COMPARISON OF THE PHYSICAL AND CHEMICAL PROPERTIES OF VARIOUS α-LACTALBUMINS

By

DONALD VICTOR SCHMIDT

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COMPARISON OF THE PHYSICAL AND CHEMICAL

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Thesis Approved:

Kurt E. Ebner
Thesis Adviser

Patrick E. Giese

George Gorin

George V. Odell

Larry Ewing

D. P. Durham
Dean of the Graduate College

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CHAPTER I

INTRODUCTION

Milk is a complex mixture of proteins, enzymes, lipids, carbohydrates, cations, anions, and water. This composition varies from species to species, which is probably due to the marked variations in needs of the young. Milk, produced by the maternal mammary glands, has to be an almost complete natural food since newborn mammals with the exception of man depend upon it solely for nourishment and for partial immunity. The successful lactation in the human is not necessary for survival since milk substitutes, such as cow milk, are readily available.

The early studies of milk proteins were directed toward their nutritive and immunological properties. Many enzymes were found in the various mammalian milks and one of these enzymes was lactose synthetase. This enzyme has been the object of a great deal of study since it is involved in the production of the disaccharide lactose (4-O-β-D-galactosyl-D-glucose), which is the only major carbohydrate constituent of milk.

Lactose synthetase was the first mammalian enzyme isolated which required two naturally occurring proteins. These were originally named the A and B protein (1). The B protein was subsequently identified as α-lactalbumin (2) and thus revealing the biological function of this protein. Hill, et al. (3) called α-lactalbumin a "specifier" protein when it was observed that α-lactalbumin changed the acceptor specificity

of the galactosyl transferase (A protein) from N-acetylglucosamine to glucose. Recently, Morrison and Ebner (4) have shown through their kinetic studies that α-lactalbumin is a modifier of the galactosyl transferase and effectively reduces the apparent K_m of glucose so that it becomes a biologically significant substrate.

The discovery of a biological function for α-lactalbumin brought renewed interest to this already well-characterized, abundant milk protein which constitutes about 20 percent of the whey protein (5). The initial studies of α-lactalbumin date back to 1899 when Wichmann (6) obtained a crystalline material from milk after removal of casein which he called "lactalbumin". Following earlier studies (7,8), Svedberg and Pederson (9,10) in 1937 studied the protein in the ultracentrifuge and named it α-lactalbumin. The physical and chemical properties of bovine α-lactalbumin have been well characterized since it is readily available and is a very stable protein. Studies on the protein have advanced to the elucidation of the complete amino acid sequence by Brew, et al. (11).

The elucidation of the previous partial amino acid sequence of bovine α-lactalbumin resulted in the discovery that α-lactalbumin and hen's egg white lysozyme (12) had very similar amino acid sequences. Both proteins also have similar or identical amino and carboxyl terminal residues and identical disulfide bonds. Both proteins are involved in similar reactions, but neither protein shows any of its related protein's enzymatic activity. It is also possible to fit the amino acid sequence of bovine α-lactalbumin into the structural conformation of lysozyme (13). This postulated structure will be verified or disproven by the x-ray analysis of α-lactalbumin now in progress.

A great deal of research has been conducted on bovine α-lactalbumin

and hen's egg white lysozyme, but little attention has been directed toward α-lactalbumin from other sources. Revolutionary advances in the methodology of protein chemistry have provided efficient methods for the isolation and characterization of proteins. Probably one of the more useful methods is the development of the high speed amino acid analyzers.

The purpose of this study through the utilization of modern characterization methods was to isolate and compare the physical and chemical properties of the α-lactalbumins from bovine, goat, sheep, pig, buffalo (India), guinea pig, and human (Caucasian, Indian (American), Negro and Japanese). The properties of the various α-lactalbumins were established in an attempt to aid in the understanding of the function of α-lactalbumin in the lactose synthetase reaction, and to give some insight into the evolutionary development of this protein.

CHAPTER II

LITERATURE REVIEW

α-Lactalbumin and Lactose Biosynthesis

Preparation of the mammary gland for lactation begins early in pregnancy, and secretion of milk begins soon after parturition. The newborn mammal obtains its nourishment and some of its passive immunity from the milk produced by the maternal mammary glands. Milk contains a great variety of compounds including lipids, carbohydrates, vitamins, salts, proteins and many other miscellaneous compounds; therefore, with this wide variety of nutritive constituents, milk is unique in being an almost complete natural food (14). This unique significance of milk proteins in mammalian nutrition and their immunological role alone justifies the study of the chemistry of these proteins. However, they also possess physiochemical properties that make them of great importance for studies in protein chemistry. Individual proteins undergo an array of interactions with themselves and with one another and they exhibit a variety of conformations. The study of their association-dissociation reactions and conformations poses many problems (15) and since milk proteins possess these properties, they have been the subject of intensive research (16).

One of the milk proteins that has received a great deal of attention is α-lactalbumin since it possesses a very unique physiological property. Bovine skimmilk contains about 70-150 mg of α-lactalbumin

per 100 ml of skimmilk and thus it is a major constituent of the whey proteins (17). Milk produced under normal conditions contains a wide variety of enzymes and many of these enzymes have either been purified, isolated from milk, or definitely identified in milk. These milk enzymes are presumed to be normal constituents of secretory epithelial cells where they participate in cell metabolism and in the biosynthesis of milk constituents (18). Milk proteins appear to be secreted by vacuoles emptying through the plasma membrane (19).

Before 1964, no biological function for α-lactalbumin was known (20). The biological role of α-lactalbumin was elucidated by Ebner and Brodbeck (1,21,22) when they first separated the soluble lactose synthetase from bovine milk into two components: the A protein and the B protein which was identified by Brodbeck, et al. (2) as α-lactalbumin.

Lactose synthetase (E.C. 2.4.1.22) is the final enzyme in the pathway of lactose biosynthesis and this milk carbohydrate makes up half the dry weight of milk. The understanding of the control of lactose biosynthesis is only achieved with a detailed knowledge of the enzymes involved. The biosynthesis of lactose occurs by the following series of enzymatic reactions (23):

1) UTP + glucose-1-P $\rightleftharpoons$ UDP-glucose + PPi

2) UDP-glucose $\rightleftharpoons$ UDP-galactose

3) UDP-galactose + glucose $\longrightarrow$ lactose + UDP .

Reaction 1 is catalyzed by the enzyme UDP-glucose pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridyltransferase, E.C. 2.7.9). Reaction 2 is catalyzed by the enzyme UDP-galactose-4-epimerase (E.C. 5.1.3.2), and reaction 3 by the enzyme lactose synthetase

(UDP-galactose: D-glucose-1-galactosyltransferase, E.C. 2.4.1.22).

The A protein of lactose synthetase was shown by Hill, et al. (3, 24) to be an excellent UDP-galactose: N-acetylglucosamine galactosyl transferase. This enzyme can also catalyze the transfer of galactose from UDP-galactose to N-acetylglucosamine on the carbohydrate side chains of proteins.

4) UDP-galactose + N-acetylglucosamine $\rightleftharpoons$ N-acetyllactosamine + UDP.

This reaction gives a protein-bound N-acetyllactosamine group and is an important step in the serial attachment of monosacharides to form the carbohydrate moiety of many glycoproteins (25). Work by Fitzgerald, et al. (26) has shown that reaction 5

5) UDP-D-galactose + D-glucose $\rightleftharpoons$ lactose + UDP

is catalyzed by the A protein alone when the glucose concentration is high. α-Lactalbumin lowers the apparent K_m of glucose so that it becomes a good substrate. Klee and Klee (27) have recently verified and extended this mechanism. Their results show that (a) the A protein can catalyze reaction 3, but only poorly because of a very high K_m for glucose; (b) α-lactalbumin decreases the K_m for glucose as well as that for GluNAc; and (c) α-lactalbumin inhibits the overall reaction at relatively high GluNAc concentrations and also at high glucose concentrations. Thus, at the concentration of glucose which is ordinarily used in assays or found in vivo, α-lactalbumin greatly stimulates lactose synthesis. At similar concentrations of GluNAc, α-lactalbumin acts as a potent inhibitor. Kinetic experiments by Morrison and Ebner (4) have shown that (a) Mn^{2+} adds firstly to the galactosyl transferase and is

at thermodynamic equilibrium with the enzyme, (b) UDP-galactose and carbohydrate (Glu or GluNAc) add next prior to product release, (c) in the absence of α-lactalbumin, the disaccharide is released prior to UDP, (d) both glucose and GluNAc are substrate inhibitors and this inhibition is increased by α-lactalbumin, and (e) α-lactalbumin functions as a modifier of an existing enzymatic reaction. The general order of addition of substrates in the absence and presence of α-lactalbumin and their release are postulated in Figure 1.

Hill (24) originally suggested that α-lactalbumin be designated as a "specifier" protein which has the ability to change the activity of an existing protein. This "specifier" protein modifies the substrate acceptor specificity of lactose synthetase to include glucose to produce lactose, while in the absence of the "specifier" protein, the enzyme produces N-acetylglucosamine. This proposal represented a new type of molecular control of a biological reaction where the "specifier" protein altered the substrate specificity of the enzyme catalyzing one reaction in its absence, while in its presence, a different but similar reaction was catalyzed. However, recent work (4) has shown that the galactosyl transferase can form lactose in the absence of α-lactalbumin, thus α-lactalbumin functions as a simple modifier of an existing reaction. In practical terms, it reduces the apparent K_m for glucose so that it becomes a better substrate.

Schanbacher and Ebner (28) attempted to demonstrate the α-lactalbumin: A protein enzyme complex through the use of sucrose density centrifugation, equilibrium dialysis, fluorescence quenching, and gel filtration techniques. They were unable to demonstrate a complex formation in the presence or absence of substrates under conditions where

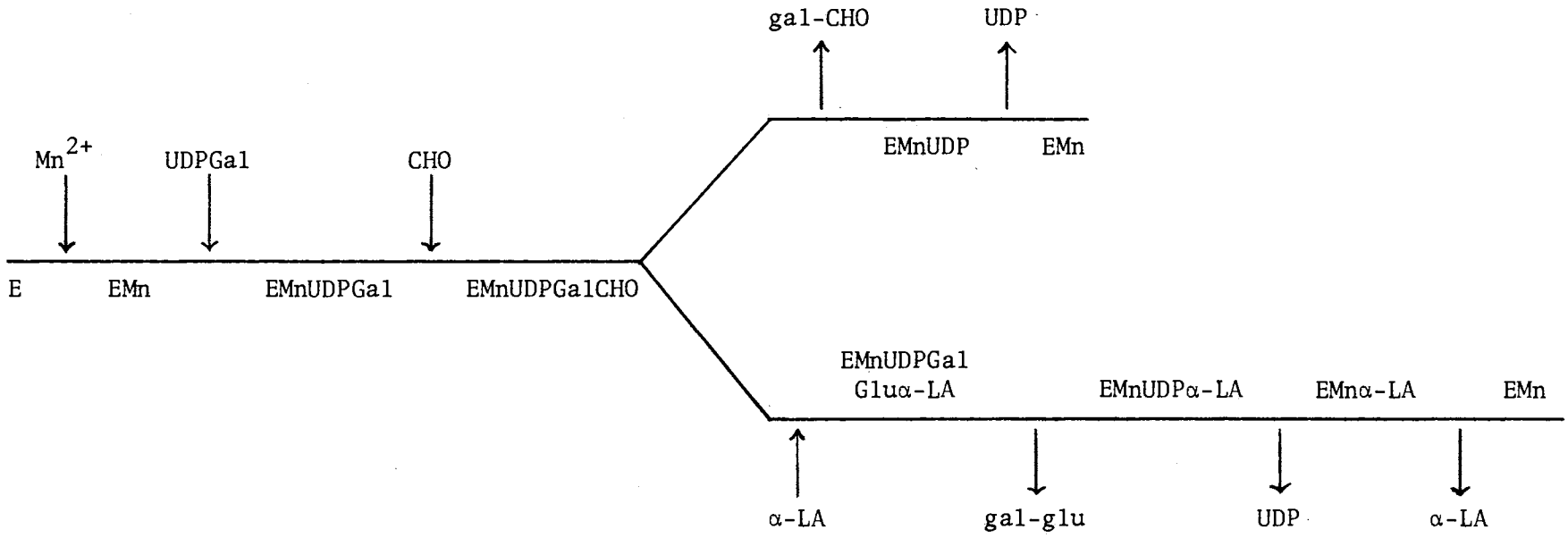

Figure 1. Schematic Representation of the Order of Addition of Substrates and Release of Products

Abbreviations are as follows: CHO, carbohydrate, either glucose or GluNAc; E, UDP-galactose: N-acetyglucosamine galactosyl transferase; α-LA, α-lactalbumin.

catalysis was occurring. Recently, Andrews (29) purified the A protein from human milk by coupling α-lactalbumin onto Sepharose. The A protein adhered to the Sepharose-α-lactalbumin column in the presence of GluNAc, but removal of GluNAc from the buffer released the A protein. Trayer, et al. (30) have used the same method to purify the A protein from bovine milk except they have used glucose as the complexing substrate instead of GluNAc. Morrison and Ebner (4) through their kinetic studies have shown that this represents a dead end complex.

Brodbeck and Ebner (1) reported they were able to titrate either the A protein or α-lactalbumin in the presence of saturating amounts of the other. According to Ley and Jenness (31), it would not be possible from their model to truly saturate α-lactalbumin with the A protein however, it would be possible to assay for low concentrations in the presence of a constant amount of A protein or to assay for A protein activity in the presence of saturating amounts of α-lactalbumin. Palmiter (32) also reported a linear response in his assay system when increasing amounts of α-lactalbumin were added, up to a point at which he felt the A subunit became limiting. At this point, the increase in reaction velocity abruptly leveled off. Recently Fitzgerald, et al. (26) observed in the lactose synthetase assay for the bovine A protein that linearity between reaction rate and A protein concentration occurred even though the level of α-lactalbumin was not saturating and that high levels of α-lactalbumin were inhibitory although linearity was still maintained.

Palmiter (33) proposed a third component, Z, which in crude preparations has the property of preventing rapid dissociation of the A: α-lactalbumin complex. During purification, Z is lost and the A and α-lactalbumin then obey the laws of mass action and chemical equilibrium.

Ley and Jenness (31) doubt existence or presence of the Z component and predict that if the experiments of Palmiter were carried to sufficiently high concentrations of α-lactalbumin, a family of hyperbolas, not straight lines, would be obtained. Similarly, the results obtained with the crude preparations of A and α-lactalbumin would not require invoking existence of the Z component to explain the flattening of the curves.

Tanahashi, et al. (2,34) have shown that purified α-lactalbumin from eight different species appear to have similar specific activities in the lactose synthetase system. Ley and Jenness (31) have shown that six α-lactalbumins from different species do not all have the same affinity for bovine A protein.

The immunological properties of α-lactalbumin from different species have been tested (34,35,36). Rabbit antisera prepared in response to bovine α-lactalbumin demonstrate a cross reaction only with the ruminant α-lactalbumins. This contrasts with all the α-lactalbumins showing lactose synthetase activity. These observations suggest there is a distinction between the enzymatic site and immunological site in these α-lactalbumins.

Published evidence of the subcellular organization of the A protein and α-lactalbumin has been summarized by Brew (37) and is summarized in this section. The A protein from the rat and bovine mammary gland is bound to the microsome fraction while α-lactalbumin is distributed between the microsomal and soluble cell fractions (38). α-Lactalbumin synthesized in a cell free system prepared from the lactating guinea pig mammary gland is enclosed in vesicles derived from rough endoplasmic reticulum (39). In homogenates prepared by pulverizing frozen bovine mammary tissue, both proteins are associated with a single particulate

fraction apparently derived from the Golgi regions of the cell (40,41).

During lactation, α-lactalbumin is synthesized on ribosomes associated with membranes of the endoplasmic reticulum and then is transferred to the lumen of the reticulum (39). Transport through the channels of the endoplasmic reticulum brings α-lactalbumin to the Golgi system where the A protein is situated and thus creates the necessary enzymatic conditions for lactose biosynthesis. Thus, lactose is synthesized in the Golgi region and is concentrated in secretory granules which travel to the cell surface and empty their contents into the lumen of the gland (37).

α-Lactalbumin is secreted along with lactose and milk proteins during the process of secretion from the Golgi region. Some A protein is also lost from the mammary gland into milk, but much less than α-lactalbumin (37). This current proposal suggests that the secretion of α-lactalbumin becomes a part of the organization and properties of lactose synthetase. Even though this mechanism appears wasteful, it has several biological advantages. The continuous flux of α-lactalbumin through the mammary cell ensures that its concentration at any time rapidly reflects changes in the rate of its synthesis. Regulation of the rate of synthesis of α-lactalbumin thus becomes an effective way of regulating the rate of lactose formation within the gland. Thus, it is a switch mechanism through which lactogenesis can be terminated. When the synthesis of α-lactalbumin ceases in response to any stimulus, the remaining protein within the cell is secreted and lactose synthesis is readily stopped. This is the current control system which has been summarized by Brew (37).

In contrast to this unique control system which indicates that

α-lactalbumin has a role in determining levels of lactose in milk, Palmiter (42) has shown that the content of lactose in milk from a large variety of animals is inversely proportional to both total protein and fat content. Thus, from this data, it seems unlikely that lactose synthesis is controlled primarily by the rate of total protein synthesis, but an alternative to this proposal is that the concentration of α-lactalbumin is determined independently of total protein content. A comparative study by Sloan, et al. (43) does not reveal any consistent relationship between α-lactalbumin concentration in milk and total protein content. The actual control mechanism of lactose biosynthesis may be similar to the control mechanisms for milk production in the mammary gland (3,24,44).

Larson and Kendall (16) have found that daily production levels of α-casein, β-casein, α-lactalbumin, and β-lactoglobulin are closely related. Little relationship existed between these four proteins and the rest of the milk proteins. Thus, total protein content may not be indicative of production levels of α-lactalbumin.

Ley and Jenness (31) also investigated what role α-lactalbumin might play in determining the lactose levels of six species. The percentage by weight of α-lactalbumin in the whey protein and the whey protein content of the milk was used to calculate the α-lactalbumin content of the milk. The linear correlation coefficient between the two sets of measurements is 0.95, indicating that perhaps α-lactalbumin does have a role in determining levels of lactose of these six species.

Some studies are appearing on the chemical modification of α-lactalbumin as related to its activity in the lactose synthetase system. Castellino and Hill (45) have modified the histidyl and methionyl

residues in α-lactalbumin. Carboxylation of methionine 90 had little effect on the activity of α-lactalbumin, but there was a progressive loss in activity, up to 60 percent, as histidyl residues were carboxymethylated.

Denton (46) has studied the effect of chemical modification of the tyrosines on the activity of α-lactalbumin in the lactose synthetase activity. Nitration, iodination and the action of tyrosinase on α-lactalbumin indicates that the tyrosines are critical for α-lactalbumin activity in lactose synthetase.

Physical and Chemical Properties of α-Lactalbumin From Various Sources

General Properties and Comparisons

Bovine α-lactalbumin is a tightly folded globular protein, having nearly 40% helical structure (47,48). Moldenhauer (49) heated 2% solutions of bovine α-lactalbumin for 40 minutes below 80°C and found no changes as evidenced by mobility on paper electrophoresis at pH 9.6. Heating above 80° resulted in an increase in the apparent negative charge. When a solution of α-lactalbumin was treated at 75° for 30 minutes in phosphate buffer at pH 6.7, there was a 14% loss of activity as measured by chromatography on DEAE, but when the same solution was heated with β-lactoglobulin, there was an 84% loss, and the suggestion was made that sulfhydryl-disulfide reactions may occur between α-lactalbumin and β-lactoglobulin (50). Wetlaufer (51) has shown that a 4% solution of bovine α-lactalbumin heated for 10 minutes at 100°C only lost 60% of the protein. Experiments by Larson (52) and Lyster (53) have shown that α-lactalbumin is the most heat resistant of the

denaturable serum proteins with over 50% of the original amount still remaining after a 77°C heat treatment for 30 minutes, a heat treatment which denatured all of the immune globulins and serum albumin.

Kronman, et al. (54,55) have studied the solvent pertubation properties of bovine α-lactalbumin in aqueous media. Their tentative conclusion, based on sucrose-ethylene glycol, and glycerol-induced difference spectra, is that three of five tryptophanyl residues are buried in the interior folds of the protein. Recently, bovine α-lactalbumin has been shown to contain only four tryptophanyl residues (13). They also found that the same fraction of tryptophanyl residues are inaccessible to dimethylsulfoxide, and that 70-80% of the chromophoric residues are exposed in 2 chloroethanol and acidic methanol. If the disulfide bonds are left intact, the fraction of exposed tyrosyls and tryptophanyls is found to be about the same in the random coil-forming denaturants such as 8 M urea. Difference spectral studies also indicate α-lactalbumin is nearly unfolded in ethylene glycol. Herskovits (56,57) examined bovine α-lactalbumin in helix promoting solvents, 2 chloroethanol and acid methanol, and found that under maximal conditions, helix formation was 20-70%.

The unit cell of crystalline bovine α-lactalbumin, as determined by X-ray diffraction spectroscopy, was an elongated parallelepiped which was occasionally arranged in rosettes (58). The assymetric unit contained four molecules of α-lactalbumin and the face centered unit contained sixteen molecules. Other shapes have been reported for crystals obtained under the same conditions (59). The various shapes probably represent habits of the same crystal form.

Several isoelectric points have been reported for bovine

α-lactalbumin. In salt-free solutions, the pI is 4.8 and in 0.5 M NaCl solution, it was 3.6 (37). Klostergaard (60) found that the isoelectric point was 5.1, which lies outside the pH range of minimum solubility. The isoelectric point, pI = 4.1 - 4.8, is listed for α-lactalbumin by the American Dairy Association (5) which probably was not based on electrophoretic measurements but inferred from minimum solubility observations of the protein over this range.

The partial specific volume of bovine α-lactalbumin, calculated from the amino acid compositon by Gordon and Ziegler (61), was 0.729 cm^3/g (58). The densities of the wet crystals are 1.213 and 1.210 g/cm^3. The partial specific volume as determined by Gordon and Semmett (62) was 0.735.

Gordon and Semmett (62) measured the optical rotation $[\alpha]^{25}D$ of bovine α-lactalbumin which was -60 ± 2^{o} at pH 6.8, 8.3, and 9.7.

The absorptivity of bovine α-lactalbumin ($E^{1\%}_{280}$) of 20.9 and 20.1 was reported by Wetlaufler (51) and Zittle and Kronman (63,64), respectively. Wetlaufler (51) also measured the E_{280}/E_{290} and found it was 1.32.

Several molecular weights have been published for bovine α-lactalbumin but these have all been superceded by Hill, et al. (11,24) who has shown that the molecular weight is 14,437 from the complete amino acid sequence. Previous molecular weights are in good agreement with this value. By sedimentation velocity the molecular weight was 15,000 (62) and 14,900 (51). Klostergaard and Pasternak (60) calculated the molecular weight as 15,500 from amino acid composition and Halwer, et al. (65) calculated the molecular weight as 16,500 by light scattering using a 0.1 N NaCl solution at pH 6.2 and 25^{o} C. Osmometry

measurements give an apparent molecular weight of 16,300 (51).

The electrophoretic mobility is -4.2×10^{-5} $cm^2 volt^{-1} sec^{-1}$ in a 1% veronal buffer, pH 8.5 (62) and -4.07 in a veronal buffer, pH 8.5 (62).

Brew and Campbell (66) have studied the physical characteristics of guinea pig α-lactalbumin and they are as follows: The molecular weight determined by gel filtration was 15,800. The crystals are irregular in form which may be due to a rapid rate of crystallization. The pI determined by electrophoresis on cellulose acetate was pH 4.8.

The physical properties of human α-lactalbumin (Japanese) were studied by Maeno and Kiyosawa (67). Electrophoresis experiments were conducted in glycine, acetate, phosphate, and veronal buffers. The patterns of α-lactalbumin were homogeneous at pH 2.0, 5.5, and 8.5, but gave two components at pH 3.5. From these experiments, the isoelectric point was calculated to be pH 4.8. The sedimentation coefficient was 1.7 S and the partial specific volume of a 1% solution was 0.72 cm^3/g. The molecular weight was calculated to be 23,000 by sedimentation velocity but they did not determine the dependence of molecular weight on protein concentration. The sedimentation velocity of bovine α-lactalbumin is dependent upon protein concentration, thus it appears Maeno and Kiyosawa's value may be too high. The molecular weight determined from amino acid composition data is about 15,000 (68). Solubility experiments show that α-lactalbumin has its lowest solubility in several buffers at pH 4.6 which is near the pI.

Bhattacharya, et al. (69) have studied the electrophoretic properties of goat α-lactalbumin. In free electrophoresis this α-lactalbumin exhibited a single symmetrical peak at pH 5.2 and its isoelectric point appears to be close to this pH. Its sedimentation coefficient, $S_{20,w}$,

is 2 S which is close to the $S_{20,w}$ values for bovine α-lactalbumin.

The properties of buffalo α-lactalbumin have been studied extensively by Bhattacharya, et al. (69) and Rao (70). Distinct differences between bovine and buffalo α-lactalbumin were found on paper electrophoresis (70). The α-lactalbumin from buffalo milk has a greater mobility than the one from cow's milk, but it was not stated which form of the bovine α-lactalbumin was used, the A or B.

Bovine α-lactalbumin A and buffalo α-lactalbumin, purified by methods (62,71) used for bovine α-lactalbumin, show very similar mobilities and patterns in free electrophoresis at several pHs. Bovine α-lactalbumin A and B and buffalo α-lactalbumin appear to have the same crystalline form, similar nitrogen contents, specific extinction coefficients at 280 nm and similar tyrosine and trypotphan contents. Their sedimentation coefficients ($S_{20,w}$) fall within the range of 1.87 and 1.99 S. The molecular weight of buffalo α-lactalbumin was calculated to be 16,200 (69).

Heterogeneity of α-Lactalbumin

McKenzie (15) and Wilkinson (72) have reviewed this subject thoroughly and portions of this review will be summarized in the following section.

In moving boundary electrophoretic experiments, Gordon and Semmett (62) have reported that bovine α-lactalbumin showed a single boundary at pH 2 and 3 (glycine-HCl buffer), pH 6.6 and 7.7 (phosphate buffer) and pH 8.5 (veronal buffer). However, in lactate buffer at pH 3.3 there were two components although in the ultracentrifuge the protein appeared homogeneous.

Zittle and Della Monica (73) found that Gordon and Semmett's preparation of α-lactalbumin was precipitated by 3.0 M ammonium sulfate. Extraction of the precipitate with 2.5 M ammonium sulfate gave a solution of a soluble form of α-lactalbumin. When the remainder of the precipitate was extracted with 1.5 M ammonium sulfate, a solution of an "insoluble form" was obtained. Zittle (63) has shown that dialysis of the total preparation produced about 75% of the "insoluble form", which can be transferred to the "soluble form" by addition of 0.1 M NaCl or dilute ammonium sulfate.

A shift of the isoelectric point, determined by precipitation and titration, from pH 4.8 in salt-free solutions to pH 3.6 in 0.5 M NaCl suggests that the chloride anion, and presumably other anions, are strongly bound by α-lactalbumin. The binding of anions suggests that the solubility transformation may be due to a slight change in physical configuration implemented by anion binding.

Klostergaard and Pasternak (37) found that bovine α-lactalbumin prepared according to the procedure of Gordon and Semmett showed various degrees of heterogeneity in boundary electrophoresis at pHs outside the isoelectric range. The protein exhibited heterogeneity in the electrophoresis experiments in the following buffers: lactate, pH 3.3; phosphate, pH 7.5; tris, pH 7.5; and barbital, pH 8.5. The degree of heterogeneity depended also on the protein concentration. In the ultracentrifuge, α-lactalbumin was homogeneous at room temperature and at 7°C.

Kronman, et al. (54,55,59,64,74,75,76,77,78) have done an exhaustive study on the heterogeneity of bovine α-lactalbumin at both acid and alkaline pH's. Kronman and Andreotti (74) examined samples prepared by the Gordon and Semmett, Aschaffenburg and Drewry, and Robbins and

Kronman methods. All samples gave a single symmetrical peak in sedimentation experiments at pH 8.6. A fast moving, trace band was observed in starch gel electrophoresis at alkaline pH. In gels containing 5 M urea at pH 3.0 a "trace" band was observed moving behind the main band. Kronman and Andreotti feel that protein-ion interactions do not account entirely for the apparent heterogeneity observed at acid and alkaline pH. It appears that the majority of observations of heterogeneity of α-lactalbumin are the result of association-aggregation processes occurring at both acid and alkaline pH's.

Kronman, et al. (54,55,59,64,74,75,76,77) examined the characteristics of several conformational changes that α-lactalbumin undergoes. The more drastic of these, which has been called acid denaturation, occurs below pH 4 and involves alteration of the tryptophan residues reflected as changes in emission (64) and absorption (75,76) spectra. Both ultracentrifuge (75) and titration (75) experiments indicate that acid denaturation involves molecular swelling, although this does not result in enhanced exposure of tryptophyl groups (54). Acid denatured α-lactalbumin shows a marked tendency to associate and aggregate (74,75).

Titration (59) and optical rotation dispersion (55) measurements both indicate a comparable structural change above pH 10. The denaturation at alkaline pH results in a swollen molecule but appears to be somewhat less complete than the acid denatured molecule. The acid denatured molecule shows characteristically low solubility, exhibits a time dependent aggregation, and an enhanced tendency to form lower molecular weight associated species (74,75). α-Lactalbumin at alkaline pH, by contrast, shows only an enhanced tendency to associate (78). The absence of aggregation at alkaline pH is most likely due to

differences in hydrophoblic and hydrophilic groups at the molecular surface for both forms of the denatured protein. The more restricted swelling and difference in optical rotaratory dispersion spectra indicates the molecule is somewhat less disrupted at a higher pH than at acidic pH values. Kronman, et al. (74,75) have defined in detail the terms association and aggregation so that the reader may be able to distinguish between these terms.

