039	6.50±4.76	0.916	26	0.717±0.064	42.66± 7.82
021R-C2	0.961	27	0.915±0.053	-10.56±3.40	0.806	28	0.804±0.116	73.64± 7.40
023B-C1	0.940	25	0.784±0.058	-4.81± 5.01	0.812	25	1.073±0.161	114.90±14.07
023B-C2	0.811	23	0.603±0.095	16.26±6.03	0.811	24	0.971±0.096	91.68± 7.23
Overall	0.944	221	0.797±0.019	-1.21±1.52	0.659	233	0.950±0.071	65.73± 5.79

R* - correlation co-efficient; N† - number of data pairs; intercept§ - nmol E1 G 24 hr⁻¹.

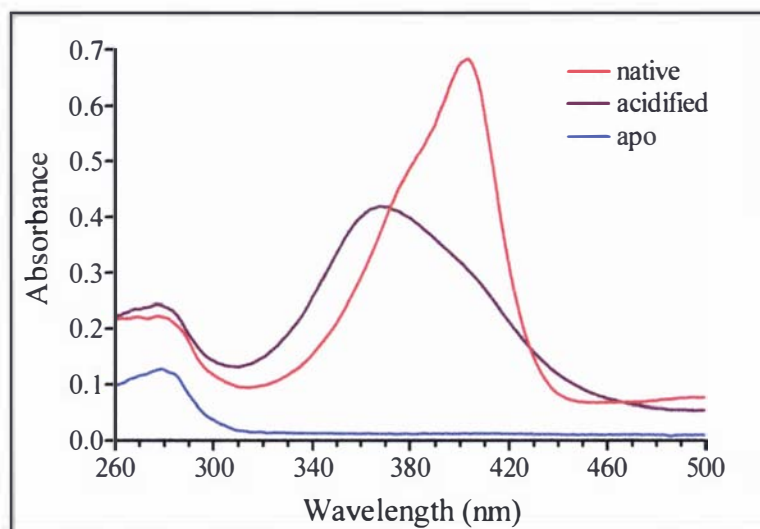
5.3.4 Data from a Typical Apo Protein Preparation

A concentrated horseradish peroxidase stock solution was prepared by dissolving 16.52 mg of commercial horseradish peroxidase powder in 1.83 mL of phosphate buffer (5 mM, pH 6.5) to give a 9.01 mg mL^{-1} solution by weight. According to the A_{404} value, the actual enzyme concentration was $158.1 \text{ } \mu\text{mol L}^{-1}$ or 6.72 mg mL^{-1} , thus the horseradish peroxidase content of the commercial sample was 75%.

During the acidification process, a total of 20 μmoles of hydrochloric acid was required to titrate 126 nmoles of native horseradish peroxidase (0.8 mL) from pH 6.71 to pH 2.01

Taking into account the volume and concentration changes from the acidification step (20 μL HCl added, 60 μL removed for assays, estimated 10 μL lost on the electrode), the methyl ethyl ketone (MEK) extraction was performed on 116 nmoles of protein (based on the A_{404} of the native enzyme) in a total volume of 750 μL . Although in most preparations, the MEK extraction was repeated a total of three times, in this example the extraction was only performed twice. Most of the colour left the aqueous layer in the first extraction, with all of the remaining colour removed in the second extraction. After the double extraction the volume of aqueous sample was estimated to be 800 μL .

The total amount of protein after the gel filtration step was estimated by the Coomassie blue method and the A_{278} absorption co-efficient to be 92.6 and 90.0 nmoles respectively. Thus the two methods agreed to within 5%. These yields corresponded to an average protein recovery from the combined MEK extraction and gel filtration step of 79% (91.3 nmoles) although yields as high as 90% were obtained for some preparations.



Spectra for the native, acidified and apo protein of this example preparation are shown in Figure 5.24.

FIGURE 5.24: Absorbance Spectra for Native, Acidified and Apo Horseradish Peroxidase

The key absorbance and peroxidase activity data from the above apo protein preparation are summarised in Table 5.2.

TABLE 5.2: Comparison of the RZ and Specific Activity of Native Horseradish Peroxidase, with its Acidified and Apo Forms

Sample	RZ (404/278)	$\bar{x}$ IR* ($\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$)	SD† (n-1)	CV‡ (%)	$\bar{x}$ IR* Relative to Native (%)
Native	3.08	1,905.7	30.86	1.6	100.00
Acidified	1.26	846.7	17.28	2.0	44.43
Apo protein	0.01	0.8	0.10	12.9	0.04

$\bar{x}$ IR* - mean initial rate; SD† - standard deviation; CV‡ - co-efficient of variation.

The native protein had an RZ value of 3.08 and a specific activity of 1,905.7 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$. The RZ and specific activity of the acidified sample were just over 40% of these native protein values. Almost all activity, and absorption at the 404 nm wavelength was removed in the apo-protein sample with only 0.04% of the native specific activity remaining.

Because of the broad range in the specific activities of the three samples, the observation that the co-efficient of variation of the specific activity data increased with a decrease in the specific activity of the sample was not unexpected. The intra-assay variation was fairly consistent for a given day, and although the level of variation reported in the above example was typical of the assay, at other times the variation was much higher. The source of this day-to-day variation was never defined.

5.3.5 Hemin and Substituted Hemin Titrations

5.3.5.1 Hemin Titration

The structure for hemin chloride is shown in Figure 5.1 (page 217).

Early hemin titrations were performed using hemin dissolved in a sodium hydroxide-tris buffer mixture. Approximately 1.5 mg of commercial hemin chloride ($M_r=652$) was weighed out accurately and dissolved in 0.6 mL of sodium hydroxide solution (0.1 M) and sonicated for 5 minutes to complete dissolution of the sample. Tris buffer (50 mM, pH 8.0) was then added to give a 0.46 mg mL^{-1} (706 μM) solution. However, it was decided that conditions were unnecessarily harsh and in later preparations the hemin was dissolved in dimethylformamide instead. In the alternative method, approximately 1.5 mg of commercial hemin chloride was weighed out accurately and dissolved in 200 μL of dimethylformamide. Tris buffer (50 mM, pH 8.0) was then added to give a 706 μM solution.

Figure 5.25 shows a typical example of a set of hemin titration curves which were obtained in the presence and absence of the horseradish peroxidase apo protein.

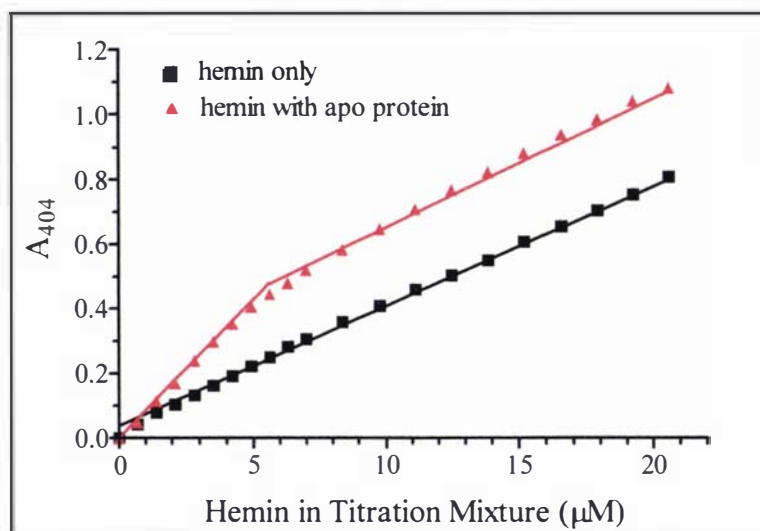


FIGURE 5.25: Hemin Titration Curve obtained in the Presence and Absence of Apo Protein

The two titration curves shown in Figure 5.25 were performed using an initial protein concentration in the apo protein titration mixture of 4.05 μM which was a little lower than the standard concentration used for the control hemin titrations (4.07 μM) of the modified hemin samples.

In the hemin only titration, the absorbance at 404 nm increased linearly with concentration and had a slope of $0.039 \Delta\text{Abs } \mu\text{M}^{-1}$.

In comparison, the hemin with apo protein titration consisted of two essentially linear phases with a small transition zone between them. The intersection point between the two gradients of the apo protein titration could be obtained by extrapolation and corresponded to an absorbance of 0.48 and a concentration of hemin in the reaction mixture of 5.58 μM .

The initial phase had a gradient of $0.086 \Delta\text{Abs } \mu\text{M}^{-1}$ and the second phase had a gradient of $0.040 \Delta\text{Abs } \mu\text{M}^{-1}$. Thus the gradient of the first phase was a factor of 2.15 higher than the second phase, and the second phase had a gradient very similar to the hemin only titration. The difference in the profiles between the with and without apo protein titrations reflects the change in hemin absorbance upon binding to the protein.

In Figure 5.24 it was shown that the apo protein had only a negligible absorbance at 404 nm compared to the native holo enzyme. Thus almost all the absorbance in a horseradish peroxidase solution at this wavelength must be due to the hemin. Although the hemin absorbs maximally at 404 nm when part of the holo enzyme, the absorbance spectra of the hemin in the free and protein bound state are quite different.

Figure 5.26 shows the absorbance spectra of a hemin chloride solution and a native horseradish peroxidase solution at the same molar concentration.

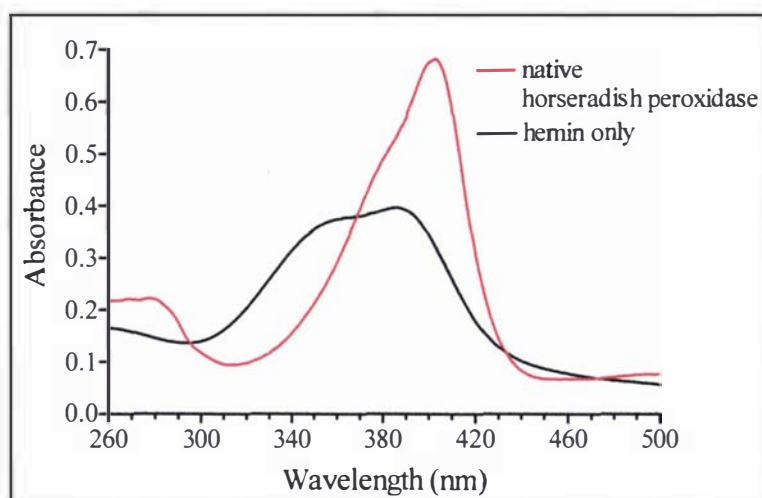


FIGURE 5.26: The Absorbance Spectra of Native Horseradish Peroxidase and Hemin Chloride obtained at a Molar Concentration of $6.32 \mu\text{M}$

The wavelength maximum and the maximum absorbance of the hemin are both lowered in the free hemin state relative to the native protein. Whereas the holo enzyme has a wavelength maximum of 404 nm, the wavelength maximum of the free hemin is 386 nm. Because of the change in wavelength maximum and absorbance maximum, the absorbance of the free hemin form at 404 nm is

dramatically lower than that observed for the native enzyme. Although, the absorption co-efficient for a compound is usually quoted using the wavelength maximum, which for the free hemin is $63,300 \text{ M cm}^{-1}$ at 386 nm, by using the apparent absorption co-efficient of the free hemin at 404 nm, the percentage of reconstituted apo protein can be estimated directly from the titration curve.

Because in the hemin only titration the absorbance increased linearly with concentration, it could be used to calculate an absorption co-efficient for free hemin at 404 nm. Using the hemin only titration data presented in Figure 5.25 the absorption co-efficient was calculated as $38,900 \text{ M cm}^{-1}$.

The total absorbance of the apo protein titration mixture is the sum of the absorbance due the reconstituted hemin and the absorbance due to the free hemin. This can be expressed by Equation 5.1.

$$Abs = \epsilon_{HRP-hemin}^{404nm} \cdot [HRP-hemin] + \epsilon_{hemin}^{404nm} \cdot [hemin]_f \quad \text{EQN 5.1}$$

The concentration of free hemin in the apo protein titration mixture is equal to the total concentration of hemin added to the reaction mixture minus the reconstituted fraction. This is expressed by Equation 5.2.

$$[hemin]_f = [hemin]_{total} - [HRP-hemin] \quad \text{EQN 5.2}$$

Equation 5.3 is derived from the substitution of Equation 5.2 into Equation 5.1 and allows the concentration of reconstituted enzyme to be calculated from the final absorbance of the titration mixture.

$$[HRP-hemin] = \frac{Abs - \epsilon_{hemin}^{404nm} \cdot [hemin]_{total}}{\epsilon_{HRP-hemin}^{404nm} - \epsilon_{hemin}^{404nm}} \quad \text{EQN 5.3}$$

When the concentration of reconstituted enzyme for the titration mixture shown in Figure 5.25 was calculated using this equation, a value of 3.93 μM was obtained which is equivalent to a total of 4.05 nmoles. The amount of apo protein in the initial reaction mixture was also 4.05 nmoles. Thus, based on the titration curve, the apparent reconstitution level of the apo protein was 100%. This result also suggested that the reconstitution is very rapid, reaching completion within the time frame of the experiment (25-35 minutes).

After the removal of any unbound hemin from the titration mixture by DE52 chromatography (DiNello and Dolphin, 1981), the reconstituted sample was assayed for activity (by the *o*-dianisidine assay), total protein content (by the Coomassie assay), and enzyme concentration (by using the A_{404}). These values were then compared with those obtained on the same day for a commercial native horseradish peroxidase preparation. This preparation was the same one which was used to prepare the apo protein used in the hemin titration.

The specific activity of the reconstituted titration mixture was 1,782 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein and 1,815 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ of reconstituted enzyme compared to the native enzyme which had a specific activity of 1,876 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein. Thus the specific activity of the reconstituted enzyme fraction was 3% less than that of the native enzyme. As the level of apo protein reconstituted with the hemin was 98%, the specific activity of the apo protein - reconstituted protein mixture was 95% compared to the same amount of the native enzyme.

Fresh titrations with native hemin were performed every time a new substituted hemin compound was tested, and the two samples processed in parallel. This allowed the activities of the new reconstituted compounds to be compared with the maximum theoretical rates and yields i.e. those obtained with the native hemin for the same batch of apo protein under the same conditions.

5.3.5.2 Hemin-Lysine-Estrone Glucuronide (6 or 7) Mono-Conjugate (HLysE1G)

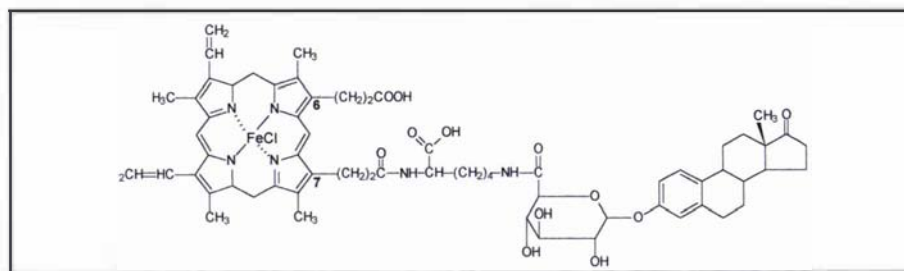


FIGURE 5.27: Hemin-Lysine-Estrone Glucuronide (6 or 7) Mono-Conjugate

The hemin-lysine-estrone glucuronide mono-conjugate (Figure 5.27) was prepared for titration by dissolving the dried sample in an aliquot of DMF followed by an aliquot of tris buffer (50 mM, pH 8.0) to give a final concentration of 706 μM by weight. The ratio of buffer to DMF used in preparing the sample was 1:4. The control titration was initiated using 1 μL increments for the first 10 μL . This was followed by 2 μL aliquots for the next 20 μL , 5 μL aliquots for the next 30 μL , and 10 μL aliquots for the final 90 μL . The control titration had a final A_{404} of 0.785 and a substituted hemin concentration (as calculated by weight) of 92 μM . The apo protein titration, which had a starting apo protein concentration of 8.1 μM , was performed using 2 μL aliquots for the first 30 μL , followed by 5 μL aliquots for the next 30 μL , and 10 μL for the last 90 μL to give a titration mixture with a final A_{404} of 1.202 and a calculated HLysE1G concentration of 141 μM . The apo protein HLysE1G titration curve and its control are shown in Figure 5.28.

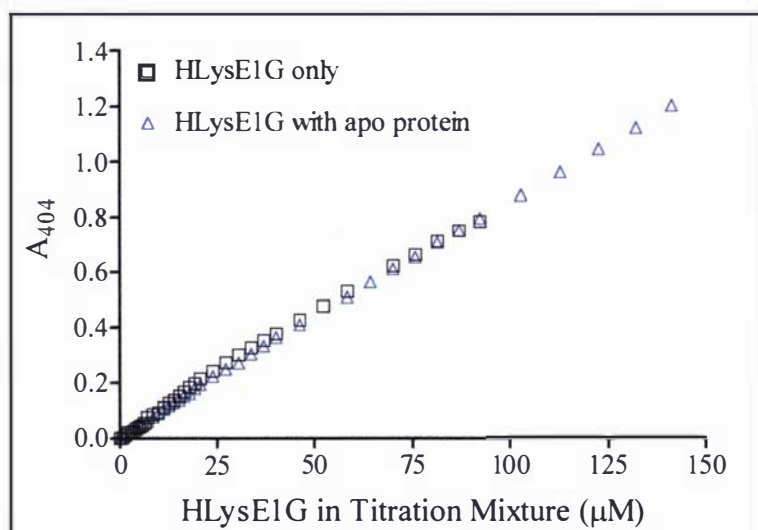


FIGURE 5.28: Hemin-Lysine-E1G Titration Curve obtained in the Presence and Absence of Apo Protein

The absorbance data for the titrations performed in the presence and absence of apo protein, both showed an essentially linear response to increases in HLysE1G concentrations, and had the same gradient. Thus there was no obvious difference in the two titration curves.

The enzyme activity of the titration mixture was 66 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$. This was equivalent to 4.5% of the

corresponding reconstituted horseradish peroxidase (i.e. hemin) rate which was performed on the same day. Because of the low activity of the titration mixture, the unbound HLysE1G was never separated from the apo protein - reconstituted enzyme mixture. Thus the percentage reconstitution of the apo protein by this substituted hemin and the activity of the reconstituted fraction were not calculated.

The HLysE1G compound was tested for cross-reactivity with anti-E1G antibody by the Ovarian Monitor E1G assay using pre-coated assay tubes. This assay showed a strong positive response.

