REGULATION OF ETHANOL METABOLISM IN ISOLATED RAT HEPATOCYTES

A thesis presented in partial fulfilment of the
requirements for the degree of
Doctor of Philosophy (BIOCHEMISTRY)
at Massey University

RACHEL AUDREY PAGE

1990

Massey University Library. Thesis Copyright Form

Title of thesis: Regulation of Ethanol Metabolism in Isolated Rat Hepatocytes

(1) (a) I give permission for my thesis to be made available to readers in the Massey University Library under conditions determined by the Librarian.

~~(b)~~ I do not wish my thesis to be made available to readers without my written consent for ______ months.

(2) (a) I agree that my thesis, or a copy, may be sent to another institution under conditions determined by the Librarian.

~~(b)~~ I do not wish my thesis, or a copy, to be sent to another institution without my written consent for ______ months.

(3) (a) I agree that my thesis may be copied for Library use.

~~(b)~~ I do not wish my thesis to be copied for Library use for ______ months.

Signed R Page

Date 23/2/90

The copyright of this thesis belongs to the author. Readers must sign their name in the space below to show that they recognise this. They are asked to add their permanent address.

NAME AND ADDRESS	DATE
Rachel Page	23/1
13 [illegible] Ferguson Street, P.N.	

ABSTRACT

The importance of an individual step in the regulation of a metabolic pathway can be quantitatively defined by determining a global coefficient called the flux control coefficient ($C^J_{E_i}$). The coefficient can be evaluated by altering the pathway flux in small increments using an enzyme-specific inhibitor. This modulation approach was applied to the study of the regulation of ethanol metabolism in isolated rat hepatocytes.

A procedure was developed for using isolated hepatocytes to measure the small decreases in rates of ethanol oxidation produced by increasing concentrations of inhibitor. The method, termed the two time point method, involved the incubation of replicate 1 ml or 2 ml samples in 10 ml erlenmeyer flasks containing cells, medium and substrates with varying inhibitor concentrations (including zero to provide a control rate).

Using this method, the apparent flux control coefficient of ADH in hepatocytes isolated from starved rats was determined using two inhibitors, tetramethylene sulphoxide (TMSO) and isobutyramide (IsB). Using the initial slope of the flux inhibition curve and the appropriate inhibition constant determined for isolated ADH, the apparent C^J_{ADH} was calculated to be 0.37 ± 0.09 (from inhibition by TMSO) or 0.43 ± 0.10 (from inhibition by IsB). The good agreement in the value obtained for the flux control coefficient of ADH, using two different inhibitors, showed that the method developed for measuring small changes in ethanol clearance rates was providing accurate, reproducible results.These flux control coefficients were calculated on the assumption that the ADH reaction is a one substrate, one product reaction. The apparent flux control coefficient of ADH was then corrected for the concentration effect of NAD^+, NADH and acetaldehyde. When substrate and product concentrations were considered, the value of the apparent C^J_{ADH} increased to a maximum of 0.53 (for TMSO inhibition) or 0.63 (for IsB inhibition). These results indicate that in starved rats the activity of the enzyme alcohol dehydrogenase is one of the major factors involved in regulation of ethanol metabolism.

The importance of ADH in fed rats was determined using TMSO. A range of values for the flux control coefficient of ADH was obtained with varying concentrations of acetaldehyde. The value of C^J_{ADH} was dependent on the concentration of acetaldehyde present in the isolated rat hepatocytes. For example the apparent flux control coefficient of ADH was about 0.02 when the acetaldehyde concentration was 138 μM and was approximately 0.5 when the concentration of acetaldehyde was 6 μM, which is analogous to conditions in starved rats. When the apparent C^J_{ADH} was corrected for the effect of substrate and product concentration the trend of decreasing

C^{J}_{ADH} with increasing acetaldehyde concentration was still observed. This indicates that the importance of alcohol dehydrogenase in regulation of ethanol metabolism, in hepatocytes isolated from fed rats, decreases with increasing acetaldehyde concentration. Obviously, as the significance of ADH in regulation of ethanol oxidation decreases, other enzymes in the ethanol pathway become more important. The most likely enzyme to have an increasing importance in the regulation of ethanol elimination, with increasing concentrations of acetaldehyde, is aldehyde dehydrogenase (AlDH).

