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A STUDY OF THE EFFECTS OF PLANE OF NUTRITION  
ON BOVINE MILK PROTEINS, WITH PARTICULAR  
EMPHASIS ON THE INDIVIDUAL WHEY PROTEINS

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF MASTER OF AGRICULTURAL SCIENCE IN ANIMAL  
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SUMMARY

The work in this thesis consists of a short term study of the effects of plane of nutrition on the synthesis and secretion of milk proteins by dairy cows, with particular emphasis on the individual whey proteins.

The treatments comprised a high and low plane of nutrition and were imposed by strictly controlled stocking rates of the experimental animals. Six sets of identical twin cows were split between the two treatment groups.

The technique of discontinuous gel electrophoresis was used for the separation of the whey proteins and a method (called "Proportional Subdivision") was developed for the quantitative analysis of the individual proteins. The four major whey proteins ( $\beta$  lactoglobulin,  $\alpha$  lactalbumin, bovine serum albumin and immunoglobulins) were isolated and quantified.

1). The treatments were deemed effective as judged by the differences which were measured in the concentrations and yields of the major milk fractions. The yields of milk, fat and protein were significantly ( $P < 0.01$ ) affected by treatment as was the protein percentage measured in skim milk, but not in whole milk. Fat percentage did not alter significantly ( $P > 0.05$ ).

2). The mastitis status of the animals was monitored using the Wisconsin Mastitis Test. It was possible therefore to show that Mastitis infections were not influencing the responses to treatment.

3). Total whey and casein proteins, each expressed as a percentage of total protein, did not alter ( $P > 0.05$ ). The caseins, expressed as a percentage of whole milk differed significantly ( $P < 0.01$ ) between treatments whereas the total whey proteins expressed similarly did not ( $P > 0.05$ ). The yields of both classes of proteins altered significantly ( $P < 0.01$ ) with treatment.

4). The concentrations of the whey proteins which are synthesised in the mammary gland ( $\beta$  lactoglobulin and  $\alpha$  lactalbumin) decreased with the low plane of nutrition, but only the difference between treatments in  $\alpha$  lactalbumin reached significance ( $P < 0.05$ ). The yields of both these proteins were reduced significantly ( $P < 0.01$ ) by the low plane treatment.

5). The concentrations of the proteins entering the mammary gland pre-formed from the blood stream (Bovine Serum Albumin and the Immunoglobulins) moved in the opposite direction to the mammary synthesised proteins in response to the low plane treatment. The difference between treatments (increase in concentration under the low plane of nutrition) was highly significant ( $P < 0.01$ ) for Bovine Serum Albumin, but failed to reach significance for the Immunoglobulins. The change in concentration was sufficient to decrease the yield of Bovine Serum Albumin under the high plane relative to the low plane treatment. The yield of Immunoglobulin was not altered significantly by the treatments ( $P > 0.05$ ).

6). The separation of the individual  $\beta$  lactoglobulin proteins (A and B) in the cows heterozygous (AB) for  $\beta$  lactoglobulin indicated that no significant changes occurred ( $P > 0.05$ ) in the concentration of the proteins between treatments. Similarly, the ratio of the two proteins (A/B) did not alter significantly ( $P > 0.05$ ) with treatment.

The relevance of these results is discussed both in relation to the synthesis and secretion of milk proteins and in relation to observations on the influence of the effect of protein composition on the manufacturing properties of milk.

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## TABLE OF CONTENTS

	<u>Page</u>
SUMMARY	ii
ACKNOWLEDGEMENTS	iv
LIST OF FIGURES	x
LIST OF PLATES	xii
LIST OF TABLES	xiii
INTRODUCTION	1
CHAPTER 1     Review of Literature	4
1:1     The Protein Composition of Milk	4
1:1:1    Nomenclature and classification of milk proteins	4
1:1:2    Milk Protein Polymorphism	6
1:1:3    Origin of Milk Proteins	8
1:1:4    Biological Functions of the Major Milk Proteins	10
1:1:4:1    Caseins	10
1:1:4:2    Alpha Lactalbumin	10
1:1:4:3    Beta Lactoglobulin	11
1:1:4:4    Immunoglobulins	12
1:1:4:5    Serum Albumin	12
1:2     Milk Protein Synthesis and Secretion by the Mammary Gland	13
1:2:1    Milk Protein Synthesis in the Mammary Gland	13
1:2:2    Protein Secretion by the Mammary Gland	15
1:2:2:1    Secretion of Proteins Synthesized in the Mammary Gland	15
1:2:2:2    Secretion of Serum Proteins by the Mammary Gland	19
1:2:2:2:1    Immunoglobulins	19
1:2:2:2:2    Serum Albumin	20

	<u>Page</u>	
1:3	Factors Affecting Milk Composition	23
1:3:1	The Effect of Nutrition on Milk Yield and on the Concentrations and Yields of the Major Milk Components	23
1:3:1:1	Energy Supplements	23
1:3:1:2	Protein Supplements	24
1:3:1:3	Stocking Rate	25
1:3:1:4	Mediation of Nutrition on Milk Synthesis	27
1:3:2	The Effect of Stress on Milk Yield and Composition	31
1:3:3	Influence of Mastitis on Milk Yield and Composition	32
1:4	Effect of Milk Proteins on Manufacturing Properties of Milk	36
CHAPTER 2	Consideration of Experimental Design and the Selection and Development of Analytical Procedures	37
2:1	Aspects of Experimental Design	37
2:1:1	General Considerations	37
2:1:2	Animals (Experimental Units)	38
2:1:3	Experimental Design	39
2:1:4	Lengths of Preliminary and Comparison Periods	40
2:2	Measurement of Response Variables	43
2:2:1	Separation of Individual Whey Proteins	43
2:2:2	Quantification of the Separated Proteins	44
2:2:2:1	Acrylamide Concentration	46
2:2:2:2	Method of Destaining Gels	46
2:2:2:3	Time of Electrophoretic Destaining	49
2:2:2:4	Separation of Intergrator Response Units of Overlapping Peaks	51
2:2:2:5	Comparison of Methods of Quantifying Protein Band Units	53
2:2:3	Determination of Total Whey Protein Concentration	56

	<u>Page</u>
2:2:4 Mastitis	57
2:2:4:1 Direct Methods	57
2:2:4:2 Indirect Methods	58
2:2:5 Milk Fat	59
2:2:6 Milk Protein	59
 CHAPTER 3      Materials and Methods	 60
3:1      Experimental Design	60
3:2      Animals (Experimental Units)	60
3:3      Experimental Procedures	63
3:3:1   Field Work	63
3:3:2   Sampling and Analysis of the Major Milk Parameters	63
3:3:2:1   Milk Yield	63
3:3:2:2   Milk Fat and Protein	65
3:3:2:3   Mastitis	65
3:4      Analysis of the Whey Protein Components	65
3:4:1   Preparation of Whey	65
3:4:2   Protein Separation	66
3:4:3   Protein Quantification	71
3:4:3:1   Standard Curves of Dye-binding by Proteins in Gels	72
3:4:3:2   Standard Curves of Dye-binding by Proteins in the Spectrophotometric Method	73
3:5      Total Whey and Casein Proteins Concentration	74

	<u>Page</u>
CHAPTER 4      Results	75
4:1      Examination of Effectiveness of Treatments	75
4:1:1    Determination of Time of Onset of Treatment Effects	75
4:1:2    Examination of the Effect of Treatments on Milk Yield and Gross Milk Composition	77
4:1:3    Influence of Mastitis on Treatment Effects	86
4:1:3:1    Effect of Basal Level of Infection and Changes in Status over Time	86
4:1:3:2    Effect of Isolated Suspicious and Positive WMT Scores	90
4:2      Effect of Plane of Nutrition on the Concentration and the Yield of Milk Proteins	93
4:2:1    Effect on Major Milk Protein Classes	93
4:2:2    Effect on the Major Individual Whey Proteins	101
CHAPTER 5      Discussion	118
5:1      Effect of Treatment on Total Milk Protein	118
5:2      Effect of Treatment on the Major Classes of Milk Proteins	120
5:3      The Effect of Treatment on Individual Whey Proteins	122
5:3:1    Mammary Synthesized Whey Proteins	122
5:3:2    Whey Proteins originating in the Bloodstream	123
CHAPTER 6      Conclusions	126
BIBLIOGRAPHY	129
APPENDICES	144

## LIST OF FIGURES

<u>Figure</u>		<u>Page</u>
1:1	Distribution of Milk Proteins	5
1:2	Diagrammatic summary of the process of Protein Biosynthesis.	14
1:3	Effect of Mastitis on the proportions of Serum Albumin and Immunoglobulin in the Total Whey Protein.	33
4:1	Effect of Plane of Nutrition on Milk Yield	79
4:2	Effect of Plane of Nutrition on Milk Fat Percentage	80
4:3	Effect of Plane of Nutrition on Milk Fat Yield	81
4:4	Effect of Plane of Nutrition on Total Milk Protein Content, expressed as a percentage of Whole Milk.	82
4:5	Effect of Plane of Nutrition on Total Milk Protein Yield.	83
4:6	Effect of Plane of Nutrition on Total Milk Protein as a percentage of Skim Milk.	84
4:7	Mean Wisconsin Mastitis Test Scores ( $\pm$ S.E.) for Individual Cows and Treatment Groups over the Preliminary and Treatment periods.	89
4:8	Effect of Plane of Nutrition on Total Whey Proteins expressed as a percentage of Whole Milk.	95
4:9	Effect of Plane of Nutrition on the Total Non Whey Proteins expressed as a percentage of Whole Milk.	96
4:10	Effect of Plane of Nutrition on Total Whey Proteins expressed as a percentage of Total Protein.	97
4:11	Effect of Plane of Nutrition on the Total Non Whey Proteins expressed as a percentage of Total Protein.	98
4:12	Effect of Plane of Nutrition on Total Whey Protein Yield.	99

<u>Figure</u>		<u>Page</u>
4:13	Effect of Plane of Nutrition on Total Non Whey Protein Yield.	100
4:14	Effect of Plane of Nutrition on $\alpha$ Lactalbumin Concentration.	103
4:15	Effect of Plane of Nutrition on $\alpha$ Lactalbumin Yield.	104
4:16	Effect of Plane of Nutrition on Bovine Serum Albumin Concentration.	105
4:17	Effect of Plane of Nutrition on Bovine Serum Albumin Yield.	106
4:18	Effect of Plane of Nutrition on Immunoglobulin Concentration.	107
4:19	Effect of Plane of Nutrition on Immunoglobulin Yield.	108
4:20	Effect of Plane of Nutrition on $\beta$ Lactoglobulin Concentration.	109
4:21	Effect of Plane of Nutrition on $\beta$ Lactoglobulin Yield.	110
4:22	Effect of Plane of Nutrition on the Concentration of $\beta$ Lactoglobulin A.	111
4:23	Effect of Plane of Nutrition on the Concentration of $\beta$ Lactoglobulin B.	112
4:24	Effect of Plane of Nutrition on the Relative Proportion of $\beta$ Lactoglobulin Variants ( $A/B$ ).	113
5:1	Effect of Plane of Nutrition on Milk SNF content.	119

## LIST OF PLATES

<u>Plate</u>		<u>Page</u>
3:1	Apparatus for Polyacrylamide Discontinuous Gel Electrophoresis (PAGE), featuring 2 X 12 gel tanks.	64
3:2a	Purity of Immunoglobulin used for the preparation of Dye-Binding Standard Curves	67
3:2aai	Scan Trace and Intergrator Plot of the Immunoglobulin Standard.	67
3:2bi	Purity of Bovine Serum Albumin used for the preparation of Dye-Binding Standard Curves	68
3:2bii	Scan Trace and Intergrator Plot of the Bovine Serum Albumin Standard.	68
3:2ci	Purity of Alpha lactalbumin used for the preparation of Dye-Binding Standard Curves.	69
3:2cii	Scan Trace and Intergrator Plot of the Alpha Lactalbumin Standard.	69
3:2di	Purity of the Beta Lactoglobulin used for the preparation of Dye-Binding Standard Curves.	70
3:2dii	Scan Trace and Intergrator Plot of the Beta Lactoglobulin Standard.	70
4:1a	Whey Protein Separation obtained by PAGE.	114
4:1b	Scan Trace and Intergrator Plot of the Gel shown in 4:1a (H.P.)	115
4:1c	Scan Trace and Intergrator Plot of the Gel shown in 4:1a (L.P.)	115
4:2a	Whey Protein Separation obtained by PAGE.	116
4:2b	Scan Trace and Intergrator Plot of the Gel shown in 4:2a (H.P.)	117
4:2c	Scan Trace and Intergrator Plot of the Gel shown in 4:2a (L.P.)	117

## LIST OF TABLES

<u>Table</u>		<u>Page</u>
1:1	Genetic Polymorphs of the Major Milk Proteins	6
1:2	Origin of some of the Minor Milk Proteins	9
2:1	Comparison of Two Methods of Gel Destaining	48
2:2	Effect of Prolonged Electrophoretic Destaining on Protein Concentration	50
2:3	Relationship between the peak proportion from two methods of separating these overlapping peaks.	52
2:4	Comparison of the Sources of Variation within each of two methods of Estimating $\beta$ Lgb Concentration from the same samples	55
3:1	Description of Experimental Animals	62
4:1	Analysis of time of onset of Treatment Effects on Mean Milk Yield - first day of treatment.	76
4:2	Influence of Plane of Nutrition on Milk Yield and Composition.	85
4:3	WMT scores of individual cows samples at the afternoon milking.	88
4:4	Analysis of individual cow WMT scores over time.	91
4:5	Effect of isolated high WMT scores on milk yield and composition.	92
4:6	Effect of Plane of Nutrition on the Yield and Concentration of the Major Milk Protein Classes.	94
4:7	Effect of Plane of Nutrition on the Concentration and Yield of the Major Whey Proteins.	102

## INTRODUCTION

Milk and other dairy products comprise a major source of man's food. The milk of the cow (*Bos taurus*) is of overwhelming importance (F.A.O. Yearbook 1974). Interest in the composition of cows milk stems largely from its importance in the human diet and from the need of milk producers to meet the legal requirements governing its sale (Rock 1961a).

Prior to about the 1850's milk had been found to contain fat, sugar, protein and minerals (Jenness and Patton 1959). The development and refinement of qualitative and quantitative techniques has subdivided these gross categories of milk composition into a vast array of molecules (Jenness 1974).

The composition of cows milk has been extensively reviewed: Cerbulis and Farrell (1975), Jenness (1974), Webb and Johnson (1965), Ling et al. (1961), Rook (1961a & b), Armstrong (1959), Jenness and Patton (1959). The Sale of Food and Drugs Act (1908) prescribed minimum compositional standards required for milk sold or intended for sale in New Zealand. These were "8.5 parts per centum of milk solids other than milk fat and 3.25 parts per centum of milk fat" (Sykes 1952).

Historically, milk produced for manufacture (as distinct from production for the sale of liquid milk) has been paid for according to the amount of milk fat it contained. However, in the last two decades, more attention has been paid to the economic importance of the milk proteins.

Attention has focussed on the inclusion of protein testing in the basis for calculating the payment a farmer receives for the milk he produces, particularly for milk destined for cheese manufacture. The Netherlands first introduced the system in the late 1950's followed by Denmark, Poland, Switzerland (Cerbulis and Farrell, 1975) and some States of America (Corwin et al. 1962). New Zealand has been investigating the inclusion of a protein component in the system of payout for milk since the late 1960's (McGillivray and Creamer 1972, Creamer 1973).

This increase in emphasis on the protein content of milk argues for an improvement in the state of knowledge relating to all aspects of the production by the cow of milk protein.

The current economic climate in New Zealand, particularly the high fixed costs of dairy farming, have emphasised the importance of milk production per unit of farm area. Dairy farmers have been forced to become less concerned with production per cow, as testified by increasing stocking rates, increased production per hectare and a decline in the average production per cow (Hutton 1972). This decline in production is indicative of chronic underfeeding of the cows, a situation which has recently been compounded by a succession of summer droughts. These conditions have resulted in the situation where the feed intake of cows is likely to be severely restricted during mid lactation.

It is important to obtain information on the effect of such underfeeding in terms of the expected changes in milk yield and composition to permit economic analysis of the effects. Such information is also relevant to the manufacturing sector of the dairy industry due to the relationships between milk composition and the manufacturing properties of the milk. Furthermore, the responses of many of the components of milk to changes in the nutritional status of the cow is incompletely understood, nor is their mode of secretion by the lactating mammary gland. It is this latter factor upon which the major emphasis of this thesis has been directed with no attempt made to analyse the economics of the changes in milk composition. Particular emphasis has been laid on the analysis of the whey protein fraction, due to the current interest in their relationships with the manufacturing properties of milk (see section 1:1:5).

An experiment was designed with the following objectives:-

1. To observe the responses by cows near peak lactation to a feed stress induced by a high stocking rate, in terms of the changes in the levels of the major descriptive categories of milk production, i.e. milk yield and the percent contents of fat and protein.
2. To investigate the effects of a feed stress on the protein composition of milk, with particular emphasis on the effects of the individual proteins of the whey fraction.

3. From the observed changes in the concentrations and yields of the individual whey proteins, to obtain information as to the control of individual protein synthesis and their mode of secretion by the mammary gland.

## CHAPTER 1

Review of Literature1:1 The Protein Composition of Milk1:1:1 Nomenclature and Classification of Milk Proteins

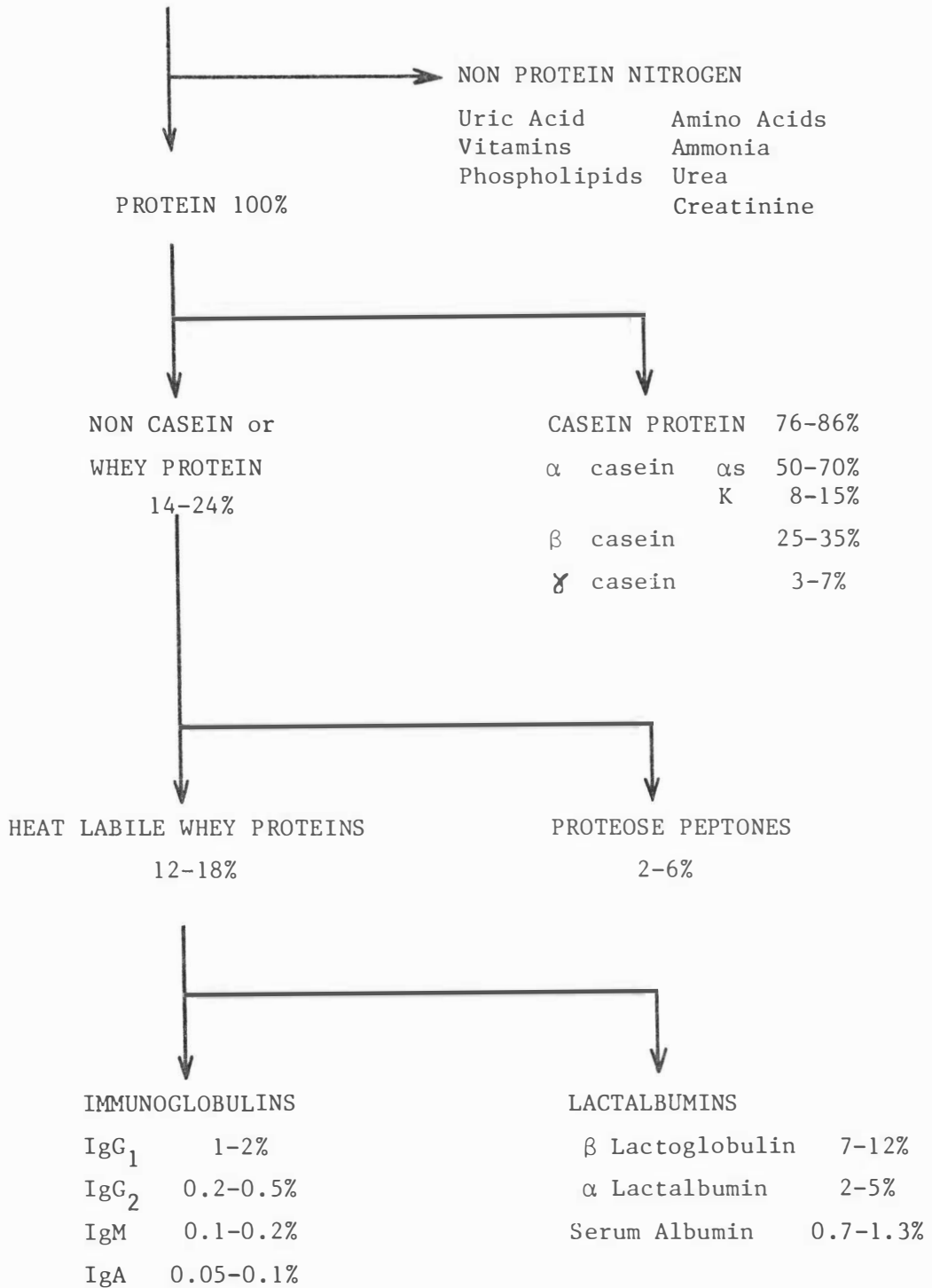
Milk proteins can be broadly separated into two fractions - caseins and whey proteins. Whereas the casein fraction of milk has been investigated since its first separation in 1830 by Bracannot, the less abundant whey proteins, which were called milk serum proteins, were thought to originate from the bloodstream and were largely ignored. Sebelien (1885) however demonstrated the complexity of the proteins of whey by separating out both globulin and albumin fractions.

Palmer (1934) isolated a single crystalline globulin component from whey which was later named Beta Lactoglobulin. Furthermore, from immune-studies he concluded that this crystalline globulin was not present in serum. Thus the description of whey proteins as "the milk serum proteins" which still persists, is a misnomer and should be avoided.

Figure I.1 shows the breakdown of the major milk proteins both qualitatively and quantitatively in relation to total milk protein.

Figure 1:1 DISTRIBUTION of MILK PROTEINS.

SKIM MILK NITROGEN 3.2% Protein



Adapted from: O'Sullivan (1971)  
and Rose et al. (1970).

1:1:2 Milk Protein Polymorphism

Polymorphism can be defined as the "Occurrence of different forms of individuals in the same species" (Kenneth 1967). The isolation of two polymorphic Beta Lactoglobulin types  $\beta$  1 and  $\beta$  2 (later changed to A and B) by Aschaffenburg and Drewry in 1955 has been followed by the elucidation of many more genetically controlled protein polymorphisms in the caseins as well as the whey protein fraction (Aschaffenburg 1968). These are summarised in Table 1:1.

Table 1:1 Genetic Polymorphs of the Major Milk Proteins

Caseins				Whey Proteins		
$\alpha$ S <sub>1</sub> ,	$\beta$	$\gamma$	K	$\beta$ Lgb.	$\alpha$ Lac.	B.S.A.
A	A <sup>1</sup>	A	A	A	A	A
B	A <sup>2</sup>	B	B	A <sub>Dr</sub>	B	B
C	A <sup>3</sup>			B		
D	B			C		
	B <sub>z</sub>			D		
	C					
	D					

From: Jenness (1970)

All of the major milk proteins are now known to occur in the form of products which reflect the action of autosomal genes transmitted by simple Mendelian inheritance (Aschaffenburg 1968). The products which represent the activity of co-dominant alleles found either singly in homozygous or paired in heterozygous animals, are distinguishable from one another electrophoretically.

The biological significance of the polymorphism of milk proteins has been intensively investigated. Relationships between variants and production traits have however been inconsistent. The most promising results have been obtained from the  $\beta$  lactoglobulin variants. Several separate studies have shown the superiority of the AA genotype (locus  $Lgb^A$ ) over the BB genotype (locus  $Lgb^B$ ) and the homozygous  $Lgb^{AA}$  animal over the heterozygote  $Lgb^{AB}$ , in terms of milk yield (Arave et al. 1971, Thatcher 1965 - cited Arave et al. 1971, Comberg et al. 1964). Thatcher also obtained significant differences favouring  $Lgb^{AA}$  in terms of S.N.F., fat corrected milk, total solids and in feed conversion efficiency. In contrast, neither Brum et al. (1968) nor Cerbulis and Farrell (1975) were able to find any significant differences between  $\beta$   $Lgb$  genotypes for the production traits of milk yield and fat percentage. Robertson (1966) - cited Arave et al. (1971) suggested three possible explanations for the lack of uniformity in the results or the lack of significant relationships between protein polymorphs and production traits. These were that:-

(i) Although the particular polymorphism may be unimportant biologically, standard tests may erroneously indicate significance due to chance alone 5% of the times.

(ii) The effects of polymorphic loci on traits may depend upon the genetic constitution of the population in which they are found.

(iii) The number of loci affecting a production trait may be small, whereas the number of polymorphs is large, leaving the possibility of picking the right one to study somewhat remote. Alternatively, in traits such as milk production where a large number of loci are likely to be involved, there is only a small probability that any one locus will have a measurable effect.

Many results have demonstrated an increased quantity of various dyes (amido black, naphthalene black, bromophenol blue, coumassie blue) bound by electrophoretically separated  $\beta$  lactoglobulin A relative to  $\beta$  lactoglobulin B. This has been evident both in the homozygous AA and BB states and in the heterozygous AB condition (Asschaffenburg and Drewry 1955, Michaelak 1967, Arave 1967, Lontie et al. 1964 - cited Kiddy et al. 1965, Kiddy et al. 1972). Whether this apparently higher concentration of protein of the A variant, particularly in the AB situation, is due to a true difference in the protein concentration, to dye-binding

differences or to a greater loss of B in the analytical methods is as yet uncertain. However the studies of Aschaffenburg and Drewry (1957) and Cerbulis and Farrell (1975) in which the  $\beta$  lactoglobulin of individual cows was isolated chemically showed that the yield of AA was greater than AB, greater than BB. Kiddy et al. (1965) however, using a chromatographic separation technique, found only a very slightly higher proportion of LgbA than LgbB in heterozygous animals. In the proposed comparisons of the same proteins from cows on different treatments, such a problem, if in fact real, will not affect the results.

### 1:1:3 Origin of Milk Proteins

The origin of the milk proteins caused considerable debate for many years and it was not until the tracer studies by Larson and Gillespie (1957) that the proteins were conclusively subdivided into two groups based on their origin. The first group (comprising about 90% of total protein in normal milk) included the  $\alpha$  casein complex,  $\beta$  casein,  $\alpha$  lactalbumin and  $\beta$  lactoglobulin. These are proteins synthesized in the mammary gland from a common free amino acid pool. The second group included the immune or gamma globulin fraction, serum albumin and gamma casein and were considered to be blood proteins entering preformed from the bloodstream. The mistaken origin of gamma casein persisted until 1972 when it was shown by Gordon et al. and Groves et al. that the molecule was identical to a large portion of the  $\beta$  casein. Milk also contains a large array of other proteins including many enzymes (Shahani et al. 1973). The origin of some of these quantitatively minor proteins is summarised in Table 1:2.

Table 1:2 Origin of some of the minor milk proteins

Protein	Ref.	Origin
Proteose Peptones	1	Component 3 -Serum Protein
	2	Components 5 & 8- Found in both Serum and Casein Micelles.
Transferrin	3	Serum
Lactoferrin	3	Mammary Gland
Ceruloplasmin	4	Serum
Lactollin	5	Unknown, but associated with lactoferrin
Fat Globule Membrane Proteins	6	Mammary Gland

1. Rose et al. (1970)

2. Jenness (1974)

3. Groves (1971)

4 Hanson et al. (1967)

5. Groves (1960)

6. Dowben et al. (1967)

Swope &amp; Brunner (1970)

#### 1:1:4 Biological Functions of the Major Milk Proteins

1:1:4:1 Caseins The loose and open structure of casein (according to Larson & Jorgenson 1974) argues against their having a specific biological function in the normal sense. Farrell & Thompson (1971) suggest that the open structure of  $\alpha S_1$  and  $\beta$  Caseins results in their being readily attacked by the proteolytic enzymes pepsin, trypsin and chymotrypsin, resulting in the caseins being an effective source of nutrients for the young. It has been suggested that both the large amounts of phosphate and calcium that are incorporated into the casein micelles and the high content of essential amino acids in casein, has conferred an evolutionary biological advantage on those animals which secrete casein for the nourishment of their young (Larson & Jorgensen 1974, Jenness 1970).

1:1:4:2 Alpha Lactalbumin The nutritive function of some of the milk proteins may be biologically of secondary importance to an enzymatic function. This appears to be the case with  $\alpha$  lactalbumin, despite its high nutritive value, particularly in relation to its high tryptophan content. Ebner et al. (1966) suggested that the B protein of the lactose synthetase complex, which catalyzes the reaction:-



had similar properties to  $\alpha$  Lactalbumin.

Brodbeck et al. (1967) subsequently showed that  $\alpha$  lactalbumin was in fact the B subunit of the enzyme and Brew et al. (1968) termed  $\alpha$  lactalbumin a "specifier protein" in view of its controlling activity on the substrate specificity of the enzyme complex. Thus the level of production of  $\alpha$  lactalbumin is considered to be a switching mechanism to control the synthesis of lactose by the cell and is a unique biochemical control mechanism in keeping with other known properties of the mammary gland (Ebner & Schanbacher 1974)

1:1:4:3      Beta Lactoglobulin      The function of  $\beta$  lactoglobulin is not as clear as is the function of  $\alpha$  lactalbumin as is emphasised by the statement of Thompson & Farrell (1974). " We feel that  $\beta$  lactoglobulin is important biologically although we don't know exactly how or why." Larson (1969) reports work in which Actinomycin D ( an inhibitor of D.N.A.- dependent R.N.A. synthesis) when applied to rat mammary cell cultures inhibited  $\beta$  casein synthesis but not that of  $\beta$  lactoglobulin.

One of several possible reasons suggested for this difference was that  $\beta$  lactoglobulin could be acting like a cell "constitutive" protein and so less easily affected by Actinomycin D than  $\beta$  casein a protein probably not concerned with survival and which the cell can more easily do without.

$\beta$  lactoglobulin may also operate as a nutritional reserve protein which would fluctuate in concentration according to the nutritional status of the animal. An investigation of changes in concentration with altered nutritional status of the cow may throw light on this hypothesis.

Farrell & Thompson (1971) showed that  $\beta$  lactoglobulin competitively inhibits the activity of bovine spleen phosphoprotein phosphatase, with the A variant having a greater effect than the B variant which was in turn more inhibiting than the C variant. These differences were assumed to be due to structural changes induced by genetic substitutions.

$\beta$  lactoglobulin does not inhibit the dephosphorylation of  $\alpha S_1$  casein (Farrell & Thompson 1971). The inhibition therefore appears restricted to low molecular weight substrates and possibly due to substrate binding by the  $\beta$  lactoglobulin. Such information has led Farrell & Thompson (1971) to speculate that  $\beta$  lactoglobulin may play a regulatory role in mammary gland phosphorous metabolism.

The fact that  $\beta$  lactoglobulin is not present in the milk of all species (camel - Kessler & Brew 1970, guinea pig - Brew & Campbell, 1967, human - Bell & McKenzie 1964) does however imply that the function (if any) of  $\beta$  lactoglobulin in the mammary gland is unlikely to be unique.

1:1:4:4    Immunoglobulins    Recent studies of ruminants have established that there are three classes of immunoglobulins - IgG, IgM and IgA and that IgG can be further subdivided into two subclasses IgG<sub>1</sub> and IgG<sub>2</sub> (Brandon et al. 1971).

The function of the immunoglobulins as a source of passive immunity for the newborn is well known (Brambell 1970). In many species the young are born with few if any antibodies in the circulation. The ability of the young to absorb them via the gut in the first few days of life furnishes a mechanism whereby the young can acquire a level of immunity.

1:1:4:5    Serum Albumin    No mention of any function for serum albumin in milk appears in the literature. It is widely accepted that serum albumin is a blood protein which enters the milk by leakage or direct infiltration through the mammary gland secretory cells.

Jeness (1974) reported that all 150 species examined to date exhibited a component with the electrophoretic mobility of the blood serum albumin of the species. This seemingly "universal" similarity between serum albumin and milk albumin supports the theory that serum albumin accidentally enters the gland and appears as a component of milk. The observations that the level of serum albumin is elevated (as are other milk components of blood origin) at times of mammary stress (Larson & Jorgensen 1974) also supports this argument.

1:2                    Milk Protein Synthesis and Secretion by the Mammary Gland

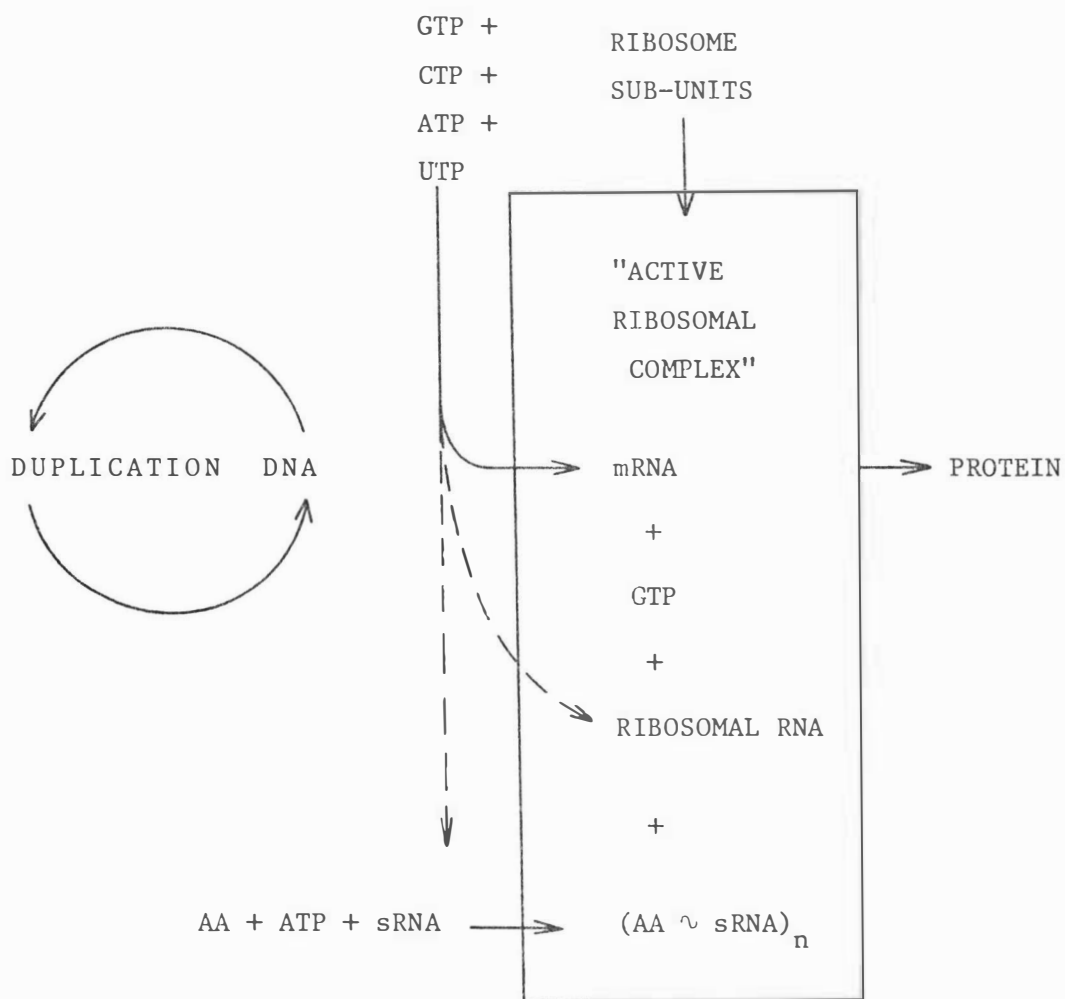
It is necessary to obtain a basic appreciation of the mechanism of protein synthesis in order to understand the influence of the treatment on the parameters measured. In this case, the individual whey proteins will receive particular attention. The present state of knowledge of the modes of secretion of the individual whey proteins must also be considered to enable valid interpretations to be placed on the ensuing results.

1:2:1                 Milk Protein Synthesis in the Mammary Gland

The basic mechanism of protein biosynthesis appears to operate in the mammary secretory cell as it does in all other cells which have been investigated ( Jones 1969, Gaye et al.1973). The process has been summarised by many authors and with particular reference to the synthesis of milk proteins by Larson (1965), Larson (1969) and Larson & Jorgensen (1974). The general mechanism of protein biosynthesis is outlined diagrammatically in Fig I:2.

Briefly, free amino acids are activated with adenosine tri-phosphate (ATP) which then associate with a specific variety of soluble ribonucleic acid (RNA) called transfer RNA (t RNA). These units are then incorporated into the polyribosomes which are complexes of ribonucleo-proteins comprising the ribosomes and containing ribosomal RNA (r RNA). There, under the mediation of a third major category of RNA called messenger RNA (m RNA), the individual amino acids are assembled into a polypeptide chain in the order determined by the sequence of bases on the m RNA chain. The three RNA species are formed by deoxy-ribonucleic acid (DNA) dependent RNA synthetases. The base sequence of the DNA forms a template for the assembly of the nucleotide base triphosphates (cytidine, guanosine, adenosine and uridine) into the RNA species and provides the mechanism of genetic control of protein synthesis. m RNA carries its genetic "message" as specific trinucleotide sequences which form a code for the individual amino acids. This "message" is transported from its site of synthesis in the nucleus to the cytoplasmic ribosomes where the protein strand is assembled.

Figure 1:2 Diagrammatic summary of the process of protein biosynthesis.



from Larson (1965).

1:2:2            Protein Secretion by the Mammary Gland

1:2:2:1        Secretion of Proteins Synthesised in the Mammary Gland

Secretory cells contain both loose ribosomes typical of non-secretory cells as well as ribosomes bound to the endoplasmic reticulum. The latter are responsible for the synthesis of proteins destined for cellular export (Gaye et al. 1973, Sacke & Healde 1974, Rolleston 1974). It is obvious that each m RNA strand must "decide" which ribosomal category to associate with; a "decision" dependent upon the function of the protein for which it codes. Blobel & Sabatini (1971) postulated that all m RNA strands whose translational products are to be transferred across a membrane contain a unique sequence of signal codons at the amino terminal end of the chain (immediately following the initiation codon). Emergence of this signal sequence (10-40 amino acid residues) of the protein chain from the large ribosomal subunit triggers the attachment of the ribosome to the membrane of the endoplasmic reticulum. Thus all translation begins on free ribosomes.

Following attachment of the ribosome to a membrane binding site, the emerging signal sequence stimulates the loose association of several membrane proteins. These proteins form a pore through the membrane which allows the emerging protein chain to pass directly to the intracisternal space of the endoplasmic reticulum. The ribosome then detaches from the membrane and the proteins which formed the pore dissociate and rediffuse into the plane of the membrane. (Blobel & Dobberstein 1975)

The peptides comprising the signal sequence are then thought to separate from the protein chain within the endoplasmic reticulum by a proteolytic mechanism.

It is possible that this signal sequence may be responsible for several of the observed types of milk proteins. The A and B

genetic variants of  $\gamma$  casein have identical amino acid sequences to the A & B variants of  $\beta$  casein respectively, but the  $\gamma$  caseins are 27 residues shorter from their N terminal end (Gordon et al 1972). It could be postulated that these 27 residues comprise the signal sequence of  $\gamma$  casein and that  $\beta$  casein is in fact  $\gamma$  casein with retention of the signal sequence.

Support for the rough endoplasmic reticulum (RER) as the site of protein synthesis has been demonstrated unequivocally by autoradiographic studies. Stein & Stein (1967) showed the localisation of tritiated leucine over the elements of the RER. Time sequence autoradiography has been used by many workers to demonstrate the pathway of protein secretion (Wellings & Philp 1964, Verley & Hollman 1966- cited Saacke & Heald 1974. Fiske et al. 1967, Heald & Saacke 1972). Activity is first revealed in the ergastoplasm (RER), followed by the golgi and the bumen of the gland.