5.3.5.3 Hemin-Lysine-Pregnanediol Glucuronide (6 or 7) Mono-Conjugate (HLysPdG)

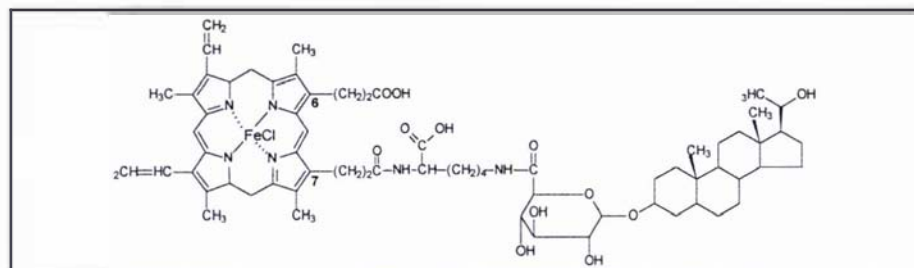


FIGURE 5.29: Hemin-Lysine-Pregnanediol Glucuronide (6 or 7) Mono-Conjugate

This sample (Figure 5.29) was prepared for titration using the same method as was used to dilute the HLysE1G sample (Section 5.3.5.2). The initial concentration of protein in the apo protein titration was 8.1 μM . The apo protein titration was performed using 1 μL increments for the first 12 μL and 2 μL aliquots for the next 30 μL . The final titration mixture had an A_{404} of 1.14 and had a theoretical total concentration of substituted hemin of 28.5 μM based on the weight of the sample. The control titration was performed using 1 μL aliquots for the first 10 μL and 2 μL aliquots for the next 20 μL to give a final titration mixture with an A_{404} of 0.732. The final theoretical concentration of substituted hemin in the reaction mixture was 20.6 μM . The HLysPdG with and without apo protein titration curves were essentially the same, both consisting of a single linear phase with the same gradient and gave a profile very similar to that obtained with the HLysE1G sample, except with a shallower gradient.

The enzyme activity of the titration mixture was 51 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ protein which was equivalent to 3% of the control hemin reconstituted horseradish peroxidase rate which was performed on the same day. On the basis of this low activity, no further work was performed on the HLysPdG titration mixture.

The HLysPdG mono-conjugate was checked for cross-reactivity using the pre-coated Ovarian Monitor PdG assay tubes and gave a positive test result for the presence of PdG.

5.3.5.4 Hemin-Methionine (6 and 7) Di-Conjugate (HMet(di))

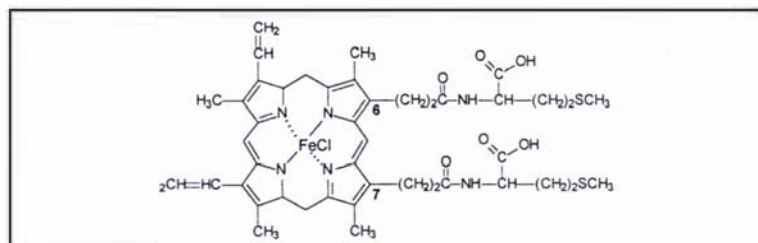


FIGURE 5.30: Hemin-Methionine (6 and 7) Di-Conjugate

This hemin di-methionine conjugate sample (Figure 5.30) was provided predissolved in DMF as a solution of unknown concentration. The apo protein was titrated using a series of 1 μL aliquots until an absorbance of 1.2 (404 nm) was reached (16 μL) and resulted in a mono-phasic and linear titration curve. The final titration mixture had a specific activity of $4.60 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$. This was equivalent to 0.7% of the reconstituted hemin - horseradish peroxidase control rate performed on the same day with the same batch of apo protein.

5.3.5.5 Hemin-Aminohexanoic Acid (6 or 7) Mono-Conjugate (HA)

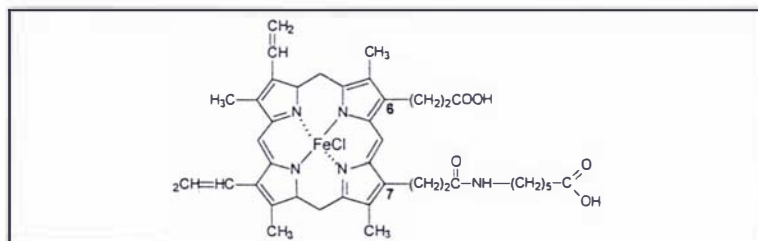


FIGURE 5.31: Hemin-Aminohexanoic Acid (6 or 7) Mono-Conjugate

The hemin-aminohexanoic acid sample (Figure 5.31) was provided predissolved in DMF as a solution of unknown concentration.

After titration the sample had a specific activity per nmol of protein of $328 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ of protein which was 53% of the control hemin rate immediately after titration. After the unreacted HA had been removed via DE chromatography, the apparent specific activity of the HA reconstitution mixture was $437 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ of protein. The level of reconstitution, based on the ratio of moles of reconstituted horseradish peroxidase (as measured by the A_{404}) and moles of total protein (as measured by the Coomassie assay), was 80% relative to the corresponding reconstituted hemin - horseradish peroxidase control. For the fraction of the total protein which reconstituted, the specific activity was $1,213 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ which was 86% relative to the reconstituted hemin control.

5.3.5.6 Hemin-Aminohexanoic Acid (6 and 7) Di-Conjugate (HA(di))

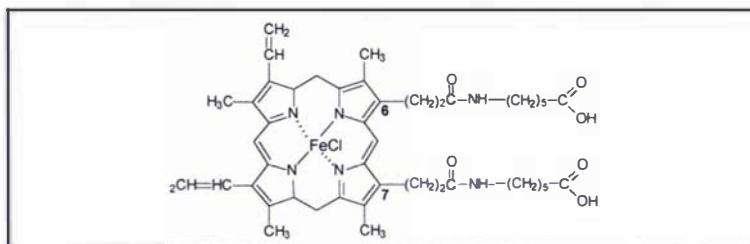


FIGURE 5.32: Hemin-Aminohexanoic Acid (6 and 7) Di-Conjugate

The aminohexanoic acid di-substituted hemin (Figure 5.32) was provided predissolved in DMF as a solution of unknown concentration, and the apo protein titration performed using an initial protein concentration of 4.06 μM . The final titration mixture had a specific activity of 69 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$, which was 9% of the specific activity of the control hemin with apo protein titration mixture. No improvement of the specific activity was obtained by leaving the titration mixture overnight.

After removal of the unbound HA(di) fraction and hemin fraction from the sample and control titration mixtures respectively by DE batch chromatography, the HA(di) sample had a total protein specific activity of 90 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ compared to the reconstituted hemin control which had a total specific activity of 628 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$. The reconstitution yield relative to the hemin control was 16% and when the specific activity was expressed per nmol of enzyme as measured by the 404 nm value the activity of the reconstituted fraction was 37% of the hemin control rate.

Figure 5.33 shows the absorption spectra for the free hemin, and mono- and di- substituted aminohexanoic acid samples.

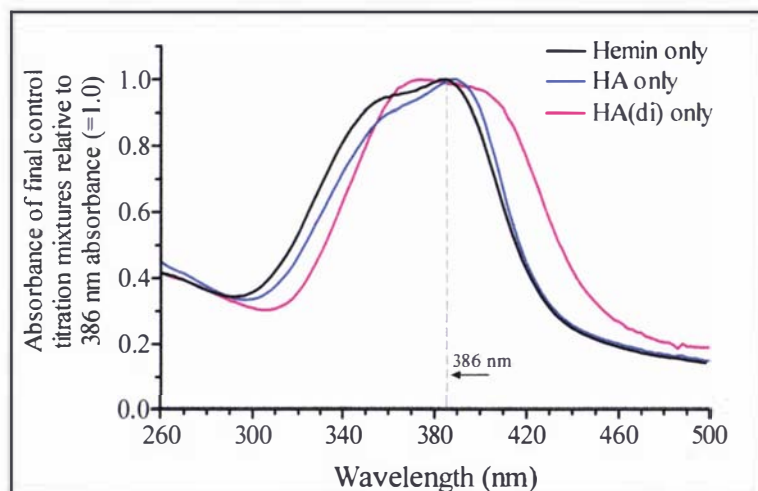
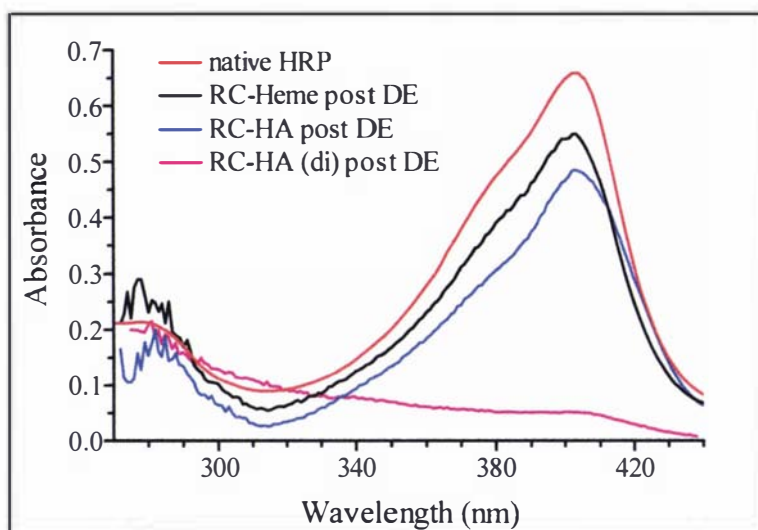


FIGURE 5.33: Absorption Spectra for Free Hemin, Mono-Substituted and Di-Substituted Hemin Aminohexanoic Acid

The hemin sample shows the characteristic hemin spectrum with an absorption maximum at 386 nm. The spectrum for the mono-substituted sample was essentially identical to the hemin spectrum except it was very slightly displaced towards a higher wavelength. The effect of the di-substitution on the hemin spectrum was more dramatic. The position of the spectrum was shifted approximately 20 nm to a higher

wavelength, the shape of the spectra became skewed towards a lower wavelength, and the absorption maximum was reduced to 360 nm.

The absorption spectra for native horseradish peroxidase and the reconstituted horseradish peroxidase protein mixtures (RC) after DE chromatography of the hemin control and the mono-substituted and di-substituted hemin aminohexanoic acid titrations are shown in Figure 5.34.



This Figure shows that the Soret band of the reconstituted samples is essentially identical between the reconstituted samples. The very low absorbance in the 404 nm region for the di-substituted hemin reflects the poor reconstitution of the protein with this sample.

5.3.5.7 Hemin-Aminohexanoate Methyl Ester (6 or 7) Mono-Conjugate (HAME)

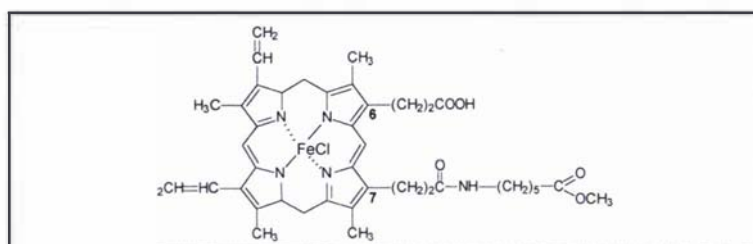


FIGURE 5.35: Hemin-Aminohexanoate Methyl Ester (6 or 7) Mono-Conjugate

The hemin-aminohexanoate methyl ester (Figure 5.35) and the hemin hexylamine (see Figure 5.36, Section 5.3.5.8) compounds were processed at the same time using the same hemin control titrations. The methyl ester was not readily soluble, and was ultimately dissolved in a 3:1 ratio of DMF to tris buffer (50 mM, pH 7.0). Although the sample didn't appear by eye to be as highly coloured as the standard hemin preparation, the titration data showed it to have as strong an absorbance at 404 nm as the hemin control sample.

The titration data of the HAME obtained in the presence and absence of apo protein is presented graphically in Figure 5.37 in the following Section with the hemin and hemin-hexylamine data which were performed at the same time. With respect to the HAME titration curves, the titration performed in the presence of apo protein had a gradient approximately 1.6 times higher than the HAME control providing strong evidence of HAME binding to the protein.

The hemin-aminohexanoate methyl ester had a specific activity after titration of $733 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$, which was 44% of the activity relative to the hemin control titration rate. However, after passage through the DE column this was reduced to 34%. The final specific activity of the reconstituted fraction corresponded to 39% of the activity of the reconstituted fraction of the hemin control.

5.3.5.8 Hemin-Hexylamine (6 or 7) Mono-Conjugate (HH)

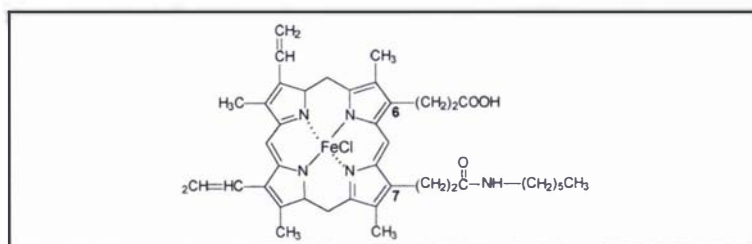


FIGURE 5.36: Hemin-Hexylamine (6 or 7) Mono-Conjugate

Due to the difficulties in preparing, drying and dissolving the hemin-hexylamine sample (Figure 5.36), the amount dissolved in DMF in preparation for the titration was unknown. The sample was added directly to the titration mixture without first diluting it with buffer.

The titration data for the hemin, hemin-hexylamine and hemin-aminohexanoate methyl ester in the presence and absence of apo protein are shown in Figure 5.37.

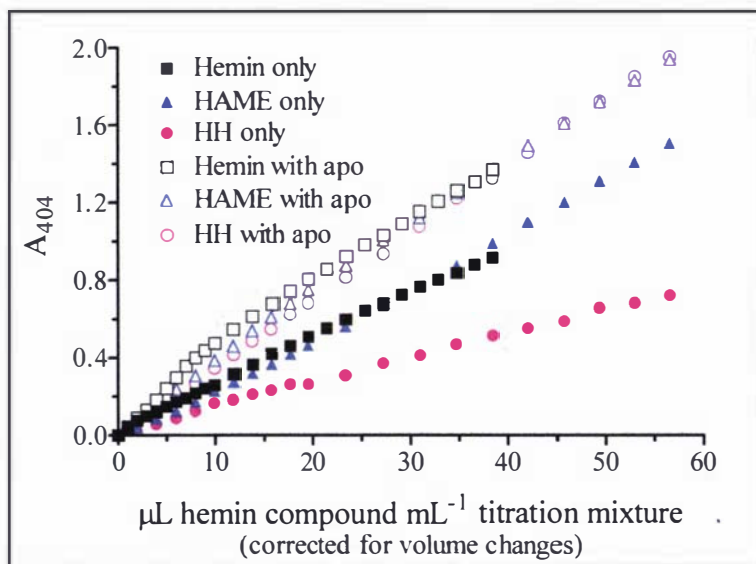


FIGURE 5.37: Hemin, Hemin-Aminohexanoate Methyl Ester and Hemin-Hexylamine Titration Curves obtained in the Presence and Absence of Apo Protein

The titration data for the hemin-hexylamine sample showed it to have a much stronger absorbance at 404 nm than a straight visual comparison of the stock sample with the standard hemin preparation would indicate, although its absorbance was not as strong as that of the hemin-aminohexanoate ester. The HH titration curve performed in the presence of apo protein had a gradient approximately 2.4 fold higher than the HH control line.

Figure 5.38 shows the absorption spectra for the hemin, HAME, and HH samples obtained in the absence of apo protein, and also in the presence of apo protein after the removal of any unbound sample by microscale DE chromatography.

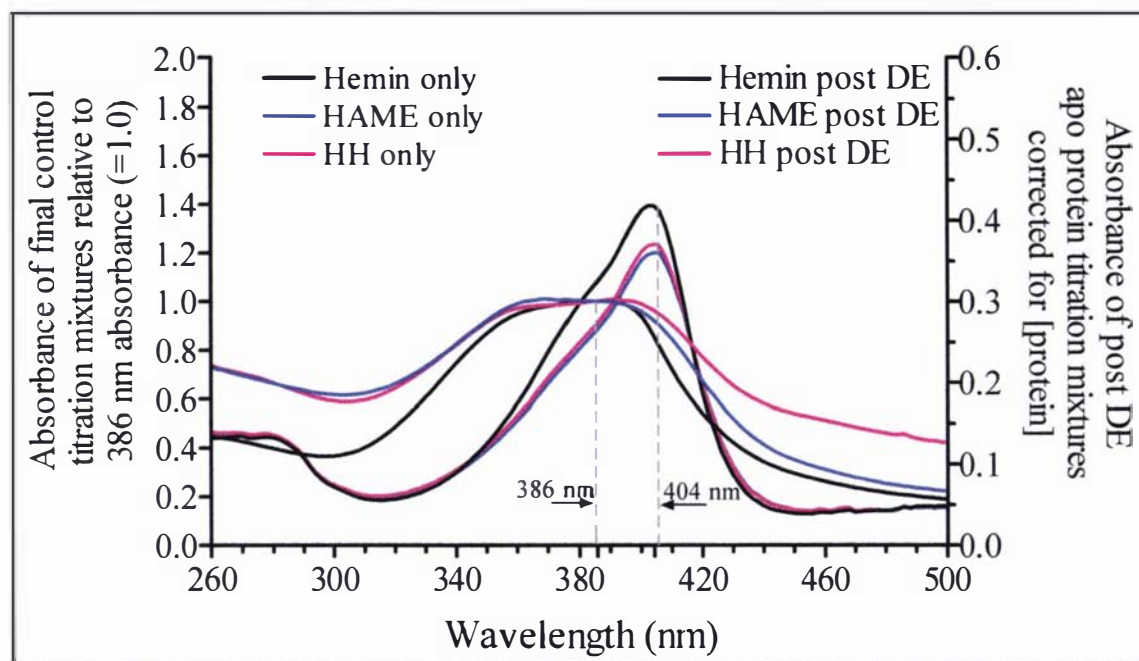


FIGURE 5.38: Absorption Spectra for Hemin, Hemin-Aminohexanoate Methyl Ester and Hemin-Hexylamine in the Free and Reconstituted Forms

This figure clearly shows the similarity of the HAME and HH spectra compared to hemin over the critical range for both the free form of the compound and when complexed with horseradish peroxidase to form the holo enzyme. As the true concentration of the substituted hemins in the titrations mixtures was not known, and to enable the different profiles to be more easily compared the protein free titrations were all expressed relative to the absorbance of the unsubstituted hemin sample at its 386 nm wavelength maximum. The two substituted hemins had the typical broad flat top spectrum of free hemin, although the overlap decreased with the distance from this absorbance plateau. The similarity of the spectra of the post DE reconstituted enzyme samples was even more striking. These are displayed in Figure 5.38 corrected for protein concentration as measured by the Coomassie Method. The superposition of the three samples at 278 nm showed the high correlation between the Coomassie method and A_{278} absorbance method for protein content. The shape of the main A_{404} peak also appeared to be identical for the three reconstitution enzyme spectra. The only difference between these three spectra was in the height of the A_{404} peak. The RZ values for the hemin post DE titration mixture was 3.14 while the RZ of both the HAME and HH post DE titration mixtures was 2.69.

