ISOLATION AND CHARACTERIZATION OF MOUSE, RAT, AND RAT TUMOR α-LACTALBUMIN

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CHAPTER I

INTRODUCTION

Milk by itself is almost a complete natural food. Enzymes, proteins, carbohydrates, lipids, cations, anions and water make it a liquid that is for many animals their sole means of nourishment for a short time. It is also a very important source of antibodies to the young and hence is responsible for partial immunity.

The major carbohydrate in most all milks is lactose and for this reason, lactose synthetase, the enzyme system responsible for the biosynthesis of lactose, has received much attention.

Lactose synthesis requires the presence of two proteins, a galactosyltransferase and α-lactalbumin (1,2). The B protein or α-lactalbumin was suggested by Hill et al. (3) to act as a "specifier" protein in that it could alter the galactosyl acceptor specificity of the A protein or galactosyltransferase from N-acetylglucosamine to glucose. More recent studies (4) indicate that α-lactalbumin may be best described as a modifier protein in that it lowers the apparent Km for glucose in the galactosyltransferase reaction.

To date many chemical and physical studies have been made on α-lactalbumin. It is now known for example that α-lactalbumin from different species are closely related in amino acid composition, molecular weights and structure (5,6,7,8). However, there are differences as determined by immunological cross reaction (9). α-Lactalbumin from

ruminant species will not cross react with antibodies made to α-lactalbumin from non-ruminant species (9). This observation is of interest since it has been shown that bovine galactosyltransferase will react with α-lactalbumin from non-ruminants to form lactose (9).

Hill et al. (5) have shown that the amino acid sequence of bovine α-lactalbumin and hen's egg white lysozyme are very similar, but again antibodies to α-lactalbumin will not react with lysozyme (10). It seems that the antibody determinant groups involved in antibody binding are quite different between the α-lactalbumin from various species and bovine α-lactalbumin and lysozyme.

The purpose of this study was to isolate α-lactalbumin from mouse and rat milk, and from rat mammary tumors and to determine if they would cross react with antibodies to bovine or human α-lactalbumin.

An attempt was also made to isolate various forms of nitrated bovine α-lactalbumin (mono, di, tri and tetra) and to determine their cross reaction with antibodies to bovine α-lactalbumin. Denton and Ebner (11) have noted a loss in activity of α-lactalbumin in the lactose synthetase reaction with nitration of tyrosyl residues. It would be of interest to determine if the tyrosyls are also involved in the antigen antibody reaction.

CHAPTER II

LITERATURE REVIEW

Lactose Biosynthesis in Mammary Tissue

The new born receives nourishment from milk and also in its early days of nursing some of its passive immunity (12). These two very important properties of milk make it a very unique and important natural food (13). It is primarily because of these two functions and the observation of many interesting protein interactions that has made the biochemistry of milk a subject of intensive studies (14).

α-Lactalbumin is one of the simpler proteins found in milk and it has received a great deal of investigation. Bovine skim-milk contains from 70-150 mg of α-lactalbumin per 100 ml and is classified as a major component of the whey proteins in milk (15).

Ebner and Brodbeck (1,11,16) were the first to describe the biological role of α-lactalbumin during their studies on the biosynthesis of lactose. Lactose synthetase (E.C.2.4.1.22.) is the enzyme system responsible for the biosynthesis of lactose, the carbohydrate that makes up half the dry weight of milk. The sequence of reactions and the enzymes (17) that catalyze the formation of lactose are listed below.

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 UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridyl transferase, E.C.2.7.9), reaction 2. by UDP-galactose-4-epimerase (E.C.5.1.3.2) and the final reaction is catalyzed by lactose synthetase (UDP-galactose: D-glucose-1-galactosyl-transferase). Lactose synthetase was shown first to exist in lactating mammary glands of cows and guinea pigs (18) and also in bovine milk (19). Ebner and Brodbeck (1) separated the soluble lactose synthetase from milk into two protein fractions and designated them as A and B proteins, according to their separation by gel filtration. The A and B proteins when assayed separately exhibited no lactose synthetase activity but when the two proteins were combined, lactose synthesis did occur. Later Hill et al. (20) showed that the A protein catalyzes the transfer of galactose to N-acetylglucosamine to form N-acetyllactosamine and that this reaction is inhibited by α-lactalbumin. They proposed that α-lactalbumin changes the galactosyl acceptor specificity of the A protein from N-acetylglucosamine in its absence to glucose in its presence. They could find no separate enzymatic activity for α-lactalbumin.

Fitzgerald et al. (21) showed that the galactosyl transferase in the absence of α-lactalbumin catalyzes the formation of lactose (reaction 3.) when the glucose concentration was very high and α-lactalbumin reduced the Km for glucose to the mM region. Klee and Klee (22) reported similar results and also stated that α-lactalbumin slightly lowered the Km for N-acetylglucosamine. One should also note that galactosyltransferase activity is important for the transfer of N-acetylglucosamine to galactosyl residues to form secreted glycoproteins in the mammary tissue (27).

Morrison and Ebner (4,23,24) have described the steady state kinetic analysis of the lactose synthetase reaction and have demonstrated that α-lactalbumin does participate in the reaction. They have shown that (a) Mn^{++}, UDP-galactose, and the carbohydrate acceptor of the galactosyl group add to the enzyme in an ordered manner and that α-lactalbumin adds after the substrates and dissociates before product release (b) Mn^{++} is not released at each turn of the catalytic cycle whereas α-lactalbumin is released with each turn: disaccharide and the UDP are the order of product release (c) at high concentrations, carbohydrate can add randomly to all enzyme forms but an active complex is not formed unless Mn^{++} and UDP-galactose have added previously. (d) the effect of α-lactalbumin is due to its ability to combine with a complex containing carbohydrate and cause displacement of an already established equilibria.

Figure 1. shows the scheme proposed by Morrison and Ebner (4) and describes how α-lactalbumin modifies an existing enzymatic reaction. There are two possible ways the reaction may proceed. When N-acetylglucosamine is the substrate and α-lactalbumin is not present, the reaction goes by the linear path and the rate depends on UDP release. However, when α-lactalbumin is present the reaction is forced to go predominately by the branched pathway. In this pathway Morrison and Ebner (4) have postulated that α-lactalbumin is the first product released and UDP release is rate limiting.