The mechanism by which the proteins move from the RER to the golgi region is not well defined. Small vesicles bud off from the endoplasmic reticulum, move to and are incorporated into the golgi apparatus (Kurosumi et al. 1968, Linzell & Peaker 1971, Jamieson & Palade 1967, Morré et al. 1971). Apart from knowing that this mechanism requires an energy input (Jamieson & Palade 1968), the reactions, forces involved and the mode of movement are unknown (Palade 1975).

It is within the golgi apparatus that much of the protein modification occurs which results in the development of the tertiary structure and properties of the individual proteins. Barry (1961) concluded that the phosphorylation of casein molecules occurs after the completion of chain synthesis. There is evidence that a pool of unphosphorylated casein exists in the mammary gland secretory cell. (Turkington & Topper 1966, Singh et al. 1967) and Bingham et al. (1972) suggested that this was sited in the golgi apparatus. Similarly Carroll et al. (1971) have proposed that

the binding of calcium occurs following phosphorylation of casein in the golgi. The golgi apparatus also appears to transform the membrane of the endoplasmic reticular vacuoles to one which has properties similar to the plasma membrane (Grove et al. 1963, Morré et al. 1970 - cited Keenan et al. 1970). Membrane input at the proximal or forming face of the dictyosome is "balanced" by the utilisation of membrane in the elaboration of secretory vesicles at the distal or maturing face of the dictyosome (Keenan & Huang 1972). Golgi vesicles pinch off from the golgi cisternae in a similar manner to that shown for the "budding" of the RER vacuoles ( Saacke & Healde 1974). These workers also suggest that the contents of one vacuole may be injected into an adjacent vacuole, or that in the maturation of golgi vacuoles, merging may occur. These vacuoles are also assumed to contain the newly formed lactose from the golgi body as well as the free B protein of its synthesis -  $\alpha$  lactalbumin. Extending this hypothesis, Linzell & Peaker (1971) proposed that lactose is the major osmoregulator in the golgi vacuole. Lactose causes water to be drawn into the vacuole to balance the tonicity of vacuolar material with plasma and with milk.

The manner by which the vacuoles move to the apex of the cell is an area of continuing debate. Brooker & Forsyth (1972) reported the existence of microtubules and filaments along which the vesicles move in a positive manner to the apex of the cell.

Palade (1975) suggests that no such organisation exists in the apical region of pancreatic cells. Others have shown that newly formed (labelled) secretory granules are distributed at random within the pre-existing granule population, so arguing against any form of organised transport ( Scheele & Palade 1975, Tartakoff et al. 1975). In contrast to these findings, Le Marchand et al. (1974) proposed that the drugs colchicine, vinblastine & vincristine (previously shown to interfere with an hepatic microtubular system, Borisy & Taylor 1967, Malawisto & Sato 1969)

either strongly inhibited or completely suppressed the release of newly synthesised proteins though total hepatic protein synthesis remained unaltered.

The contents of the secretory vacuoles are discharged into the glandular lumen by a process originally called membrane fusion but has since been changed to the more descriptive exocytosis or reverse pinocytosis (Palade 1975). As the vacuoles approach the apical cell surface, the two membranes fuse, fission occurs within this area of fusion and the two membranes become contiguous. This process permits the discharge of the secretory products whilst ensuring the maintenance of a continuous diffusion barrier between the cell sol and the extracellular medium.

The vesicles thus provide and replace cell membrane which is consumed in the secretion (process of envelopment) of the lipid droplets (Bargmann & Knoop 1959, 1960 - cited Keenan et al. 1970a, Patton & Fowkes 1967, Keenan et al. 1970b, Keenan & Huang 1972).

This cisternal packaging - exocytosis model of protein secretion has considerable acceptance over a wide variety of cells. Rothman (1975) has critically evaluated this model and summarised the problems associated with it. He suggests as an alternative, an equilibrium or membrane transport mechanism at least for the secretion of digestive enzymes by the pancreas. The central tenet of this theory is that the proteins can move bi-directionally through the membranes so that digestive enzymes can be in equilibrium with each other. Rothman however notes that this equilibrium model does not preclude the packaging- exocytosis model. The latter would seem most likely to be responsible for the secretion of the milk proteins synthesised in the mammary gland.

1:2:2:2      Secretion of Serum Proteins by the Mammary Gland

1:2:2:2:1      Immunoglobulins      Larson & Kendall (1957)

demonstrated a decrease in the levels of several of the serum globulin fractions in cows approaching parturition. This substantiated the radioactive tracer studies which indicated that the immunoglobulins found in colostrum and milk were not synthesised by the mammary gland. (Askonas et al. 1954). Dixon et al. (1961) suggested that there was a specific transfer mechanism through the cells which favoured gamma globulin and excluded to a great extent the smaller albumin molecules. Pierce & Feinstein (1965) demonstrated that this selective absorption extended to differentiating between immunoglobulin fractions. The predominance of IgG<sub>1</sub> (see Fig 1:1) in milk has been attributed to the existence of a selective mechanism of transfer which can distinguish between IgG<sub>1</sub> and other Immunoglobulin fractions (Watson & Lascelles 1973). It has been shown that this selective mechanism is much more active during colostrum formation than during established lactation and also during the early phases of involution when milk production is declining rapidly (Watson et al. 1972).

Brandon et al. (1971) also showed that the concentrations of IgG<sub>2</sub>, IgA and IgM do not alter with approaching parturition or the establishment of lactation. Lascelles (1971) - cited Brandon et al. (1971) suggested that selective transfer of IgG<sub>1</sub> required the presence of receptor sites on the basal or intercellular membrane of glandular epithelial cells. Transport vesicles forming at these sites would therefore contain more IgG<sub>1</sub> than IgG<sub>2</sub>. The electron microscope studies of Bargmann & Welsch (1969) have shown vesicular structures around the basal border of the cell. Murad (1970) also showed that proteinaceous particles appeared in the cytoplasm and were totally secreted into the glandular lumen prior to parturition. Sonoda et al. (1973) has presented cytological evidence that a pinocytotic process is responsible for IgG uptake by the fetal yolk sac from the maternal uterine lumen for transfer to the fetus. The proposed basal membrane vesicle theory of immunoglobulin transport has been used by Peaker (1975) to explain the increased sodium and decreased potassium concentrations (relative to milk) observed in colostrum. The sodium pumps shown to be present

(as indicated by the presence of Na-K activated ATPases) on the basal and lateral membranes, but not on the luminal membrane (Kimura 1969, Johnson & Wooding (in prepn)-cited Peaker 1975) would be transported with the membrane of the transport vacuole to the luminal membrane. In this position the pumps would operate to exchange sodium for potassium in milk. Once lactation begins, depletion of luminal membrane by the normal process of fat droplet secretion and its replacement by membrane of golgi origin occurs. This rapidly removes the pump mechanisms and allows the characteristic ionic balance of milk to establish.

1:2:2:2:2      Serum Albumin      The mechanism by which serum albumin is secreted into milk is not well defined. Mackenzie & Lascelles (1968) concluded that IgG<sub>2</sub> and other serum proteins must be transferred into milk by a less efficient selective mechanism than that of IgG<sub>1</sub>, or by a passive mechanism such as filtration between the cells. The latter, referred to as the leakage theory is well entrenched in the literature (Butler 1974, Mackenzie & Lascelles 1968. Lascelles et al. 1971). The leakage theory as the explanation of normal physiological levels of serum albumin in milk must be questioned on the following grounds:-

1). In established lactation a steep non-electrolyte gradient is maintained (lactose-milk : plasma = 1000:1) (Peaker 1975) as well as a moderate to high ionic gradient (Sodium = 1:10 in the cow and 1:37 in man). Clearly a "leaky" epithelium could not favour the establishment or maintenance of such differences, particularly in the case of lactose.

2). The cells do not allow molecules such as sucrose to pass from blood to milk. Linzell & Peaker (1971a) used this fact to estimate the volume of milk in the mammary gland.

Thus it can be queried whether the "normal" levels of albumin ( and other serum proteins) in milk are due to a continuous passive leakage between the cells.

Administration of oxytocin even in small and physiological amounts leads to increases in milk sodium and chloride and decreases in lactose and potassium concentrations ( Peaker 1975). Wheelock et al.(1965) also showed an increased concentration of the non-casein proteins of milk under such conditions. Linzell & Peaker (1971b) suggested that the effect of oxytocin was to disrupt the secretory epithelium sufficiently to permit the passage of a little extracellular fluid in one direction and milk in the other, via the tight junctions. Following cessation of oxytocin administration, the milk composition quickly returns to normal (Linzell 1967). Thus the possibility exists that the observed concentration of serum albumin in milk is due to leakage during and immediately after the previous milking. This secretion is subsequently diluted by the normal secretion. Such a hypothesis is supported by the work of Linzell (1967) in which it was observed that frequent milking raised milk Na & Cl but depressed K and lactose concentrations. Furthermore, Dill et al.(1972) have shown that with successive injections of oxytocin, the concentration of serum albumin consistently increased. The differential sieving of proteins on size as suggested by Mackenzie & Lascelles (1968) would result in the differing concentrations of the serum proteins (albumin, proteose peptones, IgG<sub>2</sub>, IgM) in milk according to this theory.

Another possible mechanism for the transfer of albumin to milk is via the IgG<sub>1</sub> transport vesicles as proposed by Brandon et al.(1971). If, as is proposed, the transport vesicles form at the IgG<sub>1</sub> binding sites on the basal membrane, they will contain predominantly IgG<sub>1</sub>, but do not preclude the random inclusion of other serum proteins as they form.

A further possibility is that serum albumin escapes into the lumen of the gland with or following the movement through the intercellular spaces by the leucocytes. Though leucocyte passage between the cells has never been observed, the probability of observing such an event is not high. Normal cows milk contains in the vicinity of 50,000 leucocytes per ml (Newbould 1974) and about 0.4 mg per ml of serum albumin (Rolleri et al. 1956). Thus each leucocyte would have to transport 0.008  $\mu$ g of albumin. The albumin content of interstitial fluid (approximated by lymph) is about 10 mg/ml (Lascelles et al. 1964). Sufficient serum albumin to account for the physiological quantities in milk could therefore enter the gland by as little as 0.8 nl of interstitial fluid entering the gland per leucocyte. This is however some 20 times the volume of a leucocyte so is likely to account for only a small proportion of the total serum albumin influx unless of course the progress of the leucocyte between the cells so distorted the tight junctions that considerable leakage occurred subsequently.

Analysis of the concentrations of the whey proteins may shed some light on the mechanism of entry of the serum proteins into the lumen of the mammary gland.

### 1:3 Factors affecting Milk Composition

Having defined the area which this work is considering (Milk Protein synthesis and secretion) and to some extent the problems to be solved, it now remains to establish those factors which have an influence on the field.

#### 1:3:1 The Effect of Nutrition on Milk Yield and on the Concentrations and Yields of the Major Milk Components

Large numbers of trials have been carried out to investigate the effects of level of feed intake on milk yield and the major milk components. Broster et al. (1969) have suggested that the literature on level of feeding of dairy cows should be subdivided into short-term trials which ignore changes in body reserves and long-term trials which do account for such changes.

This review will concentrate on short-term trials.

1:3:1:1 Energy Supplements Increasing the level of energy intake by supplementary feeding has been shown by many workers (Wright et al. 1974), Dawson & Rook 1972, Wilson et al. 1971, Gordon & Forbes 1970, 1971, Gardner 1969, Broster et al. 1969, Rook 1961a) to result in an increase in the yield of milk produced, though the increase occurs at an ever decreasing rate (Armstrong (1968). Huber & Boman (1966) suggested that the decline in response to increasing levels of energy supplementation was associated with an increasing rate of live-weight gain. A decrease in the plane of energy nutrition results in a decrease in milk yield (Wright et al. 1974, Greenhalgh & Reid 1969, Broster et al. 1969, Rook 1961a, Burt 1957)

The protein concentration of milk has also been shown to be raised by increased levels of dietary energy (Dawson & Rook 1972, Gordon & Forbes 1971, Rook 1961a, Rook & Line, 1961, Kirchgessner et al. 1957) and decreased by reduced levels of dietary energy (Wright et al. 1974, Dawson & Rook 1972, Kirchgessner et al. 1967). The effect of changes of energy levels are greater at low

than at high planes of nutrition (Rook 1961a) an observation which may explain many of the conflicting results and variation in the level of response reported in the literature.

Dawson and Rook (1972) have also shown that as lactation progresses, the responses to both increased and decreased planes of nutrition energy vary. The extent of the response in terms of milk yield decreased as did that of protein content. The extent of the response in lactose content increased and was reflected in an increasing level of response in the (SNF) percentage.

Increased energy intake has little effect on milk fat content (Castle 1958, 1959, 1961, Kirchgessner et al. 1967, Armstrong 1968). If however the energy supplement induces marked changes in the proportions of the volatile fatty acids (VFA) produced in the rumen, dramatic depressions in milk fat content can result (Schultz 1974, Davis & Brown 1970, Armstrong 1968).

#### 1:3:1:2 Protein Supplements

For a given level of dietary energy, any increase in the level of protein intake above requirements has little effect on milk yield (Gordon & Forbes 1970, Armstrong 1968, Blaxter 1964). Paquay et al. (1973) have shown further that any increase in protein intake over requirement induces no improvement in milk secretion but leads to enhanced urinary N losses. The protein requirement can however be raised by an increase in the level of energy intake. Similarly the results of Reid & Holmes (1956) and Logan et al. (1959) indicate that the response from additional energy can be limited by the protein content of the diet. Levels of dietary protein considerably below requirements lead to a decrease in both milk yield and protein content (Thomas 1971, Armstrong 1968, Kirchgessner et al. 1967). Small dietary protein deficits also reduce milk yield but do not affect protein percentage (Rook 1961a, Kirchgessner et al. 1967, Paquay et al. 1973).

An increase in the protein content of rations has been shown by some workers to increase milk fat percentage (Review by Kirchgessner et al. 1967), but only in situations where the ration has a high crude fibre content and is low in starch.

Prolonged deficiencies of both protein and energy (underfeeding) causes a depression in the fat content of milk (Review by Kirchgessner et al. 1967). This drop in fat percentage is however limited by the concomitant depression in milk yield. This situation often results in no change or even an increase in the fat content of milk, particularly when there is a sudden reduction in feed intake. Rook (1961a) summarised the effect of plane of nutrition on fat content as, "a change in milk yield without a compensatory change in fat yield".

Variations in the plane of nutrition (comprising both available energy and protein) have thus been shown to exert a considerable influence on total milk protein concentration as well as other milk components. It is therefore appropriate to alter the plane of nutrition of a treatment group of cows to investigate the effects on milk composition and in particular on the individual proteins of the whey.

<sup>1</sup>:3:1:3 Stocking Rate Comparitively little work has been carried out investigating nutritional effects on milk production using treatments based entirely on grazing systems. This is no doubt a reflection of the difficulties associated with such trials, particularly in the estimation of intakes and in separating the effects of quality and quantity of feed consumed. New Zealand's dairy industry which is based almost completely on grazed pasture means that adjustment of stocking rate is the most appropriate method of imposing relevant nutritional treatments on lactating cows.

Maximum feed intake and hence maximum production per animal is achieved at low grazing intensities because neither quality nor quantity of feed is limiting. Improvements in the efficiency of harvesting ( increasing stock rate) are obtained only at the

expense of a reduction in intake by the animal and a consequent decrease in its production (Greenhalgh 1975). McMeekan & Walshe (1963) showed that milk and fat yields per cow were depressed by increased stocking rates. These same parameters, however, when measured on a per unit area basis were shown to increase with the increased stocking rate. Similar effects have been noted by other workers (Flux & Patchell 1954, Patchell 1957, Flux & Patchell 1957, Munford et al. 1964, Greenhalgh & Reid 1968, Greenhalgh et al. 1966, Greenhalgh et al. 1967, Hutton & Parker 1973) over varying periods of time, stages of lactation and degrees of restriction. In general, the concentrations of total protein and SNF were depressed whilst the concentration of milk fat was increased as the stocking rate increased.

The actual way in which measurable effects due to stocking rate differences arise is not clear. The importance of selective grazing by sheep was originally pointed out by Stapledon (1927)-cited Bredon et al. (1967). Fagan (1929) stated further that cattle were selective as to the parts of plants eaten. Hennessy & Ahern (1967) reported that cattle preferentially select the top portions of irrigated pasture. Similarly Eldridge (1974) showed that when cows are grazed rotationally they select the top pasture layers first, regardless of the stocking rate.

Several workers (Davies 1925, Jones 1933, Stapledon 1934, Hardison et al. 1954) have shown that the degree of selectivity is related to the amount of palatable herbage available. Selectivity is greatest when an abundance of varied herbage exists and decreases as the supply becomes more restricted.

The effect of selectivity by the animals on the digesta has been investigated using oesophageal and rumen fistula and analysis of pasture offered and refused. Many workers have noted that the concentration of nitrogen in the faeces of grazing animals is higher than in animals fed the same material but plucked

or clipped (Review by Hardison et al.1954). Material selected by animals has been shown consistently to have higher digestibility and crude protein and less crude fibre than animals fed the same material without the opportunity to select (Bredon et al.1967, Hardison et al.1954, Arnold et al.1966).

The two observations - height of selective grazing and composition of the intake of animals permitted to graze selectively, have been related by the work of Clarke et al. - cited Eldridge (1974) They found that protein content of pasture declined from the top layer to the bottom.

The general conclusions from work published on selective grazing x pasture intake interactions is that clipped herbage provides an unreliable index of the chemical composition of herbage selected by grazing animals. Similarly the effects of pasture quality are difficult to separate from the effects of pasture quantity in stocking rate experiments with grazing animals.

Restricted grazing is thus likely to result in the combined effects of limiting available energy and protein as indicated in Sections 1:3:1:1 and 1:3:1:2. A high stocking rate treatment could be expected to depress milk yield and total milk protein percentage and result in a variable effect on fat concentration and in so doing achieve the objectives of the project as previously stated.

#### 1:3:1:4 Mediation of Nutrition on Milk Synthesis

Glucose plays a central role in the metabolism of the mammary gland (Davis & Bauman 1974). Barry (1952) showed that glucose was the main precursor of Lactose. Linzell & Peaker (1971c) proposed that the presence of lactose in the golgi secretory vesicles draws water osmotically from the cell into the vesicle. Glucose therefore, by influencing lactose synthesis largely controls the milk yield. This mechanism is also responsible for the relative stability of the lactose percentage in milk.

A shortage of glucose can be caused by underfeeding (Linzell 1967b) and without glucose in the perfusate, milk secretion almost ceases in isolated perfused goat mammary glands (Hardwick

et al. 1961). Secretion of the aqueous phase of milk resumes as soon as glucose is replaced. No other sugar or metabolite will substitute for glucose in stimulating secretion (Hardwick et al. 1963).

Linzell (1968) has shown that omitting either amino acids or acetate from the perfusate does not depress the level of secretion, but merely lowers the output of milk fat and protein.

Folley and French (1950) demonstrated that fatty acid synthesis in the mammary tissue of ruminants was uniquely different from that in non-ruminants. Subsequent studies using  $^{14}\text{C}$  substrates indicated that ruminants were unable to incorporate glucose into fatty acids (in contrast to non-ruminants). Instead, ruminants utilise plasma acetate and Beta Hydroxy Butyrate (Balmain et al. 1954, Hardwick et al. 1963, Bauman et al. 1970) derived from the fermentation of dietary carbohydrates in the rumen (Folley & McNaught 1961). Bauman et al. (1973) showed that the difference in substrate requirement was due to the absence of two enzymes of the citrate cleavage pathway (ATP citrate lyase and NADP-malate dehydrogenase).

It is now well established that on pasture diets, little glucose is absorbed from the gastro intestinal tract of ruminants due to the fact that little by passes the rumen microbial population (Lindsay 1970). Therefore ruminants depend almost entirely on the processes of gluconeogenesis for the glucose needed for specific functions, including milk synthesis (Ballard et al. 1968). Propionate and protein are the major gluconeogenic materials, but glycerol and lactate also make varying contributions (Lindsay 1970). Production of propionate in the rumen is stimulated by increased proportions of concentrate feeds in the ration (Schultze 1974, McDonald et al. 1973) whereas gluconeogenic amino acids are utilised for glucose production by the liver even when they may be limiting for protein synthesis (Purser 1970). Thus underfeeding or diets of low digestibility limits the gluconeogenic material available to the animal which, by limiting lactose production, results in a decrease in milk yield.

Mepham & Linzell (1966) showed that all essential amino acids are removed from the plasma in sufficient quantities to account for the corresponding synthesis of milk proteins by the mammary gland. The uptake of non-essential amino acids is variable and in some cases less than their output in milk (Bickerstaffe et al. 1974). It is suggested that those absorbed in excess (Arginine, Ornithine) could be deaminated, providing nitrogen for re-synthesis of limiting non-essential amino acids. Glutamate, Alanine, Glycine, Serine and sometimes Aspartate have been shown to be limiting. Verbeke et al. (1969) suggested that materials such as short chain fatty acids could provide the carbon skeletons for amination in the production of non-essential but limiting amino acids.

Halfpenny et al. (1969) showed that the protein content of milk was significantly increased with higher intakes of energy. This increase was associated with increased jugular blood concentrations on non-essential amino acids, but notably with lower essential amino acid concentrations than during the low intake control period. This they suggested reflects an increased uptake by the gland of essential amino acids to meet the requirements for increased protein synthesis.

The decrease in milk protein content resulting from low planes of nutrition may then be due to a limitation of one or more non-essential amino acids; there being some restriction on the formation of these amino acids from glucose or other usable substrates. Glutamic acid has been implicated as the major limiting precursor for milk protein synthesis, reflecting the level of production of propionate in the rumen (Halfpenny et al. 1969).

Ruminant milk lipids are characterised by the presence of substantial quantities of short and medium chain length fatty acids up to C16 (Storry et al. 1973, Bickerstaffe 1971, Dimick et al. 1970). This attribute is a result of a very active de novo synthesis from the precursors - acetate and  $\beta$  hydroxy butyrate

which the mammary secretory cell obtains from the bloodstream (Linzell 1968). Longer chain fatty acids (some of the C16 and C18 or more) are derived directly from the plasma lipids (Bishop et al. 1969). Bickerstaffe et al. (1974) showed that plasma triglycerides and blood acetate accounted for 35-80 percent and 25-50 percent respectively of the milk fat triglyceride. The majority of the triglycerides taken up by the udder were derived from the low density lipoprotein fraction.  $\beta$  hydroxy butyrate may be more important than its level of mammary uptake (Bickerstaffe et al. 1974) or level of incorporation in synthesised triglyceride (Bines & Brown 1968, Palmquist 1969) would indicate. It appears to be the preferred chain initiator in 50 percent of the fatty acids formed by de novo synthesis, i.e. those not formed by the Malonyl pathway (Dimick et al. 1970).

Net free fatty acid arteriovenous differences have not been recorded in either goats (Linzell 1968) or cows (Bickerstaffe et al. 1974). Infusion of labelled acids by these workers have shown that an exchange of fatty acids does occur between plasma and the mammary cells. The net uptake is derived from the hydrolysis of triglycerides by lipoprotein lipase which is situated on the capillary endothelium (Barry et al. 1963). In cows fasted for 24 hours, it has been shown that the level of plasma free fatty acid is increased, the release of fatty acid from triglyceride decreases and so there is a considerable net arteriovenous difference across the mammary gland under these conditions (Kronfield 1965). Underfeeding also alters the composition of the milk fat. Products of de novo synthesis (short chain fatty acids SCFA) are decreased whilst the proportion of long chain fatty acids (LCFA) increases, indicating mobilisation of body fat reserves (Remond & Journet 1970, Annison et al. 1968). This marked rise in mammary free fatty acids uptake compensates for the fall in the precursors of SCFA synthesis. The lack of glucose resulting in depressed milk yield, with the continuing fat production results in the commonly observed rise in fat percentage with underfeeding. Long term underfeeding results in depleted body reserves and a consequent decrease in fat percentage.

1.:3:2

The effect of Stress on Milk Yield and Composition

Stress resulting only from underfeeding and Social or Psychological pressures will be considered.

The effects of stress in animals are believed to be consequences mainly of the release of adrenocorticotrophin (ACTH Campbell et al. (1964)). Several experiments have demonstrated the effects of injecting ACTH in dairy cows (Campbell et al. 1964, Brush 1960, Flux et al. 1954). These include a decrease in milk yield with concomitant increases in the concentrations of milk fat and protein. Braun et al. (1970) suggested that such responses were due to a progressive impairment of glucose utilisation as the plasma corticoid concentration is increased.

Campbell et al. (1964) suggested that the changes in milk composition reported by Munford et al. (1964) resulting from restricted grazing (confinement in a concrete yard for part or all of a milking interval) could have been partially due to nervous stress.

Social or Psychological stress is becoming a problem of practical significance with increasing production pressure on animals (Church 1971). Hancock (1954) examined the behaviour of monozygotic twins and from his discussion it might be expected that separation of twins by treatments would create a source of psychological stress. Social order would have greater implications particularly when animals are confined. Schake & Riggs (1966) showed that social rank affected time spent eating and distance walked in grazing. These workers (Schake & Riggs 1969) again showed that the amount of non-productive activity increases as the food supply is reduced. Larsen (1963) showed similar results (presented by Church 1971) for grazing animals. The animal low in the social order being forced to travel much farther to obtain food or to obtain it without being molested by more dominant animals.

These results suggest that the effects of sociological stress on the animals will tend to over estimate the effects due to the treatment alone.

### 1:3:3 Influence of Mastitis on Milk Yield and Composition

Several recent reviews have summarised the effects of bacterial infection of the udder on milk yield and on the major milk components (Newbould 1974, Newstead 1973, Wheelock & Dodd 1969). Mastitis results in the depression of milk yield, the degree varying from a few percent to complete cessation of secretion. Subclinical mastitis depresses yields by as much as forty percent. This depression in yield (as determined on an individual quarter basis) persists for the whole of lactation, even when the bacteria are eliminated by antibiotic therapy (Crossman et al. 1950, Wheelock et al. 1966). The milk production from the infected quarter does recover, but often not completely, even in the succeeding lactation (Wheelock et al. 1966). Tolle (1969)-cited Newstead (1973) estimated that in the German Federal Republic the national milk yield is reduced by 4.3 percent due to subclinical mastitis. It is expected that New Zealand losses would be similar as the incidence of mastitis is comparable (Newstead 1973).

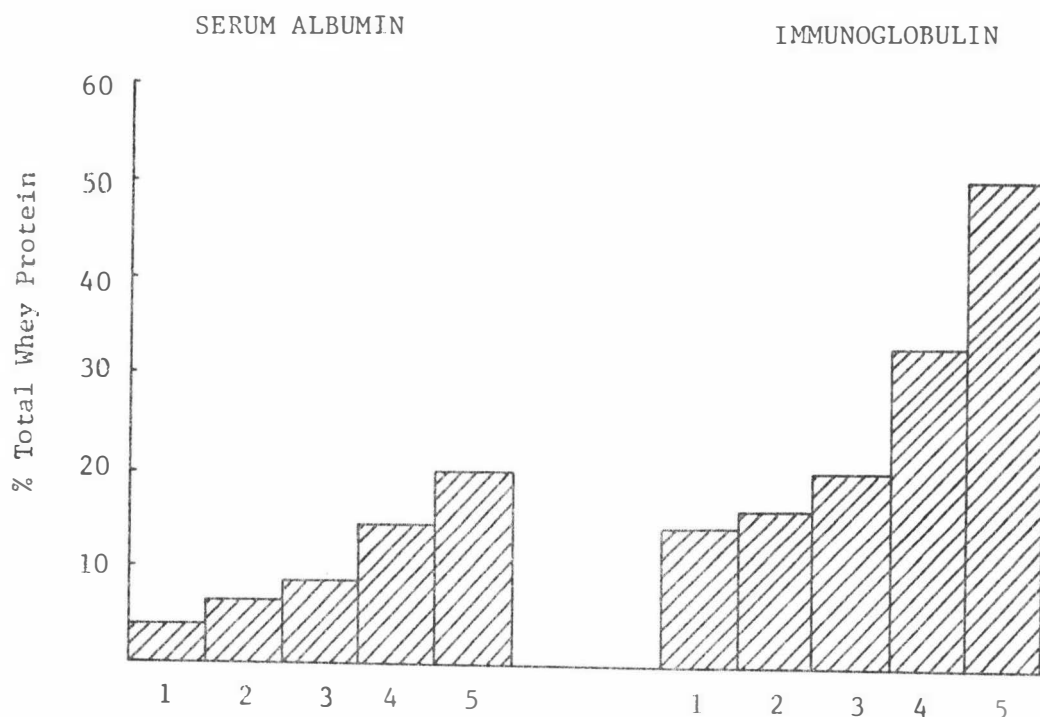
Philpot (1967) and Ashworth et al. (1967) demonstrated the changes in milk composition associated with mastitis infection. Subclinical mastitis was shown to cause a depression in the percentages of milk fat, SNF and lactose. Ashworth et al. (1967) found that total protein content was depressed but that the relative drop was less than that for fat and lactose.

The data relating to the influence of mastitis on the proteins synthesised in the mammary gland are conflicting.

Whereas Ashworth (1965) recorded a decrease in the total casein content of milk, Butkus et al. (1970) reported that casein content of milk increased in mastitic milk. Haenlein et al. (1973)

found that both  $\alpha$  S<sub>1</sub> and  $\beta$  casein decreased with mastitis but Randolph et al. (1974), whilst agreeing that  $\beta$  casein levels were decreased, found that  $\alpha$  S caseins were increased. Both groups agreed that K casein levels increased. Both Haenlein et al. (1973) and Renner (1970) found that the levels of  $\beta$  lactoglobulin and  $\alpha$  lactalbumin were reduced. Any depression in the level of proteins synthesised in the gland is offset by an increase in the level of serum proteins. This increase can be sufficient to raise the gross protein content of milk (Newstead 1973). Increases in serum albumin and immunoglobulin concentrations in milk have been consistently recorded with mastitis infections, whilst other serum proteins such as the proteose peptones (Haenlein et al. 1974) and lactoferrin (Schanbacher & Smith 1975) have also been shown to increase with udder infections. See Fig 1:3

Fig. 1:3 Effect of Mastitis on the Proportions of Serum Albumin and Immunoglobulin in the total whey protein.



Level of Severity of Mastitis Infection from Newstead (1973).

The changes in mineral composition with subclinical mastitis have also been reported with some consistency in the results. Tolle (1969) showed increasing chloride and sodium levels whilst the level of potassium decreased with increasing cell count. Such changes are characteristic according to Wheelock & Dodd (1969), Peaker (1975), Tolle (1970), Renner (1970).

It may be possible to utilise the information gained from changes in milk composition with mastitis to explain normal mammary gland function. The influx of serum proteins into milk and the changes in mineral composition have suggested to many workers that bacterial infections damage the glandular epithelial tissue and permit increased leakage between the cells, both to and from the milk.

The two alternative theories of serum protein secretion outlined in Section 1:2:2:2 (secretion associated with leucocyte invasion and secretion via IgG<sub>1</sub> vesicular transport) can also partly explain the observed changes in milk composition caused by udder infection. Secretion of serum proteins associated with leucocyte invasion would increase due to the increase in leucocyte entry stimulated by the infection. Leucocyte numbers may increase by 100 fold with severe infections (Newbould 1974). Such a rise could account for the maximum of about a 4 fold increase in serum proteins in milk even allowing for considerable non-linearity in the relationship. The time relationship between the increases in serum albumin and IgG (Schanbacher & Smith 1975) supports this leucocyte transport theory as well as the immunoglobulin vesicle transport mechanism. The shift of basal membrane ion pumps to the cell apical barrier with the stimulated vesicular transport of IgG<sub>1</sub> would also contribute to the altered mineral balance in direction observed. However as the IgG<sub>1</sub> secretion remains relatively high in quarters following infection whilst the serum albumin gradually returns to pre-infection levels (Schanbacher & Smith 1975) it is unlikely that either of these mechanisms is solely responsible for the changes observed.

The decreases in the synthesis of protein and lactose by the gland suggests that cellular activity is reduced.

The response of the mammary gland to infection would thus appear to be a temporary involution. Schanbacher & Smith (1975) have shown a second peak of IgG concentration about 9 days after infection. Prior to this, galactosyl transferase activity increased as did the concentration of  $\alpha$  lactalbumin. These changes, it is suggested, are indicative of mammary re-development as is the production of a secretion which has certain compositional characteristics normally associated with colostrum.

The effects of mastitis therefore make it essential to perform the proposed experiment with animals free of infection and to know that infection did not establish during the course of the treatments.

#### 1:4 Effect of Milk Proteins on Manufacturing Properties of Milk

The level of  $\beta$  lactoglobulin significantly affects the maximum and minimum heat stabilities of milk (Feagan et al. 1972, Feagan et al. 1970, Rose 1962). Rose (1961) and Tessier & Rose (1964) have demonstrated the capacity of added  $\beta$  lactoglobulin to increase the maximum heat stability obtainable in the pH range of normal milk.

Differences between the heat stability/pH curves of milks of differing  $\beta$  lactoglobulin genotype have been demonstrated by these workers. Feagan et al. (1972) however found that this was due to quantitative differences in the amount of  $\beta$  lactoglobulin. The addition of equal quantities of each variant to a control milk produced the same results for each - an increase in the maximum and a decrease in the minimum heat stabilities. The increase in heat stability was also shown to develop stepwise with successive additions of  $\beta$  lactoglobulin. Feagan et al. (1972) suggested that the additions to milk of  $\beta$  lactoglobulin in the form of a whey protein concentrate may be a commercial proposition for improving the heat stability characteristics of process milk.

If it is possible to demonstrate a significant nutritional relationship with  $\beta$  lactoglobulin concentration, such adjustments as suggested by Feagan et al. (1972) may be able to be avoided.

The concentration of serum albumin and immunoglobulin also have an effect on heat stability as both proteins are highly heat sensitive (Newstead 1973). Furthermore Feagan et al. (1970) have shown that the addition of serum albumin to normal milk completely destroys its heat stability. The increase in the levels of these two proteins has also been suggested as part of the reason for the reduction in heat stability due to subclinical mastitis (Newstead 1973). The plane of nutrition of the cow may also affect the concentration of these two proteins in milk so their response to a change in nutritional status is worth examining in relation to their importance as controllers of manufacturing quality.

## CHAPTER 2

Consideration of Experimental Design and the  
Selection and Development of Analytical Procedures

The objective of this Chapter is to outline the methods and techniques available for consideration at each stage of the design, sampling and analysis of the experiment. The reasons for the selection of particular methods and the results of laboratory experiments conducted to assist in the choice of analytical techniques will be presented.

2:1 Aspects of Experimental Design

The choice of optimum experimental design is complicated by the many designs and variations available and the combinations of them that have been described in the literature. However, animal experiments must be designed in relation to two basic (but conflicting) principles:

- 1). That the sensitivity of the experiment should be as high as possible.
- 2). That the size of the experiment is limited by the available resources.

2:1:1 General Considerations

To design experiments that make efficient use of resources, (Gill 1969) suggested that the following points must be predetermined:

- a) the magnitude of the experimental error variance involved;
- b) the size of the difference that one desires to detect;
- c) the significance level of the test of hypothesis;  
(Consideration of Type I error, or the rejection of the null hypothesis that is in fact true.)
- d) the power of the test of hypothesis to detect treatment differences of specified magnitude (where power is one minus the probability of Type II error; Type II error being the acceptance of a null hypothesis that is in fact false);

The first of these points must be obtained from prior data whilst the remainder are somewhat arbitrary decisions required of the researcher.

The predetermination of experimental size, whilst providing an estimate of the numbers of experimental units required, is limited in application by the following:-

- a) the availability of suitable experimental animals;
- b) the availability of resources to manage, sample or analyse the treatments;
- c) the likelihood that the error variance of the experiment will differ to that used in the calculation of numbers required;

Cochran and Cox (1957) list three factors by which the accuracy of experiments may be increased. These are:-

- (a) to increase the size of the experiment;
- (b) to refine the experimental technique;
- (c) to utilise an experimental design or method of analysis which minimizes the effects of variability;

Experimentation in biological systems tends to require large numbers of observations to overcome the effects of variation between individual units and the inability to control this variation, particularly in relation to interactive elements of the system. Various experimental techniques have been developed to overcome this problem.

2:1:2 Animals (Experimental Units) Experimental units should represent the population to which results are to apply (Lucas 1959). Representativeness is sometimes sacrificed by choosing "uniform" animals for the purpose of reducing experimental error. In this study representativeness was the objective and uniformity was sacrificed by choosing a variety of breeds and of Beta Lactoglobulin ( $\beta$  Lgb) genotypes to extend the applicability of the conclusions. Other aspects of experimental theory were relied upon to restrict the error variance.

The use of monozygous twin cows is one method by which the effect of experimental error can be greatly reduced (Lucas 1959) by eliminating genetic variance between two or more treatments

(Dick & Whittle 1951). Twin efficiency values (TEV) have been recorded for various traits based on this fact, where TEV is defined as the number of sets of randomly selected animals which a set of identical twins can replace without loss of experimental sensitivity (Martin & Wilcox 1968). Patchell (1956) reported a TEV of 3.2-5.5 for the yield of milk and milk constituents and 12.6 and 23.3 for the percentages of milk protein and milk fat respectively. He noted that these figures may be optimistic due to genotype x environment interactions and that they are subject to sampling errors. They do however, indicate which milk characteristics may be most profitably studied by using monzygous twins.

2:1:3 Experimental Design Experiments with lactating cows are usually based either on continuous treatment or on changeover designs (Broster & Curnow 1964). Though change-over designs result in considerable control of error, they suffer from several drawbacks:-

- a) carry-over or residual effects mask the results of the current treatment (Lucas 1959);
- b) whether or not carry-over effects exist, the design may result in bias of the estimate of error (Patterson & Lucas 1959);
- c) associated with the carry-over effects is the problem of determining the optimum period length, particularly in time-limited experiments;
- d) the results have limited practical application.

The continuous treatment design has the advantages of simplicity and permits nutritional problems to be examined under the conditions in which they occur. Furthermore (though not relevant to this study), continuous designs permit the examination of short, intermediate and long-term effects of the treatments (Broster & Curnow 1964).

Covariance analysis using production during a preliminary period as an independent variable, has been used to reduce the effects of variation between animals in indoor continuous trials. Reductions in the coefficients of variation by 30% to 50% have been obtained by the use of covariance designs as compared to analysis without covariance (Bartlett 1935, Burt 1958, Broster et al. 1960, Broster and Curnow 1963). Burt (1958) also found that covariance analysis reduced the coefficient of variation of milk yield of grazing cows by 10%.

Cunningham and Owen (1971) compared methods of statistically analysing performance data from a dairy cattle feeding experiment. A modified switchback design reduced error variance for milk yield and fat % by 94% and 52% respectively, compared to the completely randomised continuous design. Use of a covariance adjustment for performance in a preliminary period reduced the error variance to almost the same extent. The use of a second covariate (body weight) did not improve the precision further.

It was decided that for the present experiment, the disadvantages of switchback designs detailed previously, were sufficiently great to warrant the use of a continuous design. Furthermore, a continuous design would more adequately meet the objectives of the experiment, in that the relatively low efficiency of the continuous design could be improved sufficiently by the following:-

a) inclusion of a preliminary period in which to establish data for use in an analysis of covariance;

b) use of monozygous twins to restrict the error variance. The number of experimental animals was limited particularly by the number of samples that could be analysed. This prohibited the use of the numbers of unrelated animals that otherwise would have been required.

#### 2:1:4 Lengths of Preliminary and Comparison Periods (PP and CP)

It has been demonstrated frequently that the response to changes in the nutritional status of the cow, in terms of milk

yield and composition, occurs relatively rapidly. Vik-Mo et al. (1974) considered that abomasal infusion of glucose and casein lasting 5-7 days was sufficient to determine the effects on milk yield and composition. Storry et al. (1974) showed that both the percentage and yield of fat stabilized under treatments of both high and low roughage diets after about 7 days in feeding periods of 12 days. Stobbs and Brett (1974) found that changes in milk yield and composition due to restricted feed intake occurred within 3-6 days. Paquay et al. (1973) allowed a period of 10 days for the effects of diet to stabilize before beginning Nitrogen balance determinations. Feagan (1973) (unpublished data) reported that the  $\beta$  Lgb content of factory bulk milk doubled in 2-3 days following the change in feed quality and quantity, associated with summer drought breaking rains in South Australia.