The hemin-hexylamine sample had a specific activity after titration of $562 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ protein which was 34% of the rate obtained with the hemin control titration mixture. After chromatography of the sample through the mini-DE column, it had a specific activity of $753 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ protein and $835 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ enzyme. This was equivalent to 44% of the specific activity per nmole of protein and 50% of the specific activity per nmole of enzyme which was obtained with the corresponding

reconstituted hemin control sample. In this example, these rates also reflected the level of activity relative to the native sample as the hemin titration mixture had a similar level of reconstitution and activity as the native enzyme.

5.3.5.9 Hemin-Phenylalanine Methyl Ester (6 or 7) Mono-Conjugate (HPheME)

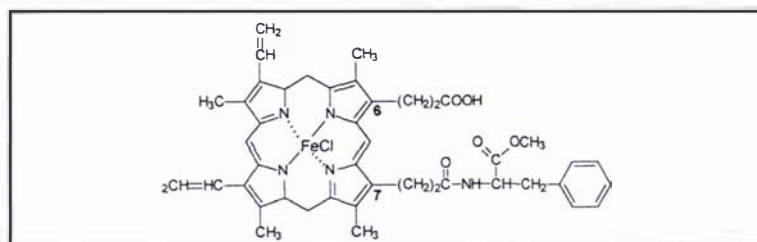


FIGURE 5.39: Hemin-Phenylalanine Methyl Ester (6 or 7) Mono-Conjugate

This hemin-phenylalanine methyl ester (Figure 5.39) was also only partially soluble in DMF. After enough DMF had been added to dissolve most of the sample, the insoluble fraction was left to settle, and the soluble fraction siphoned into a clean container. The soluble fraction was used for all titrations. The control and apo protein titrations were both performed using a total volume of 40 μL of HPheME. The HPheME only titration had a final absorbance of 1.28 and the HPheME with apo protein titration had a final absorbance of 1.75.

Immediately after titration the apo protein HPheME titration mixture had a specific activity of 59 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$, which was 5% of the hemin control titration rate performed on the same day. After passage through the DE column the specific activity of the HPheME titration mixture relative to the reconstituted horseradish peroxidase - hemin control was 5% per nmol protein, and 17.8% per nmol of enzyme.

5.3.5.10 Hemin-Methionine Methyl Ester (6 or 7) Mono-Conjugate (HMetME)

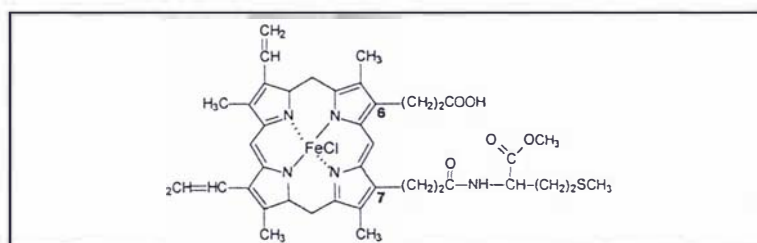


FIGURE 5.40: Hemin-Methionine Methyl Ester (6 or 7) Mono-Conjugate

The titrations with the methionine methyl ester (Figure 5.40) and the proline methyl ester (Figure 5.41, Section 5.3.5.11) were performed at the same time and were worked through together using the same hemin control titration. Both samples were only partially soluble in DMF, with the solubility of the proline sample being particularly low. The insoluble fraction was removed by centrifugation on a bench centrifuge (Gallenkamp, speed 5). Immediately after titration, the HMetME with apo protein sample had a specific activity of 82 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ which was 10% of the hemin titration control rate. Within

the limits of experimental error, storing the titration mixture at 4°C overnight had no effect on the activity.

After removal of the unbound HMetME and concentration of the subsequent sample, the titration mixture had a specific activity of $169 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ and $582 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ enzyme}$. This was equivalent to a 38% reconstitution yield relative to the hemin control and 29% relative to the native enzyme. The activity of the reconstituted fraction was 37% relative to the reconstituted hemin - horseradish peroxidase fraction and 31% relative to the native enzyme.

5.3.5.11 Hemin-Proline Methyl Ester (6 or 7) Mono-Conjugate (HProME)

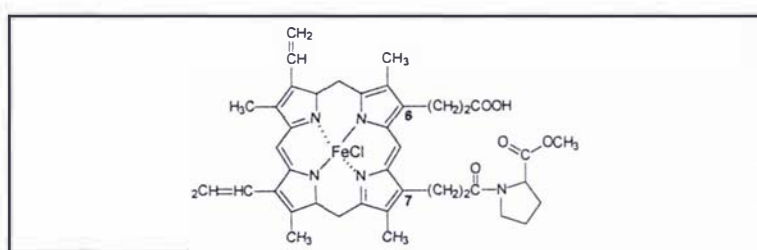


FIGURE 5.41: Hemin-Proline Methyl Ester (6 or 7) Mono-Conjugate

The preparation of the hemin proline methyl ester sample (Figure 5.41) is described in Section 5.3.5.10. Immediately after titration, the HProME with apo protein sample had a specific activity of $301 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ which was 38% of the hemin control rate. As for the HMetME titration mixture, there was no obvious change in the activity of the mixture after overnight storage at 4°C.

After removal of the unbound HProME and concentration of the subsequent sample, the titration mixture had a specific activity of $432 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ and $795 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ enzyme}$. This was equivalent to a 54% reconstitution yield of the apo protein and 71% relative to the apo protein - hemin control. For the reconstituted fraction, the enzyme activity was 43% of the native peroxidase rate and 50% relative to the reconstituted fraction of the hemin control.

5.3.5.12 Hemin-Diaminopolyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPE1G)

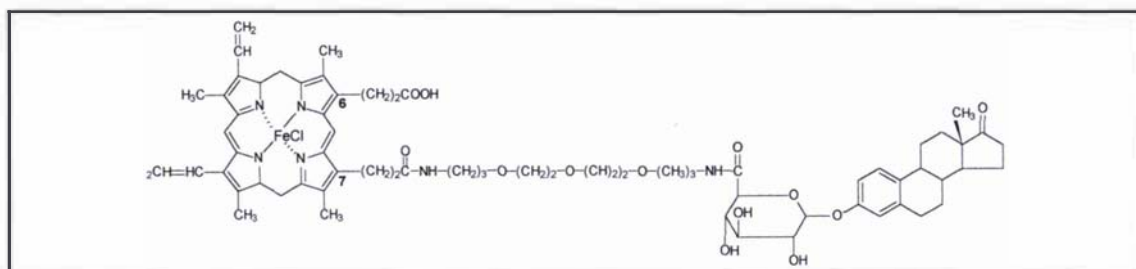


FIGURE 5.42: Hemin-Diaminopolyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate

The HPE1G sample (Figure 5.42) was prepared by dissolving it in 50 μL of DMF and then diluting a further 1 in 4 with tris buffer (50 mM). The titrations were performed using a total volume of 30 μL of dissolved sample. The titration profiles of the with and without apo protein titrations for the hemin control and HPE1G sample are shown in Figure 5.43 and the absorption spectra of the control without apo protein final titration mixtures are shown in Figures 5.46 (Section 5.3.5.14).

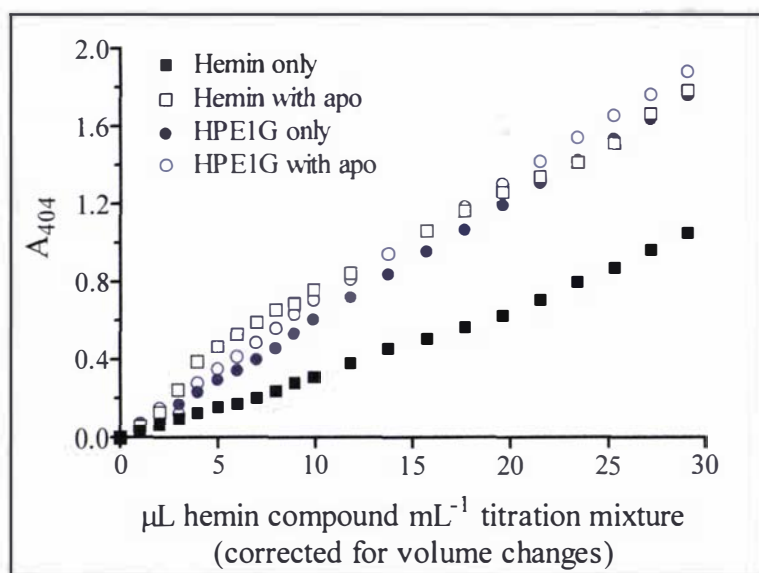


FIGURE 5.43: Hemin and Hemin-Diaminopolyethyleneglycol Estrone Glucuronide Titration Curves obtained in the Presence and Absence of Apo Protein

The final apo protein/HPE1G titration mixture had a final absorbance at 404 nm of 1.89 units which was marginally higher than the control HPE1G only titration which had a final absorbance of 1.76 units.

Immediately after titration the apo protein/HPE1G mixture had an activity of 178 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ of protein which was equivalent to 15% of the activity of the corresponding hemin control titration. The final

activities of the HPE1G sample after DE chromatography were 258 and 914 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ of protein and enzyme respectively which were equivalent to 14% and 48% of the horseradish peroxidase - hemin titration control values. The value for the specific activity of the post DE total protein sample was 52 fold higher than the apo protein control, and the final reconstitution yield of the apo protein with the HPE1G sample was 29%.

The absorption spectra of the post DE hemin and HPE1G reconstitution mixtures are given in Figure 5.47 (Section 5.3.5.14).

5.3.5.13 *Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPAE1G)*

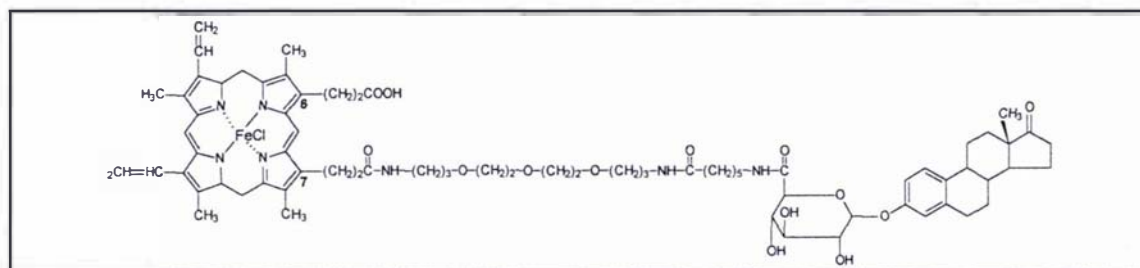


FIGURE 5.44: *Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate*

The structure of HPAE1G is shown in Figure 5.44. This sample (1.06 mg) was dissolved in 230 μL of DMF to give a light-to-medium green coloured solution. Due to the low intensity of the colour compared to the hemin control which was almost black, the HPAE1G sample was used directly in the titrations without further dilution with the tris buffer, and a total of 50 μL of the prepared sample was used in both the control and apo protein titrations.

After titration, the A_{404} value was 0.883 for the HPAE1G control and 0.983 for the apo protein/HPAE1G mixture. The specific activity of this mixture was 88 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein, compared to the control apo protein/hemin titration which had an activity of 1511 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein and the native horseradish peroxidase sample which had a specific activity of 1538 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein. Thus the reconstitution and reactivation of the apo protein appeared to be almost complete for the hemin titration. The specific activity of the hemin titration mixture relative to the native rate was 98%, while the specific activity of the apo protein/HPAE1G mixture was comparatively low at only 5.8% of the hemin control rate.

The apo protein titration mixture was left to incubate overnight at 4°C and reassayed the next day. This resulted in a 2.2 fold increase in rates relative to the previous day to 197 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein compared to the hemin control rate which was unchanged. Thus relative to the specific activity of the HPAE1G sample had increased to 13% of the control rate.

A further overnight incubation at 4°C, did not result in any further increases in specific activity. Thus, at this point further incubation steps were abandoned and the protein was purified from any unbound hemin using the DE resin and then concentrated.

The purified sample had an RZ value of 1.21, and a specific activity of 236 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ protein and 668 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ enzyme, and the specific activity of the native sample for this day's assays was 1808 $\Delta\text{Abs min}^{-1} \text{nmol}^{-1}$ enzyme. Thus, 35% of the apo protein was successfully reconstituted with the HPAE1G to give a holo enzyme with 37% of the activity of the native sample. In comparison the reconstitution yield with the hemin sample was 100% with a full recovery of the apo protein's specific activity back

to the native enzyme levels. Spectra of the HP AE1G final titration mixture control and the post DE protein/HP AE1G mixture are given in Figures 5.46 and 5.47 (Section 5.3.5.14).

5.3.5.14 Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPAAE1G)

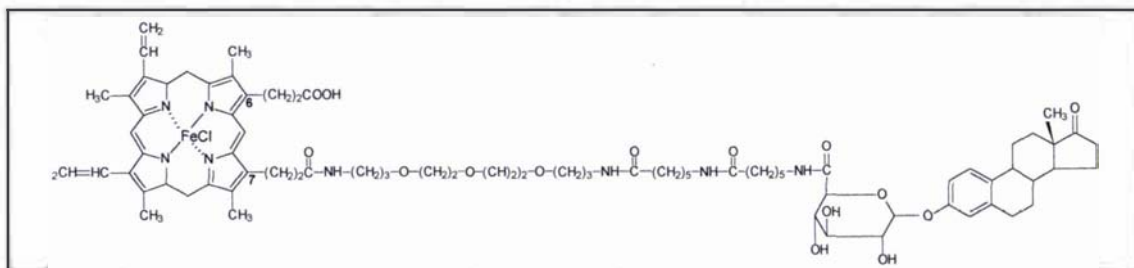


FIGURE 5.45: Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate

The HPAAE1G sample (Figure 5.45) which was used in the following titration was suspected to be contaminated with up to 20% free estrone glucuronide. This sample was analysed at the same time as the HP AE1G sample and hemin sample reported in Section 5.3.5.13. When the sample (1.63 mg) was dissolved in DMF (283 μ L), the resulting solution was a light green. Due to the apparently dilute nature of the sample it was not further diluted with tris buffer as was the usual protocol. The titration was performed using a total of 72 μ L of the prepared sample in both the control and apo protein titrations. The final absorbance (404 nm) of the two titrations was 0.891 and 0.948 respectively.

Figure 5.46 shows the spectra of the control hemin, HPE1G, HP AE1G and HPAAE1G titration mixtures.

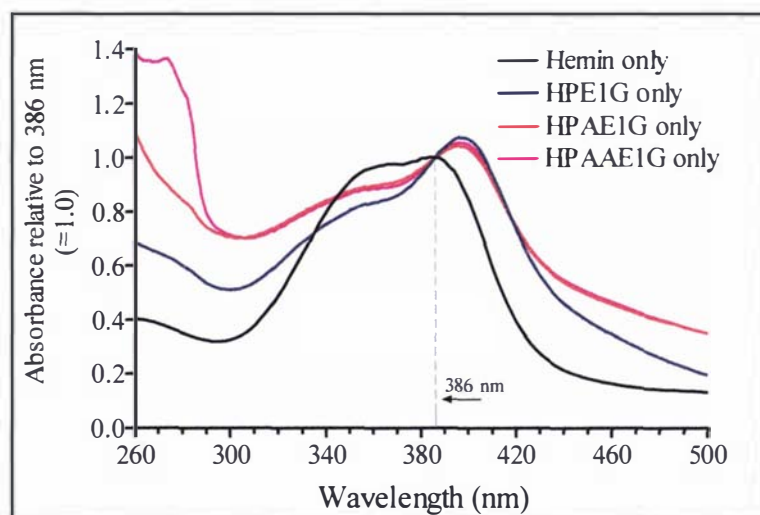


FIGURE 5.46: Absorption Spectra for Free Hemin, HPE1G, HP AE1G and HPAAE1G

This showed that the absorption maximum of hemin shifted to a higher wavelength for all three polyethyleneglycol substituted hemins examined ($\lambda_{\text{max}} = 398 \text{ nm}$) with the absorption maximum of the hemin becoming more defined.

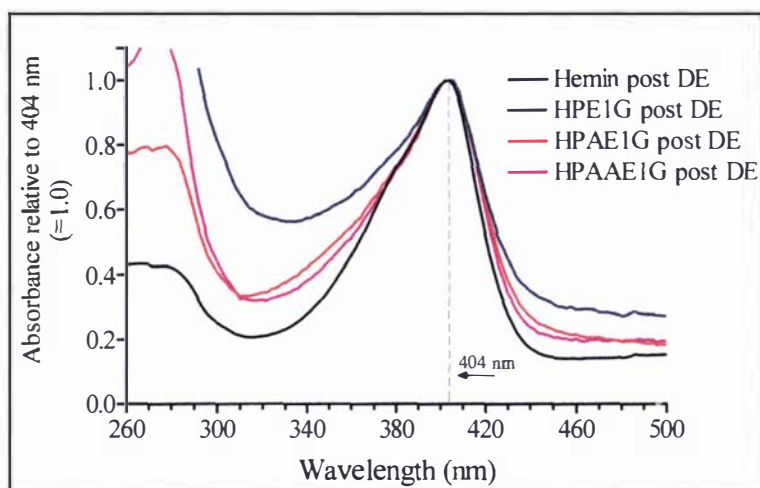
After titration, the specific activity of the HPAAE1G titration mixture was $88 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ protein which was

equivalent to only 5.8% of the specific activity of the apo protein/hemin control titration rate.

The apo protein/HPAAE1G titration mixture was left to incubate overnight at 4°C and reassayed the next day. This resulted in a 1.69 fold increase in rates relative to the previous day to 148 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ protein and was equivalent to 9.8% of the apo protein/hemin control rate.