Preliminary inhibitor studies were carried out on AlDH in hepatocytes isolated from starved rats, using the irreversible inhibitor disulfiram. The flux control coefficient of aldehyde dehydrogenase was calculated to be about 0.1. However, because of the problems encountered with the preparation of disulfiram stock solutions, an alternative inactivator of aldehyde dehydrogenase was required for the inhibitor titration experiments. Diethyldithiocarbamate (DDC), a metabolite of disulfiram, was used. The results showed that inhibition of AlDH occurred in the presence of DDC and that the minimum amount of DDC required for total inhibiton of aldehyde dehydrogenase was in the concentration range of 300 μM to 500 μM. Further studies should be carried out for determination of the initial slope of the flux inhibition curve, using DDC, so that the flux control coefficient of AlDH may be calculated.

In chapter 6, a theoretical approach for determining the importance of the shuttle systems and electron transport chain in regulation of ethanol oxidation in rat hepatocytes is discussed. A literature search on possible inhibitors for the malate-aspartate shuttle, α-glycerophosphate shuttle and electron transport chain was carried out.

The results from this study show that it is possible to evaluate the importance of some of the individual steps in the ethanol pathway by using the modulation approach devised by Kacser and Burns. However the two time point method developed for measuring the small decreases in flux through the ethanol pathway imposed by increasing concentrations of inhibitor is only sensitive enough to detect changes in flux as low as 8.0%. Enzymes that have low flux control coefficients will produce changes in flux of about 8.0%. Thus, the development of a more sensitive method for measuring the small changes in flux through the ethanol pathway is needed, or a method other than the modulation approach for calculating the flux control coefficient is required.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr K. E. Crow and Dr M. J. Hardman for their invaluable advice and encouragement when times were rough. Iwould like to say that I was lucky in having two very good supervisors. They were not only interested in the results produced during the course of my work but cared about my well being. Thanks Kathy and Mike.

I also wish to thank all my friends, especially Kathryn and John, for making my PhD years enjoyable.

Finally, Iwould like to thank my family, for their loving support, encouragement and help over the years. I could not have finished this thesis without it.

Table of Contents

		PAGE
ABSTRACT		ii
ACKNOWLEDGEMENTS		iv
TABLE OF CONTENTS		v
LIST OF FIGURES		
LIST OF TABLES		

CHAPTER 1
INTRODUCTION

1.1	Metabolism of Ethanol		1
1.2	Regulation of Ethanol Metabolism		3
1.3	Theoretical Frameworks for the Study of Metabolic Control		7
	1.3.1	Biochemical Systems Theory (BST)	7
	1.3.2	Metabolic Control Theory (MCT)	8
	1.3.3	Flux Oriented Theory (FOT)	10
	1.3.4	Comparison of Theories	11
1.4	Determination of Flux Control Coefficients		13

CHAPTER 2
MATERIALS AND METHODS

2.1	Source of Materials and Reagents		18
2.2	Hydrolytic Enzymes		18
	2.2.1	Collagenase	18
	2.2.2	Hyaluronidase	20
2.3	Krebs-Henseleit (K-H) Buffer		20
	2.3.1	Perfusion Medium	20
	2.3.2	Cell Suspension Medium	21

PAGE

2.4	Bovine Serum Albumin (BSA) For Hepatocytes		21
	2.4.1	Dialysis of BSA	21
	2.4.2	Determination of Protein Concentration	22
2.5	Preparation of Isolated Hepatocytes		22
	2.5.1	Rats	23
	2.5.2	Operative Technique	23
		2.5.2.1 *Surgical Procedure*	23
	2.5.3	Perfusion	25
	2.5.4	Collection of Hepatocytes	25
	2.5.5	*Vena Cava* Cannula	26
	2.5.6	Perfusion Lung	26
	2.5.7	Nylon Gauze Sieve	27
2.6	Cell Wet Weight		27
2.7	Criteria For Cell Integrity		27
	2.7.1	Trypan Blue Exclusion Test	28
	2.7.2	ATP Content	28
2.8	Incubation of Isolated Hepatocytes		28
2.9	Ethanol Assay		29
2.10	Acetaldehyde Assay		29
	2.10.1	Standard Acetaldehyde Solutions	30
	2.10.2	Assay Reagents	30
	2.10.3	Measurement	30
	2.10.4	Maintenance of Apparatus	31
2.11	ATP Assay		31