When glucose is the substrate the reaction also goes predominately by the branched path when α-lactalbumin is present. However, in this case the rate is not slower than the linear pathway. In the presence of α-lactalbumin glucose has approximately the same apparent Km as

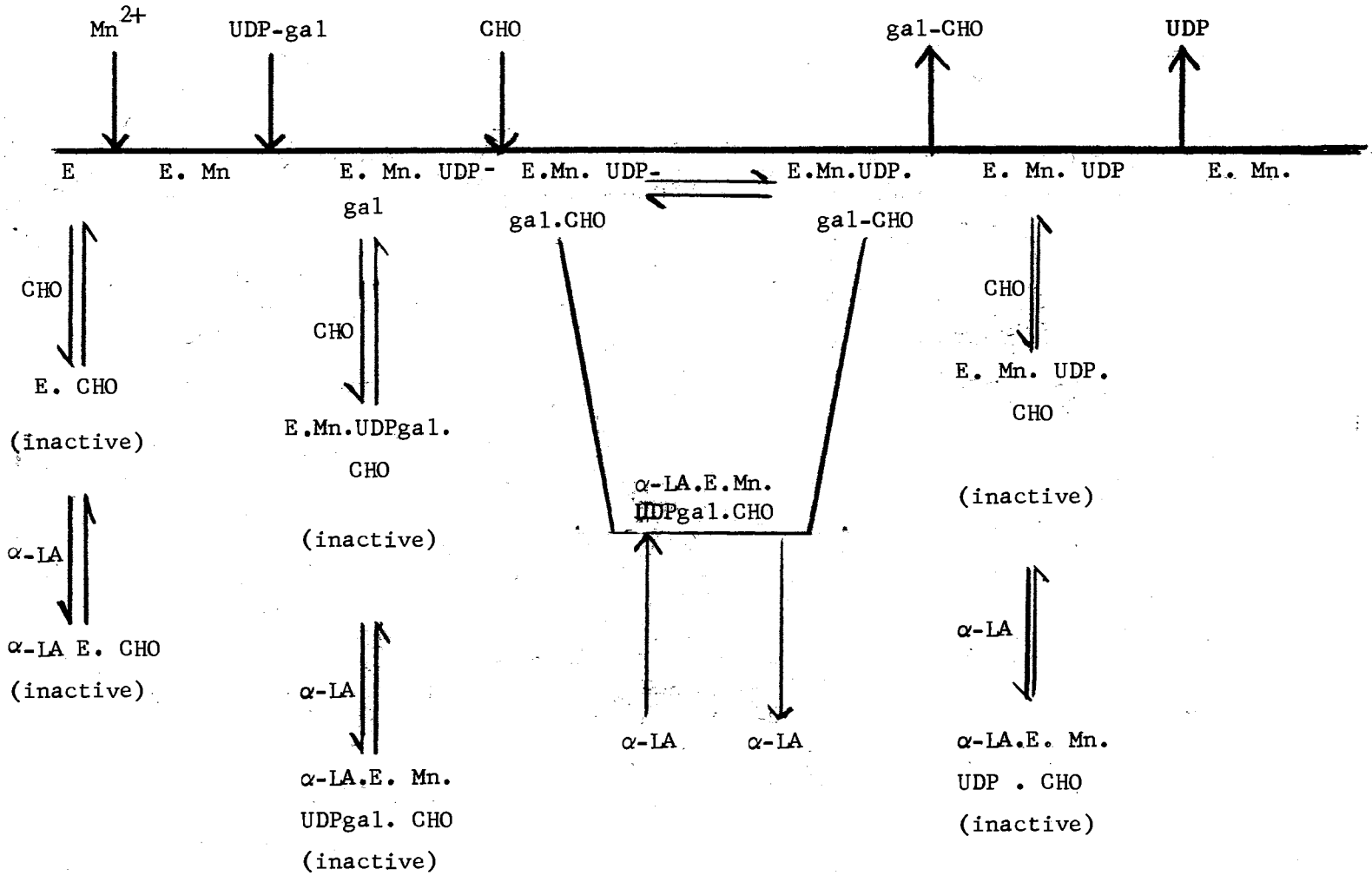


Figure 1. Schematic Representation of Order of Addition of Substrates and Release of Products of the Galactosyltransferase Reaction

N-acetylglucosamine, in the absence of α-lactalbumin.

Schanbacher and Ebner (25) were not able to demonstrate, under conditions of maximum product formation, the formation of an α-lactalbumin-galactosyltransferase complex by sucrose gradient centrifugation, equilibrium dialysis, fluoresence quenching and gel filtration techniques. Morrison and Ebner (4) have since shown that α-lactalbumin dissociates from the enzyme prior to product release and suggest this may be why Schanbacher and Ebner (25) could show no α-lactalbumin-galactosyltransferase complex under those conditions.

The subcellular organization of galactosyltransferase and α-lactalbumin was reviewed by Brew (26). Studies of the sedimentation properties and enzyme constitution of mammary tissue particles which appear to be very similar to the membrane of the Golgi, have shown that the galactosyltransferase and α-lactalbumin are present in these particles as a stable complex (26).

It is the current belief that lactose is synthesized in the Golgi apparatus and is concentrated in secretory granules that migrate to the edge of the cell (27). α-Lactalbumin is synthesized on ribosomes of the rough endoplasmic reticulm (28) and travels through the lumen of the endoplasmic reticulum to the Golgi region where it comes in contact with the galactosyltransferase (26) to synthesize lactose. After reaction with the galactosyltransferase, α-lactalbumin is secreted from the cell into the milk (26). Very little galactosyltransferase is found in milk because it is attached to the membrane of the Golgi and most of it remains in the secretory cell.

Brew (26) has proposed that because α-lactalbumin is released into milk, it could be involved in control of lactose synthesis. The

production of lactose would then be regulated by the rate of α-lactalbumin synthesis. Khatra and Brew (29) have evidence that would support this theory. They showed that in four different species, α-lactalbumin and lactose synthetase activity is directly related to the levels of lactose in the milks (27). Also, Schmidt et al. (30) have shown the same relationship in Northern fur seal milk where lactose levels and α-lactalbumin levels are both low.