Consequently, though longer experimental or CP are more common (20-60 days) in dairy feeding experiments (particularly with continuous designs), the above evidence suggested that a CP of 10 days would be sufficient to induce the expected responses. This decision was also supported by Lucas (1959) who suggested that the shortest CP compatible with the experimental purpose should be used, as experimental error increases with period length.

PP prior to CP serve not only to establish the independent variable necessary for covariance analysis but also permits the allotment of cows to treatment groups by the procedure of balancing. This is done by assigning the cows to treatment groups in such a way that the mean PP performances of each group are similar. Lucas (1959) states that though balancing has been criticized on the grounds that it leads to bias, it is acceptable in dairy experiments due to the fact that the relationship between PP and CP performance is essentially linear. Furthermore balancing is preferable to random allotment in small experiments because it guarantees a maximum efficiency factor for the experiment. Consequently it was decided to balance the

treatment groups rather than to randomly assign cows within twin pair mates to treatments.

Both Campbell et al. (1964) and Munford et al. (1964) used a PP of five days. Burt (1958) used a control period of 1-2 weeks whilst Bartlett (1935) found no advantage for a 5 week period over a 1 week PP in an indoor experiment.

The twin pairs chosen for this experiment were within a range of 8-12 weeks post-calving. At this stage of lactation, day to day changes are the major component of the variation in milk yield and composition (Munford 1974 - pers. comm.). Dick (1950) estimated that the mean day to day difference in milk yield was 8.13% with a standard deviation of 2.65% of the total daily yield. In the present experiment all cows had been run together under normal grazing conditions since calving and so had been standardized for 8-12 weeks. A 4 day PP immediately prior to the start of the CP was considered sufficient to establish the independent variable for covariance analysis under these conditions, and on which to balance the treatment groups. A point to be remembered in their establishment is that a PP which is shorter than optimum only results in removing less of the variance from the CP, whereas an excessively long PP with grazing animals may result in limiting the correction possible. The latter may arise because of a poorer relationship between the PP and the CP values resulting from movement along the lactation curve or from environmental changes over the extended period.

The conclusions arrived at as to the design of the experiment were that a continuous treatment with covariance analysis would best meet the stated requirements. A PP of 4 days and a CP of 10 days would be sufficient to measure the effects of the treatments, and the animals would be allocated to treatments by the procedure of balancing.

2:2

Measurement of Response Variables

In this section, the choice of the major analytical procedures will be presented as will the measures which were straight forward or which had no practical alternatives. Experiments which were carried out to develop analytical techniques will also be presented and the results discussed.

2:2:1 Separation of Individual Whey Proteins The determination of the quantities of individual protein fractions in a mixture of proteins (such as whey) requires an initial separation of the components. An exception to this is the Radial Immunodiffusion technique first used by Ouchterlony in 1949, cited and developed further by Mancini et al.(1965).

Fractionation of complex protein mixtures can be achieved by salting ; eg  $(\text{NH}_4)_2 \text{SO}_4$  and with alcohols or various other organic solvents. However, these techniques generally lack specificity for individual protein species and require careful control of conditions. Water soluble polymers such as polyethylene glycol (PEG) also fractionate protein mixtures (Polson et al.1964) and are apparently more suitable than other precipitation methods for the separation of milk protein components (Richter et al.1974).

Davies (1974) separated whey proteins by column chromatography (using Sephadex g-100) and then quantified the fractions using a micro-Kjeldahl procedure. Though the results (each fraction expressed as a proportion) were in good agreement with the values of Rolleri et al.(1956), (obtained from moving-boundary electrophoresis) Davies somewhat pointedly suggested that more rapid techniques should be considered.

Separation of proteins by gel-electrophoresis was first introduced by Smithies (1955). Since then a large amount of work has been done in evaluating various gels, buffers and running conditions which has been reviewed by Porter (1966) and Gordon (1972).

The principle of electrophoretic protein separation in gels is based upon two properties of the protein, molecular size and nett electrical charge. The pH of the system is adjusted so that all proteins in the sample have a mobility towards one electrode. Differing nett charges result in varying degrees of attraction to that electrode. Molecular size effects the degree of restriction for each protein as an interaction with pore size of the gel. Thus by altering the running conditions various protein mixtures can be separated, then fixed in position and stained for qualitative or quantitative analysis.

The discontinuous polyacrylamide gel electrophoresis (PAGE) method of Ornstein (1964) and Davis (1964), as modified by Hartman and Swanson (1965), has been extensively used for the separation of milk proteins, e.g. Dill et al. (1972), Newstead (1973). The excellent and rapid separation of protein fractions possible as shown in Plate 4:1a was considered suitable for this study.

2:2:2 Quantification of the Separated Proteins Davis et al. (1974) summarized the techniques available for the quantitative analysis of proteins following their separation by PAGE as:-

- (a) scanning the unstained gel in an ultra-violet monitor at 280nm.
- (b) scanning a stained gel in the visible region.
- (c) slicing out the stained bands followed by spectrophotometric analysis of the solubilized dye.
- (d) photographing the stained protein bands and using a densitometer to scan the negative.

Several disadvantages are associated with each method. For example, the first mentioned method suffers seriously from the level of baseline "noise" (Davis 1974), whilst the second is limited by the difficulties of keeping staining and destaining conditions constant (Watkin and Miller 1970). The slicing technique becomes impossible if the bands are even slightly skewed and the final

technique is dependent upon the accuracy and repeatability of the photographing and developing (Davis 1974). Though Watkin and Miller (1970) stated that the optical density (OD) of a stained band detected by any scanning machine cannot be directly translated into quantity of protein, Kruski & Narayan (1974) suggested that quantification of PAGE peaks from biological mixtures was feasible.

It was decided that the most convenient method would be that of scanning the stained gel in the visible region. Each protein peak (OD) is plotted on a chart and the area under each peak is simultaneously presented on a linear scale (Plate 2:2). By counting the integrator responses (scan units) below each peak, an indirect estimate of concentration can be obtained for that protein. A sufficient level of accuracy can be obtained by this method if the following aspects are given consideration:-

- a) constancy of acrylamide concentration (Kruski & Narayan 1968);
- b) fixed migration distance (running time) (Kruski & Narayan 1968, Fishbein 1972, Davis et al. 1974);
- c) accuracy in the dilution and loading of the protein samples (Kruski & Narayan 1968);
- d) limiting the amount of protein applied so that the relationship between the OD of the stained band and the concentration of protein is linear (Kruski & Narayan 1968, 1974);
- e) rapid fixing and staining following PAGE to restrict protein diffusion from the bands (Davis et al. 1974);
- f) constancy of staining and destaining conditions (Watkin & Miller 1970);
- g) storage of destained gels in acetic acid and in darkness to prevent bands fading prior to scanning (Davis et al. 1974);
- h) the establishment under individual circumstances of the particular dye binding relationships for each protein to be quantified (Kruski & Narayan 1974, Dolby 1961);
- i) the establishment of a set pattern of analysing the scans to overcome the problems of overlapping peaks and variable backgrounds (Kruski & Narayan 1974).

The method was modified in the light of the results of several experiments which were conducted to try and improve the technique. The technique as used is presented in Chapter 3 with the method and necessary preparations detailed in Appendix I

Laboratory experiments were conducted to investigate the effects of acrylamide concentration, method of destaining and length of the destaining period.

2:2:2:1 Acrylamide Concentration Three gel concentrations, 9, 11 and 13% of acrylamide with proportionally increased bis-acrylamide concentrations were evaluated. Eight gels of each concentration indicated, by subjective appraisal, that 13% gels gave better protein separation than 11%, which was in turn better than that obtained using 9% gels. The 13% gels were however, too brittle and prone to fracture for ease of handling. Consequently 11% gels were used throughout the analysis.

2:2:2:2 Method of Destaining Gels Two alternative methods have been described in the literature for the removal of excess stain from gels. First, diffusion, whereby the stain not bound to protein is washed from the gel matrix by frequent changes of destaining fluid (2% Acetic Acid). Secondly, electrophoresis, whereby the unbound stain migrates from the gel to the positive electrode.

An experiment was conducted to determine which of these methods would best meet the major requirements of the analysis, that is quantitiveness and speed.

Method: 12 gels were loaded, run and stained identically, by the methods outlined in Chapter 3. Two groups of 6 gels were formed and destained by one of the two methods:

a) Diffusion Destaining The 6 gels were destained in a "home-made" continuous circulation diffuser with air agitation. The circulation rate of fluid (2% Acetic Acid) was 20ml/min with diffused stain being removed by an in-line charcoal filter. Diffusion destaining was continued until it was considered that the clarity of the inter-band gel matched that of the electrically destained gels.

b) Electrophoretic Destaining The remaining 6 gels were loaded into destaining tubes and placed in the baths as for running the gels. Destaining fluid replaced the running buffer. A current of 3 $\frac{1}{2}$ m Amp per tube was run until the stain front just cleared the end of the gel.

Both groups of gels were stored and scanned as described in Chapter 3. The results are presented in Table 2:1

Table 2:1 Comparison of Two Methods of Gel Destaining

Protein	Electrical Mean Scan Units	Diffusion Mean Scan Units
BSA <sup>≠</sup>	8.3	6.8
α Lac	16.8	12.0
β Lgb	82.0	52.0

<u>t test</u>	t value	Significance
BSA	2.02	NS (P > 0.05)
α Lac	4.46	*** (P < 0.005)
β Lgb	7.73	**** (P < 0.001)

Time of Destaining - Electrical = 2.8 hr  
 - Diffusion = 69 hr

<sup>≠</sup> BSA Bovine Serum Albumin  
 α Lac Alpha Lactalbumin  
 β Lgb Beta Lactoglobulin

Conclusions        The electrical destaining method consistently resulted in higher protein concentrations than the gels destained by the diffusion method. Furthermore the time taken for diffusion destaining meant that the diffusion technique was not a practical alternative to electrical destaining.

Having established that electrophoretic destaining was the most suitable for the requirements of the analysis, it was decided to check the effect of prolonged destaining by this method. Davis (1964) suggested that prolonged electrophoretic destaining could result in a reduction in the amount of dye bound to a protein.

#### 2:2:2:3        Time of Electrophoretic Destaining

##### Method

Twenty four gels were prepared, loaded, run and stained under identical conditions and destained electrophoretically as described earlier. At the point of completion of destaining 6 gels were removed, 3 at random from each bath. Blocks of 6 gels were removed similarly after  $\frac{1}{2}$ ,  $1\frac{1}{4}$  and  $2\frac{1}{2}$  hours of extra destaining, with the current being altered with each block to maintain  $3\frac{1}{2}$ mAmp/tube throughout the experiment. Gels were stored and scanned as before and the results are presented in Table 2:2.

Table 2:2 Effect of Prolonged Electrophoretic Destaining on Protein Concentration

Protein	Extra Destaining time (hr)				F.ratio	LSD (5%)
	0	$\frac{1}{2}$	$1\frac{1}{4}$	$2\frac{1}{2}$		
	SU	SU	SU	SU		
IG $\neq$	26.6 <sup>a</sup>	23.0	18.8 <sup>b</sup>	17.5 <sup>b</sup>	4.17*	4.6
BSA	10.5 <sup>a</sup>	10.7	10.2	9.3 <sup>b</sup>	3.25*	1.0
$\alpha$ Lac	31.3 <sup>a</sup>	31.5	28.7 <sup>b</sup>	28.7 <sup>b</sup>	3.17*	2.6
$\beta$ Lgb	132.8 <sup>a</sup>	128.0	115.2 <sup>b</sup>	115.3 <sup>b</sup>	9.20**	9.2
Total	203.6 <sup>a</sup>	197.8	177.2 <sup>b</sup>	175.8 <sup>b</sup>	6.96**	16.0

SU Scan units - mean of 6 gels

\* Significant at P < 0.05

\*\* Significant at P < 0.01

Within rows (proteins) superscript "a" differs significantly (P < 0.05) to superscript "b".

$\neq$  IG Immunoglobulins

BSA Bovine Serum Albumin

$\alpha$  Lac Alpha Lactalbumin

$\beta$  Lgb Beta Lactoglobulin

Discussion Significant losses of stain occurred from all protein bands when electrophoretic destaining was prolonged for between  $\frac{1}{2}$  and  $1\frac{1}{4}$  hours. Some depression of staining of the high concentration  $\beta$  Lgb band was evident under these conditions after even  $\frac{1}{2}$  hour. It was concluded therefore that no over-destaining could be permitted to occur. To this end two factors were noted as being relevant:-

- a) that the constriction of the destaining tube should be no greater than just sufficient to retain the gel and should be constant for all tubes;
- b) that all air bubbles must be excluded from the loaded tube prior to destaining.

#### 2:2:2:4 Separation of Intergrator Response Units of Overlapping Peaks

Several methods are available which allow the intergrator response units to be separated where the scans of the protein peaks overlap; for example the  $\beta$  Lgb A & B variants in the heterozygous animal. (Plate 2:3).

The method chosen to separate the peaks was to continue the peak slopes to interception, directly below which was taken as the point of separation of the two proteins. This method resulted in peak proportions being obtained which were similar to those obtained by using the method of "Point of Inflection" of the scan. The method used did however, slightly favour the flatter of the two peaks by comparison with "Point of Inflection". The results of these two methods however, differed markedly to those from the methods of doubling the number of responses from the non-overlapping half peaks. A computer programme to generate peak area by yet another method based on peak heights, width at half peak height and peak separation was later developed by Dr. E. Cant (Dairy Research Institute, Palmerston North, New Zealand) and a comparison of its performance against the method used (Table 2:3) with 5 gels of differing peak relationships showed reasonable agreement.

Table 2:3 Relationship between the peak proportion  $\frac{\beta \text{ lgb B}}{\beta \text{ lgb A}}$  from two methods of separating these overlapping peaks.

Gel No.	I Computed Area (Cant Programme)	II Counted Area (Method Used)	% Difference ( $\frac{I-II}{I} \times \frac{100}{1}$ )
47/19	0.735	0.696	-5.3 %
11/18	0.661	0.699	+5.7 %
39/13	0.897	0.821	-8.4 %
39/10	1.035	1.152	+11.3%
40/23	0.975	0.865	-11.2%

## 2:2:2:5 Comparison of Methods of Quantifying Protein Band Units

Two methods for converting the scan units of each protein to an actual concentration of protein were considered. The first method (Direct Relationship) relied on reading the concentration of the individual protein directly from a standard curve relating scan units to concentration of the individual proteins. This method is dependent upon the quantitative application of the sample and complete recovery of the proteins during processing.

The second method (Proportional Subdivision) was designed to overcome the major problem of the first method, namely the maintenance of quantitiveness throughout the PAGE analysis. The method utilizes the scan units from each band as a proportion of total scan units from the one gel. This proportion following correction for dye binding differences between proteins is then used to partition a separate determination of protein concentration. In these experiments the separate determination of protein concentration was obtained using an adaptation of the dye-binding method of Dolby (1961)(Appendix II and Section 2:2:3) The Proportional Subdivision method was however, complicated by the observation that the dye-binding relationship for the individual proteins in the free binding system of Dolby's, differ to those of the same proteins in the gel system. Consequently another set of standard curves was required for the method. Both sets of standard curves are presented in Appendix V as well as a comparison of the relative dye-binding coefficients.

The Proportional Subdivision method decreases the demands for accurate quantitative running of each sample, but requires an extra analysis and the use of a second standard curve for each estimate. Hence it was decided to compare the two methods to determine whether in fact Proportional Subdivision actually improves the accuracy of the estimate relative to that obtained by the Direct Relationship method.

Method The effectiveness of the two methods was tested by conducting a One-Way Analysis of Variance (Snedecor & Cochran 1967 p260) within each method on the results obtained by each for the same group of samples.

Thirty seven duplicated determinations of  $\beta$  Lgb concentration were selected at random from the complete list of cows x days calculations and the results are presented in Table 2:4.

Table 2:4 Comparison of the Sources of Variation within Each of Two Methods of Estimating  $\beta$  Lgb Concentration from the Same Samples.

i: Direct Relationship

Source	df	SS	MS	F ratio
Total	73	32.7		
Between Samples	36	28.0	0.78	
Within Samples	37	4.7	0.13	6.12

ii: Proportional Subdivision

Source	df	SS	MS	F ratio
Total	73	31.8		
Between Samples	36	31.5	0.88	
Within Samples	37	0.3	0.007	120.1

Discussion            The calculation of the F ratio in these analyses serves only to demonstrate the reduction in error variance due to Method ii, Proportional Subdivision. The greater precision of the method with respect to that of the Direct Relationship method is shown by the large reduction in the within sample mean square term. In this analysis this term represents an increased repeatability of the estimates by the Proportional Subdivision method. Details of the Proportional Subdivision Method are presented in Appendix IV.

### 2:2:3 Determination of Total Concentration of Whey Protein

The Proportional Subdivision method of quantifying protein bands from PAGE outlined in Section 2:2:2:5 requires a separate determination of total concentration of whey protein. Several alternative methods were considered.

(1) Total Nitrogen (N) by Kjeldahl or Micro-Kjeldahl procedures. These measures are suitable for determining total milk protein where the proportion of non-protein-nitrogen (NPN) can be disregarded. However, in whey, NPN makes up a much larger part of total N than in milk (Roeper & Dolby 1971) and so must be considered. An extra analysis would be required to determine NPN separately if this method were to be used. Moreover, the classical micro-Kjeldahl technique of Rowland (1938) which has been widely adopted as the standard procedure for determination of whey proteins (McGann et al. 1972a) is far too time consuming in itself for a routine analysis (McGann et al. 1972b).

(2) Pro-Milk Difference Technique (PMDT). The amido black dye-binding technique has been shown to be a suitable basis for measuring protein content of herd bulk milk, quickly, conveniently and with acceptable accuracy as compared to the Kjeldahl method (Tarassuk et al. 1967, Dolby 1961, Posthumus 1960). A semi-automated protein testing instrument (Pro Milk Mark II A/SN Foss Electric, Denmark) based on the dye-binding principle was examined by Szijarto et al. (1973) and found to produce results very little different to Kjeldahl with similar repeatability. The PMDT for whey was developed by

McGann et al. (1972b) and simply involves the addition of a fixed quantity of a standard milk or milk powder to a sample of whey to raise the reading to the sensitive 3-5% protein range of the instrument scale. The difference between the two readings; standard plus whey less standard, is then corrected for the high dye-binding capacity of whey and an estimate of whey protein concentration is obtained. The method has the advantage of being a single estimate determination and has a satisfactory accuracy in relation to Kjeldahl.

The PMDT was however, unsuitable for this analysis as the whey samples had been diluted by a factor of 2.5 during their preparation. The sensitivity of the technique under these conditions would not have been sufficient.

3)

Dye-binding using Conventional Spectrophotometry. The dye-binding method of Dolby (1961) can be used for both milk and whey samples. Dolby reported that the method adjusted to analyze whey yielded protein concentrations which were in good agreement with Kjeldahl values.

It was decided to use this method (Dolby 1961) to analyze the whey protein concentration. The small volume and dilute nature of the samples necessitated some modifications to the method. The modified method as used is detailed in Appendix II.

#### 2:2:4 Mastitis

Many methods of detecting subclinical mastitis have been developed. They can be broadly subdivided into direct and indirect measures.

2:2:4:1 Direct Methods Direct tests used for the detection of subclinical mastitis include bacteriological examination (Postle et al. 1971, Natzke et al. 1972, Thomas et al. 1972) and microscopic (DMCC) (Eberhart et al. 1968, Waite & Smith 1972, Eberhart 1972) or electronic (Coulter counter) cell counting (Cullen 1965, Phipps &

Newbould 1965, Phipps 1968). The first two methods are exceedingly time consuming and due to the large dilution factors, errors of up to  $\pm 50$  percent have been recorded (Strynadka & Thornton 1937). Electronic counting of cells is accurate and rapid but requires special equipment which was not available.

2:2:4:2 Indirect Methods Most of these methods are indirect estimates of the number of leucocytes in milk and are based on the reaction noted by Whiteside (1939) in which milk from inflamed quarters, when mixed with sodium hydroxide produces a viscous mass. Various adaptations to this reaction have produced the Californian Mastitis Test (CMT) (Schalm & Noorlander 1957), the Michigan Mastitis Test (MIT) (Paape et al. 1962), the Wisconsin Mastitis Test (WMT) (Thompson & Postle 1964) and the Ruakura rolling ball viscometer, recently examined by Milne & Smythe (1976) based on the same reaction.

Indirect tests based on other factors having a relationship to udder infection have also been considered. These include the concentrations in milk of lactose, chlorides, serum albumin, the K/Na ratio (Klastrup 1975) and electrical conductivity (Greatrix et al. 1968). Many of these require a base level reading from an assumed uninfected quarter of the same cow.

The CMT and WMT measures have been the most widely accepted for measuring subclinical mastitis in research and for the monitoring or checking of the incidence of subclinical mastitis in commercial herds. Daniel et al. (1971) described the reliability of the WMT and concluded that it was a suitable objective measure for routine subclinical mastitis testing, was simple and the results showed a good correlation to direct microscopic cell counts. The WMT method as used is presented in Appendix III.

#### 2:2:5 Milk Fat

The manufacturers (A/SN Foss Electric Denmark) claim that the Milko-tester is accurate to  $\pm 0.06\%$  fat as measured against Rose Gottlieb and the reproducibility of results between duplicates is  $\pm 0.02\%$ . Gray (1976) reported that all trials at Ruakura over a three year period supported these claims. Shipe & Senyk (1973) found that the Milko-tester gave higher (0.03% fat) results than the Babcock test. The standard deviation of the Milko-tester results ( $\pm 0.032$ ). A Milko-tester was available and the above results indicated its suitability for the fat analysis in this experiment.

#### 2:2:6 Milk Protein

The amido black dye-binding method has been shown to be a suitable technique for measuring the total protein content of herd bulk milk (Posthumus 1960, Dolby 1961, Tarassuk et al. 1967). Several reports have however, suggested that dye-binding methods are less accurate for milk from individual cows (Posthumus 1960, Erb & Ashworth 1962, Butcher et al. 1967). This is apparently due to the variation in dye-binding capacity of the individual proteins which are present in differing proportions in the milk of different cows (Waite & Smith 1972). Casein binds only 75-80% of the weight of dye bound by the whey proteins. The main factors reducing the ratio of whey to casein are stage of lactation (Rook 1961a) and mastitis (Waite & Blackburn 1963). A short term trial in mid lactation will not be influenced by the former and Waite & Smith (1972) showed that the effects of the latter were so small as to constitute no objection to the use of the method.

An instrument (Pro-Milk Mark II) for semi automatic determination of dye-binding by milk proteins was available and was used for the analysis of total milk protein of the daily milk samples.

## CHAPTER 3

### Materials and Methods

The reasons behind the choice of the major methods has been covered in Chapter 2. This Chapter will detail the design and running of the experiment and the methods by which the samples were analyzed.

#### 3:1 Experimental Design

It was decided that a balanced block covariance design would be the most appropriate arrangement of the animals to meet the objectives of the study. Animals were balanced within treatment on the basis of their milk yield during a preliminary period of four days. The comparison period was ten days. A further two samples were taken at three and five days following the completion of the comparison period. These samples were included to monitor the changes in milk yield and composition during the animals re-adaption to the herd environment and were not intended to enter the statistical analysis. The return to the herd involved a considerable improvement in nutritional status for the high stocking rate group and some decrease in that of the low group.

#### 3:2 Animals (Experimental Units)

Monozygous twins were used to restrict the error variance within the limited number of animals per treatment that could be handled. The criteria for choice of sets of twins from those available were in order of decreasing importance, as follows:-

- (1) a negative ( $< 10$ ) WMT score for each quarter of both cows for the two days prior to the preliminary period. The herd was originally screened by selecting the twin sets, both members of which had a bulk milk sample WMT score of less than 10.
- (2) calving date was within a fortnight for both cows of each set.
- (3) all sets of twins were between  $1\frac{1}{2}$  and 3 months into lactation.
- (4) representation of the three  $\beta$  Lgb genotypes (AA, AB, BB).

Only the  $\beta$  Lgb genotype criteria could not be fulfilled due to low frequency of AA twin pairs and to the incidence of mastitis within them. Consequently three sets each of AB and BB genotypes were chosen. The descriptions of the experimental animals chosen is summarized in Table 3:1.

Table 3:1 Description of Experimental Animals (6 sets of Monozygous Twins)

Twin Pair No	Breed	$\beta$ Lgb Genotype	MMT Quarters	Individual Mean scores	Calving Date day/month	Stage of Lactation week
31	Friesian	BB	6,4,3,6		2/9	9
32	"	"	8,6,7,6		16/9	7
89	Jersey	AB	2,2,3,2		31/7	12
90	"	"	2,3,3,3		25/7	13
95	"	AB	3,3,5,4		24/7	13
96	"	"	2,2,2,2		31/7	12
101	Jersey X Friesian	BB	2,3,5,2		26/7	13
102	"	"	3,5,5,4		5/8	11
117	Friesian X Jersey	AB	3,5,2,2		7/8	11
118	"	"	2,2,2,3		8/8	11
133	Jersey x Friesian	BB	5,3,5,4		29/7	12
134	"	"	2,7,7,6		23/7	13

### 3:3 Experimental Procedures

3:3:1 Field Work All cows chosen for the experiment were part of the Massey University No.3 Dairy Herd (Identical Twin Herd) and had been treated as a commercial herd since calving. As a consequence, no adjustment period for change of routine was required. The four days prior to the removal of the animals from the main herd for the experimental treatments was used as the preliminary period. The cows continued to be milked at 05.30 and 16.30 hours. The stocking rate was controlled by two single wire electric fences which split the paddock longitudinally into  $\frac{3}{4}$  and  $\frac{1}{4}$  and laterally into "breaks" of these areas. In this way the relative stocking rates were fixed at 3:1. Actual stocking rates were not fixed but controlled by varying the distance the lateral fence was shifted forward for each new break. The only criterion for the decision as to size of break was that the high stocking rate group had to be obviously restricted as judged by the height of grazing and the grazing behaviour of the animals. A fresh "break" of pasture was made available to the cows after each milking. Weighing the cows was not considered feasible due to the short-term nature of the experiment and the variation in such measures both within days (Taylor 1954) and between days (Pearson - Hughes & Harker 1950). Objective assessment classified the low stocked group, as obviously feeding "ad lib" under the conditions imposed, whilst the high stocked group was moderately to severely restricted (Plate 3:1). A back fence to contain the high stocked group was not considered necessary due to the heavy grazing resulting in slow pasture regeneration. An initial habit of fence jumping (H.S.  $\rightarrow$  L.S.) by cow 32 was cured by attaching a length of wire to her "anti-suckle" nose ring for several days.

### 3:3:2 Sampling and Analysis of the Major Milk Parameters

3:3:2:1 Milk Yield Milk yield of each cow was recorded at each milking by the use of "Waikato" milk meters which collect a proportion of the total yield in calibrated flasks. Collections

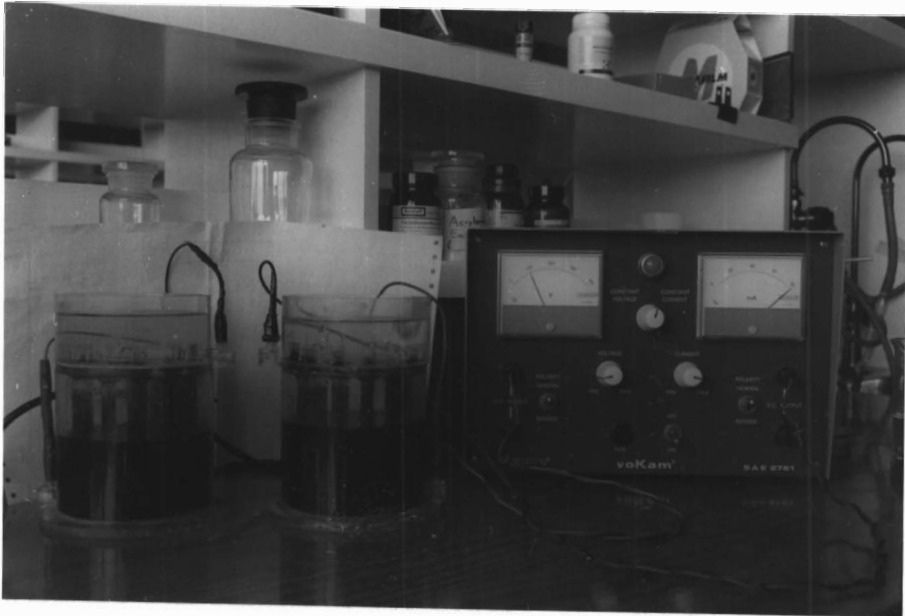


Plate 3:1 Apparatus for Polyacrylamide Discontinuous Gel

Electrophoresis (PAGE), featuring 2 x 12 gel tanks. Gel destaining is shown in progress with the stain migrating from the gel, top (negative electrode bath) to bottom (positive electrode bath).

were combined to form a daily pm/am composite sample for each cow. The conversion of weight of milk (flasks calibrated in kilograms) to volume of milk was made using the conversion factor 1ml=1.032g, being a mean calculated from the data of Jenness & Patton (1959).

3:3:2:2 Milk Fat and Protein Total fat and protein analyses were carried out using the Milko-tester and the Pro-milk Mark II (Both of A/SN Foss Electric, Denmark) respectively on the daily composite milk samples. Protein analysis was carried out on both the whole milk and the skim milk samples to ensure that any change in milk fat percentage did not conceal a significant response in terms of true protein concentration. The skim milk samples were prepared using a bench centrifuge at 2500 RPM for 10 minutes.

3:3:2:3 Mastitis The incidence of mastitis in the cows was monitored using the WMT (Appendix III). Milk samples for the test were collected at the pm milking, either from the milk meter (for cow bulk milk tests) or from the individual teats (for  $\frac{1}{4}$  milk tests). Quarter samples consisted of about 10mls of fore-milk collected following 3-4 squirts to waste during the normal washing procedure. Samples from the milk meter were collected daily and if the WMT scores were suspicious (score 11-16) or positive (score  $> 17$ ) quarter samples were collected at the subsequent pm milking. In addition,  $\frac{1}{4}$  samples were taken from all cows at every third pm milking.

### 3:4 Analysis of the Whey Protein Components.

3:4:1 Preparation of Whey Whey samples were prepared from skim milk using the method of Rowland (1938) which he showed attained the maximum precipitation of casein from milk. The whey samples were stored in 6 ml plastic vials at  $-10^{\circ}$  C until required for analysis.

3:4:2 Protein Separation Protein separation was carried out using PAGE for the reasons discussed in Chapter 2:2:1 and using the materials detailed in Appendix I. Electrophoresis of the samples was carried out using 2 x 12 gel baths which constituted a run of 24 samples. Each sample was diluted 3 fold with a 10% solution of sucrose to increase the bulk density of the sample and to reduce the amount of  $\mu$  Lgb to a level within the region of linear OD (see Appendix VI) of the scanning and intergration systems.

Samples were loaded using a 100 microlitre SGE syringe to layer the samples onto the gels underneath the running buffer. This method overcame the possibility of sample loss due to swirling which occurs when the buffer is added to the top tank of the apparatus (Plate 3:1) after the samples are loaded. The dye trace (bromophenol blue) which delineates the electrophoretic front moving in the gel, was applied in the sucrose solution rather than in the running buffer. This modification to the method prevented the possibility of mis-loading samples onto gels which arose when the method of loading the sample underneath the running buffer was adopted. Samples were run at  $3\frac{1}{2}$  mAmp per tube using a Shandon Vokam power pack and finished when the running front had just cleared the bottom of the gel, invariably taking two hours. Gels were then quickly removed from the tubes, placed in an amido black staining solution (see Appendix I) and left overnight (minimum of 12 hours) to ensure completeness of staining.

Removal of unbound stain (destaining) was carried out electrophoretically with the gels loaded into tubes with constricted ends to hold them in place. At the completion of destaining the gels were removed from the destaining tubes and stored in 2% Acetic Acid in 10mm x 138mm glass test tubes and sealed with corks.

Identification of the protein bands was made by comparison with the published PAGE separations of Richter et al. (1974), Davies (1974), and Kumar & Mikolajick (1972) and by the running of purified proteins (Plates 3:2).



Plate 3:2ai Purity of Immunoglobulin used for the preparation of  
Dye - Binding Standard Curves.

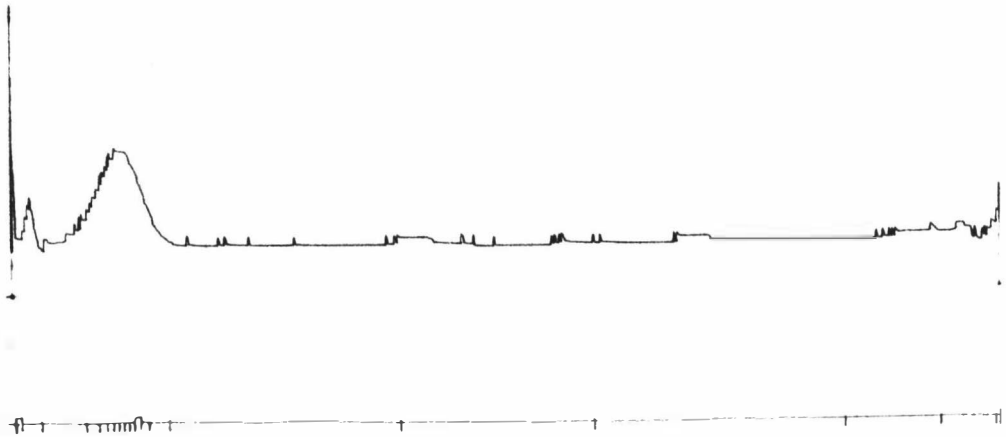


Plate 3:2aii Scan trace and Integrator plot of the above  
Immunoglobulin Standard.

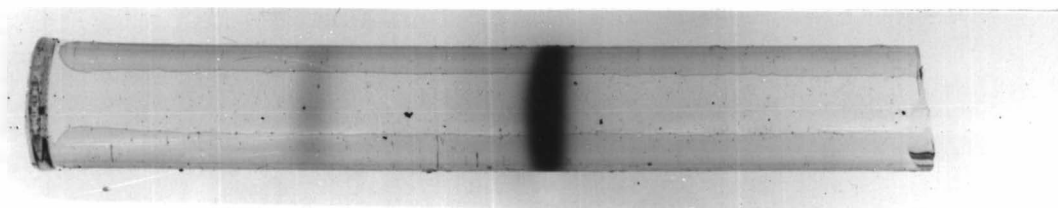


Plate 3:2bi Purity of Bovine Serum Albumin used for the preparation  
of Dye - Binding Standard Curves.

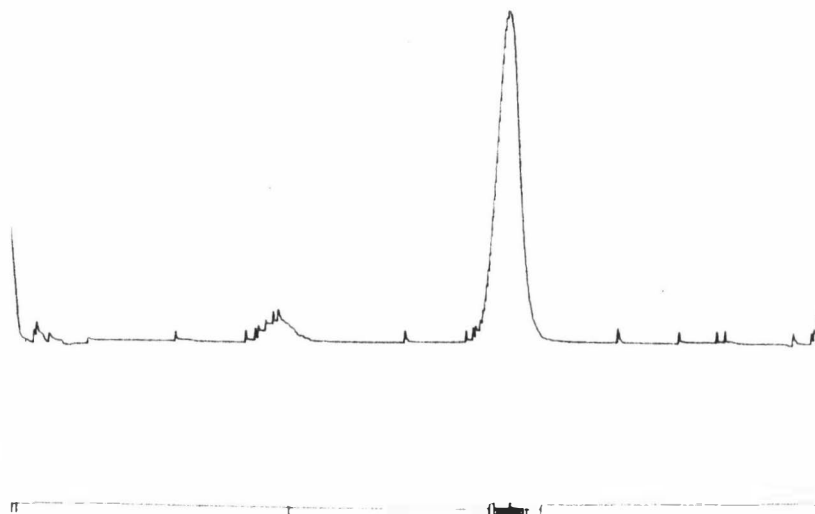


Plate 3:2bii Scan Trace and Intergrator plot of the above Bovine  
Serum Albumin Standard.

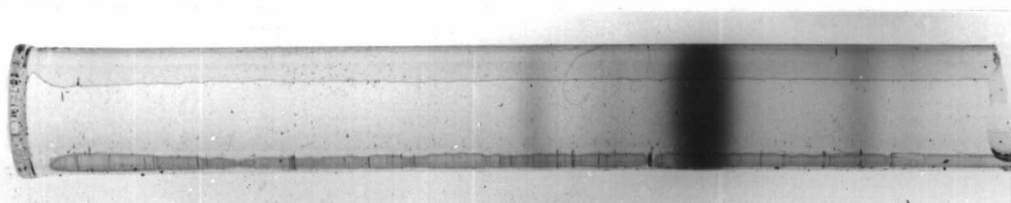


Plate 3:2ci Purity of Alpha Lactalbumin used for the preparation  
of Dye - Binding Standard Curves.

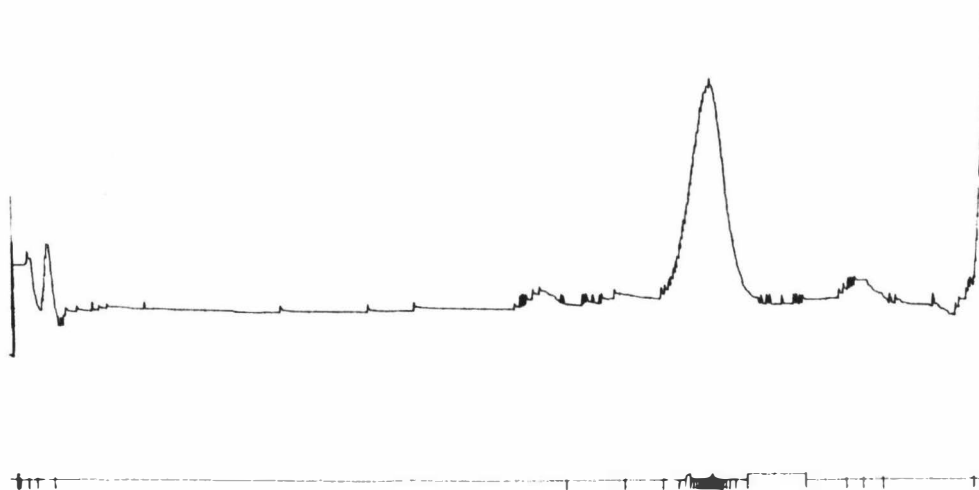


Plate 3:2cii Scan trace and Integrator plot of the above  
Alpha Lactalbumin Standard.



Plate 3:2di Purity of Beta Lactoglobulin used for the preparation  
of Dye - Binding Standard Curves.