PAGE

CHAPTER 3
DEVELOPMENT OF A METHOD FOR MEASUREMENT OF SMALL CHANGES IN RATES OF ETHANOL OXIDATION

3.1	Background to Present Work		32
3.2	Two Time Point Method		35
	3.2.1	Procedure for Addition of Cell Suspension to Flasks and Incubation of Isolated Hepatocytes	35
	3.2.2	Calculation of Ethanol Clearance Rates	37
3.3	Development of the Two Time Point Method		42
	3.3.1	Incubation Mixture and Cell Suspension	42
		3.3.1.1 *Lactate and Pyruvate*	42
		3.3.1.2 *Ethanol*	43
		3.3.1.3 *Cell Content*	46
	3.3.2	Transfer of Cells to the Incubation Vessel	47
	3.3.3	Cell Suspension Transfer and Gassing Procedure	49
	3.3.4	Incubation Vessel	50
	3.3.5	Incubation Vessel - Experimental Loss of Ethanol	52
3.4	Conclusion		55

CHAPTER 4
ALCOHOL DEHYDROGENASE

4.1	Introduction		59
4.2	Tetramethylene Sulphoxide and Isobutyramide		66
	4.2.1	Type of Inhibition	66
	4.2.2	Determination of Flux Control Coefficient	68
4.3	The Use of Isolated Hepatocytes From Fed and Starved Rats		69

PAGE

4.4	Experimental Results and Discussion		69
	4.4.1	Starved Rats	69
	4.4.2	Fed Rats	78
4.5	Substrate and Product Concentration Effect on the Flux Control Coefficient		83
	4.5.1	Correction of the Apparent C^{J}_{ADH}	83
	4.5.2	Conclusion	97

CHAPTER 5
ALDEHYDE DEHYDROGENASE

5.1	Introduction	98
5.2	Experimental Results and Discussion	100

CHAPTER 6
SHUTTLE SYSTEMS AND ELECTRON TRANSPORT CHAIN

6.1	Shuttle Systems	110
6.2	Electron Transport Chain	116
6.3	Conclusion	117

CHAPTER 7
DISCUSSION

7.1	Summary of Results	119
7.2	Problems	122
7.3	Future Work	124

PAGE

7.4 Conclusion 126

REFERENCES 127

APPENDIX 143

List of Figures

FIGURE	TITLE	PAGE
1.1	Schematic Diagram of the Interactions Between the Metabolism of Ethanol, Shuttle Systems, and Respiratory Chain	4
1.2	Steps in the Metabolism of Ethanol	6
2.1	Perfusion Apparatus	24
3.1	Graph of Expected Results Using the Two Time Point Method	34
3.2	Experimental Procedure for the Two Time Point Method	36
3.3	Extrapolation of Control Rate For Determination of the True Zero Time Ethanol Concentration	39
3.4	Graph of Ethanol Concentration vs Time for a TMSO Experiment	56
4.1	Ordered Bi-Bi Mechanism For Rat Liver ADH	61
4.2	Steady State Rate Equation For An Ordered Bi-Bi Mechanism	63
4.3	Dixon Plot for Tetramethylene Sulphoxide	73
4.4	Dixon Plot for Isobutyramide	74
4.5	Flux Inhibition Curve For Tetramethylene Sulphoxide	75
4.6	Flux Inhibition Curve For Isobutyramide	76
4.7	Flux Inhibition Curve For Tetramethylene Sulphoxide when [AcH] is low (11.3 μM)	79
4.8	Flux Inhibition Curve For Tetramethylene Sulphoxide when [AcH] is high (80.6 μM)	80
4.9	Graph of Flux Control Coefficient of ADH vs Acetaldehyde Concentration	82
5.1	Flux Inhibition Curve for Data of Crow *et al.* (1977a)	103
5.2	Flux Inhibition Curve for Cell Preparation 3	104
5.3	Graph of Normalised Flux vs Diethyldithiocarbamate Concentration	107
6.1	Schematic Diagram of the α-Glycerophosphate Shuttle	111
6.2	Schematic Diagram of the Malate-Aspartate Shuttle	112