A further overnight incubation at 4°C, did not result in any further increases in specific activity. Thus after this final incubation the titration mixture was separated from any unbound HPAAE1G and free estrone glucuronide using the DE column and then concentrated. The final mixture had an RZ value of 0.746 and a specific activity of 263 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ protein and 904 $\Delta\text{Abs min}^{-1} \text{ nmol}^{-1}$ enzyme. The final reconstitution yield was therefore 29% and the specific activity of the reconstituted fraction was 50% that of the native horseradish peroxidase sample.

The absorption spectra of the post DE hemin, HPE1G, HP AE1G, and HPAAE1G apo protein titration mixtures are shown in Figure 5.47.



This Figure clearly shows that the absorption maximum of the hemin Soret band was not changed by modification of the hemin with any of the polyethylene linkers.

FIGURE 5.47: Absorption Spectra for Horseradish Peroxidase Reconstituted with Hemin, and HPE1G, HP AE1G and HPAAE1G after DE chromatography

5.3.6 Analysis of Hemin-Polyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HPE1G) using a Reconstitution Assay Format with Apo Horseradish Peroxidase

The apo horseradish peroxidase - HPE1G reconstitution reaction was examined as a possible assay system for E1G using various polyclonal antisera, a monoclonal antibody and a range of control assays. The assay conditions for the various biological reagents were apo protein - 11.23 picomoles, native horseradish peroxidase - 0.159 picomoles, HPE1G - 139 picomoles, polyclonal antisera or monoclonal antibody - 4 μ L undiluted, and estrone glucuronide standard - 6,900 pmoles (i.e. 50 μ L 500,000 nmol 24 hr⁻¹ standard).

The effects of the various assay components on the rate of the peroxidase assay are described below and summarised in Table 5.3.

TABLE 5.3: The Effect of the Assay Mixture Components on the Rate of the Peroxidase Assay

assay mixture	mean initial rate (Δ Abs min ⁻¹)	n	CV [§] (%)	mean specific activity (IR [†]) (Δ Abs min ⁻¹ nmol ⁻¹ protein)
apo HRP only	0.276*	3	4	5
unpurified anti-E1G AS 243-4 only control	8x10 ⁻⁵	1	-	-
apo HRP + unpurified anti-E1G AS 243-4	0.967	4	14	86
apo HRP + HPE1G	0.326	7	23	29
apo HRP + HPE1G + unpurified anti-E1G AS 243-4	5.087	5	30	453
apo HRP + HPE1G + unpurified anti-E1G AS 243-4 + 500,000 nmol E1G 24 hr ⁻¹ std	4.441	1	-	396
apo HRP + HPE1G + purified anti-E1G AS 2434-4	0.655	3	15	58
apo HRP + HPE1G + unpurified anti-PdG AS ET3-4	8.908	1	-	793
apo HRP + HPE1G + monoclonal anti-E1G Ab	0.059	3	33	5
apo HRP + HPE1G + monoclonal anti-E1G Ab + 500,000 nmol E1G 24 hr ⁻¹ std	0.095	3	26	8
native HRP only	0.492*	2	2	3,096

Abbreviations: Ab - antibody, AS - antiserum, HRP - horseradish peroxidase, E1G - estrone glucuronide, std - standard, IR - initial rate; CV[§] - co-efficient of variation. NB: * denotes that these values were measured using a different protein concentration in the assay mixture and are not directly comparable to other values in the same column.

Incubation of the apo protein with the modified hemin HPE1G, resulted in a 6 fold increase in the peroxidase activity relative to the apo protein only control rate. The peroxidase activity of the apo protein - HPE1G reconstitution assay mixture was also found to be strongly activated by the addition of unpurified anti-estrone glucuronide antiserum which resulted in the assay activity increasing by a further 16 fold.

The activity of the antiserum only assay was negligible, while the sum of the activity of the apo protein - HPE1G mixture, and the apo protein - antiserum mixture equalled only 25% of the activity of the apo protein - HPE1G - antiserum mixture. Thus the 16 fold increase in peroxidase activity observed for the apo protein - HPE1G mixture in the presence of the unpurified anti-estrone glucuronide antiserum was not due to the simple summation of activities.

This activating effect of the antiserum was even higher in the presence of unpurified anti-pregnanediol glucuronide antiserum (27 fold), but was moderated by the replacement of the crude antiserum with a purified anti-estrone glucuronide antiserum (only a 2 fold increase) and strongly reversed (82% inhibition) by the presence of a monoclonal anti-estrone glucuronide antibody.

The effect of the addition of a large amount of free estrone glucuronide (50 molar excess relative to HPE1G) on the modulation of the peroxidase activity of the apo protein - HPE1G reconstitution mixtures by the unpurified anti-estrone glucuronide antiserum and the monoclonal anti-estrone glucuronide antibody was also examined. For the unpurified anti-estrone glucuronide antiserum, the antiserum mediated increase in activity appeared to be slightly reversed (13%) by the addition of a large excess of free estrone glucuronide. However this assay was performed without replicates and this may just reflect the variation of the assay. There was also a reversal (1.6 fold relative to the inhibited rate) of the monoclonal anti-estrone glucuronide mediated inhibition by the high estrone glucuronide levels. As the difference between the activity in the two experiments carried out with and without an excess of estrone glucuronide in the assay was much greater with the monoclonal antibody, and this result was derived from multiple assays (Table 5.3) it can be concluded that this reversal of the inhibition was probably not an artifact of the assay variability.

Although the incubation of the apo protein with the modified hemin HPE1G, resulted in a 6 fold increase in the peroxidase activity relative to the apo protein only control rate, this was significantly lower than the 52 fold increase observed for the post DE HPE1G titration mixture (see Section 5.3.5.12). However compared to the initial concentrations of the reaction mixture of the reconstitution assay (i.e. before dilution with assay buffer), the final titration mixture from which the post DE titration mixture was derived had a slightly higher ratio of hemin conjugate to apo protein (14 as opposed to 12), and a much higher concentration of both hemin conjugate (41.6 μM versus 1.2 μM) and apo protein (3.95 μM versus 0.1 μM).

For the various reconstitution assay conditions reported in Table 5.3 for which the peroxidase activity was measured using multiple assays, the average co-efficient of variation was 18%. This was much higher than the variation experienced with the standard peroxidase assay which usually fluctuated between 2 and 5%.

The effect of the monoclonal anti-estrone glucuronide antibody, time, and the HPE1G concentration on the peroxidase activity of the reconstitution assay were all studied in greater detail. The effect of the monoclonal antibody was examined by titrating a constant amount of apo protein (30 picomoles) and HPE1G (72 pmoles) against an increasing

concentration of antibody using the standard modified reconstitution assay method (Section 5.2.14). This assay format was non-competitive with the HPE1G being mixed with the antibody first followed by the addition of the apo protein immediately afterwards.

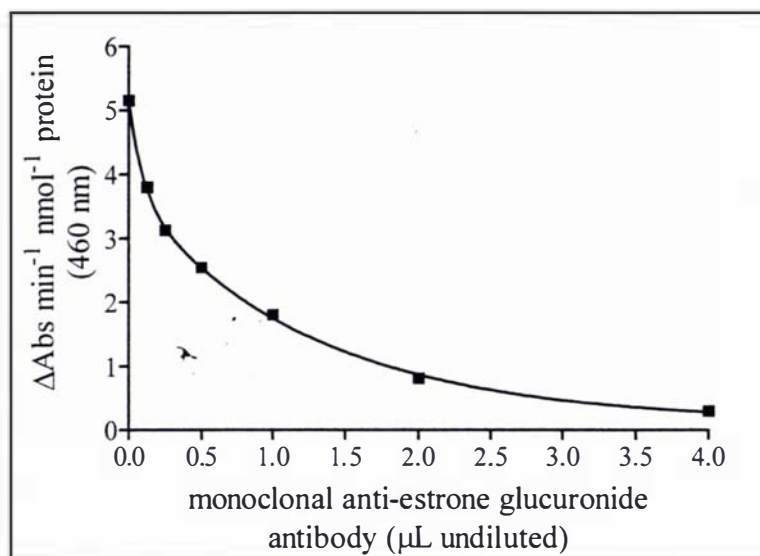


FIGURE 5.48: Titration of Apo Protein (30 pmoles) and HPE1G (72 pmoles) with Monoclonal Anti-Estrone Glucuronide Antibody in a Reconstitution Format

The titration data are presented in Figure 5.48 and show the peroxidase activity after subtraction of the apo protein only blank rate. The titration of the apo protein - HPE1G mixture resulted in a classically shaped titration curve, with nearly complete inhibition (92%) of the enzymic activity of the reconstituted peroxidase being obtained with 4 μL of the undiluted monoclonal anti-E1G antibody.

The effect of the incubation time of the apo protein with HPE1G on the peroxidase activity was examined using a variation of the modified standard reconstitution assay. The apo protein was incubated with the HPE1G at 40°C using a series of incubation periods (0 to 90 minutes) and a molar ratio of apo protein to HPE1G of 1:1 (30 pmoles). The data are presented in Figure 5.49. This graph clearly showed that the peroxidase activity

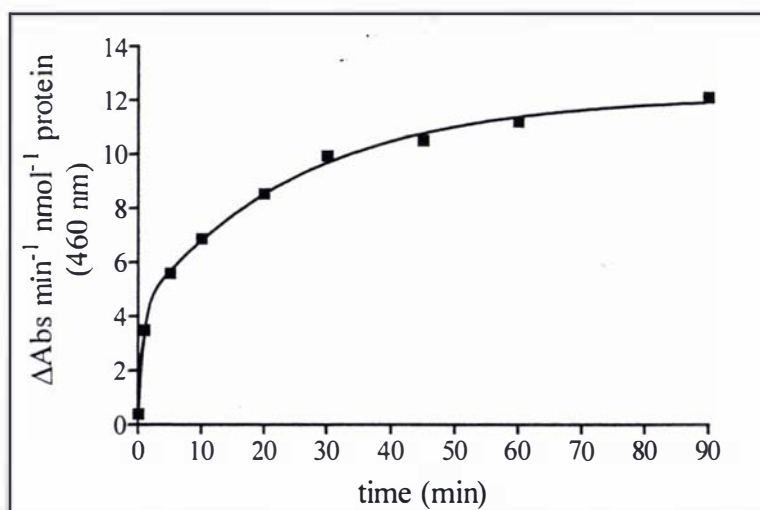


FIGURE 5.49: Effect of Incubation Time on the Activity of an Apo Protein - HPE1G Reconstitution Assay

increased hyperbolically with the length of the incubation period. Under the conditions examined, the initial increases in activity were very high with almost half the activity observed within the 90 minute incubation period being obtained within the first five minutes. Despite this rapid initial equilibration period, at the end of 90 minutes reconstitution was still continuing.

The effect of the incubation time on the peroxidase activity of an apo protein - unmodified hemin mixture was also examined. This was performed using an identical assay format to the equivalent HPE1G experiment except that the concentration of apo protein and

hemin compound had to be reduced due to the greater activity of the hemin reconstitution mixture. Thus a 1:1 ratio of apo protein to hemin compound was used as before but the amount of the two in the assay was reduced to 10 picomoles each. The resulting incubation time curve is shown in Figure 5.50.

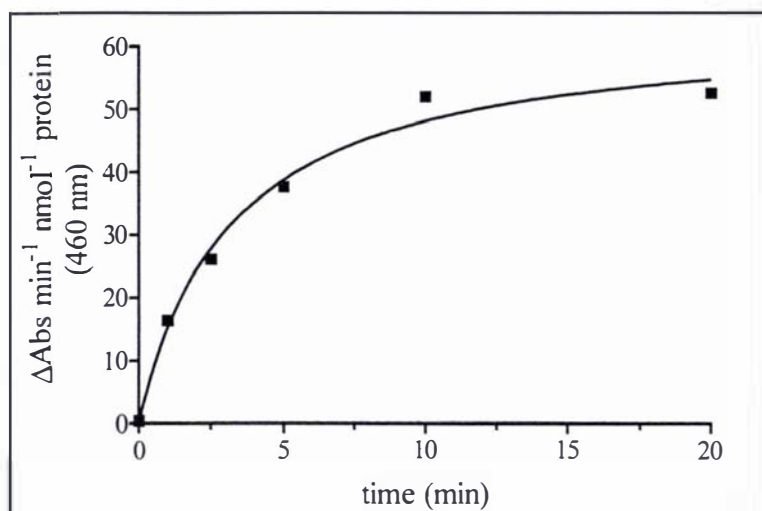


FIGURE 5.50: Effect of Incubation Time on the Activity of an Apo Protein - Hemin Reconstitution Assay

The relationship of the peroxidase activity with the reconstitution assay time was also clearly hyperbolic for the hemin sample. However, reconstitution was clearly much faster for the hemin sample, reaching close to maximum levels at the end of only 20 minutes even at the much lower concentration of protein and hemin compound in the assay mixture.

The effect of the HPE1G concentration on the peroxidase activity of the apo protein - HPE1G mixture was also examined using a variation of the modified standard reconstitution assay. This assay was performed using a 15 minute incubation time at room temperature of the apo protein with HPE1G. The effect of the HPE1G concentration was examined by titrating a constant amount of apo protein (30 pmoles) against an increasing amount of HPE1G (0-90 pmoles). The results of this titration are shown in Figure 5.51.

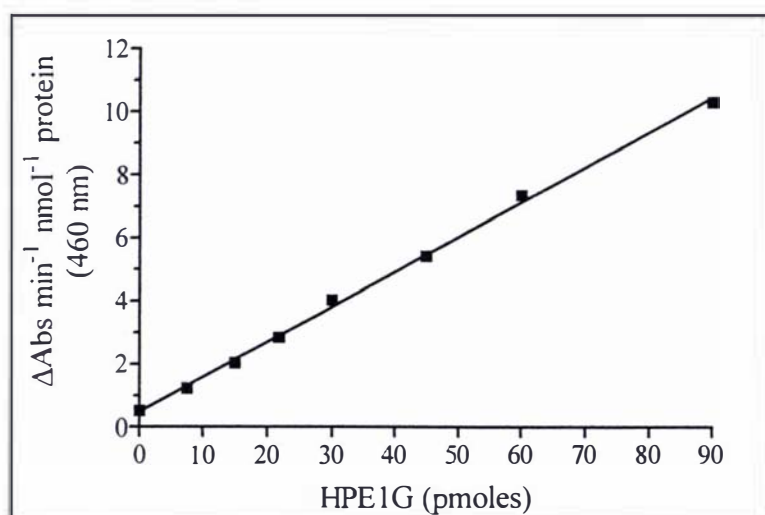


FIGURE 5.51: Titration of Apo Protein (30 pmoles) with HPE1G using a Reconstitution Assay Format and a 15 Minute Incubation Period

Although the HPE1G was never present in a vast molar excess relative to the apo protein (maximum 1:1.5), the data presented in Figure 5.51 clearly shows that under the conditions examined the peroxidase activity increased linearly with the amount of HPE1G.

The last experiment performed using a reconstitution assay format was with the free hemin, apo protein and monoclonal anti-estrone glucuronide antibody. The data presented

in Table 5.3 showed that pre-mixing the HPE1G with this same antibody before introducing the apo protein resulted in an almost complete inhibition of peroxidase activity from the apo protein - HPE1G mixture which was not reversed upon the pre-mixing of the antibody with a high concentration of estrone glucuronide. The first half of this experiment where the HPE1G was mixed with the monoclonal antibody and then the apo protein, was therefore repeated using unconjugated hemin. This experiment was performed using the modified reconstitution assay, and 60 picomoles of apo protein, 3 picomoles of hemin, 4 μL of undiluted monoclonal antibody, and a 15 minute incubation period at room temperature of the hemin - apo protein - antibody mixture. Under these conditions the peroxidase activity of the apo protein - hemin mixture was measured as $12 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ and was dramatically reduced to $0.90 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ in the presence of the antibody. Taking into account the apo protein only blank rate of $0.57 \Delta\text{Abs min}^{-1} \text{ nmol}^{-1} \text{ protein}$ this was equivalent to an inhibition of the reconstituted peroxidase activity by the monoclonal antibody of 97%. Thus the monoclonal anti-estrone glucuronide antibody had a similar effect on the peroxidase activity of the hemin reconstitution assay as it did on the HPE1G reconstitution assay.

On the basis of this result all further attempts at using a reconstitution assay format were abandoned and all other assays were performed using only the pre-reconstituted conjugates as prepared in Sections 5.3.5.12-5.3.5.14.

5.3.7 Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Polyethyleneglycol-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPE1G) by Solution Assay and Enzyme Linked ImmunoSorbent Assay (ELISA)

Table 5.4 shows the activation of the native horseradish peroxidase sample and the reconstituted HPE1G sample by the anti-estrone glucuronide antiserum and monoclonal antibody relative to the antiserum/antibody free assays.

TABLE 5.4: Activation of Native Horseradish Peroxidase and Pre-Reconstituted Horseradish Peroxidase-HPE1G by Monoclonal and Polyclonal Anti-Estrone Glucuronide Antisera

Sample	Activation by Monoclonal Antibody (without antibody = 1.0)	Activation by Polyclonal Antiserum (without antiserum = 1.0)
Native HRP	1.15	1.16
Pre-reconstituted HRP-HPE1G	1.27	1.26

These results show that the presence of both the polyclonal antiserum and the monoclonal antibody in the assay mixture caused moderate activation of the peroxidase activity of both the native horseradish peroxidase and the pre-reconstituted HPE1G sample. The activation was approximately 10% higher than for the pre-reconstituted sample.

The pre-reconstituted HPE1G sample was also analysed as a signal generator using non-competitive ELISA at four HRP-HPE1G concentrations and two different antiserum dilutions (see Section 5.2.15 for methods). This experiment showed no evidence of colour

development under any of the 8 conditions examined 6 hours after the initiation of the peroxidase activity assay. This was contrasted very strongly by the horseradish peroxidase active ester estrone glucuronide control wells which had an absorbance in excess of 1 unit for all four antiserum - enzyme conjugate combinations examined.

5.3.8 Analysis of the Pre-Reconstituted Horseradish Peroxidase Hemin-Polyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) by Enzyme Linked ImmunoSorbent Assay (ELISA)

In the non-competitive ELISA where the horseradish peroxidase active ester estrone glucuronide conjugate in the standard ELISA assay was replaced by the reconstituted hemin, HPAAE1G and HPAE1G horseradish peroxidase samples (see Section 5.2.16), there was no detectable colour development after 9 minutes from the initiation of the peroxidase assay with the *o*-phenylenediamine substrate. Over the same development period, the active ester estrone glucuronide control wells, which had a protein concentration 5 and 20 fold lower than the protein concentration of the reconstituted sample wells, were associated with strong colour development (0.83 absorbance units).