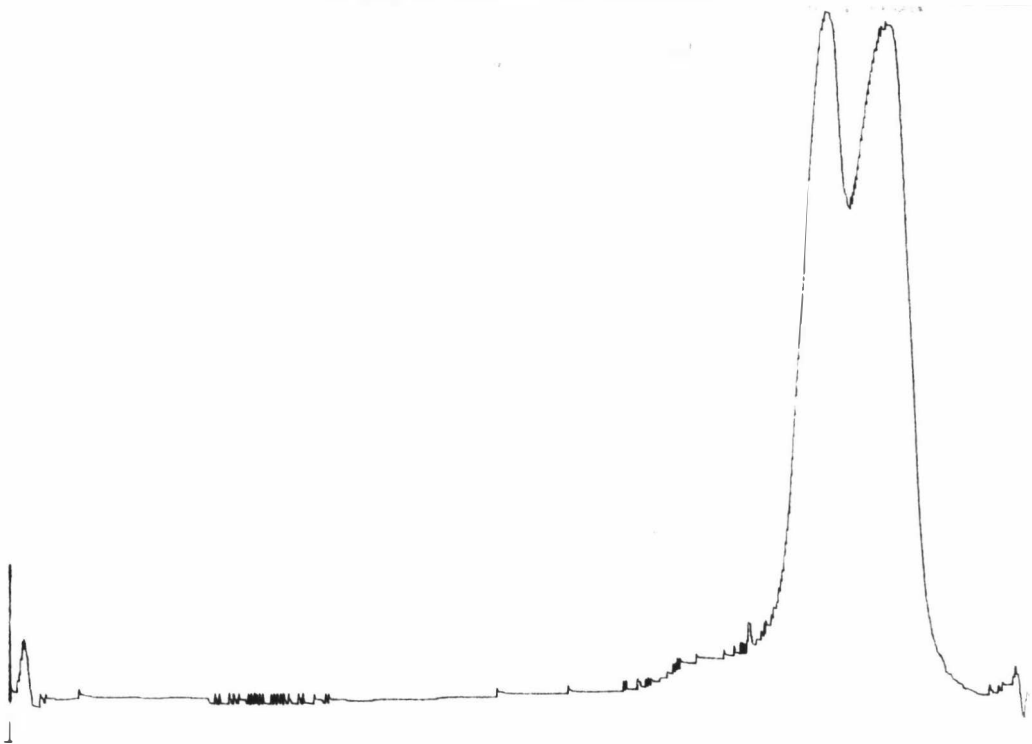


Plate 3:2dii Scan traces and Intergrator plot of the above  
Beta Lactoglobulin Standard.

3:4:3 Protein Quantification The gels were scanned in the visible region within the storage tubes using a Canalco Scanning Densitometer (Model G) in combination with a Canalco Chart Recorder and Integrator (Model 8I). Protein bands are represented by peaks of absorption plotted on the chart and the area under the peak (protein quantity) is represented by the number of intergrator scan units for each. The calibration of the system was checked for linearity and equivalence of intergrator sensitivity setting (see Appendix VI). This data indicated lack of linearity both at very low and at very high OD readings. The two intergrator sensitivity settings were virtually identical over their mid range. This led to the decision to scan the first section of each gel at intergrator sensitivity setting 4, as neither the IG nor the BSA exceeded an OD of 0.8. In the trough forward of the BSA peak, the intergrator sensitivity was re-set at 2 for the greater OD of the  $\alpha$  Lac and  $\beta$  Lgb peaks. Counts for all bands intergrated at 4 were subsequently halved. This procedure resulted in improving the accuracy of the intergration of the peak areas of proteins at lower concentrations.

Overlapping peaks were resolved by the method of continuing the initial slope of the curve into the overlapping area and the separation judged as being the point of junction of these curves.

The intergrator scan units were converted to concentrations of each protein in mg/ml by using a method developed and called Proportional Subdivision. The proportion of each protein in the whey was calculated by correcting the number of scan units by the dye-binding constant of each protein in the gel, and expressing this as a proportion of the total number of corrected scan units for the whole gel.

These proportions were then used to subdivide a separate estimate of dye-binding by the total whey proteins as determined by the spectrophotometric method (Appendix II). The results were corrected for variation in the dye-binding capacity of each protein in the spectrophotometric system. Details of this calculation are presented in Appendix IV.

The dye-binding capacities (constants) were determined for the four major whey proteins for both the gel and the spectrophotometric systems. The standard curve for the gel system related protein quantity to intergrated peak area (number of intergrator scan units), and the spectrophotometric standard curve related protein concentration to absorption at 615 m $\mu$  (Appendix V).

Pure proteins were used to prepare these standard curves. BSA (Cohn Fraction V) was obtained from Sigma Chemical Co., St. Louis, Mo. Ig (Ig G<sub>1</sub>) were prepared from bovine colostrum whey by the method of Brandon et al. (1971).  $\beta$  Lgb and  $\alpha$  Lac were prepared from bovine whey (mid lactation) by the method of Richter et al. (1974). These samples were further purified by passing them through a Sephadex G-75 (Pharmacia, Upsala, Sweden) column followed by re-concentration using the Diaflo system (Amicon Corporation, Lexington, Mass.) with a U.M.2 membrane.

Purity of the fractions was checked by running the individual samples on PAGE under the conditions previously described.

### 3:4:3:1 Standard Curves for Dye-Binding by Proteins in Gels

Six concentrations of each protein were run on PAGE (section 3:4:2) to form the standard curves, covering the expected range of each protein in the sample. Equivalent dilutions of the four proteins were run in the same gel. Each concentration was replicated four times within each run and three runs were

conducted to obtain a mean number of scan units for each protein concentration. The two highest  $\beta$  Lgb concentrations resulted in smearing of the bands, possibly due to molecular interaction. Consequently these points were discarded as were the  $\alpha$  Lac points which were obscured by this smearing. This decision was justified by the fact that in the analysis, sample dilution would be sufficient (or would be made sufficient in a re-run) to prevent such high concentrations occurring. Moreover, such concentrations were entering the region in which the linear response of the integration system was failing.

The standard curves are presented in Appendix Va with the Anovar, Regression Coefficients (Dye-binding constants) and the Y intercepts. For all four pure proteins, variance due to regression was highly significant. ( $P < 0.005$ ) and none of the Y intercepts deviated significantly from zero ( $P < 0.05$ ).

### 3:4:3:2 Standard Curve of Dye-Binding by Proteins in the Spectrophotometric Method

Dye-binding in the spectrophotometric method by each of the four pure proteins was determined as described for whey proteins - Appendix II. Six concentrations of each protein covering the expected range of sample concentrations and including zero protein content were used. Only two replicates of these points were conducted as each replicate was in effect the mean of nine determinations according to the method detailed in Appendix II. The standard curves are presented in Appendix Vb with the Anovar, Regression Coefficients (Dye-Binding Constants) and the Y intercepts. For all four proteins, variance due to regression was highly significant ( $P < 0.005$ ). It was not considered necessary to determine whether the Y intercepts deviated from zero.

The slopes (dye-binding constants) of the relationships between absorption and protein quantity for the four major proteins are presented in Appendix Vc. Both gel and spectrophotometric factors are presented as ratios relative to the dye-binding of the IG.

### 3:5 Total Whey and Casein Proteins Concentration

Due to the variation in dye-binding of the component whey proteins and the large differences in their relative concentrations expected, a direct relationship could not be derived for the total whey protein concentration from the spectrophotometric dye-binding determination of whey protein. The concentration of total whey protein was therefore estimated from the sum of the concentrations of the component proteins. Minor proteins were included using a mean value of the coefficients of the four major proteins for their dye-binding coefficient.

Casein concentration was then calculated by difference, by subtracting the total whey protein concentration from the total milk protein concentration.

## CHAPTER 4

### Results

Within this Chapter the results of the experimental treatments on the yield of milk, and on the yield and concentration of the various milk components will be presented.

Any investigation should first establish that the treatments imposed are effective. Only then can any results be accurately interpreted. Section 4:1 of this Chapter examines the effect of the treatments on the parameters of milk production which have established and widely reported responses to changing states of nutrition of the cow. Section 4:2 contains the main body of results which can be examined in the light of the interpretation of the effectiveness of the treatments obtained from Section 4:1.

#### 4:1 Examination of Effectiveness of Treatments

Milk volume, the yield and percentage content of milk fat and total milk protein have been the most common components by which the efficacy of nutritional treatments have been measured in experiments with lactating dairy cows (Broster & Smith 1969, Gordon & Forbes 1970, Broster 1972, Vik-Mo et al. 1974, Gordon 1976, Hutton & Parker 1973).

The measurement of the above parameters was undertaken in this work particularly to enable differences consistent with differing nutritional states between treatment groups, to be demonstrated.

##### 4:1:1 Determination of Time of Onset of Treatment Effects

Before analysing treatment responses it is necessary to establish when the treatments became effective according to the parameters measured. To establish the length of this period of adjustment to treatment, successive mean daily milk yields for the six cows per treatment group were analysed by Covariance using the mean of the daily milk yields over the preliminary period as the independent variable ( $\bar{x}$ ). Results are presented in Table 4:1.

Table 4:1 Analysis of Time of Onset of Treatment Effects on Mean Milk Yield - First Day of Treatment.

Milk Yield (l/day) from 6 pairs of monozygous twins during a preliminary period of 4 days and the first day of a treatment period. During treatment one cow from each set of twins was given a high plane (H.P) and the other a low plane (L.P) of nutrition.

Treatment	Preliminary Period Mean ( $\bar{x}$ ) $\pm$ S.E. l/day	Day 1 Mean Adjusted for Covariance ( $\bar{y}$ ) $\pm$ S.E. l/day	Difference $\bar{y}_{HP} - \bar{y}_{LP}$ l/day	D=LSD 5% l/day	F ratio	Significance
H.P.	11.65 $\pm$ 3.4	12.11 $\pm$ 3.7				
L.P.	11.66 $\pm$ 4.0	10.23 $\pm$ 4.0	1.89	2.12	6.14	NS

SE Standard Error

LSD Least significant difference

NS Not significant. (P > 0.05)

The change in milk yield due to treatments failed to reach significance ( $P > 0.05$ ) on day one of the treatment (day 5 of experiment) period. However, the trend of the data and the approach of the observed difference to the calculated D (LSD) suggested that it is valid to include the observations from the first day of treatment in the analysis of treatment effects. Thus the analysis period extended from Day 5 to Day 14 inclusive with days 1-4 being the preliminary period.

4:1:2 Examination of the Effect of Treatments on Milk Yield and Gross Milk Composition

Daily means derived from the six cows in each treatment group have been plotted for each parameter and are presented in Figs. 4:1 to 4:6 inclusive.

The treatment effects have been analysed by 2 way Covariance using measures of the particular response variable gained during the preliminary period as the respective independent variable ( $x$ )  
A model of the form:-

$$Y_{ij} = \mu + \alpha_i + \rho_j + \beta (x_{ij} - \bar{x}_{..}) + E_{ij}$$

has been used where:-

- $Y_{ij}$  is the value of the  $j$  th observation in the  $i$  th block
- $\mu$  is the overall mean
- $\alpha_i$  represents the treatment effects
- $\rho_j$  represents the block effects
- $\beta$  is the regression coefficient of  $Y$  on  $X$
- $E_{ij}$  is the residual

Details of the analysis of each parameter are presented in Appendix VII and the results in Table 4:2.

Significant differences ( $P < 0.01$ ) were measured between the means of treatment groups in terms of the yields of milk, fat and protein. The percentage content of both fat and protein (whole milk) did not alter significantly ( $P > 0.05$ ). The change in protein content as measured in the fat free samples was significant ( $P < 0.01$ ).

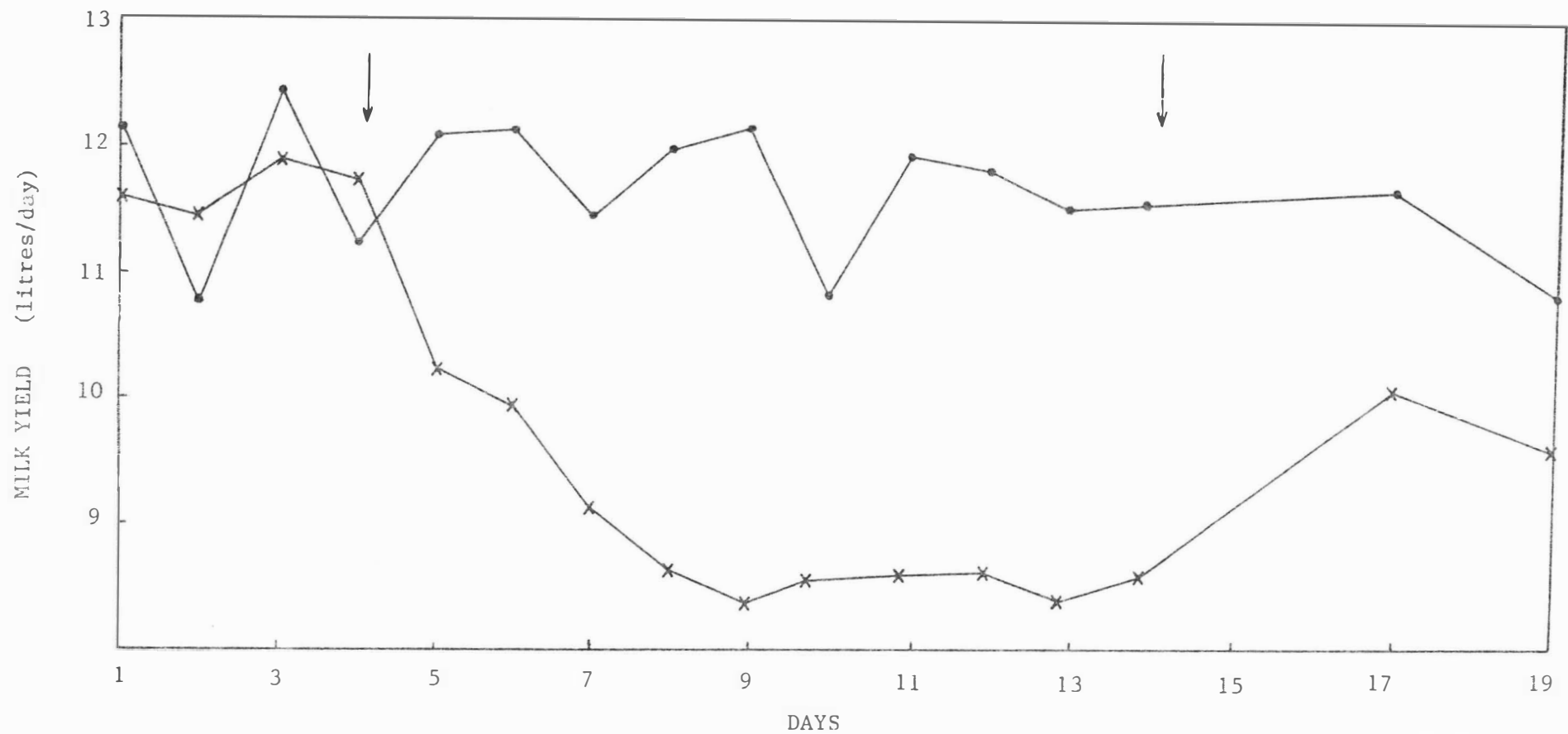


Fig. 4:1 Effect of Plane of Nutrition on Milk Yield (Litres/day)

• High plane

x Low plane

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

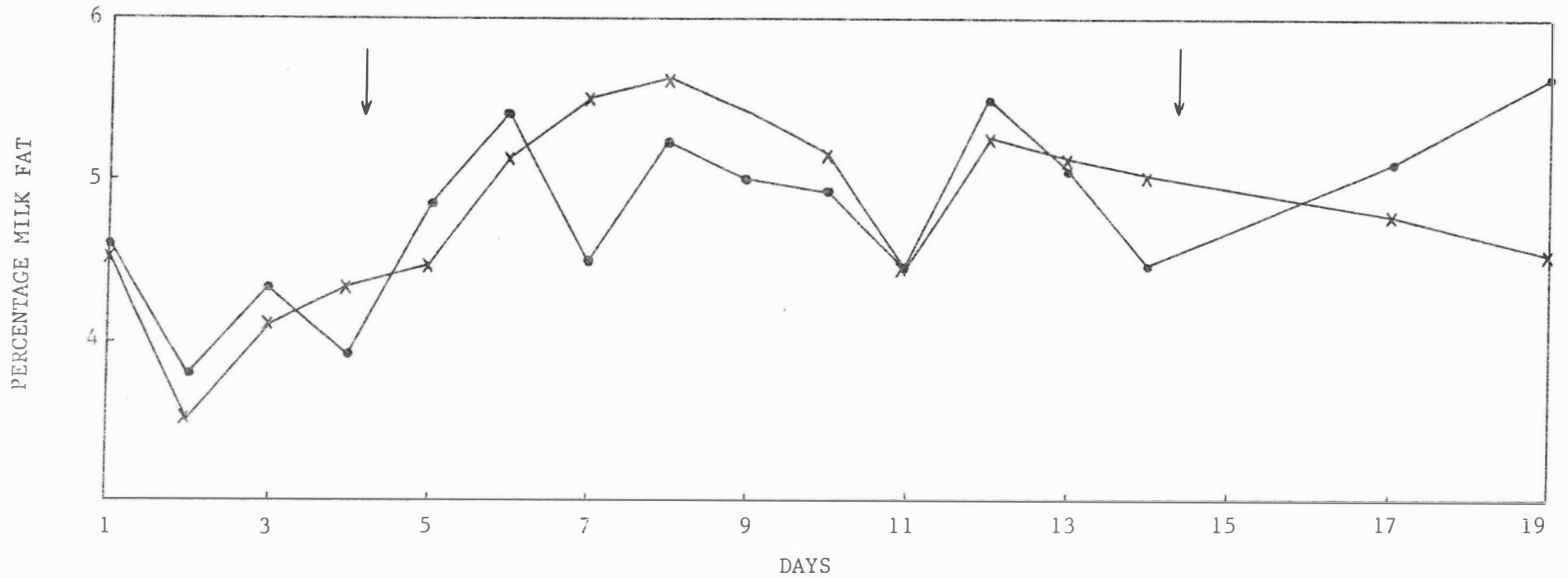


Fig. 4:2 Effect of Plane of Nutrition on Milk Fat Percentage.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

• High plane

x Low plane

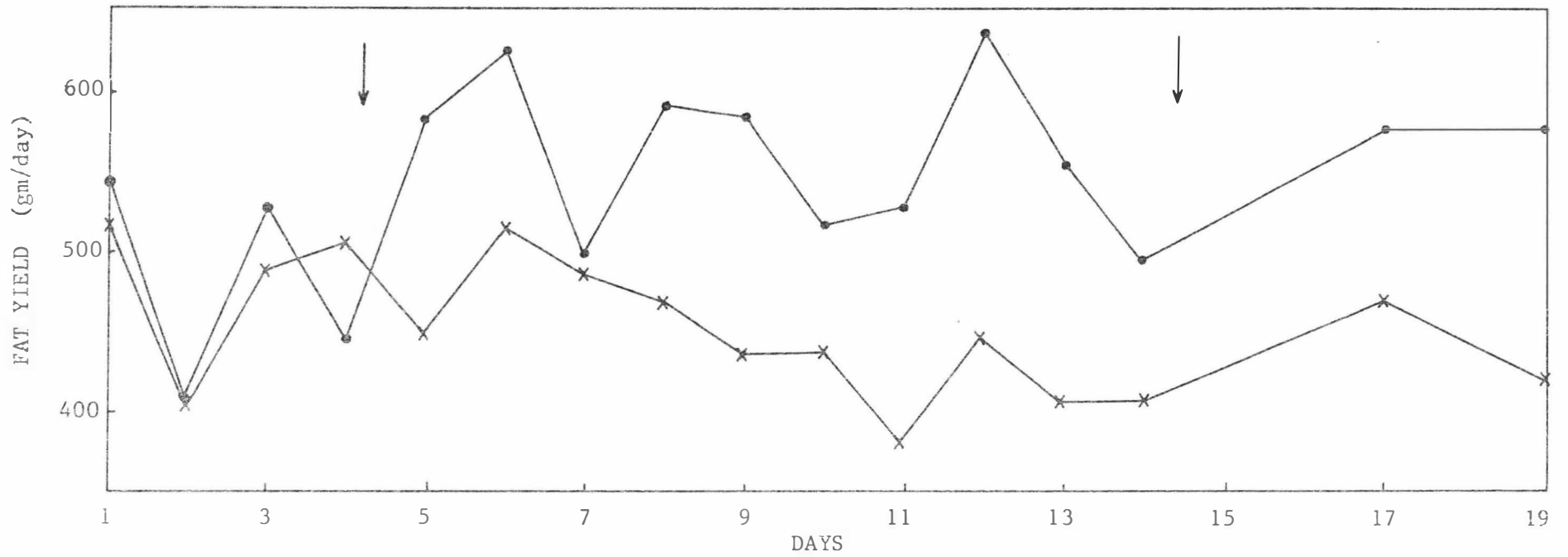


Fig 4:3 Effect of Plane of Nutrition on Milk Fat Yield (gm).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane  
 × Low plane

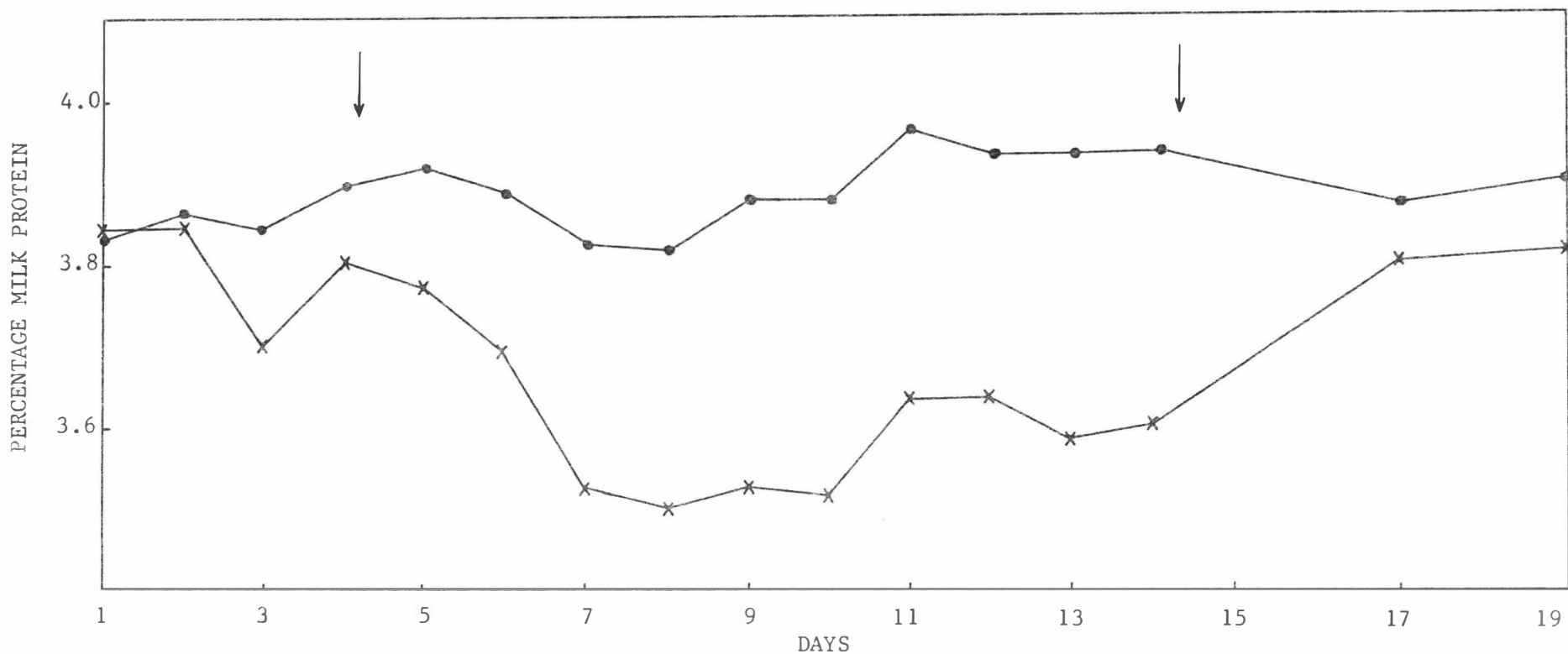


Fig. 4:4 Effect of Plane of Nutrition on Total Milk Protein Content, Expressed as a Percentage of Whole Milk.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

- High plane
- × Low plane

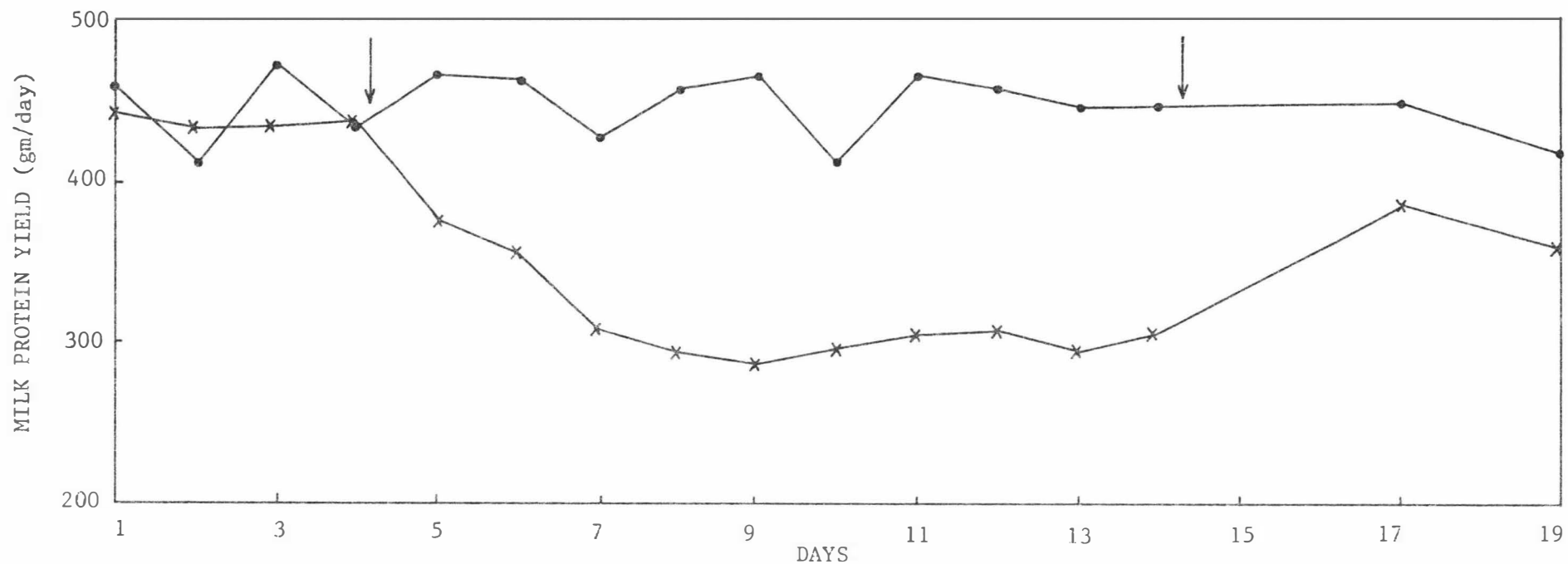


Fig. 4:5 Effect of Plane of Nutrition on Total Milk Protein Yield (gm/day)

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

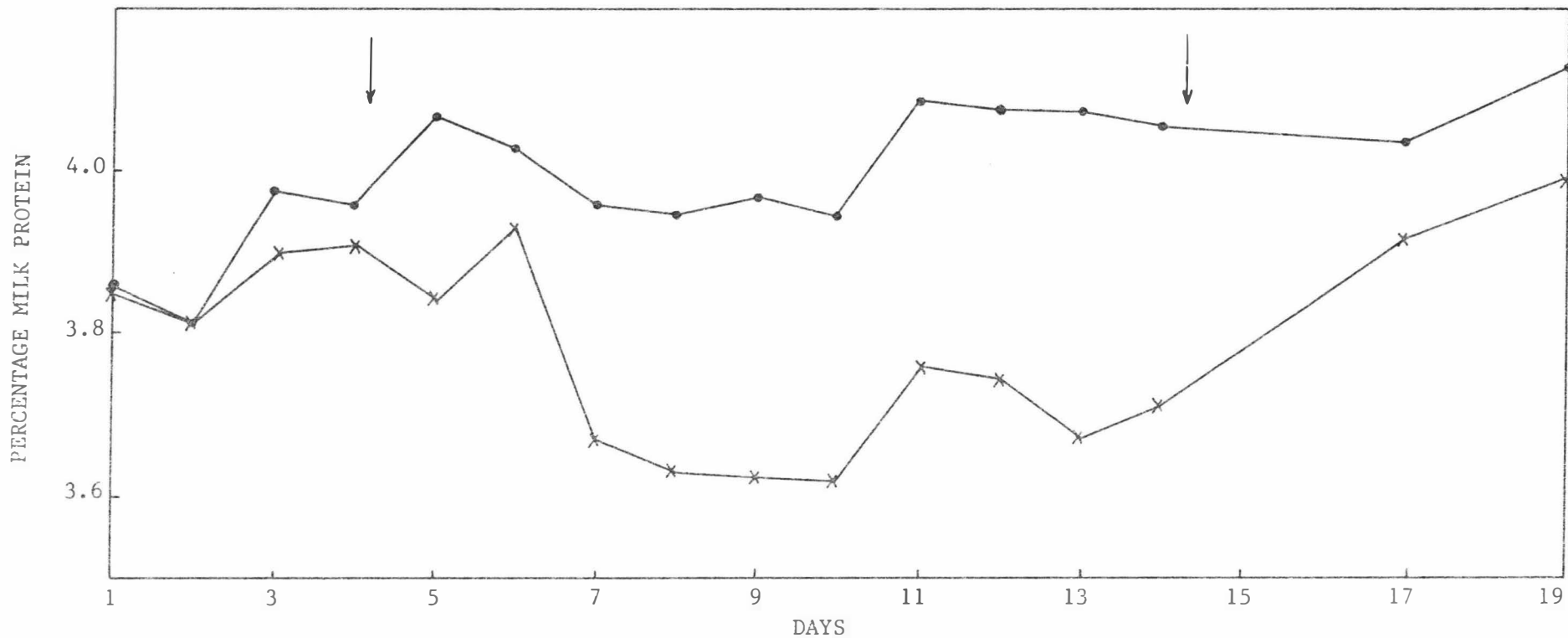


Fig. 4:6 Effect of Plane of Nutrition on Total Milk Protein as a Percentage of Skim Milk.

● High plane

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

× Low plane

Treatment period between arrows.

Table 4:2 Influence of Plane of Nutrition on Milk Yield and Composition.

Milk (l/day), fat (g/day), and protein (g/day) yield. Protein and fat concentration (%), from 6 pairs of monozygous twins for a preliminary period of 4 days, a treatment period of 10 days and a post experimental period of 4 days. During the treatment period one cow from each set of twins was given a high plane (H.P.) and the other a low plane (L.P.) of nutrition.

Parameter	Treatment	Preliminary Period Mean ( $\bar{x}$ ) $\pm$ S.E.	Treat. Period Means Adjusted for Covariance ( $\bar{Y}$ ) $\pm$ S.E.	$\frac{\bar{Y}}{\bar{x}} \times \frac{100}{1}$ %	$\bar{Y} - \bar{Y}$ H.P. L.P.	D=LSD 5% 1%	F ratio	Signif.	Mean Post Expt. Period $\pm$ S.E.
Milk Yield (litres/day)	H.P.	11.7 $\pm$ 0.73	11.73 $\pm$ 0.43	100.2		0.62			11.2 $\pm$ 0.86
	L.P.	11.7 $\pm$ 0.83	8.90 $\pm$ 0.43	76.1	2.82	1.02	161.23	**	9.9 $\pm$ 1.05
Fat %	H.P.	4.15 $\pm$ 0.16	4.93 $\pm$ 0.14	118.8		0.54			5.35 $\pm$ 0.40
	L.P.	4.13 $\pm$ 0.21	5.13 $\pm$ 0.16	124.2	-0.20	0.89	1.06	NS	4.65 $\pm$ 0.24
Fat Yield (grams/day)	H.P.	480.0 $\pm$ 31.07	555.30 $\pm$ 17.33	116.5		51.19			574.4 $\pm$ 28.05
	L.P.	476.7 $\pm$ 34.53	446.80 $\pm$ 20.42	92.6	108.55	84.89	34.66	**	444.1 $\pm$ 38.16
Protein % - whole milk	H.P.	3.84 $\pm$ 0.06	3.86 $\pm$ 0.04	100.0		0.29			3.90 $\pm$ 0.07
	L.P.	3.80 $\pm$ 0.05	3.62 $\pm$ 0.05	95.3	0.24	0.48	3.14	NS	3.80 $\pm$ 0.09
Protein Yield (grams/day)	H.P.	443.85 $\pm$ 24.68	449.78 $\pm$ 13.15	101.3		28.73			430.0 $\pm$ 26.52
	L.P.	435.26 $\pm$ 26.75	310.19 $\pm$ 12.83	71.2	139.59	47.64	181.99	**	369.3 $\pm$ 33.97
Protein % - skim milk	H.P.	3.91 $\pm$ 0.06	3.97 $\pm$ 0.03	101.5		0.13			3.95 $\pm$ 0.09
	L.P.	3.87 $\pm$ 0.06	3.77 $\pm$ 0.04	97.4	0.20	0.22	19.75	**	4.09 $\pm$ 0.09

S.E. Standard error

\* P < 0.05

LSD Least significant difference

\*\* P < 0.01

NS Not significant

Results from Table 4:2 and Figs 4:1 - 4:6 are consistent with a nutritional stress suffered by the cows in the L.P. treatment group. Broster & Smith (1969) reported depressions in milk yield (15%), fat yield (14%) and SNF % (3%) by a "low" plane of nutrition (c.f. "high" plane) over the 12-18 week stage of lactation. Similarly, Gordon & Forbes (1970) measured the effects of underfeeding lactating dairy cows (80% of estimated nett energy and 77% of digestible crude protein requirements, compared to 120% of estimated requirements for both nett energy and digestible crude protein). Reductions in milk yield (21%), fat yield (15%), protein percent (1%) and protein yield (23%) were recorded with an increase in fat percentage of 7%. Similarly, Gordon (1976) found that decreased levels of concentrate feeding and increased stocking rates at pasture, both resulted in decreased milk yield, protein % and SNF %, but did not affect fat%.

The results obtained from this experiment are in sufficient agreement with those reported from other nutritional and management experiments to accept that the treatments imposed resulted in a significant alteration in the plane of nutrition between the two treatment groups.

#### 4:1:3 Influence of Mastitis on Treatment Effects

Mastitis infections have been shown to affect both milk yield and the concentration of many milk components (Ashworth et al, 1967, Wheelock & Dodd, 1969, Renner 1970, Haenlein et al. 1973, Newstead 1973, Newbould 1974, Schanbacher & Smith 1975). It was therefore necessary to demonstrate that mastitis infections did not occur, or that they occurred but had no significant effects on the response variables, or conversely that they occurred and required a correction for the variables which were affected by the infection.

4:1:3:1 Effect of Basal Level of Infection and Changes in Status over Time Level of infection of the mammary gland is assumed to be proportional to the Wisconsin Mastitis Test score.

No clinical cases of mastitis were detected during the experiment. Table 4:3 indicates that only two positive WMT scores were noted (underlined) and that 6 were suspicious. Both positive scores occurred as isolated recordings and no cow was persistently suspicious. Analysis (paired t test) Snedecor & Cochran p.91) indicates that the difference between the means of the treatment groups was not significant ( $P > 0.05$ ). Figure 4:7 presents the individual cow mean WMT scores and treatment means for the combined preliminary and treatment periods.

Table 4:3 WMT\* Scores of Individual Cows Sampled at the Afternoon Milking.

6 pairs of monozygous twins tested on the days indicated during a preliminary and a treatment period. During the treatment period one cow from each set of twins was given a high plane (H.P.) and the other a low plane (L.P.) of nutrition.

Day	Cow Number	31	32	89	90	95	96	101	102	117	118	133	134
	Treatment	H.P.	L.P.	L.P.	H.P.	H.P.	L.P.	H.P.	L.P.	L.P.	H.P.	H.P.	L.P.
Preliminary Period													
1		5	8	2	2	3	2	1	6	3	3	4	2
2													
3													
4		5	7	3	3	6	4	4	5	6	5	11	5
Treatment Period													
5		2	2	3	2	2	2	2	16	2	3	2	2
6													
7		2	2	2	11	3	3	2	10	3	2	5	2
8		3	5	3	3	2	2	2	3	6	2	15	2
9		7	<u>18</u>	8	8	7	9	<u>18</u>	8	10	2	4	2
10		6	2	6	6	9	9	3	4	11	8	10	6
11		2	2	3	10	2	3	2	3	2	2	4	2
12		2	2	3	2	2	2	2	4	2	9	11	2
13													
14		1	2	5	5	2	5	3	3	2	2	5	2
Mean		3.5	5.0	3.8	5.2	3.8	4.1	3.9	6.2	4.7	3.8	7.1	2.7
Standard error (S.E.)		±0.6	±1.6	±0.6	±1.1	±0.8	±0.9	±1.6	±1.3	±1.1	±0.8	±1.3	±0.5

WMT score < 11 = Negative, 11-17 = Suspicious, > 17 = Positive.

Mean WMT score H.P. = 4.55 ± 0.5      t (0.05)<sub>5</sub> = 0.1338 NS  
 L.P. = 4.42 ± 0.4      Required t (0.05)<sub>5df</sub> = 2.571

WMT\* Wisconsin Mastitis Test.

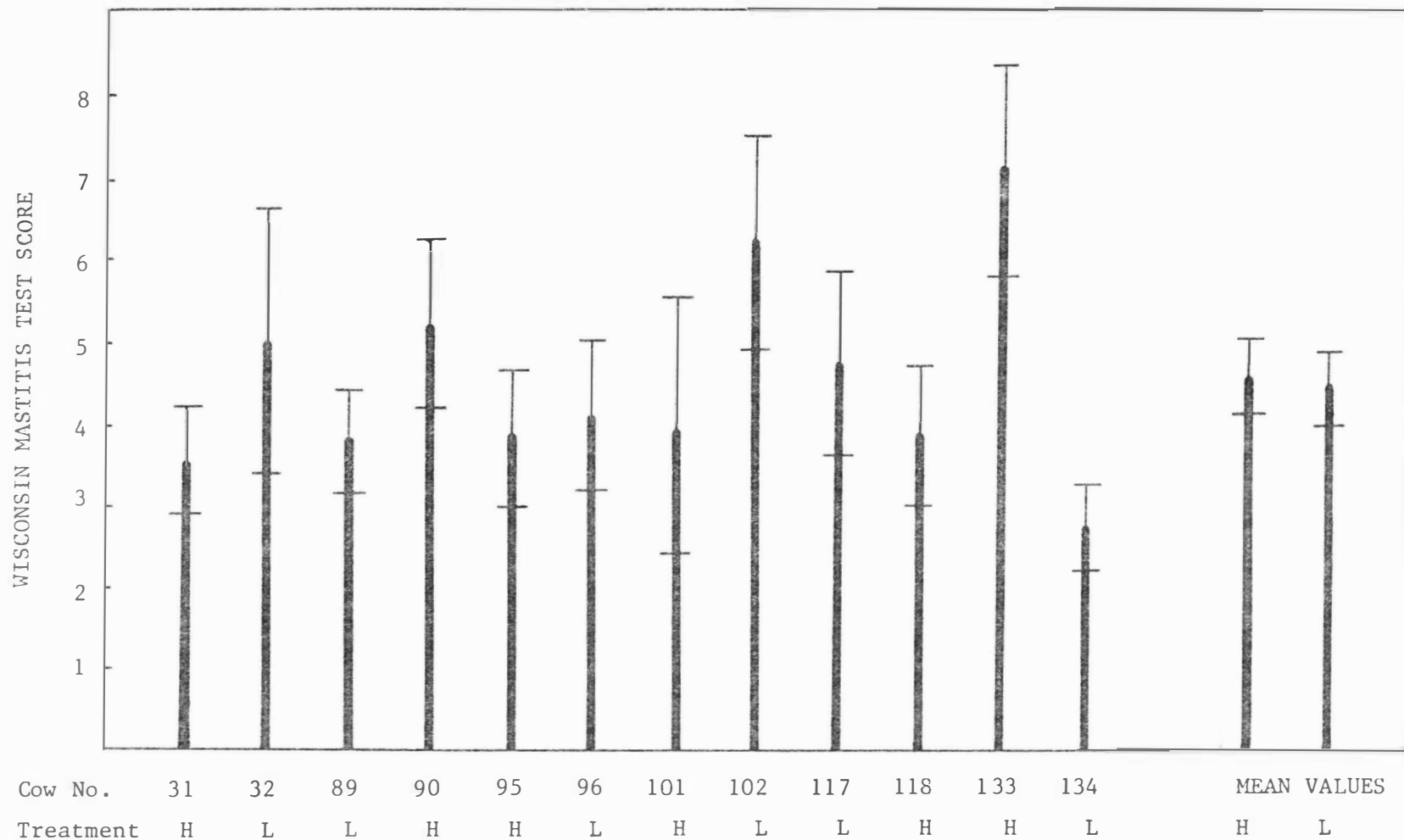


Fig. 4:7 Mean Wisconsin Mastitis Test Scores ( $\pm$  S.E.) for Individual Cows and Treatment Groups over the Preliminary and Treatment Periods.