Much work has been done on α-lactalbumin but little information exists on the heterogeneity of highly purified α-lactalbumin other than the detailed work by Kronman, et al. (54,55,59,64,74,75,76,77,78) which has been reviewed by McKenzie (15). Recently, Gordon, et al. (2) reported that a minor component present in recrystallized α-lactalbumin has the same amino acid composition as the main α-lactalbumin material, but contains one residue of hexosamine per molecule of protein.

More recently, Barman (79) has shown that bovine milk contains two α-lactalbumin components which can be separated by anion-exchange chromatography. One of these, main peak α-lactalbumin, is presumably the α-lactalbumin with a molecular weight of 14,437 (3). The minor peak is glyco-α-lactalbumin, a glycoprotein of molecular weight about 16,800 which contains 11 to 12 sugar residues per molecule of protein. This data may suggest that the heterogeneity of α-lactalbumin is at least partially due to a glyco-α-lactalbumin. Such a glycoprotein has been found to be heterogeneous and unstable (80,81). The glyco-α-lactalbumin, representing about 15% of the total molecules, is about equally active as α-lactalbumin which acts as the modifier protein in the lactose synthetase reaction (79).

Structural Studies

Hill and coworkers have elucidated the complete amino acid sequence and the location of the disulfide bonds of bovine α-lactalbumin. This work will be reviewed in the last section while work previous to the complete amino acid sequencing will be covered here.

Yasunobu and Wilcox (82) found bovine α-lactalbumin contains a single peptide chain with one N-terminal glutamic acid residue and one C-terminal leucine residue. This data was also verified by Wetlaufer (51), Weil and Seibles (83,84), and Davie (85). Weil and Seibles (84) have elucidated the amino acid sequence of four peptides but only one peptide sequence agrees exactly with Hill's complete sequence (11).

Brew, et al. (24) have suggested that the α-lactalbumins from other animal species possess amino acid compositions similar to bovine α-lactalbumin. Human, goat, sheep, dog, guinea pig, and kangaroo α-lactalbumins differ slightly from bovine α-lactalbumin in their amino acid composition. Human α-lactalbumin also contains one or two residues of glucosamine per molecule that are covalently linked in an unknown manner. Gordon (86) has analyzed the amino acid content of α-lactalbumin A prepared from milks of India zebu cows and α-lactalbumin from Indian water buffalo. He has found only minor differences between bovine α-lactalbumin A and B and buffalo α-lactalbumin.

Genetic Variants

Two genetic variants of α-lactalbumin have been identified, namely α-lactalbumin A and B (87). The A gene is responsible for synthesis of α-lactalbumin A and the B gene is responsible for synthesis of α-lactalbumin B. Blumberg and Tombs (88) have shown by paper

electrophoresis that two different types of α-lactalbumin occur in Fulani cattle. The fast moving band is called α-A and the slower band is called α-B which is typical of American and British cattle. Bhattacharya, et al. (69) have investigated the inheritance of α-lactalbumin polymorphism in Indian Zebu cattle. Three types of patterns were observed for α-lactalbumin - those only with the slow moving component, α-B, those with only fast moving component, α-A, and those with two α-lactalbumin bands, α-AB. Buffalo α-lactalbumin was similar in mobility to bovine α-A. Gene frequencies were determined on Indian Zebu, Boran (Kenya), and Fulani (Nigeria) and they were 0.24, 0.13, and 0.15, respectively. Osterhoff and Pretorius (89) reported the presence of the A variant in South African cows of European descent, with frequencies of 0.08 in Brown Swiss, 0.12 in Holstein-Freisians, and 0.05 in Jerseys.

Mawal (90) has observed polymorphism in both breeds of Gir and Gavathi cows, namely α-A and α-B. No polymorphism was observed in α-lactalbumin of two breeds of Indian buffalos, but buffalo α-lactalbumin showed a slightly higher electrophoretic mobility than that of bovine α-A. Mawal has also reported that tryptic-chymotrypic peptide pattern analysis of Indian bovine α-lactalbumin A, bovine α-lactalbumin B and buffalo α-lactalbumin show that these protein species have differing peptides.

Gordon, et al. (86) have shown that the two variants are identical in amino acid composition, except for the absence of the solitary arginine residue of α-B which is replaced by a residue of glutamic acid or glutamine in α-A. When combined with the A protein of lactose synthetase, the two variants of α-lactalbumin are indistinguishable in

their enzymatic activity (34).

Methods of Purification of α-Lactalbumin

In 1936 Pedersen (7) observed a slow moving peak in the sedimentation pattern of whey proteins, which he designated the "α-peak". Also in 1936, Kekwick (8) isolated a crystalline protein that appeared to be responsible for the α-peak. Svedberg and Pedersen (9,10), in their studies of whey proteins in the ultracentrifuge, attributed the α-peak, one of the three major components in the sedimentation diagram of whey to α-lactalbumin. Later, Sorensen and Sorensen (91) prepared a crystalline protein from the albumin fraction of whey which they designated "crystalline insoluble substance" on the basis of its insolubility in water and in dilute salt solution at pH 6-7. The protein was not characterized. Jacobsen (92) modified the Sorensen method so that it was possible for one person to prepare a considerable amount of protein in five days (15).

Gordon and Semmett (62) have prepared "crystalline insoluble substance" along the lines of Sorensen and Sorensen (91) and have confirmed many of their observations. Gordon and Semmett's purification procedure has not been used extensively since four grams of α-lactalbumin is obtained from 60 liters of skimmed milk, which is a minimal yield.

Zweig and Block (93) have developed a purification procedure to isolate α-lactalbumin and β-lactoglobulin from ferrilaction (the ferric complex of whey proteins). By this method all of the whey proteins are precipitated by the addition of appropriate amounts of ferric chloride. This precipitate is easily separated by decantation and centrifugation. This method avoids the initial steps of first salting-out the globulins,

the β-lactoglobulin, and α-lactalbumin, which requires the use of large amounts of ammonium sulfate and tedious filtrations. This crystallization is the general procedure used or described by Gordon and Semmett (62).

The reason that Zweig and Block's procedure probably has not been more widely utilized is due to one of the initial purification steps. A ferric chloride precipitate is adjusted to pH 1.3. It seems in terms of current criteria that α-lactalbumin can tolerate short periods at this low pH and recover to its native state at pH 5-6 (71). However, with increasing sophistication of physiochemical techniques, subtle changes will be detected eventually and thus, it is preferable to avoid such a step (15).

Gordon and Ziegler (62,94,95) have developed a method of preparation of α-lactalbumin which avoids any low pH steps. Their method may be summarized as follows: Casein is separated from skim milk by acidification to pH 4.6. The whey proteins are then fractionated by salting-out with ammonium sulfate at pH 6.0. A crude globulin fraction is removed at about 58% saturation. The remaining proteins, including β-lactoglobulin and α-lactalbumin, are precipitated as a crude albumin fraction at about 80% saturation. Salt is removed from the albumin fraction by dialysis at pH 5.2, whereupon the β-lactoglobulin crystallizes. The mother liquor from crystallization is adjusted to pH 4.0, and ammonium sulfate is added to about 33% saturation. Crude α-lactalbumin is precipitated. It is dissolved at pH 8.0, reprecipitated at pH 4.0, dissolved again, and crystallized by the addition of ammonium sulfate to 50-66% saturation at pH 6.6 and is recrystallized by repeating these steps.

In 1957, Aschaffenburg and Drewry (71) developed a purification method to isolate α-lactalbumin and β-lactoglobulin. The method is summarized as follows: Whole milk is warmed to 40° whereupon anhydrous sodium sulfate is added. The supernatant whey solution is cooled to 25° and strong acid is used to adjust the pH to pH 2.0. A series of pH precipitation steps and an ammonium sulfate fractionation prepares the α-lactalbumin for final crystallization which is done by the method of Gordon and Semmett (62).

Aschaffenburg and Drewry's method has been criticized for several reasons (15). It has been criticized for exposure of the preparation to a strongly acid condition, but no evidence has ever been presented that such exposure is detrimental in any way. The use of anhydrous sodium sulfate is inconvenient, since it involves undesirable heating of the protein solution to 40°. Also, sodium sulfate is not as soluble as ammonium sulfate. Careful control of temperature is required in the subsequent cooling to avoid crystallization of sodium sulfate in the whey solution. Strong acid (11 N HCl) is also used to lower the pH and this must be done very carefully to avoid local extreme acid conditions in the whey solution.

Aschaffenburg (96) has improved the preceding procedure by increasing the yield of bovine α-lactalbumin to 0.6-0.7 g/l of whey. The method is also applicable to goat's milk, yielding 0.9 g/l from a whey richer in α-lactalbumin than that of cow's milk. The method is based on observation of Fox, et al. (97) which revealed that β-lactoglobulin is largely soluble in acid whey made 3% w/v trichloroacetic acid (TCA) while the other proteins are precipitated. No indication has been found that there is any adverse effect of the treatment with TCA. A

somewhat better yield may be obtained by the method of Bleumink (98) but this procedure involves vacuum distillation of large volumes of acetone added during preparation.

Aschaffenburg's improved procedure (96) is altered at the pH 4 precipitation for the isolation of goat α-lactalbumin. The precipitate at pH 4 is dissolved with dilute ammonia solution, then adjusted to pH 5.3 and dialyzed against water at room temperature. Lozenge-shaped monoclinic crystals form which differ from needle-shaped crystals obtained from a salt-free solution in the cold by Sen (99).

Trace amounts of serum albumin still remain in these isolation procedures, and their elimination, if desired, requires suitable column treatment, e.g., on DEAE cellulose (100) or Sephadex.

Macroscopic crystals of α-lactalbumin have been grown by a procedure developed by Gordon (101). The technique was developed following an observation that α-lactalbumin is more soluble in strong ammonium sulfate solutions at 4° than at 25°. In several successful experiments, a saturated solution of the purified protein was prepared in an ammonium sulfate solution, 44-50% saturation, pH 6.6, at 0°; the solution was then filtered in the cold and the clear filtrate was allowed to stand undisturbed at room temperature. In three days, needle-like crystals, some at least 3 mm long, had formed.

Barman (79) has reported the presence of a minor component with the same amino acid composition as α-lactalbumin, but containing 11-12 sugar residues per molecule of protein in a highly purified preparation of α-lactalbumin. α-Lactalbumin was prepared from bovine milk as described by Robbins and Kronman to the step where α-lactalbumin is precipitated at pH 4.0. The fraction was then purified on a Sephadex G-100

column. The purified α-lactalbumin was subjected to anion-exchange chromatography on DEAE cellulose and two fractions were obtained. The major fraction of α-lactalbumin contained no detectable carbohydrate and the minor fraction (glyco-α-lactalbumin) had a high carbohydrate content.

In 1965, Groves (100) prepared bovine α-lactalbumin by the usual salt fractionation and recrystallization procedure and subjected it to disc electrophoresis at pH 9.5. An impurity was found moving ahead of the main band. When paper electrophoretic determinations were used extensively to identify proteins, α-lactalbumins prepared by the usual methods always showed a minor protein moving just ahead of the main protein band which could not be removed by recrystallization or other fractionation procedures (71). Wetlaufer (51) also noted a "fast" lactalbumin by paper electrophoresis and found that the fast component isolated by electrophoresis had the same sedimentation coefficient and ultraviolet spectrum as the original material.

Groves (100) also prepared bovine α-lactalbumin by the ion exchange method and no "fast" component was found and attempts to produce it by exposing the protein to conditions similar to those used in regular fractionation procedures failed. He suggested that the minor fraction is an impurity and not a transformation product of the fractionation procedure, but this minor fraction may be the glyco-α-lactalbumin which Barman (79) and Gordon (86) have isolated.

Armstrong and McKenzie (102) and Armstrong, et al. (103) studied the fractionation of bovine α-lactalbumin and β-lactoglobulin bearing in mind the severe and numerous pH adjustments and the use of strong acids and alkalis to adjust the pH (15). They started with an ammonium

sulfate bovine whey to avoid the criticism of Aschaffenburg and Drewry's method (15). Furthermore, the preparation of this fraction was carried out at 20° without undesirable heating, the ammonium sulfate dissolved readily, and there was no danger of subsequent crystallization in the whey protein solution. They preferred to use 1 N hydrochloric acid and 1 N ammonia for pH adjustment of the pH value, to lessen the danger of local excesses of acid and alkali. Also they endeavored to effect fractionation by lowering the pH value only to 3.5, avoiding any danger of dissociation (15). The crystallization of α-lactalbumin was done by the method of Gordon and Semmett (62).

Preformed ultrafiltration membranes, which serve as barriers to the diffusive transport of macromolecules, have been reported as a practical system for the rapid concentration of dilute protein solutions (104). The recent formulation of a membrane with a more "expanded structure" (approximate retention value, 30-35,000) suggested its use as a partitioning device for separation of larger sized molecular species.

Crystalline bovine α-lactalbumin prepared by the method of Robbins and Kronman (105), provided a material able to diffuse through the membrane, but reputed to contain a small quantity of albumins as a contaminant (105). Human serum albumin and bovine α-lactalbumin were mixed and subjected to the membrane filtration procedure (62). Although the membrane did not give complete separation of the two proteins, it provided a rapid and inexpensive method for separating relatively large amounts of material without resorting to gel filtration.

The purification of α-lactalbumin from human milk has to be altered from the usual procedure used for bovine α-lactalbumin. Human casein

is not as easily precipitated by acids as is cow's casein (67). In order to prepare whey protein from human milk, Deutsch (106) removed casein by adding rennin to human milk and adjusted to pH 4.8. The denaturation of whey proteins must be avoided during the treatment with rennin and the dilution of whey proteins is unsuitable for the preparation of α-lactalbumin. It is therefore desirable to remove casein from acidified milk by centrifugation.

Johansson (107) has purified human α-lactalbumin on calcium phosphate columns. After defatting the fresh or pooled frozen milk by centrifugation, the α-lactalbumin was obtained from the 50-100% ammonium sulfate fraction. This crude albumin fraction was placed on a calcium phosphate column which was eluted with stepwise increased concentrations of phosphate buffer. α-Lactalbumin off this column was shown to be homogeneous by paper electrophoresis and in the ultracentrifuge, but in moving boundry electrophoresis, a small impurity with a lower mobility was found.

More recently, Maeno and Kiyosawa (67) have purified α-lactalbumin from human milk which is summarized as follows: Centrifugation and heating to 30^{o} was used to remove the cream, and casein was removed by the usual pH 4.6 step. An ammonium sulfate fractionation at pH 6.6 removed part of the whey proteins. The supernatant solution was then adjusted to pH 4.8 to precipitate α-lactalbumin. The α-lactalbumin is dissolved in water at pH 7.0 and then precipitated again at pH 4.8. Repetition of this final step two or three times produces purified α-lactalbumin.

McKenzie (15) in reviewing purification methods for α-lactalbumin recommends the preparation of the whey protein fraction by ammonium

sulfate fractionation of milk. It is possible through careful control of pH, protein concentration and temperature to prepare fractions from which α-lactalbumin of high purity may be readily prepared. It is furthermore possible to prepare not only bovine α-lactalbumin, but also α-lactalbumins of other species, providing careful attention is given to differences between species.

Structural and Evolutionary Relationships of Bovine α-Lactalbumin and Hen's Egg-White Lysozyme

Structural Relationships

Yasunobu and Wilcox (82) in 1958 pointed out that α-lactalbumin and lysozyme have similar molecular weights, the same number of disulfide bonds, similar amino acid compositions and identical or similar amino and carboxyl terminal residues. They also noted that α-lactalbumin is readily oxidized by tyrosinase while lysozyme is not oxidized even after treatment with urea.

Brew and Campbell (66) characterized the physiochemical properties of guinea pig α-lactalbumin and noted that bovine and guinea pig α-lactalbumin have some structural features in common with hen's egg-white lysozyme. This comparison led Hill, et al. (3,24) to further compare α-lactalbumin and lysozyme. Brew, et al. (12) established the partial sequence of α-lactalbumin and compared it to lysozyme. Recently Brew, et al. (11,108,109) have determined the complete amino acid sequence of α-lactalbumin and the exact location of the disulfide bonds.

With knowledge of the complete amino acid sequence of α-lactalbumin, it is possible to make an exact structural comparison of α-lactalbumin and hen egg-white lysozyme as shown in Figure 2. As the sequences are

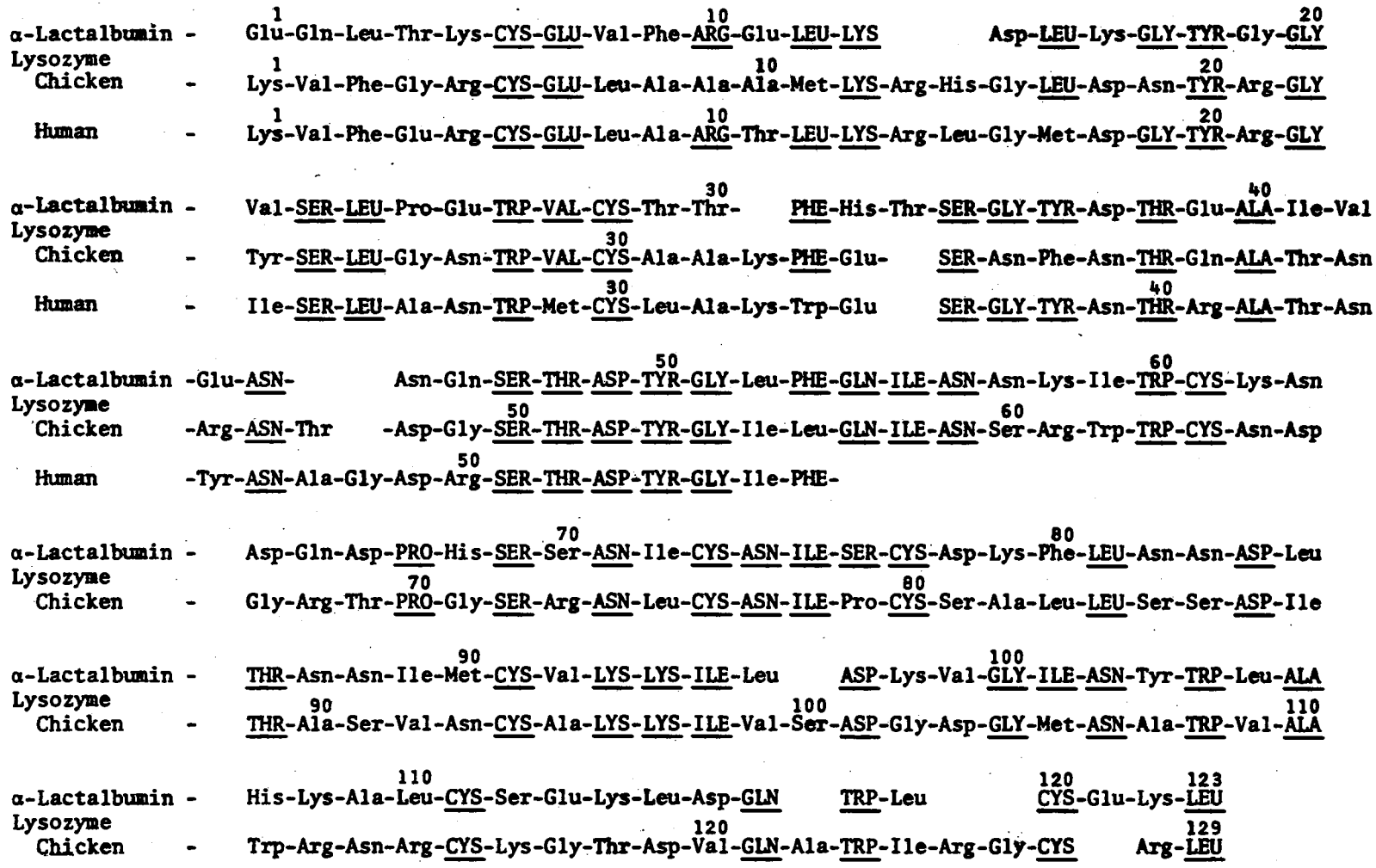

Figure 2. Comparison of the Amino Acid Sequences of Bovine α-Lactalbumin, Chicken Egg-White Lysozyme, and Human Lysozyme

aligned, a total of forty-nine residues are identical at corresponding positions in the two sequences and an additional twenty-three residues are conservative replacements. Also the arrangement of the disulfide bonds is essentially identical with that found in chicken egg-white lysozyme. Thus it is clear that the sequence of α-lactalbumin and lysozyme are sufficiently similar such that the α-lactalbumin sequence can be folded into a three-dimensional structure very similar to that of lysozyme (13). It is also noted that α-lactalbumin is quite similar in sequence to human lysozyme (12), whose partial structure is also given in Figure 1. There are six residues in α-lactalbumin which differ from residues at corresponding positions in egg-white lysozyme, but are identical with the corresponding residues in human lysozyme (11).

Brown, et al. (13) and Hill, et al. (3) have reviewed the structural relationship between lysozyme and α-lactalbumin. These reviews are summarized and extended in this section.

The proportion of identical residues of egg-white lysozyme and α-lactalbumin is greater than that between sperm whale myoglobin and either of the chains of hemoglobin (110); despite the differences in composition (111), the three globin chains are known to have very similar tertiary structures. Similar studies by Ingram (112) show that 44% of the α and β chains of human hemoglobin are identical. This compares to 40% of the residues of α-lactalbumin and lysozyme being identical. Since these globin chains have been shown to have very similar tertiary structures, and since the four disulfide bonds in α-lactalbumin and lysozyme are essentially identical (109), it is quite possible that these latter two proteins may also have very similar molecular conformations.

Blake, et al. (113) have constructed a model of lysozyme which was modified by Browne, et al. (13) to accomodate the α-lactalbumin sequence by changing the side chains that differ in the two molecules. Seven residues were deleted in the model to correct for the six fewer residues of α-lactalbumin and an additional one to help achieve maximum homology between the two structures.

The theoretical model based on known characteristics of α-lactalbumin demonstrates it is quite similar to the conformation of lysozyme. Approximately 70 side chains were changed but no nonpolar side chains were exposed to the surface and no polar side chains were buried. The deleted residues did not affect the similarity of the two structures since the deletions occurred either at the ends of helical regions or in the loops. This only caused some local rearrangement of the main chain conformation. Although several changes in side chains were made, the major hydrophobic regions in the interior of the molecule are preserved (13).

The cleft region of lysozyme which contains the active site has several critical changes in the α-lactalbumin model. There are two critical acidic side chain residues which aid in labilizing the susceptible bond in lysozyme substrates (113). One residue is preserved while glutamic acid 35 is replaced by threonine 33 or histidine 32 (113,114). Several other side chains which are important in binding lysozyme substrates are absent in the α-lactalbumin model (3). Although lysozyme does not participate in lactose synthesis, and α-lactalbumin does not act upon lysozyme substrates (13), the role of α-lactalbumin in lactose synthesis implies a functional as well as a structural similarity between α-lactalbumin and lysozyme. One enzyme is involved in

the cleavage and the other in synthesis of β, 1-4 glucopyranosyl linkage. Brew, et al. (24) have demonstrated that α-lactalbumin does not display interaction with a disaccharide substrate. This leaves a dilemma; the theoretical model leads the observer to anticipate a related activity for α-lactalbumin and lysozyme which are known to be involved in closely similar reactions. Biochemical experiments suggest they act in very different ways (13).

The predicted conformation for α-lactalbumin will be evaluated when the determination of the structure by X-ray crystallography is completed which is now in progress (Aschaffenburg, Handford and Philips, manuscript in preparation). Studies of the physical properties of α-lactalbumin and lysozyme in solution indicate conformational similarities. Kronman (64) has found a similarity in the circular dichroic spectra of the two proteins. Aune (115) and Cowburn, et al. (116) have also shown that the optical rotary dispersion spectra of the two proteins are quite similar. The predicted location of the tyrosyl and tryptophanyl residues in α-lactalbumin is verified by H^+ - titration (77) and solvent perturbation studies (54).

All possible parameters must be exhaustively studied before it can be absolutely certain that two conformations are identical, especially in solution. Krigbaum and Kugler (117) studied the molecular conformation of lysozyme and α-lactalbumin in solution through the use of small angle X-ray diffraction measurements. They found that lysozyme has a radius of gyration, R of 14.3 Å, and its equivalent scattering body is a prolate ellipsoid having dimensions 28 X 28 X 50 Å, while α-lactalbumin has R = 16.7 Å, and its equivalent ellipsoid is oblate with dimensions 22 X 44 X 57 Å. They concluded that the two proteins

have different molecular conformations in solution. It was also found that lysozyme has practically the same conformation in the crystalline state and in solution. It can be postulated from this finding that α-lactalbumin will have a similar conformation in the crystalline form and in solution; thus, α-lactalbumin and lysozyme also may have different conformations in the crystalline state.

Atassi, et al. (118) have also compared the conformation of lysozyme and α-lactalbumin. Tryptic hydrolysis of both proteins indicated both are sterically inaccessible to attack by trypsin. Nitration experiments indicated very little difference between the two proteins in the availability of the tyrosines and tryptophans. α-Lactalbumin disulfide bonds were much more susceptible to reduction than lysozyme which indicates a conformation difference.

It has been established that the antigenic reactivity of native protein is greatly influenced by conformation changes (119,120). Atassi, et al. (118) found that neither α-lactalbumin nor lysozyme will cross react with the antisera of the other protein. It was therefore concluded that the two proteins have different conformations. These workers failed to consider that the antigenic site of the two proteins may have different amino acid sequences (121), resulting in a failure of cross reaction, while still retaining similar conformations.

A profound difference between the structures of α-lactalbumin and lysozyme is the overall charge of the two proteins. Lysozyme has an isoelectric point of pH 10.5 while α-lactalbumin has a pI of 4.8. This difference in overall charge may be a specific adaptation of α-lactalbumin to a changed enzymatic role (12), and any chemical modification which increases acidity of lysozyme decreases its specific activity (122).

Canfield and McMurry (123) have characterized goose egg-white lysozyme. They have found amino acid differences, especially those for lysine, arginine, glutamic acid, half-cystine, tyrosine, and tryptophan, which indicate large portions of the primary structure must be different. The goose lysozyme is several times more active than hen lysozyme, and goose lysozyme does not act on chitin which is hydrolyzed by hen lysozyme. Both lysozymes appear to liberate reducing sugars, thus it is probable they act at the same site in cell walls. This comparison of the two lysozymes illustrates that two proteins may have the same function but may vary widely in structure. Jolles, et al. (124,125) have compared lysozyme from several sources and have arrived at the same conclusion. This points out how closely hen lysozyme and α-lactalbumin are related. The proteins have different functions and yet they are closely related in their structure.

Through the comparison of structures of proteins, it may be found that very many protein molecules may belong to a comparatively small number of families. It may be possible to derive the structure of all the members of a family relatively easily once one or two have been analyzed in detail. Several reviews (126,127,128) have been published which have evaluated the relationship of amino acid sequences, protein conformation and the biological activity of peptides.

Evolutionary Relationships

If similar proteins are compared from different species, the evolutionary relationship of the species may be traced, since viable mutations which become established in a population will have evolutionary stability, and can act as markers to identify familial resemblances.

Comparison of species differences in insulin (129), immunoglobulins (130), hemoglobin (112), and cytochrome C (129) have laid the foundation for such studies. However, an alternative approach is to compare different proteins from the same species or individual. Considerable homologies between the amino acid sequences of these different proteins might indicate a common evolutionary ancestor (131).

After millions of years of mutational assault on the genes, natural selection has produced enzymes with most amino acids unchanged. Two of the reasons for this conservation must be the necessity to define the overall shape and dynamic properties of the molecule, and the necessity to insure its resistance to denaturation under the range of conditions normal for the organism (129). Two such enzymes that have evolved through evolution are α-lactalbumin and lysozyme.

The structural similarities between α-lactalbumin and lysozyme could have arisen through evolution by either of two mechanisms. The genes could have arisen by convergence from separate origins or the genes may have diverged from a common ancestral gene (3). The accepted and most popular mechanism is divergent evolution.

Since the two proteins are similar in structure, it is assumed that the genes which control the sequences of lysozyme and α-lactalbumin have a recent common ancestor on the evolutionary tree. When a milk producing system evolved, it is possible that the gene responsible for a protein which degraded a carbohydrate linkage gave rise to α-lactalbumin through a series of mutations (132). Therefore, through gene duplication and mutation and followed by separate evolution of the products, a new enzymatic function developed. Examples of this process have been cited in the case of the pancreatic proteases (133)

and possibly other enzymes (134). However, further studies are necessary to show if this relationship exists between α-lactablumin and lysozyme.

CHAPTER III

ISOLATION AND COMPARISON OF VARIOUS α-LACTALBUMINS

Experimental Procedure

Materials and Reagents

Fresh unpasteurized whole milk was obtained from the following sources: goat (Capra Hircus) milk from Dr. Robert Noble, Oklahoma State University; pig (Yorkshire) milk from Dr. J. C. Hillier, Oklahoma State University; sheep (Rambouillet Dorsett) milk from Mr. A. Schanbacher, Cherokee, Oklahoma; human milk from Dr. James A. Merrill, University of Oklahoma Medical Center, Oklahoma City. Purified bovine (British Breeds) α-lactalbumin was obtained from W. Denton of this laboratory. Purified Japanese α-lactalbumin was supplied by Dr. T. Nagasawa, Morinaga Milk Company, Tokyo, Japan. Northern fur seal milk was supplied by the Bureau of Commercial Fisheries, Marine Biological Lab, St. Paul Island, Alaska. An unidentified milk protein was supplied by Dr. E. Kalan, Eastern Regional Research Lab, Philadelphia, Pennsylvania. Three and five times crystallized bovine α-lactalbumin was a gift from Dr. B. Larson, Illinois University. Guinea pig α-lactalbumin was a gift from Dr. P. Campbell, and buffalo α-lactalbumin was a gift from Dr. A. Sen.

