Thesis of Kelvin E. Hopper

'Comparative Studies of α-Lactalbumins and of their Relationships to Lysozymes'

Errata

p. 8 line 13 'Lehman' should read 'Lehmann'
p. 10 line 6 'Sarisekharen' should read 'Sasisekharan'
p. 22 line 5 'lysosomes' should read 'lysosomes'
p. 22 line 14 'has since shown' should read 'have since shown'
p. 27 line 14 'tryptphans' should read 'tryptophans'
p. 29 line 2 from bottom 'tryptohan 68' should read 'tryptophan 63'
p. 142 line 17 'effect' should read 'affect'
Tachyglossus aculeatus multiaculeatus

A remarkable contemporary
A COMPARATIVE STUDY OF $\alpha$-LACTALBUMINS
AND OF THEIR RELATIONSHIP TO LYSOZYMES

A thesis submitted for the degree of
Doctor of Philosophy
in the
Australian National University

by
Kelvin Edward Hopper

Department of Physical Biochemistry
John Curtin School of Medical Research
The work described in this thesis was carried out in the Department of Physical Biochemistry, J.C.S.M.R., between September, 1967 and January, 1971. This work was performed entirely by myself, except where otherwise stated in the text.

Kelvin Edward Hopper
ACKNOWLEDGEMENTS

I am sincerely grateful to Dr H.A. McKenzie for his supervision of this work and for his detailed and valuable criticism of the manuscript.

I am indebted to Professor A.G. Ogston for allowing me to undertake this research, and together with Dr H.A. McKenzie and other members of the department for providing a stimulating environment in which to work.

My thanks are extended to those people who have collected milk samples and in particular to Dr M.Griffiths of CSIRO Division of Wildlife for his interest in this work and for providing milk samples from the monotremes and kangaroo.

I wish to thank Dr D.C. Shaw for his guidance in the work on peptide mapping and to Mr L.B. James for carrying out the amino acid analyses.

I am grateful to Mr R.A. Goodman for his help in providing chemicals and apparatus and to many other colleagues who have made this period interesting and enjoyable.

Finally that this thesis is completed indicates the patience and encouragement provided by Kristine and I am deeply indebted to her.

Financial support in the form of a research scholarship from the Australian National University is gratefully acknowledged.
SUMMARY

A study has been made of the occurrence, isolation and properties of α-lactalbumins from a number of species. These results have been considered in relation to the role of α-lactalbumins in the lactose synthetase system and their homology with lysozymes.

1. The chromatographic fractionation of bovine whey proteins has been studied in detail to investigate the heterogeneity of α-lactalbumin and to isolate the major component in high purity.

2. A second α-lactalbumin genetic variant (A) has been isolated from the milk of Droughtmaster breeds of cattle and compared with the more common B variant. α-lactalbumin A differs from B by the substitution of glutamine for arginine.

3. Three minor proteins (SCI, SC2 and FC) occur together with each major bovine genetic variant. These minor proteins have been identified as α-lactalbumins from their amino acid compositions and activity in lactose synthetase. They differ from each other in their electrophoretic mobilities and solubility in water. The two proteins of lower mobility at pH 7.5 (SCI and SC2) are glycoproteins containing 12-15 per cent carbohydrate. SCI differs from SC2 by the presence of one residue of sialic acid per mole. FC is probably the same minor protein observed by other workers in zone electrophoresis of α-lactalbumin samples. It is not a glycoprotein and probably differs from the major
α-lactalbumin by containing one less amide group.

4. Bovine α-lactalbumin exhibits a bimodal pattern on elution from columns of DEAE-Sephadex or DEAE-cellulose at pH 6.3-7.8. The bimodality is not observed in zone electrophoresis, sedimentation or gel filtration. The relative size of each peak on rechromatography depends on previous treatment of the protein.

5. Using the knowledge gained in fractionation of bovine whey proteins, α-lactalbumins having high activity in lactose synthetase with bovine "A protein" have been isolated from human, porcine, ovine and kangaroo milk. The following order of reactivities for the α-lactalbumins has been determined: bovine > human > porcine > kangaroo. A bimodal elution pattern resembling that of the bovine α-lactalbumins has been found for the human but not for the pig or kangaroo proteins. This property may be related to the function in the lactose synthetase system. A minor α-lactalbumin similar to the bovine FC protein appears to occur in porcine, human and ovine milks. Ovine whey does not contain proteins of similar properties to bovine SC1 and SC2.

The lactose and lysozyme contents of milk from one subspecies of echidna (T. a. multiaculeatus) has been found to increase with time of lactation. The isolated lysozyme exhibits weak activity in lactose synthetase in the presence of bovine "A protein" and the pH dependence of the reaction closely parallels that of the "A protein" alone. The lysozyme has an isoelectric point of 12.0-12.5 and has approximately 1.3-1.5 times the specific lytic activity as hen egg lysozyme and similar activity to the human lysozyme, also isolated in
this present work. A lysozyme from a second sub-species of echidna (*T. a. aculeatus*) has been shown to be inactive in the lactose synthetase reaction and has lower electrophoretic mobility at pH 5.3 than the lysozyme from *T. a. multiaculeatus*. Platypus milk contains traces of lactose and lysozyme activity but no protein of similar function to the α-lactalbumins from other mammals. The lysozyme from *T. a. multiaculeatus* has an amino acid composition intermediate between the other lysozymes and α-lactalbumins isolated in this present study and cannot be simply classified in either group. There are also differences in optical rotatory dispersion in the region 205-350 nm of these proteins.
ABBREVIATIONS

The following abbreviations were used in this thesis.

ATP  adenosine 5'-triphosphate
CD   circular dichroism
DEAE-Sephadex  (diethylaminoethyl) Sephadex
NAG  N-acetylglucosamine
NAL  N-acetyllactosamine
NANA N-acetylneuraminic acid
ORD  optical rotatory dispersion
TPCK L-(tosylamide-2-phenyl) ethyl chloromethyl ketone
Tris tris(hydroxymethyl)aminoethane
UDP  uridine diphosphate

α A, α B  α-lactalbumin (A or B)
β A, β B, β C  β-lactoglobulin (A, B or C)
Dr  Droughtmaster

Amino acids were abbreviated according to the three letter system recommended by IUPAC-IUB (Biochem. J., 102, 23, (1967)) with the exception of asparagine (AsN), glutamine (Gln) and cysteic acid (C\textsubscript{γ}SO\textsubscript{3}H).
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>INTRODUCTION</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>I.A</td>
<td>GENERAL</td>
<td>1</td>
</tr>
<tr>
<td>I.B</td>
<td>ON THE STRUCTURE OF GLOBULAR PROTEINS</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>X-ray diffraction analysis of protein crystals</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Proteins in crystal and solution</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Solution studies</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Association and dissociation of proteins</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>The conformation of the polypeptide chain</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Amino acid sequence and the structure and evolution of proteins</td>
<td>10</td>
</tr>
<tr>
<td>I.C</td>
<td>BOVINE $\alpha$-LACTALBUMIN</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Historical</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>Covalent structure of $\alpha$-lactalbumin</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Apparent heterogeneity of $\alpha$-lactalbumin samples</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Association and aggregation reactions and changes in conformation</td>
<td>18</td>
</tr>
<tr>
<td>I.D</td>
<td>BIOLOGICAL FUNCTION OF $\alpha$-LACTALBUMIN</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>The lactose synthetase reaction</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-lactalbumin in a control mechanism</td>
<td>22</td>
</tr>
<tr>
<td>I.E</td>
<td>COMPARISON OF $\alpha$-LACTALBUMIN AND LYSOZYME</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Comparison of the amino acid sequences of bovine $\alpha$-lactalbumin and hen egg and human lysozymes</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>The three dimensional structure of lysozyme</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Biological functions of lysozymes</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>A model of $\alpha$-lactalbumin based on the structure of lysozyme</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>ORD and denaturation of $\alpha$-lactalbumin and lysozyme</td>
<td>31</td>
</tr>
<tr>
<td>I.F</td>
<td>LYSOZYMES AND $\alpha$-LACTALBUMINS FROM OTHER SOURCES</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>$\alpha$-lactalbumins from different species</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Immunological reactivities of $\alpha$-lactalbumins</td>
<td>34</td>
</tr>
<tr>
<td></td>
<td>Protein and carbohydrate composition of milks</td>
<td>35</td>
</tr>
<tr>
<td>Table of contents</td>
<td>PAGE</td>
<td></td>
</tr>
<tr>
<td>------------------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>Lysozymes from diverse sources</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Functions of α-lactalbumins and lysozymes</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td><strong>I.G</strong> AIMS OF THIS INVESTIGATION</td>
<td>42</td>
<td></td>
</tr>
</tbody>
</table>

**CHAPTER II MATERIALS AND EXPERIMENTAL**

**II.A MATERIALS**
- A.1 Protein samples 44
- A.2 Enzyme reagents 44
- A.3 Chemicals 44
- A.4 Column bed materials for chromatographic separations 46
- A.5 Glassware 47

**II.B PREPARATION AND ANALYSIS OF PROTEIN SOLUTIONS**
- B.1 Storage of protein 48
- B.2 Concentration of protein solutions 48
- B.3 Measurement of protein concentration 49
- B.4 Dry weight analyses 50
- B.5 Determination of the absorbancy index 51
- B.6 Kjeldahl nitrogen analyses 52
- B.7 Measurement of pH 52

**II.C CHROMATOGRAPHIC SEPARATIONS**
- C.1 Buffers 52
- C.2 Chromatography columns 52
- C.3 Column packing and elution 53
- C.4 Determination of molecular size from elution volumes 54

**II.D STARCH GEL ELECTROPHORESIS**
- D.1 Buffer systems 55
- D.2 Preparation of the gel slab 55
- D.3 Preparation of gels containing urea and 2-mercaptoethanol 56
- D.4 Electrophoresis 56

**II.E AMINO ACID ANALYSIS**
- E.1 Acid hydrolysis of the protein for amino acid analysis 57
- E.2 Oxidation of the protein with performic acid 58
Table of contents:

- E.3 Tyrosine and tryptophan analysis
- E.4 Amide nitrogen analysis
- II.F CARBOHYDRATE ANALYSES
- II.G PREPARATION OF PEPTIDE MAPS
- G.1 Cleavage of disulphide bridges
- G.2 Digestion with trypsin or chymotrypsin
- G.3 N-terminal analysis
- II.H ENZYME ASSAYS
- H.1 Lysozyme activity
- H.2 Lactose synthetase and NAL synthetase assays
- II.I OPTICAL ROTATORY DISPERSION
- II.J SEDIMENTATION VELOCITY

CHAPTER III ON THE FRACTIONATION OF BOVINE α-LACTALBUMINS

- III.A INTRODUCTION
- Fractionation of bovine α-lactalbumin by salt precipitation
- Column fractionation of bovine whey proteins
- III.B SAMPLES AND METHODS
- Milk samples
- Column fractionation
- III.C RESULTS
- Gel filtration of whey proteins
  (a) Fractionation of total whey protein
  (b) Gel filtration of the pH 3.5 precipitate of Method Ia
  (c) Gel filtration of the pH 3.5 precipitates of Methods IIa and IIb
  (d) Effect of dialysis, sample volume and concentration, elution rate, direction of flow and grade of Sephadex
  (e) Lactose synthetase and NAL synthetase activities in fractions from gel filtration
# Table of contents

<table>
<thead>
<tr>
<th>Chapter IV</th>
<th>Isolation and Properties of Bovine α-Lactalbumin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>IV.A</td>
<td>Introduction</td>
</tr>
<tr>
<td>IV.B</td>
<td>Experimental</td>
</tr>
<tr>
<td>IV.C</td>
<td>Results</td>
</tr>
<tr>
<td>IV.D</td>
<td>Discussion</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Chapter V</th>
<th>On the Minor α-Lactalbumin Components of Bovine Milk</th>
</tr>
</thead>
<tbody>
<tr>
<td>V.A</td>
<td>Introduction</td>
</tr>
<tr>
<td>V.B</td>
<td>Results</td>
</tr>
<tr>
<td></td>
<td>Isolation of the minor components</td>
</tr>
<tr>
<td></td>
<td>Starch gel electrophoresis</td>
</tr>
<tr>
<td></td>
<td>Amino acid analyses</td>
</tr>
<tr>
<td></td>
<td>Carbohydrate analyses</td>
</tr>
<tr>
<td></td>
<td>Reaction of α-lactalbumin slow components with neuraminidase</td>
</tr>
</tbody>
</table>
Table of contents ..

<table>
<thead>
<tr>
<th>Ultraviolet absorption spectra of minor components</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactose synthetase specifier activity of the minor components</td>
<td>122</td>
</tr>
<tr>
<td>Gel filtration and sedimentation velocity</td>
<td>123</td>
</tr>
<tr>
<td>Solubility</td>
<td>124</td>
</tr>
<tr>
<td>Peptide maps</td>
<td>125</td>
</tr>
</tbody>
</table>

V.C DISCUSSION 128

TABLE VI APPARENT HETEROGENEITY OF α-LACTALBUMIN IN DEAE-SEPHADEX CHROMATOGRAPHY 136

VI.A INTRODUCTION 136

VI.B EXPERIMENTAL 136

VI.C RESULTS 137

Fractionation of α-lactalbumin by column chromatography and starch gel electrophoresis 137

Rechromatography of fractions on DEAE-Sephadex 138

Chromatography on DEAE-Sephadex after pH 3.5 precipitation 139

Chromatography on DEAE-Sephadex after gel filtration 140

Chromatography in different buffer systems 141

Effect of bed type, temperature, and presence of magnesium chloride on elution from DEAE-Sephadex 142

Column electrophoresis 142

Electrodialysis of sample before fractionation on DEAE-Sephadex 143

Elution of α-lactalbumin A from DEAE-Sephadex 144

Elution of bovine α-lactalbumin minor components on DEAE-Sephadex 144

Activity in lactose synthetase 145

Sedimentation velocity 145

Optical rotatory dispersion 147

VI.D DISCUSSION 148
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER VII LYSOZYMES AND α-LACTALBUMINS FROM DIFFERENT SPECIES</td>
<td>160</td>
</tr>
<tr>
<td>VII.A INTRODUCTION</td>
<td>160</td>
</tr>
<tr>
<td>VII.B EXPERIMENTAL</td>
<td>162</td>
</tr>
<tr>
<td>Milk samples</td>
<td>162</td>
</tr>
<tr>
<td>Preliminary fraction</td>
<td>163</td>
</tr>
<tr>
<td>Preparation of dialysable carbohydrates from whey</td>
<td>165</td>
</tr>
<tr>
<td>VII.C RESULTS</td>
<td>165</td>
</tr>
<tr>
<td>Enzyme activities</td>
<td>165</td>
</tr>
<tr>
<td>(a) Lactose synthetase and NAL synthetase activities</td>
<td>165</td>
</tr>
<tr>
<td>(b) Lysozyme activity</td>
<td>167</td>
</tr>
<tr>
<td>The whey proteins from the placentals and marsupial</td>
<td>169</td>
</tr>
<tr>
<td>(a) Fractionation of human milk</td>
<td>169</td>
</tr>
<tr>
<td>(b) Fractionation of porcine whey proteins</td>
<td>171</td>
</tr>
<tr>
<td>(c) Fractionation of ovine whey proteins</td>
<td>172</td>
</tr>
<tr>
<td>(d) Fractionation of kangaroo milk</td>
<td>173</td>
</tr>
<tr>
<td>Milk from the monotremes; echidna and platypus</td>
<td>174</td>
</tr>
<tr>
<td>(a) Preliminary fractionation of milk</td>
<td>174</td>
</tr>
<tr>
<td>(b) Composition of the dialysable carbohydrates in the whey</td>
<td>174</td>
</tr>
<tr>
<td>(c) Preparation of echidna lysozyme and fractionation of whey proteins</td>
<td>175</td>
</tr>
<tr>
<td>(d) Starch gel electrophoresis of purified lysozymes</td>
<td>176</td>
</tr>
<tr>
<td>(e) Activity of echidna lysozyme in lactose synthetase</td>
<td>176</td>
</tr>
<tr>
<td>(f) Lysozyme activity of the lysozyme from echidna M(1+5)</td>
<td>178</td>
</tr>
<tr>
<td>(g) Fractionation of platypus whey proteins</td>
<td>179</td>
</tr>
<tr>
<td>Comparison of properties of the isolated α-lactalbumins</td>
<td>180</td>
</tr>
<tr>
<td>(a) Starch gel electrophoresis</td>
<td>180</td>
</tr>
<tr>
<td>(b) Function in lactose synthetase</td>
<td>180</td>
</tr>
<tr>
<td>(c) Absorption spectra</td>
<td>181</td>
</tr>
<tr>
<td>(d) Amino acid analyses</td>
<td>182</td>
</tr>
</tbody>
</table>
Table of contents ..

(e) Carbohydrate analyses
(f) Optical rotatory dispersion

VII.D DISCUSSION AND CONCLUSIONS

Fractionation of α-lactalbumins
Fractionation of milk from the monotremes
Comparison of compositions of the proteins
Optical rotatory dispersion
Function of α-lactalbumin in lactose synthetase
The evolution of lactose synthetase

APPENDIX I Buffers

APPENDIX II Publications

REFERENCES
CHAPTER I

INTRODUCTION

I.A GENERAL

The unique function of a globular protein depends on the conformation of the polypeptide chain, and the interaction of the amino acid side chains with each other and with other components in the medium. Investigation of the structure of proteins therefore is fundamental to a knowledge of their mode of action.

The understanding of protein structure has developed considerably in the last fifty years due to advances in many fields. These include: (i) development of efficient methods of protein purification, for example: ion exchange chromatography and gel filtration, (ii) a greater understanding of the covalent structure of proteins which has arisen largely from the development of automated methods of amino acid analysis, reliable sequencing techniques and the use of chemical modification of functional groups, (iii) application of X-ray crystallography to the study of the structure of proteins, (iv) an increased knowledge of the properties of globular proteins in solution and development and refinement of physico-chemical methods such as ultracentrifugation, absorption spectroscopy, optical rotatory dispersion, circular dichroism and electrophoresis.

Investigations of protein structure therefore may be directed along several lines which although extremely valuable in themselves, each has certain limitations which
may be overcome by a thorough analysis by several methods. More recently it has become apparent that further complementary insight into the structure of proteins can be obtained by studying their evolution, through a comparison of proteins from different sources or of genetic variants from the same or similar source. If the nature of the amino acid substitutions or other differences in composition, such as covalently bound carbohydrate, are known in detail, the differing properties may be related to these variations in structure. A knowledge of the amino acid substitutions may also be used to accurately trace the chemical evolution of the protein. Furthermore, proteins isolated from diverse sources and having a specific function may show general features such as similar isoelectric points, molecular weights, or ability to associate, which are common to all proteins of that type. Investigations of the properties of proteins from such sources would be valuable to an understanding of the function of the protein and its behaviour in the cell.

The work presented in this thesis is a study of $\alpha$-lactalbumins from several sources. $\alpha$-lactalbumin is a milk specific protein comprising 1-3 per cent of the proteins in bovine milk (McKenzie, 1967) or 20 per cent of the whey protein (Larson and Jenness, 1955). At the time of commencement of the work reported in this thesis, several detailed investigations had been carried out on the physico-chemical and chemical properties of bovine $\alpha$-lactalbumin, particularly through the efforts of Gordon, Kronman, Weil and co-workers. Recently however, interest in $\alpha$-lactalbumin
has gained considerable impetus following the initial
discovery of its function in the lactose synthetase reaction
by Ebner, Denton and Brodbeck (1966) and the proposal by
Brew and Campbell (1967a) that α-lactalbumin and lysozyme may
share a common ancestor.

Some relevant aspects of the structure of globular
proteins will now be considered followed by a brief review
of the occurrence, isolation and chemical, physical and
enzymatic properties of α-lactalbumins and of their relation
to lysozymes.

I.B ON THE STRUCTURE OF GLOBULAR PROTEINS

X-ray diffraction analysis of protein crystals

X-ray diffraction studies of protein crystals is the
only method available at present which allows detailed
knowledge of the arrangement of atoms in the molecule to
be obtained.

Following the important pioneering work of Astbury on
X-ray diffraction of wool keratin (Astbury, 1938, 1939) the
method was applied to the determination of the structure of
globular proteins. An interesting account of this early
work was presented by Hodgkin and Riley (1968). More specific
details of the method and those results obtained up to 1964
are contained in a review by Dickerson (1964). Later
through the use of multiple isomorphous replacement of heavy
metals and improved methods of computation by fourier
analysis, the structures of several globular proteins have been
presented. Knowledge of the amino acid sequences of these
proteins was essential in these earlier investigations for
the identification of side chains in the electron density maps.
The three dimensional structure of sperm whale myoglobin to 6 Å resolution was reported by Kendrew et al. (1958) and later extended to 2 Å resolution (Kendrew, 1963). Several features were apparent from the model prepared from this data: (i) myoglobin is a spherical molecule with most of the polar side chains distributed on the surface and non-polar side chains situated in the interior with the exception of a hydrophobic pocket which surrounds the haem group, (ii) there is no organized water structure within the molecule although the surface side chains and peptide carbonyl and amide nitrogen groups all have bound water molecules, (iii) the peptide CO-NH bonds are all in trans-configuration and all of the large amounts of α-helix (77 per cent) is right handed.

Subsequently, high resolution X-ray diffraction studies of a number of proteins have been reported. Perutz et al. (1968) presented the three dimensional structure of horse oxyhaemoglobin at 2.8 Å resolution and showed that each of the chains were similar in structure to the myoglobin molecule. Hen egg white lysozyme was the first enzyme for which a high resolution X-ray analysis was completed. In addition to the three dimensional structure, the binding sites of several inhibitors were ascertained and a mechanism of action of the enzyme proposed. Details of the structure of lysozyme are presented later in this chapter.

More recent analyses of several globular proteins have substantiated many of the findings made in the initial work on myoglobin. In addition, the activities of the enzymes reside in very specific areas of the molecule usually incorporated in a cleft or depression where the side chains
may interact most effectively with the substrate (Dickerson and Geis, 1969). Significantly it is now realized that myoglobin is not a 'typical' globular protein since it has an abnormally high amount of helical structure (see also Klotz, 1970).

**Proteins in crystal and solution**

A major assumption made in interpretation of the protein structure obtained from X-ray diffraction studies is that the molecule is in the same conformation in crystal and in solution. Valuable discussions on this problem are presented by Edsall (1968) and Rupley (1969). Protein crystals are generally highly hydrated structures containing large spaces and little contact between the macromolecules. In accord with this statement, Blake *et al.* (1967) conclude that the conformation of lysozyme in the crystal is not influenced greatly by neighbouring protein molecules. Studies on hydrogen-deuterium exchange (Praissman and Rupley, 1964, 1968a,b), binding of inhibitors (Butler and Rupley, 1967), small angle X-ray scattering of solutions (Krigbaum and Kügler, 1970), and prediction of structure by ORD measurements (Hamaguchi and Imahori, 1964) have shown that lysozyme exists in much the same conformation in solution and crystal. Rupley (1969) noted that although different arrangements of proteins in the crystal using different contact points may be postulated, only few are chosen. Since energy is required to alter the conformation, the most stable crystal form requiring minimum energy will be selected to form the lattice.
Solution studies

X-ray crystallography has several further important limitations as a method for studying and relating the structure and function of proteins. The structure obtained from this analysis is static and cannot show directly a mechanism of action of the protein. Studies of the protein in solution are essential to determine the groups involved in the activity unless crystals of a stable complex between protein and substrate may be prepared. Due to the very nature of enzymes this latter procedure may be very difficult or impossible although complexes with inhibitors or analogues of the substrate have been used on several occasions with considerable success (Blow and Steitz, 1970). Furthermore, the forces which allow a particular conformation of the molecule to exist are expressed in the aqueous medium. A study of the properties of the protein under varying conditions such as in the presence of denaturing agents or by changing the temperature are valuable in investigating these forces (Kauzmann, 1959; Tanford, 1968). Methods which may be used for studying the conformation of proteins in solution include optical rotatory dispersion, circular dichroism, hydrogen-deuterium exchange, infrared and difference spectroscopy and nuclear magnetic resonance spectroscopy.

Association and dissociation of proteins

Association and dissociation of proteins and interaction of single protein molecules or complexes with other molecules in solution is important in the regulation and control of metabolic processes. Waugh (1954) describes
two forms of protein interaction, namely; those occurring at such distances that the average properties of the surfaces may be used to calculate the interaction energies, and those at close range where specific local forces are important. In addition, the second process may be subdivided further to define the types of proteins interacting. (i) association-dissociation of similar protein molecules,

\[ nA \rightleftharpoons An \]

(ii) association-dissociation of different proteins

\[ A + B \rightleftharpoons C (+D) \]

The role of protein association in the biological function of enzymes is discussed by Atkinson (1966) and Koshland and Neet (1968) and a list of proteins which are known to exist as associated complexes of identical protein molecules under physiological conditions has been compiled by Klotz, Langerman and Darnall (1970).

The existence of co-enzymes and other molecules which affect the process of enzyme action is now well known. The more readily recognized systems are those in which the bound molecule interacts at the active site or with the substrate. Monod et al. (1963, 1965) have also proposed the existence of allosteric interaction where the bound component, termed an "allosteric affector", alters the conformation of the protein at the active site. The "allosteric affector" need have no relation to the substrate and may interact with the enzyme at a site removed from the protein-substrate binding site. Indeed, Monod et al. (1963) specify that the absence of any inherent relationship of
"affector" to substrate is of great biological importance. Where the single protein molecule or complex also interacts with smaller molecules or ions, the binding sites may be inter-dependent. The concept of reciprocal linked functions was first introduced by Wyman (1948, 1964) who described a mathematical treatment for such interactions which are particularly relevant to haemoglobin. He proposed that such processes may also play an important role in enzyme catalysis and that haemoglobin may be used as an enzyme model.

Changes in the arrangement of groups at points of contact of the associating molecules may have important consequences in the function of the complex. Perutz and Lehman (1968) have estimated the possible effects of substitutions occurring in abnormal haemoglobin chains. Changes at points of contact of the subunits may cause reduction in association resulting in loss of oxygen binding efficiency.

The conformation of the polypeptide chain

Estimations of permissible and most stable conformations of polypeptide chains has been studied extensively particularly by Pauling and Corey and more recently through the work of Schellman, Ramachandran, Scheraga and others. Pauling, Corey and Branson (1951) followed a set of structural principles based on the dimensions of small molecules from measurements obtained from X-ray diffraction studies, to propose several stable ordered conformations of the polypeptide chain. The following conditions were applied: (i) the bond lengths and angles must be identical to those found in simple peptides, (ii) the amide groups must be
planar with the trans configuration preferred, (iii) maximum hydrogen bonding must occur between the peptide carbonyl oxygen and amide nitrogen atoms along the structure, (iv) all amino acid residues must be crystallographically equivalent. Two helical structures; the α-helix having 3.6 residues per turn and the γ-helix of 5.1 residues per turn were found to fulfill these requirements (Pauling and Corey, 1951a). Pauling and Corey (1951b) also described an antiparallel chain, pleated sheet configuration which they proposed may be important in the structure of silk fibroin (Pauling and Corey, 1951b; Marsh, Corey and Pauling, 1955) and that such regular arrangements of the polypeptide chain may also be found in the globular proteins (Pauling and Corey, 1951c). Both helical and pleated sheet conformations have been observed to some extent in the structures of globular proteins studied so far by X-ray crystallography. Myoglobin and haemoglobin contain about 77 per cent helix and carboxypeptidase A contains a large section of anti-parallel chain pleated sheet. All other globular proteins, however, contain much less of both structures.

As more proteins are studied by X-ray diffraction analysis it is becoming obvious that extensive helical or pleated sheet structure need not be present for a stable globular protein. However certain specific requirements must be met to maintain overall low free energy of the structure and these may be satisfied by the formation of helical or other regular arrangements. A polypeptide chain of molecular dimensions and properties described by Pauling
and Corey may only assume a conformation within certain limits of the dihedral angles due to steric hindrance and restriction of rotation about the peptide bond. Several workers have studied these structures extensively and details of their work is reviewed by Schellman and Schellman (1964) and Ramachandran and Sarisekharen (1968).

Scheraga et al. (1967) have attempted to compute possible structures of the polypeptide chain, having minimum free energy, by considering the steric and electrostatic interaction of side chains and formation of intrachain bonds. Preliminary computations of structures of the decapeptide gramicidin S have shown a measure of success although more critical determinations of larger molecules may show that the native structure of a protein is not necessarily one of minimum free energy. The relation between the amino acid sequences and formation of regions of regular conformation such as α-helix has been discussed by Guzzo (1965) and Prothero (1966).

Amino acid sequence and the structure and evolution of proteins

There is increasing evidence to support the view that the amino acid sequence of globular protein determines its three dimensional structure. That the sequence of amino acids is coded for by a linear sequence of bases in the gene indicates that the information required to construct a particular conformation is implicit in such a sequence (Helinski and Yanofsky, 1966; Anfinsen, 1967; Dayhoff and Eck, 1968). The recent chemical synthesis of active ribonuclease A by Gutte and Merrifield (1970) is also relevant; the polypeptide chain was synthesized in a stepwise manner from the C-terminal and
folded into an active enzyme. However, Watson and Kendrew (1961) compared the sequences of sperm whale myoglobin and the α- and β-chains of human haemoglobin and attempted to relate the differences with the structure of myoglobin determined by Kendrew et al. (1961) and of horse oxyhaemoglobin as presented by Perutz et al. (1960). They found poor correspondence between the sequences despite the similarities of structure of these proteins. Watson and Kendrew state "It must be concluded from this that the crucial interactions which determine the tertiary structure of a protein are very complicated and are not confined to the corners between helices".

The conformations of the globular proteins are complex and appear to be determined by the difference in function of each molecule and its relation to the biological system. Therefore protein structure may be delicately controlled by natural selection. Mutations which occur in the sequence of bases in the DNA may produce changes in the sequence of amino acids in the protein but the success of the new protein mutant depends on the effect of the substituted amino acid on the function of the protein. The pressures of natural selection operate on the organism as a whole and although the mutations occur in the base sequence in the genome, the conformation of the completed protein and the requirements of the cell determine the success of the new variant. The fibrous proteins themselves have been selected for their role and a strong regular linear arrangement is essential for their success. The globular proteins however, although maintaining considerable stability and integrity, must direct their action
to a particular function which is associated specifically to a region of the surface of the molecule. They are not necessarily elongated molecules where a linear regular conformation gives strength, but must exist in an aqueous environment in close contact with other components which affect the performance of the side chains exposed to them. The stability of the globular proteins probably relies heavily on the entropy gained from removal of nonpolar side chains from the outside of the molecule and the dispersion of the regions of ordered water molecules around the hydrophobic residues (Kauzmann, 1959).

Protein evolution therefore is concerned with the viable products from this series of mutations. Substitutions which adversely affect the regions of the molecule responsible for the activity are selected against while those aiding or not affecting the function may be incorporated in future proteins. A valuable discussion on these aspects of protein evolution was presented by Smith (1967).

Naurath, Walsh and Winter (1967) describe homologous proteins as those having significant similarity in amino acid sequence, indicating that they have originated from a common ancestor. Studies on homologous proteins are well documented, (Anfinsen, 1959; Dayhoff and Eck, 1968; Jukes and Cantor, 1969). Sanger (1952) provided the first evidence for homology in his investigation of the primary structure of insulin from cattle, pigs, sheep, horses and sperm whales. Subsequently, the sequences of a number of similar proteins from different sources have been studied and the
substitutions compared with the taxonomic classification, e.g., the cytochromes c (Margoliash and Smith, 1965; Margoliash, Fitch and Dickerson, 1968) and the fibrinopeptides (Doolittle and Blomback, 1964).

If a process of evolution is to include the development of the whole expanding biological system, provision must be made for the synthesis of proteins of new function. Gene duplication followed by divergent evolution is a mechanism for allowing the population of genes to increase, thereby enabling a new gene to exist without the pressures of selection. By this process two proteins may arise: one inactive and the original protein of normal function. The inactive protein could undergo further alteration to create a new array of side chains which may perform another role in the cell. Examples such as trypsin and chymotrypsin or muscle phosphorylase and glycogen synthetase have been cited (Neurath, Walsh and Winter, 1967). Brew and Campbell (1967a) proposed that lysozyme and α-lactalbumin evolved in this way.

I.C BOVINE α-LACTALBUMIN

The physico-chemical and chemical properties of α-lactalbumin are reviewed by McKenzie (1967) and the biological function of α-lactalbumin and its relation to lysozyme by Ebner and Brodbeck (1968). More recent reviews embodying the reports published in the last three years has been presented by Ebner (1970) and Gordon (1971).
Historical

Wichmann (1899) prepared a crude whey sample from bovine milk by precipitation of casein and "globulins" on saturation with magnesium sulphate. A protein was crystallized from the whey on addition of ammonium sulphate and acid and due to its solubility in saturated magnesium sulphate it was termed a "lactalbumin".

Sjögren and Svedberg (1930) also prepared crystals from whey by a similar method after removal of casein and globulin by precipitation in 50 per cent saturated ammonium sulphate. They found that the lactalbumin showed heterogeneity on sedimentation and electrophoresis and considering the method of purification it is likely that these samples and those prepared by Wichmann were mixtures of β-lactoglobulin and α-lactalbumin (see also Pedersen, 1940).

Pedersen (1936) identified three major whey protein components in sedimentation velocity studies and designated the slowest of these the α-component. The β- and γ-components proved to be the lactoglobulin of Palmer (1936) (Cannan et al., 1942) and bovine serum albumin (Polis et al., 1950) respectively. Kekwich (see Pedersen, 1936) isolated this α-protein and was unable to detect heterogeneity in moving boundary electrophoresis and ultracentrifugation studies. Svedberg (1937) called the protein α-lactalbumin. Sørensen and Sørensen (1939) isolated a "crystalline insoluble substance" from bovine acid whey and although apparently of similar crystal form to β-lactoglobulin it differed considerably in solubility in dilute salt solution and contained more tryptophan. The protein was purified from an "albumin" fraction by crystallizing the β-lactoglobulin
at pH 5.2 and subsequent precipitation of \( \alpha \)-lactalbumin at pH 4.6. Crystals of \( \alpha \)-lactalbumin were prepared by addition of \((\text{NH}_4)_2\text{SO}_4\) to a solution of the protein at pH 6.6. Several workers have subsequently reported on the fractionation of whey proteins and their results are considered in Chapter III. Due to the large quantities of \( \alpha \)-lactalbumin in milk it was earlier thought to be present solely as a source of protein for the young. More recently, however, Ebner, Denton and Brodbeck (1966) showed that \( \alpha \)-lactalbumin functions in the enzyme system; lactose synthetase (EC 2.4.1.22).

Prior to the discovery of its function and homology with lysozyme, bovine \( \alpha \)-lactalbumin was the subject of several detailed chemical and physio-chemical investigations.

**Covalent structure of \( \alpha \)-lactalbumin**

Bovine \( \alpha \)-lactalbumin is a single chain protein of 123 amino acids which is unusually rich in tryptophan (4-5 residues) and contains four disulphide bridges but no sulphydryl groups (Gordon and Semmert, 1953; Gordon, Semmert and Ziegler, 1954; Gordon and Ziegler, 1955a; Brew, Vanaman and Hill, 1967).

Yasunobu and Wilcox (1958) used dinitrofluorobenzene and phenylisothiocyanate to identify glutamic acid as the single N-terminal residue. This result was confirmed by Weil and Seibles (1961a) and Wetlaufer (1961). The C-terminal leucine was determined by Weil and Seibles (1961a) by cleavage with carboxypeptidase and by Yasunobu and Wilcox (1958) by hydrazinolysis.

The effect of chemical modification of lysines on the digestion of \( \alpha \)-lactalbumin with trypsin was studied by Weil
and Telka (1957). This work was followed by the preparation of several further derivatives including S-sulpho-\(\alpha\)-lactalbumin (Weil and Seibles, 1959) and S-cyanoethyl-\(\alpha\)-lactalbumin (Weil and Seibles, 1961b) and the determination of the sequence of the six amino acid residues from the C-terminal end by the action of carboxypeptidase A and B.

Weil and Seibles (1954) isolated four peptides from a peptic digest and determined the sequence of these 32 residues. Later Dautrevaux et al. (1966a,b) isolated nine peptides from a tryptic digest of oxidized \(\alpha\)-lactalbumin but their sequences were not determined. Brew, Vanaman and Hill (1967) have subsequently published the partial sequence of bovine \(\alpha\)-lactalbumin following the proposal of Brew and Campbell (1967a) that this protein and lysozyme may have evolved from a common ancestor. The complete sequence has recently been published by Brew et al. (1970) and is discussed later in this chapter. Only some sections of the four peptides described by Weil and Seibles could be identified in the complete sequence.

**Apparent heterogeneity of \(\alpha\)-lactalbumin samples**

Gordon and Semmett (1953) reported that samples of \(\alpha\)-lactalbumin prepared by salt fractionation and crystallization gave normal diffusion and sedimentation curves at pH 7.5 in phosphate buffer and calculated a value of 15,100 for the apparent weight average molecular weight \(\bar{M}_{\text{app}}\) using \(\bar{v} = 0.735\) cc/g as determined by Groves. This molecular weight is in good agreement with values of 16,300 obtained by Wetlaufer (1961) from osmotic pressure measurements and 15,500 obtained by Gordon and Ziegler (1955a) from amino acid analysis. These results are
slightly higher than the value of 14,440 calculated from the amino acid sequence study of Brew, Vanaman and Hill (1967). Gordon and Semnett (1953) observed a single peak on moving boundary electrophoresis at pH 3.0 (glycine/HCl buffer), pH 6.6 and 7.7 (phosphate buffer), and 8.5 (veronal buffer), but a bimodal pattern in lactate buffer at pH 3.3.

Klostergaard and Pasternak (1957) also observed a single boundary in sedimentation velocity experiments on similar samples to those used by Gordon and Semnett but obtained bimodal descending electrophoresis patterns in moving boundary experiments at pH 3.3 (lactate), 7.5 (phosphate), 7.5 (tris), and 8.5 (veronal) (protein concentration approx. 10 g/l). The patterns were highly nonenantiographic and at pH 3.3 were protein concentration dependent. The two peaks could not be completely resolved in an extended experiment (16 h 10 V/cm) and a bimodal pattern was again observed on re-electrophoresis of material from the first peak, indicating that the two components remain inter-dependent throughout the experiment.

Wetlaufer (1961) found 30 per cent of the fast peak on moving boundary electrophoresis experiments at pH 7.5. The UV spectrum and sedimentation coefficient of the material from this fast peak was similar to that of the original sample. Wetlaufer also observed 5-10 per cent of a fast band on paper electrophoresis at pH 7.5 and concluded that the apparent heterogeneity of α-lactalbumin samples was probably due to reversible interaction with buffer ions but that minor components of different chemical composition may also contribute to the abnormal patterns. Aschaffenburg and Drewry (1957) also detected a minor fast band on paper.
electrophoresis at pH 8.6 but Robbins and Kronman (1964) observed a band moving behind the major band on starch gel electrophoresis at pH 3.0 in the presence of 5 M urea.

Zittle and Della Monica (1955) also observed two apparently different forms of \( \alpha \)-lactalbumin by extraction of an \((\text{NH}_4)_2\text{SO}_4\) paste of the protein with solutions of decreasing concentration of \((\text{NH}_4)_2\text{SO}_4\). Zittle (1956) reported that the more insoluble form (in 2 M \((\text{NH}_4)_2\text{SO}_4\)) could be transformed to the soluble form by the presence of 0.1 M NaCl or a variety of other salts and concluded that the behaviour was due to the binding of anions. These experiments are discussed further in Chapter VI.

Association and aggregation reactions and changes in conformation

In a series of papers Kronman and co-workers studied in detail the physico-chemical properties of \( \alpha \)-lactalbumin in solution, particularly at low pH. Kronman, Andreotti and Vitols (1964) and Kronman and Andreotti (1964) observed in sedimentation velocity experiments at pH 2.0 and 3.0 (5.8 g/100 ml and 1.5 g/100 ml respectively), time dependent formation of a fast sedimenting peak. The rate of formation of the fast peak decreased with decreasing pH, ionic strength and temperature. Values of 1.95 S and 18 S for \( s_{20} \) of material corresponding to the two peaks were obtained and the curve of \( M_{\text{app}} \) of the slower peak versus protein concentration had a positive slope. Kronman and Andreotti (1964) interpreted these results as association of protein molecules in rapid monomer-dimer equilibrium, superimposed on a time dependent aggregation reaction. No heavy component was observed on the alkaline side of the isoelectric point.
The aggregation reaction was accompanied by swelling of the molecule (Kronman, Andreotti and Vitols, 1964; Robbins, et al., 1967) formation of a tryptophan difference spectrum against a reference protein solution at pH 6 (Kronman, Cerankowski and Holmes, 1965; Kronman, Holmes, and Robbins, 1967) and changes in optical rotatory dispersion properties (Kronman, Blum and Holmes, 1965, 1966). Below pH 4 the absorption spectrum of α-lactalbumin in the region 270-300 nm exhibited a blue shift which was reversible on increasing the pH, and was strongly pH dependent but insensitive to changes in the ionic strength. Kronman, Cerankowski and Holmes (1965) attributed this spectral change to variation in the environment of three buried tryptophan groups. Kronman and Holmes (1965) concluded that of the five tryptophan groups in α-lactalbumin (estimated by Gordon and Semmett, 1953) two are available to solvent at 25°C and the remaining groups are only exposed on reduction of the disulphide bridges in the presence of 8 M urea. Earlier, Weil and Seibles (1959) also found that treatment of the protein with chymotrypsin and carboxypeptidase A liberated only two moles of tryptophan per mole of protein.