Table 4:4 indicates by analysis of deviation from regression that no cow altered significantly ( $P > 0.05$ ) in her WMT score during the experiment. This confirms the lack of relationship indicated by the low "r" values and the fact that  $\bar{Y}$  is equal to the calculated Y intercept (a) for all cows. Thus, change in mastitis status of the cows can be removed as a possible source of confounding results. Furthermore, the analysis by Covariance will remove base level differences in the process of adjusting treatment means.

#### 4:1:3:2 Effect of Isolated Suspicious and Positive WMT Scores

The occurrence of isolated suspicious and positive WMT scores raises the possibility that milk yield and/or compositional changes occur in conjunction with such observations. It was necessary to determine whether the data from cows on days of high WMT score should be discarded from the analysis of treatment effects. Values for the major response parameters from the high WMT samples were compared within cows to the mean of the values for the day before and the day after. Results are presented in Table 4:5.

Table 4:4 Analysis of Individual Cow WMT\* Scores Over Time.

Cow Number	31	32	89	90	95	96	101	102	117	118	133	134
Treatment	H.P.	L.P.	L.P.	H.P.	H.P.	L.P.	H.P.	L.P.	L.P.	H.P.	H.P.	L.P.
r	0.370	0.269	0.439	0.326	0.074	0.314	0.118	0.454	0.006	0.171	0.131	0.088
± S.E.r	2.04	5.23	1.84	3.434	2.722	2.787	5.288	3.930	3.642	2.778	4.501	1.579
b	0.195	0.348	0.214	0.283	0.048	0.219	0.150	0.477	0.005	0.115	0.141	0.033
± S.E.b	0.171	0.441	0.155	0.289	0.229	0.235	0.446	0.331	0.307	0.234	0.379	0.133
$\bar{Y}$	3.50	5.00	3.80	5.20	3.80	4.10	3.90	6.20	4.70	3.80	7.10	2.70
$\bar{x}$	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1	8.1
a	3.50	5.00	3.80	5.20	3.80	4.10	3.90	6.20	4.70	3.80	7.10	2.70
t (0.05) <sub>8df</sub>	1.14	0.79	1.38	0.98	0.21	0.93	0.34	1.44	0.02	0.49	0.37	0.25

For significance  $t(0.05)_{8df}$  must exceed 2.306

WMT\* Wisconsin Mastitis Test.

Table 4:5 Effect of Isolated High WMT\* Scores on Milk Yield and Composition.

The milk yield (l/day), protein concentration (%), proportion of whey protein (%) and concentration (mg/ml) of bovine serum albumin (BSA), immunoglobulin (IG),  $\alpha$  lactalbumin ( $\alpha$  lac) and  $\beta$  lactoglobulin ( $\beta$  Lgb) in 8 milk samples with high WMT scores compared with milk samples taken the day before and the day after the high score.

Parameter	No of Observations	High WMT day $\pm$ S.E.	Mean of Day before & Day after High WMT day $\pm$ S.E.	Difference	D=LSD (.05)	F** (1,7)	Significance
Milk Yield (l/day)	8	11.404 $\pm$ 1.193	11.810 $\pm$ 1.316	0.406	0.762	1.59	NS
Protein (%)	8	3.694 $\pm$ 0.127	3.704 $\pm$ 0.124	0.010	0.045	0.28	NS
Proportion of Whey Protein in Total Protein (%)	8	17.893 $\pm$ 0.758	18.501 $\pm$ 0.478	0.608	1.281	1.26	NS
BSA Conc. (mg/ml)	8	0.506 $\pm$ 0.052	0.506 $\pm$ 0.053	0.00	0.037	0.001	NS
IG Conc. (mg/ml)	8	0.515 $\pm$ 0.103	0.519 $\pm$ 0.089	0.004	0.079	0.017	NS
$\alpha$ lac Conc. (mg/ml)	8	1.623 $\pm$ 0.094	1.705 $\pm$ 0.076	0.082	0.126	<b>2.410</b>	NS
$\beta$ Lgb Conc. (mg/ml)	8	3.440 $\pm$ 0.244	3.599 $\pm$ 0.195	0.159	0.267	1.977	NS

\* Wisconsin Mastitis Test

\*\* 2 Way Anovar

S.E. Standard Error

NS Not Significant (P > 0.05)

No significant changes ( $P > 0.05$ ) in milk yield or in the components of milk considered most likely to alter with a mastitis infection, were observed. Consequently the high WMT readings can be disregarded in terms of their affecting the treatment responses. Therefore, the results from these samples were retained in the analysis of treatment effects.

#### 4:2 Effect of Plane of Nutrition on the Concentration and the Yield of Milk Proteins

##### 4:2:1 Effect on Major Milk Protein Classes

Fig. 1:1 indicates that the first separation of milk protein is into the broad groupings of Casein and Whey proteins. It is therefore logical to examine first the effect of the treatments on these two major groupings of milk proteins. Analysis of the effects of plane of nutrition on the yield and concentration of the major milk protein classes is presented in Table 4:6. Changes in the yield and concentration of the protein classes over time are presented in Figures 4:8 to 4:13 inclusive.

Table 4:6 Effect of Plane of Nutrition on the Yield and Concentration of the Major Milk Protein Classes.

Whey protein and non whey protein (casein) yield (g/day) also expressed as a percentage of the whole milk and the total protein yield from 6 pairs of monozygous twins during a preliminary period of 4 days, a treatment period of 10 days and a post experimental period of 4 days. During the treatment period one cow from each set of twins was given a high plane (H.P.) and the other a low plane (L.P.) of nutrition.

Parameter	Treatment	Mean Preliminary Period ( $\bar{x}$ ) $\pm$ S.E.	Mean Treat. Period Adjusted for Covariance ( $\bar{y}$ $\pm$ S.E.)	$\frac{\bar{y}}{\bar{x}} \times \frac{100}{1}$	Difference $\bar{y}_{H.P.} - \bar{y}_{L.P.}$	D=LSD		F ratio	Sig.	Mean Post Expt. Period $\pm$ S.E.
						5%	1%			
Whey Protein (% of whole milk)	H.P.	0.75 $\pm$ 0.05	0.75 $\pm$ 0.04	100.0%		0.12			NS	0.74 $\pm$ 0.05
	L.P.	0.77 $\pm$ 0.06	0.72 $\pm$ 0.08	93.5%	0.03	0.20	0.415			0.73 $\pm$ 0.09
Non Whey Protein (Caseins) (% of whole milk)	H.P.	3.10 $\pm$ 0.08	3.15 $\pm$ 0.08	101.6%		0.16				3.12 $\pm$ 0.07
	L.P.	3.03 $\pm$ 0.06	2.86 $\pm$ 0.08	94.4%	0.29	0.27	23.45	**		3.08 $\pm$ 0.09
Whey Protein (% of Total Protein)	H.P.	19.49 $\pm$ 0.90	19.36 $\pm$ 0.52	99.3		2.20				19.00 $\pm$ 0.86
	L.P.	20.16 $\pm$ 1.15	19.73 $\pm$ 1.28	97.9%	-0.37	3.65	0.225	NS		19.11 $\pm$ 1.90
Non Whey Protein (Caseins) (% of Total Protein)	H.P.	80.51 $\pm$ 0.90	80.64 $\pm$ 0.52	100.2%		2.04				81.00 $\pm$ 0.86
	L.P.	79.84 $\pm$ 1.13	80.27 $\pm$ 1.28	100.5%	0.37	3.38	0.261	NS		80.90 $\pm$ 1.94
Whey Protein Yield (g/day)	H.P.	84.8 $\pm$ 6.47	84.7 $\pm$ 6.97	99.9%		8.97				81.37 $\pm$ 5.18
	L.P.	85.4 $\pm$ 7.06	60.7 $\pm$ 5.06	71.1%	23.99	14.88	55.07	**		66.85 $\pm$ 5.11
Non Whey Protein (Casein) Yield (g/day)	H.P.	358.5 $\pm$ 39.4	364.0 $\pm$ 45.4	101.5%		21.71				348.1 $\pm$ 21.2
	L.P.	363.5 $\pm$ 34.6	249.6 $\pm$ 32.7	68.7	114.4	36.01	213.9	**		301.9 $\pm$ 29.7

S.E. Standard error

LSD Least significant difference

\*\* (P < 0.01)

NS Not significant.

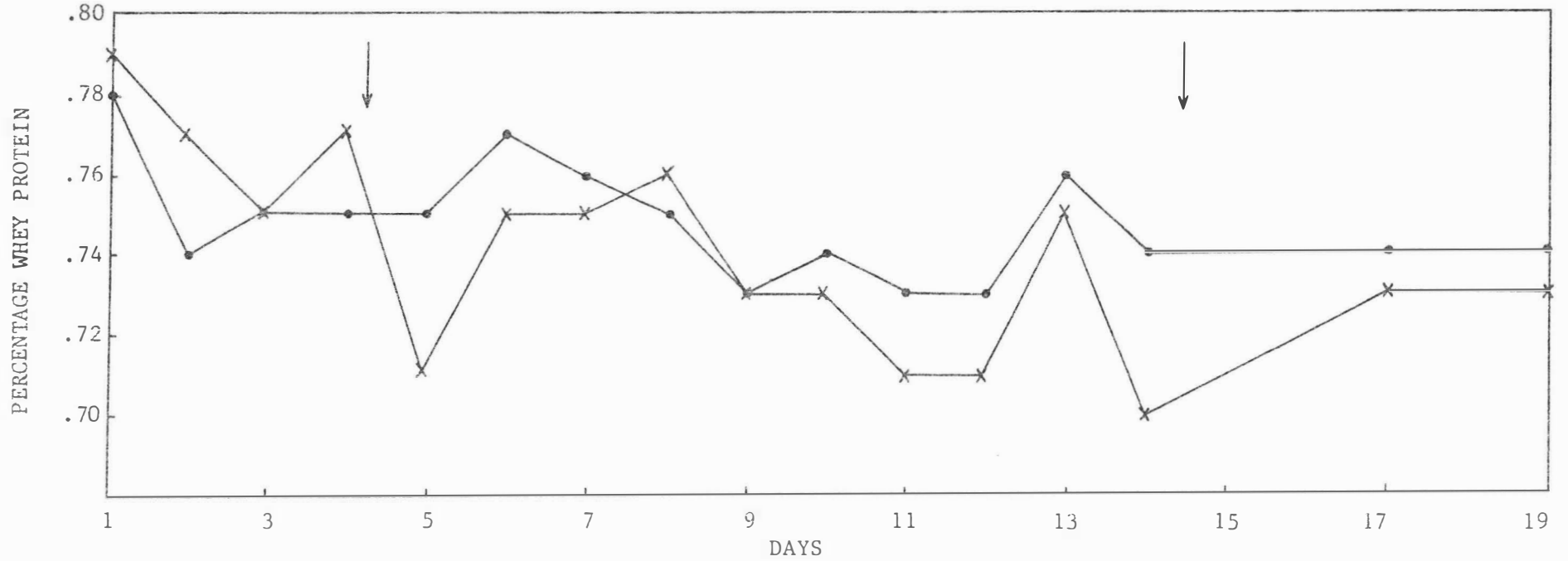


Fig. 4:8 Effect of Plane of Nutrition on Total Whey Proteins Expressed as a Percentage of Whole Milk.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

- High plane
- × Low plane

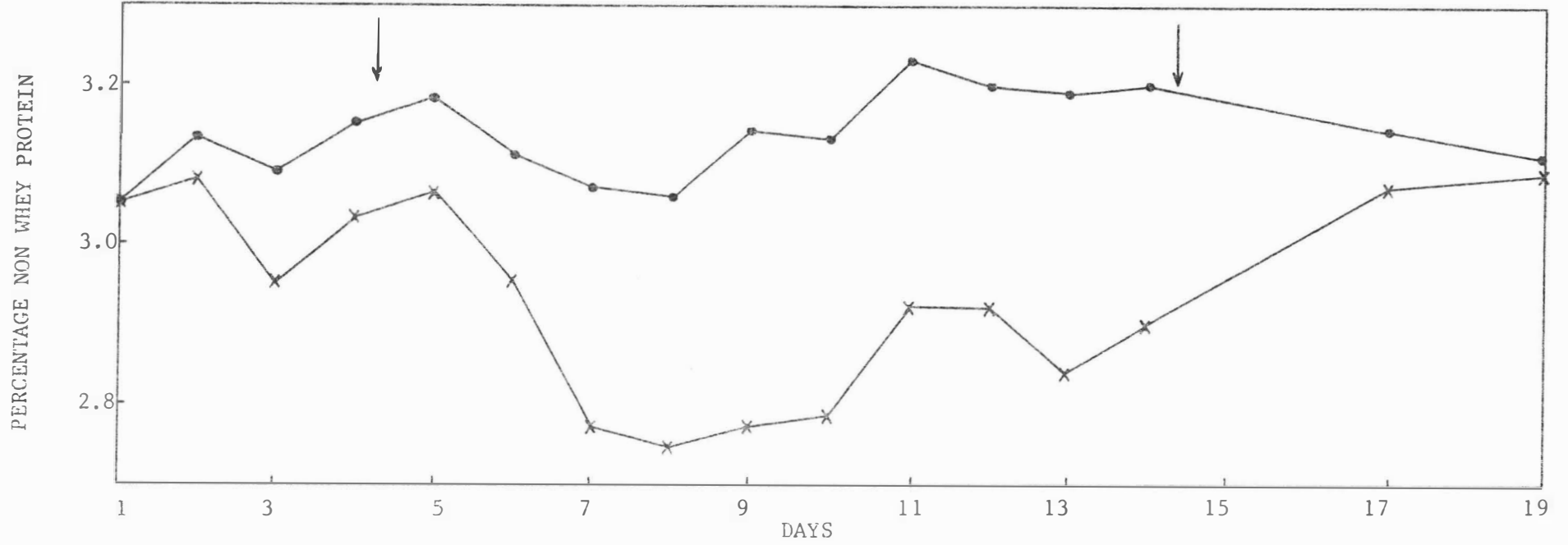


Fig. 4:9 Effect of Plane of Nutrition on the Total Non Whey Proteins Expressed as a Percentage of Whole Milk.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

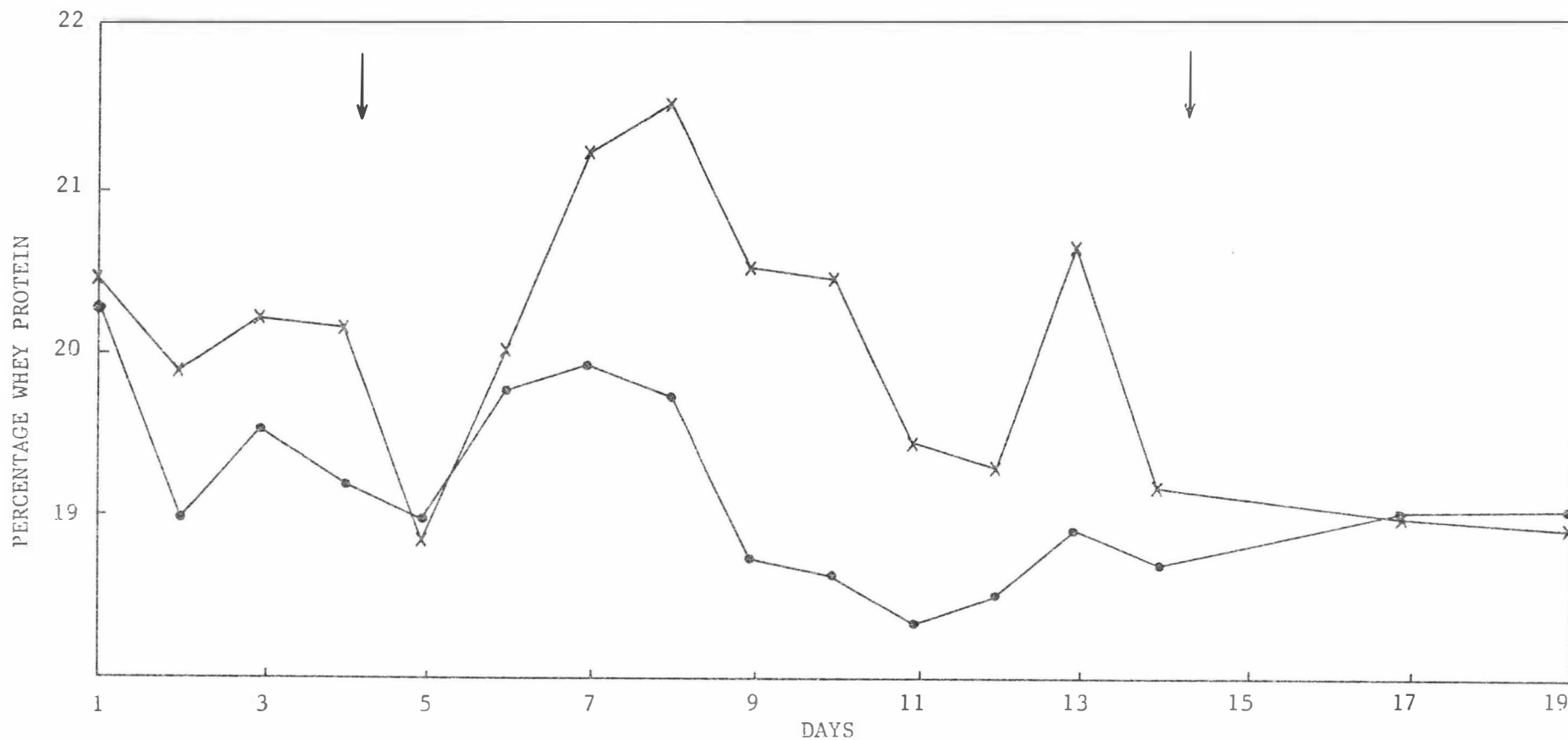


Fig. 4:10 Effect of Plane of Nutrition on Total Whey Proteins Expressed as a Percentage of Total Protein.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

• High plane

× Low plane

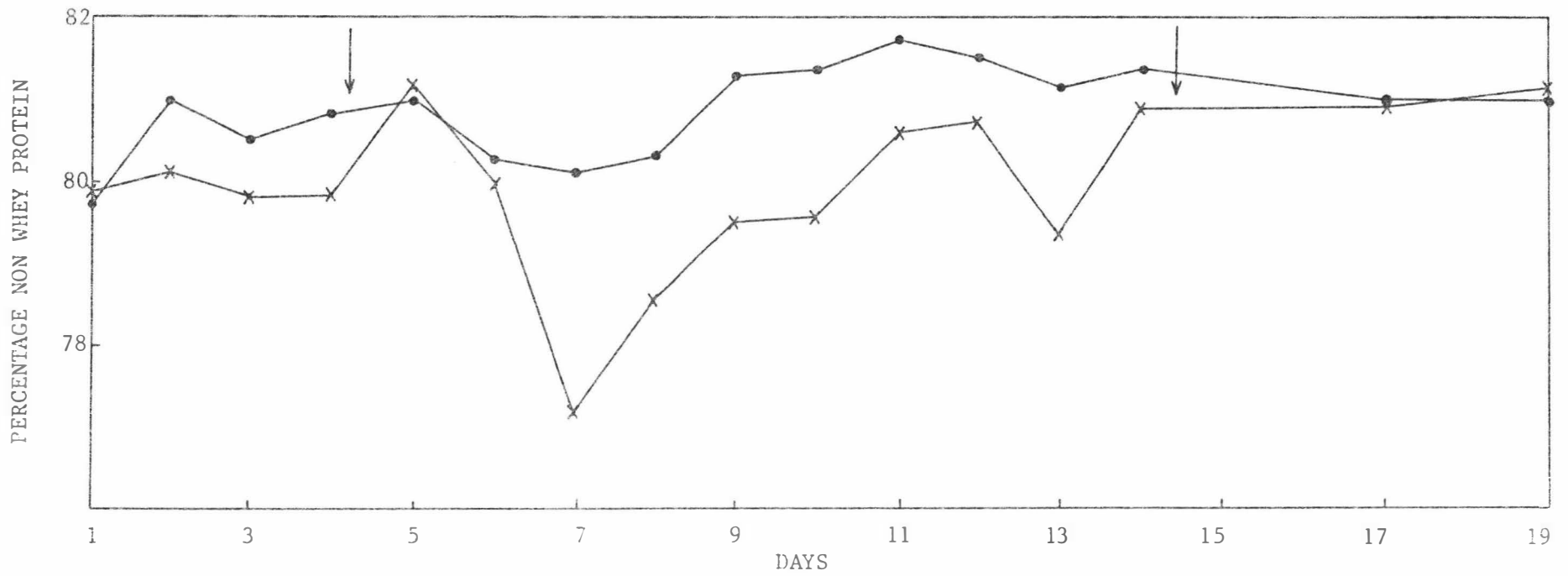


Fig. 4.11 Effect of Plane of Nutrition on the Total Non Whey Proteins Expressed as a Percentage of Total Protein.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane



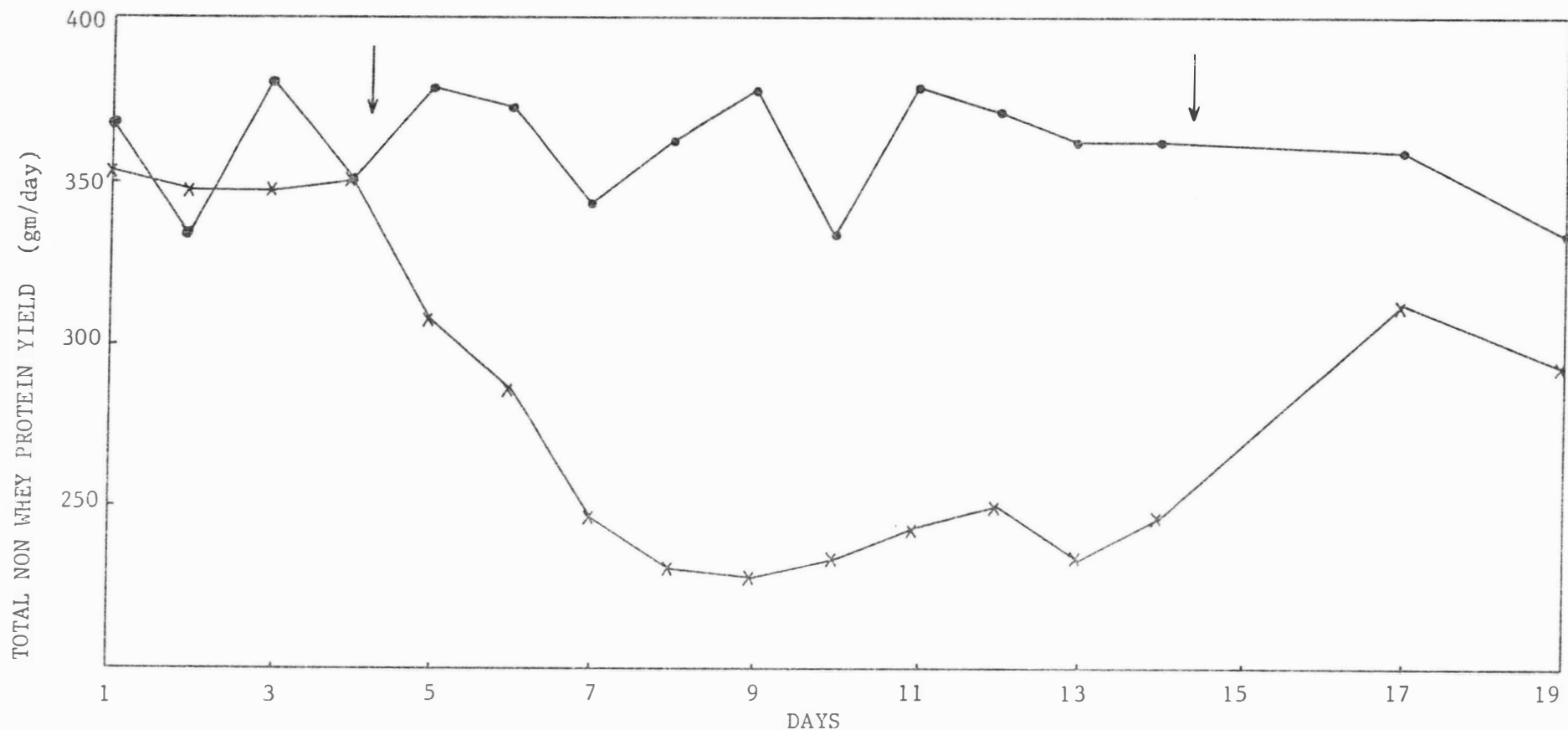


Fig. 4:13 Effect of Plane of Nutrition on Total Non Whey Protein Yield (gm/day).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

No significant ( $P > 0.05$ ) changes were observed in the proportion of total protein contributed by each of the whey and non whey protein fractions. The concentration of whey protein in whole milk did not alter ( $P > 0.05$ ) whereas the concentration of non whey protein changed significantly ( $P < 0.01$ ) with treatment. Yields of both whey and non whey proteins were altered significantly ( $P < 0.01$ ) by plane of nutrition.

#### 4:2:2 Effect on the Major Individual Whey Proteins

The effects of plane of nutrition on the four individual whey proteins ( $\beta$  Lgb,  $\alpha$  Lac, BSA, IG) which comprise the great majority of the whey protein fraction are presented in Table 4:7. Changes in the yield and concentration of these proteins over time are presented in Figs. 4:14 to 4:21 inclusive, and shown in Plates 4:1a,b, c and 4:2a, b, c.

With underfeeding, the concentration of  $\alpha$ Lac decreased ( $P < 0.05$ ) whilst the concentration of BSA increased ( $P < 0.01$ ). Changes in concentration of  $\beta$  Lgb and IG were not significant ( $P > 0.05$ ). Yields of  $\beta$  Lgb and  $\alpha$  Lac decreased significantly ( $P < 0.01$ ) with restricted feeding. Changes in the yield of BSA and IG were not significant ( $P > 0.05$ ). The increase in yield of BSA over the first 5 days of underfeeding was also non-significant as was the maximum daily difference between the treatments (Fig. 4:17).

The absorption due to  $\beta$  Lgb A and  $\beta$  Lgb B in the heterozygous (AB) cows (4 sets) did not alter significantly ( $P > 0.05$ ) nor did the relative proportions of the variants (Fig. 4:22 to 4:24 ).

Table 4:7 Effect of Plane of Nutrition on the Concentration and Yield of the Major Whey Proteins.

The concentration (mg/ml) and yield (g/day) of  $\alpha$  lactalbumin ( $\alpha$  lac), bovine serum albumin (BSA), immunoglobulin (IG) and  $\beta$  lactoglobulin ( $\beta$  lgb) from 6 pairs of monozygous twins during a preliminary period of 4 days, a treatment period of 10 days, and a post experimental period of 4 days, together with the mean yield of BSA between days 5 to 9 and on day 7. The proportion of  $\beta$  lgb A &  $\beta$  lgb B based on their relative dye-binding capacity is also presented. During the treatment period (days 5-14) one cow from each set of twins was given a high plane (H.P.) and the other a low plane (L.P.) of nutrition.

Parameter	Treat. Period	Mean Preliminary Period ( $\bar{x}$ ) $\pm$ S.E.	Mean Treat. Period Adjusted for Covariate ( $\bar{y}$ ) $\pm$ S.E.	$\frac{\bar{y}}{\bar{x}} \times \frac{100}{1}$	Difference $\bar{y}_H.P. - \bar{y}_L.P.$	D=LSD 5%, 1%	F ratio	Sig	Mean Post Exp. Period $\pm$ S.E.
$\alpha$ Lac Conc. (mg/ml)	H.P.	1.74 $\pm$ 0.05	1.81 $\pm$ 0.06	104.0%	0.29	0.21	14.70	*	1.72 $\pm$ 0.06
	L.P.	1.76 $\pm$ 0.08	1.52 $\pm$ 0.07	86.4%					1.64 $\pm$ 0.07
$\alpha$ Lac Yield (g/day)	H.P.	20.0 $\pm$ 1.97	20.93 $\pm$ 2.28	104.7%	7.52	2.73	58.17	**	19.34 $\pm$ 1.67
	L.P.	20.0 $\pm$ 2.28	13.41 $\pm$ 4.22	67.1%					15.91 $\pm$ 1.62
BSA Conc. (mg/ml)	H.P.	0.40 $\pm$ 0.04	0.44 $\pm$ 0.05	110.0%	-0.17	0.08	35.71	**	0.465 $\pm$ 0.05
	L.P.	0.42 $\pm$ 0.04	0.61 $\pm$ 0.08	145.2%					0.419 $\pm$ 0.07
BSA Yield (g/day)	H.P.	4.49 $\pm$ 0.39	4.91 $\pm$ 0.58	109.4%	-0.18	0.51	0.95	NS	5.06 $\pm$ 0.54
	L.P.	4.59 $\pm$ 0.30	5.09 $\pm$ 0.54	110.9%					3.59 $\pm$ 0.28
BSA Yield Days 5-9 (g/day)	H.P.	4.49 $\pm$ 0.39	5.07 $\pm$ 0.26	112.9%	-0.70	0.92	4.48	NS	---
	L.P.	4.59 $\pm$ 0.30	5.77 $\pm$ 0.33	125.7%					---
BSA Yield Max. Difference Day 7 (g/day)	H.P.	4.49 $\pm$ 0.39	4.97 $\pm$ 0.42	110.7%	-1.79	2.30	4.65	NS	---
	L.P.	4.59 $\pm$ 0.30	6.76 $\pm$ 1.11	147.3%					---
IG Conc. (mg/ml)	H.P.	0.61 $\pm$ 0.08	0.44 $\pm$ 0.07	72.1%	-0.21	0.24	5.55	NS	0.55 $\pm$ 0.07
	L.P.	0.56 $\pm$ 0.10	0.65 $\pm$ 0.16	116.1%					0.50 $\pm$ 0.14
IG Yield (g/day)	H.P.	6.77 $\pm$ 1.62	5.19 $\pm$ 0.82	76.7%	0.12	2.06	0.03	NS	5.94 $\pm$ 0.73
	L.P.	6.13 $\pm$ 0.78	5.06 $\pm$ 0.82	82.5%					3.89 $\pm$ 0.61
$\beta$ Lgb Conc. (mg/ml)	H.P.	4.33 $\pm$ 0.34	4.18 $\pm$ 0.32	96.5%	0.30	0.52	2.57	NS	4.01 $\pm$ 0.20
	L.P.	4.42 $\pm$ 0.39	3.88 $\pm$ 0.38	87.8%					4.08 $\pm$ 0.26
$\beta$ Lgb Yield (g/day)	H.P.	48.8 $\pm$ 4.45	46.81 $\pm$ 4.33	95.9%	13.82	5.02	58.30	**	44.09 $\pm$ 3.15
	L.P.	49.3 $\pm$ 5.10	32.99 $\pm$ 3.10	66.9%					38.26 $\pm$ 3.37
$\beta$ Lgb A Abs.	H.P.	0.171 $\pm$ 0.005	0.202 $\pm$ 0.004	117.6%	0.02	0.08	6.29	NS	0.1897 $\pm$ 0.010
	L.P.	0.178 $\pm$ 0.006	0.180 $\pm$ 0.006	101.0%					0.1989 $\pm$ 0.015
$\beta$ Lgb B Abs.	H.P.	0.127 $\pm$ 0.004	0.145 $\pm$ 0.003	114.4%	0.01	0.056	0.00	NS	0.1399 $\pm$ 0.005
	L.P.	0.130 $\pm$ 0.004	0.140 $\pm$ 0.004	107.8%					0.1386 $\pm$ 0.009
Relative Propn. ( $\beta$ Lgb A/B Abs.)	H.P.	1.363 $\pm$ 0.027	1.374 $\pm$ 0.019	100.8%	0.06	0.121	4.74	NS	1.355 $\pm$ 0.048
	L.P.	1.369 $\pm$ 0.029	1.313 $\pm$ 0.014	95.9%					1.430 $\pm$ 0.003

S.E. Standard Error. LSD Least significant difference. \* (P < 0.05) \*\* (P < 0.01) NS Not Significant.

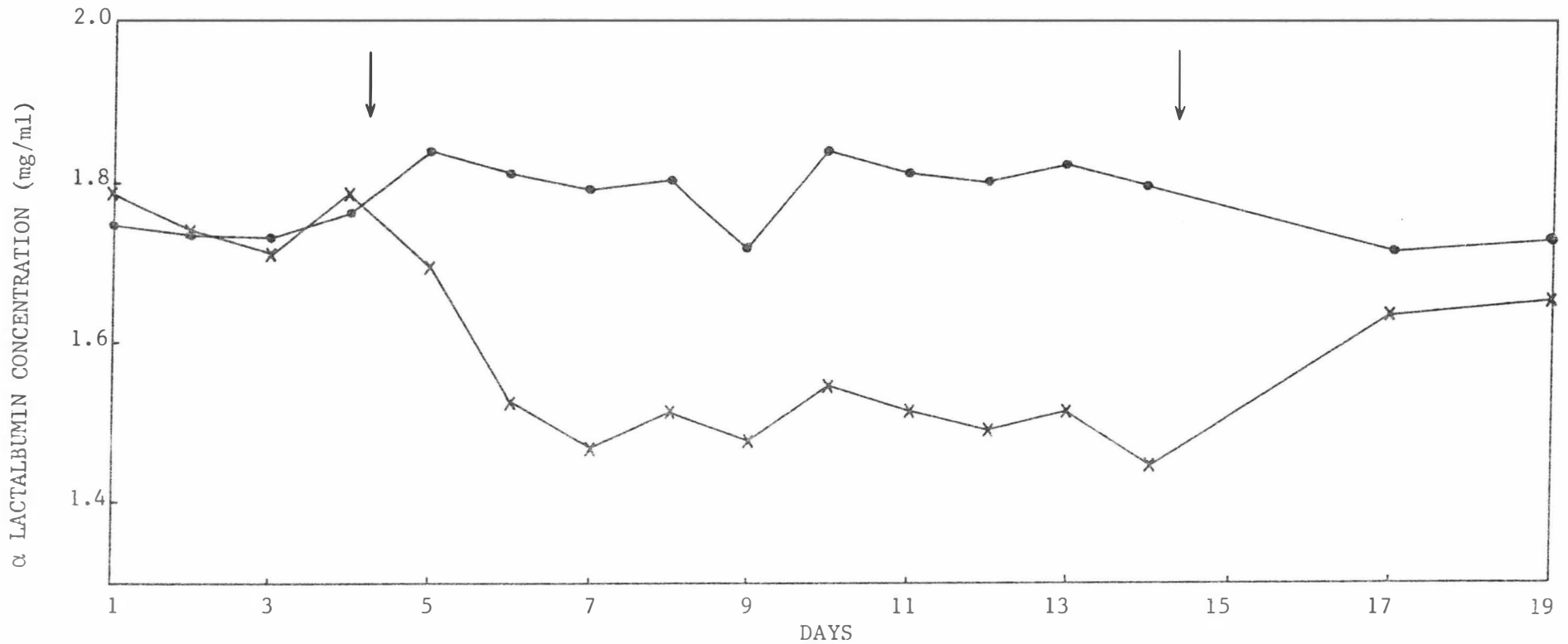


Fig. 4:14 Effect of Plane of Nutrition on  $\alpha$  Lactalbumin Concentration.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

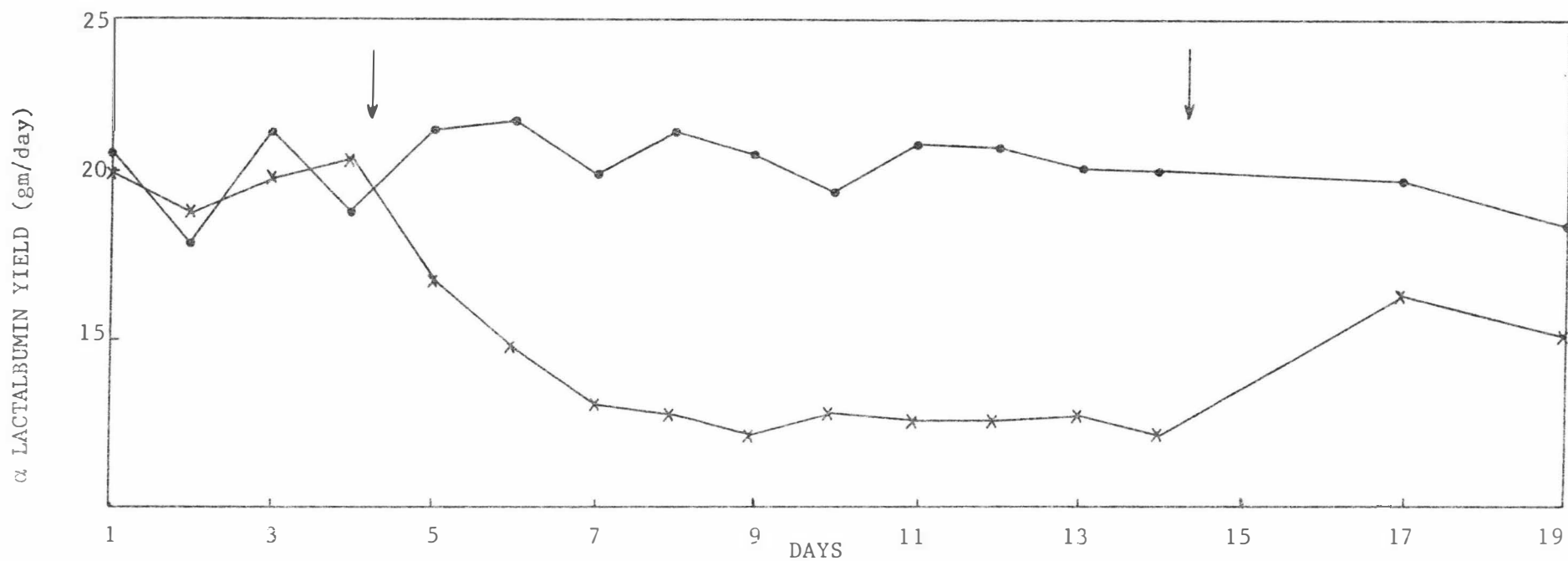


Fig. 4:15 Effect of Plane of Nutrition on  $\alpha$  Lactalbumin Yield (gm/day).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

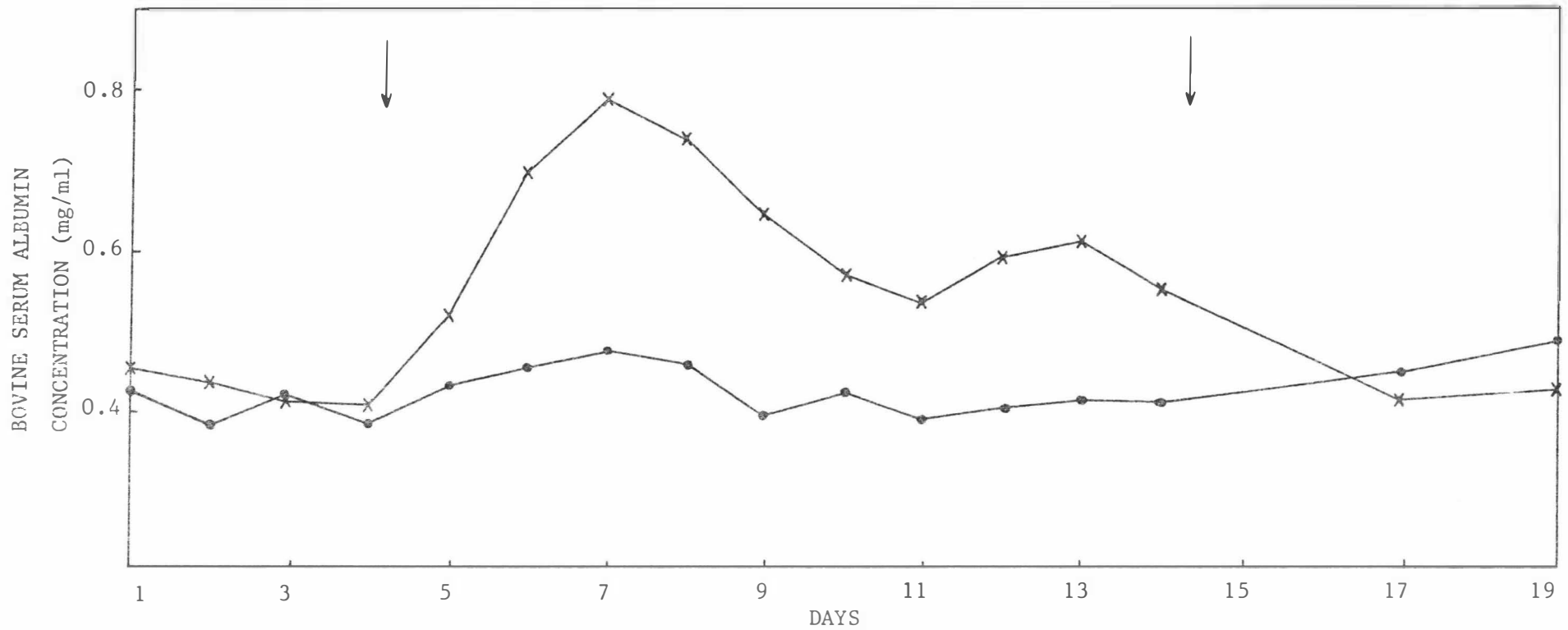


Fig. 4:16 Effect of Plane of Nutrition on Bovine Serum Albumin Concentration.