List of Tables

TABLE	TITLE	PAGE
1.1	Terminology in BST, MCT and FOT for Control Parameters	11
2.1	Materials and their Source	19
3.1	Percentage Inhibition of Ethanol Clearance Using Varying Concentrations of Tetramethylene Sulphoxide	33
3.2	Amount of Ethanol Present in Control and Inhibitor Samples Incubated for the same Time	38
3.3	Comparison of the Experimental and Extrapolated Ethanol Concentrations at True Zero Time	40
3.4	Comparison of the % Inhibition Results Obtained Using the Averaged and Extreme Extrapolated True Zero Time Samples	41
3.5	The Viability of Cells Suspended With or Without Lactate and Pyruvate	44
3.6	Ethanol Content Present Before and After Incubation Using Method 1 and 2 for Ethanol Transfer	45
3.7	Results of Ethanol Utilisation for Incubation Mixtures Containing 50 or 60 % of the Original Hepatocyte Suspension	48
3.8	Metabolic Viability and Cell Integrity For Cells Transferred by a P_{1000} Gilson Pipetman or 2 ml Detipped Glass Pipette	49
3.9	Loss of Ethanol During the 20 second Gassing Step	50
3.10	Comparison of Ethanol Disappearance Rates and Percentage Inhibition Results With Plastic or Balsa Cork Stoppers	51
3.11	ATP Content of Hepatocytes Incubated in Flasks and Test Tubes	53
3.12	Ethanol Concentration Present in Flasks for Different Periods of Incubation	54

TABLE	TITLE	PAGE
3.13	Percentage Inhibition of Ethanol Clearance for the Two Time Point and Repeated Sampling Methods	57
3.14	Advantages of the Two Time Point Method Compared to the Repeated Sampling Method	58
4.1	Characterisation of Rat Alcohol Dehydrogenase Isoenzymes	60
4.2	Kinetic Constants of Rat Liver Alcohol Dehydrogenase	61
4.3	The Relative Importance (C_E^J) of Horse Liver ADH Isoenzymes and the Mitochondrial and Cytosolic AlDH Isozymes in the Metabolism of Ethanol	64
4.4	Kinetic Constants for Inhibition of Alcohol Dehydrogenase	67
4.5	Combined Results for Inhibition by Tetramethylene Sulphoxide	70
4.6	Combined Results for Inhibition by Isobutyramide	71
4.7	Flux Control Coefficient of ADH and Inhibition Constant Determined Using the Combined Results for Inhibition by Tetramethylene Sulphoxide and Isobutyramide	77
4.8	Table of Flux Control Coefficient of ADH with their Corresponding Acetaldehyde Levels	81
4.9	Derived Equations For Substrate and Product Effect on the C_{ADH}^J	85
4.10	Substrate (A,B) and Product (Q) Concentrations of ADH Reaction	86
4.11	Effect of Both Substrates and Changing NADH Concentration on the Apparent C_{ADH}^J Determined from Liver Cells Isolated from Starved Rats	88
4.12	Effect of Both Substrates and Changing Product Concentrations on the C_{ADH}^J Evaluated from Liver Cells Isolated from Starved Rats Assuming TMSO and IsB are Uncompetitive Inhibitors	89

TABLE	TITLE	PAGE
4.13	Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Starved Rats Assuming TMSO and IsB are Noncompetitive Inhibitors	90
4.14	Effect of Both Substrates in the Absence of Products on the C^J_{ADH} Evaluated from liver Cells Isolated from Fed Rats	91
4.15	Effect of Both Substrates and Changing NADH Concentration on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is an Uncompetitive Inhibitor	92
4.16	Effect of Both Substrates and Changing NADH Concentration on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is a Noncompetitive Inhibitor	93
4.17	Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is an Uncompetitive Inhibitor	94
4.18	Effect of Both Substrates and Changing Product Concentrations on the C^J_{ADH} Evaluated from Liver Cells Isolated from Fed Rats Assuming TMSO is a Noncompetitive Inhibitor	95
5.1	% Inhibition and Acetaldehyde Concentration with Increasing Concentrations of Disulfiram in Hepatocytes from Starved Rats	101
5.2	Flux Control Coefficient of AlDH in Hepatocytes Isolated from Starved Rats	102
5.3	The % Inhibition and Acetaldehyde Concentrations Obtained Using a Range of Diethyldithiocarbamate Concentrations	106
7.1	Acetaldehyde Concentrations and Flux Control Coefficients of Alcohol Dehydrogenase for Hepatocytes Isolated from Fed and Starved Rats	120