In addition to these changes at low pH, a subtle change in accessibility of the two exposed tryptophan groups to sucrose, ethylene glycol and glycerol, was detected by difference spectroscopy on reducing the temperature from 25°C to 1°C at pH 6. At the lower temperature the 'exposed' tryptophans were no longer accessible to large molecules such as sucrose although were still available to small molecules such as D₂O. At pH 1.8-3.0 the change in
accessibility with temperature was more limited, with one group buried with respect to sucrose and glycerol.

The changes in conformation on decreasing the pH appeared distinct from those occurring with change in temperature. Kronman (1967) showed that the conformation changes occurring on reducing the pH below 4 gave rise to a long wavelength shift of 10-18 nm in the ultraviolet fluorescence of \( \alpha \)-lactalbumin. The less drastic process on changing the temperature from 25°C to 1°C at pH 6 resulted in a short wavelength shift of about 10 nm in the emission spectrum. He concluded that the change in accessibility of the two tryptophans to perturbants on decreasing the temperature was due to a contraction of crevices which contain these groups. The change in spectral properties of \( \alpha \)-lactalbumin at low pH probably results from increased freedom of rotation of the three buried tryptophans.

Similar changes in conformation to those observed at low pH were also detected above pH 10. However no aggregation reaction was found at this pH (Kronman, Holmes, and Robbins, 1967; Robbins et al., 1967).

These results are considered further in Chapter VI in relation to the apparent heterogeneity of samples of \( \alpha \)-lactalbumin which was observed in this present work.

**I.D BIOLOGICAL FUNCTION OF \( \alpha \)-LACTALBUMIN**

The lactose synthetase reaction

Watkins and Hassid (1962) reported that lactose may be synthesized by bovine or guinea-pig mammary gland particulate preparations using UDP-galactose and glucose as substrates.
They could not solubilize this lactose synthetase from these preparations but also detected the activity in bovine milk and partially purified it (Babad and Hassid, 1964, 1966). Brodbeck and Ebner (1966) were able to resolve the soluble enzyme into two protein components by gel filtration and one of these proteins, termed the "B protein", was identified as α-lactalbumin (Ebner, Denton and Brodbeck, 1966; Brodbeck et al., 1967). To avoid confusion with the two α-lactalbumin genetic variants also termed A and B the lactose synthetase proteins will be designated "A protein" and "B protein" in this thesis. Following these initial discoveries, work on the lactose synthetase reaction has developed enormously in the past three years. In reviewing the literature on this subject some reports which have been published after commencement of this thesis are also included for continuity.

Brew, Vanaman and Hill (1968) found that the "A protein" also catalyses the synthesis of N-acetyllactosamine from N-acetylglucosamine (NAG) and UDP-galactose. In the presence of α-lactalbumin and glucose this reaction is inhibited and the specificity changes so that lactose is produced as shown in the following reactions:

(a) Reaction catalysed by the "A protein"

\[
\text{UDP-galactose} + \text{N-acetylglucosamine} \rightarrow \text{N-acetyllactosamine} + \text{UDP}
\]

(b) Reaction catalysed by "A protein" plus α-lactalbumin

\[
\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}
\]

More recently Hill et al. (1969) have shown that the reactions are quite specific for glucose or N-acetylglucosamine
and only very low amounts of radioactive products were formed when other closely related saccharides were included in the reaction with UDP-[\(^{14}\)C] galactose.

The "A protein" was found associated mainly with the subcellular particles co-sedimenting with the lysozomes from homogenates of the cells and were thought to have arisen from the Golgi apparatus (Coffey and Reithel, 1968a,b). The "A protein" appears to be widely distributed in animal tissues. McGuire et al. (1965) detected an enzyme, capable of adding UDP-galactose to NAG or a glycoprotein acceptor, in goat colostrum and several preparations of rat tissues. The activity was particularly high in the mammary gland. Brew, Vanaman and Hill (1968) has since shown that this protein behaves in the same way as the "A protein" from lactose synthetase and were able to synthesize lactose when \(\alpha\)-lactalbumin and glucose were added to a particulate preparation from rat liver which possessed galactosyl transferase activity.

The "A protein" is particularly unstable and is not readily solubilized from cell preparations (Brodbeck and Ebner, 1966). Although a soluble preparation may be obtained from milk, further work on the nature of the "A protein" has been hindered by its high lability, with consequent problems on fractionation and storage. These problems are discussed further in Chapters II and III.

\(\alpha\)-lactalbumin in a control mechanism

The "A protein" is held tightly in the mammary gland and only small amounts are secreted in the milk. On the other hand, during lactation, large amounts of \(\alpha\)-lactalbumin
may be isolated from the milk (1-3 mg/ml in bovine milk). Ebner (1970) concluded that although the concentration of \( \alpha \)-lactalbumin in the mammary gland is low, there would be sufficient to inhibit the NAL synthetase activity of the "A protein". More recently, Ebner et al. (1970) reported that \( \alpha \)-lactalbumin, at its concentration in the mammary gland, does not inhibit the formation of the \( \beta (1-4) \) linkage between galactose and NAG when the NAG is joined to another residue. Therefore lactose synthesis and glycoprotein synthesis may occur concurrently. Earlier, Hill et al. (1969) found that the extent of galactose incorporation into an orosomucoid acceptor was reduced slightly in the presence of \( \alpha \)-lactalbumin.

During pregnancy and lactation the synthesis of "A protein" and \( \alpha \)-lactalbumin is asynchronous. Synthesis of "A protein" is high and \( \alpha \)-lactalbumin low during pregnancy. On parturition large amounts of \( \alpha \)-lactalbumin are synthesized, causing a change of specificity of the system to prepare lactose, possibly with some reduction in the synthesis of the link between galactose and NAG. The synthesis of the two proteins is stimulated by different combinations of the hormones insulin, prolactin and hydrocortisone but the changes in hormonal stimuli necessary to induce formation of either protein has not been established (Turkington, et al., 1968).

The lactose synthetase system appears to be unique as a control mechanism in a biochemical reaction. Interaction of an enzyme with another "specifier protein" produces a change in specificity of the enzyme to catalyse a different but
closely related reaction. Such a mechanism allows the conservation of an active site and enables the organism to control two reactions by the synthesis of a specifier protein which in this case is α-lactalbumin.

Although synthesis of large amounts of α-lactalbumin to perform the intracellular function of lactose production appears inefficient, Brew (1969) argues that the continuous flux of α-lactalbumin ensures that its concentration at any time reflects a change in rate of its synthesis. This mechanism enables direct control of lactose synthesis by regulation of α-lactalbumin synthesis.

### I. E COMPARISON OF α-LACTALBUMIN AND LYSOZYME

Brew and Campbell (1967a) noted the marked similarity of molecular weight, amino acid composition and N- and C-terminal amino acid residues of α-lactalbumin and lysozyme. They suggested that α-lactalbumin may have evolved from lysozyme by gradual modification. Support for this hypothesis was presented subsequently by Brew, Vanaman and Hill (1967) by the determination of the partial amino acid sequence of bovine α-lactalbumin. Earlier, Yasunobu and Wilcox (1957) also noted the similarity of molecular weight and tyrosine and cystine residues of bovine α-lactalbumin and hen egg lysozyme but found that the two proteins differed in susceptibility to tyrosinase.

Lysozyme is a small protein which is extremely widespread among plants and animals, and has the ability of hydrolysing certain bacterial cell walls. It is particularly abundant in the white of birds' eggs. α-lactalbumin has only
been found in milk, where it is important in the synthesis of lactose. Some relevant properties of the hen egg lysozyme and bovine \(\alpha\)-lactalbumin are summarized in Table I.1.

Hen egg lysozyme was the first enzyme and the third protein for which a high resolution X-ray diffraction study of the three dimensional structure was completed. It is now a well studied enzyme and a considerable literature has accumulated on aspects of its action and structure. Pertinent details of the protein following the elucidation of its three dimensional structure are contained in a symposium organized by M.F. Perutz (1967) for the Royal Society of London. Other reviews on lysozyme were prepared by Jolles (1960, 1964, 1967) and Feeney and Allison (1969).

Comparison of the amino acid sequences of bovine \(\alpha\)-lactalbumin and hen egg and human lysozymes

The complete amino acid sequence of bovine \(\alpha\)-lactalbumin \(B\) has recently been presented by Brew et al. (1970) and is shown in Fig. I.1 together with the sequence of hen egg lysozyme (Canfield, 1963) and the sequence of the first 57 residues from the N-terminal of human lysozyme (see Brew et al., 1970).

For maximum alignment of sequences, six gaps of a total of nine residues were placed in \(\alpha\)-lactalbumin, three gaps of one residue each in hen egg lysozyme, and one gap of one residue on this section of the human lysozyme chain. A total of 49 residues are identical in the sequences of \(\alpha\)-lactalbumin \(B\) and hen egg lysozyme and in addition, 23 residues in corresponding positions are chemically similar.
TABLE I.1 Comparison of properties of bovine α-lactalbumin and hen egg lysozyme

<table>
<thead>
<tr>
<th>Property</th>
<th>Bovine α-lactalbumin B</th>
<th>Hen egg Lysozyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of Amino-acid residues</td>
<td>123 (a)</td>
<td>129 (b)</td>
</tr>
<tr>
<td>Minimum Molecular Weight (from sequence)</td>
<td>14,437 (a)</td>
<td>14,388 (b)</td>
</tr>
<tr>
<td>Nitrogen Content (g/100g)</td>
<td>15.9 (c)</td>
<td>17.5 (k)</td>
</tr>
<tr>
<td>Number of Cystine Bridges</td>
<td>4 (a)</td>
<td>4 (b)</td>
</tr>
<tr>
<td>N-terminal Residue</td>
<td>Glutamic acid (a, c,d)</td>
<td>Lysine (b)</td>
</tr>
<tr>
<td>C-terminal Residue</td>
<td>Leucine (a,c,d)</td>
<td>Leucine (b)</td>
</tr>
<tr>
<td>Isoelectric point</td>
<td>3.8-5.5 (e,f)</td>
<td>10.5-11 (k)</td>
</tr>
<tr>
<td>Absorbancy Index</td>
<td>20.9 (g), 20.1 (e)</td>
<td>27.3 (h), 26.4 (i)</td>
</tr>
<tr>
<td>Partial Specific Volume (cc/g)</td>
<td>0.735 (c)</td>
<td>0.703 (j)</td>
</tr>
</tbody>
</table>

References.

(a) Brew, Vanaman and Hill (1967); (b) Jolles (1964);
(c) Gordon and Semmett (1954); (d) Weil and Seibles (1961a);
(e) Kronman and Andreotti (1964); (f) Zittle (1956);
(g) Wetlaufer (1961); (h) Glazer (1959); (i) Sophianopoulos et al. (1962); (j) Fevold and Alderton (1949);

Closer correspondence between the sequences of the bovine α-lactalbumin and human lysozyme rather than hen egg lysozyme is apparent from the data at hand although the sequence for the human protein has yet to be completed. Of
FIG. 1.1 Amino acid sequences of bovine α-lactalbumin B and hen egg lysozyme, and the partial sequence of the first 57 residues from the N-terminal of human lysozyme, as presented by Brew et al. (1970).
great importance in the comparison is the identical pairing of the cystine bridges in the two completed sequences. Investigations on the renaturation of α-lactalbumin after breaking these links and disruption of the structure have not been reported.

Manwell (1967) proposed that hen egg lysozyme and bovine pancreatic ribonuclease were also homologous proteins. Of the 122 residues in the sequence of ribonuclease, 16 were in identical positions in lysozyme including six residues which are also present in bovine α-lactalbumin. The positions of the disulphide links and the three dimensional structures of the two proteins, however, are quite different.

Green (1968) noted that avidin and lysozyme also had similar compositions and that tryptophans were necessary in both for binding of substrates. He suggested that these proteins may also share a common ancestor. The amino acid sequence of avidin was determined by De Lange (1970) but no areas of sequence common to lysozyme were found.

The three dimensional structure of lysozyme

Three isomorphous uranium derivatives of lysozyme were prepared and analysed, enabling fourier maps to 2 Å resolution to be constructed (Blake et al. 1965, 1967a). The molecule is roughly ellipsoidal of dimensions 45 x 30 x 30 Å. A deep cleft on one side divides the molecule into two sections which are joined by a length of helix. As found in myoglobin by Kendrew et al. (1961) most of the polar side chains are arranged on the surface while the majority of the nonpolar groups are buried in the core of the molecule. Most of these hydrophobic groups on the surface line the cleft.
This area of the molecule was later found to contain the active site and the hydrophobic residues aid interaction with the substrate. Three sections of chain (5-15, 24-34, 88-96) form a distorted \( \alpha \)-helical arrangement and Blake et al. (1967) describe the sections 24-34 and 88-96 as between \( \alpha \)-helical and 3\(_{10}\) helical. An important feature of the structure is the presence of antiparallel chain \( \beta \)-pleated sheet structure between residues 41-54 as predicted by Hamaguchi and Imahori (1964) from optical rotatory dispersion measurements of lysozyme amide derivatives.

**Biological functions of lysozymes**

Fleming (1922) noted that a substance in many tissues and secretions was capable of lysing certain bacteria, particularly a strain which he called *Micrococcus lysodeikticus*. He named the substance lysozyme. The bond between NAG and N-acetylmuramic acid is now known to be particularly susceptible to the action of lysozyme (Salton and Ghuysen, 1959). Chitin, a polymer of \( \beta \)(1-4) linked NAG is also cleaved (Berger and Weiser, 1967). Sharon and Seifter (1964) reported that lysozyme may also catalyse a transglycosylation reaction and Chipman, Pollock and Sharon (1968) have concluded that the hydrolysis of tetrasaccharide from cell walls to the disaccharide proceeds mainly by a transglycosylation mechanism. The biological functions of lysozyme and \( \alpha \)-lactalbumin are compared in Fig. 1.2.

Blake, North, Phillips and co-workers extended their study on the three dimensional structure of lysozyme to the determination of the position of binding of certain inhibitors. Johnson and Phillips (1965) prepared crystals of...
lysozyme and allowed the inhibitors to diffuse into the lattice from solution. Studies to 2 Å resolution were carried out on the complexes of lysozyme and NAG or tri-NAG (Blake et al., 1967b). The tri-NAG is bound over half of the cleft, forming hydrogen bonds with the main chain CO (107) and NH (59), tryptophans 62 and 63 and aspartic acid 101 and many non-polar interactions.

Blake et al. (1967b) then prepared a model of the complex between lysozyme and hexa-N-acetylchitohexose, based on the structure of the six residues of the hexasaccharide A to F using ABC as the trisaccharide complex. For adequate fitting of the substrate to the site, the pyranose ring of D was distorted from the normal chair to half chair configuration. On the basis of this model and chemistry of the substrates, Blake et al. proposed a mechanism for the cleavage of the hexasaccharide, between residues D and E by interaction with Glu 35 and Asp 52 of the enzyme. Chipman and Sharon (1969) have reviewed the mechanisms of lysozyme action and conclude that the process proposed by Blake et al. is also consistent with a transglycosylation reaction using small substrates.

Support for the formation of a carbonium ion on residue D in the mechanism was presented by Dahlquist, Rand-Meir and Raftery (1968) and implication of Aspartic 52 and glutamic 35 in the active site by Lin and Koshland (1969) and Parsons et al. (1969). Lysozyme has six tryptophans of which three are in the cleft, and at least two of these bind the substrate. Tryptophans 68 and 108 were found to be essential for activity (Hayashi et al. 1965, Hartdegen and Rupley, 1964).
Inactivation of lysozyme occurred when the disulphide bridges were extensively destroyed although partial cleavage left the protein active (Fraenkel-Conrat et al., 1950; Azari, 1966).

A model of α-lactalbumin based on the structure of lysozyme

In view of the similarity in amino acid sequences of α-lactalbumin and lysozyme, Browne et al. (1969) constructed a model of α-lactalbumin based on the co-ordinates of lysozyme as determined by Blake et al. (1967). The backbone structure of lysozyme was prepared and the α-lactalbumin side chains added with some slight modification to allow for differences in size and the insertion of gaps. Browne et al. (1969) concluded that such a structure of α-lactalbumin is consistent with the expected interaction between side chains and the size of added groups. However as Browne et al. (1969) point out, although the sequence of one protein may be accommodated in the three dimensional model of another, the conformations of the molecules may still be quite different, even in those regions where the amino acid sequences are similar. Although the three dimensional arrangement of residues in this model is only one possible form, the following details should be mentioned:

1. The cleft which is the active site of lysozyme is also present in α-lactalbumin although now somewhat altered by the presence of tyrosine 107. The unique glutamic acid 35 of lysozyme is absent and is possibly replaced by histidine 32 or threonine 35.

2. All eleven arginines of lysozyme are replaced, and the clusters of basic groups are absent in α-lactalbumin.
3. Chemical studies have shown that all four tyrosines are titrated normally (Robbins et al., 1967) and react with acetylimidazole (Gorbunoff, 1967). One tyrosine is less reactive with cyanogen bromide however and the model shows that all four tyrosines are on the surface but that the phenolic hydroxyl of Tyr 38 may be less accessible than the others.

4. Kronman et al. (1966) and Kronman (1968) found from solvent perturbation studies that the two exposed tryptophans become less accessible to small molecules on changing the temperature from 25°C to 2°C (see earlier, I.C). On inspection of the model, the configuration of groups around the exposed tryptophans may restrict accessibility as a result of a small conformational change. The remaining two tryptophans are exposed on denaturation of the protein (Kronman, 1967) and these are shown as internal in the model.

**ORD and denaturation of α-lactalbumin and lysozyme**

Herskovits and Mescanti (1965) have reported values of -235 and -145 for $b_o$ of α-lactalbumin and lysozyme respectively in water at pH 7 indicating that the conformation of the polypeptide chains in the two proteins may be quite different. Under these conditions Herskovits (1965) and Herskovits and Mescanti (1965) also compared the difference spectra and optical rotation of solutions of several proteins, including lysozyme and α-lactalbumin, in 8 M urea, formic acid, ethylene glycol, methanol and 2-chlorethanol. They found that the structure of α-lactalbumin is more easily altered by ethylene glycol as shown by change in $b_o$, particularly in the presence of electrolyte. They also found
from difference spectra that the tyrosine and tryptophan residues are more readily exposed in \( \alpha \)-lactalbumin than in lysozyme using the glycol medium. In 9M urea the value of \( b_0 \) for \( \alpha \)-lactalbumin at pH 7.4 and for lysozyme at pH 3.2 are both -10. Maes \textit{et al.} (1969) have reported the \( \alpha \)-lactalbumin is less stable to heat than lysozyme.

Lysozyme is particularly stable to urea at concentrations up to 8M at pH 5.4-5.8 and 25°C (Hamaguchi, 1958; Glazer, 1959) but becomes less stable as the temperature is raised. Guanidine hydrochloride is more effective than urea in disrupting the conformation of lysozyme (Hamaguchi and Kurono, 1963; Jirgensons, 1961; Tanford \textit{et al.} 1966) although even with this reagent no change in optical rotation or viscosity was observed below 3M at pH 7 and 25°C.

Thermodynamic studies of the denaturation of lysozyme by guanidine hydrochloride have been carried out by Aune (1968), Tanford (1968) and Aune and Tanford (1969a,b). In contrast, the effect of these reagents on \( \alpha \)-lactalbumin have not been reported in detail. Atassi, Habeeb and Rydstedt (1970) have found that lysozyme shows a greater tendency than \( \alpha \)-lactalbumin to resist unfolding in 2.5 M guanidine. King (1970) following the increase in size of nuclear magnetic resonance peaks, and Hopper (personal observation), following changes in optical rotation at 313-578 nm, have also found that \( \alpha \)-lactalbumin is more susceptible than lysozyme to denaturation by urea at pH 2.5-3.0 and 6.0-6.5 at 20-25°C.
I.F LYSOZYMES AND $\alpha$-LACTALBUMINS FROM OTHER SOURCES

$\alpha$-lactalbumins from different species

(a) Bovine genetic variants. There are two known genetic variants of bovine $\alpha$-lactalbumin. The B variant is found in all breeds of cattle and is the only $\alpha$-lactalbumin present in stock of European origin. The A variant which is found in some Eastern breeds and in Droughtmaster cattle is described in Chapter IV.

(b) Water buffalo $\alpha$-lactalbumin. $\alpha$-lactalbumin was observed in buffalo milk by Sen and Sinha (1961). Bhattacharya et al. (1963) found that this protein had a similar electrophoretic mobility, nitrogen content, absorbancy index and tyrosine and tryptophan contents to bovine $\alpha$-lactalbumin.

(c) Caprid $\alpha$-lactalbumin. Sen and Chaudhuri (1962) isolated a crystalline lactalbumin from goat milk using a modification of the method of Aschaffenburg and Drewry (1957) and crystallization from (NH$_4$)$_2$SO$_4$ solutions at pH 6. Crystals were also formed from salt free solutions at 2-4°C. Chaudhuri and Sen (1964) (see Gordon, 1971) compared the purified protein with bovine $\alpha$-lactalbumin and concluded from amino acid analyses, sedimentation and diffusion coefficients, electrophoretic mobilities and frictional ratios that it was an $\alpha$-lactalbumin. X-ray crystallographic analysis of goat $\alpha$-lactalbumin is in progress (Hill et al., 1969).

(d) Human $\alpha$-lactalbumin. Johansson (1958) purified a protein having a sedimentation coefficient of 1.73 S from human milk on calcium phosphate columns and indicated that it may be an
α-lactalbumin. Maeno and Kiyosawa (1962) purified the human α-lactalbumin by repeated precipitation at pH 4.8 in the presence of ammonium sulphate. The sedimentation coefficient in 0.1 M phosphate buffer at pH 7.0 was 1.75 S and the molecular weight, calculated from approach to equilibrium experiments was 23,000. From comparisons with bovine α-lactalbumin the protein was considered a 'lactalbumin'.

(e) Guinea-pig α-lactalbumin. Brew and Campbell (1967a) isolated a protein from guinea-pig whey and identified it as an α-lactalbumin from measurements of amino acid composition, N- and C- terminal residues, elution from columns of Sephadex G-100 (MW 15,800), and isoelectric point (pH 4.8). This investigation led to the current interest in the homology of α-lactalbumin and lysozyme. Brew and Campbell (1966b) extended this study to the biosynthesis of α-lactalbumin and concluded that cell free extracts of lactating mammary gland synthesize this protein.

Immunological reactivities of α-lactalbumins

The immunological cross-reactions shown by the milk of a variety of species to antisera prepared to bovine α-lactalbumin was studied by Johke et al. (1964) and Larson and Hageman (1963). The milk from ruminants; (cow, sheep, goat and water buffalo) reacted with the bovine antisera whereas the milk from non-ruminants (camel, horse, rat, mouse, guinea-pig, pig, dog, and rabbit) did not.

Similar results were obtained by Lyster, et al. (1966) on milk from ruminants and non-ruminants. It is interesting to note the inclusion of the pig in the non-ruminant group in view of the presence of an analogue of
β-lactoglobulin in pig milk (Kalan et al., 1968; Kessler and Brew, 1970; McKenzie et al., 1970).

The immunological cross-reactivity between bovine α-lactalbumin and hen egg lysozyme was studied by Atassi, Habeeb and Rydstept (1970) but no reaction was detected by double diffusion and quantitative precipitin experiments.

**Protein and carbohydrate composition of milks**

The protein composition of milk from many species have been compared by Deutsch (1947) using moving boundary electrophoresis at pH 8.6, and Sloan et al. (1964) using paper electrophoresis at pH 8.6. Unfortunately little information on the identity and nature of the individual proteins can be gained from these patterns. Of more importance to the present discussion is the study by Jenness, Regehr and Sloan (1964) on the dialysable carbohydrates from the milk of 58 species in eight orders of mammals, using paper chromatography. They identified the individual sugars by their mobilities relative to glucose (R_g) and detected lactose (R_g = 0.56) in all samples examined. Lactose was the major sugar in milk of all species except the bear (*Ursus horribilis* and *Ursus americanus*), marsupial (*Didelphis virginiana*, *Macropus rufus*, *Setonix brachyurus*) and the rabbit (*Sylvilagus floridanus*). The major free sugars in bear milk are a trisaccharide (R_g = 0.34) and higher oligosaccharide (R_g = 0.11), and in rabbit milk, glucose is present in high amount. Milk from the marsupials contained glucose, galactose and a mixture of oligosaccharides including lactose. Pilson and Kelley (1962) could detect no lactose in the milk of the California sea lion (*Zalophus*...
Milkecalifornianus), by chromatographic procedures. This is the only milk studied so far which does not contain lactose. There have been no reports on the fractionation of this milk and the nature of the whey proteins is not known.

There has been some conflicting reports on the identification of the free carbohydrates in milk of the monotreme, echidna (Tachyglossus aculeatus). Marston (1962) identified lactose in echidna milk by the microscopic appearance of crystals of the osazone derivative prepared from a protein-free filtrate of the milk. He estimated the total sugar content expressed as lactose as 2.81 per cent. Kerry and Messer (see Griffiths, 1968), using paper chromatography, reported no lactose and found very little lactase in the bile of an adult echidna (Kerry, 1969).

Lysozymes from diverse sources

Lysozymes are particularly widespread in nature. The most thoroughly understood lysozyme is that obtained from the white of hen's eggs, and due to the large amount of published work on this protein it is regarded as a convenient point of reference for other lysozymes. Table I.II lists a number of lysozymes from mammals and birds which have been isolated and studied. Some of these and other proteins will now be considered briefly.

(a) Human lysozymes. The lysozymes isolated from human organs and secretions behave similarly and are probably identical proteins (Jollès et al., 1965; Osserman and Lawlor, 1966) in contrast to those isolated from hen lung and hen egg by Jollès and Zuili (1960). The human lysozymes were found to have 2.5-3.5 times the specific activity of
## TABLE I.II Lysozymes from aves and mammals

<table>
<thead>
<tr>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Avian eggs</strong></td>
<td></td>
</tr>
<tr>
<td>Hen</td>
<td>Jollès (1964)</td>
</tr>
<tr>
<td>Duck</td>
<td>Dianoux and Jollès (1967)</td>
</tr>
<tr>
<td>Goose</td>
<td>Canfield and McMurry (1967)</td>
</tr>
<tr>
<td>Turkey</td>
<td>LaRue and Speck (1970)</td>
</tr>
<tr>
<td>Quail</td>
<td>Arnheim et al. (1969)</td>
</tr>
<tr>
<td>Pheasant</td>
<td>Arnheim et al. (1969)</td>
</tr>
<tr>
<td><strong>B. Mammalian organs and secretions</strong></td>
<td></td>
</tr>
<tr>
<td>Dog spleen and kidney</td>
<td>Jollès and Fromageot (1954)</td>
</tr>
<tr>
<td>Rabbit spleen and kidney</td>
<td>Jollès (1964)</td>
</tr>
<tr>
<td>Mice (urine*)</td>
<td>Riblet and Herzenberg (1970)</td>
</tr>
<tr>
<td>Human</td>
<td></td>
</tr>
<tr>
<td>placenta</td>
<td>Petit et al. (1963)</td>
</tr>
<tr>
<td>normal leucocytes</td>
<td>Jollès (1964)</td>
</tr>
<tr>
<td>milk</td>
<td>Jollès and Jollès (1967)</td>
</tr>
<tr>
<td>tears</td>
<td>Parry et al. (1969)</td>
</tr>
<tr>
<td>parotoid secretion</td>
<td>Bonavida et al. (1969)</td>
</tr>
<tr>
<td>urine*</td>
<td>Chandan et al. (1965)</td>
</tr>
<tr>
<td>Bovine milk</td>
<td>Osserman and Lawlor (1966)</td>
</tr>
</tbody>
</table>

* subjects suffering from monocytic and monomyelocytic leukemia
hen egg lysozyme (Osserman and Lawlor, 1966; Jolles and Jolles, 1967; Parry, Chandan and Shahani, 1969), although using the lysoplate method Osserman and Lawlor found that the human lysozyme was twelve times as active. It is a more basic protein than the hen egg lysozyme having an isoelectric point of 10.5-11.0 compared with 10.0-10.5 for the hen egg lysozyme (Parry et al., 1969). Cohen (1969) using proton magnetic resonance studies showed that their active centres were probably similar and Sharon, Jolles and Jolles (1966) found that they functioned by related mechanisms. Osserman and Lawlor (1966) found that rabbit antiserum to human urinary lysozyme did not inhibit the activity of hen egg lysozyme. They found only one common tryptic peptide but recently Canfield has found considerable homology between the two proteins (see Fig. I.1). X-ray diffraction studies have been commenced on the human urinary lysozyme (Osserman et al., 1969).

(b) Lysozymes from the milk of various species. Lysozyme activity has been detected in varying amounts in the milk from numerous species. Prickett, Miller and McDonald (1933) determined the following series in order of decreasing activity: human, monkey (rhesus) dog, cat, rat, llama > rabbit >> bovine > goat, sheep, and guinea-pig. Sloan et al. (1961) detected proteins moving to the cathode in milk from the horse, tapair, bear, dog, and mustela and suggested that they may be lysozymes. However, there was no such component in samples of human milk where lysozyme is known to be present.
Lysozymes have been isolated from human and bovine milk. Bovine milk contains 0-260 µg lysozyme/ml. (Shahani et al., 1962, Chandan, Parry and Shahani, 1965) which is 0.35 times as active as the hen egg lysozyme, in contrast to human milk which contains up to 100 mg/100 ml of a lysozyme which is 2.5-12 times as active as hen egg lysozyme. The isoelectric point of the bovine lysozyme was about 9.5. Buss (1969) reported up to 80 mg/100 ml of lysozyme in baboon milk based on equivalent specific activity of hen egg lysozyme.

(c) Lysozyme from plants and bacteria. Meyer, Hahnel and Steinberg (1946) detected lysozyme activity in the latex of papaya and fig plants. These proteins have been isolated and studied in detail (Howard and Glazer, 1967, Glazer et al., 1969). Both proteins are single chains of molecular weight 25,000-29,000 and have different affinities than the hen egg lysozyme for the usual substrates.

Lysozymes have been shown to be present in certain strains of bacteria (Ghuysen, 1957, and Richmond, 1959) and have been isolated from bacteriophage T2 (Koch and Dreyer, 1958), bacteriophage T4 (Inouye and Tsugita, 1966) and bacteriophage λ (Black and Hogness, 1969). These lysozymes also form a separate class from the animal and bird proteins. The phage lysozymes are single chains of molecular weight approximately 18,000, contain no disulphide bridges and the sequences show no obvious homology to the hen egg lysozyme. The phage lysozymes and plant lysozymes show few similarities to the mammalian proteins and have been proposed as evidence for convergent evolution (Feeney and Allison, 1969).
Functions of α-lactalbumins and lysozymes

The sequence homology of α-lactalbumin and lysozyme introduces several most interesting aspects to the study of the role of α-lactalbumin in lactose synthesis. At first sight the functions of the two proteins appear to be related and may be regarded to act in opposite sense toward a β(1-4) glycosidic linkage: lysozyme in hydrolysis and α-lactalbumin in synthesis, as shown in Fig. 1.2. Brew, Vanaman and Hill (1967) suggested that this difference in function may also be related to the isoelectric points of the proteins (lysozyme = 11, α-lactalbumin = 4-5) since all lysozymes from different sources studied so far are basic proteins and the specific activity of hen egg lysozyme is reduced by chemical modifications which increase its content of acidic groups (acetylation) or remains unchanged on treatments which increase its basisity (guanidination) (Jolles, 1964).

The mechanism of action of lactose synthetase is not clear, due primarily to difficulties in isolation and storage of the "A protein", and although X-ray crystallographic studies on α-lactalbumin are now in progress little clue may be expected from them in deciding the sites involved. The "A protein" is comparatively unstable and crystallization of an AB complex of one exists, for X-ray diffraction studies, is unlikely on present considerations. Much work has still to be carried out on the nature of the "A protein", particularly methods of preparation and storage of an active sample, and the presence of subunits. Only when a pure preparation is
FIG. I. 2 The biological functions of α-lactalbumin and lysozyme.
available can physico-chemical studies on the binding of "A" and "B proteins" be meaningful. Similarly, uncertainties also exist concerning the purity of samples of α-lactalbumin prepared by salt fractionation and crystallization. Further information regarding the nature of the apparent heterogeneity of these samples may be valuable in investigations of the function of α-lactalbumin.

The apparent structural homology between α-lactalbumin and lysozyme may prove to be extremely valuable in the study of the enzyme processes. The close relation of the sequences of the two proteins has been established from statistical analysis (Jukes and Cantor, 1969) and we may assume that they have evolved from a common ancestor. The mammalian and avian lysozymes which have been studied are closely related molecules having similar compositions and isoelectric points and in the few cases recently reported, this close relationship was vindicated in their amino acid sequences, e.g., duck lysozyme (Hermann and Jolles, 1970) turkey lysozyme (LaRue and Speck, 1970) and human lysozyme (see Brew et al., 1970).

Lysozymes are widely distributed proteins but the synthesis of lactose is peculiar to the mammary gland. The initial divergence therefore probably occurred during the differentiation of the mammary cell. The sequence of human lysozyme shows greater similarity to hen egg lysozyme than to bovine α-lactalbumin. Substitutions in α-lactalbumin therefore must have occurred at a greater rate than in lysozymes considering the great taxonomic differences between aves and mammals. Such a rapid rate of mutational change may result in
considerable variation in composition and properties of 
$\alpha$-lactalbumins from different species. Since $\alpha$-lactalbumin
and lysozyme have different highly specific functions and yet
have evolved from a common ancestor, differences in structure
may be related to differences in activity. A comparison
of the compositions and physico-chemical and biological
properties of lysozymes and $\alpha$-lactalbumins from wide selection
of sources may show important features which may be related
to the function and evolution of the proteins, and more
indirectly, the evolution of the mammary gland.

I. G AIMS OF THIS INVESTIGATION

The work described in this thesis is a comparative study
of $\alpha$-lactalbumins aimed to obtain a better understanding of
the properties of this protein in solution and to investigate:
(i) its role in the lactose synthetase reaction and factors
which affect interaction with the "A protein".
(ii) the relationship between the structures and compositions
of $\alpha$-lactalbumins and lysozymes with reference to the
evolution of $\alpha$-lactalbumin.

Critical to any study of $\alpha$-lactalbumin is the isolation
of the protein in high purity. There has been much
controversy as to the extent of true chemical heterogeneity
of bovine $\alpha$-lactalbumin and the extent of apparent
heterogeneity arising out of its interactions in transport
experiments. Thus the first part of the present work is
concerned with procedures for isolation of $\alpha$-lactalbumin
involving salt fractionation and chromatography. This was
extended to embrace a study of the fractionation of bovine
$\alpha$-lactalbumin A and B from Droughtmaster breeds, followed by
a detailed investigation of the chemical, physical and enzymatic properties of the isolated major components of bovine \(\alpha\)-lactalbumin A and B.

It was found in the course of this work that each of these major genetic variants occurs together with three minor \(\alpha\)-lactalbumin components which can be separated from the major protein by suitable fractionation methods. It was of importance to determine the nature of these minor components and their relation to the function of \(\alpha\)-lactalbumin in the lactose synthetase system. Furthermore in chromatography on DEAE-Sephadex, each of the major components can exhibit apparent heterogeneity. This was briefly studied.

The knowledge gained in the above chromatographic studies on the isolation of bovine \(\alpha\)-lactalbumins was then used in a comparative study of the \(\alpha\)-lactalbumins and lysozymes from milk of the human, pig, sheep, kangaroo, echidna and platypus. In each case the chemical properties and enzymic behaviour of the isolated proteins were investigated. Some comparative studies have been reported by other workers on the behaviour of \(\alpha\)-lactalbumin from other species in the lactose synthetase reaction. However this is the first time an extensive study has been made including the monotremes and marsupials which are unique to Australia.
CHAPTER II
MATERIALS AND EXPERIMENTAL

II.A MATERIALS

A.1 Protein samples

Milk proteins. The source of milk samples from various species and the preparation of the milk proteins are given in the relevant chapter.

Hen egg lysozyme. Samples were obtained from Worthington Biochemical Corp., Freehold, N.J.. This preparation described as twice crystallized and salt free (code LYSF), was not purified further.

A.2 Enzyme reagents


Neuraminidase. (RKD 04) Behringwerke, (Australian Hoechst Ltd.).

Pepsin. Three times crystallized, Sigma Chemical Co., St. Louis, Miss.


A.3 Chemicals

Reagents used in this study were of high purity and were selected for low heavy metal content. All water was distilled twice; the second process was carried out in Pyrex glass apparatus. Sources of the less common reagents or those requiring special purification are listed:
Ammonia. A.R. grade ammonia was distilled and collected in distilled water.

Ammonium sulphate. Special Enzyme Grade, Mann Research Laboratories, Orangeburg, N.Y.

Hydrochloric acid.
(a) Used in acid hydrolysis of proteins. "Suprapur", E. Merck, A.G. Damstadt.
(b) Used in preparation of buffers. Constant boiling mixture of water and A.R. grade HCl redistilled by Miss A. McInnes.

Imidazole. "Purum" Fluka A.G. Buchs. Recrystallized from redistilled benzene (90 gm in 110 ml benzene). Small crystals containing low amounts of contaminants which absorbed ultraviolet radiation particularly at 280 nm were prepared if solution was achieved below 35°C and crystals were formed rapidly.

Tris(hydroxymethyl)aminomethane. "Sigma 7-9". Sigma Chemical Co., St. Louis, Miss.

Urea. A.R., Mallinckrodt Chemical Works, St. Louis, Miss. Recrystallized from 70 per cent redistilled ethanol. (two litres of alcohol per 450 g of urea). The urea was dissolved below 50°C, filtered hot, and crystallized rapidly by cooling to 0-4°C.

PPO, (2, 5-diphenyloxazole), and POPOP, [1, 4-bis-2- (5-phenyloxazolyl) -benzene ]. Packard Instrument Co. Inc.

La Grange, Ill.

UDP-galactose, (D-galactose $^{14}$C (U), approximately 200 mCi/mM, New England Nuclear Corp., Boston, Mass.
Micrococcus lysodeikticus cells. Sigma Chemical Co.
St. Louis, Miss.

Acetylacetone. Redistilled at 138-140°C by Dr E.R.B. Graham.


Hydrolysed potato starch. Connaught Medical Laboratories, Toronto, Canada.

Acetone, ethanol, toluene, benzene and acetic acid were redistilled from A.R. grade reagent by Miss A. McInnes.

Dialysis casing, (Visking Co.) and "Millipore" membranes (Millipore Filter Corp.) were heated to 60°C in 3 per cent acetic acid, cooled and washed liberally with distilled water. The dialysis casing was stored in water and the filter membranes air dried.

A.4 Column bed materials for chromatographic separations
(a) Gel filtration Sephadex G-75, G-100 or G-200 fine grade cross linked dextran spherical beads, are products of Pharmacia Fine Chemicals, Uppsala, Sweden, and were used in all gel filtration separations. The Sephadex G-75, G-100 and G-200 were swollen and equilibrated in buffer under reduced pressure (water pump) for two, three and five days respectively.

(b) Ion exchange chromatography. The following ion exchange beds were used for fractionations in this study. (i) DEAE-Sephadex A-50. (Pharmacia Fine Chemicals, Uppsala, Sweden).

Fresh beads were swollen and equilibrated in buffer for two days. The used packing material was regenerated as follows: the beads were washed repeatedly with 0.5 M NaOH on a large sintered glass filter funnel until the effluent was free of chloride. The bed material was then washed with water (five
times bed volume) and then with 0.5 M HCl to introduce the chloride counter-ion. Excess acid was removed by washing with several litres of distilled water and then the desired buffer until the pH of the effluent was the same as the applied solution. (ii) DEAE-cellulose. (Selectacel) Carl Schleicher and Schuell Co., Keene N.H.. Before use the fibres were cleaned using the regeneration procedure outlined above for DEAE-Sephadex. The washing and regeneration cycle was repeated several times to remove the slight yellow colour of the resin. (iii) Amberlite IRC-50. L.R., 100-200 mesh type 1 BDH Laboratory Chemicals Division, England. The resin was cleaned by washing with 0.5 M HCl and then equilibrated with the appropriate buffer. (iv) Dowex I, 200-400 mesh, Fluka A.G. Buchs S.G.. This anion-exchange resin was used after conversion to the acetate or chloride forms by washing with 0.5 M NaOH, water, 3 per cent acetic acid (or 0.5 M HCl) and about twenty bed volumes of water.

A.5 Glassware

Laboratory glassware was of Pyrex type. This glassware was washed in hot detergent solution, rinsed with large volumes of distilled water and rinsed with a 3 per cent (v/v) solution of redistilled acetic acid. A final thorough rinsing with glass-distilled water was made. Spectrophotometer cells were washed with water and then 3 per cent acetic acid or 2 M HCl and finally with distilled water. The cells were periodically soaked overnight in a solution of pepsin (1 mg/ml) in 0.01 M HCl, to remove adsorbed denatured protein.
II. B PREPARATION AND ANALYSIS OF PROTEIN SOLUTIONS

B.1 Storage of protein

Samples containing large amounts of protein or those intended ultimately for physico-chemical studies were usually stored as pastes after precipitation of the protein with 530 g (NH₄)₂SO₄/l. A drop of toluene was placed on the inside of the lid of the jar to inhibit bacterial growth. Protein solutions were prepared by dialysis of the paste against 0.05M KCl, water or an appropriate buffer. Smaller purified protein samples for chemical analysis (amino acid analysis, peptide mapping etc.) were dialysed exhaustively against distilled water over several days, lyophilized and stored under reduced pressure over phosphorus pentoxide.