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

• High plane

x Low plane

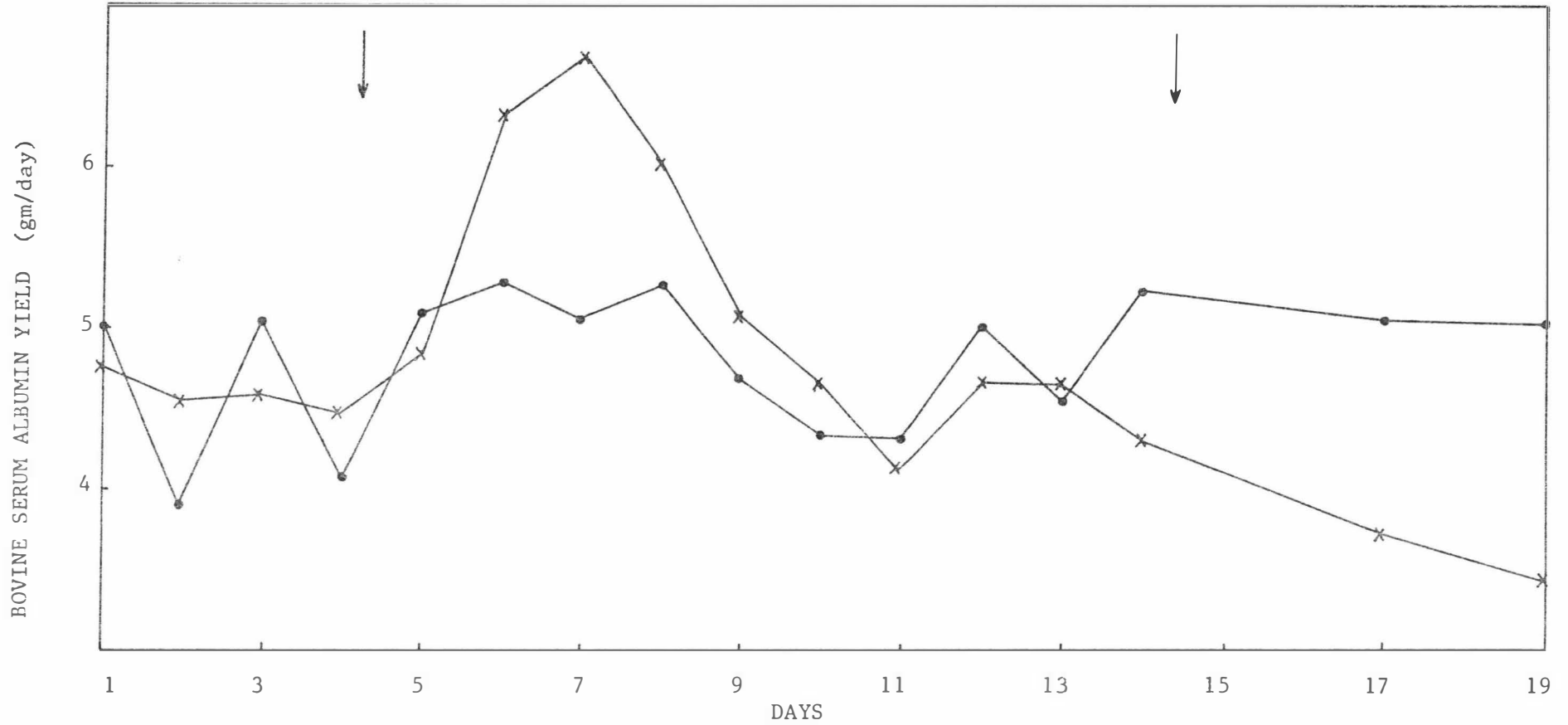


Fig. 4:17 Effect of Plane of Nutrition on Bovine Serum Albumin Yield (gm/day).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane  
 x Low plane

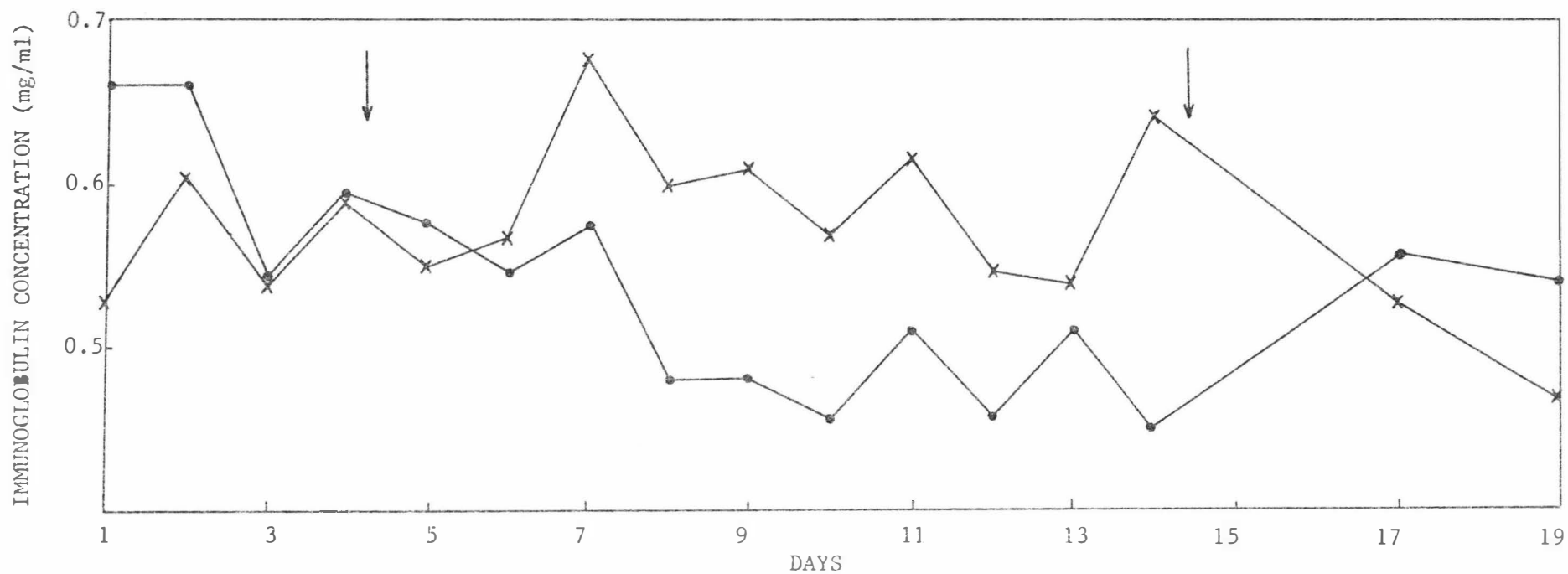


Fig. 4:18 Effect of Plane of Nutrition on Immunoglobulin Concentration (mg/ml).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

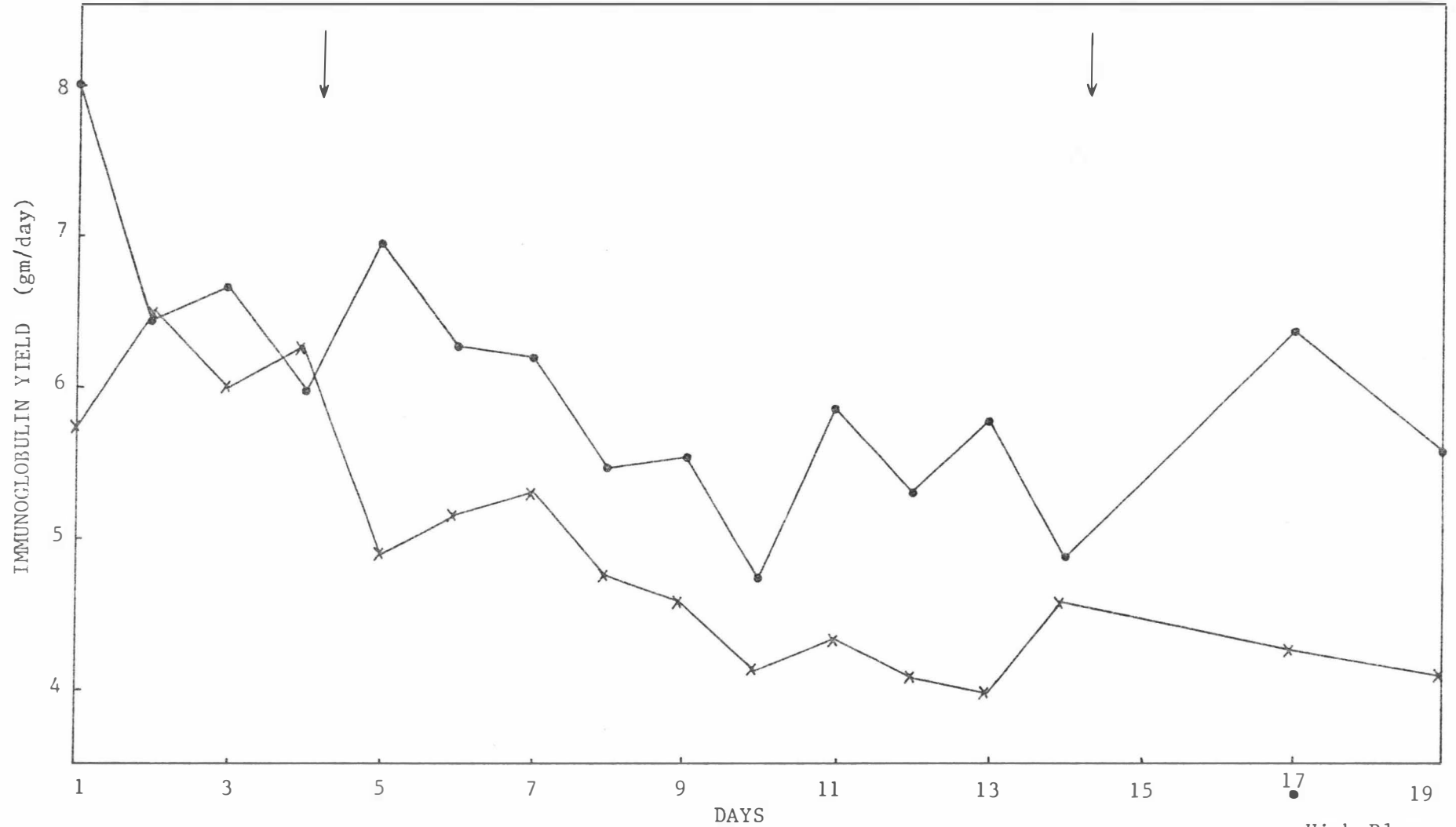


Fig. 4:19 Effect of Plane of Nutrition on Immunoglobulin Yield (gm/day).  
 Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment. Treatment period between arrows.

● High Plane  
 × Low Plane

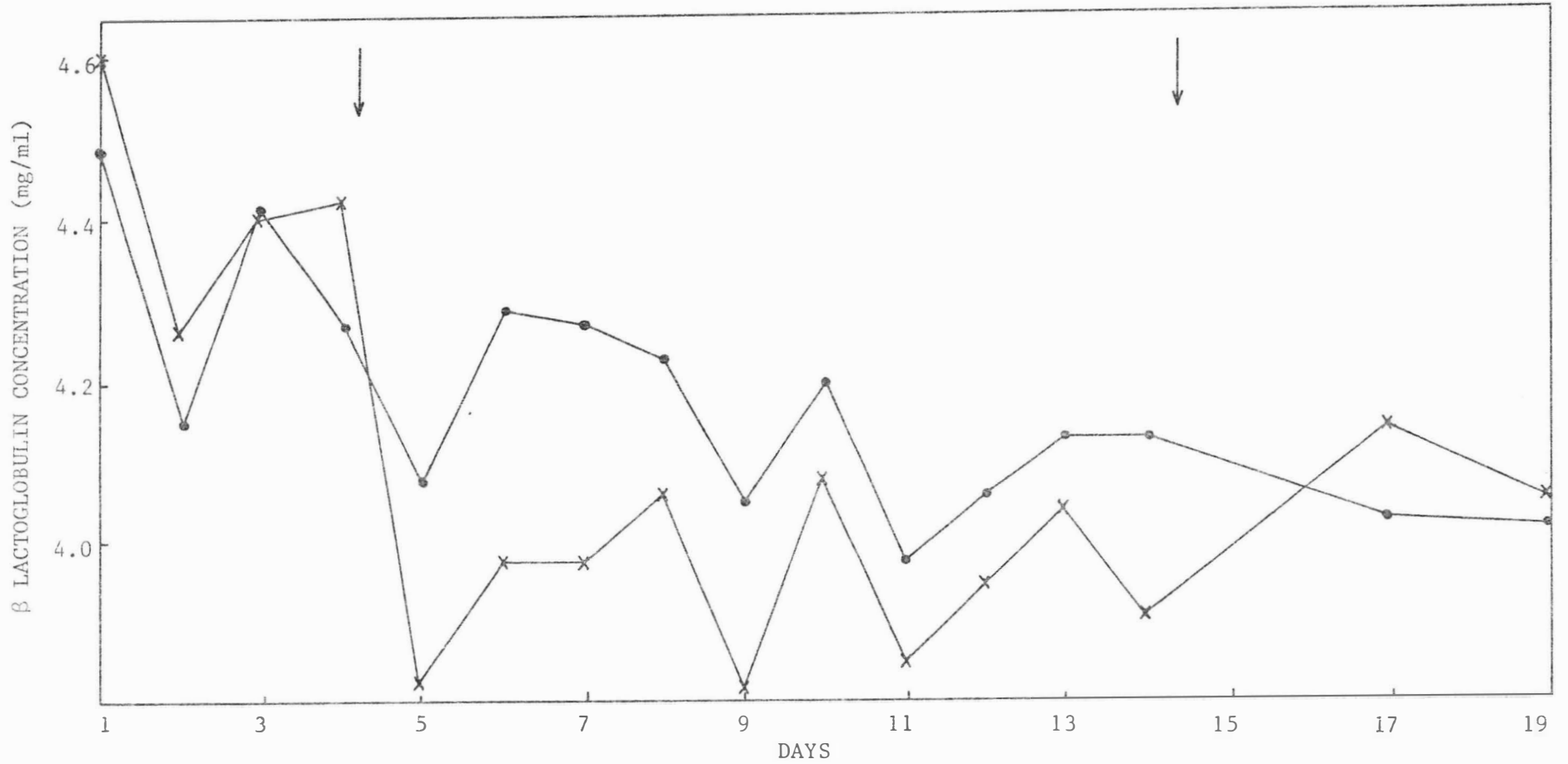


Fig. 4:20 Effect of Plane of Nutrition on  $\beta$  Lactoglobulin Concentration (mg/ml).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

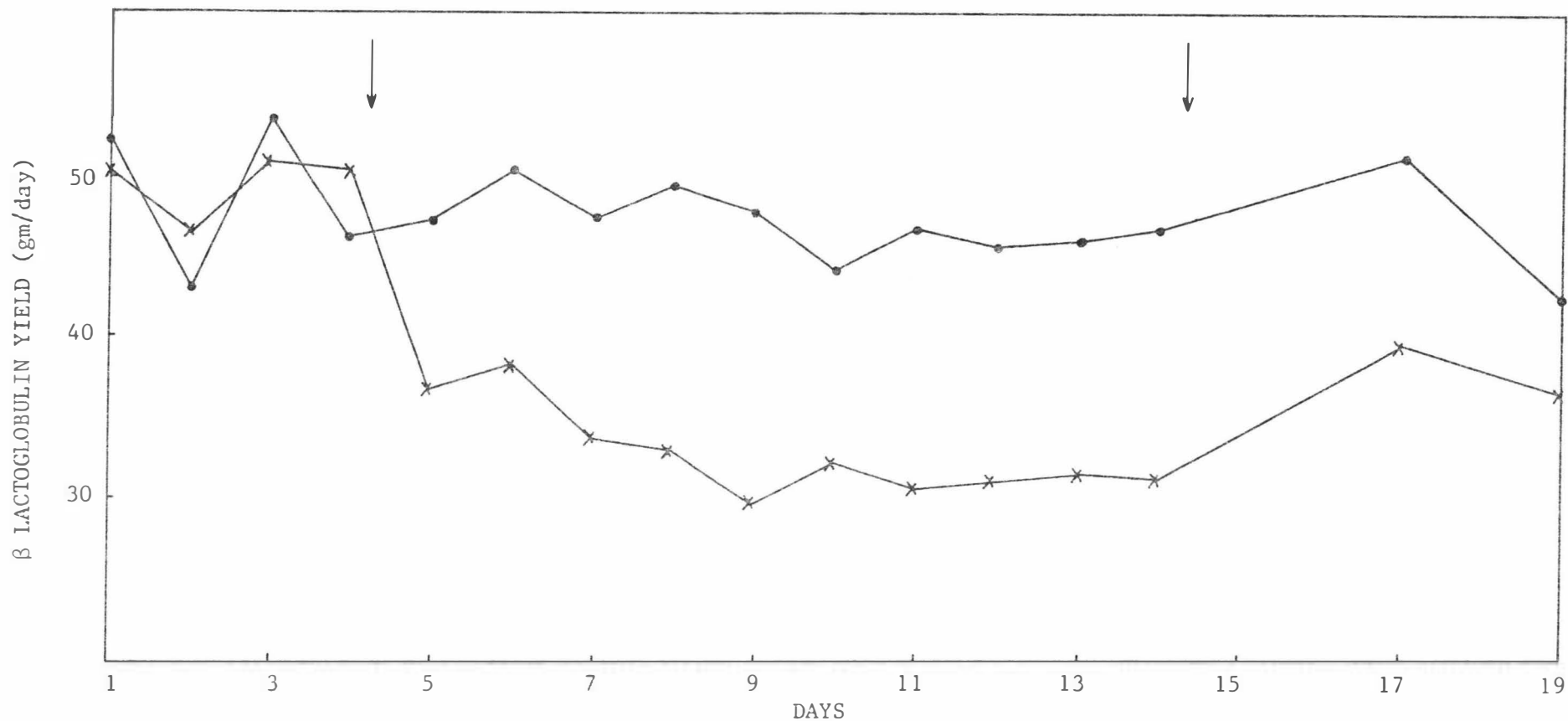


Fig. 4:21 Effect of Plane of Nutrition on  $\beta$  Lactoglobulin Yield (gm/day).

Each point represents the mean of 6 cows, their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

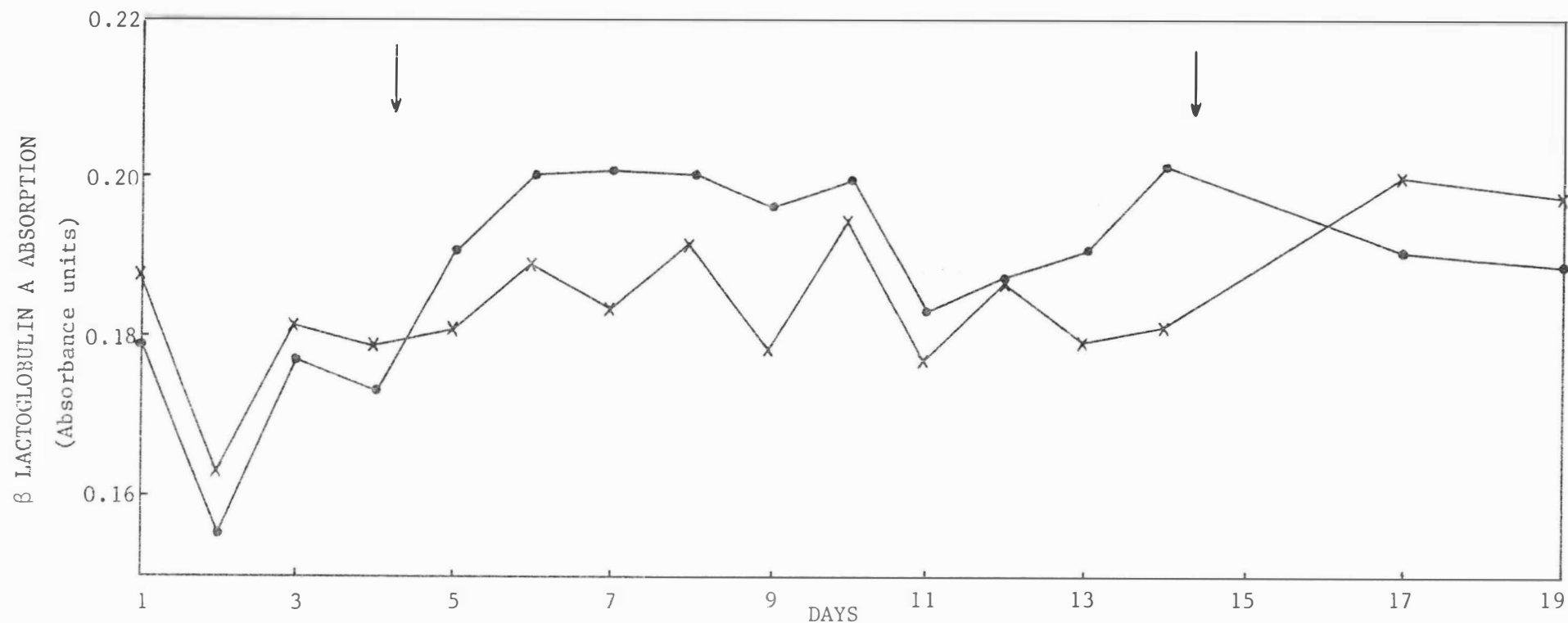


Fig. 4:22 Effect of Plane of Nutrition on the Concentration of  $\beta$  Lactoglobulin A (Absorbance Units).

Each point represents the mean of 4 cows heterozygous for  $\beta$  Lactoglobulin type (AB), their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

- High plane
- × Low plane

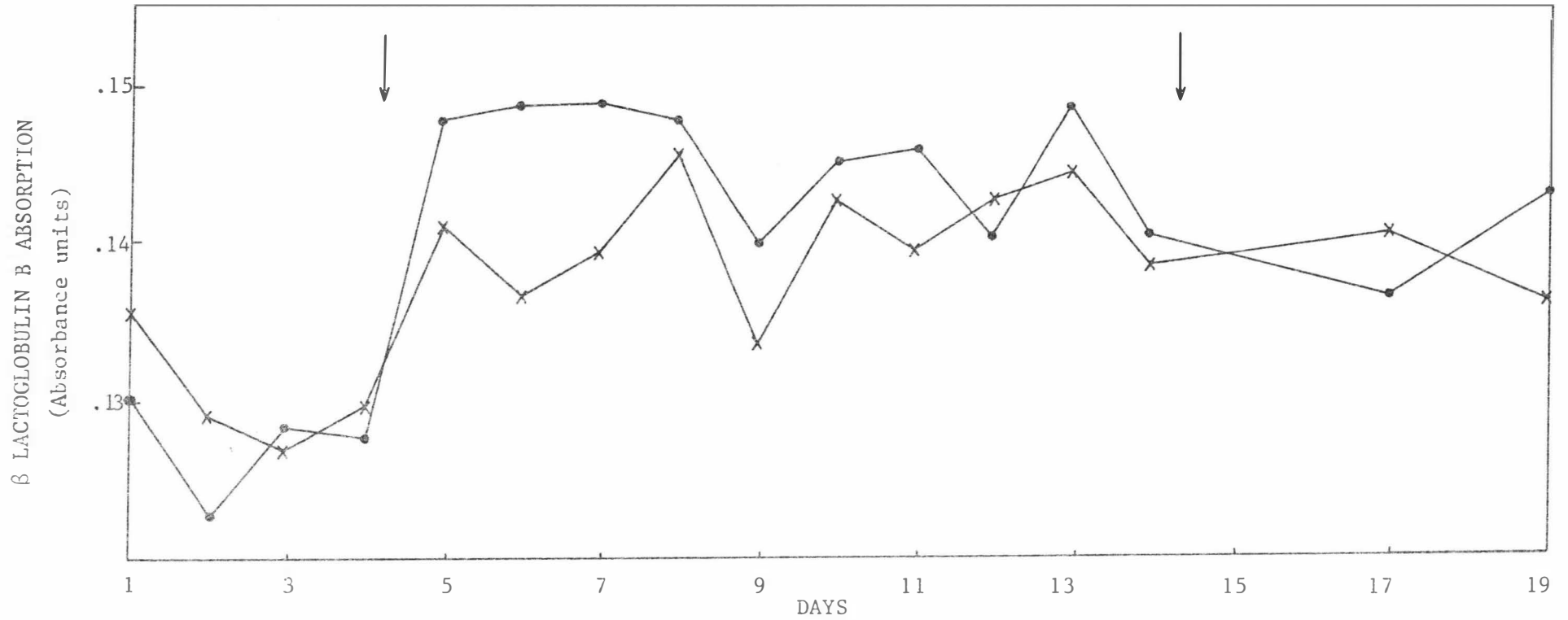


Fig. 4:23 Effect of Plane of Nutrition on the Concentration of  $\beta$  Lactoglobulin B (Absorbance units).

Each point represents the mean of 4 cows heterozygous for  $\beta$  Lactoglobulin type  $AB_1$  their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

× Low plane

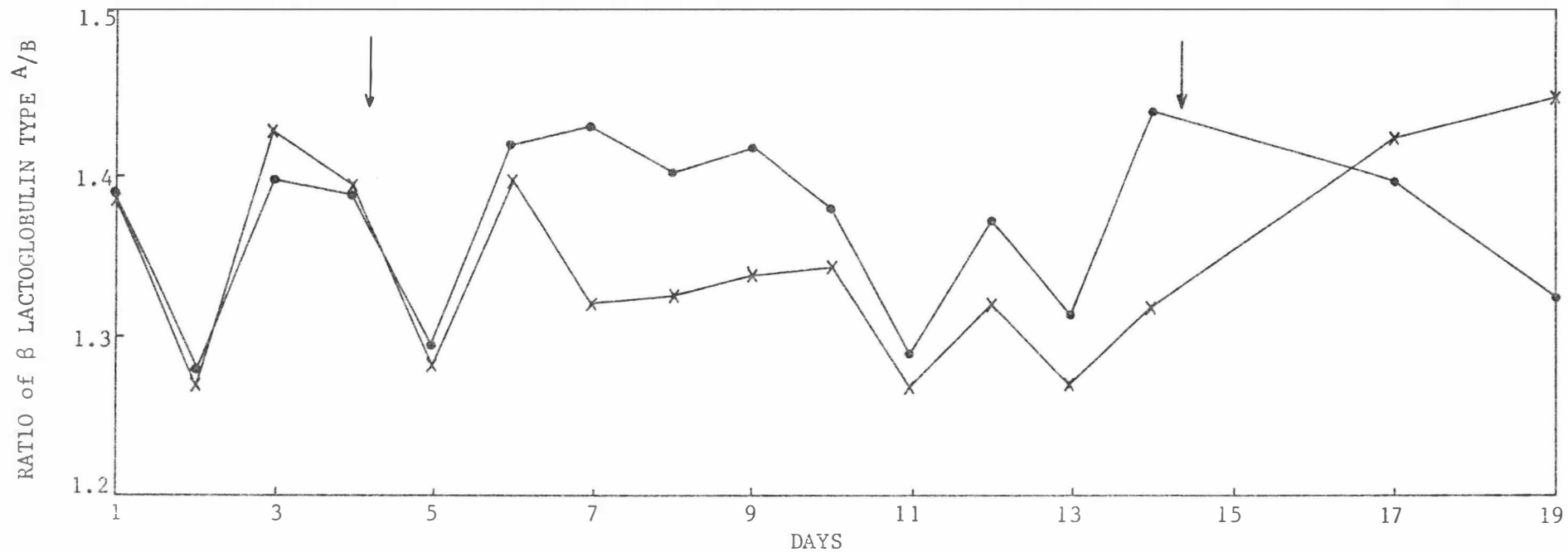


Fig. 4:24 Effect of Plane of Nutrition on the Relative Proportion of  $\beta$  Lactoglobulin Variants (A/B).

Each point represents the mean of 4 cows heterozygous for  $\beta$  Lactoglobulin type (AB), their twin mates contributing to the corresponding point in the opposing treatment.

Treatment period between arrows.

● High plane

x Low plane

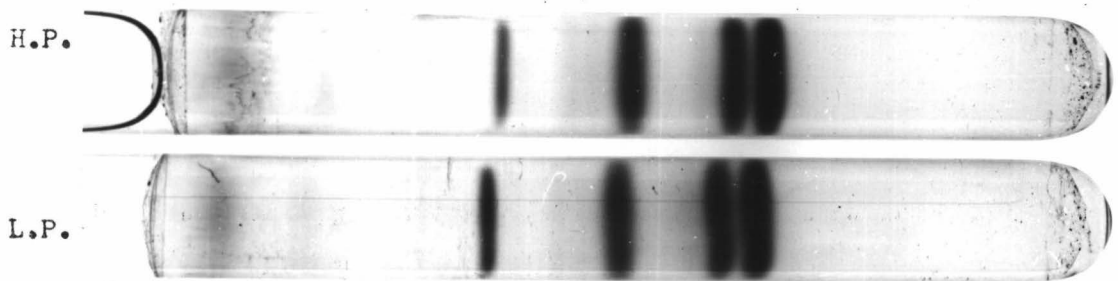


Plate 4:1a Whey protein separation obtained by PAGE (described in Chapter 3:4:2 ). Samples shown were taken at day 3 of the treatment period from a set of identical twin cows heterozygous for Beta Lactoglobulin type (AB). H.P. and L.P. refer to the sample from the cow on the High and Low plane of nutrition treatments respectively.

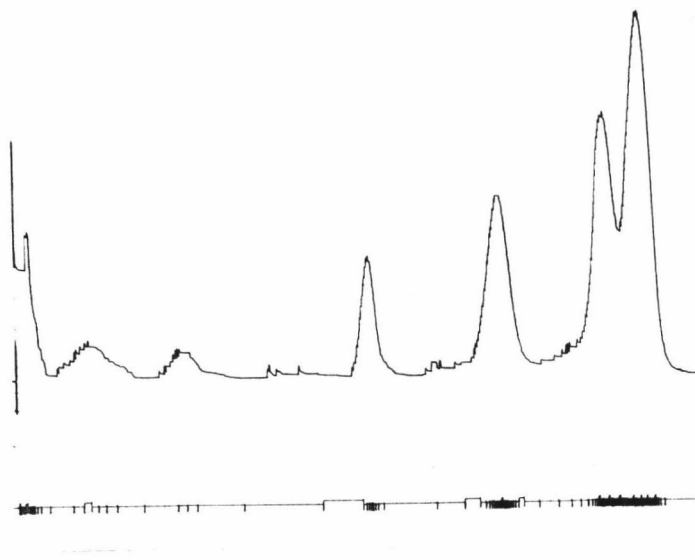


Plate 4:1b Scan trace and Intergrator plot of the gel shown  
in Plate 4:1a H.P.

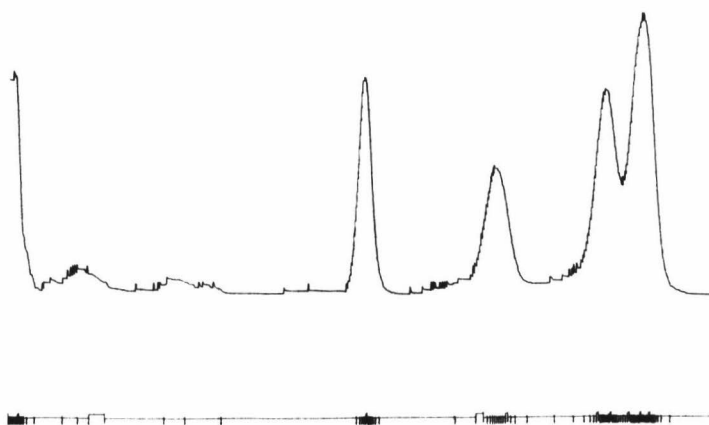


Plate 4:1c Scan trace and Intergrator plot of the gel shown  
in Plate 4:1a L.P.

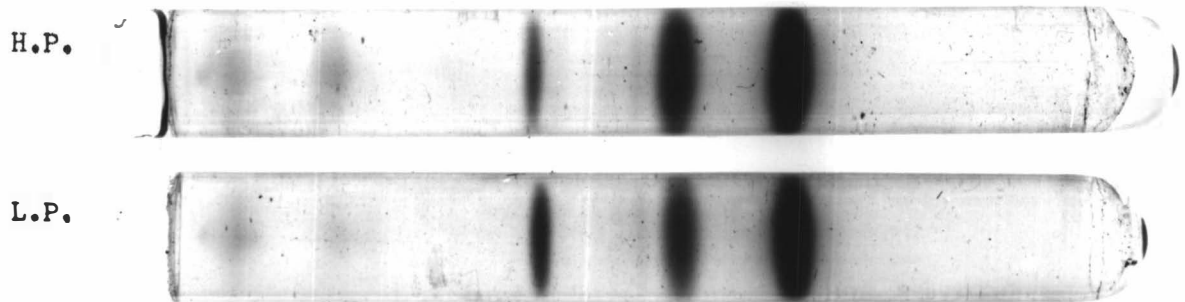


Plate 4:2a Whey protein separation obtained by PAGE (described in Chapter 3:4:2 ). Samples shown were taken at day 3 of the treatment period from a set of identical twin cows homozygous for Beta Lactoglobulin type (BB). H.P. and L.P. refer to the sample from the cow on the High and Low plane of nutrition treatments respectively.

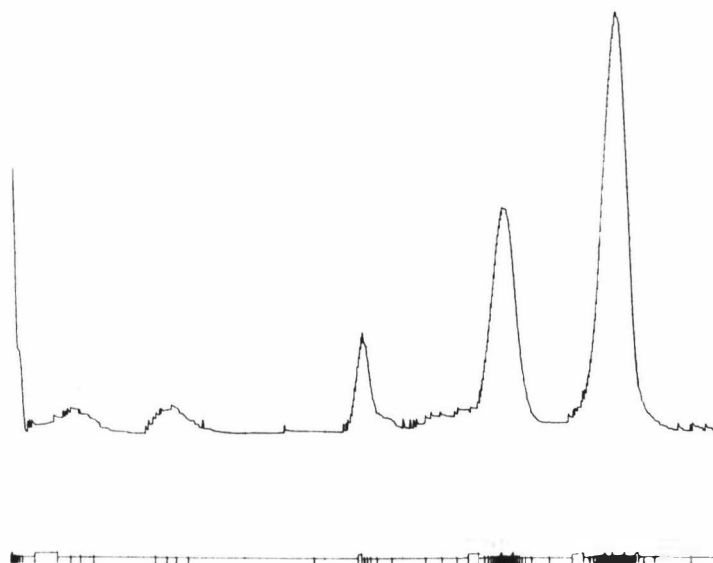


Plate 4:2b Scan trace and Intergrator plot of the gel shown  
in Plate 4:2a H.P.

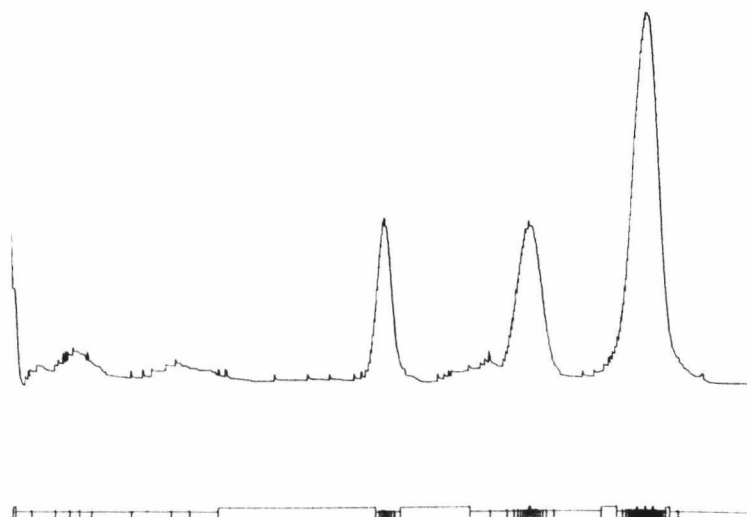


Plate 4:2c Scan trace and Intergrator plot of the gel shown  
in Plate 4:2a L.P.

## CHAPTER 5

Discussion

The effectiveness of the treatments has been examined in Chapter 4:1 in relation to the response variables by which the effect of varying the nutritional status of lactating cows is commonly recorded, i.e. Milk Yield, Fat %, Protein % and the Yields of Fat and Protein. The results indicated an effective difference between the nutritional states of the two treatment groups.

This discussion will concentrate on examining the effect of this difference between treatments on the milk protein classes and in particular on the individual whey proteins.

5:1 Effect of Treatment on Total Milk Protein

Munford et al.(1964) presented milk SNF both as the percentage of whole milk and as milk serum SNF percentage, by correcting the measure for the variation in fat content, using the formula 
$$\frac{\text{SNF} \times 100}{100 - \text{Fat } \%}$$
.

The change in total protein percentage in this experiment was measured separately in whole and skim milk in an attempt to improve the sensitivity of the protein determination. It was considered that separate determinations would be more effective than the Munford et al.(1964) correction.

The protein concentration in whole milk did not alter significantly (  $P > 0.05$ ), in contrast to the highly significant (  $P < 0.01$ ), difference measured between treatments in the skim milk samples. This difference reflects an improvement in the sensitivity of the measure, due presumably to the absence of random and treatment variation in milk fat content.