Three times crystallized β-lactoglobulin was a gift from Dr. B. L. Larson, University of Illinois. Cytochrome C_1, type III, Tris

(tris-hydroxymethylaminoethane), phosphoenolpyruvate, NADH, carboxypeptidase-A DFP, pyruvate kinase (Type I from rabbit muscle, crystalline ammonium sulfate suspension containing lactic dehydrogenase), lysozyme, TPCK treated trypsin, glyclyglycine, and α-chymotrypsinogen were purchased from Sigma Chemical Company. Insulin was purchased from Calbiochem. Starch was purchased from Connaught Medical Research Laboratories, Toronto. Fluorodinitrobenzene was obtained from K and K Laboratories. Bio-Gel P, Dowex 50W-8X, and insulin were purchased from Bio-Rad Laboratories and Sephadex G from Pharmacia. Bovine serum albumin was obtained from Mann Chemical Company. DEAE-cellulose was purchased from Brown Company (Selectacel) or from Whatman (DE-32). MN-Cellulose powder 300 was obtained from Macherey, Nagel and Company (Germany). Size 8 dialysis tubing was obtained from Union Carbide Corporation. Precoated TLC plates were purchased from Brinkman Instruments, Inc. Special Agar-Noble was purchased from Difco Labs, Detroit. Disc gel electrophoresis reagents were obtained from Canalco. UDP-galactose was purchased from California Biochemicals or was synthesized by the method of Moffat and Khorana (135).

The A-protein of lactose synthetase was prepared according to the procedure described by Fitzgerald, et al. (26). The A-protein was obtained as an ammonium sulfate solution of partially purified A-protein from the HA_I and HA_{II} step of the purification (26). All other materials were of reagent quality.

Purification Procedures for Various α-Lactalbumins

α-Lactalbumins from goat, sheep, pig, northern fur seal, and human were prepared from skim milk according to the procedure of Brodbeck,

et al. (2), with the following general modifications:

(1) The skim milk was warmed to 24° before the adjustment of pH to 4.6.

(2) The first ammonium sulfate fractionation was at 40% saturation (243 g/l).

(3) The second ammonium sulfate step was stopped at 90% saturation (375 g/l).

(4) The linear gradient for the DEAE or DE-32 column was from 20 to 300 mM Tris-HCl, pH 7.8.

The following final steps of the purification replace the crystallization procedure of Brodbeck, et al. (2). Ammonium sulfate was added to the pooled main peak off the DEAE column until the solution became slightly cloudy. It was then centrifuged at 15,000 x g for 20 minutes. The supernatant solution was made 90 percent saturation in ammonium sulfate, centrifuged, and the precipitate was dissolved in approximately 5-20 ml of water. The purified α-lactalbumin was checked for purity by starch and disc gel electrophoresis. If an impurity was detected, the final ammonium sulfate fractionation was repeated. The α-lactalbumin solution was then exhaustively dialyzed against distilled water for approximately one week until the conductivity of the protein solution was approximately 20 micromho. Disc gel electrophoresis, (see electrophoresis procedures) was used to recheck the purity of the protein preparation. The protein was lyophilized and stored at -15°C.

Before any experiment was conducted which required the dry weight of the protein, the α-lactalbumins were dried in a vacuum oven at 40° until a constant weight was obtained.

Gel Filtration Experiments

Several sizes of gel filtration columns were used. The lower ends of the columns were nearly flat and had two mm luer connectors which allowed the attachment of a syringe needle. The bottom of the column contained either a glass or a plastic porous frit disc which was wedged into the bottom of the column. In the case of the preparatory columns, glass wool was placed on the bottom and was covered by a very thin layer of sand. A disc of Whatman 1 mm filter paper was placed on top of these column bottoms. The columns were treated with a 1% solution of dichlorodimethyl silane in benzene, prior to packing (136). The instructions of the gel manuals (136,137) were followed when gel columns were packed either at room temperature or at 4^{o}. Sample solutions, containing 15 per cent sucrose, were placed on the columns by layering under the eluent and slightly above the top of the gel. A disc of Whatmann 1 mm filter paper was placed on top of the gel to prevent disturbance of the gel during sample application. The uniformity of packing and the void volume was determined by passing a solution of blue dextran (1 mg/ml in 15% sucrose) through the column. Flow rates were used which did not cause any local distortion of the blue dextran band.

Ion Exchange Chromatography

All ion exchange celluloses and resins were regenerated and packed according to their corresponding technical manuals (138,139,140). A small amount of 1,1,1-trichloro-2-methyl-2-propanol was added to all buffers to retard bacterial growth in columns which were maintained and operated at room temperature or in the cold. Several void volumes of starting buffer were eluted through each column before a sample was

applied.

Spectrophotometric Assay for Lactose Synthetase

One unit of enzymatic activity is defined as that amount of enzyme which catalyzes the formation of one millimicromole of UDP per minute and is equivalent to an A_{340}/min/ml of 0.0062. α-Lactalbumin was estimated by assuming an extinction coefficient of 2.0 at 1 mg/ml at A_{280}. α-Lactalbumin was assayed in the presence of saturating amounts of the A protein (1). Enzymatic activity was assayed spectrophotometrically at 340 nm by coupling UDP formation, a product of the lactose synthetase reaction, to NADH oxidation by means of pyruvate kinase and lactic dehydrogenase. The rate of conversion of NADH to NAD^+ was followed at 340 nm on a Cary Model 14 or Beckman DB recording spectrophotometer with water jacketed cell chambers thermoregulated at 25^o. The standard assay mixture contained 1.0 mM PEP, 0.15 mM NADH, 5 mM $MnCl_2$, 4 mM UDP-galactose, 20 mM glucose, 0.05 ml of a 1 to 10 dilution of pyruvate kinase (Sigma, Type I, 25 mg protein/ml, with 2.4 IU pyruvate kinase/mg protein), 50 mM glycylglycine, pH 8.5 and approximately 100 units of A protein, and water in a total volume of 1.0 ml. Standard curves were prepared for each group of assays (26).

Electrophoretic Procedures

The apparatus and procedures for starch-gel electrophoresis were previously described by Abbott and Johnson (141). Thin-layer gels on glass plates were used in this method. Two buffer systems were employed for the starch gel electrophoresis. An 8 mM aluminum lactate-3 M urea buffer, pH 3.3 with lactic acid and a 15 mM Tris, 3 mM citric acid,

8 mM boric acid, and 0.5 M urea buffer, pH 8.6 with NaOH were used to determine the purity and the electrophoretic mobility of the various purified α-lactalbumins.

The starch gels were stained with a nigrosine stain and destained in five percent acetic acid (142). The starch gels were then wrapped in saran wrap and stored at 4^{o}.

The most commonly used disc gels were a 7 percent, pH 9.5, separating gel which was prepared according to Canalco instructions (143). The gels were poured in a standard glass tube, 7.5 X 0.5 cm, using approximately 0.8 ml. Approximately 0.15 ml of stacking gel was layered on top of the polymerized separating gel. Gels were pre-electrophoresed for 25 minutes to remove the catalyst. If the catalyst is not removed, an oxidizing condition exists in the gel which may have an adverse effect on proteins passing through the gel. No sample gel was used. Protein solutions were 10 percent with respect to sucrose and were layered directly on top of the stacking gel. A Canalco Model 6 system was used for disc gel experiments except for the preparatory disc gel experiments. Two milliamps per gel were maintained until the tracking dye entered the separating gel. The current was then maintained at 5 milliamps with a Buchler Constant Power Supply until the dye reached the end of the gel. Long gels (140 X 5 mm) run at 4^{o} were used to achieve better separation in order to compare mobilities of the various α-lactalbumins. 1.5 milliamps per gel were maintained until the dye passed into the separating gel. The current was then held at 3 milliamps until the dye reached the end of the gel. This lower electric flux was used to prevent excessive heating.

Dissociating conditions were established in the disc gels by

placing 6 M urea in both the separating and the stacking gels. The procedure of Weber and Osborn (144) was followed when sodium dodecyl sulfate and β-mercaptoethanol were used in the disc gels.

Weber and Osborn's buffer (144) was also used to electrophorese the proteins at pH 7.3. A different buffer was used at pH 9.5 which was 50 mM H_3BO_3. Charge and size isomers were distinguished by varying the acrylamide gel concentration according to the disc gel electrophoresis method of Hedrick and Smith (145).

A Canalco prepartory disc gel electrophoresis unit was used in an attempt to separate the two forms of pig α-lactalbumin. The stacking and separating gels were the standard 7 percent, pH 9.5 gels. The experiments followed the Canalco prepartory disc electrophoresis instructions (143).

Low pH 4.3 disc gel electrophoresis was done according to Canalco instructions (143). Any alterations in disc electrophoresis procedures are reported in the results section.

Amino Acid Analysis

Amino acid analyses were obtained with a Beckman Model 120 C automatic amino acid analyzer according to the method of Moore and Stein (146). The α-lactalbumins were hydrolyzed using 3 ml of constant boiling HCl in thick walled, Pyrex, 16 X 125 mm ignition tubes. Triplicate samples were hydrolyzed at 110° for 24, 48, and 72 hour periods in the sealed, evacuated ignition tubes by placing the sealed tubes in refluxing toluene. After hydrolysis, the tubes were opened, and the samples were evaporated at 40° to dryness on a rotary evaporator. To assure removal of the HCl, 2 ml of water was added and the samples were

evaporated to dryness again. The values for threonine, serine, and methionine were determined by extrapolation of the data to zero hydrolysis time. Half-cystine was determined by several different methods. One method was by oxidizing cystine to cysteic acid with performic acid oxidation procedures (147,148). Zahler and Cleland's method (149) involved the reduction of disulfude with dithiothreitol and the determination of the monothiols with 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) in the presence of arsenite. A third method (150) involved the reduction of the disulfide with $NaBH_4$ and determination of the monothiols with DTNB. Tryptophan was determined by the spectrophotometric method of Goodwin and Morton (151) as outlined by Beaven and Holiday (152) using a Cary 14 recording spectrophotometer. The ultraviolet spectra of the proteins were determined on the Cary 14.

Carboxy- and Amino-Terminal Amino Acid Determinations

The fluorodinitrobenzene method for amino end-group analysis was accomplished according to the method of Fraenkel-Conrat, et al. (153). The dinitrophenylamino acid derivatives were separated by two-dimensional paper chromatography and identified by comparison with known derivatives.

For carboxyl-terminal studies, two methods (154,155) were combined. Carboxypeptidase A treated with diisopropylflurophosphate was used at a 1 to 200 molar ratio of enzyme to substrate. The protein substrate was treated with carboxypeptidase at pH 8.0 and 25° C. After the start of the digestion, 100 µl aliquots were removed at 30 seconds, one or two minutes and five minutes. The addition of an equal volume of the ten percent trichloroacetic acid terminated the reaction and precipitated

the residual protein which was then removed by centrifugation. The supernatant solutions were passed over a Dowex 50 H^+ - form. The column was washed with water and then eluted with 10 N ammonium hydroxide. The eluant containing the protein hydrolysate was evaporated to dryness on a rotary evaporator, dissolved in water, and spotted on cellulose 300 MN thin layer plates which were poured with a thickness of approximately 0.3 mm. Two chromatographic separations were made using two different buffers. The two buffers were isopropyl alcohol, formic acid, water (40:2:10), and t-butanol, methylethyl ketone, concentrated ammonium hydroxide, and water (50:30:10:10). With both amino- and carboxyl-terminal amino acid determinations, bovine α-lactalbumin was used as a standard since both N and C terminal amino acids were known.

Immunological Methods

Agar, used for immunodiffusion, was prepared by heating 100 ml of 0.5 M veronal buffer, pH 8.2 containing 1.25 gm of special Agar-Noble. Hot agar was poured into a petri dish and cooled at 25^{o}. Samples (10 mg/ml) were dissolved in 0.01 N NH_4OH and placed in the agar wells. During diffusion, the petri dishes were maintained at 37^{o} C.

Peptide Mapping Procedures

The method of Jones (156) was used to prepare the α-lactalbumins for peptide mapping. The proteins were reduced in urea with β-mercaptoethanol and aminoethylated with ethylenimine. The protein solution was passed over a 50 X 2 cm Sephadex G-25 (medium) column using 0.2 M acetic acid as the eluent. The protein solution was evaporated to dryness, dissolved in the digestion buffer, and digested with

trypsin using a 1 to 75 enzyme to protein ratio.

The digested solution was spotted directly on the TLC chromatograms. Commercial thin layer chromatograms, 20 X 20 cm, having a 300 MN cellulose thickness of 0.10 mm were used. Whatman 3 mm wicks were washed with one percent acetic acid and then with the electrophoresis buffer. This was to remove any ninhydrin positive impurities (157). An aliquot of the digested solution was spotted at a corner of the TLC plate (158). The electrophoresis buffer containing pyridine, acetic acid, and water (100:30:3000), pH 5.5 was sprayed onto the plate until the surface was damp. The plate was immersed in the Buchler Universal Electrophoresis Cell, 24.5 X 25 cm. The cell, maintained at 4^{o}, contained the electrophoresis buffer with an over-layer of varsol, used as a coolant. Paper wicks, extending into the buffer, were placed on each end of the plate. Glass rods, ground to give a flat surface, were placed on the wicks to insure intimate contact with the thin-layer plate. After electrophoresis for two hours at 300 volts the plate was dried with warm air not exceeding 30^{o}. This was followed by equilibration in the chromatography tank, and then chromatography in the second dimension chromatography buffer, n-butanol, pyridine, glacial acetic acid, and water (150:100:30:120). The plate was removed when the buffer reached the top of the plate, which takes approximately 10 hours and 30 minutes. The peptides were detected with spray consisting of ninhydrin, 50 mg; ethanol, 30 ml; glacial acetic acid, 10 ml; and collidine, 4 ml (158). The spots are developed in an 80^{o} oven for 5-10 minutes and then are fully developed at room temperatures for approximately 12 hours (157,158). Tracings were made of the peptide maps which were then wrapped in saran wrap and stored in the refrigerator.

Any modifications of the methods and procedures used will be reported in the results section along with procedures not mentioned in this section.

Results

Purification of Various α-Lactalbumins

α-Lactalbumins from goat, sheep, pig, human (Caucasian, Negro, American Indian) and the northern fur seal were purified according to the procedure of Brodbeck, et al. (2) with several general modifications which were described in the Methods section. Skim milk from these species was stored frozen until α-lactalbumin was isolated from the milks. The northern fur seal milk contained 0.5 percent phenol which had been added to preserve the milk during shipment from Alaska.

The precipitation of casein at pH 4.6 varied with the different milks. Maeno and Kiyosawa (67) have commented on the differences in the solubility of Japanese (human) and bovine casein. At the pH 4.6 precipitation step, casein precipitated in the goat, Indian and Caucasian milks. Only about one half of the casein precipitated in the pig and sheep milk, while no casein precipitated in the Negro milk. When the pH was increased to 7.4, the pig and sheep milks gave a precipitate which was removed by centrifugation. The casein which remained in the pig, sheep, and Negro milk was removed by the first ammonium sulfate step which was at 40 percent saturation (243 g/l).

Isolation of the whey proteins of northern fur seal milk (25 ml) was accomplished by centrifugation and ammonium sulfate precipitation. Raw milk, containing 0.5 percent phenol, was centrifuged at 15,000 x g for 25 minutes to remove the cream. The supernatant solution was

centrifuged at 110,000 x g for one hour to remove the casein which gave a very small precipitate when compared to an equal volume of bovine milk. After centrifugation, solid ammonium sulfate (561 g/l) was added to the bright yellow superantant solution to bring the concentration to 80 percent saturation. After centrifugation at 35,000 x g for 25 minutes, the precipitate was dissolved in a minimal volume of 20 mM Tris-HCl-10 mM $MgCl_2$, pH 7.4. Twenty-five ml portions of this protein solution were passed through a Bio-Gel P-30 (100-200 mesh) column in order to separate the α-lactalbumin from the A protein. The characteristic elution pattern of lactose synthetase from the various milks is shown in Figure 3 with the exception of the pig lactose synthetase which gave the elution pattern shown in Figure 4.

A final fractional precipitation with ammonium sulfate was accomplished with the purified α-lactalbumin after the DEAE column purification. Solid ammonium sulfate was added to the α-lactalbumin solution until the clear solution just started to become turbid. The turbidity developed between 60-70 percent saturation for various α-lactalbumin solutions. After centrifugation, the supernatant solutions were taken to 90 percent ammonium sulfate saturation (662 g/l). This step was repeated if any impurity was detected by starch gel electrophoresis.

Figure 3 shows a characteristic protein pattern of lactose synthetase partially purified from goat, sheep, and human milk. Lactose synthetase activity was demonstrated in peak two when it was assayed in the presence of peak one. No lactose synthetase could be shown when peak two was assayed individually. Peak two was the partially purified α-lactalbumin.

Chromatography of the pig milk on the Bio-Gel P-30 column gave the

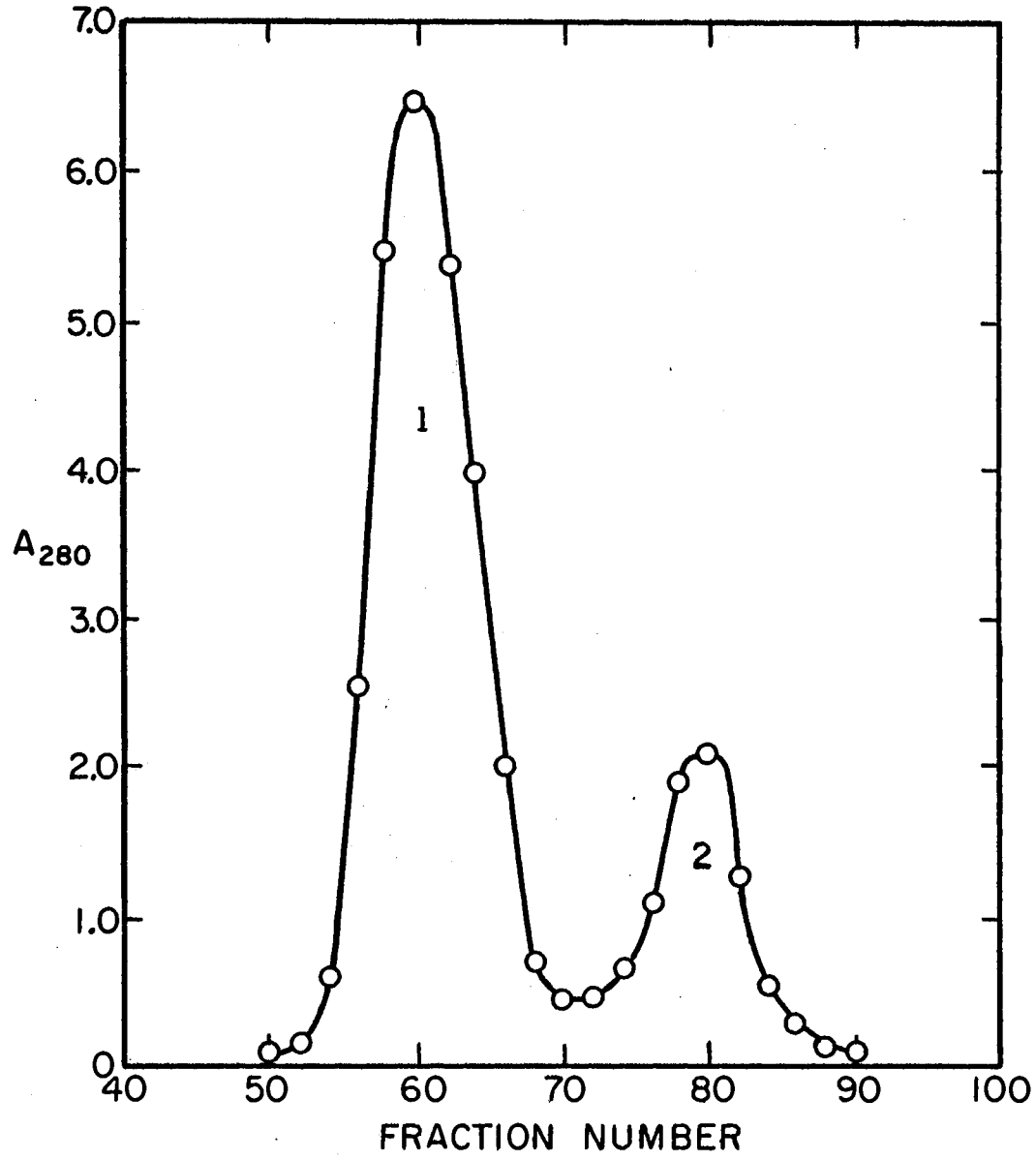

Figure 3. Typical Bio-Gel P-30 Chromatography of Lactose Synthetase From Several Sources

Typical resolution of lactose synthetase into the A protein (peak one) and α-lactalbumin (peak two) from the milk of the cow, sheep, goat, and human (Negro, Indian, and Caucasian). The columns (5 x 100 cm) were equilibrated and eluted at 4° with 20 mM Tris-HCl-5 mM $MgCl_2$, pH 7.4. Twenty-five ml portions from the ammonium sulfate fractionation were placed on the column and five ml fractions were collected after the first 200 ml passed through the column.

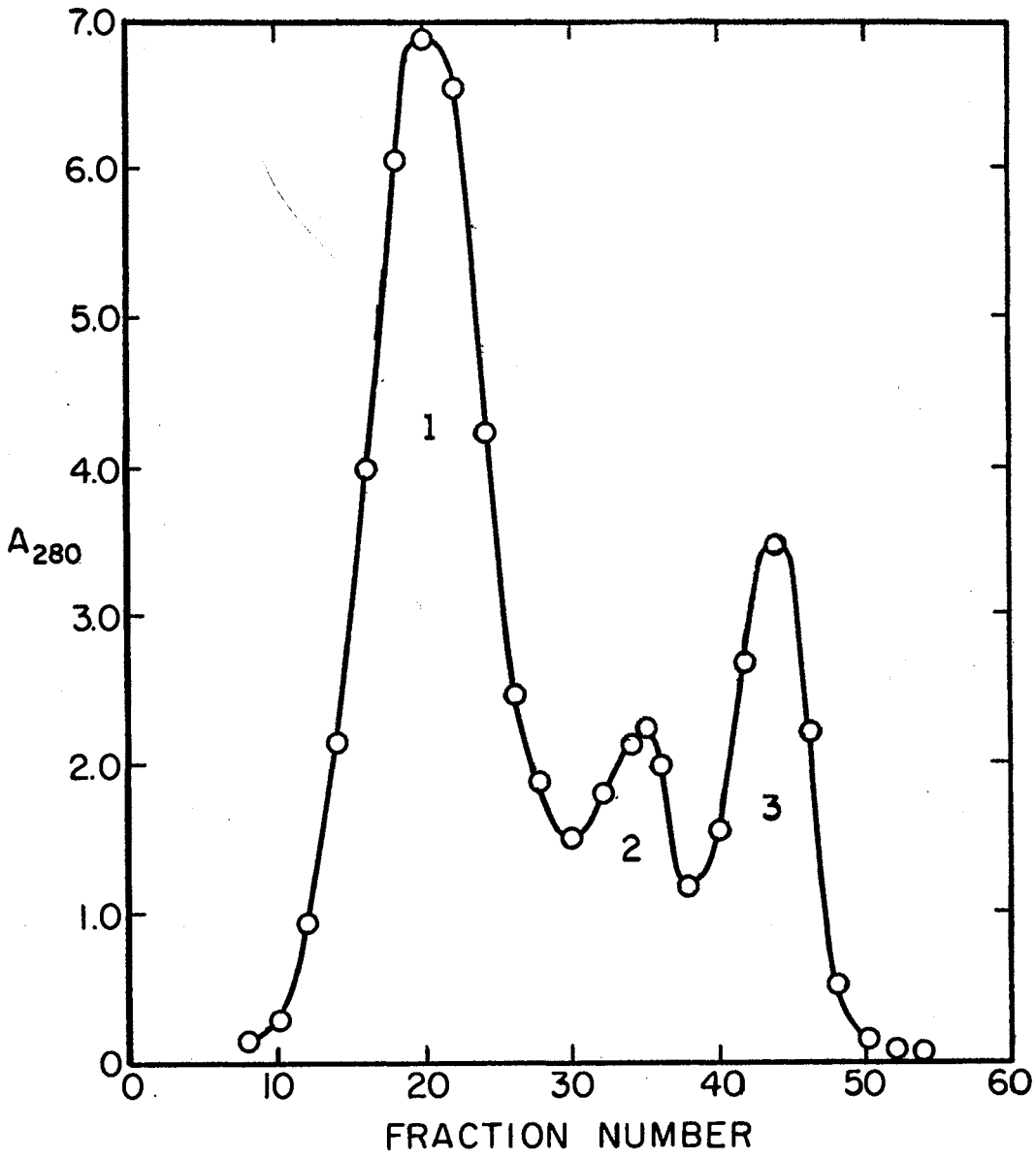

Figure 4. Bio-Gel P-30 Chromatography of Lactose Synthetase From Pig Milk

Resolution of lactose synthetase into the A protein (peak one), α-lactalbumin (peak three) and an unknown component (peak two) from pig milk. The column (5 x 100 cm) was equilibrated and eluted at 4° with 20 mM Tris-HCl-5 mM $MgCl_2$, pH 7.4 and 10.5 ml fractions were collected after the first 300 ml passed through the column.

pattern shown in Figure 4. Peak one was identified as the A protein and peak three was identified as α-lactalbumin by assay. Peak two did not have lactose synthetase activity either as the A protein or as α-lactalbumin. Shortly after this separation was completed, Dr. Kalan provided a protein isolated from the whey of pig milk. This protein, along with the protein from peak two, were assayed for lysozyme activity according to the method of Parry, et al. (159) and both were inactive. Dr. Kalan's protein, along with peak two, did not have any A protein or α-lactalbumin activity in the lactose synthetase reaction.

Starch gel electrophoresis at pH 3.3 shown in Figure 5 was used to compare bovine α-lactalbumin, pig α-lactalbumin, peak two in Figure 3, and Dr. Kalan's pig milk protein. Peak two (Figure 3) and Dr. Kalan's protein have identical but slower electrophoretic mobilities than either of the α-lactalbumins. The ultraviolet spectra of peak two and Dr. Kalan's protein have identical typical protein spectras with a small shoulder at 290 nm. It was concluded from this preliminary data that the protein in peak two of Figure 3 and Dr. Kalan's protein are possibly the same protein isolated from pig milk. The preliminary evidence presented and the data gathered by Kalan and Greenburg (160) and Kessler and Brew (161) indicate that the two proteins are possibly β-lactoglobulin in pig milk existing as a monomeric form.

Ashworth, et al. (162) have studied the gross composition of northern fur seal milk and have reported that approximately 0.14 percent of the total milk composition consists of reducing sugar. This would indicate that lactose may be present and suggests the possible presence of lactose synthetase. Preliminary experiments with gas chromatography (163) indicate that small traces of lactose are present in the fur seal

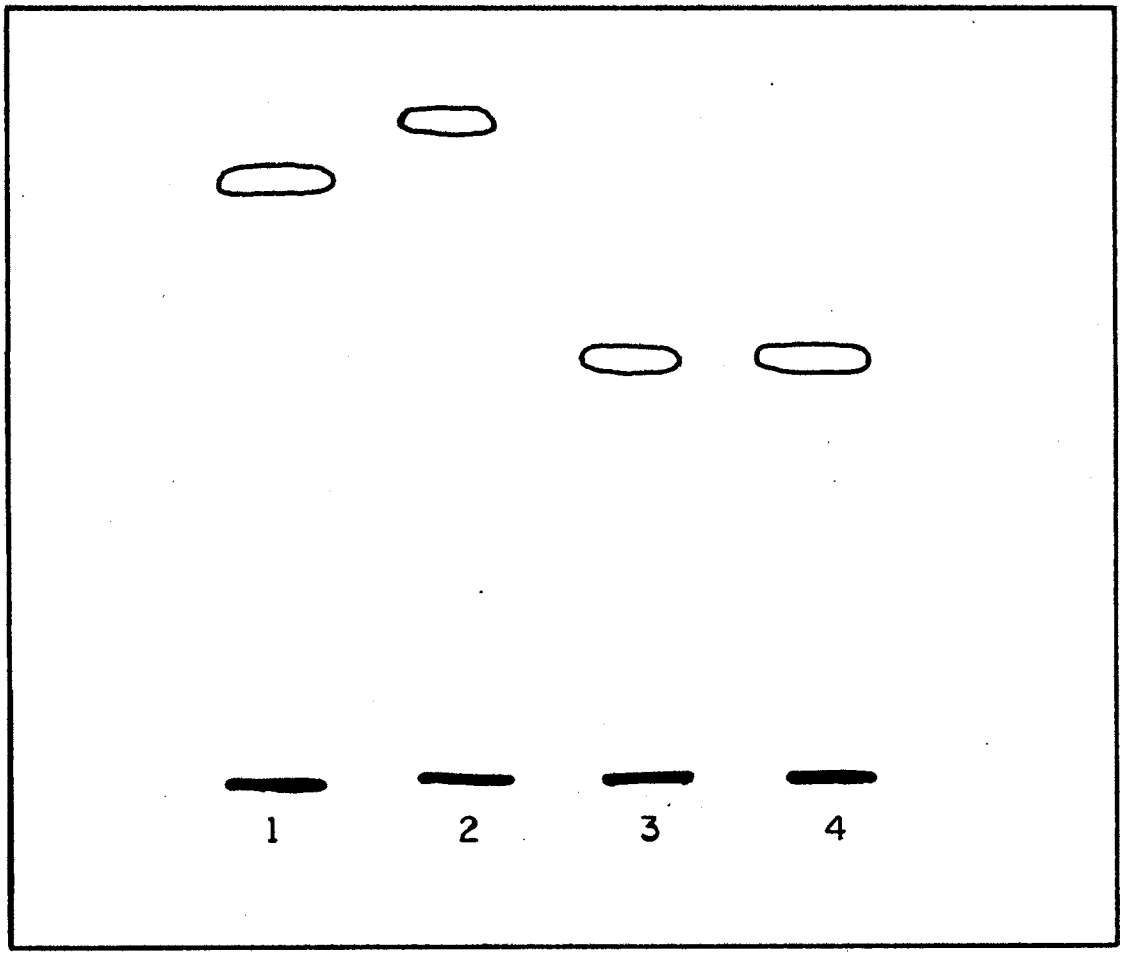

Figure 5. Starch Gel Electrophoresis of Bovine and Pig α-Lactalbumin and Two Pig Milk Whey Proteins

Electrophoretic mobilities were compared to bovine α-lactalbumin (1), pig α-lactalbumin (2), Kalan's pig milk whey protein (3), and peak two in Figure 3 (4). 100 µg of each protein was used as samples. The electrophoretic buffer, which was used to dissolve the proteins, was 8 mM aluminum lactate and 3 M urea adjusted to pH 3.3 with lactic acid. Conditions were 16 hours, 4° and 60 volts across the gel.

milk. The P-30 protein pattern of Figure 6 was the reverse of Figure 2, in which peak one is smaller than peak two. Peak two does not have the typical α-lactalbumin ultraviolet spectra, but it has the common ultraviolet spectra of a protein. Neither peak has any lysozyme activity. Preliminary evidence indicates that peak one has galactosyl transferase activity, but peak two does not stimulate, thus indicating it is not α-lactalbumin.