B.2 Concentration of protein solutions

Efficient concentration of protein solutions is essential if high recoveries are to be obtained from the numerous gel filtration steps carried out, particularly in purification of proteins in very small amount. In the early stages of this investigation, concentrated protein solutions were prepared by the following methods:

(a) Protein was precipitated with 530 g (NH₄)₂SO₄/l, centrifuged and the paste dissolved in buffer and dialysed.

(b) The protein was adsorbed on DEAE-Sephadex and eluted in a small volume with 1 M NaCl in buffer. The effluent was dialysed against a suitable buffer or solution.

(c) The solvent was separated from the protein by ultrafiltration using dialysis casing.

Some loss of protein was incurred in all three methods and (a) and (b) could not be used in the section of the work...
on the anomalous elution of $\alpha$-lactalbumin from DEAE-Sephadex described in Chapter VI. Later, ultrafiltration apparatus became available using filter membranes which were capable of retaining molecules above a certain cut-off size. Diaflo 200, 50 and 10 (Amicon Corp. Lexington, Mass.) were used with UM-2 and UM-10 membranes at a nitrogen pressure of 50 p.s.i.

**B.3 Measurement of protein concentration**

Providing the absorbancy index of the protein was known, its solution concentration was determined by absorbance measurement. Routine estimations were made using a Zeiss PMQ II spectrophotometer with matched 100-QS 10 mm or 1 mm cells. The absorption at 350 nm was subtracted from that at 280 nm as correction for scattered light.

The following values of absorbancy index at 280 nm were used for routine estimations of protein concentration:

<table>
<thead>
<tr>
<th>Protein</th>
<th>$^A_{280} \text{g/dl}^1 \text{cm}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine $\beta$-lactoglobulin  A B or C</td>
<td>9.6</td>
<td>Armstrong and McKenzie (1967)</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>6.6</td>
<td>Glazer et al., (1963)</td>
</tr>
<tr>
<td>Bovine total whey protein</td>
<td>12</td>
<td>Approximate value estimated from composition</td>
</tr>
<tr>
<td>Hen egg lysozyme</td>
<td>27.3</td>
<td>Glazer (1959)</td>
</tr>
</tbody>
</table>
Values of the absorbancy index, $A_{\lambda \text{cm}}^{1 \text{g/dl}}$, at the maximum in the 280 nm region for $\alpha$-lactalbumins from several species were calculated from measurement of dry weight/ash and absorption spectra. These experimental values are given in the relevant chapter. Other solutions of known concentration were prepared by dissolving a given weight of dry lyophilized protein in a known volume of solvent. No correction was made for bound ions which in previous estimations were determined as ash and accounted for up to 5 per cent of the dry weight. All lyophilized protein was stored over phosphorus pentoxide under reduced pressure and no loss in weight was detected on oven drying a sample of $\alpha$-lactalbumin B after storage in this way for five days.

B.4 Dry weight analyses

Aliquots of 2-5 ml containing about 10 mg of protein each in 0.05 M KCl were pipetted into tared silica crucibles which were placed in large weighing flasks with ground glass lids. The 0.05 M KCl solution prepared for dialysis was used in duplicate blanks; the actual diffusate from this dialysis was not used due to possible contamination from protein passing through the sac. The samples were taken to dryness under an infrared lamp (Phillips 13372 E/06) placed about 20 cm above the open flasks, then flasks, crucibles and lids were heated in an oven at 105-108°C for three hours. The vessels and lids were then cooled in desiccators over phosphorus pentoxide for one hour, the lids replaced and cooling continued beside the balance for a further two hours. The closed flasks plus crucibles were weighed on a Sartorius-Werke Model SM 10/100g balance to constant weight.
then reheated and reweighed until after two successive cycles the weight difference was less than 0.00005 g.

The crucibles were removed from the flasks and heated in a muffle furnace at $500 \pm 10^\circ C$ for about 12 hours. The furnace was cooled for two hours before opening, and then the crucibles cooled in the open flasks in desiccators for one hour. Cooling was continued for two hours with the flask lids tightly in place. This procedure was repeated until successive weighings agreed to within 0.00005 g. The results from the ash determination were used as correction for inorganic material which was present in the sample. The following calculation was made:

$$\text{Protein dry weight} = W_s - W_b - (A_s - A_b)$$

where $W_s$ and $W_b$ are the weights of sample and blank respectively and $A_s$ and $A_b$ are the weights of the ash from sample and blank respectively.

B.5 Determination of the absorbancy index ($A_{1\text{cm}}^{\text{g/dl}}$)

The protein solution containing approximately 5 mg protein/ml of solution was dialysed against 0.05 M KCl (five changes of two litres) and passed through a 0.45 $\mu$ Millipore filter. The absorption spectrum of a diluted sample (2.0 ml diluted to 10.0 ml) was recorded at 25°C between 350 nm and 240 nm using a Cary Model 14 spectrophotometer. (Cary Instruments, Monrovia, Calif.) with matched 10 mm Hellma 100-QS cells. The 0.05 M KCl solution prepared for dialysis was used as reference and for blanks in dry weight analyses. The absorbance of 350 nm was subtracted from that of the peak maximum occurring between 270-290 nm to correct for scattered light. This correction was never above 0.002 units.
The net maximum absorbance was converted to a value corresponding to a solution of 1 per cent protein concentration using data from dry weight analyses.

B.6 Kjeldahl nitrogen analyses

Nitrogen was determined by the micro-Kjeldahl method described by McKenzie and Wallace (1955).

B.7 Measurement of pH

Routine pH measurements were carried out using a Leeds and Northrup bench meter (No. 7666) with micro-electrode assembly. The pH scale was standardized at three points as discussed by Bates (1954) using the following buffers: (i) pH 4.008 ± 0.005 at 25°C, 10.12 g/l potassium hydrogen phthalate (0.05 M), (ii) pH 6.865 at 25°C, 3.39 g K$_2$PO$_4$, 3.53 g Na$_2$HPO$_4$ made up to 1 litre, (iii) pH 9.180 ± 0.005 at 25°C, 3.8 g/l sodium borate (0.01 M) Na$_2$B$_4$O$_7$.10H$_2$O

II.C CHROMATOGRAPHIC SEPARATIONS

C.1 Buffers

The compositions of buffers used in column fractionations of proteins are given in Appendix I.

C.2 Chromatography columns

(a) Gel filtration. The following columns were used: (i) LKB ReCyChrom (polymethylmethacrylate) columns (LKB Produkter, Sweden) of dimensions 3.2 cm bore x 40-100 cm length, fitted with reverse flow adapters, (ii) glass columns of dimensions 1.5 cm bore x 40 cm length with fixed length teflon plungers and sintered disc as bed support.

(b) Ion exchange chromatography. All experiments were carried out in glass columns, 1.5 cm bore (CR 32/20, Quickfit and
Quartz Ltd, Stone, England) with a sintered glass disc at the bottom and packed to a height of $20 \pm 0.5$ cm.

C.3 Column packing and elution

The columns were packed by gravity settling and buffer was pumped through the bed overnight to allow equilibration and further redistribution of the gel. The sample and eluting buffer were applied to the column using an LKB Peristaltic pump (Model 4912A) or Beckman Model 746 metering pump. The effluent was received in an LKB Radi Rac 3400 (10 ml or 5 ml syphon), or Beckman Model 132 fraction collector using the time or volume mode of operation. Experiments were carried out at $2 \pm 2^\circ C$ or $20 \pm 2^\circ C$ in thermostatically controlled rooms. Unless stated otherwise, elution was maintained at $0.5 \text{ ml/min.}$

Protein samples for ion exchange chromatography were dialysed against the buffer (in excess of three changes of two changes of buffer) and passed through a $0.8 \mu$ Millipore filter before being pumped onto the bed. Elution from these columns was performed using a linear gradient of NaCl in buffer which was prepared as nine lots of 200 ml solutions increasing in salt concentration from 0.02 to 0.20 M or 0.013 to 0.117 M and pumped from a Technicon Autograd gradient mixer (Technicon Inc., Tarrytown, N.Y.). The elution was generally concluded by pumping 1 M NaCl in buffer through the column. The salt concentration in the effluent was calculated from measurements of conductivity of selected fractions using a Philips QM 4249 meter with a Philips 9510 electrode.
An automatic recording of the effluent absorbance at 280 nm was obtained using an LKB Uvicord II (8300A) or Beckman DB spectrophotometer with 0.2 cm flow cells and a Beckman Potentiometric recorder in the log. mode of operation. In cases where more detailed information was required, manual determination of the absorbance of the individual fractions was carried out. The areas under the peaks in the elution profiles were measured with a planimeter.

C.4 Determination of molecular size from elution volumes

An estimate of the molecular size of some proteins was obtained from the elution volume on fractionation in columns of Sephadex G-75 or G-100, following the method of Andrews (1964)(1965). The method relies on calibration of the column by components of known molecular weight, assuming that they have a similar structure and density as the protein to be measured. Consequently, the values must be regarded as approximate, particularly when considering components of unusual composition such as glycoproteins.

\[ K_{av} = \frac{V_e - V_o}{V_t - V_o} \]

where \( V_e \) is the elution volume; \( V_o \) the void volume and \( V_t \) the total volume of the bed. Figure II.1 shows a plot of log. (molecular weight) versus \( K_{av} \) and \( V_e \), determined for a column of dimensions 3.2 cm bore x 88 cm long (total volume 704 ml) packed with Sephadex G-75 in tris/HCl buffer, pH 7.8,
FIG. 11.1. Plot of $V_e$ and $K_{av}$ versus molecular weight of proteins eluted from a column of dimensions 3.2 cm bore x 88 cm, packed with Sephadex G-75 in tris/HCl buffer, pH 7.8, $I=0.05$. The following molecular weights were used: horse heart cytochrome c, 12,400 (Margoliash, 1962); $\alpha$-lactalbumin B, 14,000 (Brew et al., 1967); Trypsin, 24,000 (Desnuelle, 1960); $\beta$-lactoglobulin C, 36,000 (McKenzie, 1967); Bovine serum albumin, 67,000 (Andrews, 1964); BSA dimer, 135,000 (Hartley et al., 1962); Blue dextran, $2 \times 10^6$. The mixture was heated with rapid stirring over a boiling water bath and was sometimes accelerated by cooling at room temperature for 30 minutes and then at 0-4°C for one hour. This gel could be used after standing for a further half hour at room temperature.
\[ I = 0.05. \] The elution volumes at the peak maximum were used in calculations.

**II.D STARCH GEL ELECTROPHORESIS**

Horizontal starch gel electrophoresis (Smithies, 1955) was performed using a starch gel slab prepared in thin trays of dimensions 20 cm x 15.5 cm x 1.5 mm as described by McKenzie (1971).

**D.1 Buffer systems**

The buffer systems used in electrophoresis are shown in Appendix I.

**D.2 Preparation of the gel slab**

A starch concentration of 15 per cent (w/v) was used for all experiments except in the buffer system of Ferguson and Wallace (1963) at pH 7.5 where 13 per cent was employed. The dry starch (13-15 g) was mixed with 20 ml of buffer in a one litre r.b. flask. The remaining buffer (80 ml) was heated to 80°C in a beaker and added to the well swirled starch slurry. The mixture was heated with rapid agitation over a gas flame until the liquid became mobile before the onset of boiling, then deaerated and the slurry poured onto the tray. The hot starch was covered with a sheet of polythene film. A glass plate was then pressed on top to distribute the liquid and extrude the excess. Heavy weights were placed on the plate and the mixture allowed to set overnight. If the gel was required for protein identification, setting was sometimes accelerated by cooling at room temperature for 30 minutes and then at 0-4°C for one hour. This gel could be used after standing for a further half hour at room temperature.
D.3 Preparation of gels containing urea and 2-mercaptoethanol

A slurry of 25 g hydrolysed starch in 30 ml of the pH 7.5 buffer described by Ferguson and Wallace (1963) was prepared in a one litre r.b. flask. The remaining buffer (57.5 ml) was heated to 70°C, mixed with 64 g of urea in a beaker and heated with stirring to 70°C. The urea solution was poured on the starch slurry and again heated to 70°C, then deaerated and mixed with 0.2 ml of 2-mercaptoethanol in a beaker. The mixture was swirled quickly and poured into the electrophoresis tray. This gel contained a final starch concentration of 17 g/100 ml slurry and a urea concentration of 7 M. To allow adequate reaction, the protein samples were reduced overnight after dilution 1:1 with a solution of the following composition: 10 ml of 10 M urea plus 0.4 ml of a tenfold dilution of 2-mercaptoethanol in water.

D.4 Electrophoresis

All electrophoresis experiments were carried out at 20°C. The samples were applied as impregnated strips of Whatman 3 MM filter paper which were inserted in slots in the gel. Electrical contact between the gel and electrode solutions was made with cellulose sponge strips ("Wettex") and a constant voltage of 150-180 V (7 V/cm) applied from a Duostat RD2 power supply for 4-5 h. After electrophoresis, the gel was inverted and immersed in dye solution (nigrosine, 0.1 per cent (w/v) in methanol, glacial acetic acid, water; 3:2:1 (by volume)) and the glass plate gently lifted from the back of the starch slab. After staining for 15 minutes the gel was drained of dye and washed in a solution of methanol, glacial acetic acid, water; 5:5:1 (by volume).
II.E AMINO ACID ANALYSIS

E.1 Acid hydrolysis of the protein for amino acid analysis

The protein (2 mg) was dissolved in 6 M HCl and sealed in a thick walled, acid washed test tube using the method of Crestfield, Moore and Stein (1963). These samples were hydrolysed at 110°C for 22 hours unless stated otherwise.

The contents of the tubes were evaporated on a Büchi rotary evaporator, dissolved in citrate buffer pH 2.2 and applied to the long and short columns of a Beckman 120B Amino Acid Analyser and eluted following the method of Spackman, Stein and Moore (1958). The analyses were carried out by Mr L.B. James of the Department of Biochemistry, Australian National University, and his valued assistance is gratefully acknowledged.

Glycine, alanine and histidine were used to compute a minimum number of residues in calculation of the compositions of proteins. The estimated molecular weight obtained by summation of the residue weights of the amino acids was compared with the approximate value determined by gel filtration. The recovery of protein as amino acids was determined from the sample dry weight and the total number of micrograms of amino acids eluting in the analysis with appropriate correction for aliquot volumes. No correction was made for inorganic contaminants and the weight of protein was considered to be the dry weight if the sample had been stored with a desiccant as described previously.

Cystine plus cysteine were estimated as cysteic acid after oxidation of the protein with performic acid and tryptophan determined on intact protein by spectrophotometric
methods. Although cystine is estimated in the amino acid analysis, some loss through conversion to cysteine or cysteic acid may occur. Cysteine is eluted co-incident with proline and an increase in the 540 nm peak height was sometimes observed. Proline was therefore determined on oxidized samples.

E.2 Oxidation of the protein with performic acid

Protein samples were oxidized by the method of Schram, Moore and Bigwood (1954) as used by Moore (1963), but the reduction of excess $\text{H}_2\text{O}_2$ with $\text{HBr}$ was omitted since excess reagent was removed during lyophilization (see Hirs, 1967). The reagent was prepared from 0.5 ml of 100 vol. $\text{H}_2\text{O}_2$ and 9.5 ml of 98 per cent formic acid, allowed to stand for one hour at room temperature and cooled to 0-4°C before use. The lyophilized protein (2 mg) was dissolved in 1-2 drops of formic acid and 1 ml of performic acid added. After four hours at 0-4°C the samples were lyophilized, 2-3 drops of deionized water added and relyophilized. These samples were hydrolysed with 6 M HCl and analysed as before. During oxidation, cystine and cysteine are converted to cysteic acid; methionine to methionine sulphone and tyrosine and tryptophan are partially or completely destroyed.

E.3 Tyrosine and tryptophan analysis

Three methods of calculation were compared, based on the absorption spectra of the intact protein in 0.95 M NaOH. Sodium hydroxide was added to the protein solution in 0.10 M NaCl to give an alkali concentration of 0.95 M and the absorption spectra between 350-240 nm recorded at time intervals after mixing using a Cary Model 14 spectrophotometer
with matched 10 mm 100-QS cells. The reference solution contained 0.95 M NaOH and 0.05 M NaCl.

Calculations followed those of Goodwin and Morton (1946), Bencze and Schmid (1957) and Armstrong (private communication). Armstrong's procedure requires measurement of the absorbance at 280 nm and 290 nm. This should be more accurate than the Goodwin and Morton method because the measurement at longer wavelength is made near the peak maximum at 290 nm rather than at 294.4 nm which is on a steep section of the curve. The two absorbance measurements are connected by simultaneous equations derived from analysis of solutions of L-tyrosine and L-tryptophan in 0.1 M NaOH.

\[
\begin{align*}
\text{moles tyrosine/ mole protein} & = \frac{+4.21 \times A_{280 \text{ nm}} - 5.34 \times A_{290 \text{ nm}}}{-5.68194} \times 15,000 \\
\text{moles tryptophan/ mole protein} & = \frac{2.28 \times A_{280 \text{ nm}} - 1.54 \times A_{290 \text{ nm}}}{5.68194} \times 15,000 
\end{align*}
\]

where \( C_p \) is the protein concentration in mg/l. This procedure was used by Bell and McKenzie (1964) and Bell, McKenzie and Shaw (1968) in determination of the amino acid composition of \( \beta \)-lactoglobulin variants.

E.4 Amide nitrogen analysis

Protein amide groups were hydrolysed by the method of Rees (1946) using 10 M HCl in closed flasks for ten days at 37°C.

The liberated ammonia was estimated by the micro-Kjeldahl procedure of McKenzie and Wallace (1955) using the total contents of each flask prepared in duplicate. The
results from amide nitrogen estimations were compared with total nitrogen analyses which were carried out at the same time.

II.F CARBOHYDRATE ANALYSES

All samples were dialysed thoroughly against water, lyophilized and a known weight of dry protein dissolved in water at pH 6.5. The protein concentration was checked by absorbance measurement where possible.

(a) Non-nitrogenous sugars were estimated by the Winzler-orcinol-sulphuric acid method using the modification of Francois, Marshall and Neuberger (1962). Sample solutions contained 2-5 mg of protein/ml. Mannose and galactose were used as standards.

(b) 2-amino sugars were estimated after acid hydrolysis of the protein in 4 M HCl for six hours at 105°C ± 1°C in evacuated sealed tubes. After hydrolysis, the contents of the tubes were adjusted to pH 5-6 with NaOH solutions and made up to 5.0 ml with water. Aliquots of 1.0 ml were taken for analysis.

Amino sugars in the neutralized hydrolysates were determined by the following methods:

(i) The Rondle and Morgan (1955) procedure as modified by Kraan and Muir (1957). The acetylacetone/Na₂CO₃ solution was adjusted to pH 9.8 (from about 9.3) with 10 per cent (w/v) NaOH. To prevent loss of the volatile 2-methylpyrrole the reaction of sugar with acetylacetone was carried out in tubes with glass stoppers which were held firmly in place while heating in a water bath.
(ii) The Cesi and Piliego (1960) procedure as described by Neuberger and Marshall (1966). This method was used for one set of analyses of the α-lactalbumin minor components. The plot of absorbance at 548 nm against amino sugar concentration of the series of standard solutions was best fitted by a curve which deviated from linearity above about 50 µg of sugar. Cesi and Piliego recommended 5-50 µg of hexosamine per analysis but Neuberger and Marshall (1966) extended this range to 10-80 µg. The results were similar to those obtained using the method of Rondle and Morgan. The reason for the non-linearity at the high concentration of hexosamine is not known.

(iii) In amino acid analysis. Glucosamine and galactosamine were also detected as peaks in the elution profile from amino acid analysis. However considerable destruction of the carbohydrate moiety occurred during hydrolysis with acid and these analyses must be regarded as qualitative. Glucosamine and galactosamine were eluted in this order after phenylalanine although this position was variable and using a different column the glucosamine was eluted before tyrosine. Calibration of the procedure using hydrolysed samples of amino sugars as standards or more gentle hydrolysis conditions was not attempted.

(c) Sialic acids were determined by the resorcinol method of Svennerholm (1957) using NANA as standard.

(d) 6-deoxyhexoses were estimated by the method of Gibbons (1955).

In each case blanks containing water and α-lactalbumin B together with reagents were treated simultaneously. The
water blank was used as reference in measurement of absorbance and the \( \alpha \)-lactalbumin blank used as correction for colour reaction of protein alone. This correction was not constant in different sets of analysis however and varied from 0-1 per cent apparent carbohydrate. The correction is discussed in Chapter V. In the estimation of 6-deoxyhexoses, measurements of absorbance at 396 nm and 427 nm of all solution before addition of the thioglycollic acid reagent were used to correct for protein colour reaction.

**II.G PREPARATION OF PEPTIDE MAPS**

**G.1 Cleavage of disulphide bridges**

Cystine was oxidized to cysteic acid using performic acid as described in the section on amino-acid analysis. After 4-5 hours reaction at 0-4°C the mixture was lyophilized, 20 ml of water added (plus 1 ml formic acid to dissolve) and relyophilized.

**G.2 Digestion with trypsin or chymotrypsin**

Lyophilized, oxidized samples of protein were dissolved in 0.5 per cent \( \text{NH}_4\text{HCO}_3 \) and aliquots of a 0.1 per cent solution of TPCK treated trypsin in 0.01 M HCl were added to give 1 mg trypsin/100 mg protein. These solutions were maintained at 37°C for two hours, then lyophilized. A tryptic digest of \( \alpha \)-lactalbumin B was allowed four hours reaction, but the resulting peptide map was the same as for the two hour hydrolysis time. Chymotryptic hydrolysis was performed using the same procedure.
When core material was to be removed as in the comparison of a-lactalbumin A and B, the digest was suspended in 1-2 ml of water and glacial acetic acid added to pH 4. The precipitated core was separated by centrifugation and the residue and supernatant analysed individually. When preliminary protein comparisons were made, the sample digest including core was dissolved in formic acid or pH 1.9 buffer and electrophoresis carried out at pH 1.9 in the first dimension. The protein digest (2 mg) was dissolved in 10 μl of buffer or acid and applied as a line (2 cm length) to Whatman 3 MM paper using an extended pasteur pipette. The paper was wet with buffer which was applied at either side of the origin and then blotted to remove the excess.

High voltage paper electrophoresis was carried out in vertical tanks designed after Ryle, et al. (1955) and Michl (1958), using mineral turpentine as cooling medium. Electrophoresis was performed at 2000 V and 100-300 mA for one hour. The first dimension was cut out as a strip and sewn onto fresh paper for the second dimension. The volatile buffer systems used in electrophoresis are described in Appendix I.

Ascending chromatographic separations were developed using the system: pyridine, isoamyl alcohol, water; 7:7:6 (by volume) in cylindrical tanks. The papers were stained with a spray of 0.01 per cent (w/v) ninhydrin in acetone. Tryptophan and tyrosine containing peptides were detected by viewing the maps before a UV lamp. In some cases the patterns were sprayed with acetone, suspended in an atmosphere of chlorine for two hours and sprayed with the
starch/KI reagent described by Rydon and Smith (1952). This method was used to supplement the ninhydrin spray and is more effective for long or acidic peptides.

The spots to be analysed were cut out, washed with acetone to remove ninhydrin, and the peptides eluted with 6 M HCl prior to hydrolysis and amino acid analysis.

G.3 N-terminal analysis

Edman degradation was carried out by the modification of Fraenkel-Conrat (1954) and Fraenkel-Conrat, Harris and Levy (1955) as described by Schroeder (1967). The peptide to be treated was located on the map by staining a small strip along the edge of the one dimensional pattern, then cut out and treated in situ with the phenyl isothiocyanate reagent. After extraction of the phenyl thiohydantoin of the N-terminal residue, the procedure was repeated to obtain the derivative of the new N-terminal residue. Specimens of glutamine and glutamic acid hydantoins were prepared by the method of Edman and Lauber (1956). A control peptide containing cysteic acid as N-terminal, and free lysine were treated in the same manner as the peptide under discussion. Chromatograms of the derivatives were prepared by ascending technique on paper and Eastman Kodak thin layer plates containing a fluorescent indicator. The solvent system: formamide, N-butylacetate, propionic acid; 7.4:194:5.8,(by volume) was used.

II.H ENZYME ASSAYS

H.1 Lysozyme activity

Lysozyme activity was estimated from the rate of clearing
of suspensions of dead lyophilized *Micrococcus lysodeikticus* cells as described by Parry, Chandan and Shahani (1965). This method was considered superior to those described by Smolelis and Hartsell (1949), Jolles (1962) and Shahani *et al.* (1962), due to its sensitivity, speed of operation and because calculations are based on initial rate and are not influenced by product inhibition. However a major limitation of the assay is that the substrate and end products are not clearly defined. The products from the reactions of isolated lysozymes with *Micrococcus lysodeikticus* were not investigated. Despite this limitation the method is rapid and reproducible and is particularly suitable for this present study where very low concentrations of lysozyme were determined. The lysophate method described by Osserman and Lawlor (1966b) is lengthy and reported values for human lysozyme were considerably higher than by measurement of transmittance of cell suspensions. The lysophate method depends on the rate of diffusion of the lysozyme through agar and therefore differences may be recorded which are not due to the specific activity of the protein.

**Method** A suspension of dead lyophilized *Micrococcus lysodeikticus* cells (50 mg/100 ml) was prepared in phosphate buffer pH 6.5, $I = 0.05$. The enzyme preparation (0.1 ml) was added to a 10 mm spectrophotometer cuvette containing 1.5 ml of cell suspension and 0.5 ml of 0.3 M NaCl or water at 25°C. The contents of the cell were mixed by inversion and the transmittance ($T$) at 540 nm recorded at 30 second intervals up to three minutes using a Zeiss PMQ II spectrophotometer with water as blank. The initial transmittance of the assay
mixture was approximately 10 per cent and increased as the suspension cleared. When the plot of T versus t was not linear up to 1 1/2 minutes the analysis was repeated on more dilute enzyme solution. The activity is recorded as the initial slope $\frac{dT}{dt}$ of the plot T versus t. Solutions of 0.1 ml containing 0.1-100 μg of lysozyme were readily assayed by this method.

H.2 Lactose synthetase and NAL synthetase assays

(a) Preparation of reaction mixture. These estimations were based on a modification of the methods of Babad and Hassid (1966) and Palmiter (1969) using incorporation of $^{14}$C] galactose into lactose or N-acetyllactosamine.

The final reaction mixture consisted of:

0.10 ml stock reaction mixture containing

- 1 μmole MgCl$_2\cdot$6H$_2$O;
- 0.5 μmole MnCl$_2\cdot$4H$_2$O;
- 3 μmole glucose;
- 0.15 μmole ATP, made up in tris/HCl buffer pH 7.1, $I = 0.05$

0.10 ml enzyme preparation or

- 0.05 ml "A protein" plus 0.05 ml "B protein"
- 0.01 ml (approx. 30 μmoles) UDP-$^{14}$C galactose

(26,000 cpm, approx. 200 mCi/mM.)

TOTAL VOLUME 0.21 ml.

In NAL synthetase assays 0.45 μmole N-acetylglucosamine replaced glucose.

The solutions were added together in indentations on a sheet of "Parafilm" and mixed by drawing into a pasteur pipette and ejecting onto the film. The reaction was commenced on addition of the UDP-$^{14}$C galactose and the solution transferred to small glass tubes (volume 0.5 ml, 1.5 mm bore) for reaction at 37 ± 0.5°C. The reaction was
terminated by applying the mixture to the top of a small column of Dowex 1 (in chloride form) prepared in a pasteur pipette with a plug of sintered polythene as bed support. These columns were packed to a height of 2 cm and washed with approximately 10 ml of water. Before application of the reaction mixture all excess liquid was forced through the pipette by gentle pressure from a rubber pipette bulb and the bed was kept dry. Similarly the sample was forced through the bed followed by two lots of 0.1 ml water. Over 90 per cent of the unbound labelled components were eluted by this procedure.

(b) Scintillation counting. Sample preparation for scintillation counting was carried out by the method of Davies and Cocking (1966). An aliquot of the reaction effluent (0.1 ml) was transferred to a glass fibre disc which was dried under an infrared lamp for 15 minutes. The discs were placed in Packard scintillation vials with 2.0 ml of scintillant of the following composition: 0.4 per cent (w/v) PPO, 0.01 per cent (w/v) POPOP in redistilled AR toluene. A Packard Tri-Carb liquid scintillation spectrometer was used to record 10 minute counts.

Each set of analyses (up to 20 reactions) contained controls without both or one enzyme. A disc containing 0.01 ml of the UDP-[14C] galactose solution (approx. 200 mCi/mM, 26,000 cpm) was measured each time samples were counted and a counting efficiency of 88 per cent determined. The background counting rate was 20 cpm determined for 10 minute counts on the vials without samples. Less than 40 per cent of the UDP-[14C] galactose was converted to products in
the assays and normally the reactions were designed to employ less than 25 per cent of the reagent.

(c) **Preparation of "A protein".** A partially purified preparation of "A protein" for "B protein" estimation was obtained by fractionation of total whey protein from fresh bovine milk on Sephadex G-75 as described in Chapter III. Since the "A protein" quickly lost activity on storage (a solution of crude preparation in tris/HCl buffer, pH 7.5 at 0-4°C lost over 50 per cent of its activity in two days) rapid purification was necessary and some impurity could not be avoided if the sample was to be used within 10 hours of milking.

(d) **Units of Activity.** Due to the presence of impurities and rapid inactivation, the concentration of "A protein", and hence its specific activity in NAL synthesis, could not be estimated. The amount of lactose or NAL synthesised was proportional to the number of disintegrations/min. of the Dowex 1 effluent of the reaction mixture containing labelled UDP-galactose. As a working unit the percentage lactose or NAL synthesised was calculated as follows:

\[
\text{Per cent lactose} = \frac{c_t - c_b}{c_i} \times \frac{0.41}{0.10} \times 100
\]

where:  
- \(c_t\) = counts per min. at time t. (normally 10 minutes)  
- \(c_b\) = counts per min. of blank without enzyme  
- \(c_i\) = counts per min. of 0.01 ml UDP-[\text{14C}] galactose  

\(26400 \pm 600\ \text{cpm}\)

Babad and Hassid (1966) defined a unit of the enzyme as the amount which catalyses the conversion of 1 per cent of UDP-[\text{14C}] galactose to [\text{14C}] lactose in one minute at 37°C.
Clearly in such analyses under these conditions the nature of variables such as the concentration of active protein or inhibition by inactive protein or other impurities, do not permit accurate comparisons of results from experiments performed at different times. Care was taken to keep conditions as constant as possible and in comparative work, estimations were carried out in the same experiment or included samples to which they could be referred.

II.1 OPTICAL ROTATORY DISPERSION

ORD spectra was recorded between 400 nm and 195 nm on a Cary Model 60 recording spectropolarimeter (Cary Instruments, Monrovia, Calif.) at 27°C using 10.0 mm and 1.0 mm Q.U.V. cells (OpticellCell Co. Inc.). The cells were cleaned by washing with 3 per cent (v/v) acetic acid and water and dried with a stream of nitrogen.

Protein samples were dialysed thoroughly against the appropriate buffer or salt solution (five changes of three litres) and passed through a 0.45 μ Millipore filter. Protein solution concentration was determined by absorbance measurements. The corrected rotations were converted to the reduced mean residue rotation \([m']_\lambda\) using the following equation:

\[
[m']_\lambda = \frac{3}{n^2+2} \times \frac{\text{MRW}}{100} \times \frac{a_\lambda}{d_c}
\]

Where \(a_\lambda\) is the observed rotation, corrected for blank, of the protein solution of concentration \(c\) g/100 ml in a cell of path length \(d\) decimeters. The mean residue weight (MRW) was calculated from the amino acid and carbohydrate composition.
of each protein. The mean residue weight of α-lactalbumin was 115.8, and for hen egg lysozyme 114.5. These values are somewhat different from those used by Herskovits and Mescanti (1965) [123.4 and 111 respectively].

Values for the Lorentz-Lorentz correction factors for refractive index dispersion, $\frac{3}{n^2+2}$, were obtained from tables presented by Fasman (1963), assuming that the refractive index of dilute salt solution was the same as that of water.

II.J SEDIMENTATION VELOCITY

Sedimentation velocity experiments were carried out in a Spinco Model E analytical ultracentrifuge with photochopper speed control and schlieren optical system equipped with a phase plate. A titanium AN-H rotor was used at a maximum speed of 68,000 rpm (333,800 g at cell centre) and the temperature regulated to 20 ± 0.1°C with the RTIC system. Experiments were performed with a single sector cell containing a Kel-F centrepiece.

Photographs of the schlieren pattern were taken at 16 min. intervals after the rotor had reached maximum speed. The position of the maximum ordinate was measured using a two co-ordinate micro-comparator (Gaertner Toolmakers Microscope Type M2001 AS-P) and the sedimentation coefficient determined from least squares calculations of plots of log x versus t, where x is the distance moved by the maximum ordinate in time t. This value was corrected to a value at 20°C in water ($s_{20,w}$) by the following equation:
Values of $n_{20}$ were obtained from Svedberg and Pedersen (1939) and $\bar{v}$ for $\alpha$-lactalbumin was taken as 0.735 (Gordon and Semmett, 1953) and corrected for temperature using the expression:

$$\bar{v} = 0.735 - 0.0004(30-t)$$

(McKenzie et al., 1967). The relative viscosities ($\frac{n_{sol}}{n_w}$) of the tris/HCl buffer and the tris/HCl buffer plus 0.05 M NaCl used in these experiments were determined with an Ostwald type viscometer having a flow time of 140 sec. $n_{rel}$ of tris/HCl buffer pH 7.5, $I = 0.05 = 1.0278$ and $n_{rel}$ of tris/HCl buffer plus 0.05 M NaCl = 1.0389.

This corrected value of the sedimentation coefficient however may only be compared with values which have been obtained under the same conditions of temperature and solvent.
CHAPTER III
ON THE FRACTIONATION OF BOVINE $\alpha$-LACTALBUMINS

III.A INTRODUCTION

Fractionation of bovine $\alpha$-lactalbumin by salt precipitation

Gordon and Semmett (1953) prepared the crystalline insoluble substance (see Chapter I) by the method of Sørensen and Sørensen (1939) and found that the diffusion and sedimentation coefficients agreed with those determined by Pedersen (1936) on the $\alpha$-lactalbumin prepared by Kekwick. Subsequently Gordon, Semmett and Ziegler (1954) showed that the solubility of $\alpha$-lactalbumin was decreased at pH 4.0-4.6 and improved separation from the albumin fraction was achieved at this pH in 1.3 M (NH$_4$)$_2$SO$_4$. This modified procedure (Gordon and Ziegler, 1955b) allowed some preliminary separation of $\alpha$-lactalbumin and $\beta$-lactoglobulin although crystallization of the latter protein was still an important step and the $\alpha$-lactalbumin was crystallized from a solution containing a considerable amount of $\beta$-lactoglobulin. They observed that the presence of $\beta$-lactoglobulin hindered crystallization of $\alpha$-lactalbumin.

Aschaffenburg and Drewry (1957) described a method of milk fractionation commencing with precipitation of casein in the presence of 20 g Na$_2$SO$_4$/100 ml at 40°C. They made the important observation that as the pH of the Na$_2$SO$_4$ whey solution was lowered to 2.0 the solubility of $\alpha$-lactalbumin and serum albumin decreased and the $\beta$-lactoglobulin remained largely in solution. After several
further precipitation steps, fractions were obtained which could be used for crystallization of α-lactalbumin and β-lactoglobulin. However this method has several disadvantages: (i) the milk must be warmed to 40°C to dissolve the Na₂SO₄ for casein precipitation, (ii) concentrated acid and alkali are used for pH adjustment with the risk of local pH excesses, (iii) inadequate studies of detailed conformation have been carried out to determine beyond doubt whether irreversible changes occur during the treatment at pH 2, (iv) the protein precipitate obtained from addition of acid or (NH₄)₂SO₄ to the Na₂SO₄ whey solution is not readily separated from the supernatant by centrifugation, (v) crystallization of salt may occur if the temperature of the whey solution falls. However the principle of the method is an important contribution to the fractionation of bovine milk proteins.

Robbins and Kronman (1964) modified the method of Gordon and Ziegler (1955b) to accommodate larger volumes, but again the separation of α-lactalbumin was far from complete. Other fractionation procedures such as precipitation and separation as ferric complexes (Zweig and Block, 1954), or with acetone (Bleumink, 1966), have been reported.

Final purification of the α-lactalbumin obtained by these methods was achieved by repeated crystallization from 50-60 per cent saturated (NH₄)₂SO₄ as described by Gordon and Ziegler (1955b). However Larson and Hageman (1963) found that samples of α-lactalbumin prepared by salt fractionation and two or three recrystallizations, did not give a single immunological response when used as an antigen. Further
crystallization steps were necessary to obtain an immunologically homogeneous product.

In this laboratory, Armstrong, McKenzie and Sawyer (1967) reported the results of an extensive investigation on the salt fractionation of bovine whey proteins and described several methods based on precipitation of α-lactalbumin and serum albumin at pH 3.5. They used solutions of lower concentration of acid and alkali than Aschaffenburg and Drewry (1957) for pH adjustment, and (NH₄)₂SO₄ rather than Na₂SO₄ to carry out the fractionation. Three major procedures, Methods Ia, IIa and IIb were described and are summarized in Fig. III.1. The pH 3.5 precipitate from Methods Ia and IIa contained the bulk of the α-lactalbumin but this was heavily contaminated by β-lactoglobulin and BSA, particularly in fractionation of milk which was homozygous in β-lactoglobulin A. The pH 3.5 precipitate from Method IIb contained large amounts of α-lactalbumin but this was less contaminated by the other whey proteins. This fraction is therefore an excellent starting material for subsequent isolation of α-lactalbumin in high purity using column chromatographic methods.

Although salt fractionation at neutral or low pH and recrystallization have been used to prepare samples of α-lactalbumin, the method suffers from several disadvantages:
(a) Paper electrophoresis patterns at pH 7.5 and 8.6 of samples of α-lactalbumin prepared by these methods contain a minor band moving ahead of the major band. Aschaffenburg and Drewry (1957) and Wetlaufer (1961) found that this pattern was retained even after repeated crystallizations. Wetlaufer also observed multiple boundaries on moving boundary electrophoresis
Whole milk
+ 264 g\((\text{NH}_4)_2\text{SO}_4\)/l

Precipitate
Casein, fat.
Supernatant (whey)

<table>
<thead>
<tr>
<th>METHOD Ia.</th>
<th>METHOD IIa.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add 1 M HCl to pH 3.5</td>
<td>+ 262 g((\text{NH}_4)_2\text{SO}_4)/l</td>
</tr>
<tr>
<td>Supernatant a</td>
<td>Precipitate(*)</td>
</tr>
<tr>
<td>Precipitate</td>
<td>dissolve in 1/10th whey volume of water. Add 1.0 M HCl to pH 3.5</td>
</tr>
<tr>
<td>Add 1/10th whey volume of 0.005 M NH₃</td>
<td></td>
</tr>
<tr>
<td>and adjust to pH 7 with 1 M NH₃</td>
<td></td>
</tr>
<tr>
<td>Solution contains α-lactalbumin with</td>
<td>Supernatant b</td>
</tr>
<tr>
<td>appreciable amounts of serum albumin and</td>
<td></td>
</tr>
<tr>
<td>β-lactoglobulin.</td>
<td></td>
</tr>
</tbody>
</table>

Solution contains α-lactalbumin less contaminated with serum albumin and β-lactoglobulin than in Method Ia.

FIG. III.1. Summary of fractionation of bovine whey protein as described by Armstrong et al. (1967). In Method IIb the precipitation at pH 3.5 (after *) is carried out at 2 ± 2°C giving a precipitate which is rich in α-lactalbumin and containing traces of serum albumin. Supernatants a and b may be fractionated further for β-lactoglobulin. The precipitate marked * is the total whey protein fraction used as starting material in most of the chromatographic separations carried out in this present study.
of similar samples and suggested that the electrophoretic heterogeneity of $\alpha$-lactalbumin may be due to interaction with buffer ions or to the presence of a minor impurity of similar composition to the major component.

(b) It is not possible to separate single $\alpha$-lactalbumin genetic variants by these methods.

(c) The salt fractionations are lengthy since the crystallization should be repeated up to five times for adequate removal of $\beta$-lactoglobulin and BSA, and the recovery of $\alpha$-lactalbumin from these steps is low. Aschaffenburg (1968) described a method for obtaining higher yields of $\alpha$-lactalbumin from bovine or caprid milk by precipitation with trichloracetic acid (3 per cent, w/v). The effect of these conditions on the conformation of the protein is not known.

(d) At pH values below its isoelectric point, $\alpha$-lactalbumin exhibits pronounced association and aggregation leading to precipitation. Kronman and co-workers have studied these changes in detail and concluded that the association and aggregation processes occurring at low pH are reversible on increasing the pH (Kronman and Andreotti, 1964; Kronman, Andreotti and Vitols, 1964). However since the samples prepared in this present study were partly intended for use in measurement of physico-chemical properties related to protein structure, a method of preparation which did not involve such pH changes was preferred.

Column fractionation of bovine whey proteins

Gel filtration and ion exchange chromatography have been used to limited extent to fractionate milk proteins. These methods may overcome the difficulties described for salt fractionation at low pH and have been used extensively in this
present study for the preparation of α-lactalbumin and for
detection and isolation of whey proteins from a number of species.