Physical and Chemical Properties of Various Species of α-Lactalbumin

The majority of research has been done on bovine α-lactalbumin although studies of the α-lactalbumins from a large number of different species have been completed to varying degrees. Bovine α-lactalbumin is a tightly folded globular protein and contains about 40% helical structure (31,32). Kronman et al. (33,34) studied the solvent pertubation properties of bovine α-lactalbumin in aqueous media and found that thiolglycolate reduction of the disulfide bonds "exposed" five tryptophan residues, and that 8M urea exposed 80% of the residues. It was later shown that α-lactalbumin contains four instead of five tryptophanyl residues (35).

Robbins et al. (36) have shown that four tyrosyls will ionize under normal conditions and Gorbunoff (37) reported they will react with N-acetylimidazole and cyanoflouride. Denton and Ebner (38) observed that the tyrosyls are indeed modified in a random fashion by iodination and nitration.

Crystalline α-lactalbumin has been shown by x-ray diffraction spectroscopy to be an elongated parallelepiped which may occasionally

be arranged in rosettes (39). The densities of the wet crystals are 1.213 and 1.210 g/cm^3. The partial specific volume determined by Gordon and Semmett (40) was 0.735 cm^3/g and that determined from the complete amino acid sequence by Gordon and Ziegler (41) was 0.729.

Wetlaufer (42) and Zittle and Kronman (43,44) showed that bovine α-lactalbumin has an absorptivity of ($E^{1\%}_{280}$) of 20.9 and 20.1 respectively.

Many different molecular weights were reported for bovine α-lactalbumin before Hill et al. (5,20) determined from the complete amino acid sequence that it was 14,600. Some of the earlier studies by sedimentation velocity determinations gave molecular weights of 15,400 (40) and 14,900 (42), and 16,500 by light scattering techniques (31).

Heterogeneity of α-Lactalbumin

Several investigators have found that α-lactalbumin is heterogeneous. Zittle and Della-Monica (43) found that after using Gordon and Semmett's procedure (40) for isolating α-lactalbumin that there was a soluble and insoluble form of α-lactalbumin. This phenomenon could possibly be explained by the fact that the isoelectric point is shifted from pH 4.8 in salt free solutions, to 3.6 in 0.5 M NaCl. It seems that α-lactalbumin strongly binds anions and this could be the reason for its change in solubility. Kronman et al. (33,34,36,44,45,46,47,28,48) studied the heterogeniety at acid and basic pH's beyond the isoelectric points for bovine α-lactalbumin. The acid form of α-lactalbumin was shown to be somewhat denatured and had a marked affinity to associate and aggregate (45,46). Titration (36) and optical dispersion (34) studies indicated that similar conformational changes occur in the basic

forms above pH 10. The acid-treated molecule has a low solubility, exhibits a time dependent aggregation and also had a tendency to form lower molecular weight associated species than that of the aggregated polymer (45,46). However at high pH only an enhanced tendency to associate was observed (48). It seems at this time that these different forms of α-lactalbumin are small and large polymers. Borman (49) has also observed heterogeniety in bovine α-lactalbumin and has separated the different forms by anion exchange chromotography and showed that one form contained carbohydrate. Glyco-α-lactalbumin from the bovine, contains 11-12 sugar residues per molecule and represents about 15% of the total α-lactalbumin and has about the same activity as α-lactalbumin in the lactose synthetase reaction.

In certain types of cattle two distinct forms of α-lactalbumin are present. Blumberg and Tombs (50) have shown that by paper electrophoresis two types were present in Fulani cattle. The fast moving band was called α-lactalbumin A and the slower band was called α-lactalbumin B which is typical of American and English cattle. These two types of α-lactalbumin have been found in a number of other cattle and are believed to correspond to two genetic variants A and B (51).

Structural Similarities Between Bovine and Other α-Lactalbumins

A great deal is now known about bovine α-lactalbumin's structure and several comparisons may be made with other species. Schmidt and Ebner (6) made a detailed study of goat, pig, and human α-lactalbumins. The ultraviolet spectra and amino acid compositions of the proteins were similar, and the molecular weights were all within the range of $14{,}500 \pm 500$. Hill and his coworkers (5) have reported the complete amino acid

sequence of bovine α-lactalbumin as well as the location of the disulfide bonds (5,52). Yasunabu and Wilcox (7) showed that it was a single peptide chain and that glutamic acid was the N-terminal residue and that leucine was the C-terminal residue. The amino acid composition of bovine α-lactalbumin is similar to human, goat, sheep, dog, guinea pig and kangaroo (6). Human α-lactalbumin has one to two residues of glucosamine that are covalantly linked in an unknown manner. Gordon (8) analyzed α-lactalbumin A from Indian Zebu cows and from water buffalo and found only minor differences between α-lactalbumin A and B and buffalo α-lactalbumin.

Mawal (43) showed that the peptide maps of Indian bovine α-lactalbumin A, bovine α-lactalbumin B, and buffalo α-lactalbumin are slightly different. Gordon et al. (8) reported that α-A and α-B are identical in amino acid composition except that α-B has an arginine where α-A has glutamic acid or glutamine. In enzyme assays for lactose formation, different species of α-lactalbumin will react with the bovine galactosyltransferase (9).

Similarities in amino acid compositions, location of disulfide bonds, molecular weights (5,52,6,7,8) and activities with the same galactosyltransferase protein (9) indicate that there are little differences between α-lactalbumins. However, there are some differences in peptide maps (53) and immunological cross reactions (9,54,55) indicating small differences in tertiary structures.

Purification of α-Lactalbumin

Pederson (56) in 1936 described a slow moving peak in sedimentation velocity experiments of whey proteins as the "α-peak" and then in the

same year Kekwick (57) isolated and crystallized the protein that appeared to be responsible for this peak. A short time after this Svedberg and Pederson (52) stated that this "α-peak" was α-lactalbumin.

Sometime later a crystalline protein prepared from the albumin fraction of whey protein was described as a "crystalline insoluble substance" by Sorensen and Sorensen (58). However, this protein was not very well characterized.