Electrophoretic Mobilities on Starch Gel

The electrophoretic mobilities of the α-lactalbumins are listed in Table I. The α-lactalbumins were separated in an aluminum-lactate buffer, pH 3.3 and a Tris-citrate buffer, pH 8.6 at 4^{o} C. 100 µl (1 mg/ml) of protein solution was placed in each Whatman 3 mm wick at the origin. All starch gels were run for 16 hours with 60 volts across the gel in a Buchler Universal Electrophoresis apparatus, 25 x 24.5 cm. The electrophoresis buffer was in the bottom of the apparatus; Whatman 3 mm wicks extended from the buffer onto the starch gel plate 2, which was suspended above the buffer. The electrophoretic mobility of bovine α-lactalbumin was designated as 100, and the rest of the α-lactalbumins were recorded as a percent of the mobility of bovine α-lactalbumin. At pH 3.3 bovine α-lactalbumin migrated 16 cm and at pH 8.6, bovine α-lactalbumin migrated 10 cm. All the human α-lactalbumins have a noticeably higher mobility than bovine α-lactalbumin at pH 3.3, but they appear to be identical in mobility at pH 8.6. The goat, sheep, and bovine α-lactalbumins have identical mobilities at pH 3.3 while the pig α-lactalbumin has a slightly higher mobility than bovine α-lactalbumin at pH 3.3. At pH 8.6, the goat and sheep α-lactalbumins moved slower

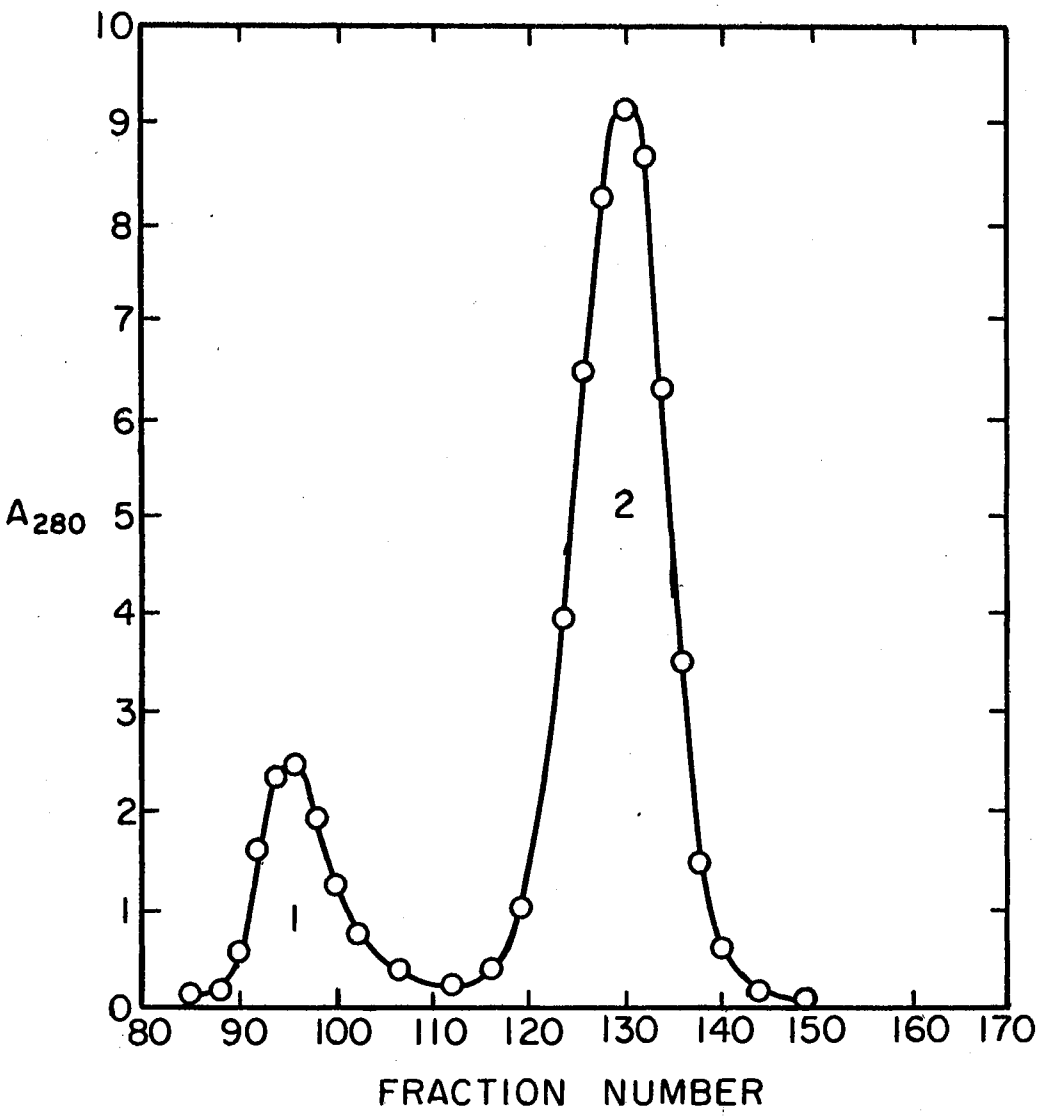

Figure 6. Bio-Gel P-30 Chromatography of Fur Seal Whey Proteins

Resolution of fur seal milk whey proteins into two fractions. 450 mg of whey proteins were placed on the column (5 x 100 cm) in 15 ml of 20 mM Tris, 5 mM $MgCl_2$, pH 7.4. The column was equilibrated and eluted with the same buffer at 4°. 5.4 ml fractions were collected after the first 45 ml passed through the column.

TABLE I

RELATIVE ELECTROPHORETIC MOBILITIES OF α-LACTALBUMIN FROM VARIOUS SOURCES ON STARCH GEL ELECTROPHORESIS

Source	pH 3.3	pH 8.6
Bovine	100	100
Indian	109	101
Caucasian	109	99
Negro	109	99
Goat	99	87
Sheep	100	84
Pig	104	107

than bovine α-lactalbumin whereas the pig α-lactalbumin has a higher mobility than bovine α-lactalbumin. All the human α-lactalbumins appear to be very similar in charge but not identical to bovine α-lactalbumin which is shown by the difference in mobility at pH 3.3. The goat, sheep, and pig α-lactalbumins appear to be quite different in charge at both pH 3.3 and pH 8.6. It may be predicted from the differecnes in electrophoretic mobilities among these proteins that there are possible structural and charge differences in the α-lactalbumins.

Spectrophotometric Properties

The spectrophotometric properties of the α-lactalbumins indicate further structural differences. Table II lists the A_{280}:A_{290} ratios

TABLE II

SPECTROPHOTOMETRIC PROPERTIES OF VARIOUS α-LACTALBUMINS

Source	$A_{280}:A_{290}$	$E^{1\%}_{280}$
Bovine	1.31	20.1
Sheep	1.35	16.7
Pig	1.36	18.1
Goat	1.36	17.3
Negro	1.45	15.2
Indian	1.43	14.1
Caucasian	1.45	15.3
Japanese	1.53	15.0
Guinea Pig	1.59	

and the extinction coefficients of the α-lactalbumins and Figures 7 and 8 compare the ultraviolet spectra of the various α-lactalbumins. The α-lactalbumins of bovine, goat, sheep and pig milk had almost identical $A_{280}:A_{290}$ ratios, but were differnt from the human milk α-lactalbumins which had similar $A_{280}:A_{290}$ ratios. The guinea pig α-lactalbumin has the largest $A_{280}:A_{290}$ ratio of any α-lactalbumin analyzed. All the proteins (Figures 7 and 8) had a pronounced shoulder at 290 nm which is characteristic of exposed tryptophanyl residues. All the proteins also had an unsymmetrical peak between 285 and 270 nm.

The extinction coefficients were obtained in a buffer which had the following composition: pH 6.9, $NaH_2PO_2 \cdot H_2O$, 5.20 g/l, Na_2HPO_4,

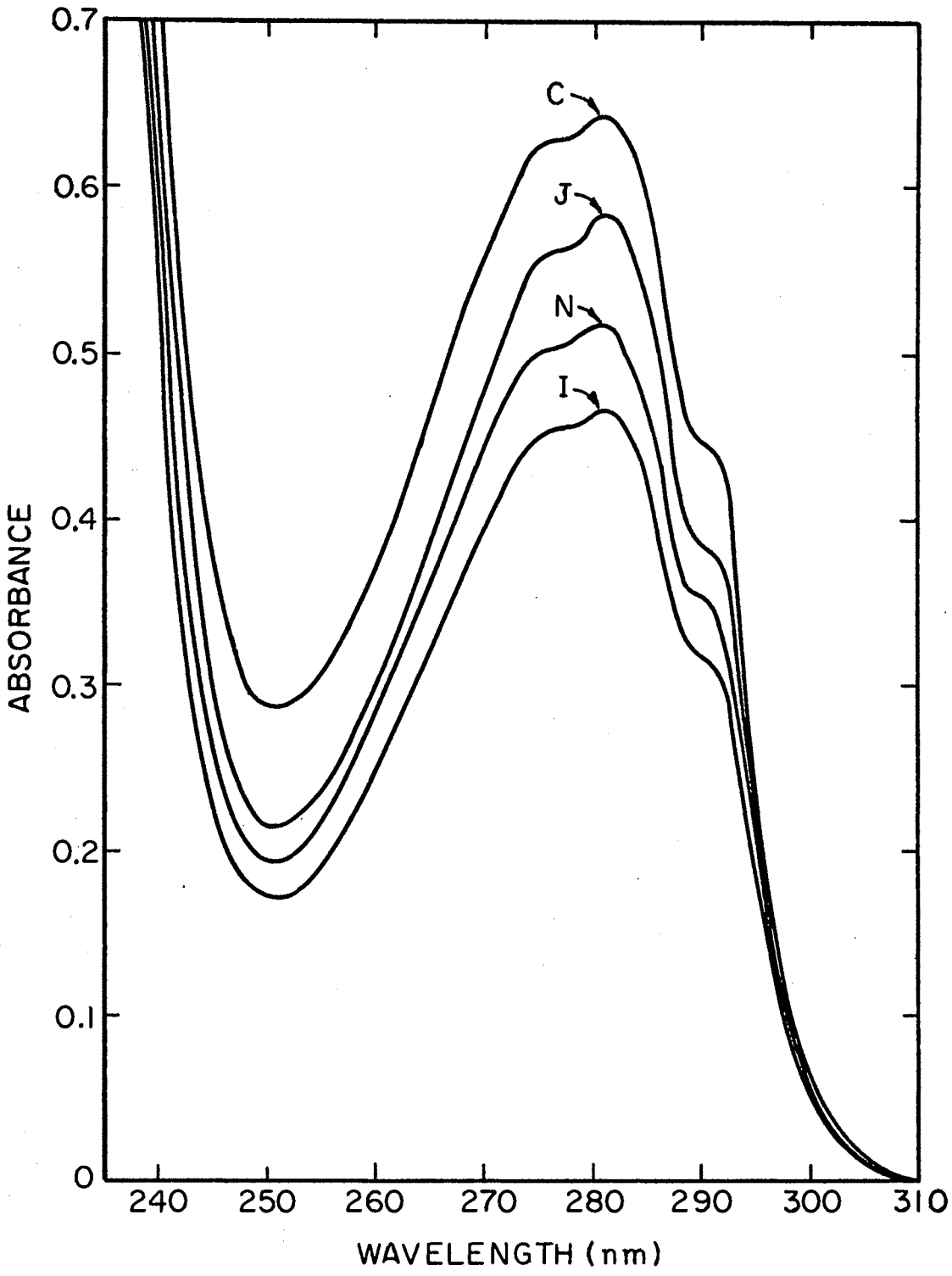

Figure 7. Ultraviolet Spectra of the Human α-Lactalbumins

The α-lactalbumins were dissolved in 0.1 N NaOH, using a concentration that gave a A_{280} = 0.4 - 0.8 for the ultraviolet spectra. The human α-lactalbumins were: Caucasian (C), Japanese (J), Negro (N), and Indian (I).

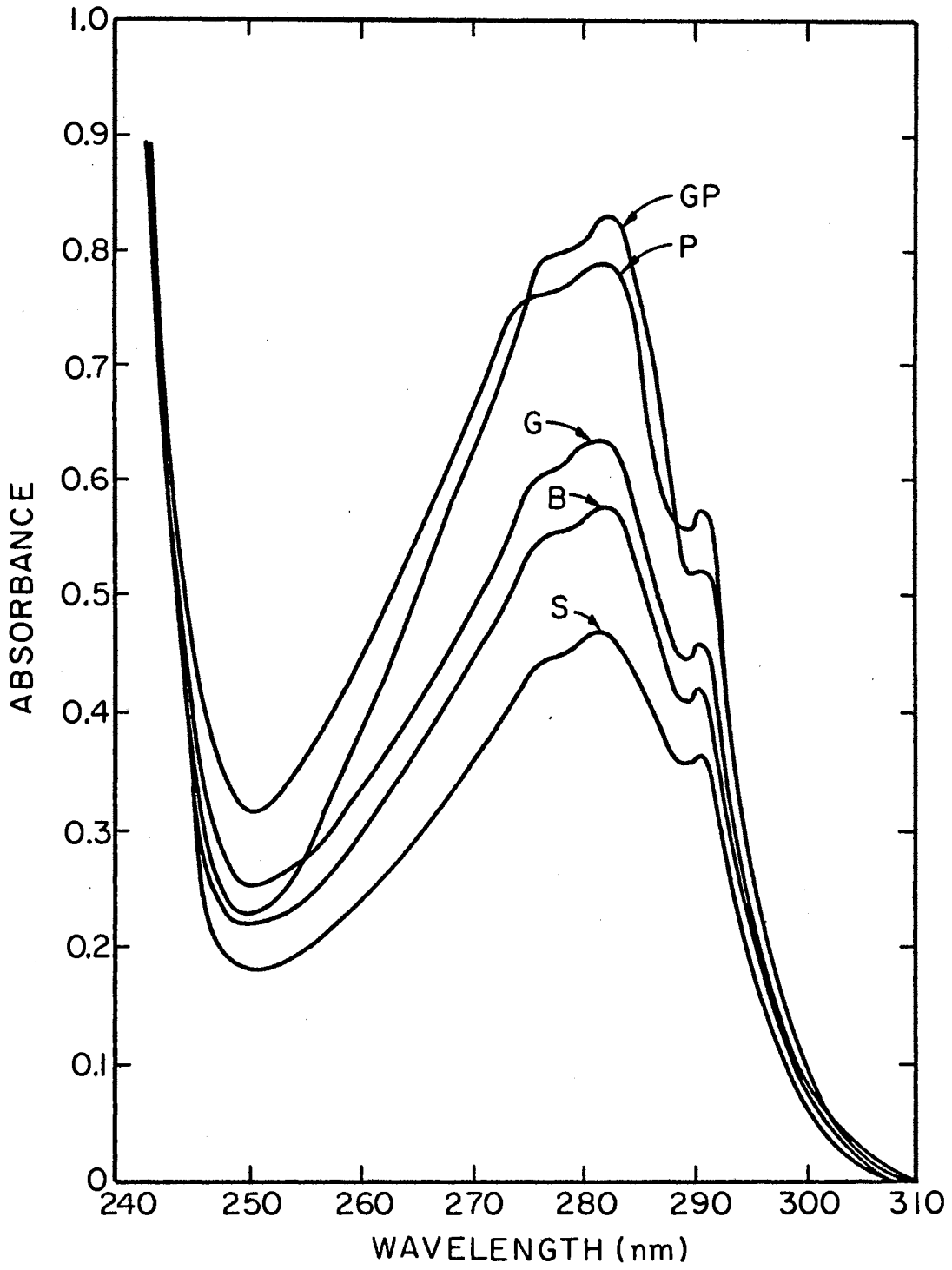

Figure 8. Ultraviolet Spectra of Goat, Sheep, Pig, Guinea Pig, and Bovine α-Lactalbumin

The α-lactalbumins from the milk of goat (G), sheep (S), pig (P), guinea pig (GP), and bovine (B) were dissolved in 0.1 N NaOH, using a concentration that gave a A_{280} = 0.4 - 0.85.

7.7 g/l. The extinction coefficient was obtained for solutions prepared by weighing α-lactalbumins which were salt-free lyophilized, and vacuum-dried at 40° to a constant weight (74). The value of 20.1 obtained for bovine α-lactalbumin was in perfect agreement with Kronman and Andreotti (74) and is very close to Wetlaufer's value (51) of 20.9.

The $E_{280}^{1\%}$ data (Table II) indicate that the bovine α-lactalbumin has the largest tyrosine and tryptophan content. The other α-lactalbumins have a smaller aromatic amino acid content with the Indian α-lactalbumin being the lowest at 14.1. The $E_{280}^{1\%}$ of guinea pig was not determined due to the lack of material and it was not reported by Brew (66) in his characterization of guinea pig α-lactalbumin.

Molecular Weights

Andrews (164) showed that molecular weight determination on gel-filtration columns provides a reasonably accurate estimation of the molecular weight of globular proteins. This technique was employed to obtain the molecular weights of the purified α-lactalbumins. Figure 9 shows a semi-log plot of the elution volume from a Sephadex G-100 versus the molecular weight for the standard proteins and the α-lactalbumins. The standard proteins used as molecular weight markers were cytochrome C, serum albumin, β-lactoglobulin, and five times crystallized bovine α-lactalbumin. Collection of the effluent was started when half of the protein solution had entered the column. A flow rate of 30 ml/hr was maintained by adjustment of the position of the buffer reservoir. The proteins were estimated spectrophotometrically on a Beckman DB, using the wavelengths recommended by Andrews (164) for the marker proteins. A wavelength of 280 nm was used for the determination of the

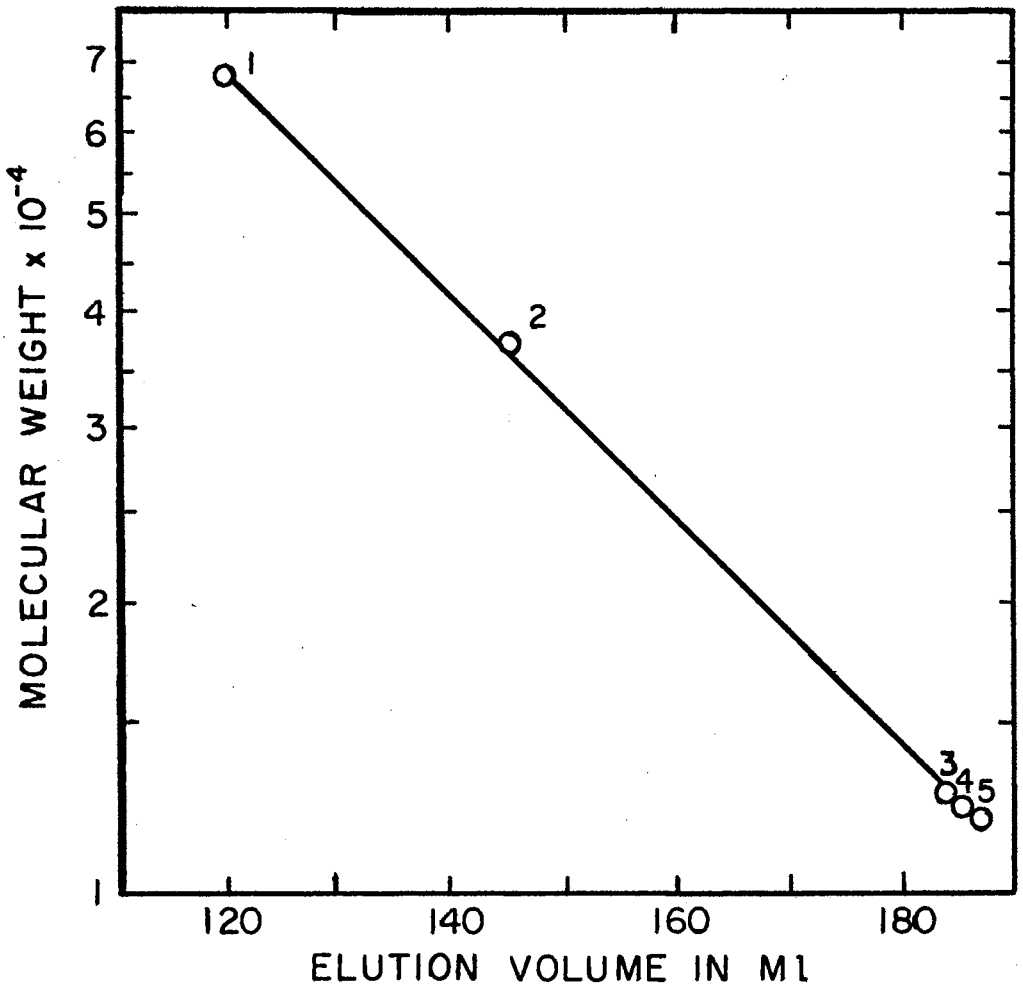

Figure 9. Molecular Weight Determinations of the α-Lactalbumins

A Sephadex G-100 (2.5 x 50 cm) column was equilibrated and eluted at 4° with 50 mM Tris-HCl-0.1 M KCl, pH 7.5. The standard proteins were: 1, serum albumin (10 mg); 2, β-lactoglobulin (10 mg); 5, cytochrome C (4 mg). Four mg of each α-lactalbumin was used and these were: 3, Japanese; 4, Caucasian, Negro, and pig; 5, Indian, sheep, goat and bovine. The standard proteins, with sucrose (15 mg) and blue dextran (4 mg) used to determine the void volume, were combined in 1 ml of the elution buffer for one run. Each α-lactalbumin with blue dextran and sucrose was placed on the column separately and run as an individual experiment.

α-lactalbumins.

The sheep, goat, bovine, and Indian α-lactalbumins along with cytochrome C came off the column at approximately the same point. Since cytochrome C has a molecular weight of 12,400, these proteins may be considered to have this molecular weight, but bovine α-lactalbumin has a molecular weight of 14,437 (12). Therefore, all these α-lactalbumins appear to have a molecular weight in the 14,500 ± 500 range. The Caucasian, Negro, pig and Japanese α-lactalbumins elution patterns indicate they also have a molecular weight of 14,500 ± 500 since they have an elution volume very close to bovine α-lactalbumin.

N-Terminal Amino Acids

The procedure of Fraenkel-Conrat, et al. (153) was utilized for the preparation and identification of the DNP-derivative of the N-terminal amino acids of the various α-lactalbumins (Table III). Five mg of protein were reacted with 1-fluoro-2,4 dinitrobenzene. The resulting DNP-proteins were hydrolyzed in 1 ml of constant boiling HCl for 10 hours in toluene at 110°. The hydrolyses were performed in thick walled, Pyrex, 16 x 125 mm, ignition tubes which were evacuated to 20 microns Hg pressure. After hydrolysis, the hydrolysate was extracted with ether. The ether phase components and the aqueous components were separated by two-dimensional chromatography. Bovine α-lactalbumin was used as a standard since the N-terminal amino acid was known. Two-dimensional chromatography (153) of the ether and aqueous extracts was useful for determining approximate relative R_f of the suspected DNP-amino acids.

Because DNP-histidine, DNP-arginine, and ε-DNP-lysine are soluble

TABLE III

TERMINAL AMINO ACIDS OF THE VARIOUS α-LACTALBUMINS

Source	C-Terminal	N-Terminal
Bovine	Leucine	Glutamic
Sheep	Leucine	Glutamic
Goat	Leucine	Glutamic
Pig	Methionine	Lysine
Caucasian	Leucine	Lysine
Indian	Leucine	Lysine
Negro	Leucine	Lysine
Japanese	Leucine	Lysine
Guinea Pig	Glutamine	Lysine

in the aqueous phase of the hydrolysate, the aqueous phase was chromatographed to establish the presence of DNP-amino acids. Upon spraying the chromatogram with ninhydrin, the yellow spots obtained from all the α-lactalbumins turned brown which indicated that the spots were ε-DNP-lysine. This finding was further verified by spraying a similar chromatogram with Sakaguchi's reagent. The spot remained yellow. By comparison, DNP-arginine turned orange and ε-DNP-lysine remained yellow (165).

The ether phase components were separated by the two-dimensional chromatographic system of Fraenkel-Conrat, et al. (153). Chromatography was carried out on 35 x 60 cm Whatman 1 mm chromatography paper. Chromatography in the two-dimensions resulted in three spots. Exposure

of the largest spot to HCl gas resulted in its disappearance indicating that it was 2,4-dinitrophenol, a reaction by-product. A second reaction by-product was 2,4-dinitroaniline which migrates just behind the solvent front in the first dimension toluene buffer system. The third spot was the DNP-N-terminal amino acid.

The two-dimensional chromatography system for goat α-lactalbumin indicated that the DNP-amino acid was either DNP-glutamic acid or DNP-aspartic acid. The descending chromatography system of Blackburn and Lowther (166) was utilized to identify the goat DNP-amino acid. Chromatography on Whatman 4 paper was used since the tertiary amyl alcohol-phthalate, pH 6, solvent systems migrates very slowly. This solvent system allowed identification of the N-terminal amino acid of goat α-lactalbumin as glutamic acid.

The two-dimensional system of Fraenkel-Conrat, et al. (153) and the tertiary amyl alcohol-phthalate solvent system was used to identify the sheep and pig DNP-amino acid. The N-terminals of sheep and pig α-lactalbumin were glutamic acid and lysine, respectively.

The two-dimensional system (153) was also used for the DNP-amino acid determination of the human α-lactalbumins. The Caucasian, Indian, Negro, and Japanese had lysine as their N-terminal amino acid. Brew (66) has shown that the guinea pig N-terminal amino acid was lysine.

C-Terminal Amino Acids

The C-terminal amino acids of the α-lactalbumins (Table III) were determined by enzymatic C-terminal group analysis utilizing carboxypeptidase A (154,155). Bovine α-lactalbumin was used to check the procedure.

Two mg of each α-lactalbumin were hydrolyzed with carboxypeptidase-A, using an enzyme to substrate ratio of 1 to 200. Portions were withdrawn from the reaction mixture (see methods) and placed in trichloracetic acid after 30 seconds, 1 or 2 minutes, and 5 minutes. These samples were passed over Dowex 50 [H^+ form] column (1/2 x 1 cm). The eluant was evaporated to dryness on a rotary shaker and the precipitate was dissolved in 0.025 ml of H_2O. Aliquots of the samples were spotted on cellulose 300 MN thin layer plates poured in the laboratory (see Methods).

Two solvent systems were used separately to determine the C-terminal amino acids. The solvents were isopropyl alcohol, formic acid, and H_2O (40:2:10) and t-butanol, methyl ethyl ketone, NH_4OH and H_2O (50:30:10:10).

After 5 minutes, four amino acids were released, but at 1 or 2 minutes, one major spot, one minor spot, and one very faint spot were present. At thirty seconds, one major spot and one faint spot were present. These findings show that the C-terminal amino acid was cleaved very easily under these conditions, since a large amount of the α-lactalbumin's C-terminal amino acids were released after 30 seconds of digestion. The next two or three amino acids were also released readily since several spots were present after five minutes of digestion.

The C-terminal amino acid of goat, sheep, Caucasian, Indian, Negro, and Japanese α-lactalbumins was leucine. Pig α-lactalbumin was the exception since methionine was the C-terminal amino acid. Brew (66) has shown that the guinea pig C-terminal amino acid was glutamine. This data indicate further structural differences among the various α-lactalbumins.

Amino Acid Analyses of Various α-Lactalbumins

Amino acid analyses were performed on hydrolyzed α-lactalbumin isolated from the milk of goat, sheep, pig, and human (Caucasian) (Tables IV, V, VI, VII). Literature values for amino acid compositions of proteins are expressed in several different units. Therefore, the amino acids' values obtained in these analyses are expressed in four different ways which are mmoles/100 g protein, g/100 g protein, assumed number of residues, and a calculated number of residues from the average molecular weight.