Préaux and Lontie (1961) and Préaux et al. (1965)
chromatographed solutions of lyophilized, dialysed whey proteins
at pH 4.7 (acetate buffer $I = 0.05$) on Sephadex G-75. They
resolved three major whey protein peaks, but the second peak which
contained β-lactoglobulin was the only fraction showing a single
band on electrophoresis. Morr, Kenkare and Gould (1964)
fractionated skimmilk on Sephadex G-75 and G-100 in phosphate
buffer (pH 7.0, $I = 0.10$) but the resulting separation of
proteins was poor.

Groves (1965) found that the α-lactalbumin fractions obtained
by stepwise elution of a bovine whey preparation from columns of
DEAE-cellulose in phosphate buffer did not exhibit the fast band
on polyacrylamide electrophoresis at pH 4.3 or 9.5. Attempts to
produce this fast band by varying the conditions of sample
preparation to simulate salt fractionation were unsuccessful.
Szuchet-Derechin and Johnson (1965) also fractionated samples of
whey proteins into several peaks on DEAE-cellulose using a pH and
ionic strength gradient in phosphate buffer. The α-lactalbumin
fraction appeared to contain little impurity in paper and starch
gel electrophoresis patterns at pH 8.4 and 9.0 respectively and
was used for further characterization by ultracentrifugation.

In 1963, Armstrong and McKenzie initiated some studies on
the chromatographic fractionation of bovine whey proteins. They
separated the $(NH_4)_2SO_4$ whey protein fraction into five peaks by
gel filtration on Sephadex G-75 in phosphate buffer at pH 7.0
and studied briefly the fractionation of whey proteins on DEAE-
Sephadex A-50. Chromatography of samples of whole whey protein
on DEAE-Sephadex A-50 using a linear gradient of NaCl in buffer
was not as successful as gel filtration, however since the proteins were eluted together in a series of overlapping peaks and profitable fractionation was not achieved in this step. They observed that anion exchange chromatography of \( \beta \)-lactoglobulin rich samples previously obtained by salt fractionation at low pH was more promising for the isolation of this protein.

Subsequently in a study of the isolation of \( \beta \)-lactoglobulin McKenzie and Murphy were concerned about the possibility of conformational changes near pH 7 and decided to use a lower pH for fractionation. The final choice of pH 6.3 (imidazole/HCl buffer, \( I = 0.043 \)) was a compromise between the changes in \( \beta \)-lactoglobulin at higher pH and possible changes in \( \alpha \)-lactalbumin at low pH, and the avoidance of the pH region of low solubility of \( \alpha \)-lactalbumin.

In view of the limitations of the methods using salt precipitation after pH adjustment and the potentiality of the column chromatographic procedures, the latter were used extensively in this present investigation of bovine whey proteins.

Chromatography of the whey protein fractions prepared by precipitation at low pH were included to determine a rapid method of isolation where detailed conformation studies are not intended. Gel filtration on Sephadex G-75 or G-100 was used as a preliminary fractionation and final purification achieved by chromatography of the \( \alpha \)-lactalbumin rich fraction on DEAE-Sephadex A-50. This anion exchange packing material was preferred to DEAE-cellulose because:

(a) DEAE-Sephadex has a higher exchange capacity due to the ability of the proteins to penetrate the gel matrix rather than be absorbed only on the surface of the particles.

(b) As a dextran gel of properties of Sephadex G-50, separation
on the basis of size as well as charge may give enhanced fractionation.

Some experience was gained in column fractionation of bovine whey proteins, particularly β-lactoglobulin, for a period of six months prior to commencement of candidature for the Ph.D. degree. The results from this preliminary work and the present study are contained in the publication by Armstrong, Hopper, McKenzie and Murphy (1970).

III.B SAMPLES AND METHODS

Milk samples

Milk samples, homozygous in α-lactalbumin A or B were obtained by hand milking or using a machine which allowed collection from individual animals. In the early stages of the work on α-lactalbumin B, milk was collected at the Moggill farm of the University of Queensland, Veterinary School, under the supervision of Dr K. Bell, Department of Preventive Medicine, or from the Camden farms of the University of Sydney, under the supervision of Dr A. Lascelles. In later work, samples were collected from cows at the Northfield Research Laboratories, South Australian Department of Agriculture, Adelaide. Each of the Adelaide cows involved was checked for sub-clinical mastitic infection at the time of sample collection. The following examinations were made: RMT (rapid mastitis test), Hopkirk test, cell count and heat stability of the casein micelles. The tests were carried out by L.F. Bailey, J.T. Feagan and A. Hehir.

The milk was cooled to 0-4°C and flown to Canberra in large dewar flasks containing several crystals of thymol to inhibit bacterial growth. Fractionation of milk from Adelaide and Camden was commenced no later than five hours after milking, while
samples from Brisbane were treated within twelve hours. Fat was separated from the milk by centrifugation at 3,000 rpm for 40 minutes in an International centrifuge (Model PR-2) using a 276a head, or in a Servall RC-2B centrifuge using a GSA rotor. In some experiments, casein and fat were removed together after addition of \(264 \text{ g}(\text{NH}_4)_2\text{SO}_4/\text{l}\). All \((\text{NH}_4)_2\text{SO}_4\) fractionations were carried out using Methods Ia, IIa or IIb of Armstrong et al. (1967) as shown schematically in Fig. III.1. The total whey protein fraction which was used frequently in this study was the Method IIa whey precipitate after addition of \(262 \text{ g}(\text{NH}_4)_2\text{SO}_4/\text{l}\) before fractionation at pH 3.5.

**Column fractionation**

The experimental techniques and apparatus used in gel filtration and ion exchange chromatography experiments are described in Chapter II. In the initial chromatography experiments, imidazole/HCl buffer pH 6.3, \(I = 0.043\) was used for the reasons set out in the introduction to this chapter. However improved fractionation may possibly be obtained at higher pH considering the relative isoelectric points of the proteins, and since \(\beta\)-lactoglobulin was not required in these preparations, the buffer system tris/HCl, pH 7.8 (at \(2 \pm 2^\circ\text{C}\)), \(I = 0.05\) was used in many subsequent experiments. In addition, use of imidazole in the buffer system may also have some disadvantages in fractionation of \(\alpha\)-lactalbumin. Shinitzky et al. (1966) reported the inhibition of hen egg lysozyme by imidazole and indole derivatives possibly through the formation of a charge transfer complex with the tryptophan residues in the enzyme. In view of the possible similarity between \(\alpha\)-lactalbumin and lysozyme such a complex between imidazole and \(\alpha\)-lactalbumin may also occur.
III.C RESULTS

Gel filtration of whey proteins

(a) Fractionation of total whey protein. More than 150 fractionations of bovine whey proteins have been performed in columns of Sephadex G-75 or G-100 in this study. A typical elution profile is shown in Fig. III.2a and the starch gel electrophoresis patterns of the fractions in Fig. III.2b. The protein concentration of each sample used for electrophoresis was adjusted to approximately 2 g/100 ml prior to application to the gel to enable minor components to be detected more easily. Thus the band intensities in each fraction give some indication of the proportion of the components in that fraction, but do not reflect the total amount of a given protein in the sample chromatographed. The individual fractions will now be discussed.

Fraction I. This peak was eluted in the void volume of Sephadex G-75 (also G-100 in later experiments) and consisted of high molecular weight material giving bands moving slower than α-lactalbumin on starch gel electrophoresis at pH 7.5 or 8.6. This fraction was frequently coloured yellow and Dr W.H. Murphy (private communication) indentified transferrin and lactoferrin in the fraction from its iron (III)-binding capacity, positive sialic acid tests, and behaviour in sedimentation velocity experiments.

Fraction II. This fraction gave a major band with a similar mobility to BSA on starch gel electrophoresis at pH 7.5 or
FIG. III.2 (a) Elution profile of bovine total whey protein ( \( \alpha \)-lactalbumin B, \( \beta \)-lactoglobulin A) on elution from a column of Sephadex G-75 (3.2 cm x 80 cm) in imidazole/HCl buffer, pH 6.3, \( I = 0.043 \). Sample applied: 10 ml, \( A_{280 \text{ nm}} = 35.4 \), approx. 0.3 g protein. Elution rate 0.5 ml/min. Protein recovery, 99 ± 1%. 

(b) Starch gel electrophoresis pattern of fractions shown in (a). Protein concentration approx. 2 g/100 ml (except Fraction V). Buffer system; pH 7.5 semi-discontinuous system of Ferguson and Wallace (1963); 7 V/cm, 4 h. The pattern marked "S" is the total whey protein. The Fraction V is dilute and the minor components can barely be detected on the gel. The fast minor band is shown more clearly in Fig. III.7b (Pattern S).
8.6. However the peak was sometimes overwhelmed by the 
β-lactoglobulin fraction which followed, and a separation was 
considered optimum when a sharp maximum was visible in the 
profile. The amount of serum albumin present varied 
considerably in preparations from milk samples of different 
animals.

Fraction III. β-lactoglobulin was eluted as a single peak 
showing little asymmetry. Some minor contaminating BSA was 
also frequently present.

Fraction IV. The major proteins eluted in this peak were found 
to be glycoproteins with an amino acid composition identical 
to the α-lactalbumin genetic variant present in the milk. A 
more detailed account of the composition and properties of 
these proteins is given in Chapter V.

Fraction V. This peak consisted predominantly of α-lactalbumin 
but included appreciable amounts of β-lactoglobulin and proteins 
from peak IV in the leading edge. Starch gel electrophoresis 
patterns of the fraction taken from within both extremities of 
the peak showed an additional fast band moving between 
β-lactoglobulin and α-lactalbumin. This protein is a third 
minor species of α-lactalbumin in bovine milk and is also 
discussed in Chapter V. Providing 20-40 per cent of the leading 
edge of the peak is rejected, a product free of appreciable 
impurities except the fast component may be isolated.

In addition to the five peaks shown in the elution profile 
of Fig. III.2a, another large peak eluting after α-lactalbumin 
has been observed in some patterns. Samples of this material
gave a negative biuret test and an absorption spectrum was recorded between 240-320 nm showing a maximum in the 278-280 nm region. The spectrum of thymol was qualitatively the same as this pattern and the material is possibly thymol which was added as preservative. Morr, Kenkare and Gould (1964) observed a similar low molecular weight non-protein peak in fractionations of skim milk on Sephadex G-75. They did not identify the compound or mention the use of such a preservative in their preparations.

(b) Gel filtration of the pH 3.5 precipitate of Method Ia

A solution of the pH 3.5 precipitate from Method Ia of Armstrong et al. (1967) was neutralized, the protein reprecipitated with 530 g (NH₄)₂SO₄/1 and centrifuged. This paste was dialysed against buffer and the resulting solution fractionated on Sephadex G-75 giving an elution profile such as shown in Fig. III.3. As expected, the β-lactoglobulin peak is smaller than in a similar fractionation of total whey protein (see Fig. III.2a) resulting in less contamination of the α-lactalbumin fractions.

(c) Gel filtration of the pH 3.5 precipitates of Methods IIa and IIb. The elution profile from gel filtration of the pH 3.5 precipitate using Method IIa was qualitatively similar to that of the Method Ia precipitate although containing much less β-lactoglobulin.

Method IIb is similar to Method IIa except that the precipitation is carried out at 0-4°C instead of 20°C and results in a fraction of α-lactalbumin which is less contaminated by other proteins. The elution profile of the pH 3.5 precipitate reflects the potentiality of this separation.
FIG. III.3 and 4. Elution profiles of solutions of the proteins in the pH 3.5 precipitates of Method Ia and IIb of Armstrong et al., 1967 from a column (3.2 cm bore x 82 cm) of DEAE-Sephadex A-50 in imidazole/NaCl buffer, pH 6.3. The samples were homozygous in β-lactoglobulin A and α-lactalbumin B.

FIG. 3. Method Ia, 10 ml applied; $A_{280 \text{ nm}}^1 \text{ cm} = 128.0$

FIG. 4. Method IIb, 8.5ml applied; $A_{280 \text{ nm}}^1 \text{ cm} = 99.0$

The major peak is α-lactalbumin and the minor peaks contain iron binding proteins, serum albumin and β-lactoglobulin.
A typical example is shown in Fig. III.4. (d) Effect of dialysis, sample volume and concentration, elution rate, direction of flow, and grade of Sephadex. From the results of fractionations of total whey protein performed in columns of Sephadex G-75 or G-100, an attempt was made to determine the effect of several parameters on the separation of these proteins. The elution volumes of the peak maxima and the approach of the elution profile to the baseline between peaks were compared for each column experiment.

A small improvement in resolution of α-lactalbumin and β-lactoglobulin was apparent when a solution of an (NH₄)₂SO₄ paste of total whey protein was dialysed against four litres of buffer and applied to the column. This pattern was compared with that for a similar sample without dialysis. Other fractionations of dialysed samples did not show striking improvement in resolution.

Protein samples of volume 5-35 ml containing the same amount of whey protein were applied to a column of dimensions 3.2 cm bore x 84 cm and eluted at 0.5 ml/min. Deterioration in separation was apparent for sample volumes greater than about 20 ml. For the column described, good results were obtained for 10 ml samples of 7-10 per cent protein concentration or 15 ml samples of 5-6 per cent protein concentration.

Using the same column, an elution rate of 0.5 ml/min. was considered maximal for good results, as some increase in tailing of peaks was apparent when the elution rate was increased to 1.0 ml/min. The effect of this increased rate was not serious however and the more rapid elution could be
used for preparative work. In the experiments described using 0.5 ml/min. the fractionation was completed in about sixteen hours allowing one separation per day.

Sephadex G-75 and G-100 (fine) were used successfully to separate the whey proteins into five peaks. A slight improvement in resolution of α-lactalbumin and β-lactoglobulin was observed with the Sephadex G-100 bed.

The separation of whey proteins appeared to be the same when elution was carried out in upward or downward direction although the sample and eluent were applied more easily if pumped in upward direction. In several experiments a selected portion of the effluent containing α-lactalbumin was recycled through the same column. The effluent from the second cycle showed better separation between peaks but these were now broader and the protein solutions more dilute. No additional peaks were observed.

(e) Lactose synthetase and NAL synthetase activities in fractions from gel filtration. These activities were estimated in the effluent from a fractionation of fresh total whey protein on Sephadex G-75 in imidazole/HCl buffer, pH 6.3, $I = 0.043$. As shown in Fig. III.5 both lactose synthetase and NAL synthetase activities were eluted in two peaks. The second peak probably contains the "A protein" alone and the first active fraction may be the "AB" complex or a higher aggregate of the "A protein". The second peak has an approximate molecular weight of 60,000 estimated from its elution volume. The second active fraction from a similar experiment was concentrated by ultrafiltration and fractionated further in a column of Sephadex G-200 in imidazole
FIG. III.5. Elution profile from Sephadex G-75 at pH 6.3 of total whey protein. 11.0 ml applied, \( A_{280}^{\text{\text{nm}}} = 61.0 \).

FIG. III.6. Elution profile from Sephadex G-200 at pH 6.3 (3.2 cm bore x 40 cm) of "Fraction II" from fractionation shown in FIG. III.5. 5.0 ml applied, \( A_{280}^{\text{\text{nm}}} = 18.0 \).

---

- Absorbance at 280 nm
- \( A_{280}^{\text{\text{nm}}} \) for N-acetyllactosamine synthetase activity
- \( A_{280}^{\text{\text{nm}}} \) for lactose synthetase activity
buffer, pH 6.3. The elution profile is shown in Fig. III.6. The fraction containing most of the activity consisted mainly of BSA as determined by starch gel electrophoresis at pH 7.5. The Fraction II from Sephadex G-75 fractionation was used frequently as source of a crude preparation of the "A protein" for enzyme assays. Further limited purification was attempted by passing this fraction through a column of DEAE-Sephadex A-50 in tris/HCl buffer, pH 7.2, $I = 0.05$. BSA adhered to the gel bed and about 30 per cent of the applied NAL synthetase activity was eluted in the first 10 ml of effluent. The activity in following fractions was not determined. The "A protein" therefore appears to have an isoelectric point above 7.2 in this buffer system although the low recovery of activity may be due to binding of active protein to the column.

Chromatography of the α-lactalbumin rich fractions on DEAE-Sephadex A-50.

Anion exchange chromatography was used in addition to gel filtration in this study to fractionate whey proteins on the basis of charge rather than size differences. The method may also facilitate removal of the fast band observed in zone electrophoresis patterns of α-lactalbumin prepared by salt fractionation and gel filtration. A clean separation of the major whey proteins was not obtained by chromatography on DEAE-Sephadex using a sample of total whey protein since the α-lactalbumin was eluted with BSA in a series of poorly resolved peaks. The fractionation of α-lactalbumin on DEAE-Sephadex was more successful if this protein was separated from BSA in the preliminary gel filtration step as described earlier.
The fractions rich in α-lactalbumin were usually concentrated by precipitation, dialysed against buffer, and pumped into the column packed with DEAE-Sephadex A-50. Dialysis was not necessary if the effluent from the gel filtration step was applied to the DEAE-Sephadex column directly or after concentration by ultrafiltration. Due to the concentrating effect of the DEAE-Sephadex, sample volumes up to 200 ml could be accommodated without serious loss in resolution.

Elution profiles containing two major protein peaks were obtained when samples of α-lactalbumin, e.g., Fraction V from gel filtration, were applied to columns of DEAE-Sephadex A-50 in tris/HCl buffer, pH 7.8 or imidazole/HCl buffer, pH 6.3. A typical elution profile is shown in Fig. III.7a. The protein from both major peaks had the same mobility on starch gel electrophoresis as shown in Fig. III.7b. The behaviour of α-lactalbumin on elution from DEAE-Sephadex is described in more detail in Chapter VI. The α-lactalbumin shown in Fraction 3 is contaminated by a small amount of the protein of Fraction 4. This is due to a slight overlap of peaks and by discarding portion of the protein in the trailing edge of the peak in Fraction 3, the protein obtained moves as a single band on electrophoresis.

Fraction 1 of the first major peak of Fig. III.7 behaves identically on starch gel electrophoresis as the forms of α-lactalbumin isolated in Fraction IV from the Sephadex G-75 fractionation (Fig. III.2). The trailing peak (4) corresponds to the fast band observed in starch gel electrophoresis, patterns of α-lactalbumin prepared by salt fractionation or gel filtration. These proteins from Fractions 1 and 4 are
FIG. III.7 (a) Chromatography on DEAE-Sephadex A-50 in imidazole/HCl buffer pH 6.3, $I=0.04$, of Fraction V from gel filtration of total whey protein (see Fig. III.2.). Column: 1.5 cm bore x 20 cm. Sample: homozygous in $\beta$-lactoglobulin C and $\alpha$-lactalbumin B, stored as $(\text{NH}_4)_2\text{SO}_4$ paste and dialysed against pH 6.3 buffer (3 changes of 2,000 ml) prior to application to the column. 25 ml applied, $A_{280\text{ nm}} = 50.0$. Protein recovery $87\pm3\%$.

(b) Diagram of starch gel electrophoresis patterns at pH 7.5 of the fractions shown in (a). Ferguson-Wallace buffer system, 7 V/cm for 4 h. The concentration of each sample was adjusted to approx. 2 g/100 ml prior to electrophoresis. Pattern "S" is the sample applied to the DEAE-Sephadex.
discussed more fully in Chapter V. Fraction 5 of Fig. III.7a is $\beta$-lactoglobulin C which was present in this sample. Some difficulty was experienced due to overlap of $\alpha$-lactalbumin and $\beta$-lactoglobulin peaks using particular combinations of genetic variants of each protein. $\beta$-lactoglobulin Droughtmaster was eluted at lower salt concentration than $\beta$-lactoglobulin C while $\alpha$-lactalbumin A was eluted at higher salt concentration than $\alpha$-lactalbumin B. Overlap of $\beta$-lactoglobulin Droughtmaster and $\alpha$-lactalbumin A is therefore a major problem in isolating this $\alpha$-lactalbumin variant and it was sometimes necessary to repeat the gel filtration step to ensure removal of all $\beta$-lactoglobulin contaminants.

The fractionation was concluded by pumping 1 M NaCl through the column to elute a small amount of UV absorbing material. The nature of this product and the material eluting before the gradient was applied were not studied further.

In cases where only $\alpha$-lactalbumin was required and pH adjustment could be tolerated, fractionation was commenced with the pH 3.5 precipitate of Method IIb. This was then eluted through a column of Sephadex G-75 and the $\alpha$-lactalbumin rich fraction chromatographed on DEAE-Sephadex. However the order of elution through these columns was sometimes reversed with satisfactory results. Elution profiles obtained from chromatography of samples of the Method IIb pH 3.5 precipitate on DEAE-Sephadex are shown in Fig. VI.4.

### Fractionation of $\alpha$-lactalbumin heterozygote

An attempt was made to separate the $\alpha$-lactalbumin A and B genetic variants from a heterozygous mixture. The partially
purified Fraction V from a previous fractionation of total whey protein on Sephadex G-75 was applied to a column of DEAE-Sephadex A-50 and eluted with a linear gradient of 0.02-0.2 M NaCl in imidazole/HCl buffer, pH 6.3. The resulting pattern was complex, consisting of three sharp peaks, each containing both variants. The third peak, eluting at 0.10 M NaCl was \(\alpha\)-lactalbumin A, only slightly contaminated by the B variant. Further fractionations of mixtures of \(\alpha\)-lactalbumin A and B were not attempted.

III. D DISCUSSION

Milk whey proteins may be fractionated and purified quickly, and with very high recovery using a combination of gel filtration and ion exchange chromatography. In addition, the procedures are particularly suited to the isolation of proteins which are available in very small amount such as the minor bovine \(\alpha\)-lactalbumins or in the fractionation of proteins from other species such as the echidna as described in Chapter VII.

A partial fractionation of the whey proteins is effected by gel filtration on columns of Sephadex G-75 or G-100 in imidazole/HCl buffer pH 6.3, \(I = 0.043\), or if \(\beta\)-lactoglobulin is not required, using the higher pH of tris/HCl buffer pH 7.8, \(I = 0.05\). Chromatography of one of these fractions (Fraction V) on DEAE-Sephadex A-50 results in \(\alpha\)-lactalbumin free of impurities detectable by starch gel electrophoresis at pH 8.6 and 7.5.
The two column fractionation procedures are complementary. The minor \( \alpha \)-lactalbumin component observed as a fast band on zone electrophoresis is separated from the major \( \alpha \)-lactalbumin by anion exchange chromatography. However the preliminary gel filtration step must be carried out to remove the other whey proteins, particularly BSA which interferes with the elution of \( \alpha \)-lactalbumin from DEAE-Sephadex.

If an efficient method of concentrating the protein solutions is used, e.g., ultrafiltration, the recovery of purified \( \alpha \)-lactalbumin from whey should be above 80 per cent provided that the fractions are carefully selected and several chromatographic steps carried out. The yield of \( \alpha \)-lactalbumin from whey by salt fractionation and one crystallization is reported to be less than 60 per cent (Aschaffenburg, 1968) and considering that the crystallization should be repeated at least three more times for removal of most of the \( \beta \)-lactoglobulin and serum albumin, the recovery of purified \( \alpha \)-lactalbumin from whey by this method is probably closer to 30-40 per cent. In addition, column methods are gentle and may be used to check the purity of the preparation, particularly if the normal absorption measurements of protein concentration are accompanied by estimations of enzyme activity in the fractions.

Larger amounts of protein may be fractionated using a sample rich in \( \alpha \)-lactalbumin prepared by preliminary salt precipitation at pH 3.5, e.g., using Method IIb of Armstrong et al. (1967). The high capacity of the DEAE-Sephadex may then be used to accommodate the increased amount of protein, bearing in mind the limitations of combinations of \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin variants. The small amount
of BSA found in the pH 3.5 precipitate of Method IIb did not interfere with the elution of $\alpha$-lactalbumin in this fractionation. However some contaminating BSA remained in the $\alpha$-lactalbumin fractions and this could be removed by gel filtration.

Three minor $\alpha$-lactalbumin components were observed and isolated in these fractionations. One of these proteins is eluted with the main $\alpha$-lactalbumin on Sephadex G-75 and after the main peak on DEAE-Sephadex. It is likely that this $\alpha$-lactalbumin minor component is similar to that in the fast band observed on starch gel electrophoresis and also reported by Aschaffenburg and Drewry (1957) and Wetlaufer (1961). The two other minor $\alpha$-lactalbumins were found to be glycoproteins which are eluted together, ahead of the main peak on Sephadex G-75 and ahead of the main peak on DEAE-Sephadex; but separated from each other. These minor proteins are termed FC, SC1 and SC2 in order of decreasing electrophoretic mobility at pH 7.5. They are discussed in Chapter V.

The behaviour of bovine $\alpha$-lactalbumin on column chromatography and zone electrophoresis is complex. In addition to the presence of three minor components, bovine $\alpha$-lactalbumin is eluted as two peaks from columns of DEAE-Sephadex using a linear salt gradient. The fractions show no heterogeneity on starch gel electrophoresis using buffer systems of pH 3.2-8.6 but again give two peaks on rechromatography on the ion exchange gel. The proportion of protein in either peak depends on previous treatment of the
protein suggesting that two interchangable forms may exist. The presence of the bimodal pattern introduces some uncertainty in estimation of the homogeneity of the final sample with possible consequences which may become apparent in a detailed investigation of the protein.

In view of these results, two forms of heterogeneity may exist in samples of α-lactalbumin prepared by salt fractionation or gel filtration.

(a) Protein impurities of different chemical composition may be present. The major contaminating proteins in samples of α-lactalbumin prepared by salt precipitation are BSA, β-lactoglobulin and three additional forms of α-lactalbumin. These may be removed by suitable chromatographic fractionations.

(b) The α-lactalbumin may be transformed by ion-binding, association-dissociation, or isomerization to closely related forms which are discernable under certain experimental conditions. The complex behaviour of α-lactalbumin on elution from DEAE-Sephadex may be included here and is considered further in Chapter VI.

The galactosyl transferase (NAL synthetase) activity is eluted in two peaks early in the gel filtration of total whey protein on Sephadex G-75. The slower of these active fractions has an apparent molecular weight of about 60,000 as estimated from the elution volume. This is in agreement with the recently reported values of 65,000-75,000 calculated for the bovine "A protein" by Fitzgerald et al. (1970) and the human "A protein" by Andrews (1969) and Nagasawa et al. (1970), but differs from the figure of
40,000-44,000 determined by Trayer et al. (1970) and Andrews (1970) for bovine and human "A protein" respectively. All of these estimations were based on the elution volumes in gel filtration and since the "A protein" is probably a glycoprotein (Nagasawa et al., 1970) considerable caution must be exercised in interpretation of the results.
CHAPTER IV

ISOLATION AND PROPERTIES OF BOVINE α-LACTALBUMIN A

IV.A INTRODUCTION

Blumberg and Tombs (1958) observed in some paper electrophoresis patterns of milk from African Zebu cattle (White Fulani), bands corresponding to either one or both of two α-lactalbumins. Bhattacharya et al. (1963) found similar bands in Indian Zebu milk. The distribution of types (AA, AB and BB) agreed closely with the predicted frequency from the Hardy-Weinberg law and the variation was concluded to be genetically determined. The faster moving Zebu α-lactalbumin at pH 8.6 was designated α-lactalbumin A and the slower variant α-lactalbumin B. The B variant has a similar mobility to the α-lactalbumin of European breeds and it has been generally assumed that the two are identical.

Following the detection by Bell, McKenzie and Murphy (1966) of β-lactoglobulin in Droughtmaster breeds of cattle, starch gel electrophoresis patterns used for typing were examined for α-lactalbumin polymorphism in these animals. In some gel patterns containing β-lactoglobulin an intensification of the band due to that protein was noticed and suspected as an indication of a fast moving α-lactalbumin variant. This was confirmed in a study of the paper electrophoresis patterns from 137 Droughtmaster cattle where all three phenotypes A, AB, and B, were observed (Bell, McKenzie and Ralston (1970) (see TABLE IV.1).
TABLE IV.I Occurrence of $\alpha$-lactalbumin types

<table>
<thead>
<tr>
<th>Animal type</th>
<th>Number</th>
<th>$\alpha$-lactalbumin A</th>
<th>AB</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Droughtmaster</td>
<td>137</td>
<td>6</td>
<td>51</td>
<td>80</td>
</tr>
<tr>
<td>Santa Gertrudis</td>
<td>36</td>
<td>-</td>
<td>8</td>
<td>28</td>
</tr>
<tr>
<td>Brahman cross</td>
<td>29</td>
<td>-</td>
<td>5</td>
<td>24</td>
</tr>
</tbody>
</table>

Droughtmaster cattle are derived from $3/8 - 5/8$ Brahman and $5/8 - 3/8$ European (predominantly shorthorn) breeds. Bell (private communication) did not detect the $\alpha$-lactalbumin A variant in starch gel electrophoresis typing experiments on milk from several thousand Australian cattle of European origin.

Protein genetic variants which have been well characterized are valuable in studies on the physico-chemical properties and function of the protein since they provide small variations in composition of known magnitude which may be related to changes in these properties. The amino acid sequences, absorbancy indices and nitrogen contents of $\alpha$-lactalbumin A and B have been compared. The work reported here, although primarily a comparison of the two proteins also supplements existing data on these properties of the B variant.

IV.B EXPERIMENTAL

Milk samples

All $\alpha$-lactalbumin A samples were obtained from the milk of one Droughtmaster animal (Cow 262, $\alpha$ A, $\beta$ ADr) from the herd at the Moggill Farm of the University of Queensland. The
a-lactalbumin B samples were prepared from milk of European breeds or a Droughtmaster animal (Cow D44, α B, β BDr).

Tyrosine and tryptophan analyses

Tyrosine and tryptophan were estimated on intact protein by the spectroscopic methods of Goodwin and Morton (1946), Bencze and Schmid (1957) and Armstrong (private communication), based on the absorption spectra of the protein in 0.95 M NaOH. The three methods usually employ 0.1 M NaOH rather than 0.95 M NaOH. This higher alkali concentration was used to ensure rapid and complete ionization of tyrosine and to open the structure. Working with a-lactalbumin B, Kronman and Holmes (1965) detected four-tryptophan residues from solvent perturbation experiments in urea solutions. They concluded that the fifth residue estimated by Gordon and Semmett (1953), was hidden from the solvent even in 8 M urea. As the existence of this fifth tryptophan was in doubt, complete opening of the structure was essential.

IV.C RESULTS

Fractionation of whey proteins

The methods outlined in Chapter III on the purification of a-lactalbumin B from dairy breeds were applied here with similar results. The elution profile from gel filtration on Sephadex G-75 of total whey protein from a Droughtmaster cow is shown in Fig. IV.1. The amount of a-lactalbumin relative to β-lactoglobulin appears to be less than in a similar fractionation of whey from dairy breeds (see Fig. III.2 of Chapter III).
FIG. IV.1 (a) Gel filtration of Droughtmaster total whey protein (α-lactalbumin A, β-lactoglobulin ADr) on Sephadex G-75 (3.2 cm bore x 88 cm) in tris/HCl buffer, pH 7.8, I = 0.05.
Sample applied: 3.4 ml, \( A_{280\,\text{nm}} \) = 11.2

(b) Diagram of starch gel electrophoresis patterns of fractions from (a). Pattern "S" is the sample applied to the column. Protein concentration approx. 2 g/100 ml. Semi-discontinuous buffer system of Ferguson and Wallace (1963) pH 7.5, 7 V/cm for 4 h.
The difference in charge of α-lactalbumin A and B which is observed as a difference in mobility on starch gel electrophoresis at pH 7.5 and 8.6 is reflected in their elution from DEAE-Sephadex A-50. The A variant is eluted at a higher NaCl concentration than α-lactalbumin B and typical elution profiles are shown in Fig. IV.2.

The A variant also has a similar set of minor components to SC1, SC2 and FC which were observed in dairy breeds, but their electrophoretic mobilities at pH 7.5 and 8.6 are correspondingly greater. Thus the behaviour of Droughtmaster α-lactalbumin A and B on chromatography on DEAE-Sephadex or Sephadex G-75 and zone electrophoresis resembles that of the α-lactalbumin from European breeds.

Activity in lactose synthesis and immunology

The α-lactalbumin A was active as the specifier protein in the lactose synthetase reaction when combined with samples of the "A protein" prepared from ovine or bovine (dairy breed) milk.

Immunoelectrophoresis was performed on the variants by Bell (private communication) who found that the two proteins were qualitatively similar using antiserum to bovine α-lactalbumin B.

Amino acid analyses

Amino acid analyses were performed on α-lactalbumin A and B from Droughtmaster cows and α-lactalbumin B from European breeds. In elution profiles of all samples, the peak corresponding to the proline-ninhydrin reaction product which normally shows absorption only at 440 nm also included considerable absorption at 570 nm at both normal
FIG. IV.2 Chromatography of \( \alpha \)-lactalbumin A and B on DEAE-Sephadex A-50 in imidazole/HCl buffer, pH 6.3, \( I = 0.043 \). The samples, which were prepared from Fraction V of gel filtration of total whey protein, were precipitated with 530 g \((\text{NH}_4)_2\text{SO}_4/l\) then dialysed prior to application to the column.

- **\( \alpha \)-lactalbumin A**
  - 140 ml of \( \lambda_{280\text{ nm}}^{\text{l cm}} = 3.3 \)

- **\( \alpha \)-lactalbumin B**
  - 11.0 ml, \( \lambda_{280\text{ nm}}^{\text{l cm}} = 92.0 \)

The results using the Armstrong (private communication) and \( (1946) \) methods of calculation indicated that tryptophan and tyrosine and tryptophan and that there are five moles of protein. The ratio of Tyr/Tyr using the Benza and Schmid (1957) method of calculation, were lower than the values determined by the other two methods.
and alternate path lengths. As this may have been due to interference from cysteine in the hydrolysate, protein samples were oxidized with performic acid and the analyses repeated. The apparent proline content was lowered as predicted and the results are shown in Table IV.II. The B variant from Droughtmaster cattle appears to have the same amino acid composition as the normal variant from dairy breeds. The A variant from Droughtmaster breeds has one less arginine (0 instead of 1) and one more glutamine or glutamic acid residue with respect to \( \alpha \)-lactalbumin.

**Tyrosine and tryptophan analyses**

The maximum value of the absorption in the region 270 nm to 300 nm for \( \alpha \)-lactalbumin A or B increased with time after mixing protein with alkali, reached a maximum after about thirty minutes and decreased. This change was presumably due to ionization of tyrosine and release and destruction of tryptophan. The estimated number of tryptophan groups and the ratio of Tyr/Trp at thirty minutes after mixing samples with alkali are shown in Table IV.III. Using the three methods of calculation, the amounts of tyrosine and tryptophan in the two proteins were similar. The results using the Armstrong (private communication) and Goodwin and Morton (1946) methods of calculation indicate equimolar tyrosine and tryptophan and that there are five moles of each residue per mole of protein. The ratio of Tyr/Trp using the Bencze and Schmid (1957) method of calculation, were lower than the values determined by the other two methods.
TABLE IV.II. Amino acid composition of α-lactalbumin variants expressed as residues per mole of protein.

Alanine = 3.0 residues was used in calculations.

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>α-lactalbumin variant and origin</th>
<th>Other workers' values for European</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A from Dr</td>
<td>A ox. from Dr</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.6</td>
<td>10.7</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.7</td>
<td>2.5</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>20.4</td>
<td>20.4</td>
</tr>
<tr>
<td>Threonine</td>
<td>6.5</td>
<td>6.3</td>
</tr>
<tr>
<td>Serine</td>
<td>6.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>14.3</td>
<td>13.8</td>
</tr>
<tr>
<td>Proline</td>
<td>2.8</td>
<td>1.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>5.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Half Cystine</td>
<td>5.3</td>
<td>5.0</td>
</tr>
<tr>
<td>Cysteic Acid</td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Valine</td>
<td>5.5</td>
<td>5.2</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Methionine Sulphone</td>
<td>0.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.3</td>
<td>6.9</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.7</td>
<td>12.1</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>3.7</td>
<td>3.9</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

* Identical from ultraviolet spectra approx. 4 residues.

(a) Gordon and Ziegler (1955a)
(b) Brew, Vanaman and Hill (1967)

Analyses marked "ox." were carried out on oxidized samples.
TABLE IV.III.  Estimations of tryptophan groups and Tyr/Trp ratios of samples of α-lactalbumin A and B in 0.95 M NaOH

The measurements shown were recorded at 30 minutes after mixing the sample with alkali.

<table>
<thead>
<tr>
<th>Method of Calculation</th>
<th>Protein</th>
<th>α-lactalbumin B</th>
<th>α-lactalbumin A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Armstrong</td>
<td>Tyr/Trp ratio</td>
<td>0.95</td>
<td>0.86</td>
</tr>
<tr>
<td></td>
<td>Tryptophan residues/mole</td>
<td>5.02</td>
<td>4.88</td>
</tr>
<tr>
<td>Goodwin and Morton</td>
<td>Tyr/Trp ratio</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>Tryptophan residues/mole</td>
<td>5.00</td>
<td>4.84</td>
</tr>
<tr>
<td>Bencze and Schmid</td>
<td>Tyr/Trp ratio</td>
<td>0.60</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Nitrogen content

The results are shown in Table IV.IV. A high amount of ash remained on heating samples of both α-lactalbumin variants at 500 ± 5°C. The ash was a white, water soluble powder. Solutions of the ash in water gave white precipitates when tested for chloride with acidified silver nitrate or for sulphate with acidified barium sulphate. The ash solution was heated on a platinum wire over a bunsen burner and produced a bright orange-yellow coloration in the flame; characteristic of sodium. This amount of ash may be considered as three moles of Na₂SO₄ or seven moles of NaCl per mole of protein.
TABLE IV. Total nitrogen content

<table>
<thead>
<tr>
<th></th>
<th>α-lactalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Total nitrogen</td>
<td>0.499 ± 0.003</td>
</tr>
<tr>
<td>mg/ml</td>
<td></td>
</tr>
<tr>
<td>Dry weight of</td>
<td>3.183 ± 0.003</td>
</tr>
<tr>
<td>protein, mg/ml</td>
<td></td>
</tr>
<tr>
<td>Ash, per cent</td>
<td>3.1</td>
</tr>
<tr>
<td>(g/100 g dry</td>
<td></td>
</tr>
<tr>
<td>weight)</td>
<td></td>
</tr>
<tr>
<td>Per cent nitrogen</td>
<td>15.67 ± 0.08</td>
</tr>
</tbody>
</table>

Gordon and Semmett (1953) reported a value of 15.86 per cent nitrogen and Zweig and Block (1954) calculated 14.94 per cent (uncorrected for ash) for α-lactalbumin B. Based on the partial amino acid sequence of Brew et al. (1967) and 15 amide groups (Gordon and Ziegler, 1955a) a theoretical nitrogen content of α-lactalbumin B is 15.95 per cent.

Absorbancy index

The ultraviolet absorption spectra of the two proteins were similar with the maximum in the 280 nm region occurring at 281.5 nm. The absorbancy indices, \( A_{\lambda}^{1\text{cm}} \) g/dl, of α-lactalbumin A and B were calculated to be 20.2 ± 0.1 and 20.9 ± 0.1 respectively, using protein solutions of concentration 0.395 and 0.544 mg/ml and 10 ml aliquots for dry weight analyses. Using 15.8 per cent nitrogen, Wetlaufer (1961) determined an absorbancy index at 280 nm of 20.9 for α-lactalbumin B, while Kronman and Andreotti (1964) calculated 20.1 from measurements in phosphate buffer at pH 6.9.
Amide nitrogen analyses

The results from amide nitrogen analyses are shown in Table IV.V. Total nitrogen analyses were carried out at the same time. These results indicate that there is one more amide group in $\alpha$-lactalbumin A than in B.

**TABLE IV.V. Amide nitrogen analysis**

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$-lactalbumin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Total nitrogen mg/ml</td>
<td>1.234 ± 0.005</td>
</tr>
<tr>
<td>Protein mg/ml</td>
<td>7.87 ± 0.08</td>
</tr>
<tr>
<td>Amide nitrogen mg/ml</td>
<td>0.153 ± 0.005</td>
</tr>
<tr>
<td>Amide nitrogen g/100 g protein</td>
<td>1.94 ± 0.08</td>
</tr>
<tr>
<td>Amide groups per mole protein</td>
<td>20.0</td>
</tr>
</tbody>
</table>

Peptide maps

Peptide maps prepared from tryptic and chymotryptic digests of oxidized $\alpha$-lactalbumin A and B were compared. The lyophilized samples after hydrolysis with trypsin were not completely soluble in the pH 4.7 buffer used for the first dimension electrophoresis. Nevertheless, the solution plus core material was applied to the paper and the electrophoresis run at pH 4.7. The strip was cut out and separations made in the second dimension by electrophoresis at pH 1.9 or by chromatography. The electrophoresis patterns of the tryptic digest showed two spots in $\alpha$-lactalbumin B.
and one in A which were not common to both proteins. The peptide unique to α-lactalbumin A was not clearly resolved from the material at the origin and it was necessary to repeat the maps using hydrolysate after removing the core at pH 4. Electrophoresis of the core from both variants was carried out at pH 8.9/1.9 but no differences were apparent between the A and B variants.

Separations were carried out on the soluble peptides by electrophoresis at pH 4.7/1.9 or pH 4.7 followed by chromatography, and the patterns are shown in Fig's. IV.3 and 4. Again three spots were not common to maps of both variants. These peptides were eluted and their amino acid compositions determined. The composition of the B difference peptide furthest from the origin was Lys, Glu, Leu, and for the second B peptide was CySO₃H, Glu, Val, Phe, Arg. The analysis of the A difference peptide was Lys, CySO₃H, (Glu)₃, Val, Leu, Phe. The composition of these peptides is consistent with the cleavage points considered in the DISCUSSION.