The results of the competition ELISA with the reconstituted hemin, HPAAE1G and HPAE1G samples and the non-competitive control active ester conjugate only data set (see Section 5.2.16) are not so simply summarised and are shown graphically in Figure 5.52.

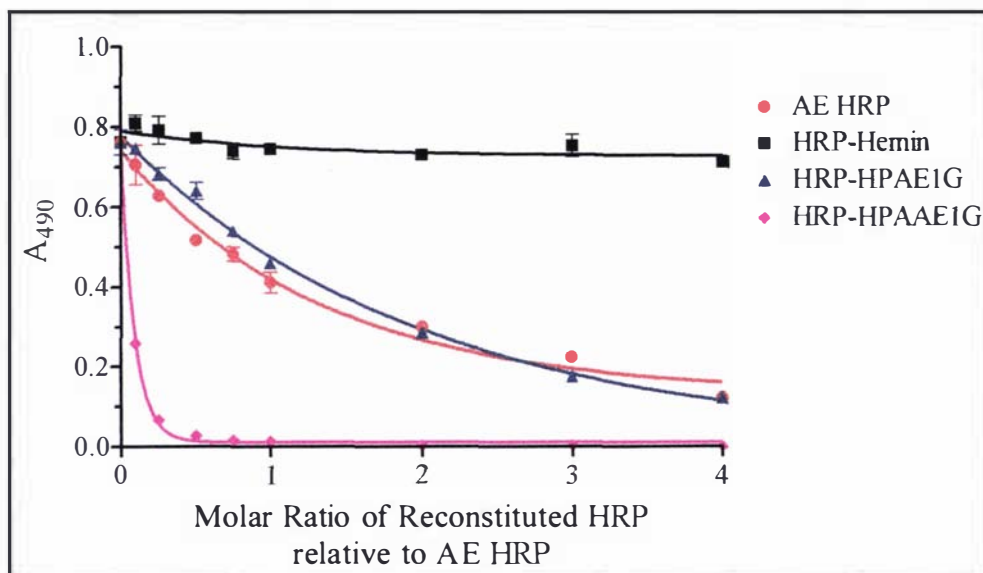


FIGURE 5.52: ELISA Plate Competition Assay between Active Ester Horseradish Peroxidase-E1G and Reconstituted Horseradish Peroxidase-Hemin, -HPAE1G and -HPAAE1G

In the competition ELISA between the active ester horseradish peroxidase estrone glucuronide conjugate and the reconstituted HRP-HPAE1G and HRP-HPAAE1G samples, the concentration of the active ester conjugate was kept constant against an increasing concentration of the reconstituted enzyme. For these wells, the absorbance decreased rapidly as the amount of the reconstituted enzyme increased relative to the active ester conjugate from a molar ratio of 0 to 4. This was in contrast to the findings for the competition ELISA between the reconstituted HRP-hemin sample and the horseradish peroxidase active ester conjugate. In this experiment, the absorbance showed only a slight decrease as the molar ratio of reconstituted enzyme to the active ester conjugate increased. In the control experiment with the active ester conjugate only, the absorbance also showed a strong decrease with the decreasing concentration of the enzyme conjugate.

The absorbance decrease was non-linear for the three competitive ELISAs with the reconstituted enzymes, and also for the non-competitive active ester only control series, with all four data sets being fitted using a one phase exponential decay equation (PRISM, GraphPad™). The absolute decrease in absorbances for the active ester conjugate control series and the competitive ELISA with the HRP-HPAE1G samples were very similar with both reaching a minimum absorbance of approximately 0.15 absorbance units. This was equivalent to a horseradish peroxidase concentration of 10.2 fmole well⁻¹ for the active ester only control and molar ratio of reconstituted enzyme to active ester of 4:1 for the HRP-HPAE1G sample. However, the rate of decrease for the competitive ELISA with the HRP-HPAAE1G sample was considerably faster, reaching an absorbance of 0.01 at a molar ratio of reconstituted enzyme to active ester conjugate of only 0.5:1.

5.3.9 Cross Reactivity of the Pre-Reconstituted Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAE1G) and Horseradish Peroxidase Hemin-Diaminopolyethyleneglycol-6-Aminohexanoate-6-Aminohexanoate-Estrone Glucuronide (6 or 7) Mono-Conjugate (HRP-HPAAE1G) in the Ovarian Monitor

In the standard Ovarian Monitor assay the level of lysozyme-estrone glucuronide conjugate binding to the anti-estrone glucuronide antibody (and hence the level of inactivation of the lysozyme activity) is determined by the amount of free urinary or standard estrone glucuronide added to the assay.

In the Ovarian Monitor cross reactivity experiments of the reconstituted horseradish peroxidase-HPAE1G and -HPAAE1G samples, the free estrone glucuronide in the standard Ovarian Monitor assay was replaced with the reconstituted samples. The molar concentrations of the reconstituted enzyme in the samples assayed were equivalent to the molar estrone glucuronide concentration of a time diluted urine sample with an excretion rate of 267 and 800 nmol E1G 24 hr⁻¹.

The Ovarian Monitor twenty minute change in transmission values for the reconstituted HRP-hemin, -HPAE1G and -HPAAE1G samples, and the estrone glucuronide standards as measured by both the pre-coated Ovarian Monitor assay tubes and the wet chemistry assay are summarised in Table 5.5.

TABLE 5.5: Cross reactivity of the Reconstituted Horseradish Peroxidase-Hemin, -HPAE1G, and -HPAAE1G Samples with the Ovarian Monitor Estrone Glucuronide Assay

Sample	Pre-coated assay tubes (ΔT average - blank*)	Wet chemistry assay (ΔT average - blank [†])
E1G Standard (0.074 nmol mL ⁻¹ $\equiv$ 267 nmol 24 hr ⁻¹)	182	136
E1G Standard (0.222 nmol mL ⁻¹ $\equiv$ 800 nmol 24 hr ⁻¹)	206	214
Post DE RC HRP-hemin (0.074 nmol mL ⁻¹ $\equiv$ 267 nmol 24 hr ⁻¹)	12	27
Post DE RC HRP-hemin (0.222 nmol mL ⁻¹ $\equiv$ 800 nmol 24 hr ⁻¹)	-4	67
Post DE RC HRP-HPAE1G (0.074 nmol mL ⁻¹ $\equiv$ 267 nmol 24 hr ⁻¹)	134	164
Post DE RC HRP-HPAE1G (0.222 nmol mL ⁻¹ $\equiv$ 800 nmol 24 hr ⁻¹)	211	305
Post DE RC HRP-HPAAE1G (0.074 nmol mL ⁻¹ $\equiv$ 267 nmol 24 hr ⁻¹)	232	290
Post DE RC HRP-HPAAE1G (0.222 nmol mL ⁻¹ $\equiv$ 800 nmol 24 hr ⁻¹)	233	315
Post DE E1G only (5.55 nmol mL ⁻¹ $\equiv$ 20,000 nmol 24 hr ⁻¹)	27	-

Blank* - 24; Blank[†] - 26

The 800 nmol 24 hr⁻¹ estrone glucuronide standard which helps define the top end of the estrone glucuronide standard curve has a steroid concentration a factor of 25 lower than the initial steroid concentration of the post DE sample ($\equiv$ 20,000 nmol E1G 24 hr⁻¹). However, after passage of this very high concentration estrone glucuronide sample through the DE column (Section 5.2.17), the rate of transmission change for this sample was reduced to close to standard curve baseline levels.

The rate of transmission change for the reconstituted horseradish peroxidase-hemin samples were also close to baseline levels. Only the reconstituted horseradish peroxidase-hemin-E1G samples showed an increase in transmission change significantly above the standard curve baseline. Dilution of the reconstituted HPAE1G samples to an estrone glucuronide concentration equivalent to the concentration of a time diluted urine with an excretion rate equal to 267 and 800 nmol E1G 24 hr⁻¹ resulted in very similar rates to the equivalent estrone glucuronide standards, while the rates observed with the similarly

diluted reconstituted HPAAE1G samples were consistently higher than those observed with the equivalent estrone glucuronide standards.

An observation which held regardless of the sample assayed (and including the estrone glucuronide standards) was that the rates measured with the pre-coated Ovarian Monitor tubes were generally lower than the rates measured with the wet chemistry assay.

The post lysis reaction wet chemistry Ovarian Monitor assay mixtures for the three reconstituted samples were also assayed for peroxidase activity using an end-point assay. These assays were performed on both duplicates of the with and without antiserum mixtures at both reconstituted enzyme concentrations. Although each assay mixture was assayed in duplicate making a total of four assays for each condition, only the average absorbance of these four assays is reported. The absorbance data are summarised in Table 5.6.

TABLE 5.6: Peroxidase Activity of the Post Ovarian Monitor Horseradish Peroxidase-Hemin, -HPAE1G and -HPAAE1G Assay Mixtures as measured in Absorbance (490 nm) using a 150 Second o-Phenylenediamine End Point Assay

Sample	0.074 nmol mL ⁻¹ ($\approx$ 267 nmol 24 hr ⁻¹)		0.222 nmol mL ⁻¹ ($\approx$ 800 nmol 24 hr ⁻¹)	
	with antiserum	without antiserum	with antiserum	without antiserum
HRP-Hemin	0.857 ($\pm$ 0.026)	0.693 ($\pm$ 0.017)	2.130 ($\pm$ 0.154)	1.919 ($\pm$ 0.051)
HRP-HPAE1G	0.136 ($\pm$ 0.010)	0.099 ($\pm$ 0.002)	0.400 ($\pm$ 0.050)	0.325 ($\pm$ 0.003)
HRP-HPAAE1G	0.254 ($\pm$ 0.006)	0.221 ($\pm$ 0.006)	0.708 ($\pm$ 0.048)	0.626 ($\pm$ 0.056)

The dilution factors required to give a concentration of reconstituted horseradish peroxidase of 0.074 and 0.222 nmol mL⁻¹ in preparation for analysis by the Ovarian Monitor assay were estimated for the three different samples from their A₄₀₄ values. For the HPAE1G and HPAAE1G reconstituted protein samples, these concentrations were, in terms of estrone glucuronide content, equivalent to the estrone glucuronide concentration of a time diluted urine sample with an excretion rate of 267 and 800 nmol E1G 24 hr⁻¹.

The absorbance data in Table 5.6 show that when an aliquot from the final reaction mixture from the Ovarian Monitor assay was assayed for horseradish peroxidase activity, the peroxidase assays performed on the 0.222 nmol mL⁻¹ Ovarian Monitor assay mixture had an activity approximately 3 times higher than that observed for the equivalent 0.074 nmol mL⁻¹ Ovarian Monitor assay mixture. This observation held regardless of the nature of the hemin compound of the reconstituted protein and whether the original Ovarian Monitor assay was performed in the presence or absence of anti-estrone glucuronide antiserum, although the relative rates were slightly lower for the HRP-hemin samples. In the case of the HRP-hemin samples, the end point absorbance of the high concentration peroxidase assays for both the with and without antiserum formats, was well in excess of 1.0. Thus the lower peroxidase activity of the high concentration hemin samples relative to the low concentration samples is probably an artifact due to the absorbance recordings for the high concentration samples being off-scale. In summary, the results showed strong evidence that the peroxidase activity of the post lysis Ovarian

Monitor reaction mixtures was directly and linearly proportional to the concentration of reconstituted protein used in the original Ovarian Monitor assay.

The data presented in Table 5.6 also showed that the peroxidase activity data obtained from the Ovarian Monitor assays performed in the presence of antiserum were consistently higher (10-37%) than those recorded for the equivalent antiserum free Ovarian Monitor assay mixtures. This activating or protective effect of the anti-estrone glucuronide antiserum on the peroxidase activity was observed for both the estrone glucuronide (HPAE1G and HPAAE1G) and non-estrone glucuronide (hemin) reconstituted protein samples and was consistent with the results presented in Section 5.3.6 on the effect of antiserum on the native enzyme and the HRP-HPE1G reconstituted sample.

The post Ovarian Monitor HRP-hemin, -HPAE1G and -HPAAE1G assay mixtures were also compared for their relative peroxidase activities. The peroxidase activity of the HPAE1G and HPAAE1G reconstituted samples relative to the hemin reconstituted sample was 15% and 31% respectively. Compared to the relative peroxidase activities of the original reconstituted samples, this is a significant reduction for both estrone glucuronide substituted proteins, which were previously observed to have 37% and 50% of the reconstituted hemin activity.

5.4 DISCUSSION

5.4.1 Establishment of an ELISA Reference Assay for Estrone Glucuronide using Timed Urine Specimens

In the development of new assay systems it is important to have access to a reliable reference method. Previously, the total estrogen method, which extracts the major urinary estrogens (estrone, estradiol and estriol) and their conjugation products from urine and then quantifies them by the Kober-Ittrich procedure (Brown *et al.*, 1968) has been used in Melbourne to validate the use of estrone glucuronide as a suitable fertility marker as well as the Ovarian Monitor estrone glucuronide assay itself (Brown *et al.*, 1988; Brown *et al.*, 1989). Because these validations have already been completed, and the total estrogen method is technically demanding and time consuming, due to a lack of resources and space the equipment has since been dismantled, and the method never adopted in New Zealand. However, the development of alternative E1G assays still requires a reference assay. Although theoretically the Ovarian Monitor could be used in this role, compared to the Ovarian Monitor assay, the ELISA microplate horseradish peroxidase immunoassay for E1G published by Dr Keith Henderson (Henderson *et al.*, 1995) has more potential for the rapid processing of large numbers of samples.

This assay was originally trialed using a monoclonal antibody and early morning urine samples with the E1G concentrations corrected for variations in the rate of urine production by measurement of creatinine. As previously discussed (Section 1.3.3.6), because the data obtained from time diluted urines are more accurate for reflecting circulating estrogen levels than early morning samples and have the advantage that they can be taken at any time of day, the use of early morning urines has been rejected for fertility monitoring by the Ovarian Monitor Research group. The standard range of dilutions required to convert an early morning urine sample to the constant excretion rate of 150 mL hr⁻¹ is between 2 to 10 fold. Thus the optimum working range for the estrone glucuronide standard curve is lower for an assay based on time diluted urine samples.

Compared to the production of a monoclonal antibody, the production of polyclonal antisera requires no cell culture, is a relatively low cost procedure and simple to perform. The use of an antibody mixture also has the advantages of high specificity and affinity (Tjissen, 1995d), and in fact the original developer of the ELISA E1G assay has since stated that he would never work with a monoclonal antibody again for these reasons (Henderson, personal communication). It was also for these reasons, and in particular the easy availability of the polyclonal antisera, that the adaptation of the early morning urine ELISA assay into a reference estrone glucuronide fertility assay also involved its conversion to a polyclonal antiserum based assay.

Like the production of the lysozyme conjugates, the production of the horseradish peroxidase-estrone glucuronide conjugate proved to be a simple procedure, although it did require a comparatively large excess of the E1G active ester to achieve an adequate level of conjugation. The degree of acylation of horseradish peroxidase by the active ester method was never determined as the heterogeneity of the native enzyme prevented a simple analytical procedure, like the 7 M urea Mono-S separation (Section 2.2.5) which was used

for the analysis of the lysozyme-E1G conjugates, to be developed. However, as the enzyme was to be used in a solid phase system where any unconjugated enzyme is removed from the sample wells during the washing step, the lack of purification and characterisation of the sample was not considered to be of major concern.

The horseradish peroxidase conjugates proved to be as reliable as the lysozyme conjugates. The enzyme conjugate was stable when stored in concentrated form and there was no difficulty in calibrating the ELISA with different batches of the signal generator. For the calibration, a standard set of chequerboard experiments allowed the rapid selection of appropriate dilutions for the conjugate, antiserum and estrone glucuronide standards. These dilutions were selected so that a standard curve could be generated which covered the range of E1G concentrations found in time diluted urine samples during the normal menstrual cycle (150 mL hr^{-1} , 35-465 nmol E1G 24 hr^{-1} 10th-90th percentile).

The greatest inconsistency in the assay was in the day-to-day colour development time. As there was no dependence on the position of the standard curves on this parameter (Figure 5.6), the duration of the peroxidase reaction was not fixed and the reaction continued to be stopped using an arbitrary assessment of the colour development. Despite this variation, for speed and accuracy, attempts were made to optimise the assay so that the colour development in the blank wells reached approximately 1.0 absorbance unit in 30 minutes.

At a dilution of 20,000 for the unpurified anti-E1G antiserum (243-4), the position of the E1G standard curve on the E1G excretion rate axis (the EC_{50} value) was independent of the concentration of the signal generator between the range of 12.75 and 102 fmole of conjugate per well (Figure 5.8). Based on the results presented in Figures 5.7 a final conjugate concentration of 51 fmole per well was chosen for the analysis of the cycles from the WHO trial.

In the Ovarian Monitor assay as the amount of signal generator increases the standard curve shifts up the concentration axis. Thus, it might have been expected that the position of the standard curve would also shift towards higher estrone glucuronide concentrations as the concentration of signal generator was increased. The fact that this did not occur shows that a simple 1:1 competition between the signal generator and the free estrone glucuronide for a limited population of available antibody binding sites as occurs in the Ovarian Monitor lysozyme system (Equation 3.6) cannot be taking place. These differences between the two systems most likely reside in the site of the immune reaction which takes place in solution for the Ovarian Monitor assay and on a solid phase for the ELISA assay. Further evidence that this is the case is supported by the work of Schramm and Paek (1992) who have studied the competitive binding kinetics of a horseradish peroxidase-progesterone conjugate and progesterone with anti-progesterone antibody using an ELISA format. From their work they concluded that the antibody density, and the relative rates of diffusion of the hapten compared to the hapten-conjugate are important determinants of the binding kinetics in a solid phase system.

Although the ELISA assay has the potential for a much higher throughput and easy automation, for a single assay the Ovarian Monitor system is much quicker requiring only

15 minutes of incubation time for the antibody with the hapten and hapten conjugate compared to 24 hours required for the ELISA. This is partly due to the much greater distances for diffusion of the conjugate and hapten to reach the antibody in the ELISA system compared to the Ovarian Monitor system. Furthermore, because the rate of diffusion of a molecule is proportional to its size and an enzyme-hapten conjugate is much larger than the free hapten, the free estrone glucuronide molecule has a much faster rate of diffusion. This means that the free estrone glucuronide and the enzyme hapten conjugate are not in a 1:1 competition for the immobilised antibody at the initiation of the binding reaction. Thus if an attempt is made to reduce the assay time in an ELISA by decreasing the antibody binding time and the binding reaction is stopped prematurely, the level of free estrone glucuronide bound will be disproportionately high compared to the equilibrium conditions.