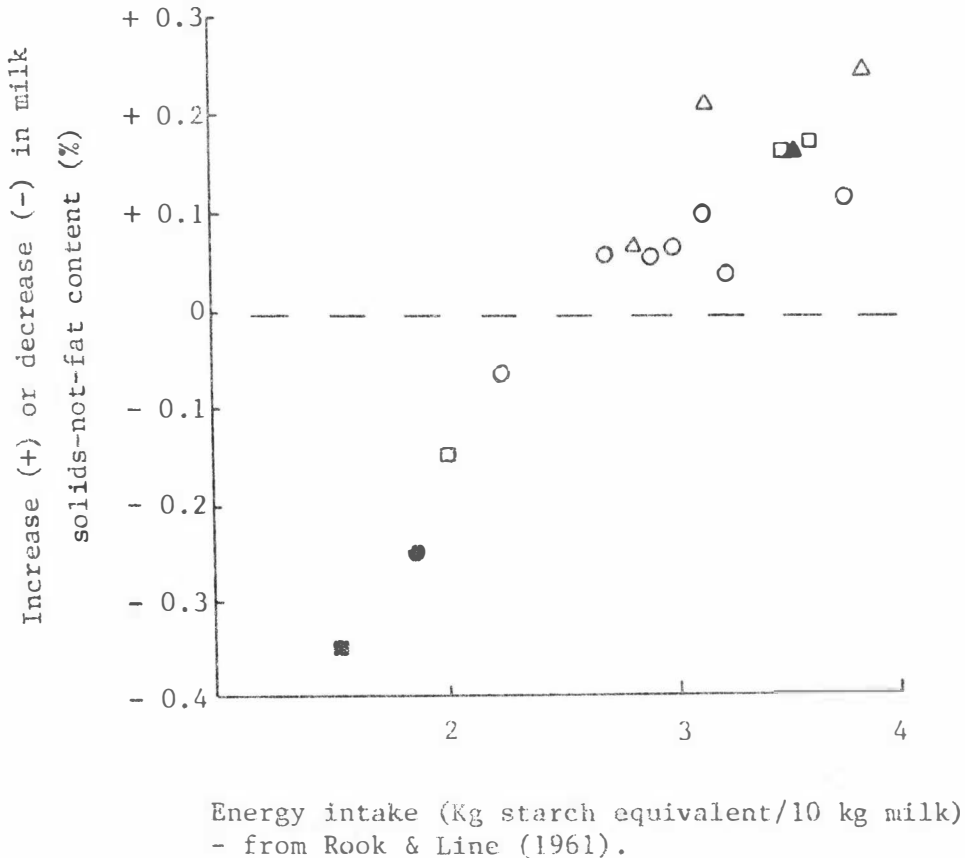
This result differs considerably to that of Munford et al.(1964) where the level of significance of the difference between treatment means of the two methods did not alter, nor did the apparent standard

deviations of the observations.

Changes in milk SNF concentration is mainly a product of changes in levels of milk protein (Balch 1972). Many reports suggest that the supply of dietary energy is considerably more important than dietary protein in affecting SNF % and therefore protein % (Rowland 1946, Bailey 1952, Burt 1957, Rook 1961, Huber & Boman 1966, Broster 1972). Fig.5:1 shows the effect of plane of energy nutrition on milk SNF % over a range of conditions described by several authors.

Fig. 5:1 Effect of Plane of Nutrition on Milk S.N.F. Content.

The Various Symbols represent results reported by different authors.



When cows are fed less than the usually accepted standard of energy requirements, milk SNF content falls and when fed above the standard there is a tendency for it to rise.

Milk protein is only affected by extreme protein underfeeding (Rook 1971, Armstrong & Prescott 1970). Furthermore these reports suggest that dietary protein in excess of recognised standards (Woodman 1957) has little effect on milk yield and composition.

The results obtained can therefore be interpreted as a major depression in energy intake being the main effect, possibly compounded by a restriction in protein intake.

#### 5:2 Effect of Treatment on the Major Classes of Milk Proteins

The proportions of both Caseins (non whey protein) and Whey Protein in total protein were similar to those reported by Szijarto et al. (1973), Ashworth (1965) and Cerbulis & Farrell (1975). The proportion to which each protein class contributed to total protein did not alter significantly ( $P > 0.05$ ) with treatment, nor did the post treatment period monitoring of the parameters indicate any changes due to the return to the herd situation of full feeding. These results are in good agreement with those reported by Haenlein et al. (1968).

Although the apparent relative changes in whey protein and casein concentration (expressed as a percentage of whole milk) due to the effect of the treatments are very similar (mean depressions of 6.5% and 6.2% respectively due to underfeeding), the significance of these responses varies greatly. Whereas the change in concentration of the whey proteins was not significant ( $P > 0.05$ ) the change in the concentration of the caseins was significant at the 1% level of probability. This difference is

attributed to the large variation in response by the whey protein class relative to the caseins. Furthermore, the observations in the post treatment period indicated an increase in the concentration of the caseins with the return to full feeding of the low plane treatment group. No such increase is recorded for the whey proteins at this time.

These observations can be explained by the observed changes in concentration of the whey proteins. Such changes are the resultant or residual of increased concentrations of the mammary synthesised whey proteins plus the decreased concentrations of the whey proteins originating in the bloodstream.

The results obtained are consistent with previously reported work. Balch & Campling (1961) demonstrated that a response in SNF content to level of Nitrogen intake (at low levels of intake) was mainly due to the changes in the levels of the casein fraction. Similarly Jenness (1974) has reported alterations in milk protein content as a result of underfeeding, to be due primarily to changes in the percentage of casein. Haenlein et al. (1968) also demonstrated changes in both the whey and casein contents of milk due to the feeding of a high energy ration. Furthermore, the changes in content observed were reversed during a post treatment period. It was also noted that the proportional change in the casein fraction was considerably greater than that of the whey protein fraction. Rook & Line ('96') showed that changes in milk protein content due to plane of energy nutrition could be accounted for almost quantitatively by changes in casein content with proportionately similar changes in the mammary synthesised whey proteins.

The yields of both whey proteins and the caseins showed highly significant responses ( $P < 0.01$ ) to treatment, reflecting the additive direction of the changes in milk yield and protein content. The return to full feeding in the post treatment period by the low

plane treatment group produced an apparent increase in the yields of both protein classes, further demonstrating the effectiveness of the treatment and its influence on both classes of milk proteins.

### 5:3 The Effect of Treatment on Individual Whey Proteins

Larson & Gillespie (1957) established that  $\beta$  lactoglobulin and  $\alpha$  lactalbumin were synthesized within the mammary gland whilst the immunoglobulins and milk serum albumin entered the milk pre-formed from the bloodstream.

5:3:1 Mammary Synthesized Whey Proteins The concentration of both  $\alpha$  lactalbumin and  $\beta$  lactoglobulin decreased under the low plane treatment, but only the change in  $\alpha$  lactalbumin reached significance ( $P < 0.05$ ). Rook & Line (1961) demonstrated a change in total milk protein content associated with a change in energy intake, to which  $\alpha$  lactalbumin and  $\beta$  lactoglobulin contributed proportionally. However, Yousef et al. (1970) found that, in response to an increased grain intake,  $\beta$  lactoglobulin increased whereas  $\alpha$  lactalbumin did not. In a cell culture experiment, Park & Chandler (1970) showed that the synthesis of both  $\alpha$  lactalbumin and  $\beta$  lactoglobulin was increased with the increasing availability of amino acids. These reports are in accord with the findings of the current work, assuming of course that the mechanisms of protein synthesis within the mammary gland operate systematically, both positively and negatively in relation to increases and decreases in the level of nutrients supplied to the gland.

Yields of both  $\alpha$  lactalbumin and  $\beta$  lactoglobulin differed highly significantly ( $P < 0.01$ ) with treatment, reflecting the common direction of movement of the changes in both the concentration of the proteins and the volume of milk secreted.

No significant changes ( $P > 0.05$ ) due to treatment were recorded in the concentration (expressed in absorbance units) of the individual  $\beta$  lactoglobulin variants A and B within the heterozygous cows. The average relative concentration of the A protein was shown to be considerably higher (36.6%) than the concentration of the B protein (within heterozygous AB animals) during the preliminary period.

These results agree with the majority of reports. Lontie et al. (1963) in a specific experiment demonstrated that  $\beta$  lactoglobulin AB milk contained more A protein than B protein, supporting the original findings of Aschaffenburg & Drewry (1957) and Rose (1962). Davis (1974) however, has shown higher concentrations of the  $\beta$  lactoglobulin B protein as did Hillier (1976). Although Cerbulis & Farrell (1975) did not separate  $\beta$  lactoglobulin variant concentration within heterozygous animals, they found that in a population of cows the average concentration of  $\beta$  lactoglobulin A was greater than  $\beta$  lactoglobulin AB which was in turn greater than  $\beta$  lactoglobulin B. This lends some support to the above observation but such data runs the danger of unequal numbers of variants confounding the results.

5:3:2 Whey Proteins originating in the Bloodstream The concentrations of both serum albumin and the immunoglobulins increased with the low plane of nutrition, but only the change in serum albumin reached significance ( $P < 0.01$ ). The non-significant ( $P > 0.05$ ) changes in the yields of these two proteins reflects the inverse form of the relationship which exists between milk yield and concentration of these proteins.

The increase in concentration of these pre-formed proteins, helps explain the non-significant ( $P > 0.05$ ) change in the concentration of total whey proteins (Table 4:6). The decreased rate of synthesis of the whey proteins originating in the mammary gland ( $\beta$  lactoglobulin and  $\alpha$  lactalbumin) is offset by increased concentrations of the preformed proteins serum albumin and immunoglobulins.

Rook (1971) suggested that an increased entry of blood proteins into milk could be associated with either an alteration in the permeability of the udder tissue or a more rapid decline in the rate of synthesis of milk than in the transfer of proteins from the blood stream.

The results obtained imply that the proteins which enter the milk preformed from the bloodstream do so at a rate which is independent of the rate at which milk is synthesised in the mammary gland, (at least in the short term) as emphasised by the increased yield of serum albumin to form a peak several days after the onset of the treatments. Thus, Rook's second suggested mechanism is not the simple answer.

The pattern of change, both in concentration and yield of the immunoglobulins is worth considering. See Figs. 4:18 and 4:19. The mean immunoglobulin concentration of the underfed group changed slightly and non-significantly ( $P > 0.05$ ) with respect to the high plane group; the average difference between the treatment group means being due as much to a decreased immunoglobulin concentration of the high plane group as to an increased immunoglobulin concentration of the low plane group. Such a pattern would fit more closely the second hypothesis (volume of secretion effect) of Rook than the first (altered udder permeability). An alternative explanation could be that the first hypothesis is in fact operating for the passively transferred immunoglobulin components ( $G_2$ , A & M) Butler (1969) but that their effective concentration change is masked by the continuation of the selective transfer of immunoglobulin  $G_1$ .

In contrast the pattern of change in the concentration of serum albumin very strongly suggests that the permeability of the udder tissue has altered dramatically. The increase in concentration of serum albumin under the low plane treatment formed an initial peak at or about day 3 of the treatment period (day 7 of experiment) and was followed by a secondary peak around day 9 of the treatment period (day 13 of the experiment). This pattern of change in

concentration was still apparent in the plot of serum albumin yield against time (Fig. 4:17) although the difference between treatments was not significant ( $P > 0.05$ ). Statistical analysis of the difference in serum albumin yield between treatments for days 1-5 of the treatment period (5-9 of the experiment) and of the maximum difference in serum albumin yield (day 3 of the treatment or day 7 of the experiment) both failed to reach significance ( $P > 0.05$ ).

Schanbacher & Smith (1975) have shown a similar peak in serum albumin concentration due to the onset of acute induced mastitis. The similarity extends to the timing of the peak (50 hours post inoculation) and to the appearance of a secondary peak some four to five days later. The authors suggest that these changes in serum albumin (and also immunoglobulin) indicate destruction of the permeability barriers of the mammary epithelium and a consequent influx of serum proteins. Though less severe, the nutritional regime (low plane) imposed in this experiment has resulted in broadly similar effects on milk yield and the concentrations of the whey proteins, as has resulted from numerous investigations of the effects of mastitis (Schanbacher & Smith 1975, Haenlein et al. 1973, Randolph et al. 1974, Lecce & Legates 1959, Rowland 1938). In the absence of mastitis (see section 4:1:3), the results indicate that the effect of a nutritional deprivation may operate on the mammary gland to a greater extent than simply by limiting precursor availability for milk synthesis. Schanbacher & Smith (1975) suggest that events in the diseased udder may be related to transitions in the normal functional cycle of the gland; an opinion which the results from this work support. It can be considered that both nutritional stress and udder disease precipitate an involution response by the mammary gland which is then reversed by some unknown condition which re-stimulates the gland and an initially colostral-like secretion is formed.

## CHAPTER 6

Conclusions

The experiment conducted resulted in a nutritional difference being established between treatment groups. The yield of milk and the concentrations of the major milk components (fat and protein) were consistent with a restricted feed intake by the underfed treatment group.

The directions of the changes in the concentration and yield of the individual whey proteins were consistent with the recognised mechanisms of synthesis and/or secretion of the individual proteins. However, the extent and pattern of the changes indicated that the processes involved may be less straightforward than originally considered. Schanbacher and Smith (1975) raise the possibility that the mammary gland might elaborate factors into its secretions which serve to regulate mammary function. Certainly the response by the serum albumin fraction would support such a hypothesis.

The addition of  $\beta$  lactoglobulin to milk has been shown to improve the heat stability characteristics of that milk (Feagan et al. 1972, Tessier and Rose 1964, Rose 1961). The lack of any significant change in the concentration of  $\beta$  lactoglobulin and the absence of any indication of a slow or steady decrease in  $\beta$  lactoglobulin concentration with time of underfeeding, suggests that nutritional manipulation would be unlikely to alter the heat stability of milk through affecting the concentration of  $\beta$  lactoglobulin. The lack of a dramatic increase in the concentration of this protein with the post experimental return to full feeding supports this view.

This conclusion does however, conflict with the observations of Feagan et al. (1972) that:-

- (a) cows produce milk with a higher heat stability (at pH 6.7-6.8) when well fed (winter/spring and summer irrigated lucerne) than when poorly fed.
- b) there exists a relationship between heat stability and  $\beta$  lactoglobulin genotype which is due to the relative quantities of  $\beta$  lactoglobulin produced by each variant.

The change in concentration of  $\beta$  lactoglobulin observed (though non-significant,  $P > 0.05$ ) achieved its maximum within 24 hours of onset of the treatments (see Fig. 4:20). This indicates that  $\beta$  lactoglobulin is an extremely labile protein, a suggestion which is in line with its tentatively suggested enzymatic function (Farrell & Thompson 1971).

The significant ( $P < 0.05$ ) difference between treatments in  $\alpha$  lactalbumin concentration is a reflection of its control function in lactation as the specifier protein of the lactose synthetase enzyme (Ebner et al. 1966, Brodbeck et al. 1967, Brew et al. 1968).

The immunoglobulin concentration did not alter as dramatically as the serum albumin fraction, nor did it alter in the pattern of serum albumin which was described in the previous Chapter (Figs. 4:16 and 4:18). The comparison of the responses to treatment of these two proteins indicates that their secretion is unlikely to be interdependent. As mentioned before, the apparent secretion of immunoglobulin may be considerably stabilized by the active transport mechanism proposed for immunoglobulin G<sub>1</sub> (Pierce & Feinstein 1965, Watson & Lascelles 1973, Peaker 1975).

These results for immunoglobulin concentration changes tend to support the "leakage" theory of serum protein secretion rather than adding to the weight of evidence against it. However, under conditions of stress (e.g. infection, nutritional insufficiency) the normal physiological operation of the gland may be impaired or altered.

The design and conduct of the experiment left room for improvement. Probably the major criticism is that the controlled grazing method of imposing the nutritional treatments could have been inefficient for the objectives of the project. Under such conditions the possibility existed for dominant cows in the underfed (low plane) group to obtain relatively more feed than the shy cows. At the extreme it would be possible to have the dominant cow or cows approaching full feeding whilst the timid cows would be severely restricted.

Under such conditions, the ranking of cows according to their social position in a herd may permit such a factor and the considerable variation associated with it to be removed statistically. The excessive display of dominance was not observed in the underfed herd, but in the absence of continuous observation may have operated as pasture became limiting. With sufficient labour and facilities, a pen feeding design where intakes could be accurately controlled would be appropriate to re-examine the levels of change in the concentrations of the whey proteins.

Regular weighing of the cows was not carried out for the reasons outlined in Chapter 2. However the monitoring of liveweight of the animals would have indicated changing gut fill and indicated whether the relative intakes of the animals was comparable.

Though economic analysis is beyond the scope of this thesis the results have indicated that short term feed restrictions have a dramatic effect on milk yield and composition. It is further indicated that with an economic value placed upon milk protein, such as is proposed in the modified basis of payment formula, feed restrictions, whether qualitative or quantitative, will have a greater economic effect than on the fat only basis of payment due to the additive nature of the changes in protein concentration and milk yield.

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41: 155.
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Appendix I Poly Acrylamide Gel Electrophoresis (PAGE)

Method: Adapted from Hartman & Swanson (1965) and Davis (1964)

Materials:Small Pore Gel Solutions.

Small Pore I - equal parts of A & C.

Solution A	1N HCl	64.4ml	}	to 200 ml with deionised water.
	Tris	49.1g		
	Temed	0.23ml		

Ionic concentration = 0.110

pH = 8.9

Solution C - for 11% Acrylamide Gels

	Acrylamide	44g	}	to 100 ml with deionised water.
	Bis Acrylamide	.73g		

Small Pore II - 0.14g Ammonium Persulphate to 100 mls with deionised water. The small pore gel was mixed as it was required as follows:

1 part Solution A	}	Small Pore I
1 part Solution C		
2 parts Small Pore II		

Large Pore Gel Solutions

Solution B	1N HCl	48 ml	}	to 100 ml with deionised water
	Tris	5.98g		
	Temed	0.46 ml		

Solution D	Acrylamide	10g	}	to 100ml with deionised water
	Bis Acrylamide	2.5g		

Solution E Riboflavin - 4mg/100ml of deionised water.

The large pore gel is photopolymerised, so stock was stored in the dark at 5°C

Running Buffer      3g Tris            )  
                          14.4g Glycine        ) to 1 litre with deionised water

Stain                    2% Amido Black 10B in 7% Acetic Acid

Destaining Solution    3% Acetic Acid

Gel Tubes 6mm Inside diam., 9mm Outside diam. Small Pore Gel 60mm

Large Pore Gel 12mm

Destaining Tubes 7mm Inside diam., 9mm Outside diam. x 85mm,  
 constricted end.

Chemicals

Acetic Acid	AnalaR
Acrylamide	Eastmann
Ammonium Persulphate	M & B
Bis Acrylamide (N N'-Methylenebisacrylamide)	Eastmann
Glycine (amino acetic acid)	J.T. Baker
Hydrochloric Acid (0.994 N against 1N NaOH)	B.D.H.
Riboflavin	Eastmann
Temed (N,N,N',N' - Tetramethylethylenediamine)	Eastmann
Tris (Tris(hydroxymethyl)methylamine)	AnalaR
Amido Black 10B	Merck

Appendix II Spectrophotometric Method for the Determination of  
Dye-Binding by Total Whey Protein

Basis of Method When a negatively charged dye (acid dye) is introduced into a solution it combines with any protein present forming an insoluble protein-dye complex. Removal of this complex and determination by spectrophotometry of the residual dye concentration yields an indirect measure which can be calibrated for protein concentration.

Method - adapted from Dolby (1961).

- 1 200  $\mu$ l of sample (whey diluted 1:2.5)
- 2 1000  $\mu$ l of dye solution - 0.6165g Amido Black 10B  
63.0 g Citric Acid  
1250 ml Water
- 3 Dye solution and sample were mixed and allowed to stand for 20 minutes.
- 4 Mixture was centrifuged, approx. 1500g x 20 min.
- 5 100  $\mu$ l of the supernatant was diluted in 3.00ml H<sub>2</sub>O.
- 6 This solution was mixed by inversion.
- 7 Solution read at 615 m $\mu$ . Optical Density (O.D.) corrected for cuvettes which were cleaned and recalibrated each 12 samples.
- 8 Blanks were prepared for each run (100 samples) by substituting 200  $\mu$ l of deionised water for the sample in step 1. O.D. of blanks by this method was 1.04 compared to those of Dolby's original method of 0.90.
- 9 O.D. Protein = O.D. Blank - O.D. Sample.
- 10 Standard curves were prepared by the same method for each of the major whey proteins - IG, BSA,  $\alpha$  Lac,  $\beta$  Lgb. See Appendix Vb.

Discussion To overcome the poor duplication within samples, each sample was precipitated in triplicate and the subsequent supernatant dilution step was also triplicated. In calculating the mean protein concentration of the nine resulting estimates, outlying observations were tested by "Tchebycheffs Inequality" (Samuelson 1968), with the probability of Type 1 error being set at 10%, i.e. 1 in 10 chance of rejecting the observations when in fact it is a valid estimate. Alternatively, all observations having greater than 10% chance of being a true estimate were accepted. On this basis, few of the outlying observations were sufficiently anomalous to be rejected. An example of the calculation is presented below.

Observations	.378	.364	.382	
	.378	.350	.390	Mean .366
	.384	.299	.378	

The outlier (.299) has a 16.6% probability of being a true observation and so is retained in the estimate.

Appendix III      Wisconsin Mastitis Test

Basis of Method      In response to inflammation, the mammary gland progressively releases leucocytes into the milk. The test reagent lyzes all cells present in milk and reacts with the nucleic acids released. A viscous slime is produced, the viscosity of which is proportional to the leucocyte numbers and in turn the degree of infection.

Method      2 mls of R.M.T. reagent\* was added to 2 ml of a representative milk sample in a plastic test tube 12.1 x 130 mm with a 3 mm vent some 65 mm from the base of the tube. 10 samples were tested at a time. The rack of 10 tubes of milk and reagent was rocked gently to mix the contents and was then inverted for precisely 15 seconds. The mixture ran from the tubes through a calibrated hole in the removable brass cap at a rate inversely related to its viscosity. The amount of mixture remaining in the tube was measured in millimetres following a draining period of 20 seconds in the upright position. The tubes and caps were rinsed twice and drained between samples.

\* Rapid Mastitis Test Reagent, I.C.I. (N.Z.) Ltd. Wellington.

<u>Grading</u>	WMT Score.	Mastitis Status.	Appropriate Cell Numbers.
	1-10 mm	Negative	< 214,000 cells/ml
	11-17 mm	Suspicious	214,000 - 435,000 cells/ml
	> 17 mm	Positive	> 5000,000 cells/ml

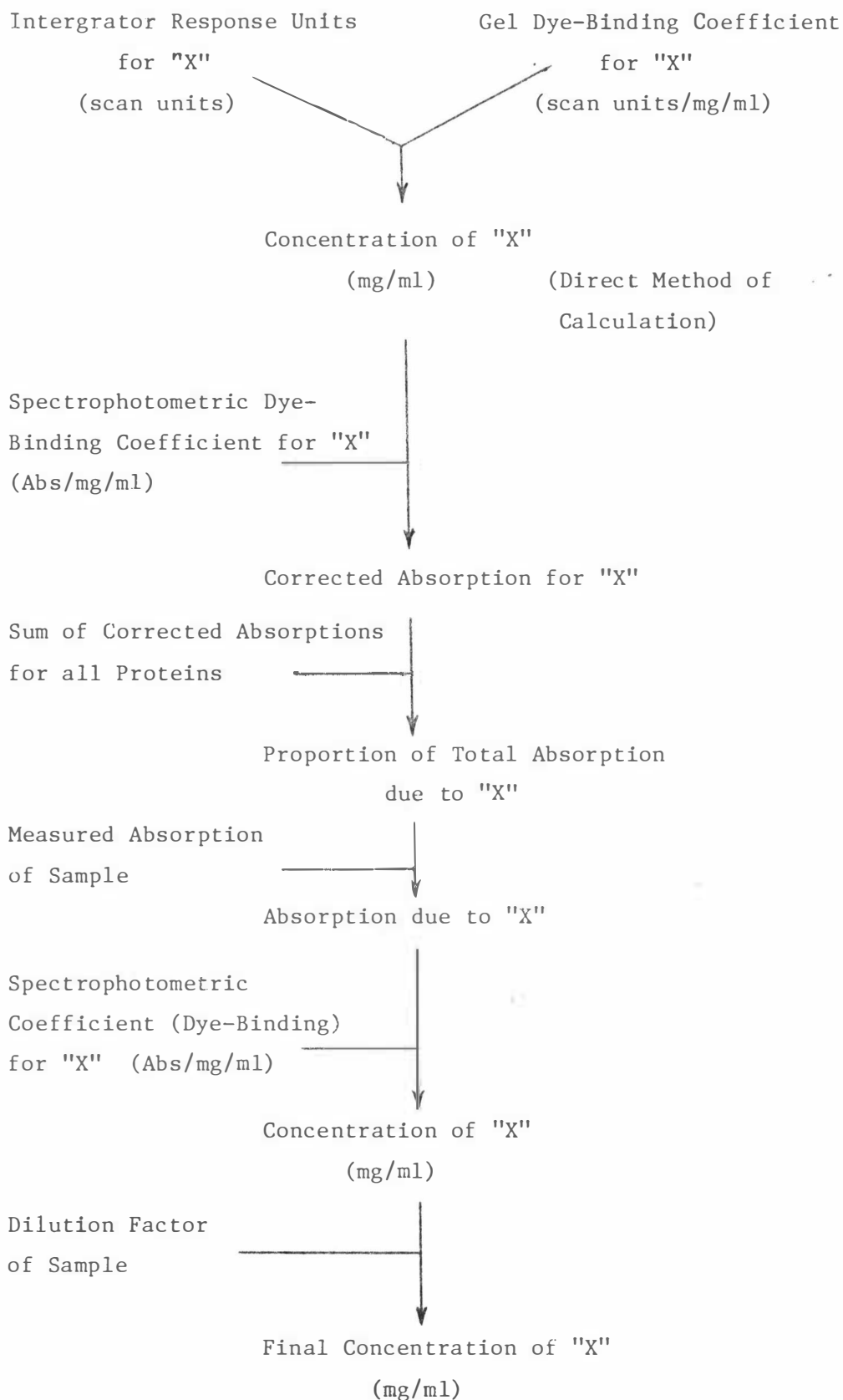
Ref. Daniel et al.(1971)

Appendix IV    Quantification of Whey Proteins by the Method of  
Proportional Subdivision

Basis of Method    The proportion of each protein in a mixture was determined by PAGE and densitometry. These proportions were then used to partition an independent estimate of the total protein content of the mixture. The method was unfortunately complicated by the varying dye-binding relationships both between individual proteins and between the two systems of estimating dye-binding in the method.

Assumptions involved in the method

1.    that the dye-binding coefficients of  $\beta$  Lgb A and  $\beta$  Lgb B are identical.
2.    that the dye-binding coefficients of the minor protein components are equal to the mean of the coefficients of the four major proteins,  $\beta$  Lgb,  $\alpha$  Lac, BSA and IG.
3.    that assumptions 1 and 2 hold for both the gel and spectrometric methods of estimating dye-binding relationships.

Outline of Method

Calculation where + A = Counts (scan units) of IG.  
 B = " " " BSA.  
 C = " " "  $\alpha$  Lac.  
 D = " " "  $\beta$  Lgb.  
 UN = " " (total) for unknown  
 minor proteins.  
 AG = Gel Dye-Binding Coefficient for A.  
 BG = " " " B.  
 CG = " " " C.  
 DG = " " " D.  
 MGB = " " (Mean) for all unknown  
 minor proteins.  
 AS = Spectrophotometer Dye-Binding Coefficient for A.  
 BS = " " " " B.  
 CS = " " " " C.  
 DS = " " " " D.  
 MSB = " (Mean) for all unknown  
 minor proteins.  
 Abs = Estimate of Sample Absorption.

True Concentration of A =  $\frac{\text{Absorption due to A, corrected for Gel dye-binding of A}}{\text{Absorption due to the sum of all proteins, each corrected for its Gel dye-binding factor.}}$

x  $\frac{\text{Spectrophotometric Sample Absorption} \times \text{Dilution}}{\text{Spectrophotometric Dye-Binding Coefficient of A}}$

$$\text{True Concentration of A} = \frac{\left[ \frac{A}{AG} \times AS \right]}{\left[ \frac{A}{AG} \times AS \right] + \left[ \frac{B}{BG} \times BS \right] + \left[ \frac{C}{CG} \times CS \right] + \left[ \frac{D}{DG} \times DS \right] + \left[ \frac{UN}{MGB} \times MSB \right]} \times \left[ \frac{ABS}{AS} \right] \times \text{Dilution}$$

Appendix Va    Standard Curves for Individual Whey Proteins  
relating Absorbance to Protein Concentration  
in the Gel Dye-Binding System.

The relationship between protein quantity and absorbance (measured as integrator scan units) was required for the determination of protein concentration. Standard curves were required for each protein due to their individual and characteristic dye-binding capacity.

Va:1 Immunoglobulins    See Graph Va:1

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	5	25,976.667	5,195.333	509.97	**
Regr'	1	25,928.337	25,928.337	2,145.96	**
Dev'n	4	48.330	12.082	1.19	NS
Within	60	611.272	10.188		
Total	65	26,587.939			

Regression Coefficient "b" (Dye-Binding Factor)

$$\text{"b" } y.x. = 435.356$$

Y intercept "a"

$$\text{"a" } = -6.251$$

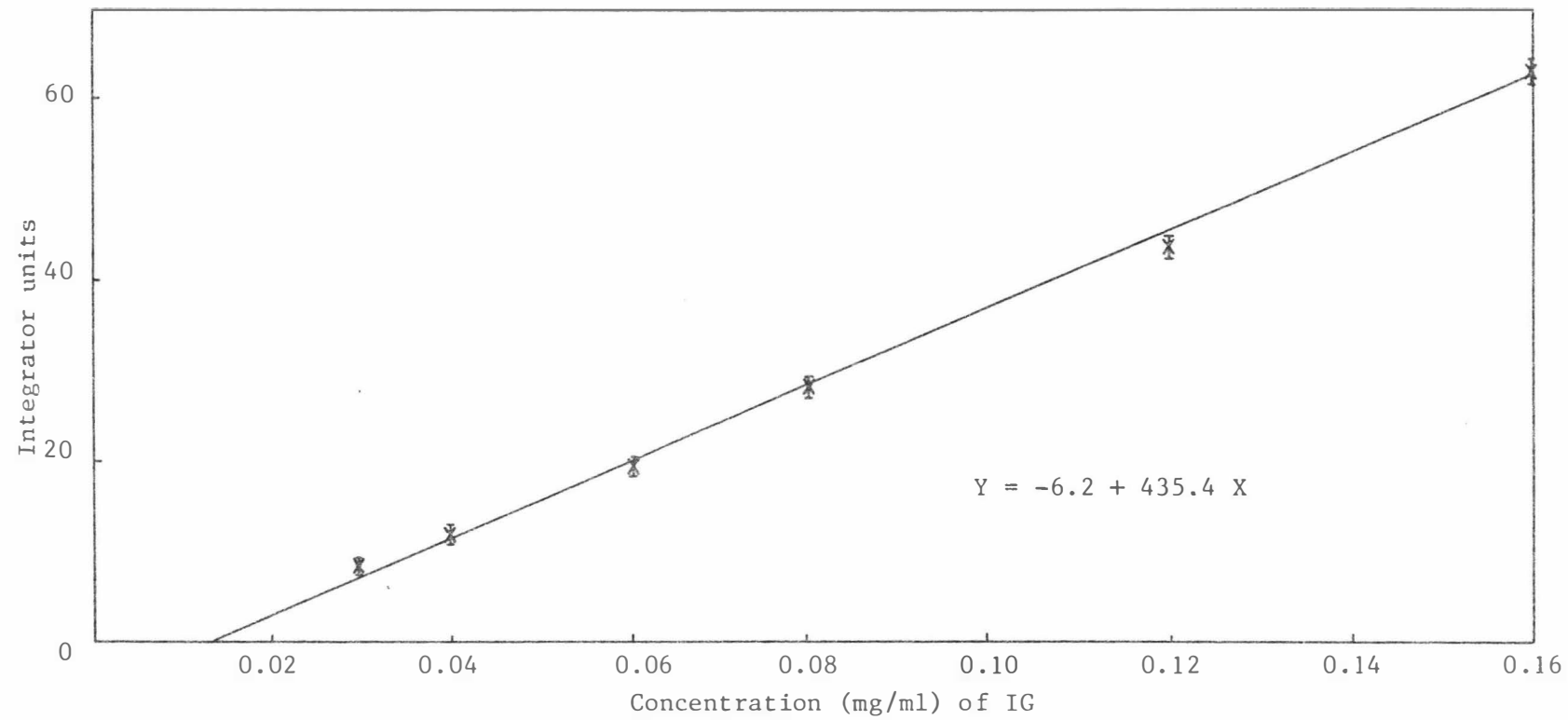
Significance of Deviation of Y intercept from  $Y = 0$ .

$$t = 0.027 \quad \text{NS}$$

$$\text{Required } t_{.01}_4 = 2.78$$

\*\* (P < 0.01)

NS (P > 0.05).



APPENDIX Va:1 Immunoglobulins standard curve (gels)

Va:2 Bovine Serum Albumin See Graph Va:2

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	5	59,007.894	11,801.579	614.86	**
Reg'r	1	58,984.023	58,984.023	9,883.84	**
Dev'n	4	23.870	5.968	0.31	NS
Within	60	1,151.636	19.194		
Total	65	60,159.530			

Regression Coefficient "b" (Dye-binding Factor)

$$\text{"b" } y.x. = 531.244$$

Y intercept "a"

$$\text{"a" } = 0.567$$

Significance of Deviation of Y intercept from  $Y = 0$ .

$$t = 0.087 \text{ NS.}$$

$$t_{.01_4} = 2.78.$$

Va:3 Alpha Lactalbumin See Graph Va:3

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	3	16,473.182	5,491.061	319.92	**
Regr'	1	16,443.655	16,443.655	1,113.80	**
Dev'n	2	29.527	14.764	0.86	NS
Within	40	686.546	17.164		
Total	43	17,159.727			

Regression Coefficient "b" (Dye-binding Factor)

$$\text{"b" } y.x. = 432.273$$

Y intercept "a"

$$\text{"a" } = -5.00$$

Significance of Deviation of Y from  $Y = 0$

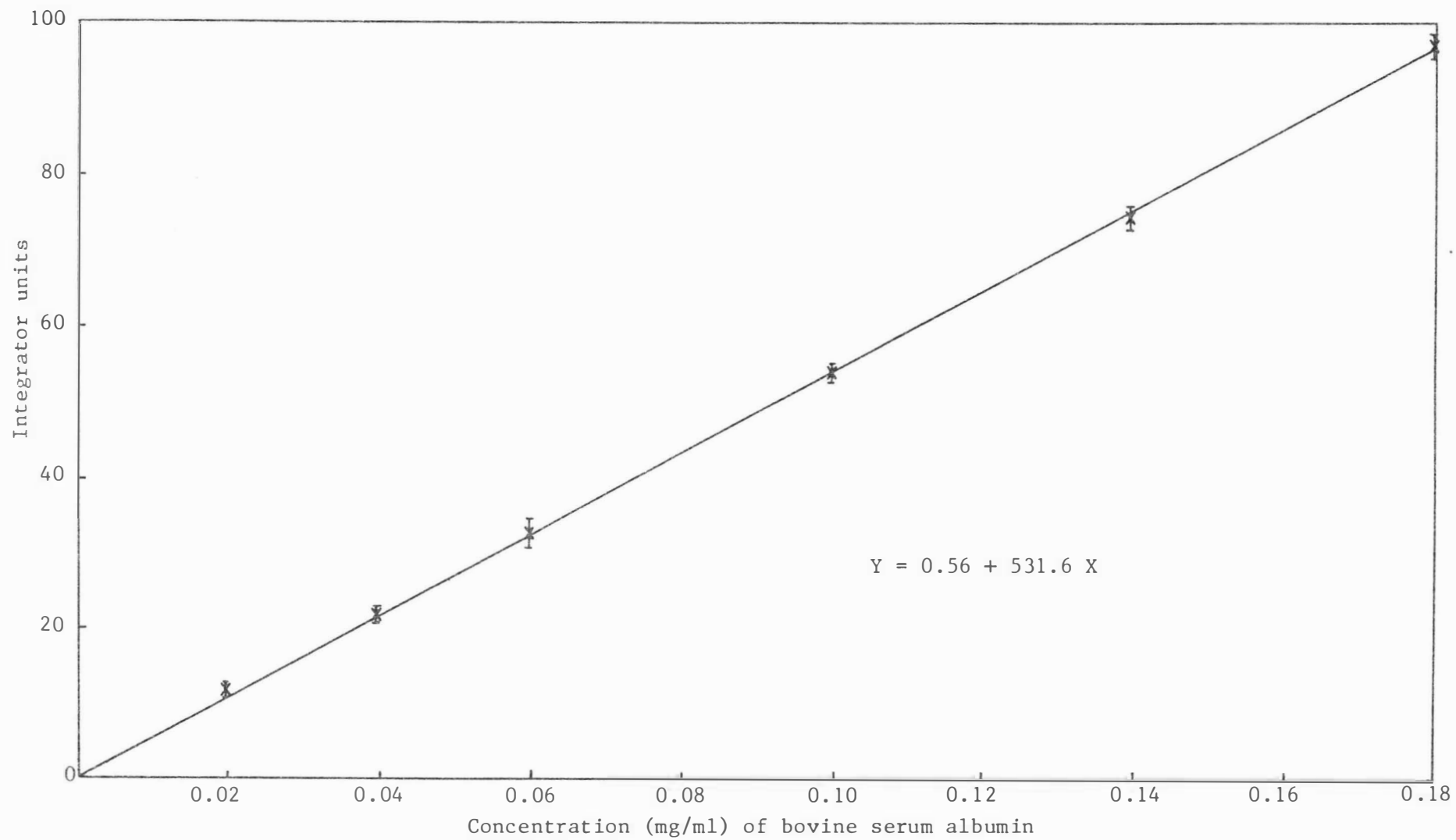
$$t = 1.572 \text{ NS.}$$

$$t_{.05_2} = 4.303.^\dagger$$

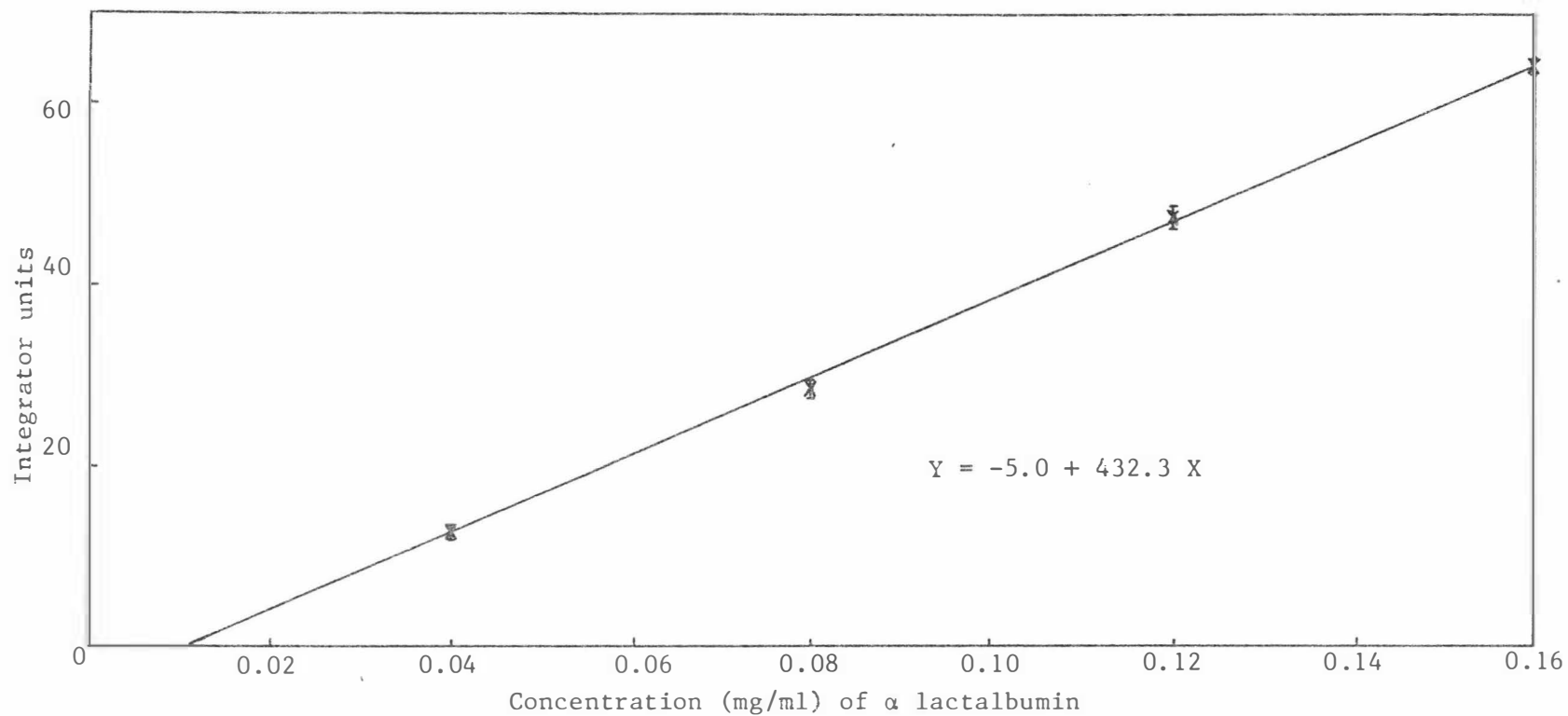
\*\* ( $P < 0.01$ )

NS ( $P > 0.05$ )

† Degrees of Freedom differ between Va:1 & 2 and Va:3 & 4 due to deletion of the two highest concentrations of both  $\alpha$  Lactalbumin and  $\beta$  Lactoglobulin. This was necessitated by these protein bands overlapping at the higher concentration.