The above difference peptides were revealed by ninhydrin staining. Subsequently the maps were viewed before an ultraviolet lamp and stained with chlorine, starch/KI reagent after oxidation of ninhydrin, but no further unique peptides were detected. Peptide maps were prepared from chymotryptic digests of both variants. The patterns contained many spots and clear difference peptides were difficult to detect. Due to the relative simplicity of the tryptic peptide maps, the chymotryptic patterns were not studied further.
FIG. IV.3 Peptide maps of bovine α-lactalbumin A and B. The first dimension is electrophoresis at pH 4.7 and the second at pH 1.9. The two peptides (open arrows) present in B, but not in A, are clearly seen. The peptide peculiar to A is more difficult to see as it has not moved far from the origin. It is more clearly seen in Fig. IV.4, where the separation in the second dimension is made chromatographically.
The eight residue tryptic peptide unique to $\alpha$-lactalbumin A was prepared in greater quantity and separated from the digest by electrophoresis at pH 4.7 and 1.9. The peptide was eluted from the paper with $\text{NH}_4\text{CO}_3$ solution (1 g/l) and digested with pepsin. Using high voltage electrophoresis at pH 1.9, the haemoglobin was separated into three components: the original octapeptide and two cleavage products. However, the yield of the two additional peptides was low and the octapeptide in greater amount using high concentration of chymotrypsin. The octapeptide with pepsin (1 mg/100 mg protein) for three hours at 37° in 0.01 M HCl gave almost quantitatively yield of the two cleavage products, having the same mobility at pH 1.9 as those from the chymotryptic digest. Fig. IV.4 shows an electrophoresis pattern at pH 6.5 of the octapeptide after digestion with pepsin. The amino acid composition of the peptide indicated by the filled arrow was: His, Leu, Lys and for the alternative peptide moving to the anode: Gla, Cys, Gly, Val, Phe. 

Using these were carried out to determine whether the substitution was one involving glutamine or glutamic acid.

FIG. IV.4 Peptide maps of $\alpha$-lactalbumin A and B. The first dimension is electrophoresis at pH 4.7 and the second is chromatography (pyridine, isoamy1 alcohol, water; 7:7:6 (by vol.) . The A difference peptide can be seen as a spot that has moved slightly towards the cathode at pH 4.7 and a little slower on chromatography than the material migrating from the core.
The eight residue tryptic peptide unique to \( \alpha \)-lactalbumin A was prepared in greater quantity and separated from the digest by electrophoresis at pH 4.7 and 1.9. The peptide was eluted from the paper with NH\(_4\)HCO\(_3\) solution (5 g/l) and digested with chymotrypsin. Using high voltage electrophoresis at pH 1.9, the hydrolysate was separated into three components: the original octapeptide and two cleavage products. However the yield of the two additional peptides was low and attempts to cleave the octapeptide in greater amount using longer reaction time and higher concentration of chymotrypsin were not successful. Hydrolysis of the octapeptide with pepsin (1 mg/100 mg protein) for three hours at 37°C in 0.01 M HCl gave almost quantitative yield of the two cleavage products, having the same mobility at pH 1.9 as those from the chymotryptic digest. Fig. IV.5 shows an electrophoresis pattern at pH 6.5 of the octapeptide after digestion with pepsin. The amino acid composition of the peptide indicated by the filled arrow was (Glx)\(_2\), Leu, Lys and for the alternate peptide moving to the anode; Glx, CySO\(_3\)H, Val, Phe.

Using these peptides two series of experiments were carried out to determine whether the substitution was one involving glutamine or glutamic acid.

1. Direct Edman degradation of the peptide (Glx)\(_2\), Leu, Lys to determine the nature of the N-terminal group.

2. Investigation of the electrophoretic mobility of this peptide at pH 6.5.
FIG. IV.5 Paper electrophoresis pattern at pH 6.5 of the tetrapeptide Lys, Leu, (Glx)\textsubscript{2} from \(\alpha\)-lactalbumin A after cleavage of the octapeptide with pepsin. The tripeptide Lys, Leu, Glu prepared from \(\alpha\)-lactalbumin B is indicated by the open arrow. The peptide which moves toward the anode in the \(\alpha\)-lactalbumin A peptic digest is the remaining cleavage product Cys, Glu, Val, Phe. Lysine is included for comparison of mobilities.
Edman degradation

Edman degradation of the peptide of composition \((\text{Glx})_2\) Leu, Lys was carried out but the phenylhydantoin derivative of the N-terminal residue could not be detected on the stained chromatogram. The two standards prepared from free glutamine and glutamic acid, and controls prepared from a peptide containing cysteic acid as N-terminal were clearly visible in the stained chromatogram. The chromatography was repeated using thin-layer plates but again the derivatives from the peptides could not be detected.

Mobility of the peptides at pH 6.5

The peptic digest of the \(\alpha\)-lactalbumin A difference octapeptide was lyophilized and electrophoresis carried out at pH 1.9. The peptide which analysed as \((\text{Glx})_2\) Leu, Lys, was cut from the sheet, sewn onto fresh paper and electrophoresis repeated at pH 6.5. Samples of free lysine and the peptide Glu, Leu, Lys, isolated from a tryptic map of \(\alpha\)-lactalbumin B, were run in parallel with the digest. A similar pattern using a sample of the whole digest of the octapeptide is shown in Fig. IV.5. Both peptides moved slightly to the negative side of the origin, probably by electroendosmosis, indicating that they are neutral at this pH.

IV.D DISCUSSION

A bovine \(\alpha\)-lactalbumin genetic variant of higher electrophoretic mobility at pH 7.5 and 8.6 than the \(\alpha\)-lactalbumin from European breeds has been isolated from milk of Droughtmaster breeds of cattle. This protein (A) is
strongly active in lactose synthetase with bovine "A protein" and its behaviour on column chromatography closely resembles that of the B variant. The two α-lactalbumins differ in amino acid composition: the A variant contains one less arginine (0 instead of 1) and one more glutamic acid (or glutamine) than α-lactalbumin B. The simplest explanation to account for the difference between the two proteins is a one base mutation in the parent nucleic acid, exchanging adenine for guanine, to give glutamine instead of arginine. The transfer to glutamic acid would involve a two base mutation according to the triplet code outlined by Crick (1967).

The sequence of amino acids about the single arginine of α-lactalbumin B as reported by Brew et al. (1967) is:

- Thr-Lys-Cys-Glu-Val-Phe-Arg-Glu-Leu-Lys-Asp-

Tryptic hydrolysis of this section of the molecule should cleave at both lysines and the arginine (shown by the lower arrows), giving two peptides which would not be present in a similar digest of α-lactalbumin A if the arginine had been replaced by glutamine or any other amino acid except lysine.

Pepsin or chymotrypsin should cleave the peptide from α-lactalbumin A or B at the phenylalanine (shown by the upper arrow) making the arginine (or Glx) the N-terminal residue of one of the peptides. The negative Edman degradation reaction of this peptide may be attributed to lack of material although the peptide which separated at pH 1.9 and 6.5 did give a satisfactory analysis as (Glx)₂ Leu, Lys, and therefore a detectable phenylisothiocyanate derivative of the N-terminal residue should have been produced. An alternative approach may be made in view of the
work reported by Schroeder (1967) on the ability of an N-terminal glutamine to form a pyrrolidone. This derivative does not react with the phenylisothiocyanate due to the absence of a terminal amino group.

The investigation of the mobility differences of peptides containing glutamic acid or glutamine was carried out at pH 6.5. In this condition the relevant peptides would have the following charges: if the Glx residue is considered as glutamine:

- CySO₃H-Glu-Val-Phe-Gln-Glu-Leu-Lys net charge 2-
- CySO₃H-Glu-Val-Phe-Gln-Glu-Leu-Lys net charge 0

If Glx was glutamic acid, the tetrapeptide (Glu)₂⁰, Leu, Lys would have a net negative charge (1-). However this peptide was neutral at pH 6.5 and moved to a similar position as the α-lactalbumin B difference peptide Glu, Leu, Lys. Thus the difference residue must be glutamine. If glutamic acid instead of glutamine was present, the peptide would have moved to the anode. The substitution of arginine for glutamine rather than glutamic acid was further supported by amide nitrogen analyses of the two proteins.

Pepsin was more successful than chymotrypsin for the digestion of the octapeptide. This is probably due to interaction of the charges on the groups in the peptide at pH 8 with the enzyme. Any ordered structure existing in the protein at this position is considered unlikely to survive the treatments outlined in preparation of a peptide of eight residues.
From the spectroscopic analyses, the tyrosine and tryptophan contents of the two \( \alpha \)-lactalbumin variants are considered to be identical. However the estimate of five tyrosine residues/mole protein does not agree with the value of four residues/mole obtained by amino acid analysis after acid hydrolysis. In addition, Brew et al. (1967) have shown four instead of five tryptophans in their sequence study of \( \alpha \)-lactalbumin B. Gordon and Ziegler (1955a) estimated five tyrosine and five tryptophan residues in \( \alpha \)-lactalbumin B by the methods of Udenfriend and Cooper (1952) and Spies and Chambers (1949) respectively but Spies (1967) has reported four tryptophans by colorimetric analysis after alkaline hydrolysis.

In view of these other analyses, \( \alpha \)-lactalbumin A and B may contain equimolar tyrosine and tryptophan but there are probably four rather than five residues of each per mole of protein. These spectrophotometric procedures used in the present study employed free amino acids as standards for calibration may be more satisfactory using suitable derivatives or peptides containing these amino acids in an attempt to simulate the actual environment in the protein. As an alternative procedure it would be interesting to study the conversion of tryptophan to kynurenine by photolysis in the presence of proflavin, then acid hydrolysis of the protein followed by amino acid analysis using the usual ion exchange chromatographic procedure. Kynurenine is resistant to the conditions of acid hydrolysis and quantitative conversion of tryptophan in lysozyme has been reported by Galiazzo, Jori and Scoffone (1968).
On the basis of these results, the two variants probably differ by direct substitution at residue 10, where glutamine in α-lactalbumin A replaces arginine in α-lactalbumin B. This conclusion is formed assuming that the remaining amino acids are in the same positions in each variant. Clearly it is possible that differences may exist which do not alter the relation of the charges of the peptides, and this problem could only be resolved by determination of the complete sequence of each protein.

Since completing this study, several workers have reported on the composition and properties of the α-lactalbumin A from Eastern breeds of cattle. Tanahashi, Brodbeck and Ebner (1968) found that the A variant from Eastern breeds and the B variant from European breeds have similar enzymic and immunological activities. Gordon et al. (1968) have determined the difference in amino acid composition between the A variant from Indian Zebu cattle and the B variant from European breeds as one more glutamic acid or glutamine and one less arginine in α-lactalbumin A than in B. The A variants from the milk of Droughtmaster cattle and Eastern breeds are therefore probably identical. However, considering the origin of the Droughtmaster breeds it is not surprising that the milk should contain those α-lactalbumins which are found in European and Eastern breeds.
CHAPTER V
ON THE MINOR α-LACTALBUMIN COMPONENTS OF BOVINE MILK

V.A INTRODUCTION

During the study on the isolation of bovine α-lactalbumin described in Chapter III, two proteins of apparent molecular weight intermediate between α-lactalbumin and β-lactoglobulin were observed in gel filtration of whey on Sephadex G-75 (see Fig. III.2a). These proteins, termed SCI and SC2, showed lower mobility than α-lactalbumin on starch gel electrophoresis at pH 7.5 and 8.6 and were eluted before α-lactalbumin on DEAE-Sephadex A-50 (see Fig. III.7a). Starch gel electrophoresis patterns of the α-lactalbumin fraction from gel filtration (Fraction V), also showed a minor band moving ahead of the major protein. This material (FC) was eluted after α-lactalbumin on chromatography on DEAE-Sephadex A-50. These relationships are summarized in Fig. V.1.

The three minor components detected in this preparative study have been isolated and all have been shown to be α-lactalbumins. Their compositions and properties are compared with those of the major α-lactalbumin variant present in the milk.

A number of workers have observed marked apparent heterogeneity on moving boundary or zone electrophoresis of samples of α-lactalbumin prepared by salt fractionation and crystallization. These observations are discussed in more detail in Chapter I. Gordon and Semmett (1953), Klostergaard
FIG. V.1. Summary of the electrophoretic and chromatographic fractionations at pH 7.5-7.8 of the three bovine minor components and α-lactalbumin from bovine milk which is homozygous in α-lactalbumin A or B.

α-lactalbumin fast component (FC): eluted coincident with α-lactalbumin on gel filtration but after α-lactalbumin using chromatography on DEAE-Sephadex A-50.

α-lactalbumin slow component 1 (SCI): eluted before α-lactalbumin on gel filtration and on chromatography on DEAE-Sephadex A-50.

α-lactalbumin slow component 2 (SC2): as for SCI but eluted ahead of SCI on chromatography on DEAE-Sephadex A-50.
and Pasternak (1957) and Wetlaufer (1961) observed bimodal moving boundary electrophoresis patterns having a fast boundary which comprised 30-70 per cent of the total protein, depending on the composition and pH of the buffer and protein concentration. A minor band of higher electrophoretic mobility than \( \alpha \)-lactalbumin on zone electrophoresis at pH 8.6 and 7.5, comprising 5-10 per cent of the total protein, was also observed by Aschaffenburg and Drewry (1957) and Wetlaufer (1961) in similar samples. They reported that this minor band was not removed by repeated recrystallization. Groves (1965) observed that samples of \( \alpha \)-lactalbumin prepared by chromatography on DEAE-cellulose in phosphate buffer did not show this fast band on polyacrylamide gel electrophoresis at pH 4.3 and 9.5. However he did not identify this protein in the column effluent nor carry out moving boundary electrophoresis experiments on the "purified" \( \alpha \)-lactalbumin sample to determine whether the "fast" components in both electrophoretic separations are affected by fractionation on DEAE-cellulose. Szuchet-Derechin and Johnson (1965) chromatographed a whole whey protein preparation on DEAE-cellulose using a gradient of pH and ionic strength and from the leading edge of the \( \alpha \)-lactalbumin peak isolated a fraction containing material which migrated between \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin on paper electrophoresis at pH 8.6. They suggested that this may be: (i) the heat stable component '5' of Larson and Jenness (1955), (ii) the heat labile component '5' of Jenness (1959) or (iii) a genetic variant of \( \alpha \)-lactalbumin or other closely related protein. In the same fraction, material moving behind \( \alpha \)-lactalbumin on
electrophoresis was considered to be $\beta_{2A}$-globulin.

Biserte et al. (1966), using preparative electrophoresis in tris/citrate buffer pH 8.6, also isolated a sample of $\alpha$-lactalbumin which was free of impurities as indicated by paper electrophoresis at pH 8.9. A protein component moving faster than $\alpha$-lactalbumin on paper electrophoresis was also separated in the preparation.

**V.B RESULTS**

Isolation of the minor components

(a) Fast component. The fast component was isolated from samples of $\alpha$-lactalbumin which had been prepared from bovine whey on Sephadex G-75. This minor protein was eluted from columns of DEAE-Sephadex A-50 at higher NaCl concentration than the major $\alpha$-lactalbumin component. A typical elution profile is shown in Fig. III.7 of Chapter III.

The fractions containing FC were carefully selected to minimise contamination by the major $\alpha$-lactalbumin. The pooled, concentrated sample was then fractionated further by gel filtration to remove small amounts of $\beta$-lactoglobulin which may also be eluted from DEAE-Sephadex at this NaCl concentration. A major protein peak at an elution volume corresponding to an approximate molecular weight of 14,000-17,000 was obtained. Several samples prepared in this way migrated as two bands on starch gel electrophoresis at pH 3.2, 5.2 and 7.5 as shown later in Fig. V.3. This apparent heterogeneity was not due to contamination by $\beta$-lactoglobulin. All samples used for further investigation showed only a single band of similar mobility to the slower of the two
shown in Fig. V.3 on starch gel electrophoresis at pH 7.5.

(b) Slow components. A sample rich in both slow components was isolated as Fraction IV from gel filtration of total whey protein on Sephadex G-75 or G-100 as shown in Fig. III.2. This fraction from several separate experiments was pooled to obtain sufficient material for further purification. The sample contained some β-lactoglobulin and considerable α-lactalbumin due to the wide selection of fractions which were combined. SC1 and SC2 were separated from each other by chromatography on DEAE-Sephadex A-50 in tris/HCl buffer pH 7.8, $I = 0.05$ eluting with a linear salt gradient. However due to overlap of the major α-lactalbumin with SC1 in this fractionation, a sample free of appreciable α-lactalbumin impurities was required for effective purification of SC1 in this step. Therefore gel filtration of the initial Fraction IV material was repeated several times giving a product which showed on electrophoresis, the two slow components generally with a trace of α-lactalbumin. As shown in Fig. V.2, SC1 was eluted in two peaks from DEAE-Sephadex using a sample prepared in this way. The bimodal patterns observed here with SC1 and previously with α-lactalbumin A or B are thought to arise by similar mechanisms.

**Starch gel electrophoresis**

Starch gel electrophoresis patterns at pH 3.2, 5.2 and 7.5 of the minor components prepared from milk homozygous in α-lactalbumin B are compared in Fig. V.3. The relative positions of the bands in patterns at pH 7.5 and 8.6 are similar. SC2 coincided with BSA when electrophoresis was carried out in the semi-discontinuous buffer system of
FIG. V.2 (a) A typical elution profile in the separation of SC1 and SC2 on DEAE-Sephadex A-50. The sample was Fraction IV from chromatography of total whey protein on Sephadex G-75 (see Fig. III.2) after three further passes through the same column to remove α-lactalbumin. Sample volume: 60 ml, $A_{280}^{1\text{cm}} = 1.5$. The fractionation was carried out in tris/HCl buffer pH 7.8, $I = 0.05$ in a column of dimensions 1.5 cm bore x 20 cm.

(b) Starch gel electrophoresis patterns of fractions from (a). "S" is the Fraction IV obtained from the initial separation on Sephadex G-75. Semi-discontinuous buffer system of Ferguson and Wallace (1963) pH 7.5, 7 V/cm, 4 h.
FIG. V.3 Starch gel electrophoresis patterns of \( \alpha \)-lactalbumin B minor components.

a. SC2  
b. SC1  
c. \( \alpha \)-lactalbumin B  
d. FC  
e. whey containing \( \beta \)-lactoglobulin AB

Buffer systems:

- pH 7.5, semi-discontinuous system of Ferguson and Wallace (1963), 7 V/cm for 4 h.
- pH 5.2, acetate buffer 7 V/cm for 9 h.
- pH 3.2, Formic acid-NaOH buffer 7 V/cm for 4 h.

A sample of \( \alpha \)-lactalbumin containing all minor components and \( \beta \)-lactoglobulin AB was used in gel filtration of total whey proteins to study the components and to determine the nature of changes in shape of the molecules due to association of protein components. Different conditions were used to obtain minimum differences in electrophoretical mobility of the protein components. The major components, \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin, are responsible for the different mobilities of each protein fraction; the minor components had similar mobilities in the cathode. At pH 5.2, SC2 has similar mobility to the whey containing \( \beta \)-lactoglobulin AB. At pH 3.2, SC2 has similar mobility to the cathode. At pH 7.5, SC2 has a net positive charge, which suggests that SC2 has possibly more positive charges than FC and SC1. At pH 5.2 and pH 3.2, SC1 and SC2 have similar mobility to the cathode. At pH 3.2, FC and SC1 have similar mobility to the cathode.
Ferguson and Wallace (1963) at pH 7.5 but these proteins were resolved in the borate system of Bell (1967) at pH 8.6.

The changes in relative mobility of the proteins with pH are interesting and the following details should be noted: (i) at pH 3.2, FC and α-lactalbumin have similar mobility to the cathode, but at pH 5.2 FC has a net negative charge while α-lactalbumin B has a net positive charge. This result suggests that FC has possibly more carboxyl groups than α-lactalbumin B, (ii) at pH 5.2, FC and SC1 have similar mobility to the anode while α-lactalbumin B and SC2 have similar mobility to the cathode. At pH 3.2 all except SC1 have similar mobility to the cathode.

To determine whether differences in overall shape, aggregation or interchain disulphide bridging were responsible for the different mobilities of the minor components and α-lactalbumin, electrophoresis was carried out in the presence of 7 M urea and 2-mercaptoethanol at pH 7.5 using the buffer system of Ferguson and Wallace (1963). The relative positions of each protein remained approximately the same as in patterns without these reagents although now the difference between bands was reduced. The minor components are therefore not the result of changes in shape of the molecules nor are they due to association of protein molecules or protein with buffer components. A difference in the compositions of the α-lactalbumins must exist to confer differing mobilities under these conditions.

A sample of α-lactalbumin containing all three minor components plus β-lactoglobulin A (as Fraction IV plus V from gel filtration of total whey protein) was run in two-
dimensional starch gel electrophoresis in borate buffer at pH 8.6. After electrophoresis in the first dimension, half of the pattern was stained with the nigrosine dye, while a thin strip of unstained pattern was inserted as sample in a second gel for the second dimension. The pattern showed five single spots, again indicating that the minor bands were not due to rapid reversible protein or ion interaction.

**Amino acid analyses**

Samples of the three lyophilized minor proteins from milk homozygous in α-lactalbumin B, and of SCI from milk homozygous in α-lactalbumin A were hydrolysed and their amino acid compositions determined. Starch gel electrophoresis patterns of these samples at pH 7.5 and 8.6 showed single bands. Each α-lactalbumin B minor component had the same amino acid composition as α-lactalbumin B which was analysed at the same time or previously in the study of the α-lactalbumin A B differences (see Table V.I). Similarly SCI from milk homozygous in α-lactalbumin A analysed as α-lactalbumin A. The amino acid compositions of α-lactalbumin A SC2 and FC were not determined.

The recovery of amino acids in the analyses was calculated from the weight of dried protein hydrolysed, the aliquot of hydrolysate taken and the residue weight of amino acids calculated from the number of micromoles detected in the analysis. The samples were lyophilized and then dried over phosphorus pentoxide under reduced pressure for several days prior to analysis. No loss in weight of a sample of α-lactalbumin was detected on drying further at 108°C. Tryptophan was not included in these estimations and no
TABLE V.I.  Amino acid analyses of $\alpha$-lactalbumin minor components expressed as residues per monomer of protein

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>B</th>
<th>B</th>
<th>B</th>
<th>B</th>
<th>A</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FC</td>
<td>SC1</td>
<td>SC1</td>
<td>SC2</td>
<td>SC1</td>
<td>SC1</td>
</tr>
<tr>
<td>Lysine</td>
<td>11.9</td>
<td>11.1</td>
<td>11.9</td>
<td>12.0</td>
<td>11.9</td>
<td></td>
</tr>
<tr>
<td>Histidine</td>
<td>2.8</td>
<td>2.7</td>
<td>3.0</td>
<td>3.0</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>1.0</td>
<td>1.0</td>
<td>0.9</td>
<td>1.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>21.5</td>
<td>20.9</td>
<td>21.0</td>
<td>21.4</td>
<td>20.3</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>6.6</td>
<td>6.8</td>
<td>6.0</td>
<td>6.7</td>
<td>7.5</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>6.3</td>
<td>6.4</td>
<td>5.9</td>
<td>6.6</td>
<td>7.7</td>
<td></td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>13.4</td>
<td>12.6</td>
<td>13.1</td>
<td>14.8</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>3.4</td>
<td>3.7</td>
<td>2.2</td>
<td>2.8</td>
<td>3.3</td>
<td>3.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>3.2</td>
<td>3.7</td>
<td>3.4</td>
<td>4.0</td>
<td>3.2</td>
<td>3.6</td>
</tr>
<tr>
<td>Half Cystine</td>
<td>5.2</td>
<td>6.4</td>
<td>6.1</td>
<td>5.9</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td>Cysteic Acid</td>
<td>7.8</td>
<td>6.8</td>
<td>7.2</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>5.7</td>
<td>5.3</td>
<td>5.4</td>
<td>5.0</td>
<td>5.7</td>
<td>5.1</td>
</tr>
<tr>
<td>Methionine</td>
<td>1.1</td>
<td>0.9</td>
<td>1.1</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine Sulphone</td>
<td>1.1</td>
<td>0.6</td>
<td>0.7</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.2</td>
<td>7.2</td>
<td>6.7</td>
<td>7.6</td>
<td>7.4</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>13.1</td>
<td>12.2</td>
<td>11.7</td>
<td>13.2</td>
<td>13.2</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>4.2</td>
<td>3.8</td>
<td>3.4</td>
<td>3.6</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>4.3</td>
<td>4.0</td>
<td>3.7</td>
<td>3.8</td>
<td>4.2</td>
<td>4.1</td>
</tr>
<tr>
<td>Number of Analyses</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

The number of residues was calculated from the micromoles of amino acids using glycine = 6.0 for the integration.
The values for methionine sulphone and cysteic acid were obtained from separate analyses of oxidized samples.
correction was made for the presence of inorganic impurities. Estimation of loss of amino acids due to breakdown in acid hydrolysis was made for samples of SCI in 6 M HCl at 108°C for 22, 48 and 72 h hydrolysis time. Threonine and serine were the most affected and the losses occurring below 22 h hydrolysis time are probably of the order of 2-5 per cent threonine and 5-10 per cent serine which would amount to less than 1 per cent of the total recovered micromoles of amino acids. The recovery of amino acids is shown in Table V.II.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Recovery, percent of dry weight of protein used in analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-lactalbumin B</td>
<td>90 ± 3</td>
</tr>
<tr>
<td>α-lactalbumin B FC</td>
<td>83 ± 3</td>
</tr>
<tr>
<td>α-lactalbumin B SCI</td>
<td>70 ± 3</td>
</tr>
<tr>
<td>α-lactalbumin B SC2</td>
<td>67</td>
</tr>
</tbody>
</table>

(trypotphan is not included in these estimations)

Carbohydrate analyses

Hexosamine, hexose, sialic acid (as N-acetyleneuraminic acid) and 6-deoxyhexoses (as fucose) were estimated in samples of the three α-lactalbumin minor components, using the methods described in Chapter II. Analyses were carried out on four occasions using different preparations of the lyophilized proteins which had been dried over phosphorus pentoxide. The results are shown in Table V.III.
TABLE V.III Carbohydrate analysis of \( \alpha \)-lactalbumin minor components

A. Hexosamine * (as per cent glucosamine)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Preparation</th>
<th>Residues/ mole protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>FC</td>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>SC1</td>
<td>5.2</td>
<td>6.5</td>
</tr>
<tr>
<td>SC2</td>
<td>5.8</td>
<td>6.7</td>
</tr>
<tr>
<td>( \alpha )-lactalbumin B</td>
<td>0.6</td>
<td>1.1</td>
</tr>
</tbody>
</table>

B. Hexose (as per cent galactose)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Preparation</th>
<th>Residues/ mole protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>FC</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>SC1</td>
<td>5.7</td>
<td>4.8</td>
</tr>
<tr>
<td>SC2</td>
<td>6.2</td>
<td>5.7</td>
</tr>
<tr>
<td>( \alpha )-lactalbumin B</td>
<td>1.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

C. Sialic acid (as per cent NANA)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Preparation</th>
<th>Residues/ mole protein†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I</td>
<td>II</td>
</tr>
<tr>
<td>FC</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>SC1</td>
<td>1.84</td>
<td>1.86</td>
</tr>
<tr>
<td>SC2</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>( \alpha )-lactalbumin B</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

D. Fucose

<table>
<thead>
<tr>
<th>Protein</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>FC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.11</td>
</tr>
<tr>
<td>SC1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.56</td>
</tr>
<tr>
<td>SC2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.77</td>
</tr>
<tr>
<td>( \alpha )-lactalbumin B</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.10</td>
</tr>
</tbody>
</table>

NOTE:
The duplicate analyses agreed to ± 0.2 per cent carbohydrate.

* Analysis I followed the method of Cessi and Piliego (1960);

† Calculations were based on a molecular weight of 15,000 for \( \alpha \)-lactalbumin B and FC and 17,000 for SC1 and SC2.
Some variation occurred in the hexosamine and hexose analyses particularly for the \( \alpha \)-lactalbumin B control. These samples were purified by the method described in Chapter III and no protein impurity was observed on starch gel electrophoresis at pH 7.5. One set of analyses (I) was carried out by the method of Cessi and Piliego (1960), using a small distillation apparatus to separate the volatile 2-methylpyrrole formed from the reaction of the amino sugar and acetylacetone reagent. However hexosamine (0.6 per cent) was again detected in the analysis of \( \alpha \)-lactalbumin B. In contrast, using the method of Rondle and Morgan (1955), two preparations (III and IV) appeared to contain less than 0.2 per cent hexosamine. In view of these results it is concluded that neither \( \alpha \)-lactalbumin B nor FC contain covalently bound carbohydrate. The positive hexose and hexosamine results must therefore arise from contamination by free sugars which have been retained in the chromatographic and dialysis steps. Rawitch and Kolb (1970) have interpreted anomalous electrophoresis patterns of samples of \( \alpha \)-lactalbumin as due to the binding of amines including glucosamine. The positive hexosamine estimations in both \( \alpha \)-lactalbumin B and FC may be due to noncovalently bound sugars.

In addition, peaks corresponding to glucosamine and galactosamine were observed in the amino acid analysis elution profiles of samples of hydrolysed SC1 and SC2. Samples of FC or \( \alpha \)-lactalbumin A or B showed no amino sugars in the analysis column effluent. Both SC1 and SC2 contained similar amounts of glucosamine or galactosamine using the phenylalanine calibration constant to determine the number of
micromoles of amino sugar and alanine as 3.0 residues for the integration. These results are shown in Table V.IV.

**TABLE V.IV** Estimation of glucosamine and galactosamine in samples of SC1 and SC2 as determined in amino acid analysis

<table>
<thead>
<tr>
<th>Protein</th>
<th>Glucosamine residues</th>
<th>Galactosamine residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-lactalbumin B SC1</td>
<td>1.38</td>
<td>0.53</td>
</tr>
<tr>
<td>$\alpha$-lactalbumin B SC2</td>
<td>1.25</td>
<td>0.53</td>
</tr>
</tbody>
</table>

The effect of hydrolysis time on the amount of glucosamine detected in the analysis and recovery of amino acids from a sample of $\alpha$-lactalbumin B SC1 are shown in Table V.V.

**TABLE V.V** Effect of hydrolysis time on recovery of amino acids and glucosamine in a sample of SC1, based on amino acid analysis

<table>
<thead>
<tr>
<th>Time of hydrolysis (6 M HCl, 108°C)</th>
<th>Recovery of dry weight of protein as amino acids (per cent)</th>
<th>Glucosamine Micromoles</th>
<th>Residues per mole</th>
</tr>
</thead>
<tbody>
<tr>
<td>22 h</td>
<td>72.0</td>
<td>0.046</td>
<td>1.38</td>
</tr>
<tr>
<td>48 h</td>
<td>71.9</td>
<td>0.024</td>
<td>0.69</td>
</tr>
<tr>
<td>72 h</td>
<td>71.0</td>
<td>0.011</td>
<td>0.32</td>
</tr>
</tbody>
</table>
A summation of the measured contributions to the weight of protein is shown in Table V.VI.

**TABLE V.VI Composition of bovine α-lactalbumins**

<table>
<thead>
<tr>
<th>Amino acids (in analysis)</th>
<th>Estimated per cent of component. (g/100 g dry weight of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>α-B</td>
</tr>
<tr>
<td>Amino acids (in analysis)</td>
<td>90</td>
</tr>
<tr>
<td>Carbohydrate residues:</td>
<td></td>
</tr>
<tr>
<td>Hexosamine</td>
<td>0.0</td>
</tr>
<tr>
<td>Hexose</td>
<td>0.0</td>
</tr>
<tr>
<td>NANA</td>
<td>0.0</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.0</td>
</tr>
<tr>
<td>Tryptophan (as 4 residues)</td>
<td>5.2</td>
</tr>
<tr>
<td>TOTAL</td>
<td>95</td>
</tr>
</tbody>
</table>

The reason for the low total recovery of weight in these analysis of SC1, SC2 and FC is not known. The samples were dialysed, lyophilized and dried in a similar manner. Some samples of α-lactalbumin have shown a high amount of ash (see Chapter IV) and the higher solubility of SC1 and SC2 may be due to increased ion binding.

**Reaction of α-lactalbumin slow components with neuraminidase**

The results from estimations of the compositions of the minor α-lactalbumin components indicated that SC1 differs from SC2 by the presence of one residue of NANA per mole of protein. The higher electrophoretic mobility of SC1 in the pH range 7.5-8.6 may therefore be due to the presence of NANA in that protein. To test this hypothesis, the two slow components were treated with neuraminidase from *Vibrio cholerae* using the conditions as discussed by Gottschalk (1966, page 216).
As shown in Fig. V.4 the electrophoretic mobility of SCl after treatment with neuraminidase is similar to that of SC2 which is unaffected by this treatment.

**Ultraviolet absorption spectra of minor components**

The absorption spectra between 350-220 nm of the α-lactalbumin B minor components in 0.05 M KCl were compared. Qualitatively the four spectra were similar, with the maximum in the 280 nm region occurring at 281.5 nm. The absorbancy index, $A_{281.5}^1$ at 281.5 nm for α-lactalbumin B SCl was calculated from dry weight/ash and absorbance measurements. A value of 15.8 ± 0.3 was determined using a solution of concentration 0.0672 ± 0.0012 g/100 ml and 10 ml aliquots for dry weight analyses. This result may be compared with a value of 20.9 ± 0.1 for α-lactalbumin B as described in Chapter IV.

**Lactose synthetase specifier activity of the minor components**

A bovine "A protein" preparation was isolated from fresh whey by fractionation on Sephadex G-75 as described in Chapter III and used without further purification. Aliquots of 0.05 ml of solutions of α-lactalbumin B FC, SCl and SC2 and α-lactalbumin A SCl of approximate concentration 0.1 g/100 ml were combined with 0.05 ml of the "A protein" preparation and 0.1 ml stock glucose reaction mixture and the reaction allowed to proceed for 10 minutes at 37°C. All four minor proteins were active in this reaction.

Solutions of SCl and α-lactalbumin B of protein concentration 0.0076 g/100 ml were prepared by dilution with water and aliquots of 0.01-0.05 ml used in separate reactions to compare the activities of equal weights of the
FIG. V.4 Starch gel electrophoresis patterns of α-lactalbumin B SCI after treatment with neuraminidase. The neuraminidase (300 units of activity in 0.2 ml) was added to 0.2 ml solutions of α-lactalbumin B SCI and SC2 in water (0.7 g/100 ml) plus 1.0 ml acetate buffer, pH 5.5, I=0.10, containing 0.004 M CaCl₂. The reactions were allowed to proceed at 37°C for 7 h and 22 h. Water (5 ml) was added to the solutions which were then concentrated by ultrafiltration to remove salt and small reaction products. This step was repeated and the retentates lyophilized. Two drops of tris/HCl buffer, pH 7.8, were added and the digests compared by electrophoresis at pH 7.5 in the semi-discontinuous buffer system of Ferguson and Wallace (1963), 7 V/cm for 4 h.

SAMPLES:

- a. SC2 untreated
- b. SCI treated for 22 h at 37°C
- c. SCI treated for 7 h at 37°C
- d. SCI untreated
- e. α-lactalbumin B untreated
two proteins in lactose synthetase. Additions of 0.05-0.01 ml of water were made to give a constant volume of the reaction mixture. As shown in Fig. V.5 the two proteins have similar activities under these conditions.

Gel Filtration and sedimentation velocity

The slow components and α-lactalbumin are clearly resolved on elution from columns of Sephadex G-75 (3.2 cm bore x 88 cm) at effluent volumes of 400-410 ml and 450-470 ml respectively (see Fig. III.2a). Values of $K_{av}$ were calculated as 0.36 and 0.46 respectively, giving an approximate molecular weight of the SC1 or SC2 of 25,000 ± 1,000 using the calibration shown in Chapter II. This may be compared with a value of 15,000 ± 1,000 for α-lactalbumin B, estimated by the same method.

Sedimentation velocity experiments were carried out on α-lactalbumin B, B SC1, and B SC2 in tris/HCl buffer, pH 7.5 $I = 0.05$. The sedimentation coefficients, corrected to $s_{20,w}$ are shown in Table V.VII.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$s_{20,w}$</th>
<th>Protein concentration</th>
<th>$I$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Svedbergs</td>
<td>g/100 ml</td>
<td></td>
</tr>
<tr>
<td>SC1</td>
<td>1.72</td>
<td>0.72</td>
<td>0.05</td>
</tr>
<tr>
<td>SC2</td>
<td>1.71</td>
<td>0.75</td>
<td>0.05</td>
</tr>
<tr>
<td>α-lactalbumin B</td>
<td>1.67</td>
<td>0.65</td>
<td>0.10</td>
</tr>
</tbody>
</table>
FIG. V.5 Reaction of α-lactalbumin B and α-lactalbumin B SC1 in lactose synthetase assay. Reaction was carried out at 37°C for 10 min. using aliquots of α-lactalbumin B or SC1, both of protein concentration 0.0076 g/100 ml calculated from absorption measurements. A\text{\textsubscript{281 nm}} for α-lactalbumin was 0.160 and for SC1; 0.120.

The reaction mixture contained:

a. 0.10 ml UDP-[\textsuperscript{14}C] galactose (approx. 200 mCi/mM, 26,400 cpm)

b. 0.10 ml stock glucose, Mn(II), ATP solution
c. 0.05 ml "A protein" preparation from bovine whey
d. 0.00 - 0.05 ml α-lactalbumin B or SC1 with addition of water to make up to 0.05 ml

In this analysis, 2,000 cpm represents conversion of 31% of the UDP-[\textsuperscript{14}C] galactose to lactose.

Estimations of range of error is based on the results of triplicate analyses of α-lactalbumin B.
Solubility

When the pH 3.5 precipitate of Methods IIb or Ia (Armstrong et al., 1967) were used as starting material for the fractionation on Sephadex G-75 the amounts of slow components were reduced. The total whey protein samples without pH adjustment contained relatively more of SC1 than SC2 and the pH 3.5 precipitate of Method Ia contained a reduced amount of SC1 and only a trace of SC2. Only small amounts of SC1 and no SC2 were present in the Method IIb pH 3.5 precipitate. The yield of FC in samples of \(\alpha\)-lactalbumin appeared to be unaffected by pH precipitation. Initially Method Ia or IIb pH 3.5 precipitates were used to prepare \(\alpha\)-lactalbumin and therefore only SC1 was isolated from these fractionations. Subsequently, total whey protein samples were used and the SC2 protein detected although SC1 was still present in greater amount. The high solubility of SC1 and SC2 under normal conditions of low pH salt fractionation of bovine whey proteins would explain why these minor proteins have not been observed previously in preparations of \(\alpha\)-lactalbumin.

The difference in solubility of the \(\alpha\)-lactalbumin was also observed when the lyophilized proteins (10 mg) were dissolved in water (10 ml). The solubility appears to follow the series of electrophoretic mobility:

- **FC**  
  Largely insoluble in water forming a fine dispersion which settles on standing. The dispersion remained on addition of 1 M acetic acid, to pH 2.
\(\alpha\)-lactalbumin B  Largely insoluble in water as for FC but is soluble on addition of 1 M acetic acid, to pH 2

SC1  Protein dissolves slowly in water

SC2  Protein dissolves readily in water.

All samples dissolved readily on addition of 0.1 M NH\(_3\) to pH 6.5

Although the samples were prepared under identical conditions and precautions taken to minimize damage to the protein during lyophilization (e.g., formation of small crystals on freezing, prevention of thawing during evacuation) changes in solubility may also be due to the presence of denatured protein. The difference in solubility was consistently observed however in preparation of solutions for analyses.

**Peptide maps**

An attempt was made to determine whether the FC, SC1 and SC2 were members of a family of \(\alpha\)-lactalbumins differing by the extent of modification at a particular site on the chain. In addition, the site of attachment of the carbohydrate on the SC1 and SC2 polypeptide chains should also reveal that section of the molecule which is not involved in interaction with the "A protein" or substrates.

Purified samples of the \(\alpha\)-lactalbumin and SC1 of both genetic variants and \(\alpha\)-lactalbumin B SC1 were digested with TPCK treated trypsin and peptide maps prepared by electrophoresis at pH 1.9 in the first dimension and chromatography (isoamyl alcohol, pyridine, water, 35:35:30 (by volume) in the second dimension. The digestions and
electrophoresis of all samples were carried out under identical conditions. Ninhydrin stained spots which were not common to the α-lactalbumin, SCI or FC were cut out, the peptide eluted and hydrolysed for amino acid analysis.

Core material which precipitated in the pH 1.9 buffer did not cause serious smearing at the origin and the resulting peptide maps of α-lactalbumin A and A SCI are shown in Fig. V.6. Several spots were unique to maps of both slow components or α-lactalbumins. The results from analysis of these peptides is shown in Table V.VIII together with their probable origin from the sequence data of Brew, Vanaman and Hill (1967). Carbohydrate analyses were not performed on peptide material although no hexosamine was detected in any of the amino acid analyses.

Peptide 1 was observed in maps of both genetic variants of SCI and only trace amounts in maps of α-lactalbumin A, B or FC. Peptide 3 was visible in maps of SCI but not in maps of α-lactalbumin or FC. Spots 2 and 5 analysed as the same section of chain (95-98). The recovery of amino acids in analysis of peptides 1 and 3 was very low considering the strong colour of the pattern. From their compositions they may be identified as abnormal peptides (99-114) which have retained an intact cleavage point for tryptic hydrolysis at lysine 108. The analyses were not clear however and small amounts of impurities would interfere markedly with the results at such low concentration. The identity of peptides 1 and 2 remains to be determined unequivocally.