The other factor which complicates an ELISA format compared to a solution assay is that in an ELISA the antibody may not be equally available to both the free E1G and the signal generator. This is because the binding is dependent on the orientation and packing of the immobilised antibody. Due to steric constraints the enzyme-hapten is much more restricted in its antibody binding sites than the much smaller hapten. This is illustrated in Figure 5.53 which shows the antibody-antigen complex formation at four different antibody densities on the solid phase using both horseradish peroxidase-E1G and free E1G as the antigen.

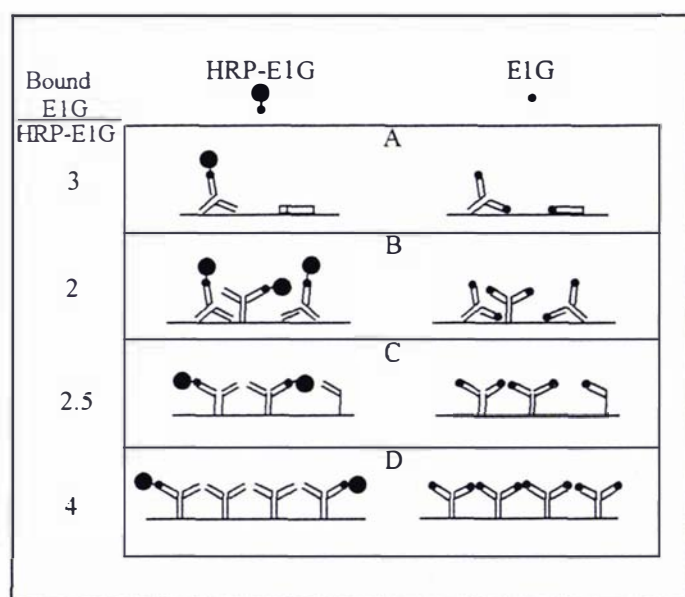


FIGURE 5.53: Model of Antibody-Antigen Complex Formation for Horseradish Peroxidase-Estrone Glucuronide Conjugate and Estrone Glucuronide at Different Antibody Densities on the Solid Phase

Antibody Density Increases from A-D

Modified from Schramm and Paek, 1992

This model shows that regardless of the antibody density, there are always more sites available to the hapten than the enzyme-hapten conjugate and that the proportion of E1G binding relative to the hapten conjugate was highest at very low and high antibody densities. This model also shows that every binding site the enzyme-hapten can bind to, the hapten can bind to as well, but the enzyme hapten cannot access all the free E1G binding sites. This means that the antibody can take up more of the small hapten before the horseradish peroxidase-E1G signal generator starts to be displaced. If even in the absence of competition from E1G, only a small fraction of the immobilised antibody binding sites accessible to the signal generator are occupied by the enzyme conjugate, then increasing the conjugate concentration will obviously increase the

enzyme conjugate, then increasing the conjugate concentration will obviously increase the

amount of conjugate bound. If this increase in conjugate concentration is only moderate then although the signal will increase proportionally, the fraction of total occupied binding sites will not be sufficient to affect the sensitivity of the dose response curve. This model is completely consistent with the results observed, with the sensitivity of the standard curves increasing with the dilution of the antiserum but not with the conjugate concentration (Figures 5.8 and 5.11).

The increase in sensitivity as the antiserum dilution increased from 10,000 to 40,000 showed that as the total concentration of binding sites was decreased less E1G was required before the binding of the signal generator was affected. This effect would be accentuated by the fact that relatively less of the sterically hindered binding sites would be immobilised on the wells as the total concentration of antibody in the coating solution was decreased. Thus the buffering effect of this population which is inaccessible to the enzyme conjugate would also be decreased. The fact that the 40,000 and 80,000 dilutions gave standard curves in essentially the same position was also consistent with the model presented in Figure 5.53, as it showed that the ratio of available conjugate and hapten antibody binding sites was at the minimum at this point (Scenario B) i.e. the point from which further increases or decreases in antibody density resulted in a relative increase in the ratio of free hapten binding.

The mid-points of the standard curves at the different dilutions of antiserum were 46, 33, 20 and 21 nmol E1G 24 hr⁻¹ for the 10,000, 20,000, 40,000 and 80,000 dilutions respectively. Although the most sensitive standard curves were obtained with the 40,000 and 80,000 antiserum dilutions, the fact that less of the signal generator was bound under these conditions (Figure 5.10) meant that an excessively long reaction time (90-150 minutes) was required for the activity assay to get an absorbance change of around 1 unit. Although this increased sensitivity could be useful in normal laboratory use as a reference assay for the menstrual cycle it is desirable to use the urine samples as collected by the women without further dilution since this decreases the amount of labour involved in setting up the ELISA plates and the urine volume was already at a practical minimum

of 5 μ L. For this reason the antibody dilution of 20,000 was chosen for the work reported in this chapter.

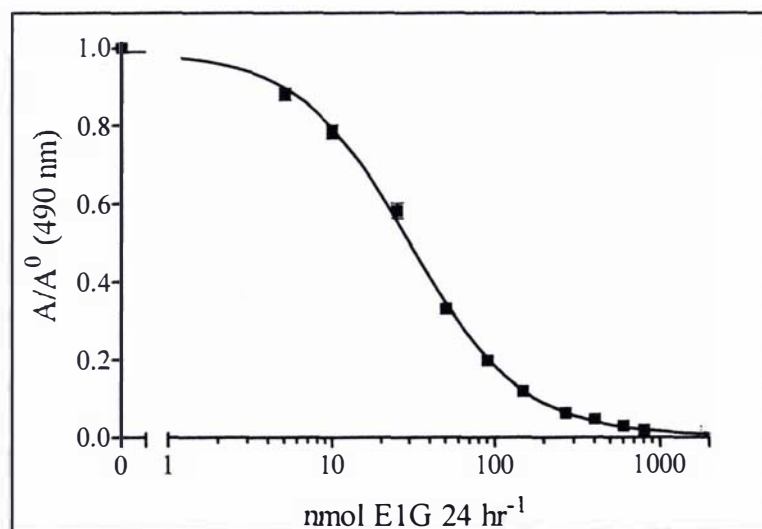


FIGURE 5.54: Inter-assay Variation of the Nine Standard Curves used for the Measurement of Estrone Glucuronide Excretion Rates from Nine Cycles from the World Health Organisation on the Ovarian Monitor

The ELISA standard curves generated with the optimised procedure were very reproducible. The inter-assay variation is shown in Figure 5.54 with the error bars reported as standard errors of the mean. The assay sensitivity which was defined as the amount of estrone glucuronide producing a

percentage Abs/Abs⁰ value two standard deviations from the zero standard was calculated as 2.4 pg E1G per well which was slightly less than that reported by Henderson (Henderson *et al.*, 1995) of 3.6 pg E1G per well, and the mean EC₅₀ value for the 9 standard curves was equivalent to an excretion rate of 30 nmol E1G 24 hr⁻¹ or 18 pg well⁻¹. Thus the optimised ELISA conditions resulted in a standard curve which showed great sensitivity over the range of E1G excretion rates experienced over the transition period into the fertile phase making it particularly suitable for determining the beginning of fertility.

The E1G excretion rate profiles for the WHO Ovarian Monitor trial cycles obtained from these standard curves were classical. All nine cycles showed an initial baseline period followed by a rise in E1G excretion rates into a clearly defined peak followed by a sharp fall. Thus the pattern of E1G excretion rates used by the Ovarian Monitor to define the beginning of fertility (an increase from baseline) and a period of peak fertility (peak day and day of fall) could be successfully applied to the data obtained by the ELISA method for all nine cycles.

Although, the textbook pattern of circulating estrogens (Figure 1.3) shows a single pre-ovulatory peak, split pre-ovulatory peaks in estrogen excretion as was observed for cycle 021R-C2 (Figure 5.19) are not uncommon with approximately 20% of the cycles in the reference database for total estrogens upon which the Ovarian Monitor assay was originally referenced, showing a double peak (Blackwell and Brown, 1992).

The most striking feature of the ELISA results was their close agreement with both the radioimmunoassay data (RIA) collected by the World Health Organisation reference laboratory at Hammersmith in London (Figure 5.22) and the Ovarian Monitor data (Figure 5.23) despite the fact that the ELISA data were collected some four years later. The correlation between the ELISA and RIA data for the individual cycles was particularly high ($r \geq 0.94$) with the data showing only small differences in the intercepts. However, due to the variations in the slopes, the excretion rate data for the two methods were only superimposed for one cycle (014X-C1, Figure 5.16). With the exception of this cycle, the slopes were all less than 1 and for the pooled data the RIA values were on average 20% less than the ELISA values. The fact that the slopes of the correlations, both for the individual cycles and the pooled data (Table 5.1), were less than 1.0 must be due to bias in either one or both of the sets of data relative to the actual E1G excretion rates.

All immunoassay data suffer from the possibility of bias (Cekan, 1987) but it is not clear in this case whether the differences in absolute values for the daily E1G excretion rates given by the two methods reflect a bias in the ELISA assay, the RIA assay, or in both. This constitutes one of the major difficulties in using competitive binding assays as a reference method since in the absence of a method which is completely specific to the hapten, it is not possible to know where the bias lies. However, it is generally considered that the lower the value given for an analyte by an assay system the more likely it is that the value is correct (Cekan, 1979). On this basis the RIA data would be regarded as the most valid. However, one of the major factors which determines assay bias in direct assays such as used here is non-specific binding (Cekan, 1987) which is a function of the assay antiserum. The WHO antiserum cross reacted equally well with estrone glucuronide and estrone and best of all with estrone sulphate (237%) whereas the antiserum used in the ELISA assays

showed very little cross reactivity with estrone (1.9%) and only 14.3% with estrone sulphate (Binnie, 1999). Thus, on these criteria the sheep antiserum used in this work would be judged as having the highest specificity for estrone glucuronide and hence would be expected to give rise to lower non-specific binding effects. However, each of the assay systems used different amounts of urine as well. For the RIA analysis, the samples were diluted 100 times and then 200 μL was taken for analysis. This corresponds to 2 μL of the original time diluted sample whereas for the ELISA, the system was optimised for 5 μL of the time diluted urine sample. Hence more urine was added to the ELISA which may increase any non-specific binding effects relative to RIA and could account for the apparently higher E1G excretion rates obtained despite the lower cross reactivity of the ELISA antiserum.

Of the three assays the non-specific binding effects would be expected to be greatest for the Ovarian Monitor assay as unlike the RIA and ELISA protocols there is no washing step, and there is proportionally much more urine added to the assay (50 μL). Thus the high baseline of the Ovarian Monitor assay was consistent with expectations.

Despite this variation in baseline, the overall correlation between the ELISA and Ovarian Monitor assays was high and the fact that the slopes for the individual correlations were close to 1.0 suggests that the assay response was the same for the two systems. This similarity between the Ovarian Monitor assay and the ELISA assay probably also partly reflects the fact that the two systems used the same antiserum, unlike the RIA assay.

The differences or apparent displacements for the Ovarian Monitor data (as seen for cycle 023B-C2 for example, Figure 5.21) were largely found in the intercepts of the correlation plots. For the nine cycles analysed the intercept varied from 6 to 126 nmol E1G 24 hr^{-1} , however this value was approximately constant for cycles collected from the same woman. For example, for trial participant 014X the intercepts were 54 and 64 nmol E1G 24 hr^{-1} and were 91 and 114 nmol E1G 24 hr^{-1} for participant 023B. These intercepts can be considered as a measure of the urine bias or 'urine blank' effect. Although the urine bias varies between individuals, because the Ovarian Monitor uses a change from baseline levels as opposed to absolute E1G excretion rates to define the beginning of fertility this makes the method universally applicable. As is clear from the Figures 5.12 to 5.21 there was a close parallel between the ELISA and Ovarian Monitor data when the baseline shift was taken into account with the beginning of fertility as defined by the first rise being obvious in all cases. In fact, the pattern of rising and falling E1G excretion rates on which a woman bases her natural family planning was faithfully reflected by all three methods, and in almost all cases the peak E1G excretion day was unambiguously defined between them, as were the lengths of the three phases.

Unfortunately the assay tubes used in the WHO study for the Ovarian Monitor data were not properly calibrated due to major downsizing of the Melbourne laboratory and construction which affected the quality control at a time when the *M. lysodeikticus* sample had just been changed. The extra sensitivity of this batch was not noticed until well into the study and was responsible for some of the very high baselines observed for some of the cycles. However, even with the properly optimised tubes the baselines although lower were still relatively high compared to the ELISA and RIA data. The variable urine bias in

the Ovarian Monitor data meant that it could not be correlated against methods without this extreme urine bias (i.e. the ELISA and RIA results) by the Deming Regression method (Deming, 1943; Martin, 2000) since this method assumes error in both sets of data when in this case the error from the Ovarian Monitor data was notably higher. Thus for these correlations, the standard least squares method which assigns all of the variation to the Y axis data was more suitable.

When the Deming regression model was used to correlate the pooled Ovarian Monitor data against the ELISA assay (Figure 5.23), the Deming fit virtually removed the Y intercept but at the expense of assigning a slope of nearly 2. Clearly, this was inconsistent with the individual cycle data (Table 5.1) where the E1G values were serially correlated. For this reason it is more informative to compare the different assay methods for the individual cycles (as done in Table 5.1) than to pool the data which is a common but questionable practice. In support of this contention, with the exception of one cycle (009D-C2), the correlation co-efficients for the individual cycles measured with the Ovarian Monitor were much higher than for the pooled data. The least squares fit to the pooled data agreed better than the Deming regression with the individual cycle fits since it gave a mean intercept of 66 nmol E1G 24 hr⁻¹. It should be noted that outliers (or occasional aberrant points which were not repeated in the original data collection) can influence the values returned for the slopes of the correlations plots.

It is noteworthy that the Ovarian Monitor data were originally validated by comparison with the Brown total estrogen method (Brown *et al.*, 1968) which measures the sum of estrone, estradiol and estriol and does not suffer from an assay bias. The correlation of the Ovarian Monitor data against the total estrogen method for 1012 urines collected in an infertility clinic (Brown *et al.*, 1988) was $Y = 3.3X + 52 \text{ nmol E1G 24 hr}^{-1}$ ($r=0.89$) where X is the total estrogen value in units of $\mu\text{g 24 hr}^{-1}$. This correlation coefficient was similar to a corresponding plot of urinary estrone versus total urinary estrogens ($r=0.87$, $N=200$) using the Kober-Ittrich procedure in both cases. Thus the fact that the ELISA data correlated well with the Ovarian Monitor data (after subtraction of the baseline values) suggests that the ELISA may be a better reference assay than the RIA and that a negative urine bias exists in the RIA data as a result of non-specific binding. However, it is clear that the lower RIA results cannot be due to sample deterioration since they were performed four years before the ELISA assays were run. The estrone glucuronide material in the urine samples had not been lost by bacterial hydrolysis after storage at -10°C since the anti-E1G antibody used in the ELISA assay is highly specific for E1G (Binnie, 1999). On the other hand, since the WHO E1G antiserum cross reacted equally well with estrone (Sufi, personal communication), if significant bacterial hydrolysis had occurred, converting estrone glucuronide into estrone, the ELISA results should have been lower than the corresponding RIA data rather than higher as observed experimentally.

Thus, the ELISA is a robust assay which gives excellent daily E1G excretion rates throughout a menstrual cycle when used with time diluted urine samples and in fact the matrix effect caused by using urine samples directly in the assay is likely to be smaller than it appears considering that the WHO reference assay itself may suffer from a negative assay bias. The menstrual cycle profiles were similar to those reported previously and gave an accurate identification of the start of the fertile period as judged by the pre-ovulatory rise

in E1G (Blackwell and Brown, 1992; Brown *et al.*, 1989). Although the data were not averaged as is often the case (see Henderson *et al.*, (1995) for example), the hormone values for the cycles measured by the ELISA clearly agreed with the pattern shown in Figure 1.3 and showed how reliably the ELISA data could be used to reflect the body's hormonal status when generated with time diluted samples.

In conclusion, this ELISA which uses an active ester horseradish peroxidase-estrone glucuronide conjugate as signal generator may be used with as a laboratory reference assay against which to compare any new assay systems developed with the reconstituted horseradish peroxidase-estrone glucuronide conjugates. Although more work needs to be done to complete the validation, its performance was judged satisfactory for use as a benchmark in this work against which any new signal generators could be compared.

5.4.2 Reconstitution of Synthetic Hemins with Apo Horseradish Peroxidase

As outlined in the introduction to this chapter (Section 5.1.6), there are several literature examples of modified hemins reconstituting with apo horseradish peroxidase or other hemin dependent apo proteins to form an active enzyme (Tamura *et al.*, 1972; Harris *et al.*, 1993; DiNello and Dolphin, 1981; Adak and Banerjee, 1998; Fernandez *et al.*, 1993). The ability of these modified compounds to reconstitute with activity into a hemin apo protein has met with varying levels of success depending upon the nature of the modification and the enzyme. The prosthetic group of the glucose oxidase enzyme, flavin adenine di-nucleotide (FAD), has also been the subject of modification and reconstitution studies. This molecule has proved to be much more amendable to large scale modifications and reconstitution in keeping with its much more open position in the holo-enzyme.

In the PGLIA reconstitution patent of Hornby and Morris which used as its example, apo glucose oxidase reconstituting with modified FAD molecules (Hornby and Morris, 1988), the use of hemin-hapten conjugates as potential PGLIA candidates was also described. However the viability of producing large molecular weight hemin-hapten conjugates, and their ability to reconstitute into a hemin apo enzyme, had never been tested. This study has now been performed using apo horseradish peroxidase and thirteen hemin compounds modified by substitution at the propionic acid side chain. The results are summarised in Table 5.7 which shows the percentage reconstitution of the samples and their activities relative to a reconstituted horseradish peroxidase-hemin control.