Zwieg and Block (59) isolated α-lactalbumin by ferric chloride precipitation of the whey proteins and subsequently isolated α-lactalbumin and β-lactoglobulin from this precipitate (40). However, this method was not often used because it required adjusting the solution to pH 1.3. Gordon and Ziegler (40,60,61) used several methods for isolating α-lactalbumin that are the basis of some of the current procedures. In their procedure, casein is precipitated from skim milk by lowering the pH to 4.6. The whey proteins are precipitated with ammonium sulfate and then α-lactalbumin, β-lactoglobulin, and other proteins besides the crude globulins are precipitated at 80% saturation. Then, another ammonium sulfate fraction at 30% saturation precipitated crude α-lactalbumin. The protein was redissolved and crystallized by ammonium sulfate.

Later, Brodbeck and Ebner (1,2) used column chromatographic procedures such as DEAE cellulose and Bio-Gel P-30 to remove minor protein contaminants.

Comparison of α-Lactalbumin and Hen's Egg-White Lysozyme

α-Lactalbumin has many characteristics that are similar to lysozyme. Yasunobu and Wilcox (7) reported that the two proteins have similar

molecular weights, the same number of disulfide bonds, similar amino acid composition and similar C and N-terminal amino acid residues. They also observed that α-lactalbumin was easily oxidized by tyrosinase whereas lysozyme was not affected. The complete amino acid sequence of both proteins is known and the comparison is presented in Figure 2. The underlined residues are the identical positions in the amino acid sequences. Forty-nine of the residues are the same at corresponding positions and twenty-three are conservative structural replacements. It has been shown that when α-lactalbumin is theoretically folded into a three dimensional structure, its structure is similar to that of lysozyme (35). There is also a very close structural similarity to human lysozyme.

Even though α-lactalbumin and lysozyme do not form the same product, they do act at the same carbohydrate linkage (20,35). Lysozyme acts to cleave β-1-4-glucopyranosyl linkages and α-lactalbumin combines with the galactosyltransferase to form these linkages.

Many of the physical properties of the two proteins are also very similar. Kronman (44) showed that the circular dichroic spectra and optical rotary dispersion spectra (62,63) were similar for both proteins. Krigbaum and Kugler (64) examined the conformation of α-lactalbumin and lysozyme in solution by small angle x-ray diffraction measurements and showed that the two proteins have different conformations in solution which would suggest that they could also then be different in the crystalline state. However, these results have been challenged (65) and the apparent difference may be due to a contaminant or dimer formation.

The overall charge of the two proteins differ markedly since

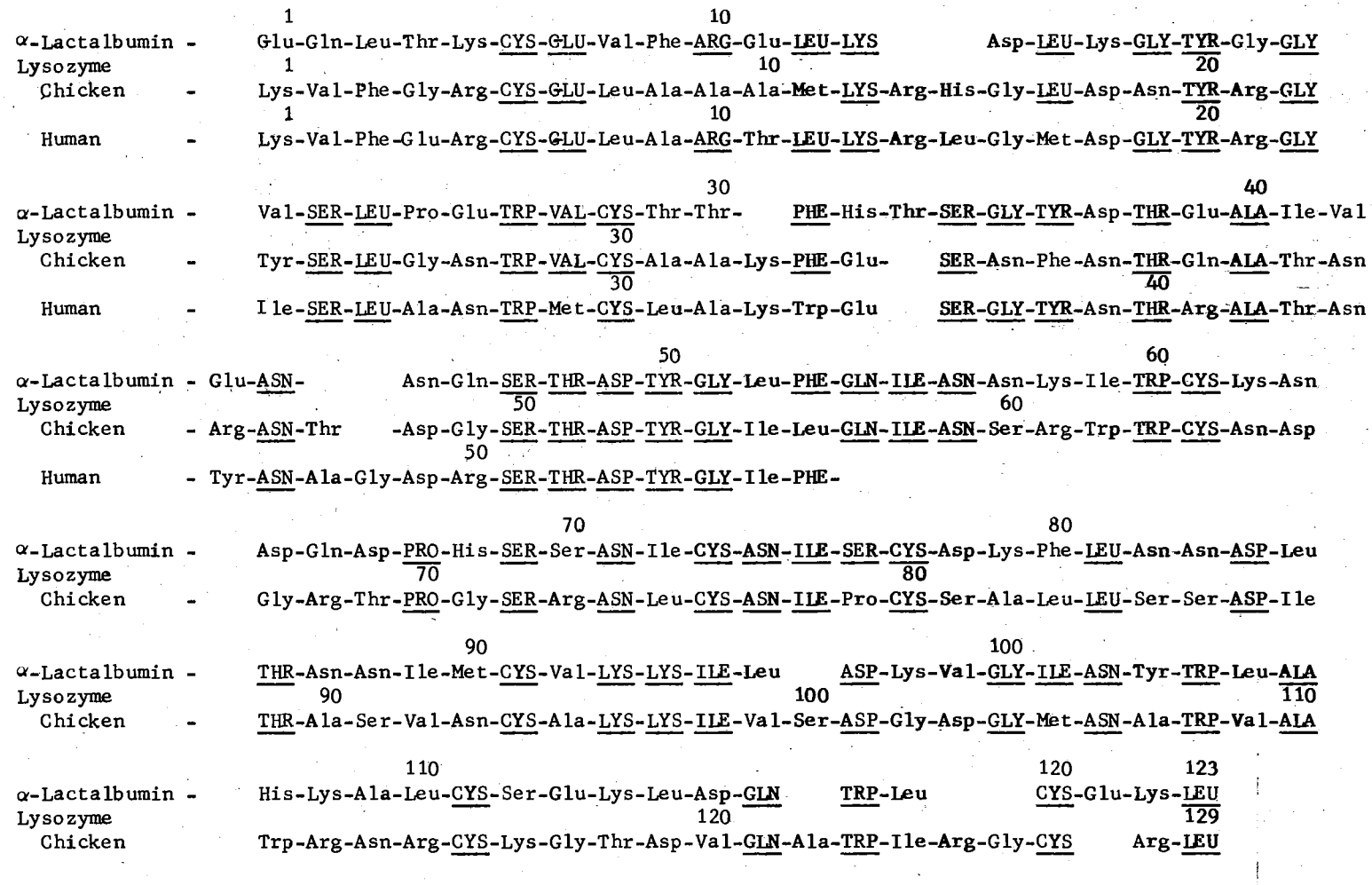


Figure 2. Comparison of the Amino Acid Sequences of Bovine α-Lactalbumin, Chicken Egg-White Lysozyme, and Human Lysozyme.

lysozyme has an isoelectric point at pH 10.5 whereas α-lactalbumin has an isoelectric point at 4.8.