There were two peptides (4 and 6) present in maps of α-lactalbumin A/B but not in patterns of SCI. One of these
FIG. V.6 Peptide maps of tryptic digests of (a) α-lactalbumin A and (b) α-lactalbumin A SC1. First dimension: electrophoresis at pH 1.9; second dimension: chromatography (isoamyl alcohol, pyridine, water; 7:7:6, by vol.). Peptide maps for the α-lactalbumin B proteins were similar to those shown except for the A B differences. The compositions of the peptides are shown in Table IV.6.
TABLE V.VIII. Amino acid composition of peptides from patterns shown in Fig. V.6

The results are expressed as residues per whole number of glycine or leucine residues.

<table>
<thead>
<tr>
<th>Residue</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.5</td>
<td>1.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.2</td>
<td>0.0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Cysteic Acid</td>
<td>0.2</td>
<td>0.0</td>
<td>ND</td>
<td>1.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.4</td>
<td>3.0</td>
<td>1.0</td>
<td>5.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.2</td>
<td>0.1</td>
<td>0.3</td>
<td>3.8</td>
<td>0.0</td>
</tr>
<tr>
<td>Serine</td>
<td>1.1</td>
<td>0.1</td>
<td>0.9</td>
<td>4.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>0.9</td>
<td>0.0</td>
<td>0.7</td>
<td>6.0</td>
<td>0.2</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.0</td>
<td>0.0</td>
<td>1.0</td>
<td>5.0</td>
<td>0.3</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.0</td>
<td>0.1</td>
<td>0.6</td>
<td>2.5</td>
<td>0.3</td>
</tr>
<tr>
<td>Valine</td>
<td>0.3</td>
<td>0.0</td>
<td>0.3</td>
<td>2.5</td>
<td>0.0</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.2</td>
<td>1.2</td>
<td>0.6</td>
<td>1.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.5</td>
<td>1.0</td>
<td>0.6</td>
<td>2.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.3</td>
<td>0.0</td>
<td>0.0</td>
<td>1.3</td>
<td>0.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>1.6</td>
<td>0.0</td>
</tr>
<tr>
<td>Micromoles isoleucine</td>
<td>0.001</td>
<td>0.007</td>
<td>0.0015</td>
<td>0.0052</td>
<td>0.013</td>
</tr>
<tr>
<td>Probable related Peptide from sequence</td>
<td>99-114</td>
<td>95-98</td>
<td>99-114</td>
<td>17-58</td>
<td>95-98</td>
</tr>
<tr>
<td>Amino acid used for integration</td>
<td>Gly</td>
<td>Leu</td>
<td>Gly</td>
<td>Gly</td>
<td>Leu</td>
</tr>
</tbody>
</table>

(ND = not determined)
spots (4) at the origin was identified from its amino acid composition as section 17-58 of the sequence and probably contains much of the core material due to the high incidence of uncharged residues. Peptide 6 was not analysed.

Differences were also observed in patterns of α-lactalbumin A and α-lactalbumin A SCl tryptic digests using electrophoresis in both dimensions (pH 4.7/1.9). Several difference peptides were observed and were considered to be the same as peptides 1, 2 and 6 of Fig. V.6. No peptides were analysed.

Peptide maps of FC using the system of electrophoresis at pH 1.9 or 4.7 in the first dimension followed by chromatography in the second dimension, were identical to patterns of α-lactalbumin prepared at the same time. This result at pH 4.7 is in contrast to the observation that FC and α-lactalbumin had different net charges on starch gel electrophoresis at pH 5.2 although similar mobilities at pH 3.2. The difference in electrophoretic mobilities was interpreted as a difference in the number of carboxyl groups in the two proteins and therefore should be detected in the peptide maps at pH 4.7.

V.C DISCUSSION

The three minor components detected in the study on the fractionation of bovine whey proteins discussed in Chapter III, have been found to be α-lactalbumins. These three minor α-lactalbumins are present for each major genetic variant, therefore, milk which is heterozygous with respect to α-lactalbumin A B contains eight α-lactalbumin components.

The α-lactalbumin B FC, SCl and SC2 and α-lactalbumin A SCl were identified by their ability to function in the
lactose synthetase reaction with bovine "A protein" and from their amino acid compositions. Proteins corresponding to α-lactalbumin A SC2 and FC were also observed in starch gel electrophoresis patterns and chromatographic fractionations of whey proteins from bovine Droughtmaster breeds and these are considered to be genetic variants of α-lactalbumin SC2 and FC.

From initial observations, in gel filtration experiments, SCl and SC2 were thought to be dimers of α-lactalbumin. However SCl and SC2 sediment at similar rates to α-lactalbumin in tris/HCl buffer, pH 7.5 at 68,000 rpm having sedimentation coefficients $s_{20,w}$ of 1.71 S, 1.72 S and 1.67 S respectively. On closer analysis of the gel filtration data an approximate molecular weight of SCl and SC2 was calculated to be 25,000 ± 1,000 which is less than twice the monomer molecular weight. Since these two proteins contain 12-15 per cent carbohydrate and that their relative electrophoretic mobilities are unchanged in gels containing 7 M urea and 2-mercaptoethanol, it may be concluded that they are not associated α-lactalbumin molecules. Calculation of the minimal molecular weight of SCl from composition data may be made as follows:

<table>
<thead>
<tr>
<th>Amino acids (Trp = 4 residues)</th>
<th>Contribution to Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetyl hexosamine 6 x 203</td>
<td>14,300</td>
</tr>
<tr>
<td>Hexose 6 x 162</td>
<td>1,218</td>
</tr>
<tr>
<td>N-acetylneuraminic acid 1 x 291</td>
<td>972</td>
</tr>
<tr>
<td>Fucose 1 x 146</td>
<td>291</td>
</tr>
<tr>
<td>TOTAL</td>
<td>16,927</td>
</tr>
</tbody>
</table>
Andrews (1965, 1966) found that many glycoproteins behave abnormally on gel filtration and estimates of the molecular weight up to twice that determined by other methods may be obtained. He suggested that the glycoproteins were more hydrated structures having lower densities.

Praeux et al. (1965) also detected two proteins which were eluted between α-lactalbumin and β-lactoglobulin from Sephadex G-75. They found that these proteins reacted with antiserum of bovine α-lactalbumin B and suggested that they were polymers of this protein. These components are probably the same as the SC1 and SC2 described here and are therefore not polymers of α-lactalbumin as proposed by these workers.

FC is eluted coincident with α-lactalbumin on gel filtration but these two proteins are separated by chromatography on DEAE-Sephadex A-50. FC is active in the lactose synthetase reaction in the presence of "A protein" and has an identical amino acid composition to α-lactalbumin. On repeating the electrophoresis or chromatography on samples of FC, a pattern is obtained which is characteristic of this protein and not of the major α-lactalbumin. Furthermore, the electrophoretic mobility difference between FC and α-lactalbumin was also observed in the presence of urea and 2-mercaptoethanol. FC, therefore must differ in composition from the major α-lactalbumin.

From consideration of the mobilities of the α-lactalbumin components at pH 3.2, 5.2, 7.5 and 8.6 it appears that a carboxyl group present in FC is masked or not present in α-lactalbumin. This would be consistent with the difference being due to loss of an amide group from the major
$\alpha$-lactalbumin. At the time of writing, amide nitrogen determinations had not been carried out on both proteins. In contrast, high voltage electrophoresis of tryptic digests of FC and $\alpha$-lactalbumin at pH 4.7 showed no difference peptides indicating that at this pH the charges on the peptides from both proteins are similar.

Several workers have reported a protein of high electrophoretic mobility in zone electrophoresis patterns of samples of $\alpha$-lactalbumin prepared by (NH$_4$)$_2$SO$_4$ precipitation and crystallization (Aschaffenburg and Drewry, 1957; Weitlaufar, 1961). The fast component which is visible in electrophoresis patterns of $\alpha$-lactalbumin prepared in the present study by gel filtration and subsequently isolated is thought to be the same protein observed by others.

Gordon et al. (1968) have isolated a minor $\alpha$-lactalbumin of higher electrophoretic mobility than the major component from a sample of $\alpha$-lactalbumin prepared by salt fractionation and crystallization. They reported that this protein differed from the major $\alpha$-lactalbumin by the presence of one hexosamine residue per molecule. The fast material of Gordon et al. (1968) and the FC appear to have similar mobility in relation to $\alpha$-lactalbumin although the former contains one or two residues of hexosamine. In this present study, small amounts of amino sugars were detected in analyses of both FC and $\alpha$-lactalbumin and if the value for the latter sample is used to correct for the colour reaction of protein, the FC contains no hexosamine. No proteins other than FC and $\beta$-lactoglobulin were observed moving ahead of $\alpha$-lactalbumin in starch gel electrophoresis patterns at pH 7.5.
On the present knowledge of the structure of glycoproteins of this size and composition the increased mobility of FC cannot be explained in terms of the presence of one hexosamine residue. Blacklow et al. (1968) reported that in all glycoproteins of this type studied so far, the carbohydrate is joined to the polypeptide chain through a glycosidic link to serine, threonine or hydroxylysine or to the amide nitrogen of asparagine. The presence of hexosamine covalently bound to the protein through such a linkage should not cause the difference in mobility observed here between FC and α-lactalbumin. However the results from peptide maps have also not confirmed the view that it is due to the loss of an amide group. Furthermore the relative electrophoretic mobilities of FC and α-lactalbumin B were not changed in the presence of urea and 2-mercaptoethanol and therefore is probably not the result of a conformation change, or ion interaction. Clearly the nature of the difference between these two proteins requires further investigation possibly involving more detailed peptide mapping over a wider pH range and hydrogen ion titration and amide analysis.

An attempt was made using peptide maps to determine the position of the attached carbohydrate to the SCl polypeptide chain. Two possible sites were detected.

The major difference peptide (1) in the maps of SCl had an amino acid composition which indicated that it was a length of chain (99-114) still containing a potential cleavage point for trypsin. Such a loss in susceptibility to digestion could arise by steric blocking of the lysine or arginine by a carbohydrate moiety. Using the diagram of the model of
lysozyme (Blake et al., 1965) the sequence 99-114 is in a very exposed area and is well separated from other sections of the chain. If the structure of α-lactalbumin resembles closely that of hen egg lysozyme, the chain should be readily cleaved by trypsin unless extensively blocked. Gel filtration experiments have shown that the presence of carbohydrate has resulted in a considerable increase in the overall volume of the molecule in solution; consequently trypsin digestion at sites which are close to the attached carbohydrate may be sterically hindered.

The major difference peptide in maps of α-lactalbumin A or B which was not in the patterns of SCl was identified as section 17-58 of the chain. However this peptide is probably not the same as that observed in maps of SCl (peptide 1) due to the differences in composition, particularly aspartic acid.

As mentioned earlier, the carbohydrate moiety of a glycoprotein is most frequently attached to serine, threonine or asparagine in the polypeptide chain. Neuberger and Marshall (1969) also found that the sequence of amino acids about such an asparagine frequently included serine or threonine as the second residue from the site of attachment toward the carboxy terminal end of the chain. They proposed that this was the method of recognition of the site to the coupling enzyme. Several such sites may be present in the sequence of α-lactalbumin B proposed by Brew, Vanaman and Hill (1967). For example, Asx(45), AsN(74) and Asx(84). None are present in the sequence 99-114 although residue 102 is an asparagine and residue 112 is serine. Residue 45 is in the difference peptide which was isolated from the tryptic digest.
of α-lactalbumin B. On present evidence, residue 45 appears the most likely site of attachment.

All bovine α-lactalbumin B minor components were active in the synthesis of lactose and the specific activity of SC1 was similar to that of the major component. Therefore the position of the carbohydrate in SC1 is of some importance. On inspection of the model of lysozyme, (Blake et al., 1965) the section 99-114 is on the surface at the top and away from the cleft which is the active site of that protein. Residue 45 is situated on the edge of the cleft.

The function of the minor α-lactalbumins is not known. A number of glycoproteins are synthesized in the mammary gland before or during lactation and Brew (1969) has proposed that lactose synthesis is superimposed on this glycoprotein synthesizing system. The synthesis of α-lactalbumin and transport past the "A protein" probably follows the path taken for other glycoproteins prepared in the mammary gland. If a suitable recognition site on the α-lactalbumin chain is available, carbohydrate may also be attached to it intentionally or accidentally if it was not lethal to its function in lactose synthetase. SC1 differs from SC2 by the presence of one residue of sialic acid. Therefore SC2 may simply be partially synthesized or degraded SC1. The FC is considered to be not directly related to SC1 or SC2 since it is probably not a glycoprotein.

The SC1 and FC may be synthesized by the mammary gland to perform specific functions. The four α-lactalbumin components form a series of decreasing solubility in water which is paralleled by increasing electrophoretic mobility at pH 7.5
135 and 8.6. The solubility of a protein reflects its surface charge distribution and ability to form aggregates. Such a range of proteins of slightly different properties may allow the mammary gland to produce lactose under varying conditions. Until more is known of the state of association of \( \alpha \)-lactalbumin and the importance of this process to its function in lactose synthesis the question remains.

Alternatively, the carbohydrate moiety may enable the protein to perform another enzymic function or to have differing immunological properties. However, Préaux \textit{et al.} (1965) have found that the two proteins eluting between \( \alpha \)-lactalbumin and \( \beta \)-lactoglobulin on gel filtration reacted with antisera to bovine \( \alpha \)-lactalbumin B. These proteins are probably SC1 and SC2 described here. Recently, Barman (1970a) has reported on a bovine milk "glyco-\( \alpha \)-lactalbumin" of composition and properties on gel filtration, sedimentation velocity and activity in lactose synthetase, which are similar to those of SC1 and SC2 discussed here. However no indication of the purity of the sample of the glycoprotein was given and from comparison with the results obtained in this present work the preparation probably consisted of SC1, SC2 and some of the major \( \alpha \)-lactalbumin component. He suggested that \( \alpha \)-lactalbumin is secreted as a glycoprotein but is then partially degraded by milk glycoside hydrolases to yield \( \alpha \)-lactalbumin and the fast component described by Gordon \textit{et al.} (1968). The fast component (FC) described in this present work is probably not a glycoprotein and is therefore not considered to be a breakdown product of SC1 or SC2.
CHAPTER VI

APPARENT HETEROGENEITY OF α-LACTALBUMIN IN
DEAE-SEPHADEX CHROMATOGRAPHY

VI.A INTRODUCTION

The fractionation of bovine whey proteins and isolation of α-lactalbumin by column chromatography as described in Chapter III is summarized in Fig. VI.1. The α-lactalbumin rich fraction from gel filtration (Fraction V) is eluted as two peaks from DEAE-Sephadex A-50 using a linear salt gradient in buffer at pH 6.3-7.8. Conditions affecting this bimodal elution on anion exchange chromatography have been investigated further to a limited extent and the results are considered in relation to other forms of apparent heterogeneity of samples of α-lactalbumin described by other workers and the lactose synthetase reaction.

VI.B EXPERIMENTAL

The source of milk samples and details of fractionation procedures have been described in Chapter III. The results outlined here were obtained from over 60 fractionations of α-lactalbumin on DEAE-Sephadex A-50. Experiments designed to test the effect of specific conditions of sample preparation and elution were performed immediately before or after a control fractionation. Unless stated otherwise, the following conditions were employed:

\[ \text{temperature } 2 \pm 2^\circ C \]
Whole milk

+ 264 g (NH₄)₂SO₄/l

Supernatant.

Whey.

+ 262 g (NH₄)₂SO₄/l.

Precipitate dialysed against appropriate buffer. Solution applied to column of Sephadex G-75

α-lactalbumin rich fraction (V). Protein precipitated with 530 g (NH₄)₂SO₄/l and dialysed against buffer. Solution applied to column of DEAE-Sephadex and eluted with a linear NaCl gradient in buffer.

FIG. VI.1. Fractionation of bovine whey proteins and isolation of α-lactalbumin by column chromatography.
column type glass columns 1.5 cm bore packed to a length of 20 ± 0.5 cm

gradient 0.013-0.117 M NaCl. Linear gradient in buffer pumped from a Technicon Autograd gradient mixer

elution rate 0.5 ml/min.

Bed material was regenerated in bulk and portions of the same lot used in comparative experiments.

The proportion of protein in each fraction was compared using the ratio of areas enclosed by peaks of the elution profile, assuming that the absorbancy index at 280 nm for α-lactalbumin was constant throughout. Where peaks were not clearly resolved in the elution profile, bell-shaped curves were constructed. A typical pattern is shown in Fig. VI.1. The area bounded by this peak and the baseline was measured with a planimeter,

\[
\text{Area ratio} = \frac{\text{units of area under peak 1}}{\text{units of area under peak 2}} = \frac{A_1}{A_2}
\]

VI.C RESULTS

Fractionation of α-lactalbumin by column chromatography and starch gel electrophoresis

The elution profiles normally obtained on column fractionation of total whey protein and isolation of α-lactalbumin on Sephadex G-75 and DEAE-Sephadex are shown in Fig. VI.1.

Rechromatography of α-lactalbumin from Fraction V after gel filtration, on Sephadex G-75 gave a single symmetrical peak but this protein was eluted in three
peaks from a column of DEAE-Sephadex A-50 with a linear gradient of NaCl in tris/HCl buffer pH 7.5-7.8, $I = 0.05$ or imidazole/HCl buffer pH 6.3, $I = 0.043$. The small third peak eluting at a high salt concentration was identified as a minor $\alpha$-lactalbumin component (FC) and is described in Chapter V. Despite the lack of good evidence on the chemical difference between FC and $\alpha$-lactalbumin, this minor protein is not considered to be the result of an interaction process. The two minor proteins SC1 and SC2 were not present in the sample applied to the column. On starch gel electrophoresis in five different buffer systems from pH 3.2-8.6, concentrated samples of protein from either major peak from anion exchange chromatography gave a single identical band of similar mobility to the $\alpha$-lactalbumin in the starting material. The compositions of these buffer systems are given in Appendix I. A single band was also observed when electrophoresis was carried out in the tris/HCl (pH 7.5) or imidazole/HCl (pH 6.3) buffers used for chromatographic fractionation. Patterns obtained using the formic acid-NaOH system (pH 3.2), semi-discontinuous Ferguson and Wallace system (pH 7.5) and boric acid-NaOH system (pH 8.6) are shown in Fig. IV.2. Samples of protein from either peak had identical amino acid compositions.

Rechromatography of fractions on DEAE-Sephadex

In order to determine whether the bimodal pattern was due to rapid reversible interaction between protein and buffer components or whether two chemically dissimilar $\alpha$-lactalbumins are resolved in the fractionation, protein
samples from each peak were concentrated and chromatographed under identical conditions. Two peaks were again eluted by either preparation but the proportion of protein in each peak appeared to depend on the method used to concentrate the protein solution. When protein from either peak was precipitated with $530 \times (\text{NH}_4)_2\text{SO}_4 / l$, centrifuged, dialysed against buffer (3 changes over 2–3 days) and rechromatographed on DEAE-Sephadex A-50, the second peak was the major fraction. Alternatively in this case the solution from the same fractionation was concentrated by ultracentrifugation, dialysed, and chromatographed as above, the first peak predominated. Such a sequence of fractionations is shown in Fig. VI.3.

Chromatography on DEAE-Sephadex after pH fractionation

Several procedures used for the fractionation of $\alpha$-lactalbumin depend on precipitation at low pH.


When the neutralized, dialysed paste containing $\alpha$-lactalbumin was chromatographed on DEAE-Sephadex A-50 samples were concentrated by ultracentrifugation: $A_{280\text{ nm}} = 16.2$. Electrophoresis was carried out for 4 h at 7 V/cm.

pH 8.6: Boric acid - NaOH system

pH 7.5: Semi discontinuous buffer system of Ferguson and Wallace (1963)

pH 3.2: Formic acid - NaOH system.

Details of the compositions of these buffers are given in Appendix I.
samples from each peak were concentrated and rechromatographed under identical conditions. Two peaks were again given by either preparation but the proportion of protein in each peak appeared to depend on the method used to concentrate the protein solution. When protein from either peak was precipitated with 530 g (NH₄)₂SO₄/l, centrifuged, dialysed against buffer (3-6 changes over 5-20 days) and rechromatographed on DEAE-Sephadex A-50, the second peak was the major fraction. Alternatively if either protein solution from the same fractionation was concentrated by ultrafiltration, dialysed, and chromatographed as before, the first peak predominated. Such a sequence of fractionations is shown in Fig. VI.3.

**Chromatography on DEAE-Sephadex after pH 3.5 precipitation**

Several procedures used for the fractionation of α-lactalbumin depend on precipitation at low pH (see Armstrong et al., 1967, Aschaffenberg and Drewry, 1957). When the neutralized, dialysed paste containing α-lactalbumin after precipitation at pH 3.5 (e.g., by Method IIb) was fractionated on DEAE-Sephadex, two peaks were again observed but the proportion in each varied with the treatment after acidification and neutralization. The effect of ammonium sulfate precipitation or dialysis, after pH adjustment is shown in Fig. VI.4. These elution profiles were obtained from samples of pH 3.5 precipitate prepared at 2 ± 2°C by Method IIb of Armstrong et al. (1967). Dialysis of the neutralized, concentrated solution gave a large first peak while ammonium sulphate precipitation and dialysis favoured formation of the second peak.
FIG. VI.3  Effect of dialysis, or precipitation with 530 g (NH₄)₂SO₄/l on chromatography of α-lactalbumin B on DEAE-Sephadex A-50 in tris/HCl buffer pH 7.8, I=0.05 at 20°C. Dialysis was carried out against 3 changes of 2,000 ml buffer over 4-6 days.
FIG. VI.4. Elution profiles of the proteins in the solutions of the pH 3.5 precipitates prepared by Method IIB of Armstrong et al. (1967) from columns of DEAE-Sephadex A-50 in imidazole/HCl buffer pH 6.3, 0-4°C.

(a) Protein in the neutralized solution was precipitated with 530 g (NH₄)₂SO₄/1 and the paste dialysed against buffer (4 changes of 2,000 ml), 13.6 ml applied, A₂₈₀ nm = 52.2.

(b) Neutralized solution of the pH 3.5 precipitate, dialysed against buffer (3 changes of 2,000 ml), 20 ml applied A₂₈₀ nm = 56.5.
Similar behaviour was observed using a purified sample of α-lactalbumin which was stored as an ammonium sulphate paste, dissolved in water and precipitated at pH 3.5.

Complete conversion of protein to give one or other peak was not achieved by any method and the effluent between peaks always contained a considerable amount of protein.

Chromatography on DEAE-Sephadex after gel filtration

From about twenty fractionations on DEAE-Sephadex A-50 of samples of α-lactalbumin after initial purification by gel filtration of total whey protein (Fraction V), the following observations were made.

In all cases where the protein was precipitated with (NH₄)₂SO₄ after gel filtration and then dialysed, \( \frac{A_1}{A_2} \) was less than 1.0. The samples were usually dialysed against three changes of 2,000 ml of buffer over 3 days, but when dialysis was carried out against eight changes of buffer over ten days, a major second peak was again observed. If the protein solution from gel filtration was concentrated by ultrafiltration and fractionated, the first peak invariably was the larger of the two. In such cases the value of the area ratio \( \frac{A_1}{A_2} \) varied considerably from 1.8 to 35. No consistent dependence of the area ratio on sample concentration, or volume or amount of protein was observed. However different milk samples were used in these preparative fractionations and other variables such as length of storage as (NH₄)₂SO₄ paste were not constant. A sample of α-lactalbumin was prepared by gel filtration of total whey protein solution. Equal portions of this solution were applied to columns of DEAE-Sephadex after
different treatments as shown in Table VI.I. An increase in the size of the first peak was evident on increasing the sample volume from five to 400 ml but the effect was less pronounced than on treatment with acid.

TABLE VI.I Effect of conditions of sample preparation on elution of α-lactalbumin from DEAE-Sephadex

<table>
<thead>
<tr>
<th>Treatment of Sample After Gel Filtration</th>
<th>Sample Volume (ml)</th>
<th>A1</th>
<th>A2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. As eluted from Sephadex G-75, concentrated by ultrafiltration</td>
<td>5.0</td>
<td>3.0</td>
<td></td>
</tr>
<tr>
<td>2. Precipitated with (NH₄)₂SO₄ and dialysed against 4 x 2,000 ml buffer</td>
<td>5.0</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>3. Precipitated and dialysed as in 2</td>
<td>400*</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>4. Precipitated and dialysed as in 2 and then brought to pH 3.5 with 1 M HCl, stirred for 2 h at 0-4°C. The paste was dissolved at pH 7 and dialysed against 1,000 ml of the final diffusate</td>
<td>5.0</td>
<td>1.55</td>
<td></td>
</tr>
</tbody>
</table>

* Sample diluted with final diffusate

Chromatography in different buffer systems

No difference in relative size of peaks was observed when tris/HCl buffer pH 7.8, \( I = 0.05 \) or imidazole/HCl buffer pH 6.3, \( I = 0.043 \) were used to fractionate samples of α-lactalbumin previously prepared by gel filtration in these buffers.
However, when phosphate buffer pH 7.3, $I = 0.05$ was used in both chromatographic steps, the pattern from DEAE-Sephadex changed in favour of the second peak as shown in Fig. VI.5.

**Effect of bed type, temperature, and presence of magnesium chloride on elution from DEAE-Sephadex**

Elution of $\alpha$-lactalbumin from a column of DEAE-cellulose gave a similar pattern to a control sample fractionated on DEAE-Sephadex although in the former case the protein was eluted at a lower ionic strength. Chromatography on DEAE-Sephadex at 20 ± 2°C rather than at 2 ± 2°C did not effect the resulting elution profile. Similarly when a sample was dialysed against 0.02 M MgCl$_2$ and eluted in the presence of 0.005 M MgCl$_2$ no difference was observed to a control fractionation not containing this salt.

**Column electrophoresis**

A sample of $\alpha$-lactalbumin, prepared by chromatography on Sephadex G-75 and DEAE-Sephadex was precipitated with 530 g (NH$_4$)$_2$SO$_4$/l and dialysed against imidazole/HCl buffer at pH 6.3, $I = 0.043$ (three changes of 2,000 ml).

Half of the sample was chromatographed on DEAE-Sephadex under normal conditions in imidazole/HCl buffer giving a bimodal pattern of area ratio 0.30. The remaining solution was applied to the top of a column of Sephadex G-75 (3 cm bore x 40 cm) prepared in imidazole/HCl buffer pH 6.3, $I = 0.043$), in a column electrophoresis apparatus (LKB Model 3340). The protein was washed into the bed with 10 ml of buffer, and the electrode compartments filled by the method described by the manufacturers.
FIG. VI.5. Elution profiles obtained from chromatography on DEAE-Sephadex A-50 of solutions of α-lactalbumin prepared from total whey protein by gel filtration. The Fraction V was concentrated by ultrafiltration and the solution dialysed against 3 changes of 2,000 ml buffer.

Fractionations carried out in phosphate buffer, pH 7.4, $I=0.05$ 10.0 ml applied, $A_{280}^\text{nm} = 11.36$

Fractionations carried out in tris/HCl buffer, pH 7.8, $I=0.05$ 9.5 ml applied, $A_{280}^\text{nm} = 11.07$

The second peak was also increased in the phosphate buffer using DEAE-cellulose instead of DEAE-Sephadex A-50.
A constant current of 40 mA was applied for seven hours from a Beckman Constat power supply.

On completion of electrophoresis the protein was eluted from the column with buffer at approximately 0.5 ml/min. One sharp protein peak was eluted at a volume of 75 ml (bovine serum albumin was eluted at 138 ml without electrophoresis). The protein fractions were pooled, concentrated by ultrafiltration, dialysed against imidazole/HCl buffer (three changes of 2,000 ml) and fractionated on a column of DEAE-Sephadex in the buffer using the normal procedure. The elution profile was very similar to the control pattern having an area ratio of 0.25.

Electrodialysis of sample before fractionation on DEAE-Sephadex

A sample of α-lactalbumin was prepared by gel filtration, the protein precipitated with 530 g (NH₄)₂SO₄/l and dialysed against tris/HCl buffer pH 7.8, \( I = 0.05 \) (eight changes of 2,000 ml). Half of the sample was applied to a column of DEAE-Sephadex and eluted under normal conditions giving a bimodal elution pattern of area ratio 0.36. The remaining solution was placed in the cell of a BTL "Chromatographic Desalting Apparatus" (Baird and Tatlock (London) Ltd.) with tris/HCl buffer, pH 7.8, \( I = 0.05 \) in the electrode compartments. The protein and electrode solutions were separated by washed dialysis membrane. A current of 40 mA was passed between electrodes for 15 min., the electrode solutions replenished and the voltage applied for a further 15 min.
This sample was applied to a column of DEAE-Sephadex and eluted under conditions described above, giving an identical elution profile to that obtained for the control of area ratio 0.36.

**Elution of \(\alpha\)-lactalbumin A from DEAE-Sephadex**

\(\alpha\)-lactalbumin A from Droughtmaster breeds of cattle differs from \(\alpha\)-lactalbumin B by the presence of one more glutamic residue and one less arginine (0 Vs 1, see Chapter IV). The A variant therefore has one less positive charge per mole at pH 6.3-7.8.

As shown in Fig. IV.2 a bimodal pattern was also observed in the fractionation of \(\alpha\)-lactalbumin A on DEAE-Sephadex, but this protein was eluted at higher salt concentration than the B variant. The salt concentration required for elution of peak 1 of \(\alpha\)-lactalbumin A was similar to that required for elution of peak 2 of the B variant. Therefore if the two peaks of a given fractionation represent two different chemical entities, the difference in charge between them may be unity.

**Elution of bovine \(\alpha\)-lactalbumin minor components on DEAE-Sephadex**

The isolation and some properties of the three bovine minor components are discussed in Chapter V. When SCI and SC2 were present as minor impurities in samples of \(\alpha\)-lactalbumin they were eluted from DEAE-Sephadex as single peaks immediately before the first \(\alpha\)-lactalbumin peak. However, when a sample of SCI and SC2 containing only small amounts of \(\alpha\)-lactalbumin was chromatographed (e.g., in separation of SCI and SC2 during the isolation
procedure), SCI was eluted in two peaks at 0.025-0.060 M NaCl. The protein from these two peaks was identical on starch gel electrophoresis at pH 7.5. Under similar conditions, \( \alpha \)-lactalbumin itself is eluted between 0.03-0.075 M NaCl when chromatographed alone. SC2 was eluted in a single peak before SCI in the fractionation.

Similar behaviour was also evident in fractionation of FC. When present as minor contaminant of the major \( \alpha \)-lactalbumin, FC was observed as a single peak after the second major protein peak, as shown in Fig. III.7 and VI.1. However, when chromatographed alone, FC was distributed throughout 500 ml of effluent in a broad irregular peak at a NaCl concentration greater than 0.10 M.

**Activity in lactose synthetase**

Samples of \( \alpha \)-lactalbumin B, prepared by gel filtration and anion exchange chromatography, were precipitated at pH 3.0 or with 530 g (NH₄)₂SO₄/l. Both samples after dialysis were active in the lactose synthetase reaction with bovine "A protein". The relative reactivity was not determined.

**Sedimentation velocity**

Sedimentation velocity experiments were carried out using samples of \( \alpha \)-lactalbumin B which were prepared to give a prominent first or second peak on chromatography on DEAE-Sephadex (see Fig. VI.3).

The methods of preparation and sedimentation coefficients obtained are shown in Table VI.II. Protein solutions were dialysed against tris/HCl buffer, pH 7.5 (at 20°C), \( I = 0.05 \) containing 0.05 M NaCl (four changes
TABLE VI.II  Sedimentation velocity

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Sedimentation Coefficient.</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-lactalbumin sample prepared from first peak of fractionation on DEAE-Sephadex. The protein was precipitated with 530 g $(NH_4)_2SO_4/1$ and treated as follows:</td>
<td>(Svedbergs)</td>
</tr>
<tr>
<td>1. Protein sample dialysed against tris/HCl buffer pH 7.5, $I = 0.05$ plus 0.05 M NaCl (four changes of 2,000 ml)</td>
<td>1.67</td>
</tr>
<tr>
<td>$A_{281.5 \text{ nm}} = 13.3$, 0.65 g/100 ml</td>
<td></td>
</tr>
<tr>
<td>2. Protein sample dialysed against tris/HCl buffer pH 7.5, $I = 0.05$ (two changes of 2,000 ml) and applied to a column of DEAE-Sephadex A-50. Samples from each of the two protein peaks were concentrated by ultrafiltration and dialysed against buffer plus salt as in 1.</td>
<td></td>
</tr>
<tr>
<td>a. First peak. $A_{281.5 \text{ nm}} = 13.0$</td>
<td></td>
</tr>
<tr>
<td>0.63 g/100 ml</td>
<td>1.72</td>
</tr>
<tr>
<td>b. Second peak. $A_{281.5 \text{ nm}} = 14.0$</td>
<td></td>
</tr>
<tr>
<td>0.69 g/100 ml</td>
<td>1.63</td>
</tr>
</tbody>
</table>
of 2,000 ml). The three sedimentation patterns were similar, each containing a single peak which showed no apparent asymmetry.

**Optical rotatory dispersion**

ORD spectra of samples of α-lactalbumin B in 0.05 M KCl were recorded at 27°C between 400 nm and 200 nm.

The α-lactalbumin was previously fractionated on DEAE-Sephadex and the protein in the first peak used to prepare the following samples:

(a) The solution was dialysed against 0.05 M KCl at pH 5.9 (four changes of 3,000 ml) and passed through a 0.45 μm Millipore filter.

(b) The protein was precipitated with 530 g (NH₄)₂SO₄/1 and the paste dialysed as in (a). Both final solutions were at pH 5.9.

The ORD curves for values of [m'] calculated from the recorded spectra are shown in Fig. VI.6. The magnitude of -[m'] at the trough at 233 nm was less for samples after precipitation. This change was consistent on repeating the spectra with fresh solutions and the difference here is likely to be significant. Some variation was also apparent at 220-215 nm but more analyses are required to assess the importance of this difference. The pattern for the sample after precipitation also contained an additional peak at 273 nm. However this region of the spectra was always very irregular and although the patterns shown are different in this region the variation may not necessarily be real. These patterns were calculated from measurements on 4-6 repeated scans of the same solution.
FIG. VI.6 Optical rotatory dispersion of solutions of bovine α-lactalbumin B after chromatography on DEAE-Sephadex. --- Protein precipitated with 530 g (NH₄)₂SO₄/l and dialysed against 0.05 M KCl. A₂₈₁.₅ nm = 1.05, final pH 5.9.

---- Protein solution from chromatography was dialysed against 0.05 M KCl. A₂₈₁.₅ nm = 1.01, final pH 5.9.

Dialysis was carried out against 4 changes of 3 l of 0.05 M KCl over 4 days and the solutions passed through a 0.45 μ Millipore filter.
VI.D DISCUSSION

In the study of the fractionation of bovine whey proteins described in Chapter III a bimodal elution pattern of \( \alpha \)-lactalbumin was observed on DEAE-Sephadex chromatography. This bimodality was investigated further in an attempt to determine the nature of the processes involved and their relation to other forms of apparent heterogeneity that have been observed in samples of \( \alpha \)-lactalbumin in this work and by others.

Bovine \( \alpha \)-lactalbumin is eluted in two peaks from columns of DEAE-Sephadex or DEAE-Cellulose using a linear NaCl gradient in tris/HCl buffer pH 7.1-7.8, imidazole/HCl buffer pH 6.3, or phosphate buffer at pH 7.4. Protein from both peaks have similar amino acid compositions and migrate as a single band of similar mobility to the protein in the applied sample on zone electrophoresis in a variety of buffer systems over the pH range 3.2-8.6. On rechromatography of protein from either peak on DEAE-Sephadex, simple patterns are not obtained and they reflect the type of treatment given to the sample. Precipitation with 530 g(NH\(_4\))\(_2\)SO\(_4\)/l favoured formation of the second peak while pH 3.5 precipitation or ultrafiltration and dialysis after fractionation on DEAE-Sephadex or Sephadex G-75 gave predominantly the first peak.

The elution of \( \alpha \)-lactalbumin from columns of DEAE-Sephadex using a linear salt gradient is complex and no simple mechanism would appear to account for all the results. The problem is made more complex by the failure to observe similar behaviour on zone electrophoresis, sedimentation velocity or gel filtration.
The appearance of the patterns and the inability to isolate one form or another on rechromatography suggests that a system involving chemical and/or physical interaction is involved. In such systems, providing the column has adequate resolution, each peak does not necessarily represent a chemical entity for reactions that are rapid or intermediate in rate compared with the chromatographic process. Refractionation of the protein from the individual peaks from such systems, using identical conditions to those in the original experiment, should again give a bimodal pattern. However a simple result is not obtained on refractionation of \( \alpha \)-lactalbumin on DEAE-Sephadex. Although a bimodal pattern is again observed, the size of the peaks depends to some extent on the method of sample preparation prior to dialysis and chromatography. Thus the size of each peak depends on certain features of the protein in the sample which are stable under conditions of sample dialysis and concentration.

Ion exchange chromatography depends on those charged groups on the surface of the protein which are accessible to the polar side-chains of the resin. Therefore two protein molecules which have the same net charge, but differ in the number and distribution of polar groups on the surface, may be separated by ion exchange chromatography. If a polar side-chain is buried but still available to solvent, the electrical double layer of counter-ions will still exist and both protein molecules may move in an electric field with similar velocities. A change in the number or distribution of surface charges may occur by several mechanisms, for example, through binding of ions or noncharged molecules to the
surface, by association-dissociation of protein molecules or by a conformation change.

Gel filtration or sedimentation velocity experiments did not give complex patterns and the sedimentation coefficients were similar using samples which should give one or other major peak on elution from DEAE-Sephadex. Consequently, the bimodal elution patterns are probably not due directly to polymerization reactions of \( \alpha \)-lactalbumin molecules in slow or rapid equilibrium. However, some dependence of the elution profile on sample volume was evident, and the profile for the \( \alpha \)-lactalbumin minor components depended to some extent on the presence of the major \( \alpha \)-lactalbumin.

Uncharged buffer components may interact with the protein although different types of molecules should bind with different affinities. Elution profiles using tris/HCl buffer pH 7.8 or imidazole/HCl buffer pH 6.3 were identical but elution in phosphate buffer favoured formation of the second peak, although still giving a bimodal pattern. Considering the low concentration of noncharged components in phosphate buffer, a mechanism involving such components appears unlikely.

Samples of \( \alpha \)-lactalbumin prepared by gel filtration or chromatography on DEAE-Sephadex without subsequent precipitation with \((NH_4)_2SO_4\) were eluted in patterns containing a major first peak. This behaviour suggests that the bimodality is due to the presence of bound ammonium or sulphate ions which are removed by these treatments. The second peak should have a higher negative charge and therefore the bound species would be anionic. However a major second peak was observed in patterns of samples which were dialysed to
different extents (1-8 changes of 2,000 ml buffer), or
electrodialysed after extensive dialysis (8 changes of 2,000
ml over several days). If specific binding of protein to
anion occurred and that the complex was stable, as shown in
DEAE elution patterns before and after column electrophoresis,
the bimodality should be detected in these zone electrophoresis
experiments.

One mechanism which may account for most of the results
is that a reaction takes place to expose or bury a charged
side-chain as follows:

\[
N_1 \xrightarrow{1} \frac{1}{2} N_2
\]

Where \(N_1\) and \(N_2\) are two forms of \(\alpha\)-lactalbumin differing
in accessibility of a charged side chain in an intermediate
rate equilibrium (under the conditions of elution) relative to
the rate of fractionation. These two forms may be partially
resolved by suitable fractionation methods such as anion
exchange chromatography. In this reaction \(N_2\) is considered the
more negative form and is eluted after \(N_1\) from DEAE-Sephadex.

Reaction 1 is catalysed by precipitation of the protein
with \((NH_4)_2SO_4\) and then dialysis. Interaction of
\(\alpha\)-lactalbumin with other ions may also be important in effecting
this transformation.

Reaction 2 is catalysed by exposure of the protein to
low pH or occurs on contact with the DEAE-Sephadex and possibly
the Sephadex G-75 gel. This effect of elution from DEAE-
Sephadex and gel filtration is indicated by formation of the
first peak on subsequent fractionation on DEAE-Sephadex and
is also suggested from the skew appearance of the second peak
in many elution profiles but almost complete symmetry of peak 1, i.e., conversion of $N_2$ to $N_1$ occurs during elution.

A small change was observed in this present study in the ORD patterns of samples of $\alpha$-lactalbumin after fractionation on DEAE-Sephadex and dialysis or precipitation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis. A decrease in the amplitude of $-\text{[m']}_{233}$ at 233 nm was observed for the sample after $(\text{NH}_4)_2\text{SO}_4$ precipitation and dialysis. This difference may be interpreted as a change in the amount of ordered structure, particularly $\alpha$-helix, in the proteins (Simmons et al., 1961), although side-chain Cotton effects occurring near 233 nm, may also affect the size and shape of the trough (Yang, 1966). Kronman et al. (1966) found considerable variation between ORD patterns at pH 2.0, 6.0, 7.5 and 11.5 in 0.15 M KCl. The side chain Cotton effects in ORD patterns between 255 nm and 300 nm above pH 6.0 were absent at pH 2. These effects were reversible on adjusting the pH from 2 to 5.6. The curves obtained in this present study for the sample after dialysis are in good agreement with that at pH 6.0 reported by these workers. The magnitude of $-\text{[m']}_{233}$ for the sample after precipitation was less than they reported for the sample at pH 6.0.