TABLE 5.7: The Reconstitution Characteristics of Thirteen Modified Hemin Compounds with Apo Horseradish Peroxidase

Sample [§]	S.A* of titration mixture (%)	relative to hemin	
		level of reconstitution (%)	S.A* of the reconstituted fraction (%)
HLysE1G	4.5	-	-
HLysPdG	3.0	-	-
HMet(di)	0.7	-	-
HA(di)	9	16	37
HA	53	80	86
HAME	44	85	39
HH	34	85	50
HPheME	5	28	18
HMetME	10	38	37
HProME	38	71	50
HPE1G	15	29	48
HPAE1G	6-13 [¶]	35	37
HPAAE1G	6-10 [¶]	29	50

Abbreviations: S.A* - specific activity NB: [¶] - the first value of the range is the specific activity of the titration mixture immediately after titration, and the last value of the range is the specific activity of the titration mixture 2 days later. Sample [§] - see Results (Sections 5.3.5.2-5.3.5.14) for structures

The aim of the hemin substitution work was to produce a hemin-hapten conjugate which could be used as an immunoassay reagent for home fertility monitoring. To measure the urinary fertility markers, estrone glucuronide and pregnanediol glucuronide by such a system requires the synthesis of a steroid glucuronide-hemin conjugate. However, a stable linkage between the propionic acid side chain and the free carboxyl group of the glucuronide moiety cannot be achieved readily by simple chemistry and a linker is required. Analysis of the environment of the propionate groups in the holo enzyme (Figure 5.3) also suggested that a linker would be essential for reconstitution. From this model it can be seen that the access of the propionate side chains to the enzyme surface is via a short protein channel, thus it is highly unlikely that a hemin-steroid glucuronide could reconstitute with the apo protein without a spacer arm to separate the hemin from the bulky steroid glucuronide.

This conclusion is supported by several hemin modification studies on the reconstitution of modified hemins with apo horseradish peroxidase. According to Fernandez *et al.* (1993) conversion of both propionate groups into their equivalent methyl and ethyl esters resulted in inactive complexes being formed in which the hemin was non-specifically bound to the protein. This was slightly contradictory to the findings of Adak and Banerjee (1998) who

reported that the hemin dimethyl ester could be reconstituted successfully but that the reconstituted enzyme was without peroxidase activity. Their results led them to conclude that the propionates were essential for the correct orientation of the hemin in the binding site and were consistent with the findings of a much earlier study by Tamura *et al.* (1972) who also observed the hemin di-ester to bind to the apo protein without forming an active enzyme. In Tamura's study, the effect of modifying one or both of the hemin propionate groups were compared for their ability to produce an active enzyme. Compared to the specific activity of peroxidase reconstituted with unmodified hemin, the reconstituted hemin di-methyl ester had no activity (0.01%) and the mono-substituted methyl ester retained up to 18% of the unmodified rate. On the basis of these results it was concluded that at least one free carboxyl group was essential for catalytic activity.

Other experimental evidence on the importance of the hemin carboxyl groups was provided by DiNello and Dolphin (1981) who compared the specific activities of horseradish peroxidase reconstituted with three different di-substituted hemins. When the enzyme was reconstituted with hemin in which both propionate groups were converted into their equivalent amides the specific activity was only 7% of that of enzyme reconstituted with the free hemin. The specific activity was also reduced to 7% when both the propionate groups were converted into butanols. However, when the propionic acid side chains were converted into butyric acid groups the specific activity increased to 32% of the hemin reconstituted rate. Thus modifications to the propionate side chains which retained a carboxyl group appeared to be less destabilising.

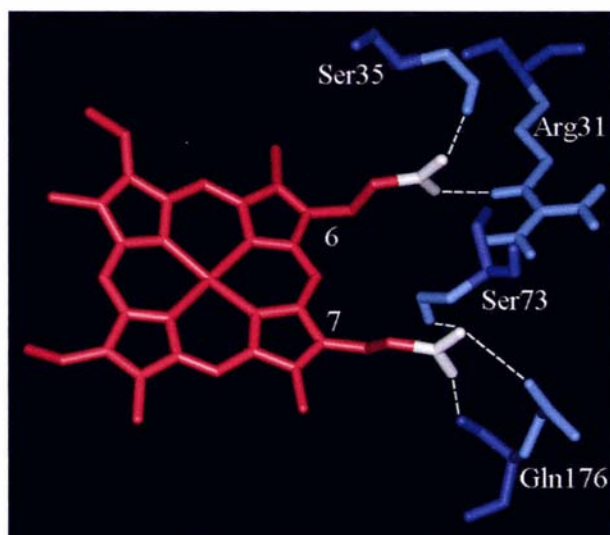


FIGURE 5.55: Hydrogen Bonds of the Hemin 6 and 7 Propionate Groups to Amino Acids Arginine 31, Serine 35, Serine 73 and Glutamine 176 of Horseradish Peroxidase

Hemin - red; amino acid backbone - medium blue; amino acid side chain - light blue

A possible explanation for these results is provided by the crystal structure of the horseradish peroxidase hemin binding pocket (Figure 5.55) which shows the 6-propionate group to be clearly occluded by the presence of arginine 31. Substitution at this position would therefore require the arginine side chain to move by at least 90 degrees to accommodate the linker and allow reconstitution to occur. However, as shown in Figure 5.55 this movement would also destroy the hydrogen bonding between arginine 31 and the 6-propionate group which is in turn likely to weaken or prevent the binding of any 6-substituted hemin to the apo protein. On the other hand the 7-propionate is hydrogen bonded to serine 73 and glutamine 176 but as can be seen from the structural model, these

residues do not directly occlude the carboxyl group. Hence modification at this position with a linkage capable of hydrogen bonding to these same two residues may still provide significant stabilisation of the bound hemin. One obvious potential candidate for this role

is an amide bond. An amide bond at this position could potentially mimic the effect of the carboxylate group as long as the linker attached to the amide nitrogen does not come into collision with the rest of the residues making up the channel to the surface.

The interactions between the linker and the channel will also help to determine the stability of the reconstituted enzymes. However, in the absence of detailed modelling studies an empirical approach as carried out here seemed to be the easiest option.

The simplest method of forming a stable linkage with one or both of the hemin propionate groups is to react it with a primary amine to form an amide bond. Thus the ideal bi-functional reagent for linking with the propionic acid groups of the hemin and the glucuronide group of the steroid was a di-amide moiety. On the basis of the available literature evidence, and the absence at that time of the crystal structure, it was concluded that a minimum of one intact propionate group might be essential for activity. Thus, the ability of a linker to provide a substitute carboxyl group close to the amide linkage with the hemin, was considered to be a desirable feature in the initial development. One of the most readily available molecules which has two primary amine groups, and a free carboxyl group is the amino acid lysine. The fact that the two amino groups are separated by a linear chain of five carbon atoms and that the carboxyl group is located close to one of the amino groups (α) made it appear to be an ideal linkage candidate for the production of reconstitutable hemin-steroid glucuronide conjugates.

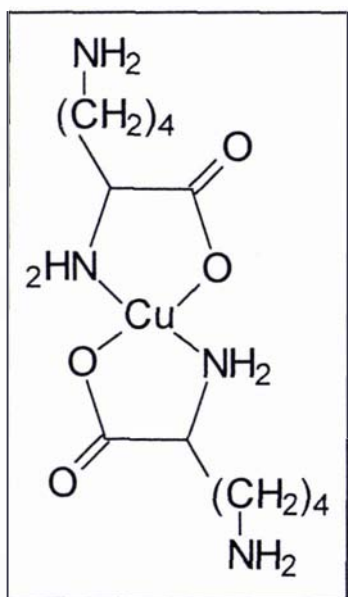


FIGURE 5.56: Bis-Copper Lysine Complex

Selective modification of the bi-functional lysine reagent was achieved by using a bis-copper lysine complex (Pleurdeau *et al.*, 1981). In this complex (see Figure 5.56), the copper is ionically bound to the α -amino - α -carboxyl ends of two lysine residues, leaving only the ϵ -amino groups available for conjugation to the steroid glucuronide by standard active ester methodology. After conjugation, the copper was removed by passing the reaction mixture through a chelex 100 resin and the now mono-conjugated lysine residue was exposed to a hemin *p*-nitrophenol active ester derivative to form a hemin-lysine-steroid glucuronide complex (Wu, 1996). The hemin-mono-lysine-steroid glucuronide conjugates were produced by limiting the exposure of the hemin-di-*p*-nitrophenyl active ester reagent to the linker, removing any unreacted *p*-nitrophenol by hydrolysis and then purifying the mono-substituted conjugate from a mixture of di- and mono-conjugates, unreacted hemin and other reaction products by high pressure liquid chromatography (Zhao, 1998).

The mono-conjugated hemin-lysine-E1G derivative did reconstitute with the apo protein but only to an extent of 4.5%. Thus it was not considered worthwhile to continue with this sample. Similar work was also carried out in parallel with a preparation of a pregnanediol glucuronide-lysine-hemin mono-conjugate, but the reconstitution with this sample was even lower (Table 5.7). The very low activities observed with the apo protein titration

mixtures of these samples (Table 5.7) suggested that either the α -carboxyl group was destabilising the binding or that the molecular leash was too short for efficient reconstitution thus introducing high energy steric interactions, and the use of a lysine leash was abandoned.

On the basis of these initial results, it was decided to concentrate on optimising the chemical nature of the linker first. Thus the next eight modified hemins examined (see Table 5.7) were synthesised without the steroid glucuronide component. However, the synthetic strategy used for the lysine derivatives was found to be unsuitable for this task. When it was applied to the synthesis of the hemin-methionine (HMet) and 6-aminohexanoic acid (HA) conjugates it resulted in a reaction mixture heavily dominated by the di-substituted isoforms. These two conjugates, were initially mistaken for their equivalent mono-substituted conjugate forms, the initial targets of the synthesis, and the later discovery of their true identities was a sobering testimony to the difficulty in synthesising, purifying and identifying the mono-substituted hemins in the absence of a suitable analytical method. This problem was overcome when ready access to electrospray mass spectroscopic analysis became available. Thus all subsequent substituted hemin compounds had their structures properly characterised by ESMS before being trialed in the reconstitution studies.

Why under the same conjugation conditions, the hemin-aminohexanoic acid and -methionine derivatives were associated with such a high proportion of di-substituted conjugate compared to the good yields of mono-substituted lysine-estrone glucuronide derivatives cannot be known for certain. However, the fact that the reaction mixtures dominated by di-substituted conjugates were conspicuously lacking in mono-substituted hemins in the presence of significant amounts of unreacted hemin suggested that for these samples the conversion of the hemin into the mono-substituted conjugate was rapidly followed by its conversion into the di-substituted form. Thus, it appeared unlikely that the proportion of mono-substituted hemin could be increased by simply reducing the linker to hemin ratio. It was suggested that the effect was solubility related (Zhao, 1998) with the samples having high rates of di-substitution being more soluble in the conjugation solvent than the only partially soluble hemin. In this scenario, conjugation at one propionate group of the partially soluble hemin with a compound of higher solubility would increase the hemin conjugate's overall solubility, thus aiding substitution at the other propionate group. However, the real significance of the results obtained with the hemin-di-*p*-nitrophenyl active ester was not the cause of the unexpected conjugate distribution, but the lack of universal application of the method for the production of mono-substituted hemin compounds in high yield. The low rates of mono-substitution for the hemin-aminohexanoic acid and -methionine samples acted as a driving force in the development of new methods for the synthesis of hemin conjugates.

An alternative procedure developed by Dr. Yinqi Wu and Zhandong Zhao (Zhao, 1998) for producing mono-substituted conjugates was to perform the conjugation on the mono-substituted hemin *p*-nitrophenyl active ester. Previously, preparation of the mono-substituted conjugates involved the di-methylation of the propionic side chains, followed by their partial hydrolysis to yield a mixture of free hemin and mono- and di-acylated derivatives. The mono-methylated conjugates were then purified, converted

into their *p*-nitrophenyl active ester equivalents and then conjugated to a hapten using standard active ester chemistry. However, this method was synthetically demanding and gave low yields of the final product. In the new procedure the mono-*p*-nitrophenyl active ester was prepared by activation of the hemin to the corresponding acyl chloride followed by reaction with *p*-nitrophenol to give a reaction mixture in which the mono-conjugate predominated. Although the reaction mixture also contained some hemin di-*p*-nitrophenyl active ester and unreacted hemin, the separation of the mono-substituted products from the other material was much simpler compared to the other methods. While the purification steps of the other methods usually required HPLC, in the new procedure the separation of the mono-substituted product was readily achieved on a large scale by utilising simple flash silica chromatography.

The main advantages of the new method were its simplicity, and its universal applicability for the synthesis of hemin mono-hapten conjugates in high yield. All further hemin conjugates which are discussed in this thesis were prepared for analysis by this newly developed procedure. Although the new method allowed the synthesis of mono-substituted hemin conjugate it could not distinguish between the 6- and 7-propionate positions and all reconstitution titrations were performed on a mixture of the 6- and 7-mono-substituted hemins. However, as discussed above, it was assumed that the apo protein would select between the 6 and 7 isomers on structural grounds and hence produce a reconstituted peroxidase with the 7 mono-conjugate. The nature of the interactions between the apo protein and the linker warrants further study since there was the suggestion from the data (Table 5.7) that a hydrophobic linker may best fit into the cavity shown in Figure 5.3.

Of the thirteen hemin conjugates examined (Table 5.7), the di-substituted methionine conjugate had the lowest overall activity recorded for an unpurified titration mixture as expected on the basis of the crystal structure and the previous modification experiments. The activity of the titration mixture was even lower than that of the two short chain lysine steroid conjugates. Although the reconstituted fraction was usually separated from the free hemin conjugate by anion exchange chromatography, due to the low activities returned by the titration mixtures of these three samples, this separation was never performed for these samples. Without the purification step, the specific activity of the reconstituting fraction and the overall reconstitution yield for these samples could not be defined. However even without this information, the results did allow the first comparison between high molecular weight hemin modifications and peroxidase activity to be made and were used as a structural point of reference for the design of the other hemin conjugates.

Clearly, retention of an α -carboxyl group in the form of a lysine or methionine linker did not aid activity relative to the straight chain linkers such as 6-aminohexanoic acid or 6-hexylamine. For example the titration mixture of the di-substituted aminohexanoic acid hemin had double the activity of the mono-substituted lysine linkers and more than ten times the activity of the di-substituted methionine linkers. Thus despite the conclusions drawn from Figure 5.55 it is possible to form an active enzyme even when the 6-propionate group is modified. The fact that a di-substituted hemin without a free carboxyl group could be more active than a mono-substituted conjugate in which the modified propionate group was replaced with an amino acid linker showed clearly that a substitute carboxyl group was not the most essential requirement for the formation of an active enzyme. Based on this

observation the emphasis shifted to producing a much wider range of mono-substituted straight chain linkers.

Of the hemin conjugates tested the mono-substituted aminohexanoic acid-hemin (HA) had the highest overall reconstitution yield (80%) and specific activity (86%) relative to a hemin reconstitution control. However as there were only very small volumes ($<50\ \mu\text{L}$) of the mono- and di-HA samples and their respective hemin controls available for measuring the 404 absorbance, the enzyme concentrations from which the yields and rates were calculated were not as accurate as those obtained for the other samples examined. For these samples absorbance spectra were obtained using equipment designed and built by Dr Simon Brown (Institute of Fundamental Sciences, Massey University, Palmerston North) for measurement of ultraviolet-visible spectra of small volumes of mitochondrial preparations with the noise of the profile reflecting the limits of the instrumentation at these minimal volumes.

There was no evidence for interactions between the HA linker and the hemin ring system since the hemin and HA spectra were similar in the region of the Soret band (Figure 5.33). In comparison, there were some minor changes for the HA(di) compound but when incorporated into the apo protein as an active peroxidase (albeit only slightly) the visible absorption spectra of both compounds were identical with those of the reconstituted hemin control indicating that the modified hemin was bound normally into its binding crevice in the apo protein. These conclusions also held for all of the other mono-substituted hemins except for HPE1G, HPAE1G and HPAAE1G conjugates.

The yield of reconstituted enzyme for the hemin control was only 50% in this experiment. As the reconstitution yield for the hemin control was usually in the order of 95-98% this was very low. Due to variations in the batches of apo protein and the day-to-day variations in the peroxidase rates, the reconstituted proteins were always compared relative to the enzymic activity of a native horseradish peroxidase control processed at the same time. However as the reconstituted peroxidase control for the mono-aminohexanoic acid reconstitution experiment was the only sample which exhibited this low yield, it is difficult to determine whether this was due to poor quality apo protein or to an error in its measurement. If it was due to poor quality apo protein or simple assay variation then the calculated yields and relative specific activities for the reconstituted mono-aminohexanoic acid sample would be correct. However, if the low yields recorded for the level of reconstitution were incorrect then the percentage yields and specific activities for the hemin-mono-aminohexanoic acid conjugate would be over-estimated explaining the apparently high yields and rates obtained. Thus, these figures must be interpreted with caution.

Variations on the aminohexanoic acid linker (HA) which were examined included the replacement of the carboxylic acid group with a methyl ester (HAME) and also with a methyl group (HH). The specific activities per nmol of reconstituted enzyme and the reconstitution yields were very similar for these latter two samples. As all modifications to the aminohexanoic linker were at the non-hemin binding end it was concluded that the nature of this end of the linker was relatively unimportant, but that a hydrophobic linear linker appeared to have increased reconstitution yields and specific activities.

Methyl esters of the amino acids phenylalanine, methionine and proline were also examined and gave three very different results. Relative to each other, the phenylalanine methyl ester had the lowest reconstitution yields and the lowest specific activity followed by the methionine methyl ester and then the proline methyl ester. In fact of the ten substituted samples for which the reconstitution yield and the specific activity of the reconstituting fraction was calculated, the HPhME sample had the lowest specific activity, and was second only to the di-substituted aminohexanoic acid sample for low yield. This result is not entirely unexpected as the phenylalanine sample has a bulky phenyl ring which could interfere with the reconstitution by coming into contact with the apo protein. The surprisingly result was the fact that the proline methyl ester with its bulky hydrocarbon ring gave one of the highest yields and specific activities. However closer examination of the position of the 7 propionic acid channel in the horseradish peroxidase crystal structure showed it to contain a small hydrophobic pocket close to the estimated position of the proline ring in the reconstituted enzyme. If there is a favoured orientation for the proline ring then there may also be a binding pocket, or an array of hydrogen bonding groups, in a position to interact with the methyl ester. Since the HProME-hemin compound was prepared from a DL-proline mixture this implies that one isomer could be favoured over the other. However, the apo protein is free to bind to the isoform which it finds less destabilising and as long as the substituted hemin is present in a reasonable excess relative to the apo protein a high rate of conjugation could still theoretically be obtained. This process of 'natural selection' would be similar to that utilised by the apo protein in the presumed preferential binding to the hemin modified at the 7-position.