Triplicate samples were hydrolyzed for 24, 48, and 72 hours. Each triplicate analysis was averaged for each time period and the amount of deviation from the average number was also recorded. These average values were combined for a final average value or an extrapolated value. An extrapolated value to zero time was used if it was apparent that amino acid destruction was occurring during hydrolysis. The 72 hour hydrolysis value was used if the amino acid value increased with hydrolysis time.

The remaining values in Tables IV, V, VI, and VII were obtained in the following manner. The average or extrapolated mmoles/100 g of protein was multiplied by each amino acid's molecular weight, which gave the g/100 g of protein values. The molecular weight of each amino acid was then divided by the g/100 g of protein value, which gave a minimal molecular weight. An assumed number for each residue was chosen which, when multiplied by the minimal molecular weight, gave a calculated molecular weight. It was assumed that the various α-lactalbumins had molecular weights similar to bovine α-lactalbumin since the molecular weight determination on the gel filtration indicated all the

TABLE IV

AMINO ACID ANALYSIS OF GOAT α-LACTALBUMIN

Amino Acid	mmoles/100 g Protein[1] 24 hr	48 hr	72 hr	Average of Extrapolated Values	g/100 g Protein	Minimal Molecular Weight	Calculated Molecular Weight	Assumed No. of Residues	Calc. No. of Residues for Average Molecular Weight of 14082
Lys	73.2 ± 2.6	74.3 ± 1.0	70.5 ± 1.4	74.3	10.86	1346	14806	11	10.5
His	16.1 ± 0.7	17.3 ± 0.7	16.0 ± 0.9	16.5	2.56	6062	12124	2	2.3
Arg	5.1 ± 0.5	6.8 ± 0.1	6.0 ± 0.3	6.0	1.04	16751	16751	1	0.8
Asp	131.0 ± 3.6	135.8 ± 1.4	125.3 ± 9.3	135.8	18.07	737	14003	19	19.1
Thr[2]	32.2 ± 1.4	32.9 ± 0.5	30.7 ± 2.1	33.0	3.93	3031	15155	5	4.6
Ser[2]	28.5 ± 1.0	28.5 ± 0.5	24.6 ± 0.5	29.6	3.11	3379	13516	4	4.2
Glu	79.1 ± 5.2	83.2 ± 1.6	78.8 ± 3.9	83.2	12.24	1202	14424	12	11.7
Pro	21.1 ± 0.9	23.2 ± 2.3	23.5 ± 2.2	23.5	2.71	4248	12744	3	3.3
Gly	29.2 ± 0.2	30.7 ± 0.5	30.1 ± 1.6	30.7	2.30	3264	13056	4	4.3
Ala	30.0 ± 1.3	32.2 ± 1.5	31.4 ± 1.3	32.2	2.87	3104	15520	5	4.5
Val	31.4 ± 0.3	35.3 ± 0.4	36.1 ± 3.0	36.1	4.23	2769	13845	5	5.1
Met	Trace								
Ile	42.4 ± 1.3	46.5 ± 0.2	47.4 ± 2.2	47.4	6.22	2109	14763	7	6.7
Leu	77.7 ± 2.8	81.0 ± 1.5	81.5 ± 4.0	81.5	10.69	1227	14724	12	11.5
Tyr	23.5 ± 1.5	24.0 ± 0.5	23.0 ± 0.6	23.6	4.28	4233	12699	3	3.3
Phe	22.9 ± 2.4	24.0 ± 0.5	24.1 ± 1.3	23.6	3.90	4236	12708	3	3.3
NH_3	82.9 ± 3.6	89.3 ± 0.5	87.3 ± 0.6	81.0	1.38	1232	14784	12	11.4
Try[3]				36.3	7.41	2756	13780	5	5.1
1/2 Cys[4]	54.5			54.5	9.22	1835		(8)	7.6
1/2 Cys[4]	52.7			52.7	8.92	1897	Total	121	119.3
1/2 Cys[5]	7.4 Residues								

[1]Average triplicate for each time period.

[2]Obtained by extrapolation to zero time.

[3]Determined by spectrophotometric method of Beaven and Holiday (152).

[4]18 hour hydrolysis (147,148).

[5]Average residues determined by DTNB methods (149,150).

TABLE V

AMINO ACID ANALYSIS OF PIG α-LACTALBUMIN

Amino Acid	mmoles/100 g Protein[1] 24 hr	48 hr	72 hr	Average of Extrapolated Values	g/100 g Protein	Minimal Molecular Weight	Calculated Molecular Weight	Assumed No. of Residues	Calc. No. of Residues for Average Molecular Weight of 14218
Lys	64.6 ± 0.5	64.1 ± 0.6	63.3 ± 3.4	64.0	9.36	1562	14058	9	9.1
His	16.0 ± 0.2	15.8 ± 0.1	15.5 ± 0.7	15.8	2.45	6334	12668	2	2.2
Arg	8.5 ± 0.4	9.6 ± 0.4	7.3 ± 0.3	8.5	1.48	11771	11771	1	1.2
Asp	127.4 ± 5.1	124.6 ± 0.6	118.7 ± 9.0	123.6	16.45	809	14562	18	17.6
Thr[2]	37.9 ± 0.1	36.3 ± 0.1	32.0 ± 2.4	42.5	5.06	2354	14124	6	6.0
Ser[2]	32.6 ± 0.1	29.8 ± 0.2	25.2 ± 0.5	38.1	4.00	2627	15762	6	5.4
Glu	82.7 ± 0.6	83.1 ± 0.4	78.1 ± 5.1	81.3	11.96	1230	14760	12	11.6
Pro	22.1 ± 0.7	22.0 ± 0.3	22.9 ± 2.2	22.3	2.57	4480	13440	3	3.2
Gly	45.3 ± 0.6	45.7 ± 1.1	42.0 ± 2.9	44.3	3.33	2254	13524	6	6.3
Ala	20.4 ± 0.1	20.2 ± 0.2	19.8 ± 2.0	20.1	1.79	4977	14931	3	2.9
Val	13.5 ± 0.2	14.1 ± 0.0	13.1 ± 0.9	13.6	1.59	7368	14736	2	1.9
Met	18.7 ± 0.5	16.4 ± 0.7	20.1 ± 0.9	19.4	2.89	5163	15489	3	2.8
Ile	59.3 ± 0.6	60.5 ± 0.4	53.7 ± 1.0	57.8	7.58	1731	14579	9	8.2
Leu	71.0 ± 0.6	72.6 ± 0.6	64.8 ± 1.3	69.5	9.12	1438	14380	10	9.9
Tyr	22.0 ± 0.2	22.2 ± 0.1	20.6 ± 0.8	21.6	3.91	4634	13902	3	3.1
Phe	22.8 ± 0.1	23.4 ± 0.1	21.6 ± 1.0	22.6	3.73	4429	13287	3	3.2
NH_3	72.4 ± 2.6	73.7 ± 0.6	77.1 ± 3.2	68.5	1.16	1465	14650	10	9.7
Try[3]				39.2	8.01	2550	15300	6	5.6
1/2 Cys[4]	50.1			50.1	8.48	1996		(8)	7.0
1/2 Cys[4]	48.1			48.1	8.14	2079	Total	120	118.1
1/2 Cys[5]	7.1 Residues								

[1]Average triplicate for each time period.

[2]Obtained by extrapolation to zero time.

[3]Determined with the spectrophotometric method of Beaven and Holiday (152).

[4]18 hour hydrolysis (147,148).

[5]Average residues determined by DTNB methods (149,150).

TABLE VI

AMINO ACID ANALYSIS OF SHEEP α-LACTALBUMIN

Amino Acid	mmoles/100 g Protein[1] 24 hr	48 hr	72 hr	Average of Extrapolated Values	g/100 g Protein	Minimal Molecular Weight	Calculated Molecular Weight	Assumed No. of Residues	Calc. No. of Residues for Average Molecular Weight of 14219
Lys	67.7 ± 1.4	69.4 ± 2.6	70.6 ± 2.6	70.6	10.32	1417	14170	10	10.0
His	15.0 ± 1.0	14.5 ± 2.4	16.3 ± 0.9	16.3	2.53	6134	12268	2	2.3
Arg	5.3 ± 0.4	5.0 ± 0.8	5.6 ± 0.9	5.3	0.92	18936	18936	1	0.8
Asp	119.3 ± 6.2	122.9 ± 5.0	128.3 ± 3.1	128.3	17.08	779	14022	18	18.3
Thr[2]	30.7 ± 1.1	29.0 ± 0.4	29.0 ± 0.7	32.0	3.81	3127	15635	5	4.5
Ser[2]	27.0 ± 0.0	24.6 ± 0.5	23.7 ± 0.9	28.3	2.97	3538	14152	4	4.0
Glu	75.0 ± 2.0	75.2 ± 3.5	78.3 ± 3.6	78.3	11.52	1277	14047	11	11.1
Pro	16.3 ± 2.4	17.3 ± 1.4	14.3 ± 0.9	15.9	1.83	6291	12582	2	2.3
Gly	28.3 ± 1.6	29.5 ± 3.6	29.7 ± 0.4	29.3	2.20	3412	13648	4	4.2
Ala	32.7 ± 0.6	33.3 ± 0.6	35.0 ± 1.0	35.0	3.12	2855	14275	5	5.0
Val	25.3 ± 3.6	27.5 ± 1.2	29.0 ± 1.0	29.0	3.40	3446	13784	4	4.1
Met	1.5 ± 0.5	2.3 ± 0.1		1.9	0.28	53293			
Ile	40.0 ± 1.3	42.7 ± 1.1	44.6 ± 1.1	44.6	5.85	2242	13452	6	6.3
Leu	70.7 ± 3.6	73.0 ± 2.4	73.6 ± 1.1	73.6	9.65	1359	14949	11	10.5
Tyr	21.0 ± 1.0	21.7 ± 0.6	21.7 ± 0.6	21.6	3.91	4634	13902	3	3.1
Phe	21.3 ± 0.8	22.4 ± 0.6	22.3 ± 0.6	22.0	3.63	4551	13653	3	3.1
NH_3	76.3 ± 1.8	84.6 ± 1.8	90.3 ± 5.6	70.0	1.19	1429	14290	10	10.0
Try[3]				28.6	5.84	3497	13988	4	4.1
1/2 Cys[4]	50.4			50.4	8.53	1984		(8)	7.5
1/2 Cys[4]	54.5			54.5	9.22	1835	Total	111	111.2
1/2 Cys[5]	7.6 Residues								

[1]Average triplicate for each time period.

[2]Obtained by extrapolation to zero time.

[3]Determined with spectrophotometric method of Beaven and Holiday (152).

[4]18 hour hydrolysis (147,148).

[5]Average residues determined by DTNB methods (149,150).

TABLE VII

AMINO ACID ANALYSIS OF HUMAN (CAUCASIAN) α-LACTALBUMIN

Amino Acid	mmoles/100 g Protein[1] 24 hr	48 hr	72 hr	Average of Extrapolated Values	g/100 g Protein	Minimal Molecular Weight	Calculated Molecular Weight	Assumed No. of Residues	Calc. No. of Residues for Average Molecular Weight of 14302
Lys	68.4 ± 4.9	66.3 ± 2.6	66.8 ± 2.6	66.8	9.76	1498	14980	10	9.5
His	10.7 ± 1.4	11.1 ± 0.5	10.9 ± 0.9	10.9	1.69	9183	9183	1	1.6
Arg	6.5 ± 0.3	4.9 ± 0.2	5.8 ± 0.6	5.7	0.99	17597	17597	1	0.8
Asp	97.6 ± 2.6	100.2 ± 1.2	94.4 ± 3.7	97.4	12.96	1027	14378	14	13.9
Thr[2]	38.0 ± 1.1	37.2 ± 1.9	34.2 ± 1.7	41.0	3.81	3127	15635	5	4.6
Ser[2]	40.6 ± 1.5	36.6 ± 2.5	32.1 ± 1.8	45.7	4.80	2189	15323	7	6.5
Glu	95.3 ± 6.0	92.6 ± 1.5	89.7 ± 2.9	92.5	13.61	1081	14053	13	13.2
Pro	17.1 ± 2.2	20.1 ± 1.3	19.9 ± 0.5	19.0	2.19	5257	15771	3	2.7
Gly	35.1 ± 1.8	36.0 ± 1.2	33.5 ± 2.1	34.9	2.62	2865	14325	5	5.0
Ala	31.5 ± 1.0	32.5 ± 0.3	31.3 ± 2.2	31.8	2.83	3148	15740	5	4.5
Val	10.0 ± 0.7	11.7 ± 0.3	11.7 ± 0.8	11.1	1.30	9012	9012	1	1.6
Met[2]	12.2 ± 0.7	11.7 ± 0.6	10.9 ± 0.9	13.0	1.94	7692	15384	2	1.9
Ile	70.2 ± 3.2	68.7 ± 1.4	68.5 ± 3.4	69.1	9.06	1448	14480	10	9.9
Leu	86.7 ± 3.6	82.7 ± 1.4	82.3 ± 5.1	83.7	10.98	1195	14340	12	12.0
Tyr	23.6 ± 0.4	23.3 ± 0.5	21.9 ± 0.6	22.9	4.15	4366	13098	3	3.3
Phe	23.6 ± 0.4	23.8 ± 0.6	22.6 ± 1.6	23.3	3.85	4291	12873	3	3.3
NH_3	71.7 ± 7.0	66.1 ± 1.4	70.0 ± 3.8	69.3	1.17	1453	14520	10	9.8
Try[3]				17.9	3.66	5580	16740	3	2.6
1/2 Cys[4]	47.5			47.5	8.04	2104		(8)	7.1
1/2 Cys[4]	51.6			51.6	8.78	1938	Total	116	113.8
1/2 Cys[5]	7.8 Residues								

[1]Average triplicate for each time period.

[2]Obtained by extrapolation to zero time.

[3]Determined with the spectrophotometric method of Beaven and Holiday (152).

[4]18 hour hydrolysis (147,148).

[5]Average residues determined by DTNB methods (149,150).

α-lactalbumins had very similar molecular weights. Therefore, assumed residue numbers were chosen which gave a calculated weight close to the molecular weight of bovine α-lactalbumin. An average molecular weight was calculated from the calculated molecular weights. The average molecular weight was divided by each assumed residue value to give the calculated amino acid residues. The calculated results are presented in Tables IV, V, VI, and VII.

Included in these tables are values for tryptophan determined by ultraviolet spectral analysis and the values for half-cystine (see Methods). Three methods were used for the determination of half-cystine. Performic acid was used to oxidize the disulfide linkages to cysteic acid (147,148). The protein was then hydrolyzed with acid and analyzed for cysteic acid on the automatic amino acid analyzer. This procedure gave unsatisfactory results, since only six half-cystines were determined for bovine α-lactalbumin which actually has eight half-cystines. The performic acid procedure was altered in several ways in order to eliminate possible over or under oxidation of the protein. Experiments involving equal and excess molar concentrations of performic acid in the protein solution were carried out at 4° and 25° with oxidation times from 30 minutes to overnight. Different methods of removing the performic acid were: evaporation on a rotary evaporator with or without the addition of HBr and by dilution with water and removal of the diluted solution by lyophilization. Assuming 90 percent recovery as cysteic acid (147), only 6.8-7.4 of the eight residues of half-cystine could be accounted for in the bovine α-lactalbumin. The cysteic acid values listed in Tables IV, V, VI, and VII were calculated by dividing the experimentally determined values by 0.9 since the published

procedure reports 90 percent recovery (147).

The methods utilizing reduction of the disulfide bond with dithiothreitol or $NaBH_4$ (149,150) also gave unsatisfactory results. The reduced disulfide was reacted with DTNB which gave a yellow color and was measured spectrophotometrically at 412 nm. An extinction coefficient of 13,600 and 12,000 was used in the dithiothreitol (DTT) reduction and $NaBH_4$ methods, respectively. Recently, J. Robyt of Iowa State University reported at the West Central States Biochemistry Conference, 1970, a study of the chemistry of Ellman's reagent (DTNB) and its use for the determination of disulfides in proteins. Robyt, through a thorough study of the chemistry of Ellman's reagent, has determined the extinction coefficient of the sulfur reagent at 412 nm to be 11,400. By using the extinction coefficients of the published procedures, the determination of the disulfide bonds in bovine α-lactalbumin were low, 6-7 residues, but by using the extinction coefficient of 11,400, the half-cystine content of bovine α-lactalbumin averaged between 7-8 residues. Therefore, the extinction coefficient of 11,400 was used for the determination of the disulfide bonds listed in Tables IV, V, VI, and VII.

It must be noted that these half-cytine values were tentative values based on the poor results obtained for the half-cytine values of the standard protein of bovine α-lactalbumin. Therefore, the half-cystine values were not used in calculating the average molecular weight. J. Robyts' unpublished procedure appears to be superior to Zohler and Cleland's method (149) for disulfide determination in proteins and therefore, this procedure may give better results for the determination of half-cystine values in the α-lactalbumins.

The average calculated molecular weights of the α-lactalbumins

were 14,302 (human, Caucasian), 14,219 (sheep), 14,218 (pig), and 14,082 (goat) which are very close to 14,437 which is the known molecular weight of bovine α-lactalbumin. The assumed number of residues determine the average molecular weights. The total assumed residues of 120 (pig) and 121 (goat) are very close to bovine α-lactalbumin's 123 residues, while the assumed residue of 116 (Caucasian) and 111 (sheep) are 7 and 11 residues, respectively, smaller than bovine α-lactalbumin.

Table VIII compares the amino acid residues of bovine, goat, sheep, pig and Caucasian α-lactalbumins. Most of the residues vary by only one or two residues. The tryptophans vary among the α-lactalbumins which cause their extinction coefficients to vary also. The aspartic and glutamic acid values for the goat, sheep, pig, and Caucasian α-lactalbumins also represent the asparagine and glutamine residues. The greatest difference of amino acid residues is between bovine, pig and human (Caucasian) α-lactalbumin. Human (Caucasian) has 14 aspartic residues whereas bovine has 21 aspartic and asparagine residues. Also the bovine has six valine residues while pig and human (Caucasian) have 2 and 1 residues, respectively. These amino acid analyses point out further structural differences among the α-lactalbumins.

The microkjeldahl procedure of Gonzalez, Cadavid and Paladini (167) was used to determine the nitrogen content of three α-lactalbumins. The α-lactalbumins contained the following nitrogen percentages: goat, 15.03%; pig, 14.88%; and sheep, 14.70%.

Heterogeneity in the Various α-Lactalbumins

The starch gel electrophoresis method was used as a criteria for purity before any of the physical and chemical properties of the

TABLE VIII

AMINO ACID COMPARISON OF BOVINE, GOAT, SHEEP, PIG, AND HUMAN (CAUCASIAN) α-LACTALBUMINS

Amino Acid	Bovine	Goat	Sheep	Pig	Human (Caucasian)
Lys	12	11	10	9	10
His	3	2	2	2	1
Arg	1	1	1	1	1
Thr	7	5	5	6	5
Ser	7	4	4	6	7
Pro	2	3	2	3	3
Gly	6	4	4	6	5
Ala	3	5	5	3	5
Val	6	5	4	2	1
Met	1	Trace	Trace	3	2
Ile	8	7	6	9	10
Leu	13	12	11	10	12
Tyr	4	3	3	3	3
Phe	4	3	3	3	3
Trp	4	5	4	6	3
1/2 Cys	8	(7-8)	(7-8)	(7-8)	(7-8)
Asp	9	19	18	18	14
Asn	12				
Glu	8	12	11	12	13
Gln	5				
NH_3		12	10	10	10

α-lactalbumins were studied. All the α-lactalbumins appeared as one sharp band on the starch gels at both pH 3.3 and 8.6. B. Colvin, a post-doctoral fellow in Dr. K. E. Ebner's group, compared rabbit antisera to pig α-lactalbumin and detected a major and minor band in the immunodiffusion pattern of Figure 10. The two bands indicated that the pig α-lactalbumin had an impurity present or that there were two species of α-lactalbumin present which could be either ionic or molecular weight variants.

The immunodiffusion experiment indicated that a more sensitive technique was needed as a criteria for purity in order to detect the presence of multiple species of α-lactalbumin. Such a sensitive technique is disc gel electrophoresis. All the purified α-lactalbumins subjected to disc gel electrophoresis at pH 9.5, using 7 percent gels, are shown in Figure 11. The α-lactalbumins of pig, sheep, and goat separate into two bands on the disc gels, while the α-lactalbumins of the humans, bovine (not shown in Figure 9), buffalo (India), and guinea pig have only one band on the disc gels. The relative migration of the various α-lactalbumins on the disc gels in relation to bovine α-lactalbumin migration were: bovine, 100; human (Caucasian, India, and Negro), 100; trailing band of sheep and goat, 79; leading band of sheep and goat, 86; trailing band of pig, 104; leading band of pig, 115.

Very faint diffuse bands appear ahead of the main bands in Figure 11 in all the α-lactalbumins except guinea pig. These faint bands are probably very small amounts of impurities.

Several explanations can be given for the two protein bands. The protein bands may represent a simple monomer and dimer. a large impurity, two different ionic species with the same molecular weight, two

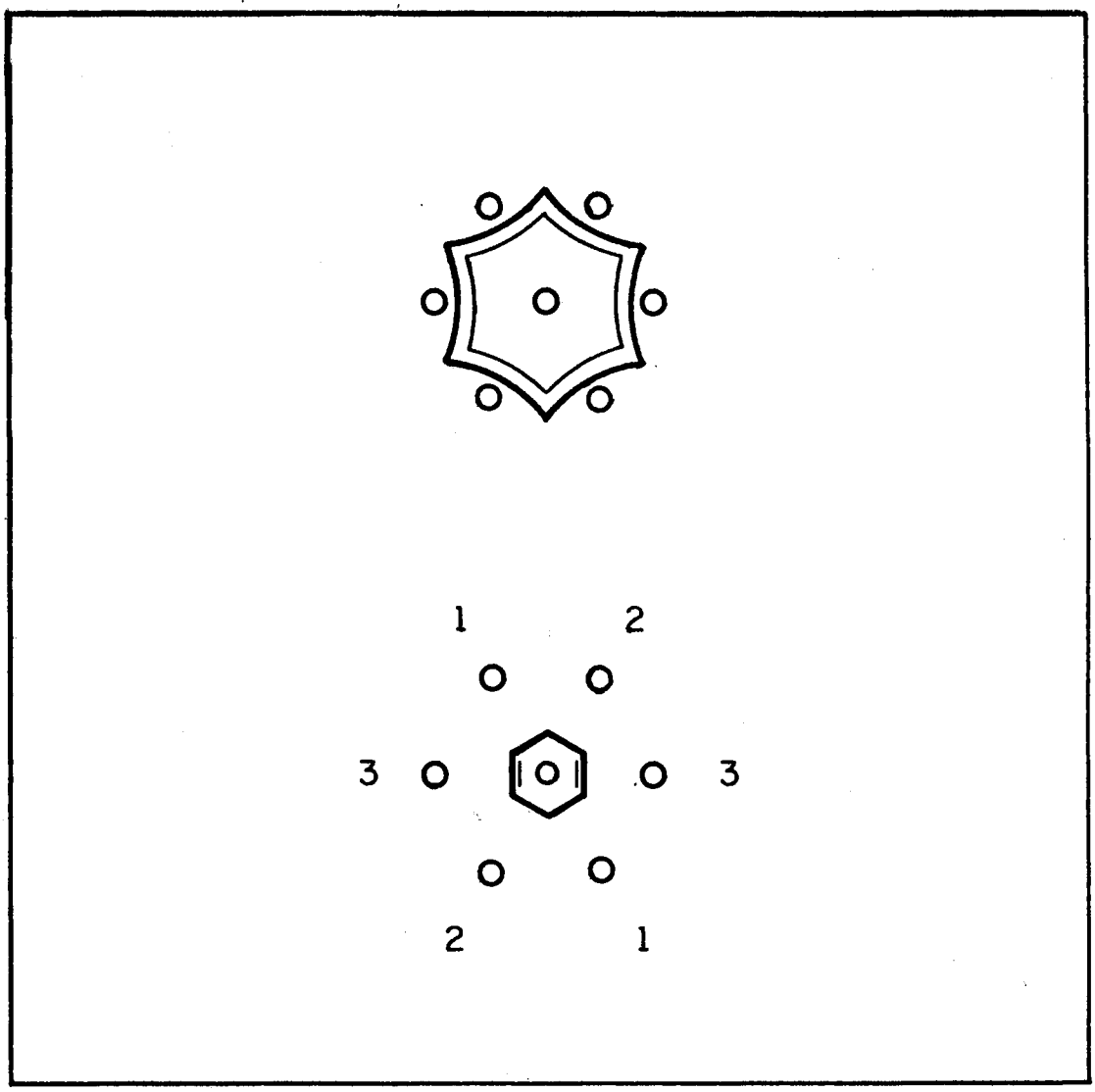

Figure 10. Immunodiffusion Patterns of Pig α-Lactalbumin Complexing With Rabbit Antisera for Pig α-Lactalbumin

The top diagram contained 200 μg in 0.01 N NH_4OH of pig α-lactalbumin in the center wells, with rabbit antisera to pig α-lactalbumin in the outside wells. The bottom diagram contained rabbit antisera to pig α-lactalbumin in the center well. The outside wells contained pig α-lactalbumin from the following sources: (1) peak one from DE-32 column, (2) peak two from DE-32 column, (3) pig α-lactalbumin purified previous to DE-32 column.

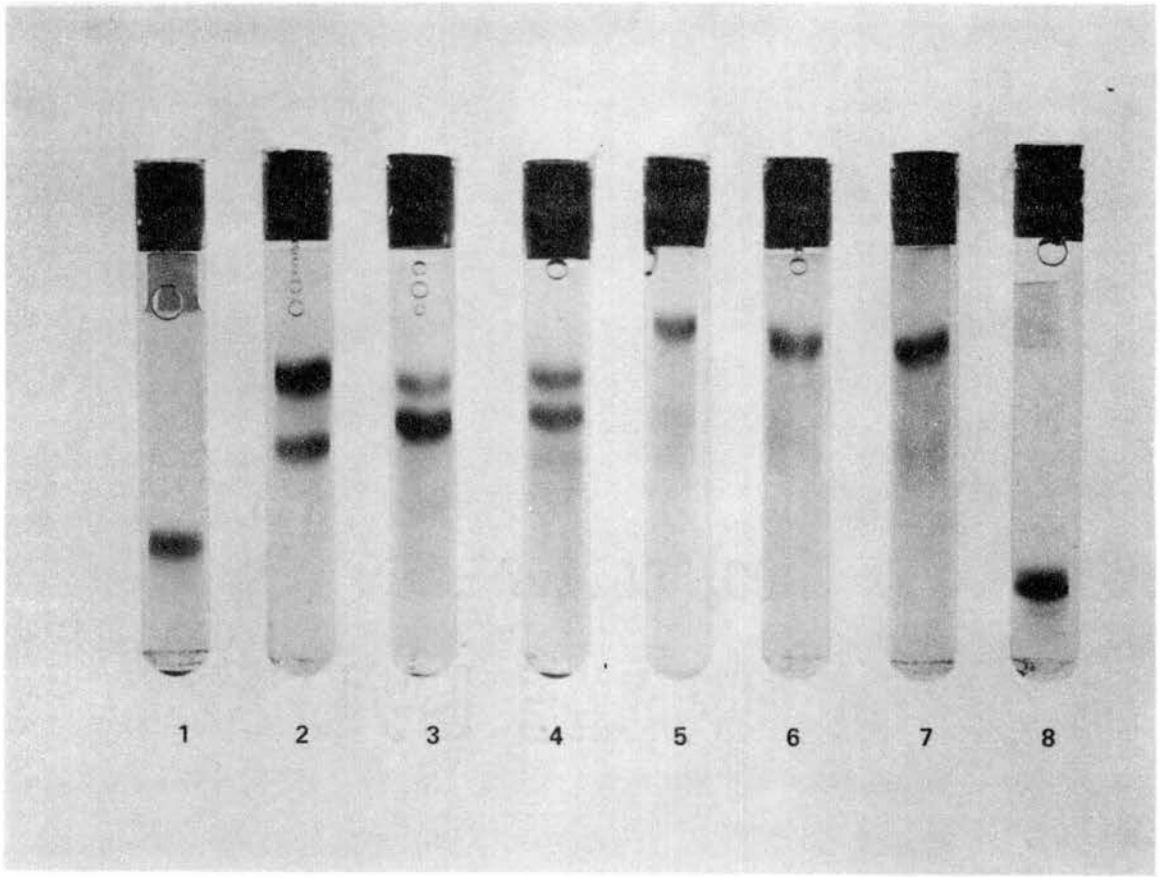

Figure 11. Characteristic Patterns of the Various α-Lactalbumins on Disc Gel Electrophoresis

Standard 7 percent gels were used for the disc gel electrophoresis study. The α-lactalbumins were (1) guinea pig, 40 µg; (2) pig, 60 µg; (3) goat, 40 µg; (4) sheep, 45 µg; (5) Caucasian, 30 µg; (6) Indian, 30 µg; (7) Negro, 30 µg; (8) buffalo, 35 µg.

different molecular weight species, and an association or dissociation phenomena caused by the pH and/or the buffer used in the standard 7 percent disc gels at pH 9.5. Therefore, an extensive study with disc gel electrophoresis was carried out in order to identify which of the preceding situations existed for the two protein bands.

The first possibility that was investigated was the buffer and/or the pH effect. The Tris-glycine, pH 9.5 buffer system of the disc gels may have caused the multiple bands; therefore, different pHs and buffers were used to verify or eliminate this possibility. A 7 percent, pH 4.3 disc gel was prepared according to Canalco specifications (143). All the α-lactalbumins at pH 4.3 shown in Figure 12 have a single band. The α-lactalbumins were also run on disc gels using phosphate buffer at pH 7.3 (see Methods). The proteins again showed only one band as shown in Figure 13. This would indicate that, at pH 4.3 and 7.3, the two protein bands of pig, goat, and sheep have identical charges or do not associate or dissociate to give a monomer-dimer pattern.