From a comparison of the elution of $\alpha$-lactalbumin A and B from DEAE-Sephadex, the first and second peaks have an apparent charge difference of unity. If the bimodal pattern is due to resolution of two forms of the protein differing slightly in structure, one polar side chain may be affected but need not necessarily be directly involved in the conformation change itself. Robbins et al. (1965) found that all 12 lysines were amidinated readily with ethylacetimidate...
hydrochloride at pH 8.6, and Lin (1970) found that of the 21 carboxyls in α-lactalbumin, 20 were modified by the carbodiimide reagent. An additional 0.6 residues (located at residues 63-79) were modified by [14C] glycinamide in denaturing conditions.

Apparent heterogeneity in samples of α-lactalbumin has been detected by several workers using a number of techniques. It is of some importance to compare the behaviour observed here on elution from DEAE-Sephadex with the results from these previous investigations.

Several other workers have fractionated samples containing α-lactalbumin on DEAE-cellulose and in all cases a bimodal peak can be observed in the elution profiles (Yaguchi, Tarassuk and Hunziker, 1960; Groves, 1965). Yaguchi et al. (1960) attribute the two peaks to two molecular forms of α-lactalbumin in solution. Groves (1965) did not comment on the bimodality.

Zittle and Della Monica (1955) investigated the fractionation of α-lactalbumin by extraction from an (NH₄)₂SO₄ paste using a gradient of decreasing (NH₄)₂SO₄ concentration. They found that two forms of the protein could be separated in the extraction. The insoluble form predominated in samples which had been dialysed, while the soluble form was the major component in samples prepared from an (NH₄)₂SO₄ paste. Zittle (1956) found that the dialysed sample of α-lactalbumin in the insoluble form (in 2 M (NH₄)₂SO₄) was slowly converted to the soluble form by addition of NaCl or a variety of other salts to 0.1-0.5 M at pH 5.6-9.8. No apparent heterogeneity was observed in sedimentation velocity experiments. Only one peak was
observed on moving boundary electrophoresis in veronal buffer pH 8.6, \( I = 0.1 \) but several peaks were present in the ascending limb at \( I = 0.01 \). The ascending and descending patterns were non-enantiographic at both ionic strengths but the peaks appeared unrelated to the changes in solubility. A rapid pH change occurred on addition of the salt to the dialysed protein solution but the solubility transformation itself was slow. Zittle suggested that the solubility transformation involves rapid anion binding followed by a slow conformation change.

Kronman and co-workers studied in detail the association and aggregation reactions of \( \alpha \)-lactalbumin at pH values below its isoelectric point. At low pH, \( \alpha \)-lactalbumin undergoes a rapid monomer-dimer reaction followed by a slow aggregation process at a rate which depends on pH, temperature, and the concentration and type of anionic species in solution. The effectiveness of a given anion to cause aggregation is related to its ability to bind non-specifically to other proteins and may affect the aggregation simply by reducing the positive charges on the surface. However, Kronman, Andreotti and Vitols (1964) showed that anion binding alone is not responsible for the aggregation but that other time dependant conformation changes must take place. No aggregation reactions were observed on ultracentrifugation above 5.2

Kronman, et al. (1964) also studied the solubility of samples of \( \alpha \)-lactalbumin after acid treatment. Protein which was exposed to acid and then brought to pH 6.6 by rapid addition of phosphate buffer showed a slow increase in
formation of the soluble form in 2.1 M (NH₄)₂SO₄. Almost maximal conversion to the soluble form occurred after 4-5 h at pH 6.6 after previous treatment at pH 2.0. Robbins et al. (1967) noted that exposure to acid (pH 2-3) or alkali (pH 10.5) left the protein more susceptible to precipitation in the pH range 3.5-5.3. These workers showed that titration of α-lactalbumin was completely reversible above pH 6 but on back titration from pH 2.0, time dependant precipitation occurred from pH 3.5 to 5.3 at 25°C. If each point was titrated rapidly and no precipitate allowed to form the forward and backward curves coincided in this region. Kronman et al. (1964) identified their aggregate below pH 4 with the less soluble component of Zittle (1956) and describe the solubility transformation as a reversal of a previous aggregation occurring during preparation at low pH.

Aggregation of protein molecules depends on the nature of their surfaces, and methods of protein purification utilizing precipitation rely on attaining optimum conditions for aggregation. Salting-out probably results from a reduction in the activity of the water causing disruption of the solvent-protein interactions and greater contact between hydrophobic areas on the protein molecules. Precipitation at low pH is influenced by a reduction in the number of surface charges and repulsion of protein molecules should be minimal at the isoelectric point. The solubility transformation described by Zittle (1956) and the aggregation reactions of Kronman et al. (1964) may be related to the bimodal elution of α-lactalbumin from DEAE-Sephadex. Treatment with acid, changes the protein to a form which is
more prone to aggregation and precipitation and which is eluted first on fractionation from columns of DEAE-Sephadex. This form is stable on dialysis against water or buffer, but is converted to another more soluble form (N₂) by increasing the ionic strength. Both Zittle and Kronman and co-workers indicated that the slow processes involved a change in conformation of the protein.

If the two peaks on anion exchange chromatography represent two different forms of α-lactalbumin arising from minor differences in structure, one aspect of the comparison with these other forms of heterogeneity described by Kronman, Zittle and co-workers is not readily explained. The solubility transformation occurs with 0.1 M salt using isoelectric protein (Zittle, 1956) or by buffering with phosphate buffer (Kronman et al., 1964), but will only occur in high concentrations of (NH₄)₂SO₄ in the present study. Zittle (1956) observed that 0.1 M ethanolamine chloride (pH 9.8) did not produce the solubility transformation, and Rawitch and Kolb (1970) detected "anomalous starch gel electrophoresis patterns" of α-lactalbumin in buffers containing hydroxylated amines such as tris and glucosamine. The behaviour was not observed in phosphate buffer or in tris buffer above pH 7 and the anomalous pattern was reverted to normal on dilution of the buffer or addition of another salt to the system. They attribute this behaviour to the binding of the tris cation to the protein. Hen egg lysozyme is inhibited by imidazole and indole derivatives and Shinitzky et al. (1966) have concluded that this is due to the formation of a complex between the protonated imidazolium
ring and the tryptophan residues in the active site. No loss of lytic activity was observed in tris/HCl buffer rather than phosphate buffer (personal observation) although amino sugars do act as inhibitors (Rupley, 1967).

In this present study therefore, the reaction of N₁ to N₂ may be inhibited by certain amines including tris, imidazole or the DEAE side chains. Fractionation in phosphate buffer in the absence of amines therefore should give a major second peak, as observed. The presence of the small first peak on elution in phosphate buffer may be due to interaction with the DEAE-Sephadex on initial binding to the column causing some formation of N₁. Further experiments on DEAE-Sephadex in several buffer systems using samples of α-lactalbumin before and after acid treatment would be needed to determine the effect of buffer components on the elution profile.

Multiple boundaries have been observed in frontal electrophoresis patterns by Gordon and Semmett (1953), Klostergaard and Pasternak (1957) and Wetlaufer (1961) using a variety of buffer systems between pH 3.2 and 8.6 (see Chapter I). A minor fast band was also observed in zone electrophoresis at pH 7.5 and 8.6 by Aschaffenburg and Drewry (1957) and Wetlaufer (1961). However the protein in the minor fast band on starch gel electrophoresis has been isolated in this present work and found to be an α-lactalbumin, probably of different chemical composition to the major component and not due to an interacting system in rapid equilibrium. This protein (FC) is discussed in Chapter V and is considered to be the same fast band reported by previous workers.
Wetlauffer (1961) found that the $\alpha$-lactalbumin samples showed no apparent heterogeneity in sedimentation velocity experiments at pH 6.0 and the UV absorption spectra of the fast fraction from moving boundary electrophoresis was similar to that of the bulk material. Klostergaard and Pasternak (1957) showed that the two peaks could not be separated in an extended moving boundary electrophoresis experiment in lactate buffer at pH 4.8, $I = 0.10$ and re-electrophoresis of protein from the first peak also gave a bimodal pattern. However at pH 4.8 $\alpha$-lactalbumin may also undergo association and aggregation reactions (see earlier, also Chapter I) and therefore the results must be interpreted with caution. Considering these workers' results, the bimodal pattern on moving boundary electrophoresis is probably not due to the presence of the minor $\alpha$-lactalbumin component (FC) but is an interaction effect. These patterns may reflect an ion binding reaction which is independant of the process observed here on fractionation on DEAE-Sephadex. The relation between the two forms of experiments remains to be determined.

Lactose synthetase is formed when $\alpha$-lactalbumin is combined with the "A protein" presumably to give an active complex. The nature of the side chains involved in the interaction of these proteins is not known at present. Adsorption of $\alpha$-lactalbumin to ion exchange resins should also be sensitive to the surface charge distribution and accessibility of particular groups and a greater understanding of the exposed side chains of $\alpha$-lactalbumin may yield valuable information on the binding of the two proteins. A preliminary
analysis showed that protein samples after \((NH_4)_2SO_4\) precipitation or treatment with acid were both strongly active in the lactose synthetase assay with "A protein". However the relative reactivities at lower concentration of \(\alpha\)-lactalbumin was not determined.
CHAPTER VII
LYSOZYMES AND \(\alpha\)-LACTALBUMINS FROM DIFFERENT SPECIES

VII.A INTRODUCTION

A comparative study of the function, composition and solution properties of \(\alpha\)-lactalbumins from different species may provide valuable information on their mode of action in lactose synthetase and their structural and evolutionary relationship to the lysozymes.

The very close correspondence between the classification of species based on phylogenetic data and such a series based on a comparison of the amino acid sequences of homologous proteins from these species has been aptly demonstrated for a number of proteins (see Dayhoff and Eck, 1968). The classification from phylogenetic and zoological data therefore is a convenient basis on which to direct a comparison of lysozymes and \(\alpha\)-lactalbumins. The sequences of proteins obtained from species which are close together in this scheme should be more similar than a third prepared from a more distant species. Such a classification is shown in Fig. VII.1.

The monotremes, marsupials and placentals differ in several respects including their mode of reproduction and the development of the young at birth. The fossil record has indicated that the marsupials and placentals probably diverged about 100 million years ago (Frith and Calaby, 1969). However due to the lack of direct fossil evidence, the relation of the evolution of the monotremes to the marsupials, placentals and reptiles is obscure. The monotremes are mammals
FIG. VII.1 Classification of mammals based on phylogenetic and taxonomic data (De Beer, 1964).

Milk from those mammals shown in boxes were studied in this present work.
but show more features reminiscent of the reptiles than do other members of the class. They are oviparous, in common with reptiles, but the earlier development resembles that of the marsupials to some extent. The echidna egg is incubated in an abdominal pouch and after hatching, the young feeds on milk which is secreted at two patches of skin in the pouch. Griffiths (1968) has thoroughly reviewed the current knowledge of the echidna.

In the present study the milk from four placental species were investigated and \( \alpha \)-lactalbumins isolated. The fractionation and properties of bovine \( \alpha \)-lactalbumins are described in Chapters III-VI and these proteins are considered to be a convenient point of reference for the study of the proteins from other species. Human milk contains a considerable amount of lysozyme (Chandan, Shahani and Holly, 1964) in addition to \( \alpha \)-lactalbumin and is therefore an excellent source of both proteins. Porcine milk was included in this study as an example of variation over shorter range from the bovine \( \alpha \)-lactalbumin. Some preliminary work on ovine milk was included primarily to detect analogues of the minor \( \alpha \)-lactalbumin glycoproteins which were isolated in the bovine milk fractionations (see Chapter V). Milk from one marsupial (red kangaroo, *Macropus rufus*) and two monotremes (echidna, *Tachyglossus aculeatus* and platypus, *Ornithorhynchus anatinus*) were studied.

Bailey and Lemon (1966) reported that two specific whey proteins in the milk of the red kangaroo increased in concentration on resumption of development of a quiescent
blastocyst resulting from post-partum mating. Lemon and Bailey (1966) then proposed that the hormonal stimuli responsible for resumption of embryonic development also causes the appearance of these new specific whey proteins in the milk. Kangaroo milk has only few specific proteins and it was therefore of some interest to determine whether either of these proteins was an α-lactalbumin. The hypothesis of Lemon and Bailey has since been shown to be incorrect and the secretion of these proteins is not associated with the development of a quiescent blastocyst (Lemon and Poole, 1969, Hopper, McKenzie and Treacy, 1970).

VII.B EXPERIMENTAL

Milk samples

Initially, pooled human milk was received from the Canberra Community Hospital. However the lysozyme activity in these samples was lower than that reported by other workers and the variation in lysozyme content was thought to be due to the stage of lactation. Subsequently, milk from a single mother at least three months post-parturition was obtained by Dr K. Mattocks at the St. George Hospital, Sydney or from a donor in Canberra who most generously provided samples.

Porcine and equine milk samples were provided by Dr K. Bell at the Department of Preventive Medicine, Veterinary School, University of Queensland. Ovine milk was obtained from one sheep from the Department of Experimental Pathology, Australian National University. Milk samples from echidnas, platypuses and red kangaroos were
generously provided by Dr M. Griffiths of the CSIRO, Division of Wildlife, Gungahlin. A sample of platypus milk was obtained from an excised mammary gland provided by the Zoology Department, Australian National University. Unless stated otherwise the samples were taken at a late stage of lactation. To obtain high yields of kangaroo milk the joey was usually removed from the mother on the evening prior to milking and oxytocin (20 IU) was injected intramuscularly five minutes before milking. The kangaroo was anaesthetized by administration of ether and milk was expressed from the larger of two teats in the pouch. Milk from two sub-species of echidnas were used in this investigation (for sub-species classification, see Griffiths, 1968). Five members of the sub-species *T. aculeatus multiaculeatus* (Rothschild) were obtained from Kangaroo Island, South Australia, and one, *T. aculeatus aculeatus* (Shaw) from Inverell in Northern New South Wales. Details of the stage of lactation etc. are shown in Table VII.I. Milk letdown was aided by intramuscular injection of 5-7 IU oxytocin/kg body weight just before milking (Griffiths, 1965a). Milk was expressed by squeezing the mammary glands and collected with a pipette. Samples of 1-20 ml of milk were obtained at each milking.

**Preliminary fractionation**

Milk samples were cooled and stored at 0-4°C until fractionated. With the exception of the platypus and echidna milks, the fat was removed by centrifugation at 6,000 rpm for 40 minutes at 0-4°C in a RC2B centrifuge (GSA rotor) and the casein precipitated with 262-300 g
### TABLE VII.1 Location of capture and stage of lactation of echidnas

Data provided by M. Griffiths

<table>
<thead>
<tr>
<th>Animal code</th>
<th>Location</th>
<th>Date</th>
<th>Weight (gm)</th>
<th>Approx. age of young (days)</th>
<th>Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>M4</td>
<td>Kangaroo Island</td>
<td>Oct.1969</td>
<td>80</td>
<td>30</td>
<td>a</td>
</tr>
</tbody>
</table>

**T. aculeatus**

| M2          | "              | Sep.1970   | 97          | 40                          | b    |
| M3          | "              | Sep.1970   | 213         | 60                          | b    |
| M(1+5) †    | "              | Sep.1969   | 300         | 70-80                       | a    |

**T. aculeatus**

| A1          | Northern N.S.W. | Oct.1969   | 300         | 70-80                       | a    |

**T. multiaculeatus**

| (1)         | Southern N.S.W. | Mar.1970   | late season | c                            |
| (2)         | "              | Dec.1969   | mid season  | c                            |

**NOTE:**

* After an incubation period of 10-10.5 days (Griffiths, McIntosh and Coles, 1969)

** Three classes of diet are differentiated:

a. Synthetic diet containing one egg, 100 ml cow's milk and 25 g termites (see Griffiths, 1965b).

b. Homogenized mixture containing "Glucodin", meat (beef, top-side) and termites.

c. Natural food when animal was captured before milking.

† Sample M(1+5) was obtained from two echidnas at the same stage of lactation.
(NH₄)₂SO₄/l. All gel filtration and anion exchange chromatographic separations were carried out as described in Chapter II.C. Amberlite IRC-50 was packed in glass columns of dimensions 1.5 cm bore x 20 cm.

Preparation of dialysable carbohydrates from whey

Qualitative paper chromatography was used to identify and compare the dialysable carbohydrates in some samples of whey from echidnas and platypuses. The whey (approximately 3-6 ml) was transferred to ultrafiltration apparatus (Amicon Diaflo, UM-2 membrane) where it was diluted with 5 ml of water. After concentration to 2 ml, a further 5 ml of water was added and the process repeated. The filtrate was passed through a column of Dowex 50 (H⁺ form), lyophilized and 0.20 ml of water added. Samples of 0.01 ml were applied as spots to Whatman No. 4 chromatography paper and developed for 16-20 hours with n-butanol, pyridine, water; 6:4:3 (by volume) or ethylacetate, acetic acid, water; 10:4:3 (by volume). Reducing sugars were detected with the aniline hydrogen phthalate reagent of Partridge (1949). Samples of glucose, galactose, mannose, fucose and lactose were chromatographed with the samples.

VII.C RESULTS

Enzyme activities

(a) Lactose synthetase and NAL synthetase activities

These assays were carried out on skimmilk (or whey in the case of the monotremes) within four hours of milking. The method is outlined in Chapter II.H.2 using a reaction time of 10 minutes at 37°C. The results are shown in Table VII.II.
TABLE VII.II Lactose synthetase and NAL synthetase activities in milks

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample volume (ml)</th>
<th>Time post partum (days)</th>
<th>Lactose synthetase (cpm)</th>
<th>NAL synthetase (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human skim milk</td>
<td>0.10</td>
<td>100-200</td>
<td>2285</td>
<td>1936</td>
</tr>
<tr>
<td>Bovine skim milk</td>
<td>0.01*</td>
<td>late</td>
<td>1943</td>
<td>1459</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
<td>late</td>
<td>2262</td>
<td>1719</td>
</tr>
<tr>
<td>Kangaroo skim milk</td>
<td>0.01*</td>
<td>200</td>
<td>864</td>
<td>1303</td>
</tr>
<tr>
<td></td>
<td>0.05*</td>
<td></td>
<td>2700</td>
<td>2265</td>
</tr>
<tr>
<td>+ 0.05 ml bovine α-B</td>
<td>0.05</td>
<td></td>
<td>2383</td>
<td>1997</td>
</tr>
<tr>
<td>Platypus (1) whey</td>
<td>0.05*</td>
<td>late season</td>
<td>977</td>
<td>540</td>
</tr>
<tr>
<td>+ 0.05 ml bovine α-B</td>
<td>0.05</td>
<td></td>
<td>975</td>
<td></td>
</tr>
<tr>
<td>+ 0.05 ml bovine &quot;A protein&quot;</td>
<td>0.05</td>
<td></td>
<td>521</td>
<td></td>
</tr>
<tr>
<td>Echidna M (1+5) whey</td>
<td>0.05*</td>
<td>70-80</td>
<td>266</td>
<td>1090</td>
</tr>
<tr>
<td>+ 0.05 ml bovine α-B</td>
<td>0.05</td>
<td></td>
<td>1280</td>
<td></td>
</tr>
<tr>
<td>+ 0.05 ml bovine &quot;A protein&quot;</td>
<td>0.05</td>
<td></td>
<td>307</td>
<td></td>
</tr>
</tbody>
</table>

NOTE:
* Diluted to 0.10 ml
** Blank counts without addition of "A or B proteins" were 17-25 cpm in all sets of analyses
In addition, the effects of added bovine α-lactalbumin B (0.05 ml of 0.007 g/100 ml) or bovine "A protein" preparation on these assays of echidna M(1+5) and platypus (1) wheys and kangaroo skimmilk were determined. As shown in Table VII.II this echidna whey had low inherent lactose synthetase activity which increased markedly on addition of bovine α-lactalbumin B. A small increase was detected on adding the bovine "A protein" although this may be attributed to the residual activity of the "A protein" alone. The NAL synthetase activity of the echidna whey dropped from 1090 cpm to 320 cpm on storage for two days at 0-4°C.

(b) Lysozyme activity

The lysozyme activities of samples of skimmilk or whey were determined by the method outlined in Chapter II.H.1. The results are shown in Table VII.III. Due to the high activity recorded with some echidna whey samples using a sample volume of 0.10 ml, dilutions of the whey solutions were used in some assays. Estimations of the approximate weight of lysozyme per volume of milk are based on measurement of 0.10 ml aliquots of solutions of hen egg lysozyme in the concentration range 0-10 mg/100 ml and assuming that the specific activities of these proteins are identical.

Both human and equine milks have high lysozyme activity and the human protein was subsequently purified. Samples of horse milk became available late in this work and they were not studied further. Starch gel electrophoresis patterns of horse milk at pH 7.5 showed a protein moving to the cathode in a similar position to that observed with human milk.

The lysozyme activity in samples of whey from the echidnas (sub-species T. a. multiaculeatus) appeared to
<table>
<thead>
<tr>
<th>Time post partum (days)</th>
<th>Sample volume diluted to 0.10 ml</th>
<th>ΔT Δt for lysozyme content (mg/100 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine skimmilk</td>
<td>0.10</td>
<td>0.38 0.08 &lt; 1</td>
</tr>
<tr>
<td>Porcine skimmilk</td>
<td>0.10</td>
<td>0.03 0.03 &lt; 1</td>
</tr>
<tr>
<td>Kangaroo skimmilk</td>
<td>0.10</td>
<td>0.3 0.3 1-2</td>
</tr>
<tr>
<td>Equine skimmilk</td>
<td>0.10</td>
<td>1.7 1.7 10</td>
</tr>
<tr>
<td>Echidna whey M4</td>
<td>30</td>
<td>0.05 1.4 2.8 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10 4.5 4.5 25</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M2 0.05 0.5 1.0 6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M3 0.01 2.4 24 160</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>M(1+5) 0.01 2.6 26 150</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.10 14.6 14.6 83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A1 70-80</td>
</tr>
<tr>
<td>Platypus whey (1)</td>
<td>0.01</td>
<td>7.3 73 ca450</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.01 7.6 76 ca450</td>
</tr>
<tr>
<td>Platypus whey (2)</td>
<td>0.10</td>
<td>0.4 0.4 1-2</td>
</tr>
</tbody>
</table>

Platypus whey

(l) late season 0.10 0.4 0.4 1-2

(2) mid season 0.10 0.4 0.4 1-2

increase as lactation progressed and reached very high values after 40-50 days. The lysozyme and other whey proteins were separated on a column of Sephadex G-25 and the lysozyme activity of the fractions coincided with absorbance measurements at 280 nm over the whole peak. The lysozyme was eluted in a single peak at 0.5 M NaCl and the lysozyme activity of the fractions coincided with absorbance measurements at 280 nm over the whole peak.
increase as lactation progressed and reached very high values at late lactation (80-90 days) compared with those milk samples from placentals and marsupials and the platypus.

The whey proteins from the placentals and marsupial

(a) Fractionation of human milk

Since both lysozyme and α-lactalbumin were to be isolated in these fractionations the procedure used for bovine milk described in Chapter III was modified slightly. The method is shown in Fig. VII.2. The treatment of skimmilk with Amberlite IRC-50 was quite effective in removing the lysozyme and no activity was detected in the effluent or washings. The lysozyme and other proteins retained by the cation exchange resin were eluted with 1 M NaCl in acetate buffer, pH 5.0, and most of the activity was concentrated in the first 10 ml of dialysed effluent.

(i) Isolation of human lysozyme The lysozyme rich effluent was dialysed against acetate buffer pH 5.0, I = 0.05 and fractionated on a column of Sephadex G-75. A major protein peak containing the lysozyme activity was eluted at 420-450 ml of effluent. The pooled lysozyme fraction was then applied to a column packed with Amberlite IRC-50 in the acetate buffer and eluted with a linear gradient of NaCl in buffer. The lysozyme was eluted in a single peak at 0.3 M NaCl and the lysozyme activity of the fractions coincided with absorbance measurements at 280 nm over the whole peak.

(ii) Fractionation of the whey proteins Preliminary fractionation of the human whey proteins after removal of lysozyme was achieved by gel filtration on Sephadex G-75. A large peak was eluted at the void volume, followed by a
Fat discarded

Effluent and washings. Some casein was precipitated with 260 g (NH₄)₂SO₄/l and removed by centrifugation.

All protein was precipitated with 530 g (NH₄)₂SO₄/l and the paste dialysed against tris/HCl buffer pH 7.8, I = 0.05 (3 x 2,000 ml) and fractionated on Sephadex G-75

Human milk (450 ml) centrifuged 40 minutes, 6,000 rpm, 0-4°C

10 ml of a slurry of Amberlite IRC-50 was added and stirred for 30 minutes. This slurry was then applied to the top of a column packed with the same resin in acetate buffer pH 5.0 and allowed to drain. The column was washed with buffer at 0.5 ml/min. until the effluent contained little absorbance at 280 nm

Retained protein was eluted with 1 M NaCl in acetate buffer pH 5.0. The protein in active fractions was precipitated with 530 g (NH₄)₂SO₄/l, centrifuged and the paste dialysed against acetate buffer for fractionation on Sephadex G-75.

FIG. VII.2 Fractionation of human milk
smaller peak containing serum albumin and a large symmetrical peak at an elution volume of 420-450 ml corresponding to a molecular weight of 15,000 ± 1,000. Samples from this last fraction were active in lactose synthetase in the presence of bovine "A protein" and starch gel electrophoresis patterns at pH 7.5 of this fraction contained a major band and a faint faster band. The protein eluted in this peak was precipitated with 530 g (NH₄)₂SO₄/1, dialysed against imidazole/HCl buffer pH 6.3 and applied to a column of DEAE-Sephadex A-50. As shown in Fig. VII.3, two major peaks and one smaller peak were eluted with the NaCl gradient. The protein from the two major peaks moved as single identical bands on electrophoresis at pH 7.5. The factors responsible for the bimodal pattern were not studied further although the behaviour appears very similar to that observed with the bovine α-lactalbumins and described in Chapter VI. The nature of the minor component eluting after the major α-lactalbumin was also not determined although the relative mobility of these two proteins was similar to that observed for bovine α-lactalbumin and FC. It is possible therefore, that the anomalous behaviour of bovine α-lactalbumin on elution from DEAE-Sephadex, and the presence in the whey of a minor α-lactalbumin of higher electrophoretic mobility at pH 7.5 than the major component, also exist in their human counterpart.

(b) **Fractionation of porcine whey proteins**

Porcine α-lactalbumin was not readily separated in a pure form by gel filtration due to the presence of a protein which has recently been identified as a low molecular weight
FIG. VII.3 (a) Elution profile of a sample of human α-lactalbumin on chromatography on DEAE-Sephadex A-50 in imidazole/HCl buffer pH 6.3, $I = 0.043$. The sample was obtained from gel filtration of total whey protein. The protein was precipitated with 530 g (NH$_4$)$_2$SO$_4$/l and the paste dialysed against the imidazole/HCl buffer (4 changes of 2,000 ml) before application to the column. Sample: 7.3 ml of $A_{280} = 34.0$.

(b) Diagram of starch gel electrophoresis patterns of fractions obtained in (a). Ferguson and Wallace buffer system pH 7.5, 7 V/cm for 4 h.
analogue of β-lactoglobulin (Kessler and Brew, 1970; Bell et al., 1970). This latter protein has a molecular weight of approximately 18,500 and consequently was not readily resolved from the α-lactalbumin by gel filtration. However in contrast to the difficulties experienced in the fractionation of bovine whey proteins on DEAE-Sephadex, the porcine α-lactalbumin was clearly separated from other components by this method in tris/HCl buffer pH 7.8, I = 0.05. A typical elution profile is shown in Fig. VII.4.

The major protein in Fraction 1 was identified as an α-lactalbumin from its activity in lactose synthetase in the presence of bovine "A protein". Two minor bands may be seen in the electrophoresis pattern of Fraction 1. The slower minor band was removed by gel filtration but the fast band increased in amount on storage of the protein as an (NH₄)₂SO₄ paste and was only removed by subsequent fractionation on DEAE-Sephadex. The amino acid composition of material isolated by chromatography and corresponding to the fast band on electrophoresis was identical to that of the major α-lactalbumin. An attempt was made to determine whether formation of this fast component occurs on (NH₄)₂SO₄ precipitation as does the second peak in the fractionation of bovine α-lactalbumin on DEAE-Sephadex. However no difference in elution profiles was observed using samples after dialysis, or after (NH₄)₂SO₄ precipitation and then dialysis. Both patterns contained a single symmetrical peak.

(c) Fractionation of ovine whey proteins

A sample of ovine whey was fractionated on Sephadex G-75 in tris/HCl buffer pH 7.8, I = 0.05. The elution profile was
FIG. VII.4 (a) Chromatography of a sample of porcine whey proteins on DEAE-Sephadex A-50 in tris/HCl buffer pH 7.8, $I = 0.05$, 0-4°C. The whey protein (NH$_4$)$_2$SO$_4$ paste was dialysed against pH 7.8 buffer (4 changes of 2,000 ml) and 12.2 ml applied, $A_{280 \, \text{nm}} = 33.8$.

(b) Starch gel electrophoresis pattern of fractions obtained in (a). Sample "S" is porcine skim-milk. The major protein in Fraction 1 was identified as $\alpha$-lactalbumin from its activity in lactose synthetase. Fraction 4 contains serum albumin and the major band in Fraction 2 and 3 is a low molecular weight analogue of $\beta$-lactoglobulin. Ferguson and Wallace buffer system pH 7.5, 7 V/cm, 4 h.
similar to that obtained with bovine total whey protein shown in Fig. III.2 except that peak IV was absent from the ovine whey profile. The α-lactalbumin fraction was not fractionated further although one faint fast moving band was observed in starch gel electrophoresis patterns of this fraction at pH 7.5. This minor band did not arise from contamination by β-lactoglobulin.

(d) Fractionation of kangaroo milk

Starch gel electrophoresis patterns at pH 7.5 of skimmilk from kangaroos at a late stage of lactation (approx. 180 days) contain three prominent fast moving bands (a, b and c) which are not present in patterns of blood serum. Of these three bands, a and c appear only at late lactation while b is present at all stages of post-partum. Using samples from a preliminary fractionation of kangaroo whey proteins on DEAE-Sephadex, carried out by Dr G.B. Treacy, the protein in band b was identified as an α-lactalbumin from its activity in lactose synthetase with bovine "A protein". As shown in Fig. VII.5, the b protein was readily isolated by this method.

The b and c proteins were not clearly resolved by gel filtration of whey on Sephadex G-75 in tris/HCl buffer at pH 7.8 and the approximate molecular weights, calculated from the elution volumes were $15,000 \pm 1,000$ for b, and $17,000 \pm 1,000$ for c. The roles of a and c proteins are not known at present.
FIG. VII. 5 (a) Chromatography of kangaroo whey proteins on DEAE-Sephadex A-50 in tris/HCl buffer pH 7.8, I=0.05. The casein was precipitated with 262 g (NH$_4$)$_2$SO$_4$/l and the whey (22 ml) dialysed against the pH 7.8 buffer (3 changes of 2,000 ml). The "band b" protein isolated in a preliminary fractionation carried out by Dr G.B. Treacy was identified as an $\alpha$-lactalbumin. Consequently in later fractionations to isolate this protein (as shown) the gradient elution was terminated by application of 1 M NaCl before elution of the "band a" protein.

(b) Starch gel electrophoresis patterns of kangaroo blood serum (B), skimmilk (S), and the "a, b and c" proteins. The sample of "band a" protein was kindly provided by Dr Treacy. Buffer system of Ferguson and Wallace (1963) pH 7.5; 7 V/cm, 4 h.
Milk from the monotremes; echidna and platypus

(a) Preliminary fractionation of milk

The echidna and platypus milks were viscous liquids and separation of the whey from the casein and fat was readily achieved by centrifugation at 20,000 rpm in a Servall RC-2B centrifuge for 30 min. at 0-4°C. As observed previously by Griffiths (private communication) four clearly separated layers were obtained. The whey was a clear pink (echidna) or pale yellow (platypus) liquid held between a firm plug of casein at the bottom of the tube (identified by McKenzie and Taylor from studies of miscelle structure) and a thick layer of solid fat and some liquid, which appeared to be an oil, at the top. Most of the whey solution could be drawn from the tube by pasteur pipette.

(b) Composition of the dialysable carbohydrates in the whey

Paper chromatography patterns of samples of the dialysable components from echidnas M(1+5), M4 and A1 and platypus (1) are shown in Fig. VII.6. From a comparison of these patterns, the following details were noted.

(i) The major free neutral sugar component in the milk of T.a. multiaculeatus at late lactation is lactose, which appears to increase in concentration as lactation proceeds. In addition, the glucose content of milk from echidnas M(1+5) was high and several spots, probably corresponding to higher oligosaccharides, were detected.

(ii) Echidna A1 (T.a. aculeatus) contained a trace of lactose and some poorly defined spots moving between lactose and the origin. These patterns were very similar to those of
FIG. VII.6 Paper chromatography of the dialysable carbohydrates from platypus and echidna wheys. System (a): ethyl acetate, acetic acid, water; 10:4:3 (by vol.). System (b): n-butanol, pyridine, water; 6:4:3 (by vol.). Samples: 1. platypus 1 (6 ml whey); 2. standard mixture containing fucose, mannose, glucose, galactose and lactose; 3. echidna M4 (3 ml whey); 4. echidna Al (3 ml whey); 5. echidna M(1+5) (6 ml whey). These chromatograms were developed for 16 h and stained with the aniline hydrogen phthalate reagent of Partridge (1949).
T. a. multiaculeatus at early lactation. It should be noted that the echidnas M4, M(1+5) and Al were fed on the same diet of termites and custard containing glucose and lactose and that echidnas M(1+5) and Al were at approximately the same stage of lactation.

(iii) Platypus milk contained only traces of lactose but large amounts of a component of the mobility of fucose and two saccharides moving behind lactose. The composition of the free neutral carbohydrates from the milk of these two species in the class Prototheria are therefore quite different.

(c) Preparation of echidna lysozyme and fractionation of whey proteins

Two methods were used to effect a preliminary separation of the echidna lysozyme from the other whey proteins: (i) retention of lysozyme on Amberlite IRC-50 and elution with salt as described for the human milk fractionation, (ii) application of the whey solution to a column of DEAE-Sephadex in tris/HCl buffer, pH 7.8; the majority of the whey proteins were retarded by the column and the lysozyme was eluted with other basic proteins.

The second procedure was found to be the more successful since the lysozyme was eluted in a small volume without the high concentration of salt which interferes with the lysozyme assay. Subsequent elution of the adsorbed whey proteins was achieved using a linear gradient of NaCl in tris/HCl buffer, pH 7.8. Such a fractionation of 15 ml of whey from echidna M(1+5) is shown in Fig. VII.7. Estimations of lactose synthetase activity in the presence of bovine "A protein" were performed on selected fractions after dialysis against
FIG. VII.7 (a) Chromatography of a sample of whey from echidna M(1+5) on DEAE-Sephadex A-50 in tris/HCl buffer, pH 7.8, I=0.05, at 0-4°C. Whey sample was not dialysed but applied directly to the column; 16 ml, \( A_{280}^{\text{nm}} = 67.4 \). (b) Starch gel electrophoresis pattern of fractions from (a). Ferguson and Wallace buffer system, pH 7.5, 4 h at 7 V/cm. "S" is the whey sample applied.

The lysozymes from echidna and human milks were compared with hen's egg lysozyme by electrophoresis at pH 5.25, 8.45 and 11.35. These patterns at pH 5.25 are shown. Three patterns are no different.
the tris/HCl buffer. No activity was detected in any of the fractions which were eluted with the salt gradient.

The lysozyme rich fraction was chromatographed on a column (3.2 cm bore x 76 cm) packed with Sephadex G-75 in acetate buffer pH 5.0, $I = 0.05$. A major protein peak was eluted between 340-390 ml where the lysozyme activity coincided with the absorbance measurements at 280 nm. Smaller peaks of absorbance were observed at 190 ml and 470 ml. The fractions in the major peak were pooled and applied to a column of Amberlite IRC-50 (H$^+$ form) and the bed was washed with 200 ml of acetate buffer pH 5.0, $I = 0.05$. No lysozyme activity was detected in the effluent until elution was commenced with 1 M NaCl. The lysozyme rich fraction was dialysed against water (6 changes of 2 l.) and lyophilized. The yield of this purified lysozyme from 15 ml of milk was 6 mg.

(d) Starch gel electrophoresis of purified lysozymes

The lysozymes from echidna M(1+5) and human milks were compared with hen egg lysozyme by electrophoresis at pH 5.25, 8.45 and 11.35. These patterns at pH 5.25 are shown in Fig. VII.8 together with the distances moved in all three systems. By comparison of these results the echidna lysozyme has an isoelectric point of approximately 12.0-12.5. In the three patterns the echidna and human lysozymes contained no detectable protein impurities.

(e) Activity of echidna lysozyme in lactose synthetase

In view of the high lactose content of the milk and the apparent absence of an $\alpha$-lactalbumin in the salt effluent from the DEAE-Sephadex fractionation, the purified lysozyme from
echidna M(1+5) was tested for lactose synthetase activity in the presence of bovine "A protein". This activity was found to be positive and increased with increasing concentration of the lysozyme.

However, on repeating the estimation on a similar sample of lysozyme from the sub-species T. a. insulicauda, no activity could be detected. A sample of whey from echidna fractionation on Sephadex G-75 in acetate buffer, 5 V/cm for 1.2 cm bore a 28 cm radioactive protein profile containing a third peak at the void volume, followed by a low peak containing serum albumin and a large third peak between the void and 380-440 ml. The lysozyme activity was not present throughout this third peak. Lactose synthetase activity in the presence of bovine "A protein" was positive in all the pooled, concentrated fractions but the activity appeared to be unrelated to the lysozyme activity. This was confirmed when a sample of echidna M(1+5) and human milk lysozymes and hen egg lysozyme at pH 5.25, acetate buffer, 7 V/cm for 4 h. 

FIG. VII.8 (a) Starch gel electrophoresis patterns of echidna M(1+5) and human milk lysozymes and hen egg lysozyme on electrophoresis in gels at pH 5.25, 8.45 and 11.35 using the acetate, boric acid-NaOH and phosphate-NaOH buffer systems respectively, as described in Appendix I.

From these results the echidna lysozyme should have zero electrophoretic mobility at pH 12.0-12.5. Isoelectric points of 10.5-11.0 and 10.0-10.5 have been reported for human milk lysozyme and hen egg lysozyme respectively (Parry, Chandan and Shahani, 1969)
echidna M(1+5) was tested for lactose synthetase activity in the presence of bovine "A protein". This activity was found to be positive and increased with increasing concentration of the lysozyme.

However on repeating the estimation on a similar sample of lysozyme from the sub-species *T. a. aculeatus*, no activity could be detected. A sample of whey from echidna Al was fractionated on Sephadex G-75 in acetate buffer pH 5.0 (3.2 cm bore x 88 cm). The elution profile contained a small peak at the void volume, followed by a large peak containing serum albumin and a large broad peak at an elution volume of 380-440 ml. The lysozyme activity was distributed throughout this third peak. Lactose synthetase activity in the presence of bovine "A protein" was positive in all the pooled, concentrated, protein fractions but the activity appeared to be unrelated to the lysozyme activity. This was confirmed when the lactose synthetase active material could be removed from the lysozyme rich fraction by passage of the solution through DEAE-Sephadex and by retention of the lysozyme on Amberlite IRC-50 and elution with salt. This dialysed concentrated lysozyme rich fraction did not increase the lactose synthetase activity of samples of bovine "A protein".

However the lysozyme from a second animal of the sub-species *T. a. multiaculeatus* (M3) was prepared by a similar method and again showed marked activity as an α-lactalbumin in lactose synthetase in the presence of bovine "A protein". These results are shown in Fig. VII.9.

Human milk contains large quantities of α-lactalbumin but samples of the human lysozyme prepared and assayed under
FIG. VII.9 (a) Lactose synthetase assays of bovine "A protein" in the presence of increasing concentrations of lysozymes from:

- echidna M(1+5)
- echidna A 1
- human milk
- hens eggs

The reaction mixture contained 0.10 ml stock glucose mixture and 0.05 ml "A protein" preparation, plus 0.01-0.05 ml of solutions of the lysozymes in water (approx. 0.6 g dried protein/100 ml) plus 0.01 ml UDP-[14C] galactose (200 nCi/ml). Aliquots of water (0.05-0.01 ml) were added to give a constant volume of reaction mixture. Reaction was carried out for 10 minutes at 37°C.

(b) Starch gel electrophoresis pattern of lysozymes from i T. a. multiaculeatus
ii T. a. aculeatus

Acetate buffer, pH of gel = 5.25, 7 V/cm for 4 h.
identical conditions did not affect the lactose synthetase reaction of the bovine "A protein". Electrophoresis patterns at pH 5.25 of the lysozymes from the echidnas M3 and Al were also compared. As shown in Fig. VII.9 the lysozyme from *T. a. multiaculeatus* had slightly greater mobility to the cathode than the lysozyme from *T. a. aculeatus*. The lysozyme preparation from echidna Al contained some slow moving impurity and a faint faster band.

The pH dependance of the lactose synthetase reaction in the presence of bovine "A protein" and lysozyme from echidna M(1+5) was investigated briefly. The stock solutions were adjusted to the desired pH by additions of dilute HCl or NaOH (using 0.02 ml for pH adjustment). The pH dependance of the lactose synthetase reaction in the presence of echidna lysozyme closely paralleled that due to the residual activity of the "A protein" alone, having maximum activity at pH 6.1-6.3 and zero activity at pH 4.2 and 9.5.