The other modified hemin samples which were examined were all based on polyethyleneglycol (PEG). This molecule was chosen as a linker because it was believed to minimise any destabilising interactions with the protein due to its linear and hydrophilic nature. It was also chosen because of its 10 carbon chain which would hopefully minimise any unfavourable interactions between the steroid glucuronide and the apo protein and also minimise any steric constraints between the enzyme linked steroid glucuronide moiety and the anti-steroid glucuronide antiserum. All three polyethylene linkers were examined with the steroid glucuronide moiety attached. The HPAE1G sample contained an extra 5 carbon chain between the PEG molecule and the steroid glucuronide by the addition of a linear aminohexanoate group to the end of the non-hemin end of the PEG linker while the HPAAE1G linker was extended by 10 carbons by the linear addition of two aminohexanoate linkers. All three samples reconstituted with reasonable yields (29-35%), while the activity of the reconstituting fractions ranged from 37%-50%. However, the rate of reconstitution of the aminohexanoic acid samples (HPAE1G and HPAAE1G) appeared to be significantly slower than for HPE1G.

The fact that the specific activities of the E1G-linker-peroxidase enzymes were 40-50% of the native enzyme control and similar to the activities generated with the protein reconstituted with the other modified hemins of Table 5.7 showed that there was no major structural reorganisation required to allow reconstitution to occur. This was confirmed by the fact that the Soret bands for all three enzymes were similar to that of the reconstituted hemin control (Figure 5.47). An interesting difference was seen however for the visible spectra of the estrone glucuronide substituted hemins (Figure 5.46). The three polyethyleneglycol linked E1G-hemin compounds showed shifts similar to those seen in the

reconstituted enzymes. That is, a new sharper peak at around 404 nm with a broad shoulder at approximately 360 nm. Although the precise details are not known these changes suggest that there is an interaction between the polyethyleneglycol side chains, or the steroid moiety, with the hemin ring which mimics the changes occurring on binding to the apo protein. The most likely suggestion is that part of the linker-E1G interacts with the iron atom of the hemin ring which is made possible by the length and flexibility of the linkers.

These results provided the first direct evidence that a hemin moiety with a large hapten attached could be reconstituted with apo horseradish peroxidase to give an active enzyme. They were also resulted in very stable enzymes, with the HPE1G-peroxidase retaining its activity for over a year.

For the present purposes, the three E1G-linker-peroxidase enzymes were judged to be satisfactory for the studies on the use of such conjugates as signal generators in immunoassays for E1G. The concept of linking E1G (and hence a whole range of other glucuronides) to horseradish peroxidase via hemin modification is therefore proven to be completely successful. To date all horseradish peroxidase based immunoassays have utilised a mixture of uncharacterised multi-hapten substituted enzyme. This is the first time that a horseradish peroxidase enzyme conjugated to a single hapten with a known structure has been synthesised.

5.4.3 The Reconstitution Solution Assays

In principle there are two main ways in which a substituted hemin may be used to assay for estrone glucuronide. If it is not possible for the anti-estrone glucuronide antibody and the apo protein to bind simultaneously to the modified hemin then a reconstitution assay of the type described by Hornby and Morris (1979), should be possible. On the other hand if both proteins can bind together to the substituted hemin this would make a reconstitution assay impossible, but the system should still be suitable for ELISA and lateral flow membrane assays. As found previously by Hornby and Morris, with glucose oxidase, pre-mixing of the apo protein with either the native prosthetic group or the modified prosthetic group (for example hemin-E1G) allowed sufficient protein to be reconstituted that it could be used as a signal generator in a reconstitution assay. The enzymic activity of the reconstitution mixture using HPE1G as the hemin substrate was a linear function of the HPE1G concentration as desired for an analytical system (Figure 5.51).

Obviously, for use in a homogeneous enzyme immunoassay for E1G, the peroxidase activity of the reconstitution assay system must be inhibited by the anti-E1G antibody in the absence of E1G but not in its presence. This requirement means that the apo protein, which binds to the hemin portion of HPE1G, and the anti-E1G antibody, which binds to the E1G moiety must not be able to bind simultaneously i.e. the binding of one must inhibit the binding of the other. Although in the initial titration experiments (Figure 5.48) with the monoclonal E1G antibody it first appeared that the requirement of mutual exclusivity was being met since increasing volumes of the antibody (which was added first to the pre-incubation mixture) caused increasing inhibition of the peroxidase activity to a maximum of 92%. Unfortunately, this inhibitory effect was not reversed by adding high concentrations of free

E1G with the monoclonal antibody and more importantly a similar inhibition effect was observed with the control hemin reconstitution assay (Table 5.3). The simplest explanation is that the inhibition was due to a non-specific binding of the hemin and HPE1G to the antibody in such a way that the hemin or hemin portion of HPE1G was not available for binding in the heme pocket of the apo protein. This could occur if the iron atom of the hemin ring was co-ordinated to appropriately positioned ligands such as the imidazole ring of histidine or the ϵ -amino group of lysine. Whatever the mode of non-specific binding it must be sufficiently strong to be the preferred mode of binding in the face of a specific antibody-E1G interaction as previously utilised in the ELISA assay for E1G reported by Henderson (Henderson *et al.*, 1995).

Surprisingly, the polyclonal antiserum for E1G did not appear to possess the same non-specific binding capacity for a hemin molecule. In the direct reconstitution assays with the HPE1G sample an initial pre-incubation with a polyclonal anti-estrone glucuronide antibody had no inhibitory effect on the peroxidase activity of the final reaction mixtures. In fact the enzymic rate was increased by a factor of two by the partially purified polyclonal antibody.

There are several possible scenarios which can be used to explain these results. The simplest explanation is that the polyclonal antibody was not recognising the estrone glucuronide molecule of free HPE1G and hence had no effect on the reconstitution assay and that the polyclonal antibody also lacked the non-specific hemin binding site of the monoclonal antibody. However, this seems unlikely given the efficiency with which the antibody inhibited the E1G-lysozyme conjugates (Section 3.3.7). Alternatively, it may be

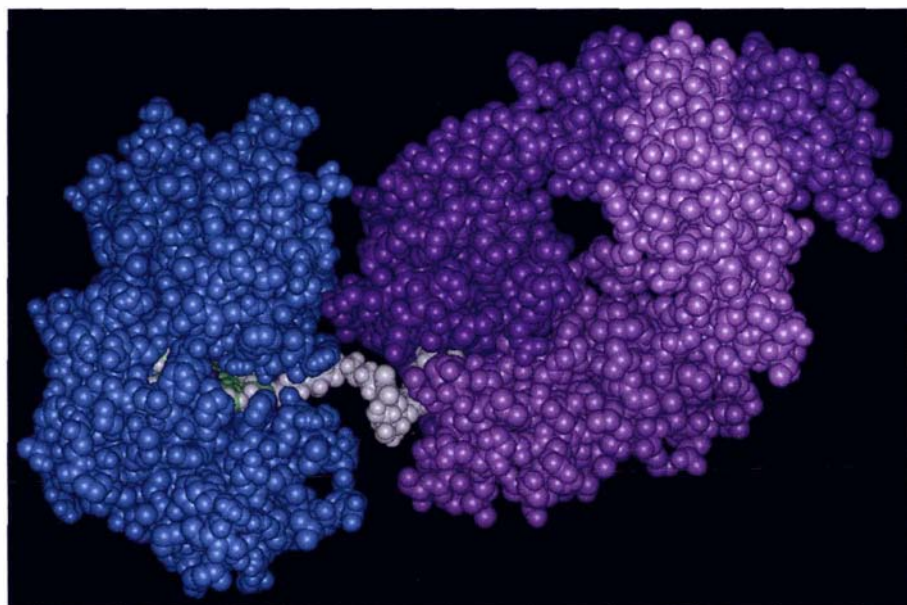


FIGURE 5.57: Steric Occlusion of the Substrate Binding Site of Horseradish PeroxidaseHemin-Polyethyleneglycol-Estrone Glucuronide Conjugate by Antibody Binding to the Steroid Moiety

Enzyme: general protein - medium blue, Hemin - green and white; Estrone glucuronide - white; Antibody: heavy chain, purple, light chain - lilac

that the antibody is binding to the estrone glucuronide group on the substituted hemin without influencing the peroxidase activity. This would occur if the formation of the antibody-substituted hemin compound did not inhibit the insertion of the hemin group into the apo protein (Figure 5.57) to form an active peroxidase. Finally, the failure of the anti-estrone glucuronide antibody to inhibit the peroxidase activity of a reconstitution assay mixture would also be observed if the substituted hemin samples were contaminated with free estrone glucuronide in the substituted hemin sample. In this situation, if sufficient E1G were present as a contaminant from the synthesis, it would severely reduce the amount of antibody available to bind to the estrone glucuronide substituted hemin compound and hence any antibody generated inhibition of the peroxidase activity. However, this seems unlikely as when the eluent of a high concentration standard of estrone glucuronide was collected from an anion exchange (DE) column, it was shown by the standard E1G Ovarian Monitor assay to contain no E1G. Thus, this work showed that the DE column was an effective method for removing free estrone glucuronide. As all the reconstitution, Ovarian Monitor and ELISA assays with the reconstituted enzyme were performed using samples which had been passed through the DE column this eliminated the possibility of any activity being the result of contaminating free E1G in the original sample.

Another unexpected result was the large activation (up to 25 times) of the steady state rates in the presence of the crude polyclonal antiserum in the incubation mixture of the reconstitution assays. A possible explanation for this observation is that protein components in the crude antiserum increase the solubility of the HPE1G molecule so that reconstitution during the pre-incubation time is more extensive. That the effect was not related to specific binding of the E1G moiety by the antibody in the serum was shown by the fact that the activation of the peroxidase activity was even higher for a crude anti-PdG antiserum. Part of the activating effect by the partially purified anti-E1G antiserum (Table 5.3) is probably due to the protective effect against inactivation by hydrogen peroxide as reported by Metelitz *et al.*, (1995) in the presence of anti-HRP antibodies. Whatever the explanation of the small activating effect it is clear that the presence of a specific antibody for E1G does not prevent binding of the HPE1G-antiserum complex to the apo protein to form an active peroxidase. Thus, it must be concluded that the polyclonal antibody and the apo protein can bind simultaneously to the HPE1G moiety and a reconstitution assay is not possible with this hemin conjugate. Hence it seems likely that the length of the polyethylene glycol linker in HPE1G is too long to allow a rapid homogeneous reconstitution enzyme immunoassay for E1G to be established. Obviously further work can be done on designing linkers of different lengths which may allow this assay format to be established.

5.4.4 The ELISA Assays with the Reconstituted Enzymes

Since the reconstitution assay was unsuccessful it was decided to compare the performance of the pre-reconstituted signal generators with the usual active ester peroxidase-E1G conjugate. However, the preliminary ELISA experiments where the active ester horseradish peroxidase estrone glucuronide conjugates were replaced with the reconstituted hemin-estrone glucuronide enzymes HPE1G-HRP, HPAE1G-HRP and HPAAE1G-HRP, showed absolutely no colour development upon addition of the peroxidase substrate. Given

the results discussed in the previous Section this result was entirely unexpected and disappointing. If both the apo protein and the anti-E1G antibody can bind to these substituted hemins then it follows that the reconstituted enzyme conjugates should be capable of binding to the anti-E1G antibodies immobilised on the ELISA plates. That is, if the reconstitution assay is not viable because binding of the antibody to the modified hemin doesn't cause inhibition of the reconstitution reaction, then the ELISA assay should work.

It may be therefore that despite the previous work the antibody does not recognise the estrone glucuronide when it is part of an intact hemin-peroxidase complex, or alternatively that the steroid group does bind to the antibody but due to conformational changes or steric constraints the peroxidase activity is inactivated. For example conjugate binding to immobilised antibody may cause conformational changes at the hemin binding site in the apo protein, or binding of the active site face of the peroxidase via the substituted hemin group to the solid phase bound antibody may prevent access of the organic substrate to the substrate channel due to steric constraints on the ELISA plate surface. Other possibilities are that only hemin-estrone glucuronide is bound to the antibody, since the strain of antibody binding causes the substituted hemin to dissociate from the apo protein, or that during the overnight incubation of enzyme at low concentrations the reconstituted enzyme spontaneously dissociates either in solution or in the antibody bound state. In either case there would be no peroxidase activity.

To try and distinguish between these possibilities the competition ELISA experiment between the active ester estrone glucuronide conjugate and the reconstituted hemin-estrone glucuronide conjugates was carried out (Figure 5.52). In these experiments the level of peroxidase activity in the wells decreased as the ratio of the reconstituted hemin-E1G enzyme to active ester conjugate concentration was increased. This provided conclusive evidence that some estrone glucuronide containing component of the reconstituted sample was actively competing with the active ester conjugate for the available antibody binding sites. The shapes of the inhibition curves strongly suggest that it is in fact the intact reconstituted enzyme molecules which are binding to the immobilised polyclonal anti-E1G antibodies and preventing the signal generator (i.e the active ester conjugate) from binding and thus reducing the amplitude of the absorbance signal. The reconstituted HPAE1G- and HPE1G- enzymes (HPE1G profile not shown) apparently competed on an equal basis with the signal generator. This was shown by the fact that the titration curves for these two reconstituted enzymes were the same and were also similar to the line obtained with the active ester only control sample. This control curve was obtained by using decreasing amounts of conjugate in the same proportions as were predicted to be available in the competition experiments if the rules of equal competition were operating.

Compared to the other samples the HPAAE1G-enzyme conjugate cross reacted to a much greater extent which would not be expected to be the case if the cross reactivity shown in these experiments was due to dissociation of the hemins during the overnight incubations. On the other hand, it would be anticipated on the basis of the structures of the reconstituted conjugates (see Figure 5.42 for example and Figure 5.57) since the E1G would extend much further from the protein surface than for the active ester conjugate and the reconstituted

HPAE1G and HPAE1G enzyme conjugates. However, even the HPAAE1G-HRP conjugate did not generate a colour when used as a signal generator.

Further evidence for binding between the reconstituted horseradish peroxidase conjugate samples and the anti-estrone glucuronide antibody was provided by the Ovarian Monitor estrone glucuronide assays. In these solution assays the addition of the reconstituted enzyme samples to the assays resulted in an increase in lytic activity from the baseline rate; that is it had the same effect as the addition of free estrone glucuronide (see Table 5.5) at the concentrations expected on the basis of their structures. The fact that the post lysis reaction mixtures from the Ovarian Monitor assays still retained most of its peroxidase activity strongly suggests that at least for the Ovarian Monitor assay, it was the estrone glucuronide group of the reconstituted protein, not any dissociated hemin-estrone glucuronide which was binding to the antibody.

5.5 CONCLUSION

The results reported in this chapter represent a paradox that has not yet been explained satisfactorily. The idea that haptens can be linked to the hemin of horse radish peroxidase to give defined conjugates with good activity and in good yield has been completely vindicated. The chemistry used for the preparation of the hemin-linker-analytes is general and represents a new technology which could be used to custom make a conjugate for any desired analyte glucuronide. The enzymes are stable and pure in the sense that all of the enzymic activity is due to the conjugate, there being no native horseradish peroxidase remaining in the samples. For the linkers used in this study, simultaneous binding of the anti-E1G antibody and the apo protein to the E1G-linker-hemin compounds was clearly possible as indicated by the lack of antibody inhibition in the reconstitution assay system. Despite this observation, no colour was produced in the ELISA assays when the new conjugates were used as signal generator - a result which was disappointing and surprising.

The weight of the evidence, although necessarily indirect, is that the conjugates bind to the surface immobilised antibodies but for some reason the enzymic activity is destroyed in the process. Even with HPE1G (the shortest linker used) structural models suggest that there is no major steric problem in fitting the antibody and the apo protein together onto the HPE1G moiety (Figure 5.57). The model shown in Figure 5.57 was based on the horseradish peroxidase crystal structure and the Fab antibody co-ordinates from a progesterone-anti-progesterone Fab structure (Arevalo *et al.*, 1993). Although the actual conformation of the linker is not known the representation shown was arrived at by optimising the torsional angles for the polyethylene glycol unit to minimise any apo protein antibody interactions. This model possibly underestimates the likely protein-protein interactions since the Fab structure used in the modelling may not be a good model for an anti-E1G antibody. For example, it is known that the anti-E1G antibodies cross react better with the steroid glucuronides than they do with the free steroid (Binnie, 1999; Henderson *et al.*, 1995). Hence, the glucuronide ring, shown free in Figure 5.57, would in fact be buried in the antibody binding site. This would have the effect of swinging the antibody towards the apo protein. Of course the torsion angles may then be adjusted to take this into account and again minimise any steric clashes.

It must be admitted also that the effect of the polysaccharide units on this binding is unknown since the crystal structure for horseradish peroxidase was carried out on an enzyme expressed without the carbohydrate attached. However, the structural data are in agreement with the reconstitution results. Models made with HPAE1G and HPAAE1G showed almost no contact at all between the antibody and the apo protein and would not be affected by taking into account binding of the antibody to the glucuronide ring. Thus, from a structural perspective there does not seem to be any reason why the anti-E1G antibodies could not bind to the exposed E1G moieties.

If this is so then the question remains as to why the conjugates do not act as signal generators. This is an important question and the answer will determine the future of this research.

There seem to be two major possibilities.

- i) In the reconstituted conjugates the polyethylene linker hydrogen bonds strongly to the protein surface so that the E1G moiety is not presented for binding to the E1G binding site of the antibody.
- ii) The conjugate does bind to the immobilised antibody but some steric blocking occurs which either prevents access of substrate to the bound conjugate or the substituted hemin group dissociates from the apo protein to give an inactive enzyme.

It is surprisingly difficult to determine which is the correct explanation in the absence of colour generating properties from the reconstituted enzymes. The experiment which appears to hold out the most hope is to examine the binding properties of the conjugates to antibodies immobilised on biosensor chips. If the conjugates bind an optical signal is produced from which the actual rates of binding and dissociation can be calculated. This experiment has yet to be done.

If the conjugates do bind to surface immobilised antibodies and are inactivated this raises the possibility of utilising the conjugates in new homogeneous enzyme immunoassays for estrone glucuronide. If a suitable polymer matrix such as a latex bead can be found which allows rapid binding in assay tubes then no colour will be generated in the absence of exogenous estrone glucuronide but a concentration dependent colour will appear as the level of estrone glucuronide increases in the assay. In effect this would give a rapid assay for estrone glucuronide where again all of the components could be dried in an assay tube and the same Monitor methodology could be utilised to fulfill the goal of providing access to the important information on fertility discussed in Chapter One with a rapid, user friendly home monitor test.

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