APPENDIX Va:2 Bovine Serum Albumin standard curve (gels).



APPENDIX Va:3  $\alpha$  Lactalbumin Standard Curve (gels)

Va:4 Beta Lactoglobulin See Graph Va:4

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	3	631,579.886	210,526.629	588.43	**
Regr'	1	629,374.041	629,374.041	570.64	**
Dev'n	2	2,205.846	1,102.923	3.08	NS
Within	40	14,311.091	357.777		
Total	43	645,890.977			

Regression Coefficient "b" (Dye-binding Factor)

$$\text{"b" } y.x. = 534.86$$

Y intercept "a"

$$\text{"a" } = 19.05$$

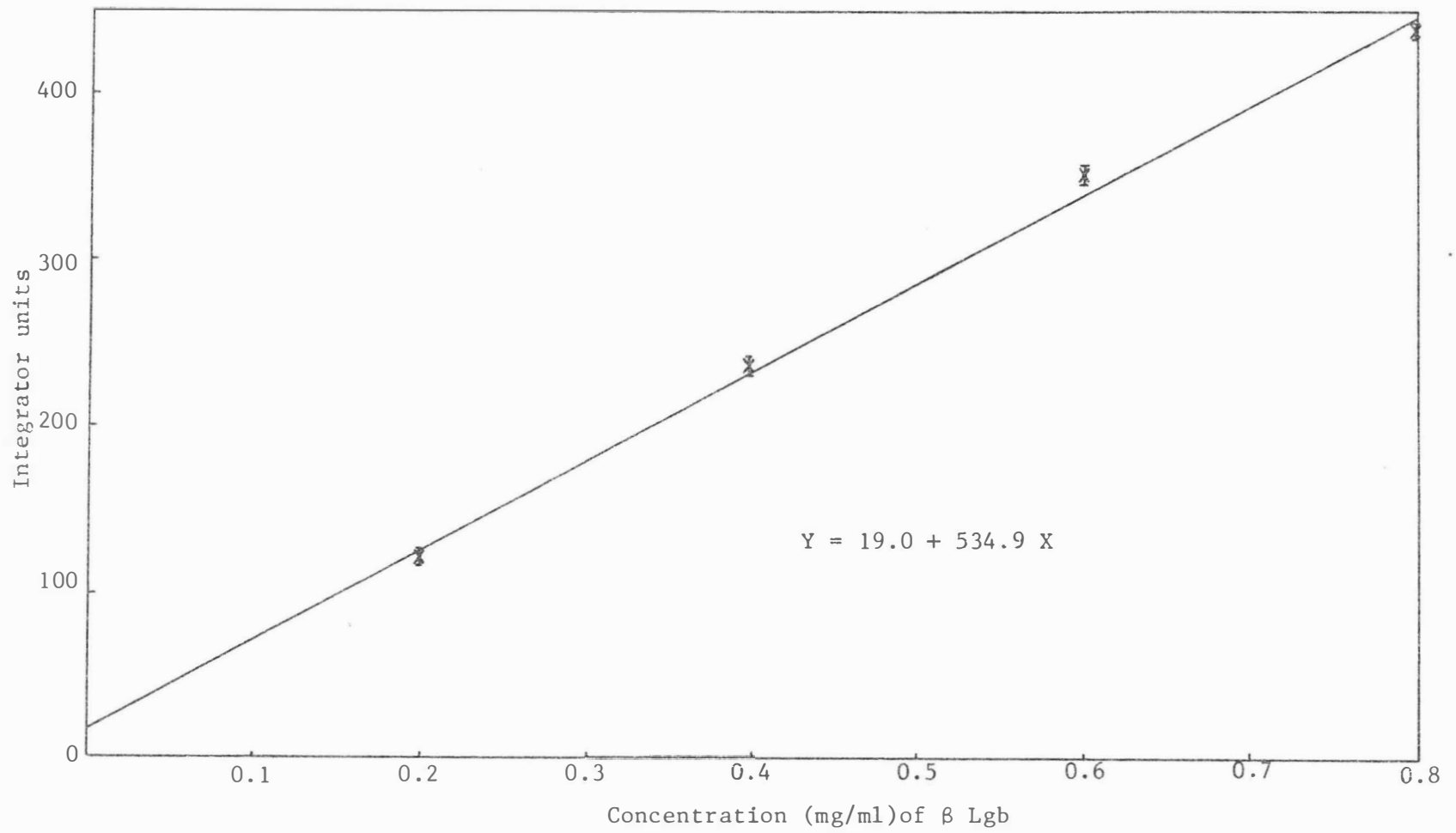
Significance of Deviation of Y from Y = o.

$$t = 2.4224 \text{ NS.}$$

$$t(0.05)_2 = 4.303.$$

\*\* (P < 0.01)

NS (P > 0.05)



APPENDIX Va:4  $\beta$  Lactoglobulin Standard Curve (gels)

Appendix Vb    Standard Curves for Individual Whey Proteins relating  
Absorbance to Protein Concentration in the  
Spectrophotometric Dye-Binding System.

The relationship between absorbance (optical density at 615nm) and protein concentration was required for the analysis of protein concentration. As each whey protein exhibits a characteristic dye-binding capacity, standard curves were required for each.

Vb:1    Immunoglobulin    See Graph Vb:1

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	5	0.7256	0.1451	342.61	**
Regr'	1	0.7216	0.7216	895.85	**
Dev'n	4	0.0040	0.0010	2.38	NS
Within	6	0.0025	0.0004		
Total	11	0.7282			

NS Not significant

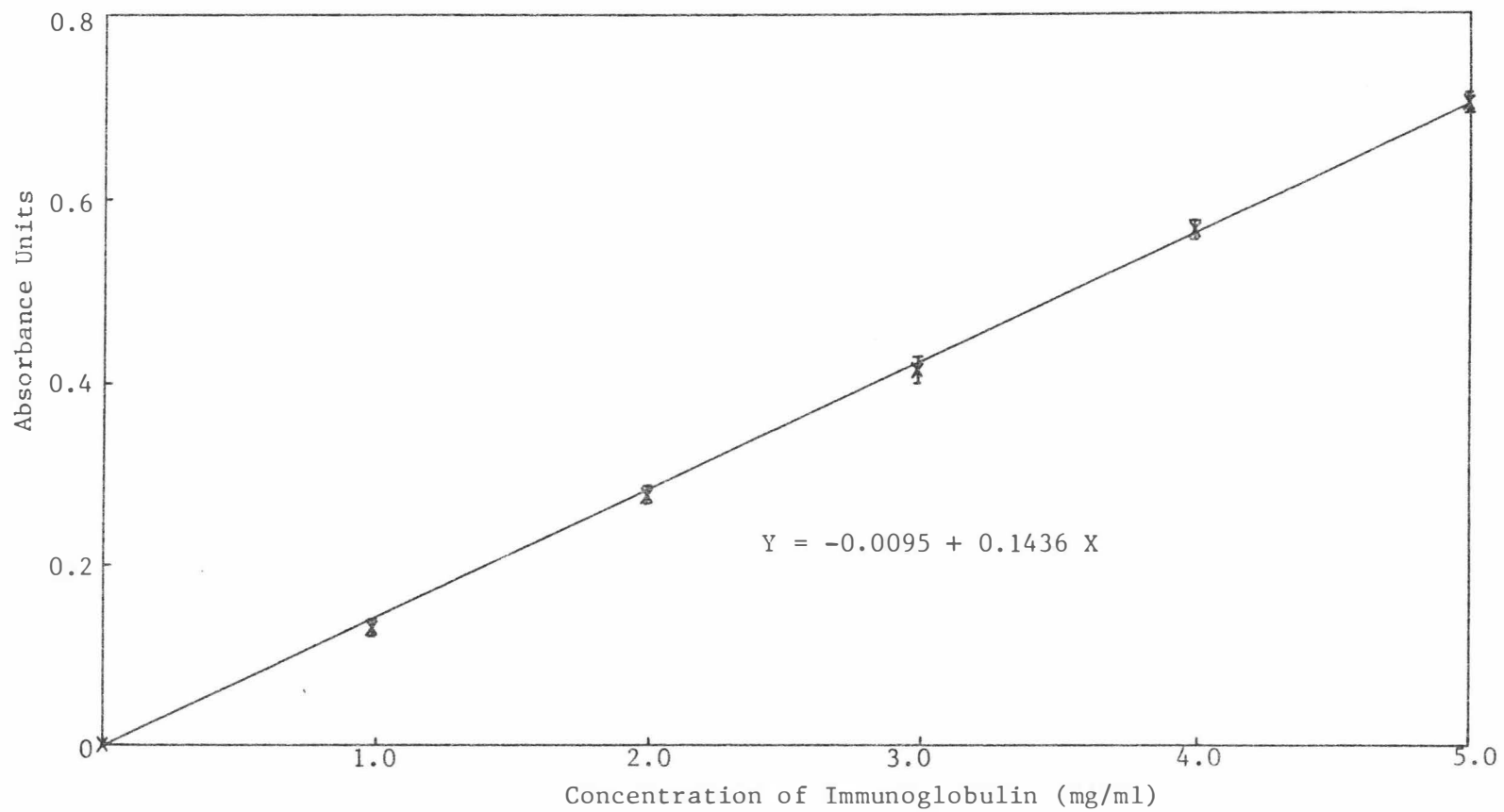
\*\* (P < 0.001)

Regression Coefficient "b" (Dye-binding factor)

$$\text{"b" } y.x. = 0.1436$$

Y intercept "a"

$$\text{"a" } = -0.0095.$$



APPENDIX Vb:1 Immunoglobulins Standard Curve (for spectrophotometer)

Vb:2 Bovine Serum Albumin See Graph Vb:2

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	5	1.8229	0.3646	3,616.88	**
Regr'	1	1.8222	1.8222	9,971.42	**
Dev'n	4	0.0007	0.0002	1.81	NS
Within	6	0.0006	0.0001		
Total	11	1.8229			

Regression Coefficient "b" (Dye-binding Factor)

$$"b"_{y.x.} = 0.2282$$

Y intercept "a"

$$"a" = 0.0061.$$

Vb:3 Alpha Lactalbumin See Graph Vb:3

Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	5	1.0328	0.2066	558.25	**
Regr'	1	1.0315	1.0315	3,327.52	**
Dev'n	4	0.0012	0.0003	0.84	NS
Within	6	0.022	0.004		
Total	11	1.0350			

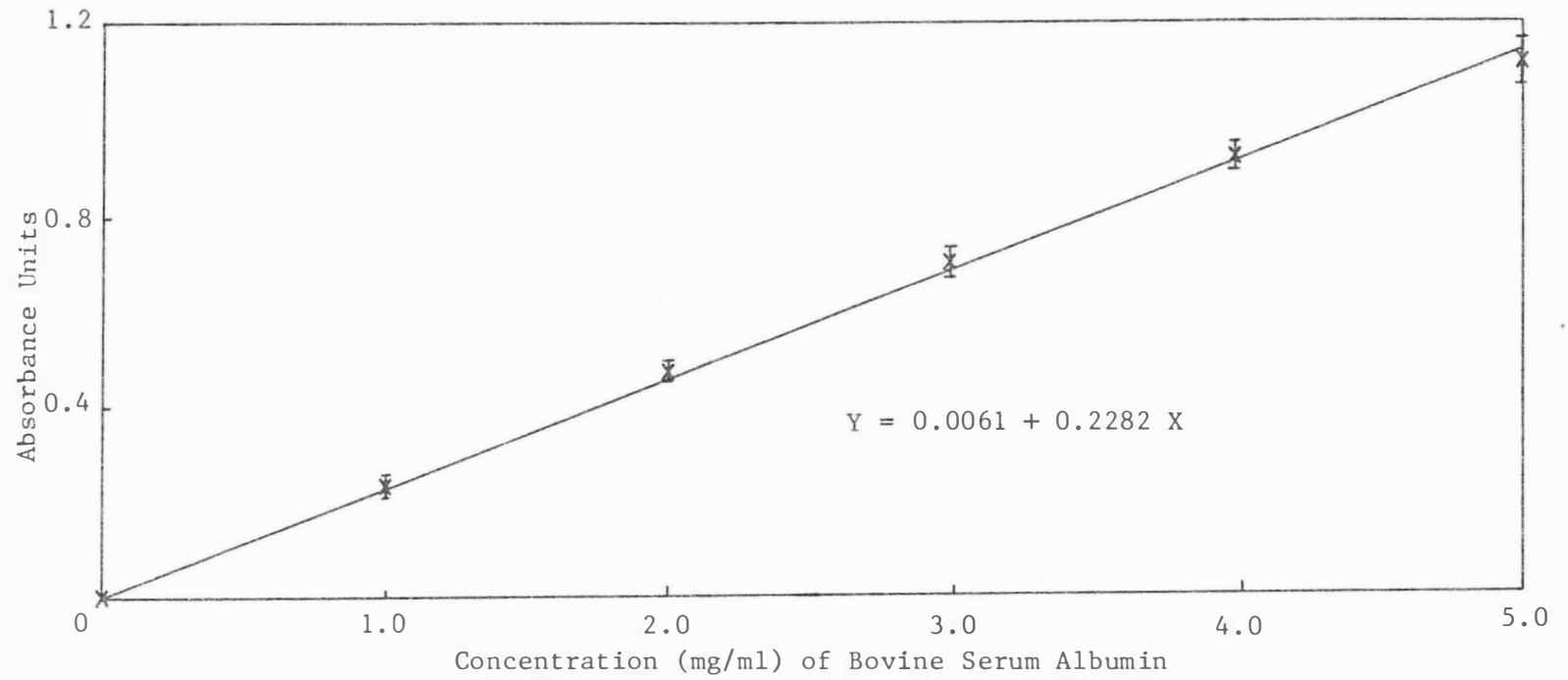
Regression Coefficient "b" (Dye-binding Factor)

$$"b"_{y.x.} = 0.225$$

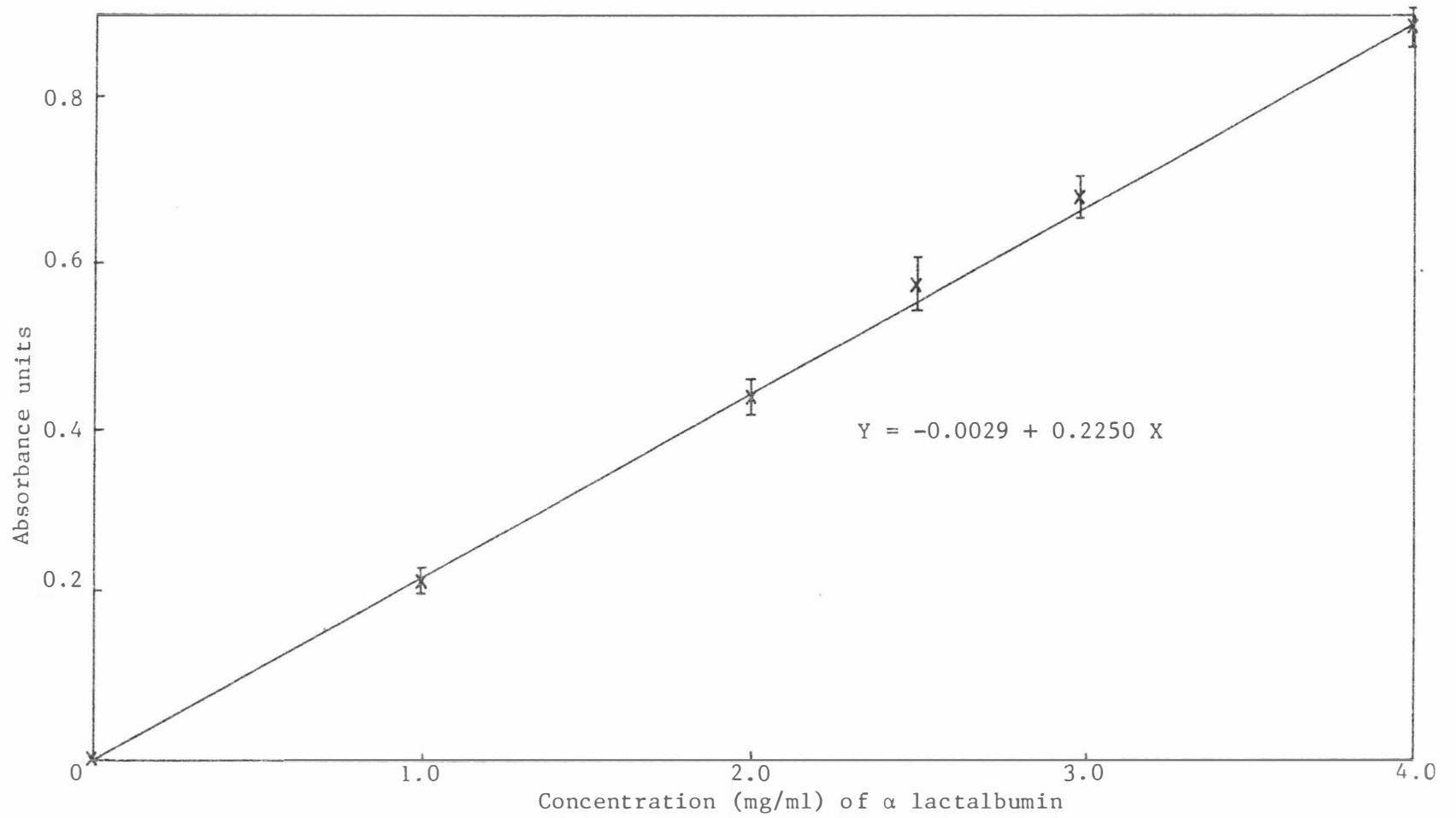
$$b_{y.x.} = 0.2250$$

Y intercept "a"

$$"a" = -0.00292$$



APPENDIX Vb:2 Bovine Serum Albumin Standard Curve (spectrophotometer)



APPENDIX Vb:3  $\alpha$  Lactalbumin Standard Curve (spectrophotometer)

Vb:4 Beta Lactoglobulin (AB) See Graph Vb:4

The regression analysis and establishment of the  $\beta$  Lactoglobulin dye-binding factor was carried out on protein separated from bulk milk. Consequently the sample contained roughly equal proportions of the two common  $\beta$  Lactoglobulin variants, A & B.

#### Regression Analysis

Source	df	SS	MS	F ratio	Sig.
Between	5	1.0627	0.2125	1,554.80	**
Regr.	1	1.0614	1.0614	3,293.70	**
Devn'	4	0.0013	0.0003	2.36	NS
Within	6	0.0008	0.0001		
Total	11	1.0635			

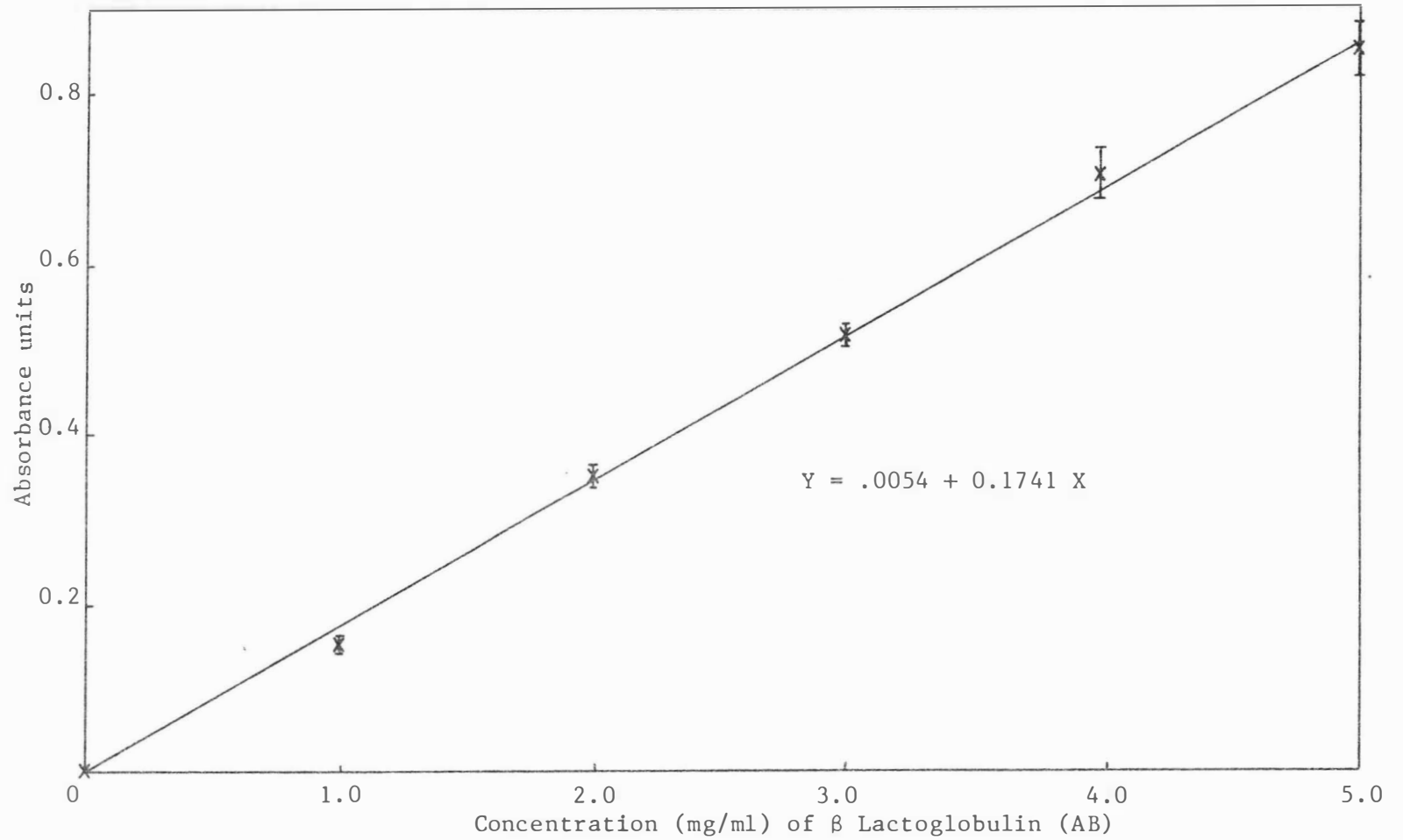
Regression Coefficient "b" (Dye-binding Factor)

$$\text{"b" } y.x. = 0.1741$$

Y intercept "a"

$$\text{"a" } = -0.0054$$

The above relationships were assumed to hold for both the AB and the BB  $\beta$  Lactoglobulin conditions throughout this experiment.



APPENDIX Vb:4  $\beta$  Lactoglobulin (AB) Standard Curve (spectrophotometer)

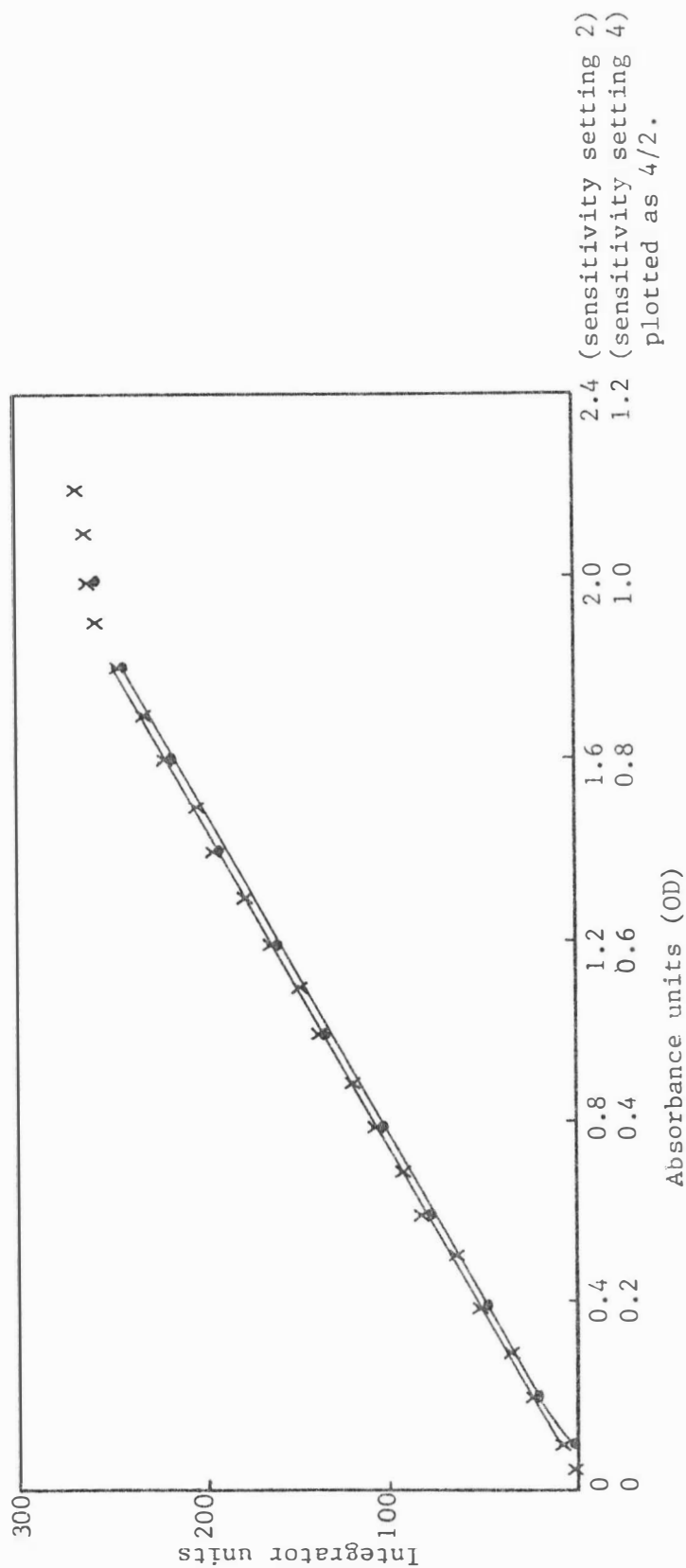
Appendix Vc    Relative Dye-Binding of Whey Proteins in the Gel  
and Spectrophotometric Systems.

Protein	Spectrophotometric System		Gel System	
	Regression Coefficient	Relative Coefficient*	Regression Coefficient	Relative Coefficient*
IG	0.1436	1.0	435.35	1.0
BSA	0.2282	1.588	531.24	1.220
$\alpha$ Lac	0.2250	1.567	432.27	0.993
$\beta$ Lgb (AB)	0.1741	1.212	534.86	1.229

\* Dye-binding coefficient of the individual protein relative to that of Immunoglobulin.

Appendix VI Linearity of Scanning/Integrator System

The linearity of the scanning densitometer and integrator system was checked at two integrator sensitivity settings.



APPENDIX VI Calibration of scan integrator against absorbance at two levels of sensitivity.

Appendix VII Covariance Analysis of the Effects of Treatment on Response Parameters.

NS = Not significant, \* = Significant at 5%, \*\* = Significant at 1%.

VII:1 Milk Yield - Day 1 of Treatment. (For Table 4:1).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	140.40	139.26	159.03					
Blocks	5	137.23	134.13	133.05					
Treatment	1	0.001	-0.04	10.58					
Error	5	3.17	5.17	15.40	4	6.97	1.74		
Tr. & Err.	6	3.171	5.13	25.98	5	17.68			
To Test Adjusted Means					1	10.71	10.71	6.14	NS

VII:2 Milk Yield - Full treatment effect. (For Table 4:2).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	140.40	122.31	136.01					
Blocks	5	137.23	122.18	111.46					
Treatment	1	0.0001	-0.06	23.95					
Error	5	3.17	0.19	0.61	4	0.59	0.15		
Tr. & Err.	6	3.170	0.135	24.56	5	24.55			
To Test Adjusted Means					1	23.95	23.95	161.2	**

VII:3 Fat Percentage. (For Table 4:2).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	3.53	4.53	9.53					
Blocks	5	2.76	4.33	8.91					
Treatment	1	0.0003	-0.01	0.12					
Error	5	0.76	0.20	0.50	4	0.45	0.11		
Tr. & Err.	6	0.76	0.19	0.62	5	0.57			
To Test Adjusted Means					1	0.12	0.12	1.06	NS

VII:4 Fat Yield. (For Table 4:2).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	152,772.4	127,111.5	147,912.8					
Blocks	5	148,664.5	122,640.2	103,918.4					
Treatment	1	32.2	1,093.3	37,146.4					
Error	5	4,075.8	3,378.0	6,848.0	4	4,048.4	1,012.1		
Tr. & Err.	6	4,108.0	4,471.3	43,994.4	5	39,127.7			
To Test Adjusted Means					1	35,079.3	35,079.3	34.7	**

VII:5 Protein Percentage - Whole Milk. (For Table 4:2)

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	0.623	0.800	1.365					
Blocks	5	0.602	0.733	0.996					
Treatment	1	0.011	0.055	0.267					
Error	5	0.010	0.012	0.101	4	0.086	0.021		
Tr. & Err.	6	0.021	0.067	0.368	5	0.155			
To Test Adjusted Means					1	0.069	0.069	3.19	NS

VII:6 Protein Yield. (For Table 4:2).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	142,294.9	113,135.5	149,751.8					
Blocks	5	138,338.8	110,437.8	91,519.0					
Treatment	1	221.6	3,548.8	56,826.1					
Error	5	3,734.5	- 851.2	1,406.8	4	1,212.8	303.2		
Tr. & Err.	6	3,956.1	2,697.6	58,232.9	5	56,393.4			
To Test Adjusted Means					1	55,180.6	55,180.6	181.9	**

VII:7 Protein Percentage - Skim Milk (For Table 4:2).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	0.807	0.958	1.525					
Blocks	5	0.794	0.899	1.153					
Treatment	1	0.005	0.034	0.282					
Error	5	0.008	0.025	0.090	4	0.019	0.005		
Tr. & Err.	6	0.013	0.059	0.372	5	0.111			
To Test Adjusted Means					1	0.092	0.092	19.75	**

VII:8 Whey Protein Percentage of Whole Milk. (For Table 4:6).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	0.173	0.164	0.228					
Blocks	5	0.164	0.157	0.201					
Treatment	1	0.001	-0.0005	0.0003					
Error	5	0.008	0.008	0.026	4	0.019	0.005		
Tr. & Err.	6	0.009	0.0075	0.0263	5	0.021			
To Test Adjusted Means					1	0.002	0.002	0.415	NS

VII:9 Non Whey Protein Percentage of Whole Milk. (For Table 4:6).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	9.913	0.354	0.658					
Blocks	5	0.262	0.276	0.366					
Treatment	1	0.017	0.065	0.249					
Error	5	9.634	0.013	0.042	4	0.042	0.011		
Tr. & Err.	6	9.651	0.078	0.291	5	0.291			
To Test Adjusted Means					1	0.249	0.249	23.45	**

VII:10 Whey Protein Percentage of Total Protein. (For Table 4:6).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	65.51	47.49	61.12					
Blocks	5	58.87	39.62	45.39					
Treatment	1	1.32	2.16	3.54					
Error	5	5.32	5.71	12.19	4	6.05	1.51		
Tr. & Err.	6	6.64	7.87	15.73	5	6.39			
To Test Adjusted Means					1	0.34	0.34	0.22	NS

VII:11 Non Whey Protein Percentage of Total Protein. (For Table 4:6).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	63.63	46.73	61.11					
Blocks	5	58.10	39.25	45.36					
Treatment	1	1.02	1.90	3.55					
Error	5	4.50	5.58	12.20	4	5.27	1.32		
Tr. & Err.	6	5.52	7.48	15.75	5	5.62			
To Test Adjusted Means					1	0.34	0.34	0.26	NS

VII:12 Non Whey Protein Yield. (For Table 4:6).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	2,755.3	1,985.1	4,003.5					
Blocks	5	2,652.6	2,086.3	2,071.2					
Treatment	1	1.07	- 43.7	1,775.6					
Error	5	101.6	- 57.5	156.7	4	124.1	31.0		
Tr. & Err.	6	102.7	-101.2	1,932.3	5	1832.5			
To Test Adjusted Means					1	1708.4	1708.4	55.1	**

VII:13 Non Whey Protein Yield. (For Table 4:6).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	108,688.9	85,713.2	106,589.5					
Blocks	5	105,558.9	82,971.5	67,329.2					
Treatment	1	245.7	3,077.5	38,544.7					
Error	5	2,884.3	- 335.8	715.6	4	676.5	169.1		
Tr. & Err.	6	3,130.0	2,741.7	39,260.3	5	36858.7			
To Test Adjusted Means					1	36,182.2	36,182.2	213.9	**

VII:14 Concentration of  $\alpha$  Lactalbumin. (For Table 4:7)

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	0.275	0.136	0.469					
Blocks	5	0.219	0.129	0.151					
Treatment	1	0.0008	-0.014	0.241					
Error	5	0.055	0.022	0.076	4	0.068	0.017		
Tr. & Err.	6	0.056	0.008	0.317	5	0.317			
To Test Adjusted Means					1	0.249	0.249	14.70	*

VII:15 Yield of  $\alpha$  Lactalbumin. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	272.70	227.57	416.00					
Blocks	5	263.45	235.40	228.64					
Treatment	1	0.016	-1.66	171.61					
Error	5	9.24	-6.17	15.74	4	11.63	2.91		
Tr. & Err.	6	9.25	-7.83	187.35	5	180.73			
To Test Adjusted Means					1	169.10	169.10	58.17	**

VII:16 Concentration of Bovine Serum Albumin. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	0.0936	0.1525	0.3741					
Blocks	5	0.0880	0.1329	0.2362					
Treatment	1	0.0016	0.0139	0.1230					
Error	5	0.0040	0.0056	0.0149	4	0.0070	0.0017		
Tr. & Err.	6	0.0056	0.0195	0.1379	5	0.0697			
To Test Adjusted Means					1	0.0627	0.0627	35.71	**

VII:17 Yield of Bovine Serum Albumin. (For Table 4:7)

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	7.297	10.579	18.865					
Blocks	5	6.642	9.959	17.800					
Treatment	1	0.029	0.078	0.209					
Error	5	0.626	0.542	0.856	4	0.387	0.097		
Tr. & Err.	6	0.655	0.620	1.065	5	0.479			
To Test Adjusted Means					1	0.092	0.092	0.95	NS

VII:18 Yield of Bovine Serum Albumin Days 5-9. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	7.297	11.053	21.694					
Blocks	5	6.642	10.449	18.482					
Treatment	1	0.029	0.224	1.728					
Error	5	0.626	0.380	1.483	4	1.252			
Tr. & Err.	6	0.655	0.604	3.211	5	2.654	0.313		
To Test Adjusted Means					1	1.403	1.403	4.48	NS

VII:19 Maximum Difference in Daily Yield of Bovine Serum Albumin  
between Treatments. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	7.297	11.871	50.446					
Blocks	5	6.642	12.403	32.929					
Treatment	1	0.029	0.480	7.976					
Error	5	0.626	-1.013	9.541	4	7.904	1.976		
Tr. & Err.	6	0.655	-0.533	17.517	5	17.084			
To Test Adjusted Means					1	9.180	9.180	4.65	NS

VII:20 Concentration of Immunoglobulins. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Source	11	0.4714	0.6060	0.0481					
Blocks	5	0.4499	0.5839	0.7789					
Treatment	1	0.0071	0.0129	0.0234					
Error	5	0.0143	0.0347	0.1458	4	0.0616	0.0154		
Tr. & Err.	6	0.0214	0.0218	0.1692	5	0.1471			
To Test Adjusted Means					1	0.0855	0.0855	5.55	NS

VII:21 Yield of Immunoglobulins. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	32.817	33.238	43.998					
Blocks	5	30.608	29.605	35.148					
Treatment	1	1.252	2.161	3.729					
Error	5	0.957	1.471	5.121	4	2.860	0.715		
Tr. & Err.	6	2.209	3.632	8.850	5	2.880			
To Test Adjusted Means					1	0.020	0.020	0.03	NS

VII:22 Concentration of  $\beta$  Lactoglobulin. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	8.057	6.466	7.346					
Blocks	5	7.866	6.326	6.645					
Treatment	1	0.026	-0.054	0.110					
Error	5	0.166	0.194	0.591	4	0.365	0.091		
Tr. & Err.	6	0.192	0.140	0.701	5	0.599			
To Test Adjusted Means					1	0.234	0.234	2.57	NS

VII:23 Yield of  $\beta$  Lactoglobulin. (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	11	1,376.0	905.8	1,446.0					
Blocks	5	1,332.0	949.2	803.3					
Treatment	1	0.9	-23.2	594.7					
Error	5	43.1	-20.2	48.0	4	38.5	9.6		
Tr. & Err.	6	44.0	-43.4	642.7	5	599.9			
To Test Adjusted Means					1	561.4	561.4	58.3	**

VII:24 Absorbance (Concentration) of  $\beta$  Lactoglobulin A in AB cows.

(For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	7	0.00293	0.00282	0.00606					
Blocks	3	0.00268	0.00279	0.00520					
Treatment	1	0.00008	-0.00018	0.00038					
Error	3	0.00016	0.00021	0.00048	2	0.00021	0.00010		
Tr. & Err.	4	0.00024	0.00003	0.00086	3	0.00085			
To Test Adjusted Means					1	0.00064	0.00064	6.29	NS

VII:25 Absorbance (Concentration) of  $\beta$  Lactoglobulin B in AB Cows.

(For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	7	0.00198	0.00154	0.00292					
Blocks	3	0.00188	0.00135	0.00227					
Treatment	1	0.00002	0.00003	0.00005					
Error	3	0.00009	0.00016	0.00059	2	0.00031314	0.00016		
Tr. & Err.	4	0.00010	0.00019	0.00064	3	0.00031329			
To Test Adjusted Means					1	0.00000015	0.00000015	0.001	NS

VII:26 Proportion  $\frac{\beta \text{ Lactoglobulin A}}{\beta \text{ Lactoglobulin B}}$  (For Table 4:7).

Source	df	$\Sigma x^2$	$\Sigma xy$	$\Sigma y^2$	Deviations from Regression				
					df	SS	MS	F ratio	Sig.
Total	7	0.05657	0.05119	0.06082					
Blocks	3	0.05513	0.05093	0.05066					
Treatment	1	0.00007	-0.00066	0.00655					
Error	3	0.00137	0.00091	0.00361	2	0.0030	0.0015		
Tr. & Err.	4	0.00144	0.00025	0.01016	3	0.0101			
To Test Adjusted Means					1	0.0071	0.0071	4.74	NS