The possible buffer effect at pH 9.5 was eliminated by substituting a 50 mM borate buffer (pH 9.5) for the standard Tris-glycine buffer. The α-lactalbumins of sheep, pig, and goat retained their two protein band pattern while bovine α-lactalbumin retained its single protein band. The results obtained in the different buffers and pH values indicate that the two protein bands are a result of the ionic conditions at pH 9.5.

Dissociating conditions were also used in the disc gels to determine if association was occurring, thus producing a higher molecular weight species at pH 9.5. Six molar urea was placed in the 7 percent disc gels at pH 4.3 and 9.5. The urea did not have any effect on the

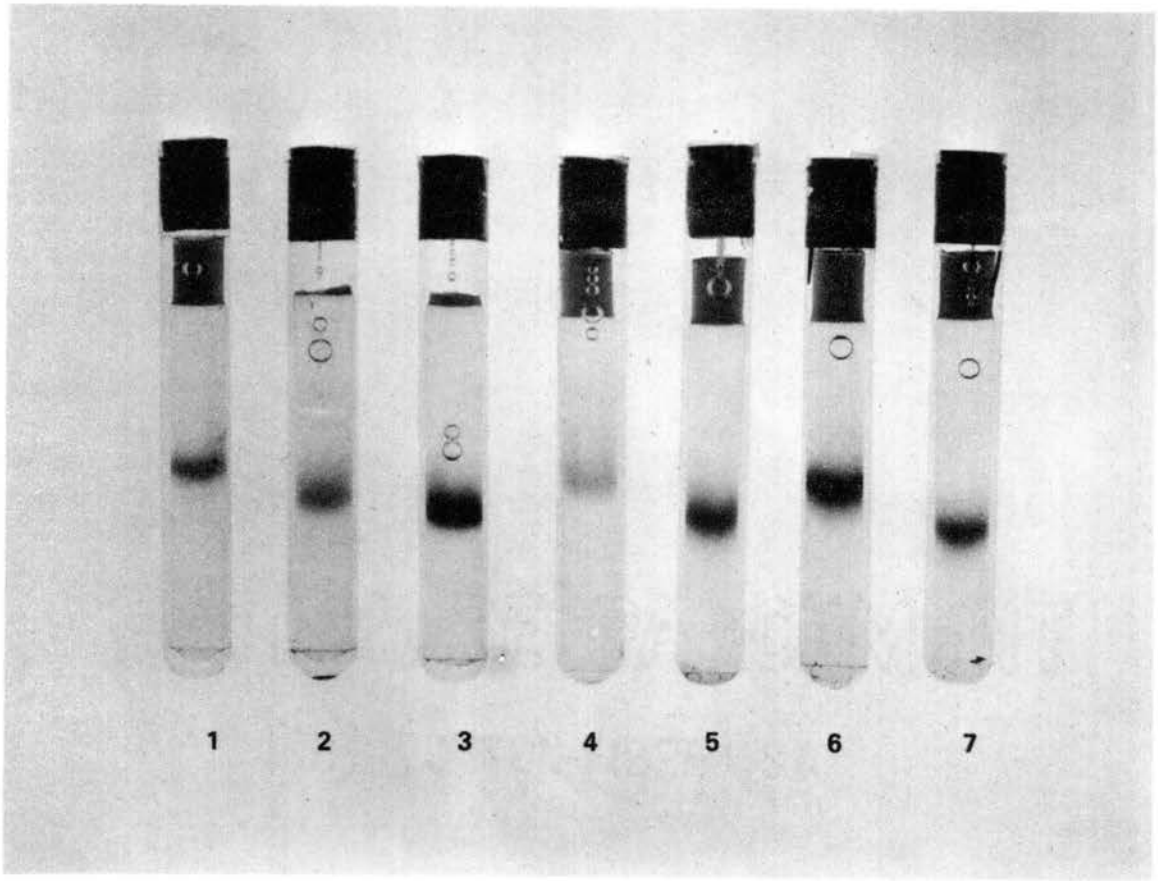

Figure 12. Disc Gel Electrophoresis of the Various α-Lactalbumins at pH 4.3

The effect of low pH, 7 percent disc gels was determined on the following α-lactalbumins: (1) bovine-3x crystallized, (2) sheep, (3) goat, (4) pig, (5) Caucasian, (6) Indian, (7) Negro.

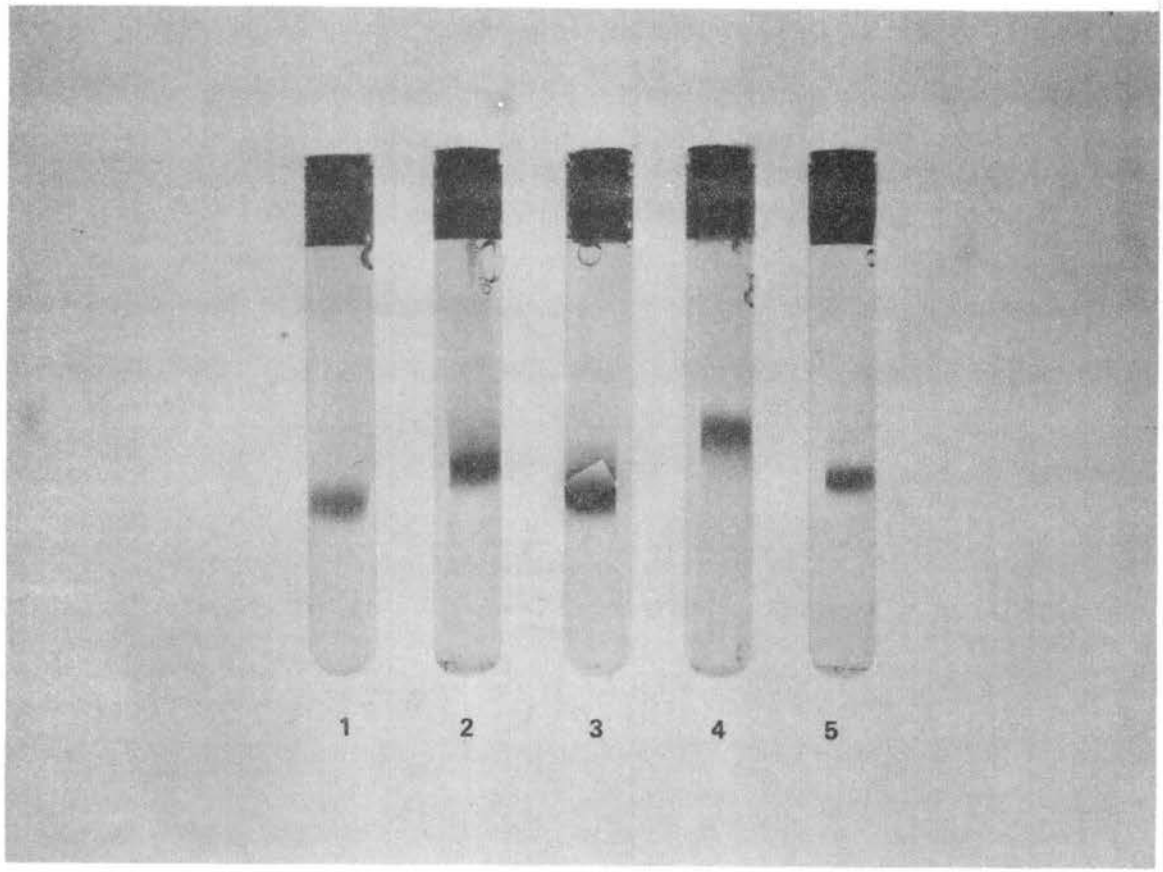

Figure 13. Disc Gel Electrophoresis of the Various α-Lactalbumins at pH 7.3

The effect of pH 7.3, 7 percent disc gels was determined on the following α-lactalbumins: (1) pig, (2) sheep, (3) goat, (4) Negro, (5) bovine.

protein patterns which were the same as the gels without urea.

Sodium doceyl sulfate (SDS) was also used as a dissociating agent in the gels (see Methods). Figure 14 shows the protein pattern in SDS. One major band was present in α-lactalbumins of goat, sheep, pig and bovine, but all the gels have two faint trailing bands. It is suspected that these minor bands were due to larger molecular weight impurities than the molecular weights of the α-lactalbumins. Even the three and five times crystallized bovine α-lactalbumins contain the same minor bands which in the standard pH 9.5 gels have one major band with a very minor leading band. This minor leading band also occurs in the other purified α-lactalbumins. The difference in protein migration in the SDS system is due solely to the molecular weight. Therefore, the faint leading protein band in the standard pH 9.5 gels may have a greater charge and a larger molecular weight than the α-lactalbumins. Thus, this same contaminating protein or proteins in the presence of SDS have the same charge as the α-lactalbumins, but due to their larger molecular weight, they migrate slower than the α-lactalbumins.

The major band of the α-lactalbumins in Figure 14 can be considered the combination of the two bands in the standard pH 9.5 gels if the minor trailing bands are due to differing molecular weight impurities, which was assumed to be the case. Therefore, the two protein bands in the standard pH 9.5 gels will appear as one band in the pH 9.5 SDS system since both the proteins have the same molecular weight and the same ionic charge due to the SDS. Further verification of this explanation will be elaborated on in the different percent disc gel study.

The method of Hedrick and Smith (145) was used to determine if the two bands on standard pH 9.5 disc gels were size or charge isomers.

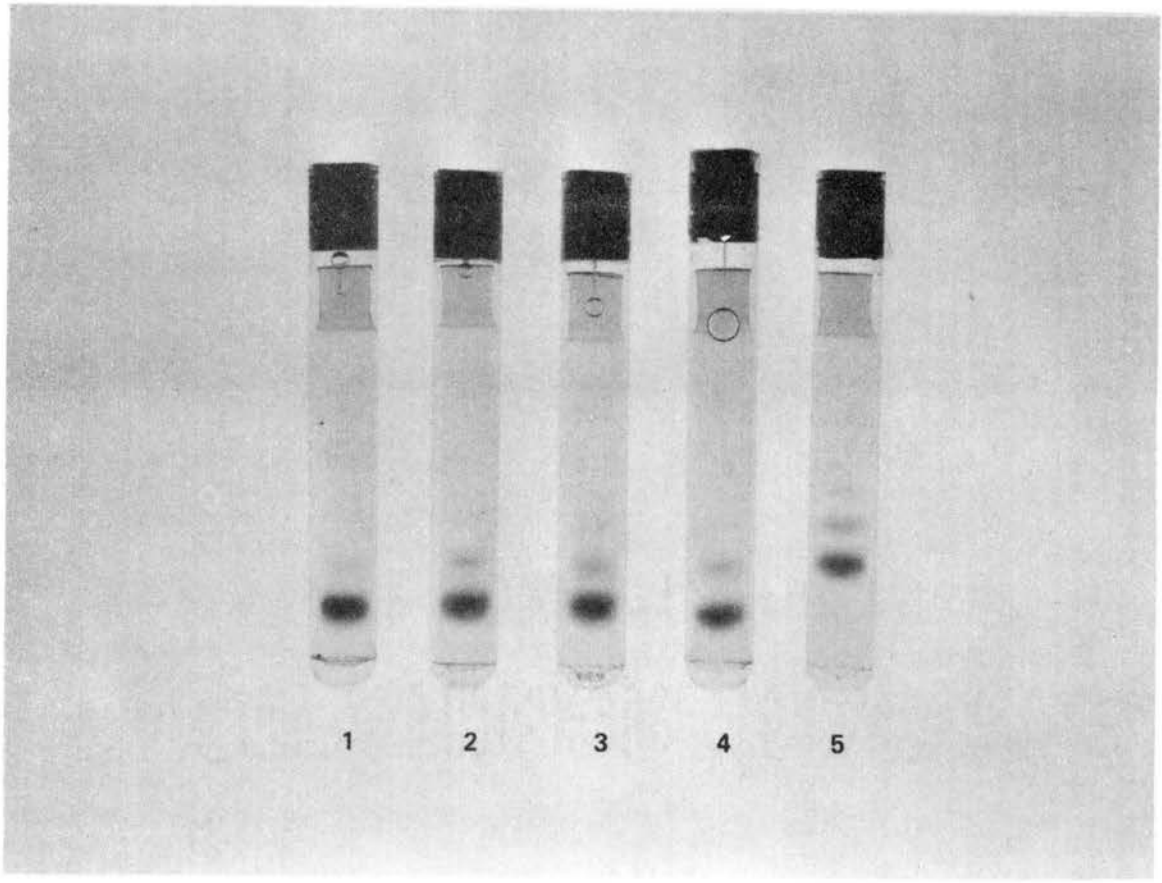

Figure 14. The Effect of SDS on the Various α-Lactalbumins in Disc Gel Electrophoresis at pH 9.5

The disc gels and buffer contained 0.1 percent SDS. The α-lactalbumins previously incubated in SDS (see Methods) were as follows: (1) pig, (2) goat, (3) sheep, (4) bovine, 5x crystallized, (5) bovine, 3x crystallized.

When the acrylamide gel concentration is plotted versus the logarithm of protein mobility relative to the dye front, charge isomeric proteins give a family of parallel lines, while size isomeric proteins yield a family of nonparallel lines which extrapolate to a common point in the vicinity of zero percent gel concentration. If the proteins differ in both size and charge, nonparallel lines are shown which intersect at gel concentrations other than zero percent gel concentration. The R_m, which is the ratio of protein migration to dye migration, was calculated by measuring the distance that the protein bands and the marker dye, marked with a small wire in the gel, had migrated from the interface of the stacking and separating gel. The α-lactalbumins of goat, sheep, pig, human (Caucasian, Indian, Negro), and bovine were run on differing percent gels varying from 3 to 12 percent.

Pig α-lactalbumin (Figure 15) represents the protein band patterns that the α-lactalbumins of goat and sheep also showed. The two major protein bands retain their relative distance throughout the 3 to 12 percent disc gel runs. The migration of the protein bands (R_m), plotted in Figure 16, display parallel lines which indicates that the two protein bands are charge isomers.

The faint leading band in the lower percent gels in Figure 15 has a decreasing R_m relative to the two major bands as the gel percent increased. Also, several very faint bands which trail the two major bands appear in the higher percent gels. These trailing bands are probably present in the two major bands in the lower percent gels since they do not appear as bands in the lower percent gels. All of these faint protein bands, when plotted on a graph similar to Figure 16, show a family of nonparallel lines which extrapolate to a common point in the vicinity

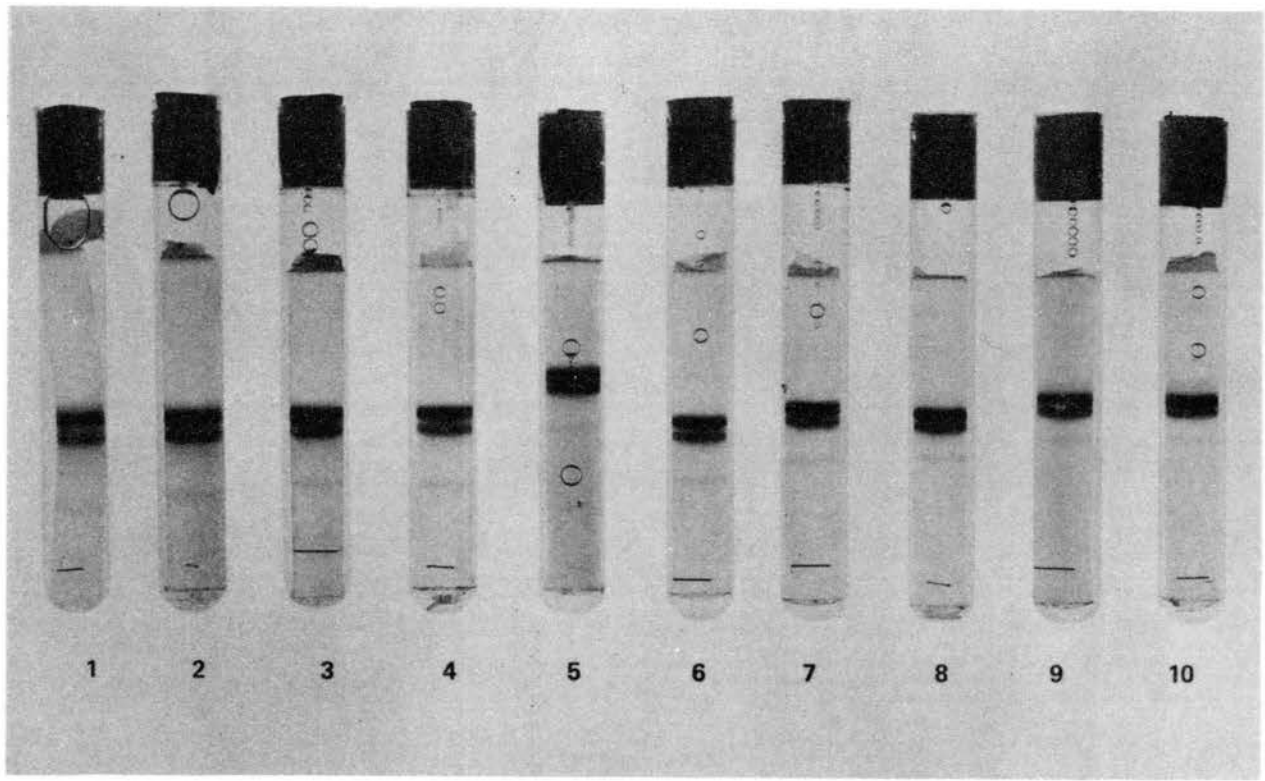

Figure 15. Disc Gel Electrophoresis of Pig α-Lactalbumin on Gels Varying From Three to Twelve Percent Acrylamide

The direction of migration was from the top of the figure with the dye band marked by a wire at the bottom of the gel. Gel percents are as follows: Tube 1, 3 percent; Tube 2, 4 percent; Tube 3, 5 percent; Tube 4, 6 percent, Tube 5, 7 percent; Tube 6, 8 percent; Tube 7, 9 percent; Tube 8, 10 percent; Tube 9, 11 percent; Tube 10, 12 percent.

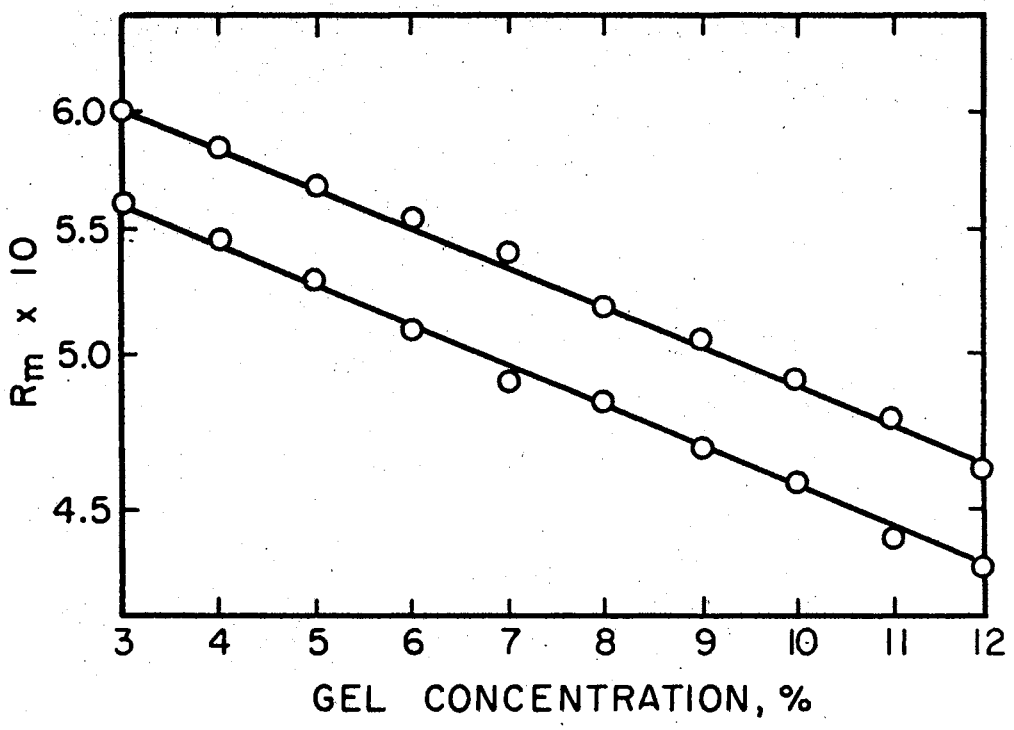

Figure 16. Relative Migration of Pig α-Lactalbumin in Varying Percent Gels on Disc Electrophoresis

The effect of different gel concentrations on the mobility of the two major bands of pig α-lactalbumin. The parallel lines indicate the two bands are charge isomers.

of zero percent gel concentration. Therefore, these faint bands, which are also present in the α-lactalbumins of goat, sheep, bovine, Caucasian, Indian and Negro, are size isomeric proteins. These size isomeric proteins are impurities which will be verified in a later section.

The different percent disc gel experiments reveal the fact that proteins cannot be considered pure on a standard 7 percent gel by showing a single, sharp protein band, when, in fact, the single band may be a composite of two or more different proteins. Therefore, different percent gels can be used as a final criteria for purity of a protein.

The next step in the identification of the two protein bands of the goat, sheep and pig was to assay each band in the disc gel for α-lactalbumin activity in the Lactose Synthetase reaction. Each protein sample was run in duplicate; one disc gel was photographed and the other disc gel was sliced into separate sections with a razorblade gel slicer. Each gel section was placed in a separate tube containing 0.1 ml of 20 mM glycine, 0.1 M KCl, pH 8.5. A stirring rod was used to macerate the gels. Another 0.1 ml of the above buffer was added, and the gel solution was frozen and thawed several times to help break up the gel matrix. The macerated gels were kept at room temperature for ten hours to enhance the diffusion of the protein into the buffer. The 50 μl aliquots of the gel solutions were assayed for α-lactalbumin activity in the Lactose Synthetase reaction (see Methods).

Figures 17, 18, and 19 show the α-lactalbumin activity in the Lactose Synthetase reaction of the two bands of pig, goat, and sheep. The activity peaks are qualitative since it appears that not all of the protein was recovered from the gel slices. Therefore, the specific activity of each α-lactalbumin band could not be determined. The

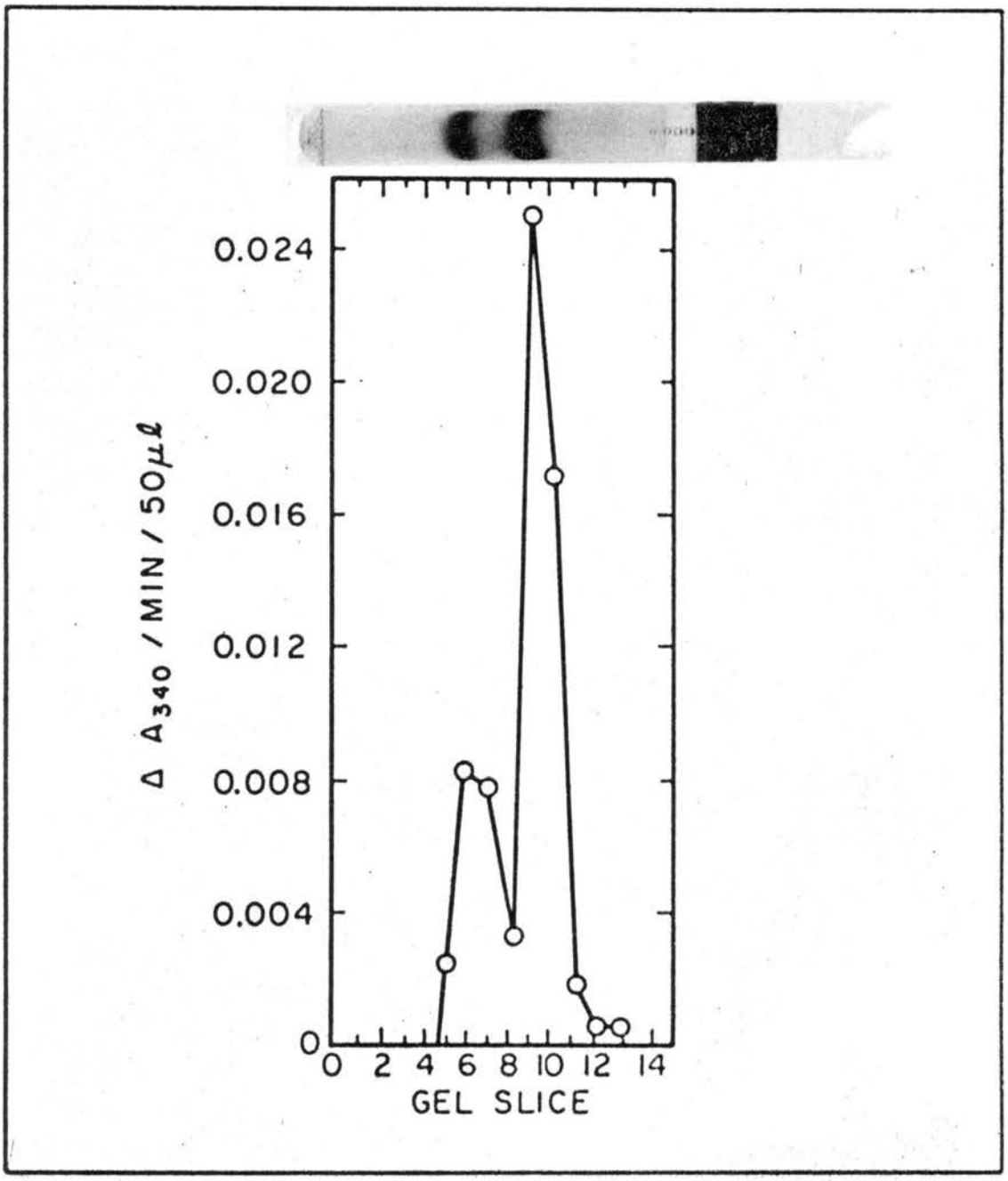

Figure 17. Lactose Synthetase Activity of Pig α-Lactalbumin in the Corresponding Disc Gel

Pig α-lactalbumin (75 μg) was run on a 7 percent disc gel at pH 9.5. The gel was sliced into 0.3 cm sections. Each section was macerated with 0.2 ml of 20 mM glycine, 0.1 M KCl, pH 8.5, and an aliquot assayed. At the top of the figure is a duplicate gel run simultaneously but stained with amido blue-black.

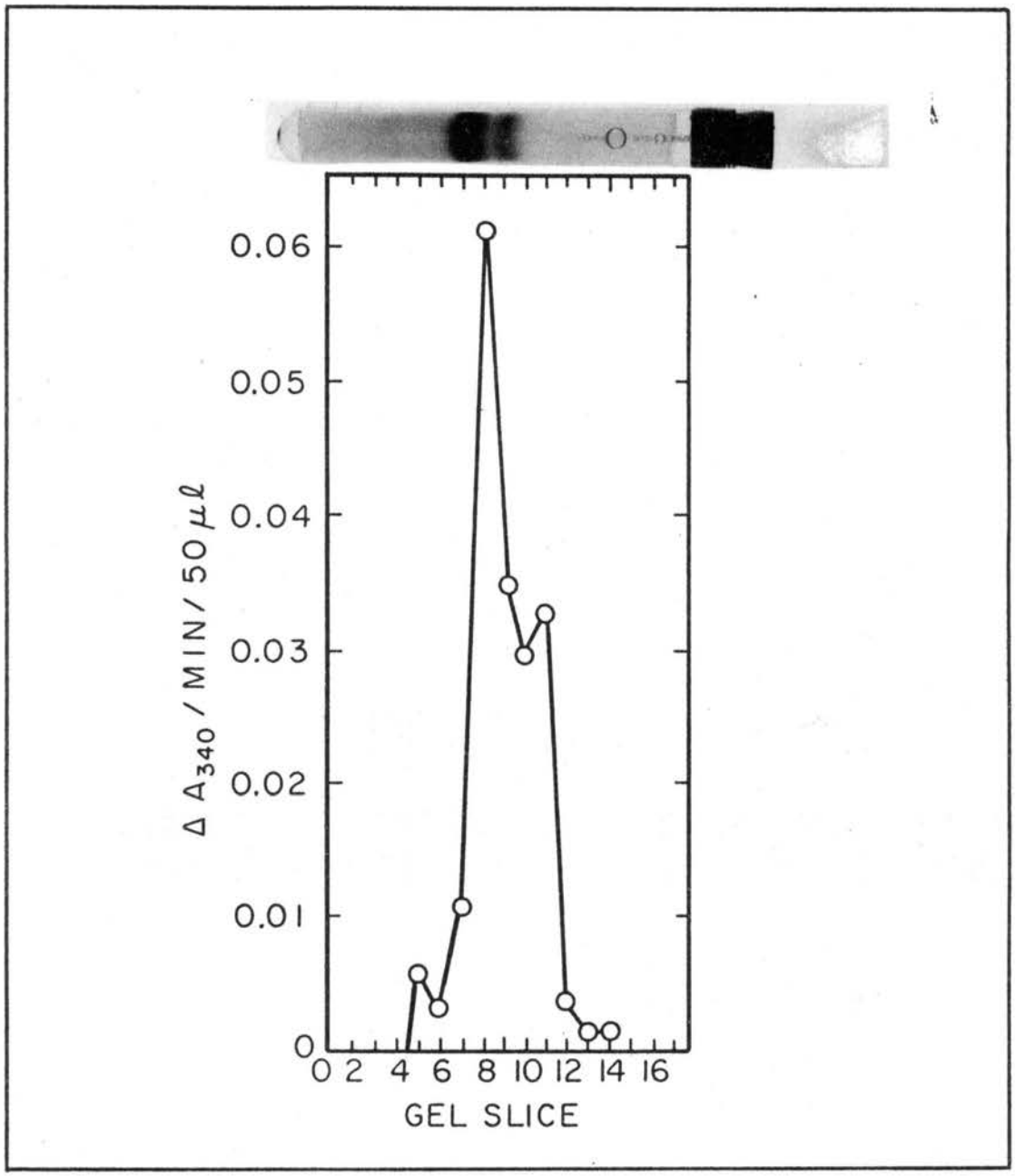

Figure 18. Lactose Synthetase Activity of Goat α-Lactalbumin in the Corresponding Disc Gel

Goat α-lactalbumin (75 μg) was run on a 7 percent disc gel at pH 9.5. The gel was sliced into 0.3 cm sections. Each section was macerated in 0.2 ml of 20 mM glycine, 0.1 M KCl, pH 8.5, incubated at 25° for 10 hours, and an aliquot was assayed. At the top of the figure is a duplicate gel run simultaneously but stained with amido blue-black.