The Evolution of α-Lactalbumin and Lysozyme

Since α-lactalbumin and lysozyme are closely related in structure, the hypothesis has been made that they arose from a common ancestral origin. After millions of years of changes in genes, enzymes that survive have amino acid sequences that are relatively unchanged in the active centers. Two reasons for this might be (a) a need to define an overall shape and dynamic properties of the modecule and (b) a need to protect the enzyme against denaturation under conditions normal for the enzyme's location in an organ (66).

Genes for α-lactalbumin and lysozyme could have evolved by convergence of two separate origins or they could have come from a common ancestral gene (3). It is currently believed that they evolved by the latter process. It may very well be that when a milk producing system evolved, a gene mutation occurred. A gene for a carbohydrate degrading enzyme (lysozyme) could have mutated to form a protein α-lactalbumin that would help to produce carbohydrate linkages (67).

A similar situation may have occurred with pancreatic proteases (68). Hartley et al. (68) have made a study of trypsin, chymotrypsin A and B, and porcine elastase and found that all the enzymes react with diisopropyl phosphorofluoridate at a unique serine residue and that the amino acid sequence immediately surrounding this residue is very similar in all three enzymes. Also the sequences around the disulfide bonds and in some other locations of the different enzymes are similar. Therefore they propose that because the sequences are similar for these three

enzymes of widely different specificity, they could also have a common evolutionary ancestral protein.

Immunological Studies

Only a few studies have been made on the immunological properties of α-lactalbumin. It has been shown by Tanahashi et al. (9,54,55) and others that there was no cross reaction between antisera to α-lactalbumin of ruminant and non-ruminant species. Pig, guinea pig and human α-lactalbumins do not react with antisera to bovine α-lactalbumin. However, the ruminant α-lactalbumins (bovine, buffalo, sheep and goat) all react with antibodies to bovine α-lactalbumin (9). Similar results were obtained with bovine A and B, water buffalo, and goat α-lactalbumin by Sen et al. (69). It is interesting to note however that Tanahashi (9) did find that all the α-lactalbumins reacted with bovine galactosyltransferase to form lactose. It has also been suggested by Jenness (70) that rat, cow, goat, deer, pig and human α-lactalbumins all have a different affinity for bovine galactosyltransferase.

At present, the α-lactalbumin antibody binding site is not known. Tanahashi et al. (9) have suggested that the immunological site and active sites are different because of the lack of immunological cross reaction between ruminants and non-ruminants while these same species will all react with bovine galactosyltransferase. Attassi and Habeeb (71,72) reported that when tyrosines 20 and 23 of lysozyme are nitrated with tetranitromethane there was a decrease in antibody and enzyme activity. When the $(NO_2)_2$-lysozyme was reduced to $(NH_2)_2$-lysozyme by reduction with sodium hydrosulfite most of the antigenicity was returned

but no enzymeatic activity was returned. Measurements of changes in conformation showed that they were very similar in the two states and they therefore postulated that the antibody binds at tyrosines 20 and 23, and also that these tyrosyl residues are not located at the active catalytic site. It has also been shown with lysozyme that N-acetyl-glucosamine an inhibitor of lysozyme, does not bind at tyrosines 20 and 23 (73). However Denton and Ebner (38) have shown that in α-lactalbumin tryptophan and histidine may also be destroyed and if this reaction would occur in lysozyme, their antibodies may also be binding at these residues.

All tests to date, for the cross reaction of α-lactalbumin antibodies with lysozyme and vice-versa have shown that they do not cross react (74,10). Arnon and Maron (10) made a very thorough investigation and could show no cross reactions by (a) antigen binding capacities, using ^{125}I-labled antigens (b) passive cutaneous anaphylaxis (c) micro-complement fixation and (d) inactivation of a lysozyme bacteriophage conjugate. This last method is very sensitive and could show if 0.0001% of anti-α-lactalbumin antibodies were cross reacting with lysozyme (10). This technique involves forming a T_4 bacteriophage-lysozyme conjugate (75) which still permits the phage to cause lysis. When lysozyme is mixed with lysozyme antibodies its activity is inhibited but mixture with anti-α-lactalbumin causes no inhibition.

Therefore even though α-lactalbumin and lysozyme have very similar amino acid sequences they do not cross react immunologically because they have different conformations, and or antigenic determinants. However they are so structurally similar that one may relate immunological studies reported on lysozyme to research on α-lactalbumin.

CHAPTER III

ISOLATION AND CHARACTERISTICS OF α-LACTALBUMIN

Experimental Procedure

Materials and Reagents

Unpasturized whole milk was obtained manually from Swiss White Albino Mice, and Fischer Rats. Fresh bovine skim milk was obtained from the Oklahoma State University Dairy. R.C.3230 AC tumors were obtained originally from Squibb Laboratories, New Brunswick, New Jersey and were maintained by transpantation in virgin female Fisher rats.

Oxytocin, Tris (tris-hydroxymethylaminoethane), glycine, phospho-enolpyruvate, NADH, pyruvate kinase (from rabbit skelantal muscle Type I), and glycylglycine were purchased from Sigma Chemical Company. Sodium pentobarbital was obtained from Haver-Lockhart Laboratories and Freund's Complete Adjuvant from Pentex Incorporated. Glucose was acquired from Fischer Scientific Laboratories. Biogel-P was purchased from Bio-Rad Laboratories and Sephadex G from Pharmacia. DEAE-cellulose 32 was obtained from Whatman. Special Agar-Nobel was from Difco Labs, Detroit and antiboides were purchased from Antibodies Incorporated, California. Reagents used for disc gel electrophoresis were purchased from Canalco. Trypan blue was from Allied Chemical.