(f) **Lysozyme activity of the lysozyme from echidna M(1+5)**

Accurate estimates of the specific activity of the echidna lysozyme was not possible due to lack of material and that further dialysis and lyophilization were not practicable. However if an assumed absorbancy index $(A_{\text{1 cm}}^{\text{1 g/dl}}$ at 281.5 nm) of 25 was used for the echidna lysozyme (see later) this protein is approximately 1.3-1.5 times as active as the hen egg lysozyme. The extinction coefficient of the human lysozyme is 25.7 (Parry, Chandan and Shahani, 1969) and from similar measurements, is 1.5-1.7 times as active as hen egg lysozyme.
The pH optimum of the reaction of lysozyme from echidna M(1+5) with cell suspension was 5.6-6.3. A pH optimum of 6.35 was reported for human milk lysozyme (Parry, Chandan and Shahani, 1969); 7.9 for bovine milk lysozyme and 6.2 for the hen egg lysozyme (Chandan, Parry and Shahani, 1965).

The activities of the hen egg and echidna lysozymes showed little dependance on ionic strength in the range 0.05-0.15. These lysozyme estimations were carried out at pH 6.5 using the procedure outlined in Chapter II.H.2 with additions of NaCl to give final concentrations of 0.0-0.3 M NaCl. The phosphate buffer alone has an ionic strength of 0.05.

(g) Fractionation of platypus whey proteins

Starch gel electrophoresis patterns at pH 7.5 or platypus whey contain fewer bands corresponding to milk specific proteins than the other milks studied. The most prominent band has a high electrophoretic mobility at pH 7.5.

A sample of platypus whey was fractionated on Sephadex G-75 in tris/HCl buffer pH 7.8, giving an elution profile containing three peaks in similar positions to those in the fractionation of echidna whey. The third peak was smaller than in the echidna preparation and consisted mainly of the major fast band observed on electrophoresis. No lactose synthetase activity in the presence of bovine "A protein" was detected in any of the fractions. These proteins were not studied further.
Comparison of properties of the isolated α-lactalbumins

(a) Starch gel electrophoresis
   The patterns at pH 7.5 of the human, bovine, porcine, ovine and kangaroo α-lactalbumins are shown in Fig. VII.10.

(b) Function in lactose synthetase
   Solutions of the human, porcine, bovine and kangaroo α-lactalbumins were used to test the relative reactivities in lactose synthesis in the presence of bovine "A protein". The results are shown in Fig. VII.10. It must be stressed that these results are not intended to reflect the reaction conditions found in vivo, using the relevant "A protein", but should provide a convenient comparison of the α-lactalbumins and may be used to indicate the differences in properties which influence activity with a particular "A protein". The analyses were carried out concurrently under normal conditions on solutions having absorbances, $A_{280 \text{ nm}}$, of 0.112.

   The labelled products of the lactose synthetase reaction using bovine "A protein" with bovine α-lactalbumin B or kangaroo α-lactalbumin, and NAL synthetase of bovine "A protein", were separated by paper chromatography as described earlier and detected by autoradiography using Kodak X-ray film exposed for 14 days. Standards were detected by staining the chromatogram with the aniline hydrogen phthalate reagent of Partridge (1949). The synthetic lactose which was detected by autoradiography and identified was not visible on staining with the reagent. A spot of a labelled component in the pattern of the NAL synthetase reaction products moved ahead of lactose but was not identified as
FIG. VII.10 (a) Starch gel electrophoresis pattern of α-lactalbumins at pH 7.5 using the Ferguson and Wallace (1963) buffer system, 7 V/cm for 4 h. Protein concentration 1.0 g/100 ml. The sample of ovine α-lactalbumin was not fractionated on DEAE-Sephadex and a minor band is visible moving ahead of the major band.

(b) Lactose synthetase estimations of bovine "A protein" in the presence of increasing concentrations of α-lactalbumins. The analyses were carried out at 37°C for 10 min. The reaction mixtures contained additions of water or α-lactalbumin solutions having an absorption (A280 nm) of 0.112.

Samples:
1. kangaroo α-lactalbumin
2. human α-lactalbumin
3. porcine α-lactalbumin
4. bovine α-lactalbumin
5. ovine α-lactalbumin
N-acetyllactosamine.

(c) Absorption spectra

The absorption spectra of the human, bovine, porcine and kangaroo α-lactalbumins, the human and echidna milk lysozymes and the hen egg lysozyme were qualitatively similar with the maximum in the 280 nm region occurring at 281.5 nm. The absorbancy indices of the human and porcine α-lactalbumins were determined as described in Chapter II.B.5. These values for $A_\lambda^1 g/dl$ at 281.5 nm are $16.3 \pm 0.2$ and $17.5 \pm 0.2$ for the human and porcine α-lactalbumins respectively.

Due to the low amounts of kangaroo α-lactalbumin and human and echidna lysozymes, accurate determination of the absorbancy indices of these proteins could not be made. An approximate value of 15 for $A_\lambda^1 g/dl$ at 280 nm of the kangaroo protein was estimated from absorption measurements of stock solutions which were prepared for carbohydrate analyses. No corrections for ash were made. Absorbancy indices ($A_\lambda^1 g/dl$) at 280 nm for human lysozyme (25.7) and hen egg lysozyme (27.3) were determined by Parry, Chandan and Shahani (1969) and Glazer (1959) respectively and these values were used here. An assumed value of 25 was used for the echidna lysozyme, based on those reported for human milk and hen egg lysozymes.

All spectra contained a broad peak over the range 279-282 nm and the value of absorbance at 280 nm was only slightly less than that at 281.5 nm. In recording elution profiles therefore a value of 280 nm was used in accordance with normal practice.
(d) **Amino acid analyses**

Oxidized and unoxidized samples of the α-lactalbumins and lysozymes were analysed as described in Chapter II.E. The compositions are listed in Table VII.IV. The sample of ovine α-lactalbumin was prepared by gel filtration and therefore contains the minor protein observed as a fast band on electrophoresis. Sufficient protein was available for only one analysis of echidna lysozyme and this result must be regarded as tentative, particularly for those residues in high amount.

(e) **Carbohydrate analyses**

Hexose, hexosamine, sialic acid (as NANA) and 6-deoxyhexoses (as fucose) were estimated in duplicate samples of human, porcine, bovine (B) and kangaroo α-lactalbumins. The bovine α-lactalbumin was prepared as described in Chapter III and contained no visible impurities on starch gel electrophoresis at pH 7.5. The results are shown in Table VII.V.

The following observations were made: (i) the kangaroo α-lactalbumin may contain one hexosamine residue but the other three proteins probably do not. A peak corresponding to glucosamine (0.4-0.6 residues) was observed in amino acid analyses of the kangaroo α-lactalbumin. No hexosamine was detected in similar analyses of the other proteins. (ii) hexose was present in all samples including bovine α-lactalbumin B and was particularly high in the human and kangaroo samples. The variability in the amount of carbohydrate in bovine α-lactalbumin B in earlier analyses (see Chapter V)
TABLE VII.IV Amino acid compositions of α-lactalbumins and lysozymes

<table>
<thead>
<tr>
<th>Residues</th>
<th>α-lactalbumins</th>
<th>lysozymes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bovine</td>
<td>Ovine</td>
</tr>
<tr>
<td>Lys</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>His</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Arg</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Asp</td>
<td>21</td>
<td>22-23</td>
</tr>
<tr>
<td>Thr</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Ser</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>Glu</td>
<td>13</td>
<td>12-13</td>
</tr>
<tr>
<td>Pro</td>
<td>2*</td>
<td>2*</td>
</tr>
<tr>
<td>Gly</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Ala</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Cys/2</td>
<td>8*</td>
<td>8*</td>
</tr>
<tr>
<td>Val</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Met</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Ile</td>
<td>7-8</td>
<td>7</td>
</tr>
<tr>
<td>Leu</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Tyr</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Phe</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Trp</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Max. number of residues</td>
<td>123</td>
<td>116</td>
</tr>
<tr>
<td>Number of analyses</td>
<td>8</td>
<td>2</td>
</tr>
</tbody>
</table>

NOTE:
* Estimated on oxidized samples
** Data from Canfield (1963)
### TABLE VII.V Carbohydrate compositions of α-lactalbumin samples

<table>
<thead>
<tr>
<th></th>
<th>Bovine</th>
<th>Porcine</th>
<th>Human</th>
<th>Kangaroo</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Res</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexosamine (as N-acetyl-glucosamine)</td>
<td>0.20</td>
<td>0.36</td>
<td>0.30</td>
<td>0.54</td>
</tr>
<tr>
<td>% Res</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.4</td>
</tr>
<tr>
<td>Hexose (as galactose)</td>
<td>1.00</td>
<td>0.33</td>
<td>1.80</td>
<td>2.30</td>
</tr>
<tr>
<td>% Res</td>
<td>0.8</td>
<td>0.3</td>
<td>1.8</td>
<td>2.0</td>
</tr>
<tr>
<td>NANA</td>
<td>0.00</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>% Res</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Fucose</td>
<td>0.09</td>
<td>0.09</td>
<td>0.12</td>
<td>0.14</td>
</tr>
<tr>
<td>% Res</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

**NOTE:**
Residues were calculated per mole of protein assuming a molecular weight of 15,000. Estimations were carried out in duplicate and results are within ± 0.2 per cent carbohydrate.
indicated that it was probably not covalently linked to this protein but present as an impurity. (iii) sialic acid and fucose were absent in all samples.

(f) Optical rotatory dispersion

ORD curves of solutions of human and echidna milk lysozymes, and human, bovine, porcine and kangaroo \( \alpha \)-lactalbumins, dialysed against 0.05 M KCl (four changes of 2 l.) were recorded between 400 nm and 205 nm as described in Chapter II.I. The solutions were adjusted to pH 6.5 with dilute NaOH to prevent precipitation of the protein, and the protein concentrations (approx. 0.07 g/100 ml) were determined from absorption measurements.

The curves of the echidna and human milk lysozymes are similar but are different from the \( \alpha \)-lactalbumin patterns which also show certain similarities within this group of proteins. The patterns for the two lysozymes and bovine \( \alpha \)-lactalbumin B are shown in Fig. VII.11. The magnitude of \(-[m']\) at 233 nm and the wavelength at the point of zero rotation in the region 230-210 nm are listed in Table VII.VI.

In the region 350-260 nm the curves for the \( \alpha \)-lactalbumins differ from the lysozymes, particularly by the presence of a shallow trough at 295 nm and a less pronounced increase in rotation at 292-288 nm. The human \( \alpha \)-lactalbumin showed more similarity to the lysozymes in this region than the other proteins.

Between 260-205 nm all curves, with the exception of the echidna lysozyme, were alike, although the magnitude of \(-[m']\) was generally greater for the lysozymes than for the
The patterns of other α-lactalbumins were qualitatively similar to the bovine pattern and all contained a characteristic peak at 295 nm.
\(\alpha\)-lactalbumins. The pattern of the echidna lysozyme contained a more definite inflection at 225-205 nm than was present in the other curves.

**TABLE VII. VI Mean residue rotation at 233 nm of \(\alpha\)-lactalbumins and lysozymes**

<table>
<thead>
<tr>
<th>Protein</th>
<th>([m']) at 233 nm</th>
<th>Wavelength of zero rotation in 230-210 nm region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine (\alpha)-lactalbumin</td>
<td>5100</td>
<td>221</td>
</tr>
<tr>
<td>Porcine (\alpha)-lactalbumin</td>
<td>4300</td>
<td>216</td>
</tr>
<tr>
<td>Human (\alpha)-lactalbumin</td>
<td>4800</td>
<td>224</td>
</tr>
<tr>
<td>Kangaroo (\alpha)-lactalbumin</td>
<td>5600</td>
<td>220</td>
</tr>
<tr>
<td>Echidna lysozyme</td>
<td>6000</td>
<td>221</td>
</tr>
<tr>
<td>Human lysozyme</td>
<td>5800</td>
<td>215</td>
</tr>
<tr>
<td>Hen egg lysozyme</td>
<td>5800</td>
<td>217</td>
</tr>
</tbody>
</table>

**VII.D DISCUSSION AND CONCLUSIONS**

In this study, lactose synthetase activity was detected in human, bovine, ovine, porcine and kangaroo milks. Accordingly, \(\alpha\)-lactalbumins having strong activity with bovine "A protein" in this reaction were isolated from these milks, using anion exchange chromatography and gel filtration. In addition, a unique lysozyme with weak activity as an \(\alpha\)-lactalbumin with bovine "A protein" was isolated from echidna milk. The chemical and enzymic properties of these proteins have been compared with the lysozymes from human milk and hen's eggs. Platypus milk contains only low amounts of lysozyme and lactose and some
apparent lactose synthetase activity which is not increased on 
addition of bovine \( \alpha \)-lactalbumin or "A protein".

**Fractionation of \( \alpha \)-lactalbumins**

Although the \( \alpha \)-lactalbumins are of similar size and 
with the exception of the echidna lysozyme have roughly 
similar anodic electrophoretic mobilities at pH 7.5 their 
behaviour on column fractionation was complex and the method 
used for their isolation differed slightly in each case. 
The differences were largely due to the changes in the 
protein composition of the wheys generally, but in addition, 
the properties of the \( \alpha \)-lactalbumins on fractionation also 
varied markedly. Particular attention was paid in these 
experiments to determine whether the minor bovine 
\( \alpha \)-lactalbumin components described in Chapter V and the 
anomalous elution of bovine \( \alpha \)-lactalbumin from DEAE-Sephadex 
described in Chapter VI were features common to all species. 
Both phenomena were found to some extent in these other 
systems studied. Porcine milk contains a small amount of an 
\( \alpha \)-lactalbumin component of higher electrophoretic mobility 
at pH 7.5 than the major \( \alpha \)-lactalbumin. This minor protein 
increased in amount in samples of purified \( \alpha \)-lactalbumin 
which was stored as an (NH\( \text{4} \))\(_2\)SO\(_4\) paste, but the conversion 
did not occur immediately on precipitation. The relative 
mobility of the major and minor porcine \( \alpha \)-lactalbumins was 
similar to that of the bovine \( \alpha \)-lactalbumin and PC.

Minor protein components of higher electrophoretic 
moieties than the major \( \alpha \)-lactalbumins were also detected 
by starch gel electrophoresis at pH 7.5 and fractionation on 
DEAE-Sephadex of human and ovine wheys. These minor components
were not identified as α-lactalbumins but their size and
electrophoretic mobilities relative to the major α-lactalbumin
were similar to that of the bovine and porcine minor
components. Only one protein band was observed moving ahead
of α-lactalbumin in electrophoresis patterns at pH 7.5 of
kangaroo milk. This protein increased in concentration as
lactation proceeded and had different amino acid composition
to the α-lactalbumin. This major protein is therefore not
analogous to the minor protein observed in the bovine milk.

Faint bands moving behind the major α-lactalbumin were
observed in samples of porcine and kangaroo wheys but not in
human or ovine wheys. These proteins did not function as
α-lactalbumins. The absence of the minor proteins in ovine
milk, which in other respects resembles bovine milk,
suggests that glycoproteins such as bovine SC1 and SC2 are
not essential to the function of the mammary gland. This
supports the view that the glycoprotein synthesis in
bovine milk was caused by accidental addition to a
susceptible site on the protein. It is possible that the
site of attachment of the carbohydrate moiety may be
determined by considering the amino acid substitutions
between the ovine and bovine α-lactalbumins. However the
compositions of the two proteins show many differences and
further possible changes in the distribution of amide groups
make such a comparison impossible, at this stage.

The anomalous bimodal elution of bovine α-lactalbumin
from DEAE-Sephadex was also observed with the human protein
but not with the porcine or kangaroo α-lactalbumins. It
is interesting to note that the bovine and human
α-lactalbumins also have greater reactivity in lactose synthetase than the porcine or kangaroo proteins. The variation in activity therefore may be the result of the ability of the human and bovine proteins to function by similar mechanisms which are slightly different from that of the porcine and kangaroo α-lactalbumins. This subtle change in mechanism may be related to the anomalous elution of the bovine and human proteins from DEAE-Sephadex. The ability of the protein to undergo this transformation may be advantageous when using the bovine "A protein" and indeed such a change may be involved in the mechanism of action of lactose synthetase generally. The activity of ovine α-lactalbumin and its behaviour on chromatography on DEAE-Sephadex were not studied.

The relative reactivities of the α-lactalbumins estimated from the slopes of the plots of concentration of α-lactalbumin, using a constant amount of bovine "A protein", versus radioactive products, is as follows: bovine > human > porcine > kangaroo. The kangaroo α-lactalbumin was about a third as active as the bovine protein in these assays. The relative reactivities of the bovine, human and porcine α-lactalbumins is unexpected considering the phylogenetic differences between these mammals. Tanahashi, Brodbeck and Ebner (1968) have also reported a similar order of relative reactivities using single concentrations of α-lactalbumins. Ley and Jenness (1970) however have recently reported that the relative reactivities and affinities of porcine and human α-lactalbumins for bovine "A protein" does follow the expected phylogenetic divergence of these mammals.
The reason for this discrepancy is not clear. The relative reactivities of these proteins is of considerable importance in these comparative studies and the results await clarification.

**Fractionation of milk from the monotremes**

Echidna and platypus milks have compositions which differ somewhat from the other milks studied. These milks contain caseins and large amounts of fat, but the kinds of whey proteins and free carbohydrates differ considerably from samples from other species. Free carbohydrates having an $R_G$ of lactose were present in only small amounts in the milk of the platypus and one sub-species of echidna (*T. a. aculeatus*) but reaches high proportions at late lactation in another sub-species of echidna (*T. a. multiaculeatus*). In addition, lactose synthetase activity in the milk of *T. a. multiaculeatus* increased considerably on addition of bovine $\alpha$-lactalbumin B and therefore this mammal is capable of synthesizing lactose by a mechanism which is similar to that in the placentals and marsupials. The lactose synthetase specifier activity in milk from *T. a. multiaculeatus* was found to reside in a lysozyme of high isoelectric point. This protein increases in quantity with lactation.

The milk from *T. a. aculeatus*, also at a late stage of lactation, contained a lysozyme in high amount but only traces of lactose. Small amounts of lactose synthetase activity in the presence of bovine "A protein" were present in some fractions also containing lysozyme but the two activities in these fractions were not coincident. The starch gel electrophoresis patterns of the wheys from the two sub-
species of echidnas were almost identical except for the presence of some different minor proteins of high electrophoretic mobility in each.

The lysozymes from milk of both sub-species of echidnas were isolated and found to differ in their ability to function in lactose synthetase with bovine "A protein". The lysozyme from *T. a. aculeatus* had no activity but the protein from *T. a. multiaculeatus* had weak activity. In addition the lysozyme from human milk, which contains large amounts of α-lactalbumin, showed no activity.

Examination of the two echidna lysozymes by electrophoresis revealed that the protein from *T. a. multiaculeatus* has higher electrophoretic mobility to the cathode at pH 5.2 than that from *T. a. aculeatus*. No heterogeneity could be detected in the patterns of the lysozyme from *T. a. multiaculeatus* at pH 5.3, 8.5 and 11.4 and this sample was used for further analysis.

The lysozyme from *T. a. multiaculeatus* is unique since it has lysozyme activity and also the ability to promote synthesis of lactose in the presence of bovine "A protein". The closely related lysozyme from *T. a. aculeatus*, without lactose synthetase specifier activity is also extremely interesting since a small change in composition and structure must account for the difference in functions of the proteins.

Several questions must be raised in considering the action of this echidna lysozyme in lactose synthetase. (a) The concentration of this active protein in the milk of echidna M(1+5) appears to be low in relation to the
amount of lactose produced. The high glucose in the artificial diet of these animals may account for this discrepancy. Some lactose synthetase specifier activity was detected in fractionation of the whey from echidna Al (T. a. aouleatus). However the protein responsible for this activity could not be identified in the fractions.

(b) All α-lactalbumins previously studied have isoelectric points below 7 and appear to interact with the bovine "A protein" which has a higher isoelectric point (as deduced from its failure to bind to DEAE-Sephadex at pH 7.2). Interaction of the "A protein" with another protein having an isoelectric point of 12 forming a complex to synthesize lactose by a similar mechanism appears unlikely unless local regions of high opposite charge are involved. Furthermore, the lysozyme which is active in lactose synthetase has a higher electrophoretic mobility to the cathode at pH 5.2 than the protein without this activity. Although the difference in function may not be related to the change in mobility, the two proteins must differ in composition by only few amino acids and the activity must be affected by minor changes in specific groups. It is also interesting to note that the α-lactalbumins of higher electrophoretic mobility to the anode, for example, kangaroo and porcine α-lactalbumins, are also less active in lactose synthesis with the bovine "A protein" than are the bovine and human α-lactalbumins. It is apparent from these results that the function of α-lactalbumin
in lactose synthesis requires specific properties of the protein and is not dependant on strong electrostatic attraction between molecules of unlike charge. However the activity may be reduced when $\alpha$-lactalbumins of markedly different isoelectric points, e.g., kangaroo and echidna proteins are used in the reaction with bovine "A protein". Jollès (1964) has reported that lysozyme activity may also be related to the isoelectric point of the protein; a high isoelectric point favours high activity. However the apparent specific activity of the echidna lysozyme (assuming a value of 25.0 for $A_\text{l}^1 \text{g/dl}$, of isoelectric point 12-12.5 is only 1.3-1.5 times greater than that of the hen egg lysozyme and less than that of the human milk lysozyme which have isoelectric points of 10.5 and 11.0 respectively.

Comparison of compositions of the proteins

The amino acid compositions of the $\alpha$-lactalbumins appear to form a group having certain details in common but with the exception of the echidna lysozyme are different from the lysozymes. These compositions are summarized in Table VII.VII which also includes analyses of guinea-pig $\alpha$-lactalbumin and avian and animal lysozymes reported by others. Particularly noticeable between groups are differences in lysine, histidine, arginine, glutamic acid, glycine, alanine, valine and leucine. The composition of the echidna lysozyme has features characteristic of both the lysozymes and $\alpha$-lactalbumins and on this basis cannot be classified simply in either group.

It is difficult to draw extensive conclusions from composition data of the proteins which differ by more than
### TABLE VII.VII  Amino acid compositions of α-lactalbumins and lysozymes

<table>
<thead>
<tr>
<th>Residue</th>
<th>placentals (a)</th>
<th>kangaroo (b)</th>
<th>echidna (c)</th>
<th>animal (d)</th>
<th>avian (e)</th>
<th>goose (f)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lys</td>
<td>11-13</td>
<td>9</td>
<td>15</td>
<td>5-8</td>
<td>6-8</td>
<td>11</td>
</tr>
<tr>
<td>Arg</td>
<td>0-2</td>
<td>2-4</td>
<td>3</td>
<td>6-12</td>
<td>9-14</td>
<td>6-7</td>
</tr>
<tr>
<td>Glu</td>
<td>12-15</td>
<td>17</td>
<td>9</td>
<td>9-11</td>
<td>3-5</td>
<td>10</td>
</tr>
<tr>
<td>Gly</td>
<td>4-7</td>
<td>6-7</td>
<td>10</td>
<td>10-13</td>
<td>12-14</td>
<td>14</td>
</tr>
<tr>
<td>Ala</td>
<td>3-6</td>
<td>6</td>
<td>8</td>
<td>11-15</td>
<td>11-12</td>
<td>10</td>
</tr>
<tr>
<td>Val</td>
<td>2-6</td>
<td>5</td>
<td>3</td>
<td>6-9</td>
<td>6-7</td>
<td>7</td>
</tr>
<tr>
<td>Leu</td>
<td>12-16</td>
<td>10</td>
<td>9-10</td>
<td>7-10</td>
<td>8</td>
<td>4-5</td>
</tr>
<tr>
<td>Phe</td>
<td>3-4</td>
<td>4-5</td>
<td>2</td>
<td>2-3</td>
<td>1-3</td>
<td>2</td>
</tr>
</tbody>
</table>

**NOTE:**

(a) Includes bovine, porcine, ovine and human (from the present study) and guinea-pig (Brew and Campbell, 1967a),

(b) includes rabbit and dog spleen and kidney (Jolles, 1967) and human (from the present study),

(c) includes hen (Canfield, 1963), duck (Jolles, 1967), quail and pheasant (Arnheim, Prager and Wilson, 1969),

(d) Canfield and McMurry (1967).
one or two substitutions unless the changes are of some prominence and reflect the general nature of the proteins. Such a change may be the difference in the number of arginine residues. With the exception of the kangaroo protein all α-lactalbumins have 0-4 but the lysozymes contain 6-14 arginines.

Inspection of the amino acid sequences of the bovine α-lactalbumin and hen egg lysozyme shows that all arginines of the latter are replaced; four by lysine two by glutamic acid, one each by serine, leucine and glycine and two are deleted. Furthermore the arginines in lysozyme form close clusters of positively charged groups on the surface of the molecule (Browne et al., 1969). Substitution of these amino acids by acidic or nonpolar residues should alter the surface charge distribution considerably. It is interesting to note that the kangaroo and echidna proteins contain 2-4 and 3 residues of arginine respectively, and have lower activities in this lactose synthetase reaction than the other α-lactalbumins.

The effect of the marked but consistent differences in the number of glycine, alanine, valine and leucine residues is not known but would be expected to influence the structure of the proteins considerably particularly if the substitutions are made with polar or bulky side chains. Inspection of the sequences of bovine α-lactalbumin and hen egg lysozyme (see Fig. I.1) shows that many such substitutions exist. No estimates of the number of tryptophan residues were obtained except by comparison of the absorbancy indices which were determined for several
proteins. The human, porcine, and kangaroo α-lactalbumins probably have less tryptophans than the bovine protein or the lysozymes. Ovine α-lactalbumin contains no methionine and therefore this amino acid is not essential for lactose synthetase activity.

Optical rotatory dispersion

Again the α-lactalbumin and lysozymes behave as two groups of proteins showing consistent differences in certain details of the ORD curves particularly in the 300-260 nm region and depth of trough at 233 nm.

The patterns in the 300-260 nm region are complex, containing several maxima and minima which probably arise from superimposed Cotton effects at absorption maxima of the side chain chromophores, in addition to a large background rotation. The curves in this region for the human and echidna lysozymes contained several sharp peaks and troughs with a prominent steep increase in \(-[m']\) at 292-288 nm. In the bovine, porcine and kangaroo α-lactalbumin patterns this Cotton effect is less pronounced and this difference is probably due to variation in the number and environment of tryptophan side chains. In addition, the α-lactalbumin pattern contains a trough at 298-296 nm and peak at 295 nm which is particularly prominent in the bovine patterns but is absent in the lysozyme curves. Other differences between the lysozymes and α-lactalbumins in the 300-260 nm region are probably the result of variation in the interactions of the aromatic or cystine side chains with neighbouring sections of the chain.
Meyers and Edsall (1965) have reported complex ORD patterns for carbonic anhydrase B and C, having features in common with these patterns for the lysozymes and \( \alpha \)-lactalbumins, particularly the steep section of the curve at 294-289 nm and the troughs at 289 nm and 298 nm. They found that these complex patterns could be eliminated in the presence of acid (pH 1.6), urea or guanidine hydrochloride and considered that these features were associated with the tryptophan absorption band with some contribution from tyrosines. Glazer and Simmons (1965) have shown that a Cotton effect at 280-290 nm in hen egg lysozyme is eliminated without change in the depth of the 233 nm trough, and the protein inactivated if treated with sodium dodecyl sulphate. Since tryptophans are essential in the active site of lysozyme they conclude that changes in the environment of these groups are responsible for this change.

With the exception of the echidna lysozyme the patterns in the 260-200 nm region are similar with small but consistent differences in the depth of trough at 233 nm occurring between the \( \alpha \)-lactalbumins and lysozymes. The value of \(-[m']\) of the 233 nm trough for the human and hen egg lysozymes is about 500-1,000\(^\circ\) greater than that for the \( \alpha \)-lactalbumins. The difference between these measurements is probably indicative of more helical content in the lysozymes.

Using an absorbancy index of 25.0 to calculate the protein concentration of solutions of the echidna lysozyme, a value of \(-[m']\)\textsubscript{233} nm of 6,000\(^\circ\) was obtained. This value is consistent with those determined from the spectra of the other lysozymes. However the pattern of the echidna
lysozyme contains a more pronounced inflection at 220-210 nm than the other lysozymes or α-lactalbumins. This difference may be due to changes in the conformations of the polypeptide chains and may be interpreted as less β-pleated sheet structure relative to α-helix in this lysozyme than in the other proteins (Sarker and Doty, 1966; Fasman, 1966; Greenfield, Davidson and Fasman, 1967).

It may be concluded therefore that the lysozymes do show some minor differences from the α-lactalbumins in the amount of helical or other ordered structure and the number and environment of the aromatic side chains.

Aune (1968) also compared the ORD curves of bovine α-lactalbumin (pH 6.1, 0.1 M KCl) and hen egg lysozyme (pH 4.5, 0.1 M KCl) at 25°C. The curves were similar between 206 nm and 233 nm but considerable differences were observed in the region between 250 nm and 320 nm. Aune attributes these differences as due to short range side chain effects possibly arising from the different numbers of aromatic chromophores. Kronman (1968) obtained similar results from ORD and CD measurements between 185 nm and 240 nm, and tentatively concluded that the backbone conformations are comparable. This conclusion was also reached very recently by Cowburn et al. (1970) from CD spectra. Cowburn et al. have also compared the proton magnetic resonance spectra of bovine α-lactalbumin and hen egg lysozyme and found large differences, particularly in the upfield region of the spectra, arising from changes in the interactions of aromatic and aliphatic side chains. On inspection of the structure of lysozyme and the model of α-lactalbumin,
Cowburn et al. conclude that the discrepancies could be accounted for by specific sequence differences and that the model proposed by Brown et al. (1969) is essentially correct. In contrast, Krigbaum and Kügler (1970) have concluded from small angle X-ray scattering of solutions, that the two proteins may be quite different in solution. Lysozyme has a radius of gyration of 14.3 Å with an equivalent scattering body of 28 x 28 x 50 Å, whereas for α-lactalbumin \( R = 16.7 \) Å and has dimensions 22 x 44 x 57 Å. Such an expanded structure of α-lactalbumin may account for the greater reactivity of the tryptophans (Barman, 1970), carboxyls (Lin, 1970) and disulphides (Atassi, Habeeb and Rydstedt, 1970) compared with those of lysozyme. However these results may also be due to increased flexibility of the molecule or different sitting of the groups in the structure.

**Function of α-lactalbumin in lactose synthetase**

The conclusions which may be reached, using comparative studies, on the mechanism of action of lactose synthetase is limited at this stage but as a consequence of this preliminary study, work in this area should be more fruitful, particularly from studies of the echidna proteins. A number of features of the reaction have become apparent in this study, and these will now be discussed and compared with more recent reports by others.

(a) Proteins having a wide range of isoelectric points are capable of promoting lactose synthesis with the bovine "A protein". If the echidna lysozyme functions in lactose synthetase by a mechanism which is similar to the
α-lactalbumins, strong electrostatic attraction of protein molecules of unlike net charge is not essential for activity.

Two approaches may then be made: (i) formation of a complex between the "A" and "B proteins" may occur through attraction of small local regions of high charge, or through hydrophobic bonding, (ii) the two proteins do not form a stable complex but the lactose synthetase process depends on rapid interaction employing specific groups on the surfaces of the molecules. Mechanism (ii) may require electrostatic or hydrophobic bonding to form the transition complex but the α-lactalbumin is then released when reaction is complete. One aspect of the present work may be important in this context. The bimodal pattern on elution of α-lactalbumin from DEAE-Sephadex may be the result of a transformation reaction which also occurs on binding to the "A protein". In its function in lactose synthetase, the α-lactalbumin may undergo a conformational change to alter the groups in contact with the "A protein" perhaps in a triggering mechanism to release the α-lactalbumin after reaction.

Brew et al. (1968) proposed that α-lactalbumin is a specifier protein acting on the "A protein" to change the specificity of an existing active site to accept glucose instead of NAG. The interaction may occur by an allosteric mechanism to alter the configuration of the active groups to accept glucose or to provide a new binding site. Alternatively the α-lactalbumin may introduce groups of its own to the active centre of the "A protein".
The lactose synthetase reaction of bovine "A protein" in the presence of echidna lysozyme shows an identical pH optimum to the residual activity occurring in its absence. Similar results were obtained by Brew et al. (1968) and Fitzgerald et al. (1970) when comparing the effect of pH, Mn(II), UDP-galactose and substrate on the lactose synthetase and NAL synthetase reactions of bovine "A protein" and \( \alpha \)-lactalbumin. It is likely therefore that the echidna lysozyme functions by a similar mechanism to the other \( \alpha \)-lactalbumins by affecting the processes occurring at the active site of the galactosyl transferase.

It is interesting to note however that a stable complex of "A" and "B proteins" has not been isolated, although Trayer et al. (1970) and Andrews (1970) have found that "A protein" was retarded on columns of Sepharose-4B to which \( \alpha \)-lactalbumin was covalently linked. The "A protein" was adsorbed in the presence of glucose or NAG and eluted when these components were ommitted from the buffer. Ley and Jenness (1970) have found that the plots of activity with \( \alpha \)-lactalbumin concentration follow simple Langmuir theory, giving a hyperbolic curve enabling an affinity constant of the two proteins to be calculated. On the basis or this conclusion it would not be possible to saturate the "B protein" with "A protein".

Recently, several further reports have been published dealing with the kinetics or the reactions of the "A protein" in the presence of \( \alpha \)-lactalbumin. Fitzgerald et al. (1970) reported that the Michaelis constant \( (K_m)\) of "A protein" for glucose is lowered in the presence of \( \alpha \)-lactalbumin
enabling lactose synthesis to take place at the low glucose concentration found in the mammary gland. Klee and Klee (1970) showed that the $K_m$ for NAG is also lowered in the presence of $\alpha$-lactalbumin and the relative affinities of glucose and NAG for the "A protein" and their concentration in the mixture determines the active substrate. At high NAG concentrations therefore, NAL synthesis is inhibited while at low NAG concentrations NAL synthesis is stimulated by $\alpha$-lactalbumin as found earlier by Brew et al. (1968). Since the relative affinities for glucose or NAG in catalysis and in inhibition are similar, Klee and Klee (1970) suggest that there is only one active site.

Two investigations on the effect of chemical modification of bovine $\alpha$-lactalbumin on the lactose synthetase reaction have recently been reported. Although a plausible model of $\alpha$-lactalbumin containing a cleft was constructed by Browne et al. (1968) these studies have shown that the functional groups are not common to both proteins.

Lin (1970) has modified the carboxyl groups of bovine $\alpha$-lactalbumin and hen egg lysozyme with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and glycinamide. He found: (i) there is no carboxyl group in $\alpha$-lactalbumin with the unique properties of glutamic 35 in lysozyme, (ii) the modified protein was not active in lactose synthesis or inhibition of NAL synthesis, and the functional groups could not be protected from the reagent by UDP-galactose, glucose or NAG. Some protection was afforded by the presence of "A protein". Lin also mentioned that treatment of the tryptophan groups of $\alpha$-lactalbumin with
hydroxynitrobenzyl bromide yielded an inactive protein.

Inspection of the model of α-lactalbumin indicates that histidine 32 may have replaced the glutamic 35 of lysozyme. Castellino and Hill (1970) however found that neither the three histidines nor the single methionine are essential for activity. About 40 per cent of the specifier function of α-lactalbumin remained when all three histidines were carboxymethylated.

The evolution of lactose synthetase

Lactose is a major carbohydrate constituent of almost all mammalian milks and its primary function is probably concerned with the transport of glucose from the mother to the young.

The development of a mammary gland and hence the class of mammals is of relatively recent origin and considering the widespread distribution of both the galactosyl transferases ("A protein") and lysozymes, these proteins probably existed before the differentiation of the mammary cell. A likely path of the evolution of α-lactalbumin therefore is from a primitive lysozyme which interacted with an existing galactosyl transferase to change its function slightly to produce lactose instead of the link between galactose and NAG in glycoprotein synthesis. Gene duplication was followed by divergent evolution to increase the efficiency of this α-lactalbumin in lactose synthesis and to produce a new protein of a single specific function.

The rate of successful mutational change in the α-lactalbumins after the initial divergence was probably greater than that of the lysozymes but slowed considerably
before the major divergence of the mammalian species. Consequently the compositions of the α-lactalbumins from placental mammals are all very similar, but differ from the lysozymes which as a group are also very similar to one another. The composition of the marsupial α-lactalbumin is slightly different as expected from its earlier divergence from the other mammalian species.

The isolation of a protein having both lysozyme and α-lactalbumin functions is extremely important in studies on the evolution of α-lactalbumin and the lactose synthetase reaction. The monotremes are unique mammals, having features in common with reptiles, marsupials and placentals. In addition, the method of secretion of the milk is different and may be considered as rudimentary. In this sense, the monotremes may be expected to reflect an early stage of development of the mammary gland and the presence of such an unusual protein as the echidna lysozyme may be an indication of this character. Alternatively, the lactose synthetase reaction may be in a state of regression in favour of another system of glucose transport. However the dual nature of the lysozyme and the possible course of evolution of α-lactalbumin suggests that the lactose synthetase system in the echidna is in development.

Lactose is not an essential component of platypus milk although fucose and higher oligosaccharides are present. A galactosyl transferase of similar activity to the bovine and echidna proteins is absent and the electrophoresis patterns are relatively simple compared with the other milks examined. These properties indicate that the platypus milk
is considerably different from that of other mammals and may represent a different approach to the problem of nutrition of the young.

Clearly, the existence of such unique fauna is a strong argument in favour of strict principles of conservation. The value of studies of aspects of the Australian wildlife cannot be overemphasized and it is surprising that it has received so little attention so far, both from scientists and society generally. Such studies however are not without physical difficulties and the problem of limited availability of samples can only become worse as some species face extinction.
APPENDIX I

1. **Buffers used in starch gel electrophoresis.** The pH values shown are those of the starch gel after setting.

**pH 3.3** Formic acid - NaOH system (Smithies, 1962).

- **Electrode:**
  - 0.08 M NaOH
  - 0.16 M formic acid

- **Gel:**
  - 0.01 M NaOH
  - 0.05 M formic acid

**pH 5.3** Sodium acetate - acetic acid, continuous system (Arnheim, Prager and Wilson, 1969)

- **Electrode:**
  - 0.20 M sodium acetate
  - 0.026 M acetic acid

- **Gel:**
  - 1 part electrode buffer: 2 parts water

**pH 7.5** Semi-discontinuous system (Ferguson and Wallace, 1963)

- **Electrode buffer pH 8.6**
  - 0.10 M LiOH
  - 0.38 M H$_3$BO$_3$

- **Gel buffer pH 7.6** prepared by mixing two stock solutions
  - 10% v/v
    - 0.076 M H$_3$BO$_3$
    - 0.02 M LiOH
  - 90% v/v
    - 0.003 M citric acid
    - 0.016 M tris

**pH 7.7** Phosphate continuous buffer system using a modification of Ashton (1957)

- **Electrode:**
  - 0.138 M Na$_2$HPO$_4$
  - 0.018 M NaH$_2$PO$_4$

- **Gel:**
  - 20 ml of electrode buffer diluted to 500 ml
pH 8.5 Borate continuous buffer system (Bell, 1967)

Electrode: 0.075 M NaOH
0.30 M H₃BO₃

Gel: 0.0112 M NaOH
0.028 M H₃BO₃

The gel buffer was prepared from stock solution

pH 8.6 Veronal buffer used for paper electrophoresis, and also for starch gel electrophoresis when diluted fifty-fold for preparation of the gel.

0.015 M diethylbarbituric acid
0.073 M sodium diethylbarbiturate

pH 11.3 Potassium phosphate - sodium hydroxide (Arnheim, Prager and Wilson, 1969).

Electrode: 0.013 K₃PO₄ plus NaOH to pH 11.7

Gel: 0.006 M K₃PO₄ plus NaOH to pH 11.7

pH of gel buffer drops to pH 11.3 after preparation of gel.

2. Buffers used for column chromatography

pH 5.0
0.076 M acetic acid
0.05 M NaOH

pH 6.3
0.050 M imidazole
0.043 M HCl

pH 7.4
0.005 M KH₂PO₄
0.015 M K₂HPO₄

pH 7.8 at 2°C \( \frac{d\mathrm{pH}}{dT} = -0.028 \) units/deg.
0.053 M tris
0.05 M HCl
3. Volatile buffers used for high voltage paper electrophoresis in preparation of peptide maps

pH 1.9  87 ml acetic acid + 25 ml formic acid/l
pH 4.7  25 ml acetic acid + 25 ml pyridine/l
pH 6.5  100 ml pyridine + 4 ml acetic acid/l
pH 8.9  20 g ammonium carbonate/l

(D.C. Shaw, private communication).

4. Chromatographic systems for qualitative comparison of sugars

(a) n-butanol, pyridine, water; 6:4:3 (by vol.)
(b) ethyl acetate, acetic acid, water; 10:4:3 (by vol.)

APPENDIX II

Publications dealing with some aspects of the work in this thesis

A COMPARISON OF BOVINE $\alpha$-LACTALBUMIN A AND B.
by: K. Bell, K.E. Hopper, H.A. McKenzie, W.H. Murphy
and D.C. Shaw.

ON THE COLUMN CHROMATOGRAPHY OF BOVINE WHEY PROTEINS.

COMPARATIVE STUDY OF $\alpha$-LACTALBUMINS AND LYSOZYMES.
Paper read at the Australian Biochemical Society
Conference at Melbourne in May, 1970.
REFERENCES


Academic Press, N.Y.