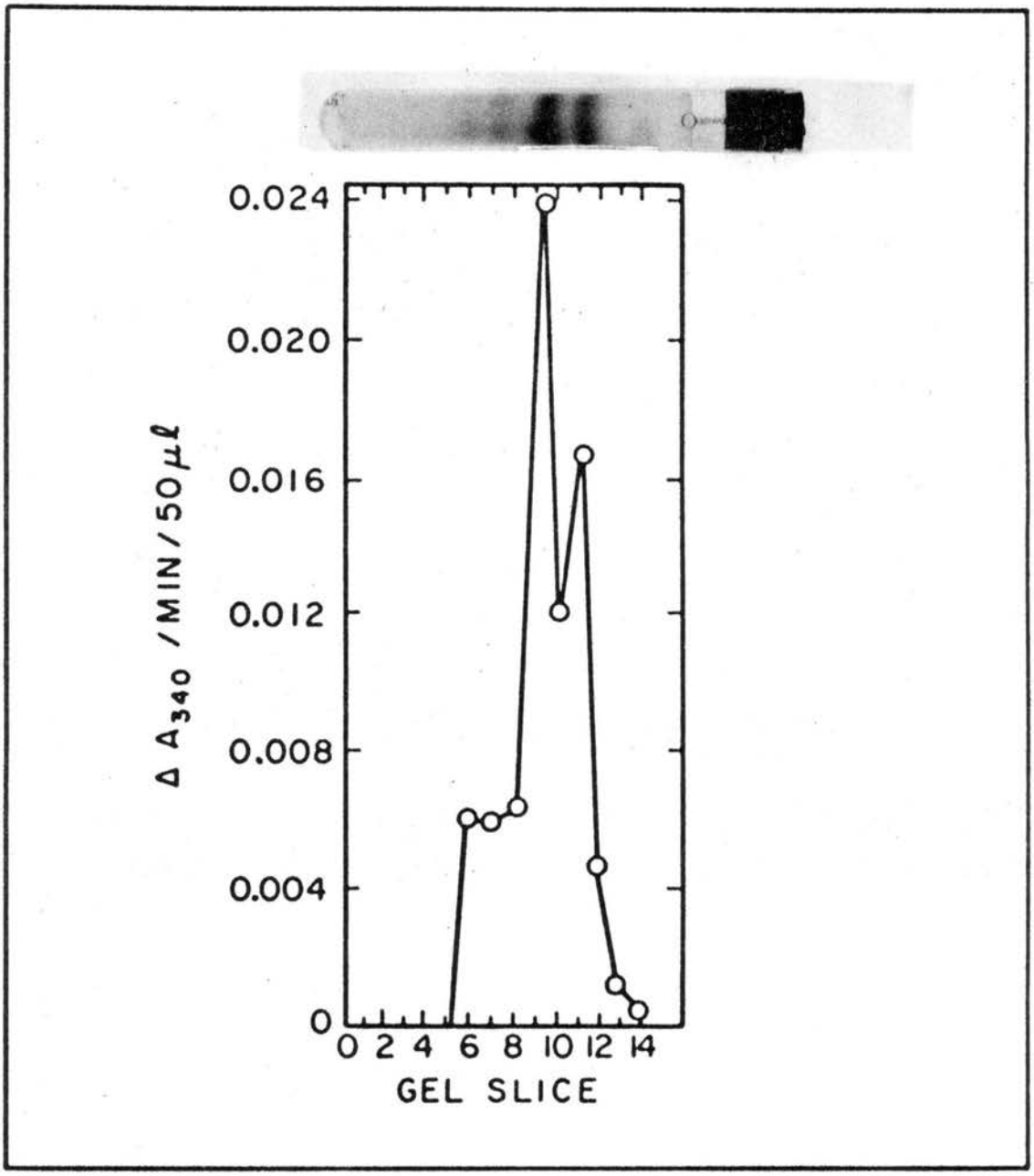

Figure 19. Lactose Synthetase Activity in Sheep α-Lactalbumin in the Corresponding Disc Gel

Sheep α-lactalbumin (60 μg) was run on a 7 percent disc gel at pH 9.5. The gel was sliced into 0.3 cm sections. Each section was macerated in 0.2 ml of 20 mM glycine, 0.1 M KCl, pH 8.5, and an aliquot was assayed. At the top of the figure is a duplicate gel run simultaneously but stained with amido blue-black.

activity peaks appear to correspond with the relative quantities of the protein in each protein band. For example, in Figure 17, 75 μg of pig α-lactalbumin was placed on the disc gel and it was estimated that the top and bottom bands contained 55 μg and 20 μg, respectively. The specific activities of the top and bottom bands were 0.42 and 0.16, respectively. The ratios of protein concentration and the specific activities of the two protein bands are similar. Similar data were also obtained for the goat and sheep α-lactalbumins.

It was suspected that one of the two protein peaks was a glyco-α-lactalbumin since Barman (79) had reported a minor component in a highly purified preparation of α-lactalbumin with the same amino acid composition as α-lactalbumin but containing 11-12 sugar residues per molecule of protein. The method of Zacharius, et al. (168) was used to check for the presence of glycoproteins in the protein bands on the standard disc gels. None of the α-lactalbumins from sheep, goat, pig (both proteins), pig (first protein peak), pig (second protein peak), human (Caucasian, Negro, Indian), bovine (purified by Brodbeck (1) procedure), and bovine (5 times crystallized) showed any glycoprotein bands on the disc gels. Therefore if any glyco-α-lactalbumin is present in these sources, it evidently has been removed during the purification procedure.

The next step in the identification of the two protein bands on the disc gel patterns of α-lactalbumin from goat, sheep, and pig was to attempt to separate the two protein bands by column chromatography. Pig α-lactalbumin was placed on a DE-32 column (1.6 x 23 cm) at 25^{o} and eluted with a linear glycine buffer gradient (1000 ml of 10 mM glycine and 1000 ml of 0.8 M glycine) at pH 9.5. These conditions were used since the two proteins were separated on the disc gels under similar

conditions. Patterns on the standard disc gels showed that the two proteins were separated partially on this column with the second peak being much larger than the first peak, but only 35 percent of the protein placed on the column was recovered in the two peaks.

A potassium phosphate buffer gradient (125 ml of 5 mM KH_2PO_4 and 125 ml of 200 mM KH_2PO_4) at pH 8.8 and pH 9.5 with the DE-32 columns (2 ml disposable syringes) was also used in an attempt to obtain separation with good recovery of the two protein bands of pig α-lactalbumin. A single peak with a 70 percent recovery was obtained with both gradients.

Preparative disc gel electrophoresis was also used (see Methods) in an attempt to separate the two protein bands of pig α-lactalbumin. A large and a small peak was obtained on the preparative disc gels, but the patterns on the disc gels indicated the two peaks were not completely separated.

Partial separation of the two protein bands of pig α-lactalbumin was achieved (Figure 20) on a DE-32 column at 4^{o} with a pH 9.5 glycine buffer. The standard disc gels' patterns in Figure 21 show that the first protein peak and the second protein peak off the DE-32 column (Figure 20) are the trailing and leading bands, respectively, of the typical two band pattern (see Figure 11) on the standard disc gels. The first protein peak (Figure 20) still contains a small amount of the second protein peak which is shown by tube 1 in Figure 21. Samples from the leading edge of protein peak one showed the same disc gel pattern (not photographed) of the disc gel 1 pattern shown in Figure 21. The second peak (Figure 20) has a very small amount of the first peak present as shown by tube 6 in Figure 21, but the trailing edge of the second

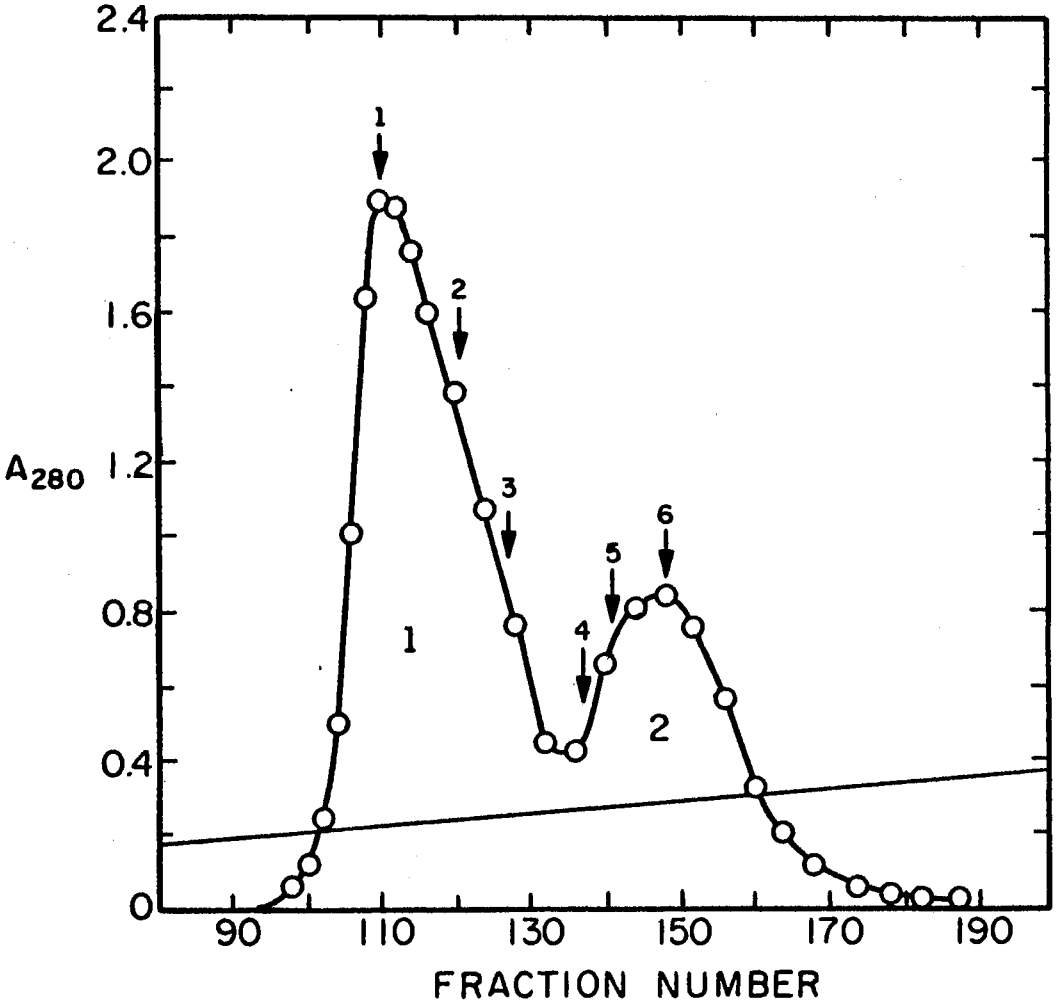

Figure 20. Elution Profile of Pig α-Lactalbumin From DE-32 Column Chromatography at pH 9.5

The DE-32 column (2 ml of DE-32 in a 5 ml disposable pipette) was equilibrated with 5 mM glycine, pH 9.5 and washed with 40 ml of the same buffer. Pig α-lactalbumin (25 mg) was dissolved in the same buffer and was eluted with a linear gradient (—) from 5 mM (80 ml) to 0.8 M (80 ml) glycine, pH 9.5. 0.7 ml fractions were collected. The numbers above the peaks correspond to the disc gels in Figure 21.

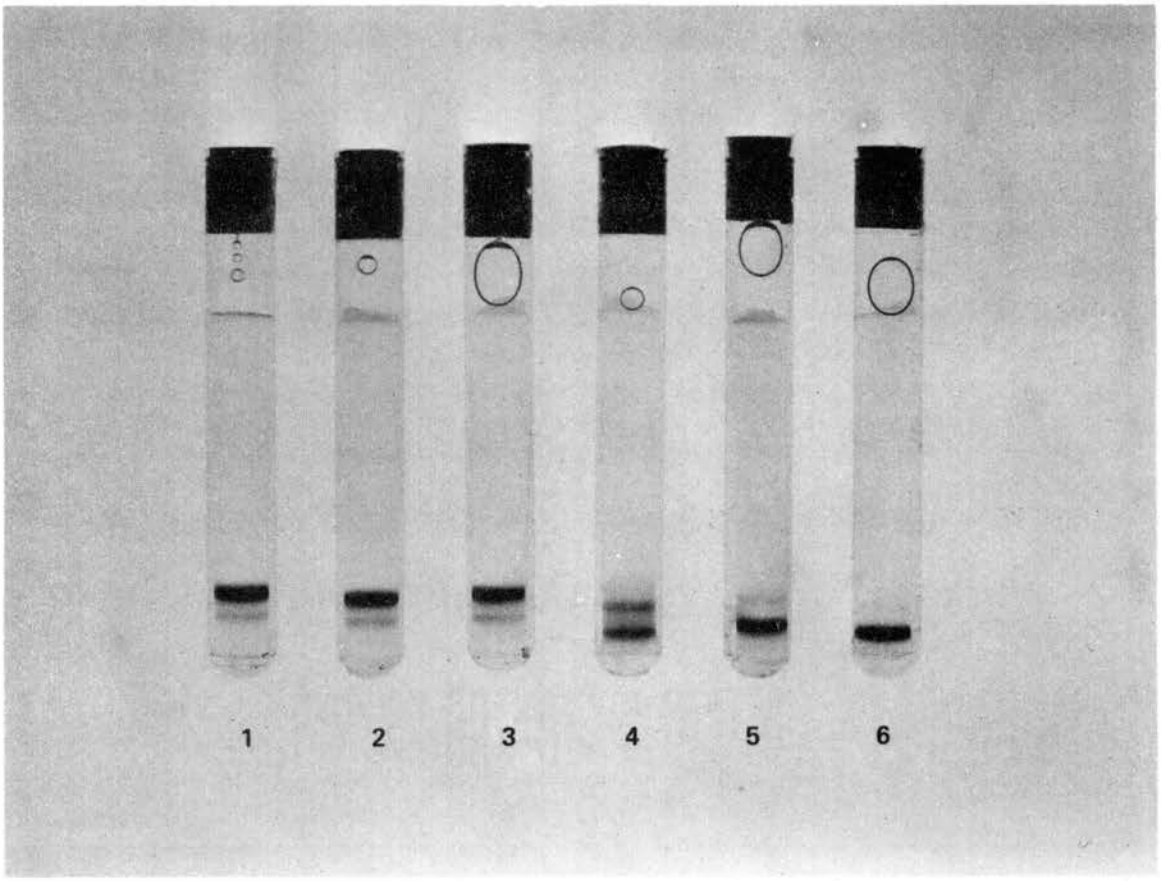

Figure 21. Disc Gel Electrophoresis of Pig α-Lactalbumin From a DE-32 Column

The disc gel numbers correspond with the numbers above the elution pattern of the column described in Figure 20. Aliquots at these numbers were taken from the fraction and electrophoresed on the above 7 percent disc gels.

peak contained only the second band on the disc gels which was not included in Figure 21.

Several of the DE-32 columns (Figure 20) were utilized in order to isolate a pure protein from the first and second protein peaks. By pooling the trailing half of peak two, a pure protein was obtained which demonstrated the protein pattern of the disc gel in Figure 21, but without the trailing faint protein band. Pooled fractions of peak one demonstrated the protein pattern shown in the disc gels 1, 2, and 3 (Figure 21), which showed that peak one contained a small amount of peak two.

The pooled fractions of peak one from three DE-32 columns were rechromatographed on a second DE-32 column which is shown in Figure 22. The numbers indicate the fractions which were electrophoresed on the standard disc gels. Fractions 1, 2, 3, 4, 5, and 6 (Figure 22) showed a pattern similar to disc gels 1, 2, and 3 in Figure 21. Fractions at 7, 8 and 9 (Figure 22) showed patterns similar to disc gels 4, 5, and 6, respectively, in Figure 21. Fraction 10 (Figure 22) showed a single band similar to disc gel 6 in Figure 21, but without the trailing faint band. Peak one was pooled and chromatographed on a third DE-32 column which also had an elution profile similar to Figure 22. The standard disc gels indicated that protein peak one still contained a small amount of protein peak two. Therefore, it appeared that the protein of peak two could not be completely removed from the protein of peak one under these column conditions.

The pooled fractions of protein peak one of pig α-lactalbumin off the third DE-32 column were rechromatographed with conditions identical to the ones used in Figure 22 except that the buffer gradient was at

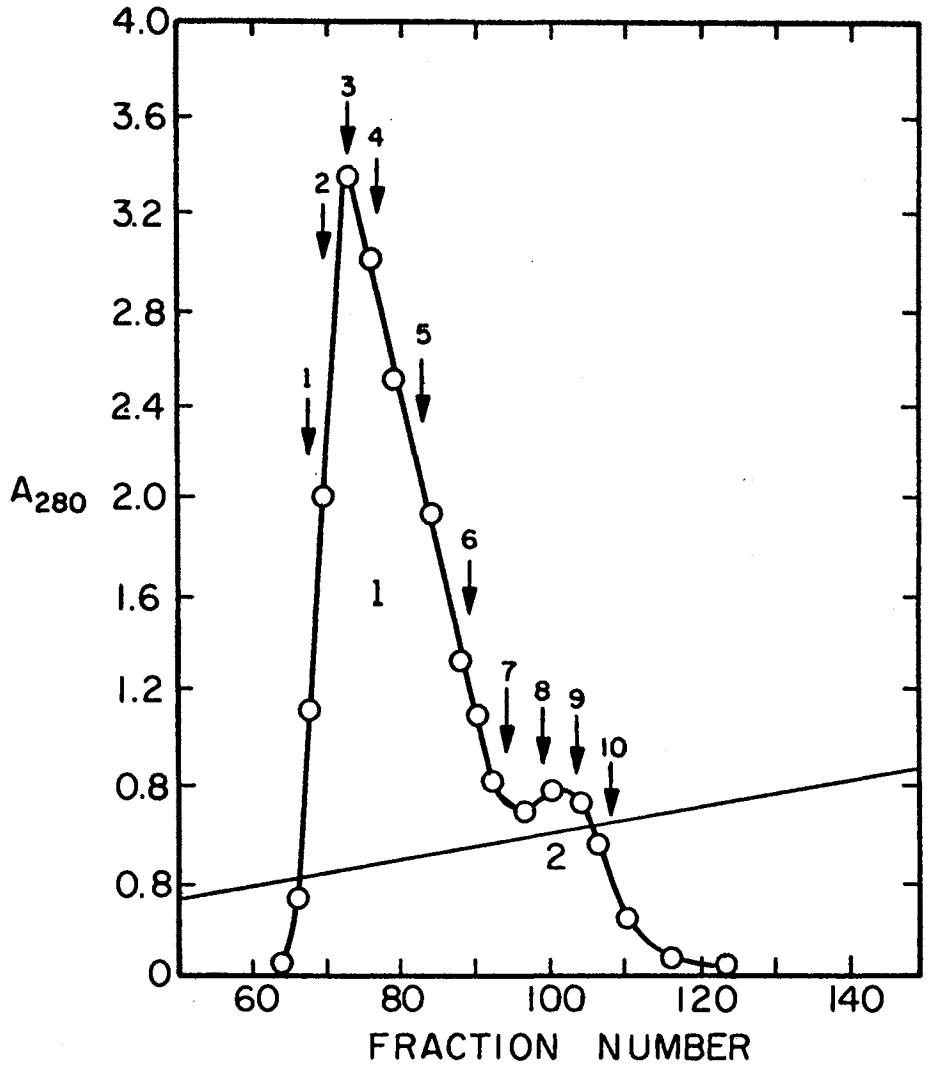

Figure 22. Rechromatography of Peak One of Pig α-Lactalbumin on a Second DE-32 Column at pH 9.5

The second DE-32 column (2 ml of DE-32 in a 5 ml disposable pipette) was equilibrated with 5 mM glycine, pH 9.5 and washed with 40 ml of the same buffer. Pooled fractions of peak one from Figure 20 and two similar columns were dialyzed against water and the starting buffer. The pooled first peaks were eluted with a linear gradient (—) from 50 mM (80 ml) to 0.8 M (80 ml) glycine, pH 9.5. 0.7 ml fractions were collected. The numbers above the peaks are the fractions which were separated on the standard disc gels (see text).

pH 10.0. A single peak was obtained from this column and the 7 percent disc gel, which was similar to disc gel 1 in Figure 21, showed that a very minor amount of peak two was still present in peak one. No further attempts were made to purify peak one, since the minor amount of peak two would not appear on a peptide map.

The α-lactalbumins of sheep and goat were chromatographed on DE-32 columns identical to the column in Figure 20. A single peak was obtained for both α-lactalbumins from this DE-32 column. Due to lack of material, no further attempts were made to separate the two protein bands of goat and sheep α-lactalbumins.

Pig α-lactalbumin, before it was chromatographed on the pH 9.5, DE-32 columns, showed two bands in the immunodiffusion experiment (Figure 10). This pig α-lactalbumin and peak one and two of pig α-lactalbumin off the DE-32 columns were separated in a second diffusion experiment which is also shown in Figure 10. The precipitin bands indicate that there is no immunological difference between peak one and two off the DE-32 columns and the pig α-lactalbumin which contains both protein bands in the standard disc gels. The minor precipitin band was evidently an impurity in the pig α-lactalbumin which was injected into the rabbit to elicit antisera production. This minor impurity was removed on the DE-32 columns during the separation of the two pig α-lactalbumin proteins.

The α-lactalbumins of the humans (Negro, Caucasian, Indian), goat and sheep were also checked for immunological activity with the rabbit antisera to pig α-lactalbumin. None of these α-lactalbumins reacted with the antisera thus indicating the non-ruminant α-lactalbumins do not have identical immunological sites with pig α-lactalbumin.

Peptide Mapping

Fingerprinting techniques have become increasingly important in the rapid characterization of proteins. Most studies have used separation on paper which requires at least 1 mg of digest at the origin. In contrast, thin layer methods on cellulose use only 0.05-0.5 mg per plate at the origin. This procedure has, for peptide separation, advantages of speed, increased sensitivity, and a high degree of resolution.

The α-lactalbumin from goat, sheep, pig human (Caucasian, Negro, Indian), bovine, guinea pig, and buffalo were reduced, aminoethylated, and hydrolyzed with trypsin. An aliquot of the digest solution, 2.5-10 µl (0.05-0.2 mg), was spotted at the origin on the cellulose thin layer plates (see Methods). All the protein digests were electrophoresed in the first dimension for two hours at 300 volts. The electrophoresis was always conducted at right angles to the direction in which the cellulose layer was spread. Next, the protein digests were chromatographed in the second dimension for 10.5 hours, which was the amount of time required for the solvent front to migrate the full distance of the thin layer plate.

The peptide maps showing the greatest similarity were grouped together in Figures 23, 24, and 25. The peptide spots were numbered in order that a general comparison could be made between the various α-lactalbumins. The very faint peptide spots appear as broken circles.

Bovine α-lactalbumin was used as a standard protein since the amino acid sequence is known for this protein. Trypsin hydrolyzes the peptide bonds at the arginine, lysine, and aminoethylated cysteine residues. The peptide bonds at the aminoethylated cysteine residues are hydrolyzed by trypsin since the side chain resembles the side chain of

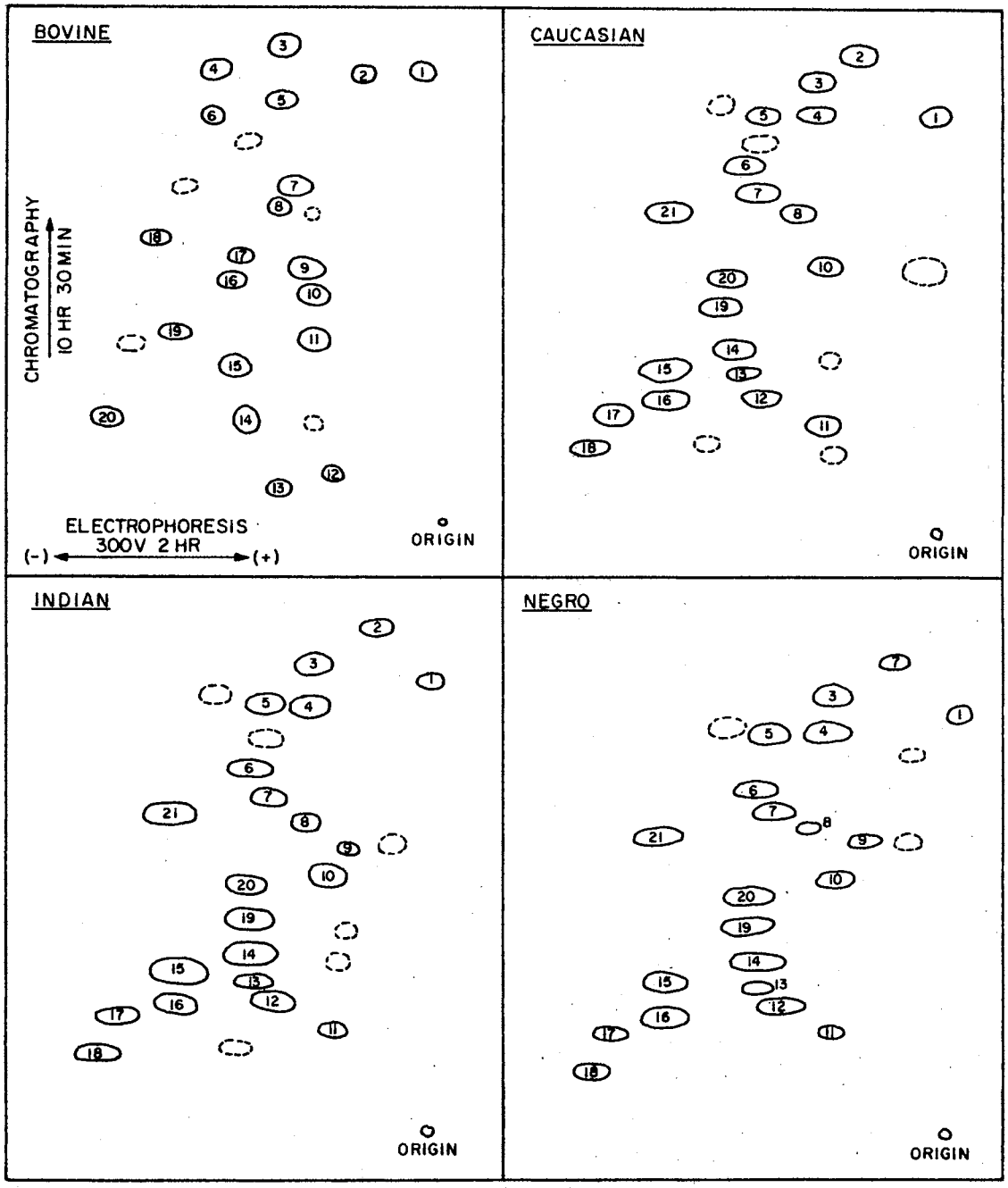

Figure 23. Peptide Maps of Tryptic Digests of the α-Lactalbumins From Bovine and human (Caucasian, Indian, Negro)

The peptides were separated in the first dimension by thin layer electrophoresis (pH 5.5, 2 hours, 300 V, 4°) and in the second dimension by ascending chromatography in n-butanol-pyridine-glacial acetic acid-water, 150:100:30:120 for 10.5 hours at 25°.