The A, C, and G solutions used for disc gel electrophoresis were mixed as follows using Canalco regeants. The A solution contained

24.0 mls 1N HCl, 18.5 gm Tris,0.115 gm Temed and was made up to 100 mls with water (final pH 8.8-9.0), C solution contained 28 gm acrylamide, 0.735 gm Bis made to 100 mls. with water and the G solution or catalyst was 0.07 gm ammonium persulfate per 50 mls H_2O and was prepared each week.

Milking Procedures

The procedure used was similar to the one described by Feldman (76). The animal was first injected intraperitoneally with sodium pentobarbital (.05 mg/gm wt). After the animal became unconscious the oxytocin was injected intraperitoneally (0.1 ml for mice and 0.2 ml for rats undiluted). Two to four minutes later warm de-ionized water (68°C) was rubbed on the teats and then a suction apparatus was applied to each teat. The collection apparatus consisted of a small vaccine bottle with two lines of tubing running out the top. One tube was connected to a water aspirator and the other to a small soft plastic suction cup (76) by polyethylene PE-200 tubing. Very low suction levels were maintained to keep from hemorrhaging the tissue around the teat. Mothers were milked twelve to fourteen days after they had their litter and were separated from the young overnight before milking.

Approximately 1 to 3 mls of milk and water were obtained from each mouse and about 8 mls from a rat. The milk was kept cold (4°C) during milking and frozen after collection.

Isolation of α-Lactalbumin from Different Sources

α-Lactalbumin was isolated from mouse and rat milk by the method Schmidt (30) used on Northern fur seal milk. The milk was centrifuged

at 15,000 x g for 25 min at 4°C and then the cream was removed with a spatula. The supernatant was centrifuged at 100,000 x g for one hour to remove the casein. Then an 80% (0.516 g/ml) saturation of ammonium sulfate at 4°C was made on the supernatant from the last centrifugation. This was centrifuged at 35,000 xg for 25 minutes and the supernatant discarded after checking for α-lactalbumin activity. The precipitate was disolved in a minimum volume of 20 mM Tris buffer, pH 8.0 amd 0.1 M KCl. Ten to twenty-five mls of this placed on a P-30 (100-200 mesh) column (4.8 x 110 cm). The α-lactalbumin peak was located by A_{280} readings and enzymatic assays and the pooled sample was placed on a DEAE-32 cellulose column. The column consisted of a 10 or 50 ml disposable syringe and was equilibrated in 5 mM Tris of pH 8.0 with 0.1 M KCl before loading the sample. The column was eluted with a linear gradient from 5 mM to 150 mM Tris buffer with a constant KCl concentration. The protein was pooled in the same manner as with the P-30 column and dialyzed against de-ionized water using cellulose casing, size 18, prepared by boiling in de-ionized water for 20 minutes and rinsing in de-ionized water, for two to four days with at least four changes of water. The dialyzed solution was lyophilized and stored in the presence of Drierite at -15°C. Purity was checked by disc gel dlectrophoresis. If protein impurities were present they were removed by adding ammonium sulfate to the protein solution until it became slightly turbid which usually was at about 60-70% saturation, 0.361 g/ml-0.436 g/ml. After standing for 10 minutes the solution was centrifuged at 35,000 x g for 25 minutes and the supernatant solution was taken to 90% saturation (0.201 g/ml-0.134 g/ml) with ammonium sulfate (6). After centrifugation at 15,000 x g for 25 minutes the precipatate was dissolved in a minimum

of de-ionized water, dialized as before, and lyophilized. α-Lactalbumin was isolated from the R 3032 AC tumors in approximately the same manner. Tumors were removed from the rats at about 21 days after implantation. They were manually sliced into mm^3 pieces and homogenized in a Sorvall Omni-Mixer. The homogenizing buffer (0.15 M KCl, 0.005 M $MgCl_2$ and 0.005 M EDTA) was used at 5 ml/gm of tissue. Homogenization was at 4^oC at a speed of 8 reached in 30 seconds and held for an additional 30 seconds. α-Lactalbumin was isolated from the homogenized tissue by the same procedure used for mouse and rat milk.

Bovine α-lactalbumin was isolated from skim milk by Aschaffenburg and Drewry's (77) procedure up to the step where it was placed in a P-30 column. Fresh skim milk was allowed to come to room temperature and then 20 gm/ml of anhydrous Na_2SO_4 were mixed with the milk. The resulting solution was filtered in large funnels through Whatmam No. 1 filter paper and two layers of cheese cloth overnight at 25^oC. The filtrate (F_1) was measured and adjusted to pH 2.0 by very slowly adding concentrated HCl. The mixture was centrifuged at 25^oC, 13,000 x g for 20 minutes and the supernatant solution was discarded. Dilute NH_4OH (1 M) (one tenth of the F_1 volume) was used to dissolve the precipitate after which it was adjusted to pH 3.5. The mixture was centrifuged at 4^oC, 13,000 x g for 20 minutes and the supernatant solution was discarded. The precipitate was dissolved in 1 M NH_4OH (one quarter the volume of the centrifuged solution F_2) adjusted to pH 4.0 and stored overnight. The F_3 solution was centrifuged at 4^oC, 10,000 x g for 25 minutes and the precipitate was saved. Ammonium sulfate (11.5 g/100 ml) was added to the supernatant solution and after stirring for 15 minutes the mixture was centrifuged at 10,000 x g for 15 minutes. The pellet from

this centrifugation was combined with the other precipitate and they were dissolved in minimal amounts of dilute NH_4OH. This solution was divided into 10-25 ml portions and frozen until they were placed on a Bio Gel P-30 column (4.8 x 110 cm) equilibrated and eluted with 50 mM phosphate buffer, pH 8.0 and 0.1 M KCl. A_{280} readings were recorded for each fraction and each protein peak was checked for activity. The tubes in the active peak were pooled and placed on a DEAE-32 column (50 ml disposable syringe). Pooled α-lactalbumin samples were either diluted with de-ionized water or dialyzed against de-ionized water before placing on the DEAE cellulose which was equilibrated with 5 mM phosphate buffer pH 8.0. A linear gradient of 5 mM to 200 mM phosphate, pH 8.0 (500 mls of each) was used to elute the column. Fractions were assayed for A_{280} and activity to locate the α-lactalbumin and the pooled α-lactalbumin was extensively dialyzed against water and lyophylized. The protein was stored at -15°C in bottles containing Drierite.

Nitration and Isolation of Bovine α-Lactalbumin

All four tyrosyls in α-lactalbumin were nitrated (38) in a 10 mg/ml solution of α-lactalbumin in 50 mM Tris, 0.1 M KCl pH 8.0. Fifty-three microliters of tetranitromethane (1:10 dilution with 95% ethanol) were added and the solution was allowed to remain at room temperature with constant stirring for 110 minutes. The reaction was stopped by dialysis against water or buffer at 4°C.

Large polyacrylamide gels (17 x 1 cm) at a concentration of 4% were used for separating the nitrated forms of α-lactalbumin. The gels were loaded with 1.5 mgs of nitrated α-lactalbumin and were subjected at 15 m a per gel until the tracking dye ran off the end of the gel. The

gels were run for one hour at the same amperage before loading the sample. See the electrophoretic methods section for complete details.

After electrophoresis, the gels were stained with .025% coomasie brilliant blue in 7% acetic acid until bands first started to appear. The gels were removed and each band was cut out, cut into fine pieces and incubated in 10 mls of 50 mM ammonium bicarbonate pH 7.6 and 0.1 M KCl at 37°C for about three hours. At the end of three hours, the solution was removed from the gels and they were washed at 37°C for three hours and the procedure was repeated. The three washes were pooled and stored frozen. The protein-dye solution was dialyzed against de-ionized water for two days with at least four changes, and then lyophilized and dissolved in no more than three mls of 50 mM NH_4HCO_3 pH 7.6. This solution was made to contain 10% sucrose and placed on a Sephadex G-25 column equilibrated and eluted with 50 mM NH_4HCO_3. The protein was monitored by A_{280} readings and the peak fractions were pooled, lyophilized and stored at -15°C.

Immunological Techniques

The Ouchterlony double diffusion technique described by Campbell et al. (78) was used to demonstrate antibody-antigen precipitations. One gram of Agar-Noble was soaked in 98 mls borate-saline buffer (6.184 gms Boric acid, 9.536 gms Borax, 4.384 gms NaCl made to 1 liter with de-ionized water) and then the flask containing the solution was heated in boiling water until the agar was dissolved. One ml of 1% aqueous merthiolate and 1 ml of 1% aqueous trypan blue were added to the agar. Approximately 8-10 mls were stored in test tubes at room temperature.

For preparation of the plates, the tubes were heated in boiling water until the agar melted. One tube was emptied into a disposable petri dish (8.5 x 1.5 cms) and allowed to gel for approximately 30 min. Then a dye punch (Gelman Inst. Co., No. 71692) containing two sets of wells was placed on the solidified agar and another tube of agar was poured into the petri dish. When the second layer solidified, the dye was removed. The dye has a pattern of one well surrounded by eight or six wells. Antibody solution was placed in the center well (.01 mls) and dilutions of antigen or different antigens were placed in the outside wells. The antigens were in solutions in which the specific protein was stable. After incubation at 37°C for about 18 hours a precipitin band was formed between an outside well and the center well. The plates were stored in a desicator containing 7% acetic acid.

A more sensitive test to show the presence of antibodies was the ring test (78). The sera was first clarified by centrifuging at 60,000 x g for 30 min. Then the tubes were arranged in the following manner.

	1	2	3	4	5	B borate buffer
TOP	B	B	Ag	B	Ag	N normal sera
BOTTOM	Ab	AG	N	N	Ab	Ab antisera
REACTION	-	-	-	-	+	Ag antigen

The tube was slanted before the top solution was poured on very slowly. After about three minutes a positive reaction in only the fifth tube should occur if there was a antigen-antibody reaction. The bottom layer should be 4-5 mm in height and the top layer 2-3 mm in height, in 5 x 0.5 cm tubes.

Young, mature New Zealand White rabbits, about four pounds in weight

were used to prepare antibodies (78). A 10 mg/ml solution of α-lactalbumin in 50 mM phosphate buffer was added to an equal volume of Freunds complete adjavant and mixed in a two ml glass syringe. Each footpad was injected with 0.1 ml of this solution once every seven days.

Blood was taken from the inner marginal vein (78), and allowed to set at room temperature for about two hours. The clot was loosened with a needle and refrigerated for 24 hours at 4°C. The straw colored sera was poured off and the clot was centrifuged in conical centrifuge tubes at 1,000 x g for 30 min. The resulting sera was stored frozen in two ml aliquots.

Enzymatic Assays for α-Lactalbumin

Assays for α-lactalbumin were by the method of Fitzgerald et al. (21). α-Lactalbumin was assayed in the presence of saturating amounts of A protein (galactosyltransferase) one unit being defined as the amount of enzyme required to form one nanamole of UDP per minute and equals a A_{340}/min/ml of 0.0062 under the conditions of the assay. The rate of the reaction was followed by coupling the production of UDP to NADH oxidation by adding PEP and pyruvate kinase to the reaction mixture. Reaction rates were measured on either a Cary Model 14 or a Hitachi Perkin-Elmer Coleman 124 Spectrophotometer. Assay mixtures contained 1.0 mM PEP, 0.05 ml of a 1 to 10 dilution of pyruvate kinase (Type I containing lactic dehydrogenase, 25 mg/ml with 2.4 I.U. per mg pyruvate kinase), 50 mM glycylglycine pH 8.5 and approximately 25 units of galactosyltransferase. One hundred microliters of solutions from fractions off columns were added and the final solution was diluted to one ml with water. An endogenous rate with only galactosyltransferase

was determined before each assay.