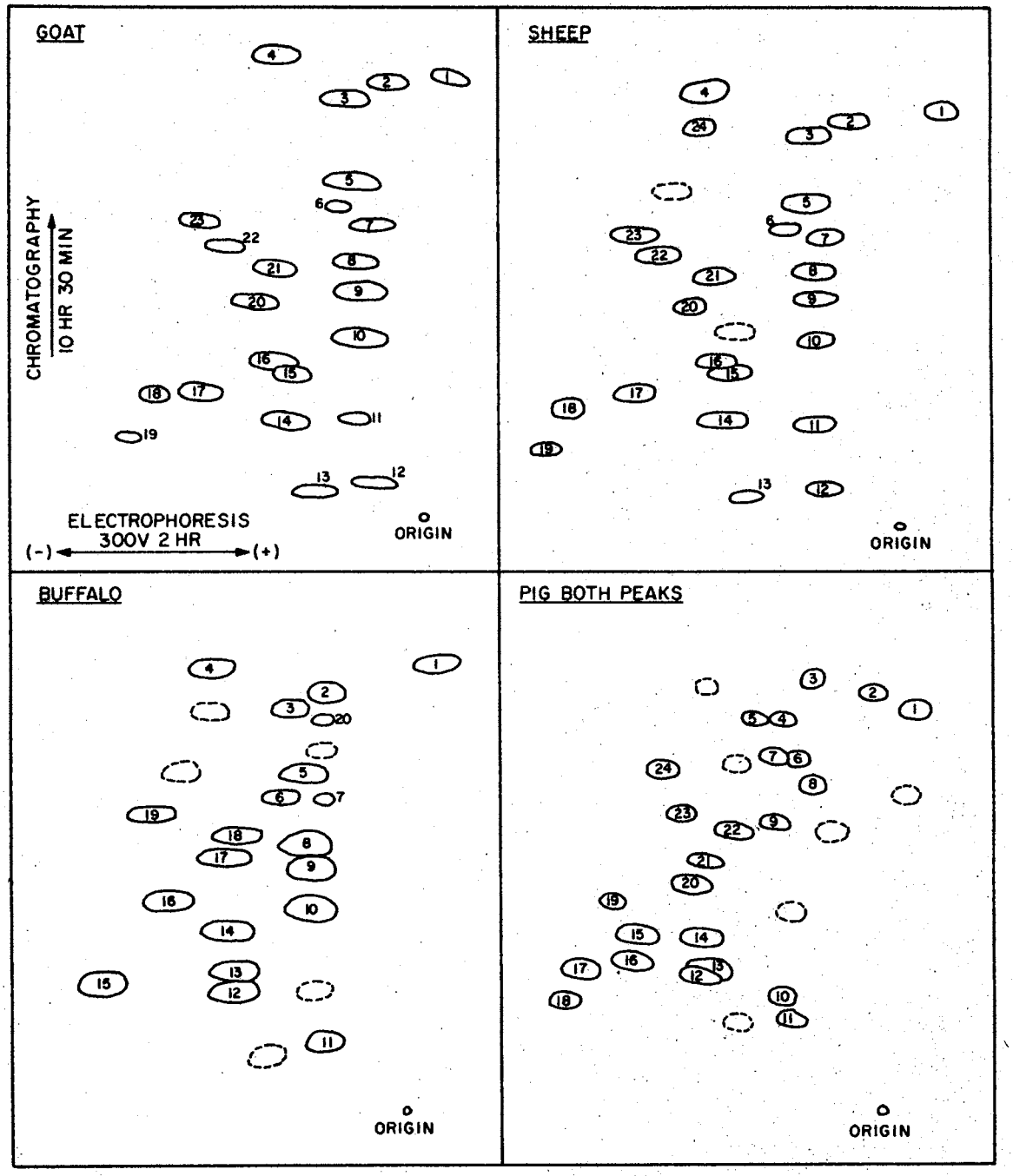

Figure 24. Peptide Maps of Tryptic Digests of the α-Lactalbumins From Goat, Sheep, Buffalo, and Pig (Both Proteins)

The peptides were separated in the first dimension by thin layer electrophoresis (pH 5.5, 2 hours, 300 V, 4°) and in the second dimension by ascending chromatography in n-butanol-pyridine-glacial acetic acid-water, 150:100:30:120 for 10.5 hours at 25°.

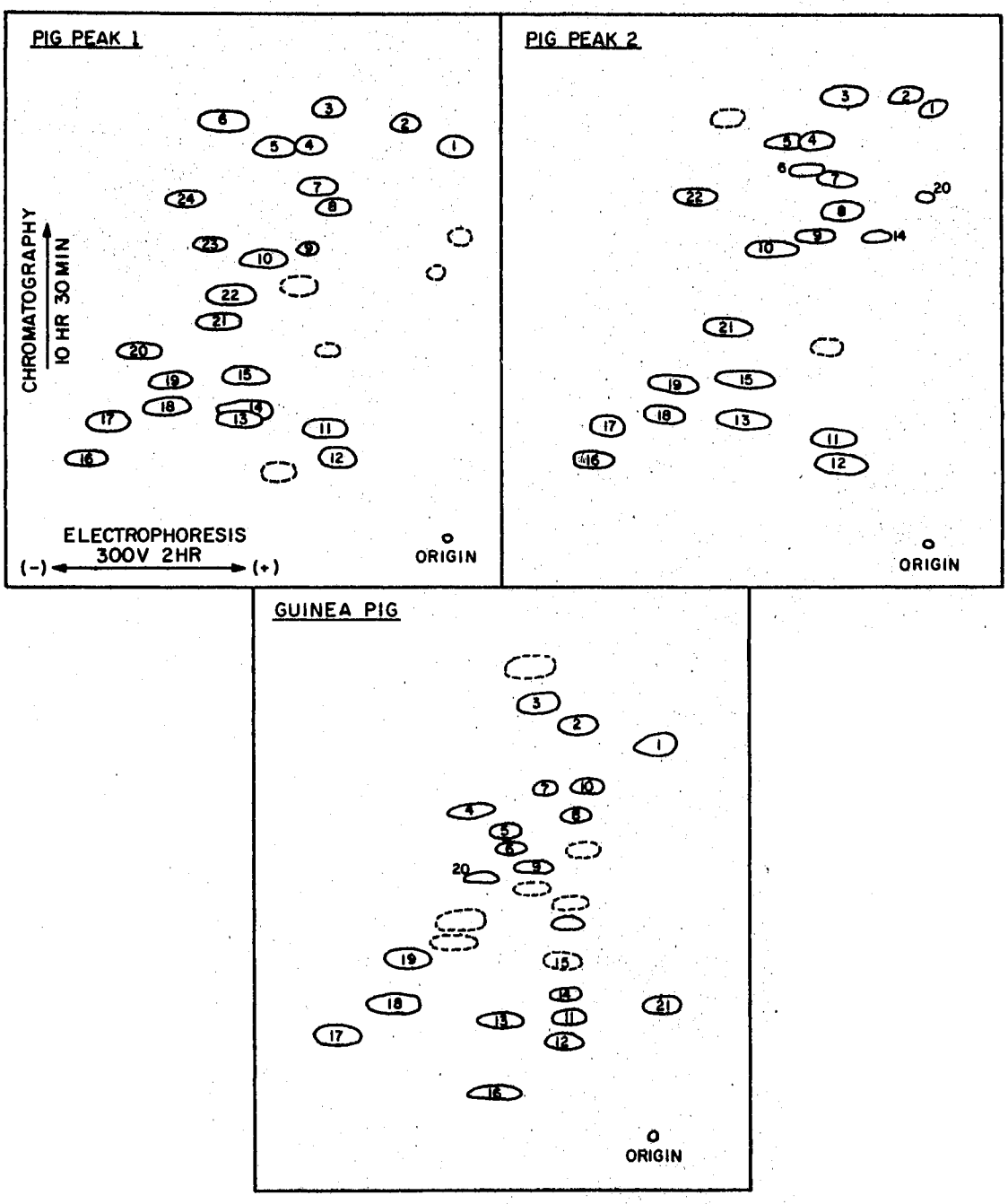

Figure 25. Peptide Maps of Tryptic Digests of the α-Lactalbumins From Guinea Pig, Pig (First Peak), and Pig (Second Peak)

The peptides were separated in the first dimension by thin layer electrophoresis (pH 5.5, 2 hours, 300 V, 4°) and in the second dimension by ascending chromatography in n-butanol-pyridine-glacial acetic acid-water, 150:100:30:120 for 10.5 hours at 25°.

lysine. Therefore, 22 peptides are produced by the trypsin cleavage of bovine α-lactalbumin. There are two identical peptides released; therefore, at least 21 peptides should appear on the peptide maps. Bovine α-lactalbumin in Figure 23 had 20 major peptide spots with four faint peptide spots. A spot also appeared at the origin which probably was the core protein consisting of 30 amino acids. Therefore, the 21 anticipated peptides were detected in the peptide map. The human α-lactalbumins (Caucasian, Indian, and Negro) (Figure 23) had almost identical peptide maps and showed a similarity to the bovine α-lactalbumin peptide map. All the humans had 21 major peptides plus a peptide at the origin, but the Caucasian α-lactalbumin lacked peptide 9. The faint peptide spots also appeared almost identical in number and position on the peptide maps. Amino acid analysis indicates that the human (Caucasian) α-lactalbumin should have 20 peptides produced by trypsin hydrolysis. Hence, the peptide map is in good agreement with the amino acid analysis. These peptide maps indicate that the human α-lactalbumins have very similar amino acid sequences.

Figure 24 shows the α-lactalbumin peptide maps of goat, sheep, pig (both proteins) and buffalo. These proteins have very similar peptide maps. All maps have core proteins which remained at the origin. The α-lactalbumins of goat and sheep have almost identical peptide maps. The only difference is that the sheep protein has one more peptide spot (24) and two faint peptide spots which might have appeared in the goat peptide map if more digest solution would have been spotted on the thin layer plate.

The buffalo α-lactalbumin peptide map has 19 major peptides, a peptide at the origin and five very minor spots. Seventeen of the

nineteen peptides have the same location as the peptides of sheep α-lactalbumin. Also three of the five faint peptide spots are in the same location as the sheep peptide map. Therefore, these two peptide maps indicate the sheep and buffalo α-lactalbumins have similar amino acid sequences.

The pig α-lactalbumin (both proteins) is quite different from the other three α-lactalbumins in Figure 24. The peptide map has 23 peptides, a core protein spot at the origin, and six faint peptide spots. Peptide 8, 9, 11, 14, 16, 17, 18, 20, and 21 are the only peptides which correspond with nine of the sheep peptides. The pig α-lactalbumin peptide map indicates that it has the most dissimilar amino acid sequence of the four α-lactalbumins in Figure 24.

The peptide maps of pig α-lactalbumin peak one and peak two off the DE-32 columns show that the two proteins are different in their amino acid sequences (Figure 25). Peptides 20, 22, and 23 of peak one protein definitely do not appear in the peptide map of peak two protein. Peptide 6 of the peak two protein peptide map definitely is not present in the peak one peptide map. Peak one peptide map has two faint peptide spots which do not match any peptides in the peak two peptide map. Peptides 20 and 14 in the peak two peptide map could possibly be similar to the two faint peptide spots in the same area in the peptide map of peak one. Therefore, these two peptide maps show that the two proteins definitely have four peptides which are dissimilar. Hence, the difference of charge of these two proteins in electrophoresis experiments is due to these four dissimilar peptides. It is also noted that the peptides in the peptide maps of peak one and two are present in the peptide map of pig α-lactalbumin which contains both proteins (Figure 24).

The peptide map of guinea pig α-lactalbumin (Figure 25) does not show close similarity to any of the peptide maps in Figures 23, 24, and 25. There are 20 major peptides, a core protein at the origin, and seven minor peptide spots. Fifteen of the peptide spots do appear identical to the pig α-lactalbumin peptide map with both proteins.

These peptide maps have been an extremely useful tool in comparing the similarities and differences in the structures of the various α-lactalbumins, but these maps can only be used as a qualitative comparison of the proteins. Studies of many other proteins with peptide maps have shown that up to 60 percent of the digest remains at the origin and may even escape detection by the ninhydrin reagent. Consequently, the peptides may only represent 40 percent of the protein. Also, unspecific peptides present in relatively low yield (10 to 20%) may give stronger colors with the ninhydrin reagent than some larger peptides, the genuine products of trypsin digest, which are present in 80 to 90 percent yields. It becomes clear, therefore, that only general qualitative conclusions should be drawn from the visual inspection of peptide maps developed with the ninhydrin reagent.

CHAPTER IV

DISCUSSION

The name "lactalbumin" has been applied to various impure crystalline protein preparations derived from milk (6). Several components in bovine whey were found by Pederson (7) and he called the slowest moving peak in the ultracentrifuge the "α-peak". Svedberg (9) was the first to use the term α-lactalbumin for the slow moving component. Gordon, et al. (62,94,95) modified the procedures of Sörensen and Sörensen (91) for the isolation of a "crystalline insoluble substance" from bovine whey and named the crystalline preparation α-lactalbumin.

Several procedures for the isolation of bovine α-lactalbumin have been reported. These procedures have been used to isolate α-lactalbumins from various other sources. The general procedure of Brodbeck, et al. (2) and a few modifications were used to isolate the various α-lactalbumins which were studied in this dissertation, with the exception of the α-lactalbumins from buffalo, guinea pig, and human (Japanese) which were donated.

Maeno and Kiyosawa (67) reviewed and commented on the differences of the solubility of human (Japanese) and bovine casein. It was found that at the usual pH 4.6 of the casein precipitation step, none of the casein in the human (Negro) milk would precipitate and about one-half of the casein precipitated in the pig and sheep milk. Since only a small amount of northern fur seal milk (25 ml) was available, the casein

was removed by centrifugation. Removal of the casein by centrifugation avoids the low pH precipitation step, but it is very time consuming if large volumes of milk are required.

The advantage of the present isolation procedure used for α-lactalbumin is that it avoided any low pH steps. The methods of Zweig and Block (93) and Aschaffenburg and Drewry (71) used a precipitation step at pH 1.3 and 2.0, respectively. No evidence exists that these low pH steps adversely affect the α-lactalbumin, but such a low pH step should be avoided if an alternate purification procedure is available. Currently, C. Merriman in Dr. K. E. Ebner's group is conducting a thorough study of the stability of bovine α-lactalbumin over the pH range from 2 to 12.

Starch gel electrophoresis and ultracentrifugation studies have in the past been the criteria for the purity of a protein. As each α-lactalbumin was isolated the protein was subjected to starch gel electrophoresis at pH 3.3 and 8.6. A single protein band at these pH's was used as an indication that the α-lactalbumins were free of contaminating proteins. The immunological experiment shown in Figure 10 indicated there was one or more proteins present in the purified pig α-lactalbumin which had not been detected by starch gel electrophoresis. Disc gel electrophoresis was a new tool at this time and was used for the detection of more than one protein in a purified protein preparation.

The disc gels showed that the various purified α-lactalbumins were not pure except for guinea pig α-lactalbumin which was prepared by the method of Brew and Campbell (66) according to the procedure of Aschaffenburg and Drewry (71). A very minor band ahead of the main band or bands was always present in the purified α-lactalbumins. In

previous purifications, this leading minor band has been postulated to be serum albumin or to be a reversible interaction with buffer or to be an association phenomena (15). Recently, Barman (79) has reported a minor component with the same amino acid composition as α-lactalbumin but 11-12 sugar residues per molecule of protein were observed. Therefore, the leading band on the disc gel was postulated to be the glyco-α-lactalbumin. The gel study with varying percent acrylamide showed that the minor band was not the glyco-α-lactalbumin but that it was a larger molecular weight protein which had a greater charge than the various α-lactalbumins.

The disc gel patterns also revealed that the α-lactalbumins of goat, sheep, and pig contained two major proteins. The method of Hedrick and Smith (145), which measures protein migration in different percent gels, identified the two major proteins as charge isomers with the same molecular weight.

The varying percent acrylamide gel experiments were a powerful tool for establishing the purity of a protein. The starch gels showed only one protein band for the various purified α-lactalbumins. The standard disc gels showed only the minor leading band and the major one or two bands of the various α-lactalbumins. When these proteins were separated on disc gels varying from 3-12 percent acrylamide, several very minor trailing bands were observed, especially in the human α-lactalbumins. In the lower percent gels, 3-6 percent, a very faint diffuse band appeared ahead of the major band, and in the standard 7 percent gel it was not detectable. The higher percentage gels (8-12 percent) contained very faint protein bands trailing the main band. Hence, a minor contaminant was present, particularly in the human

α-lactalbumins. These minor contaminants were not apparent in the α-lactalbumin bands in the 7 percent gel since they had similar relative migrations. This example demonstrates the fact that a protein may not be pure even if it appears as a single protein band in the 7 percent gels. This technique appears as a better criteria for protein purity than ultracentrifuge patterns.

An important aspect in the study of the physical and chemical properties of the various α-lactalbumins is how the various α-lactalbumins interact with the A protein in the lactose synthetase reaction. Tanahashi, et al. (34) have studied the relative enzymatic and immunological activity of the various α-lactalbumins of lactose synthetase. They have found that the α-lactalbumin from the bovine, buffalo, and human (Japanese) have identical specific activities which was set at an arbitrary number of 100. In these assays the α-lactalbumins of sheep, goat, pig, and guinea pig have specific activities of 93, 88, 84, and 75, respectively. The α-lactalbumins from the non-ruminants did not react with antisera to bovine α-lactalbumin while the α-lactalbumins from the ruminants reacted with this antisera. These observations indicate that the various α-lactalbumins have different physical and chemical properties. Further characterization of these proteins revealed why these proteins may have differeing enzymatic and immunological activities.

Bovine α-lactalbumin is a well characterized milk protein due to two reasons: (1) it occurs in large quantities in whey (0.7 to 1.5 g/l) (169) and is easily crystallized and (2) the protein's physiological function was identified originally by Brodbeck, et al. (2) as a protein component of the enzyme lactose synthetase. Recent work has shown that

it is a modifier (4) of a general galactosyl transferase.

All of the chemical and physical properties studied have indicated that the various α-lactalbumins have similar structures. The spectrophotometric properties of the various α-lactalbumins indicate a large content of aromatic amino acids in the proteins. Wetlaufer (51) and Zittle (63) have reported the absorptivity of bovine α-lactalbumin as an $E^{1\%}_{280}$ of 20.9 and 20.1, respectively. These values indicate that bovine α-lactalbumin has a high content of aromatic amino acids. The α-lactalbumins of pig, sheep, and goat have an $E^{1\%}_{280}$ in the region of 16-18 while the human α-lactalbumins have an $E^{1\%}_{280}$ in the region of 14-15 (Table II). These values are lower than bovine α-lactalbumin but they still indicate a high degree of exposed aromaticity in their protein structures.

The A_{280}:A_{290} ratios of the various α-lactalbumins is a further indication of the relatedness of the proteins in their aromatic amino acid content and exposure of tryptophanyl residues. The A_{280}:A_{290} ratio of 1.31 for bovine α-lactalbumin was in good agreement with Wetlaufer's value of 1.32. The α-lactalbumins of sheep, pig, and goat have almost the identical A_{280}:A_{290} ratios as the bovine α-lactalbumin. The human α-lactalbumin has ratios of 1.45 except for the Japanese α-lactalbumin which has a ratio of 1.53. The total aromatic amino acid content of the following α-lactalbumins was: bovine, 12; goat, 11; sheep, 10; and Caucasian, 9. These values indicate there is not any good correlation between the aromatic content and A_{280}:A_{290} ratio. Thus the aromatic amino acids are evidently buried in the protein structure which changes the spectrophotometric properties of the aromatic amino acids.

The tyrosines which are partially responsible for the previously

mentioned properties are involved in the activity of bovine α-lactalbumin in lactose synthetase. Denton (46) has modified chemically the tyrosines of bovine α-lactalbumin by nitration and iodination, and has found that the tyrosines are critical for the activity of α-lactalbumin in the lactose synthetase reaction. It was not determined which of the four tyrosines were chemically modified. The α-lactalbumins of the goat, sheep, pig, and human (Caucasian) contain three tyrosines. This difference in tyrosine content may alter the specific activity of these α-lactalbumins in the lactose synthetase assay.

The ultraviolet spectra (Figures 7 and 8) of the various α-lactalbumins indicate further similarity of the proteins. All the proteins have a shoulder at 290 nm which is characteristic of exposed tryptophanyl residues in a protein. The 290 nm shoulders of the human α-lactalbumins are not as predominant as the 290 nm shoulder of the other α-lactalbumins. This may indicate that the tryptophanyl residues of the human proteins are not as exposed as the tryptophanyl residues of the other α-lactalbumins.

Kronman, et al. (55) through solvent perturbation studies have suggested that two of the four tryptophanl residues of bovine α-lactalbumin are buried and two are exposed at 25°. At the time of these experiments it was thought that α-lactalbumin had five tryptophan residues but this has been changed to four residues by Brew, et al. (11) by their study on the amino acid sequence. Browne, et al. (13) have constructed a three dimensional model of bovine α-lactalbumin based on the coordinants of hen's egg-white lysozyme and have suggested that tryptophans 108 and 123 are exposed while tryptophans 28 and 63 are

buried in the protein structure. Hence only two exposed tryptophan residues are probably responsible for the 290 nm shoulder in the various α-lactalbumins if all the proteins possess similar conformations.

The electrophoretic mobilities of the various α-lactalbumins on starch and disc gel electrophoresis indicated the presence of excessive acidic groups. The α-lactalbumins of bovine, goat, sheep, pig, and human (Caucasian) contain the following amounts of excess acidic groups: 18, 17, 16, 18, and 15, respectively. The dicarboxylic amino acids and their amides were not distinguished in the amino acid analysis except for bovine α-lactalbumin (Table VIII) and hence the degree of amidation was not known for the other α-lactalbumins.

The various α-lactalbumins are anionic at pH 7 which agrees with the observation that some of the α-lactalbumins have been crystallized as the ammonium salt. The various α-lactalbumins will not dissolve in water when they are completely deionized, but will dissolve as ammonium salts.

The electrophoretic mobilities on the starch gels at pH 3.3 show that the human α-lactalbumins have identical charges and higher mobilities than the α-lactalbumins of bovine, goat, and sheep which have mobilities similar to each other. Pig α-lactalbumin has an electrophoretic mobility between the human α-lactalbumins and the α-lactalbumins of bovine, goat, and sheep. Electrophoretic mobilities on the starch gels at pH 8.6 show that the α-lactalbumins of bovine and humans are identical, while the α-lactalbumins of goat and sheep have noticeably smaller mobilities. Pig α-lactalbumin has a higher mobility than any of the other α-lactalbumins at this pH. Similarity in their electrophoretic mobilities are further evidence that the human

α-lactalbumins haver very similar protein structures. Pig α-lactalbumin appears to be the most dissimilar protein of the other α-lactalbumins. The electrophoretic mobilities do not correlate with the number of excess acidic groups which suggests that there is a varying degree of amidation of the aspartic and glutamic acid residues.

Several molecular weight determinations have been made on bovine α-lactalbumin. Sedimentation velocity studies have yielded molecular weights of 15,100 (62) and 14,900 (51). A value of 15,500 (65) and 16,500 (65) was determined from the amino acid composition and by the light scattering method, respectively. Osmometry measurements gave an apparent molecular weight of 16,300 (51). All of these values have been superceded by Brew's, et al. (11) value of 14,437 which was determined from the complete amino acid sequence of bovine α-lactalbumin.

The various α-lactalbumins had essentially identical elution volumes in the gel-filtration experiments. This indicates these proteins have similar and possibly almost identical molecular weights (14,500 ± 500) since molecular weight determination by gel-filtration provides a reasonably accurate estimation of the molecular weight of globular proteins (164). Furthermore, the molecular weight of the various α-lactalbumins determined from the amino acid composition studies shows that they are similar.

The C-terminal amino acids of the α-lactalbumins are leucine except for pig (methionine) and guinea pig (glutamine). Again the pig α-lactalbumin has another structural property which makes it different from other α-lactalbumins.

Brew, et al. (11) have determined the amino acid composition of bovine α-lactalbumin and its complete amino acid sequence. High

contents of asparagine (12), lysine (12) and leucine (13) and tryptophan (4) and a low content of arginine (1), methionine (1) and proline (2) are reported. Eighteen acidic groups were in excess over the basic groups. The amino acid compositions of goat, sheep, pig, and human (Caucasian) α-lactalbumin were within one to three residues of bovine α-lactalbumin, with few exceptions. The α-lactalbumins of pig and human (Caucasian) contained two and one residues of valine, respectively, while bovine α-lactalbumin contained six valine residues. The other significant difference was that human (Caucasian) α-lactalbumin contained fourteen aspartic residues which also represent asparagine while bovine α-lactalbumin contained 21 aspartic and asparagine residues. There is also a significant difference in the tryptophan residues. The α-lactalbumins of goat, pig, and human (Caucasian) contained 5, 6, and 3 tryptophans, respectively. The variations in the amino acid contents undoubtedly affect the structure of the α-lactalbumins which may be partially responsible for the different specific activities of the various α-lactalbumins in the lactose synthetase reaction.

The disulfide values are considered as approximate values. Several modifications of the performic acid procedure for disulfide determination were used but only six half-cystines were found for bovine α-lactalbumin which actually has eight half-cystines. Two procedures were used which utilized the reaction of dithiothreitol with the reduced disulfides. These methods also gave low values for the half-cystines of bovine α-lactalbumin. It appeared that there was no single accurate or precise method for the determination of the disulfides in the α-lactalbumins, and hence the values were recorded as estimates.

The variation observed in the amino acid content of the various

α-lactalbumins is also seen in other like-proteins isolated from several sources, for example, chicken egg-white and human lysozyme as shown in Figure 1. The chicken egg-white lysozyme shows a marked similarity in amino acid sequence to bovine α-lactalbumin and a model of bovine α-lactalbumin has been constructed on the coordinates of hen's egg-white lysozyme. It has been postulated that the two proteins have a common evolutionary origin. The X-ray analysis of goat α-lactalbumin is currently under way (Philips, Oxford), and when completed, will permit a direct comparison of the tertiary structure of lysozyme and α-lactalbumin. Only after the sequencing and X-ray analysis of several α-lactalbumins and lysozymes are completed may a positive structural and thus evolutionary relationship be ascribed to these similar proteins.

Heterogeneity has been observed among preparations of various α-lactalbumins prepared by several different procedures. The presence of multiple proteins is due to two possibilities: (1) protein contaminants and (2) more than one form of α-lactalbumin. Barman (60) has reported the presence of, in a highly purified preparation of bovine α-lactalbumin, a minor component with the same amino acid composition as α-lactalbumin, but contained 11-12 sugar residues per molecule of protein. No carbohydrates were detected in any of the α-lactalbumins by staining the disc gels with a carbohydrate stain. Consequently, the minor bands present in the purified α-lactalbumins were minor protein contaminants except for the two major proteins in the α-lactalbumins of goat, sheep, and pig.

Two genetic variants of bovine α-lactalbumin have been identified which are α-lactalbumin A and B. The α-lactalbumin B form occurs in the American and British cattle. Blumberg and Tombs (88) have shown

that two different forms of α-lactalbumin occur in Fulani cattle. The two major protein bands of the α-lactalbumins of goat, sheep, and pig may be a similar case. The different percent gel study indicated the two proteins are charge isomers with the same molecular weight. Also, the two proteins of pig α-lactalbumin have different peptide maps. Therefore, it is possible that two genes are present in the pig, sheep, and goat which are responsible for the two α-lactalbumin forms.

The peptide maps are a qualitative comparison of the amino acid sequences of the various α-lactalbumins. The differences in amino acid compositions of the various α-lactalbumins indicated that the peptide maps would have different patterns. The peptide maps of the human α-lactalbumins show these proteins possess the greatest similarity of structure of any of the α-lactalbumins. The peak one and peak two proteins of pig α-lactalbumins were almost identical except for three peptides which did not appear in one or the other map. This was final proof that the two protein bands of pig α-lactalbumin on the disc gels were structurally different proteins.

The peptide maps of goat and sheep α-lactalbumin showed the greatest similarity in the ruminant peptide maps which was expected since the amino acid compositions of the two proteins are quite similar. The amino acid composition of pig α-lactalbumin showed that six amino acids varied by only two or three residues from the goat and sheep α-lactalbumins. These differences are the apparent cause for the differences observed in the peptide maps of the pig, goat and sheep α-lactalbumin.

These studies are a general study of the physical and chemical properties of the various α-lactalbumins. The logical conclusion of

these studies would be the determination of the amino acid sequences and finally the elucidation of the structure by X-ray crystallography. These studies have shown also that there are some structural differences in the various α-lactalbumins studied but these differences are not sufficient for loss of activity of α-lactalbumin as a modifier in the lactose synthetase reaction, since all α-lactalbumins tested to date show activity.

SUMMARY

The purification procedure utilized in this study removes any glyco-α-lactalbumin which may be present in the various milks. The DEAE cellulose column step is essential for the preparation of highly purified α-lactalbumin.

The properties which were investigated indicated both structural similarities and differences of the various α-lactalbumins. The electrophoretic mobilities on both starch and polyacrylamide gels indicated that the human α-lactalbumins were identical in charge while the rest of the α-lactalbumins had similar but not identical mobilities. The spectrophotometric properties indicated that the α-lactalbumins had similar structures since all the proteins had 290 nm shoulders and identical 270-280 nm peaks. The amino acid composition of the various α-lactalbumins also was similar suggesting related structures. The N-terminal amino acid of the ruminant α-lactalbumin was acidic (glutamic acid) whereas the non-ruminant was basic (lysine). The C-terminal amino acids were identical for all the α-lactalbumins (leucine) except for pig (methionine) and guinea pig (glutamine). The study of the mobility of the various α-lactalbumins on the disc gels revealed that the α-lactalbumins of the goat, sheep and pig contained charge isomers of the same molecular weight. Both proteins were active in the lactose synthetase assay. The two α-lactalbumins from the pig were separated on DE-32 with a pH 9.5 phosphate gradient. Peptide maps of the two pig α-lactalbumins showed a small difference in their peptide pattern thus

indicating a difference in their protein structures. The peptide maps of the human α-lactalbumins showed that these proteins had almost identical peptides. The goat and sheep α-lactalbumin peptide maps were very similar while the pig α-lactalbumin peptide map was quite different. The overall pattern of all the α-lactalbumin peptide maps indicated that the proteins had similar structures. When comparing all the properties, the human α-lactalbumins were very similar whereas the pig α-lactalbumin was the most dissimilar of all the α-lactalbumins.

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VITA

Donald Victor Schmidt

Candidate for the Degree of

Doctor of Philosophy

Thesis: COMPARISON OF THE PHYSICAL AND CHEMICAL PROPERTIES OF VARIOUS α-LACTALBUMINS

Major Field: Biochemistry

Biographical:

Personal Data: Born in Tecumseh, Nebraska, January 9, 1943, the son of Victor A. and Leona L. M. Schmidt.

Education: Attended the public school of Sterling, Nebraska; graduated from Sterling High School, Sterling, Nebraska, in May, 1961; received the Bachelor of Arts degree from Peru State College, Peru, Nebraska, with majors in Chemistry and Physical Science in May, 1965; completed requirements for the Doctor of Philosophy degree in May, 1971.

Professional Experience: Served as a research assistant at Oklahoma State University from September, 1965 to December, 1970.

Honorary Societies: Phi Lambda Upsilon, Associate Member Sigma Xi.