Disc Gel Electrophoresis

Both large (17 x 1 cm) and small (5.5 x 0.5 cm) gels were separated in Tris-Glycine buffer (3.0 gm Tris, 14.4 gm glycine made to 1 l with de-ionized water). Standard 7% gels were prepared by mixing solution A, C and G (catalyst) together in a 1:1:2 ratio. Materials at the beginning of Chapter III describes the preparation of the A, C and G solutions. The 4% gel was made by mixing A, water, C and G solutions in a ratio of 10:4.28:5.72:20. Eight to ten to 1.0 to 1.2 mls of this final solution were added to the large or small tubes and they were put in the dark to solidify for 30 min. No stacking gels were used. The discs were placed in a Canalco electrophoretic apparatus and pre-electrophoresis was used to remove the catalyst. The small gels were operated at 5 ma per gel and the large gels at 15 ma per gel for 60 min. At the end of this period, 30-60 μg (1 mg/ml solution) of protein was loaded on the small gels or 0.3-1.5 mg (20mg/ml solution) was loaded on large gels. The protein solutions were made 10% in sucrose prior to adding the sample. The tracking dye solution (0.005% Bromophenol Blue in de-ionized water) was made 10% in sucrose, and was added to one of the gels, usually the control. The gels were developed at the same amperage and stopped just before the tracking dye reached the end of the disc.

The gels were removed and stained, for four hours or overnight with 0.5% Aniline Blue Black in 7% acetic acid. They were electrically destained (0.5 amperes until color was gone) and stored in 7% acetic acid.

Carbohydrate staining was by the Zacharius et al. (79) method. Gels were immersed in 10% trichloracetic acid for 30 min and then rinsed lightly with de-ionized water. They were next immersed in 1% periodic acid (in 7% acetic acid) for 50 min and washed overnight with de-ionized water with 3-5 changes. They were then immersed in Fuchsin-Sulfite stain in the dark for 50 min and washed three times for 10 min each, with 0.5% meta-bisulfite. The gels were finally washed with de-ionized water until the stain was removed and then stored in the dark in 7% acetic acid.

Gel Filtration and Ion Exchange Chromotography

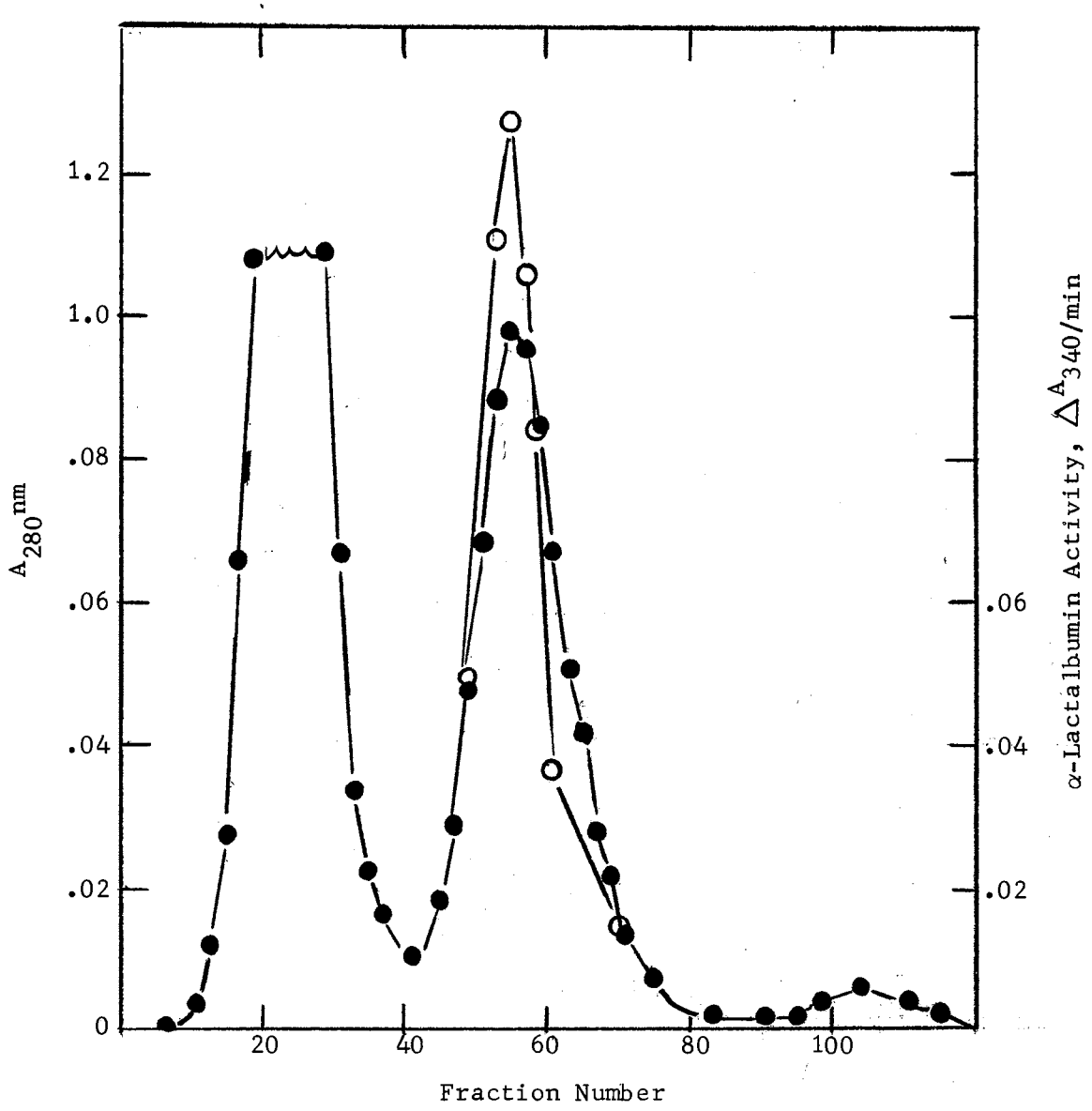
Various size columns of Sephadex (G) and Bio-Gel (P) were prepared by procedures described in respective technique manuals. All columns were fitted with glass or plastic frits and 2 mm luer connectors. Void volume of each column was determined from the elution position of Blue Dextran.

Results

Isolation of α-Lactalbumin from Mouse Milk

α-Lactalbumin was isolated from rat and mouse milk by the procedure Schmidt et al. (30) with some small variations first used for Northern fur seal milk. This procedure was also used to isolate α-lactalbumin from homogenized rat mammary tumors. The procedure details are described under methods, p. 19.

A typical protein elution pattern for the whey proteins of mouse milk separated on a P-30 column is presented in Figure 3. There are

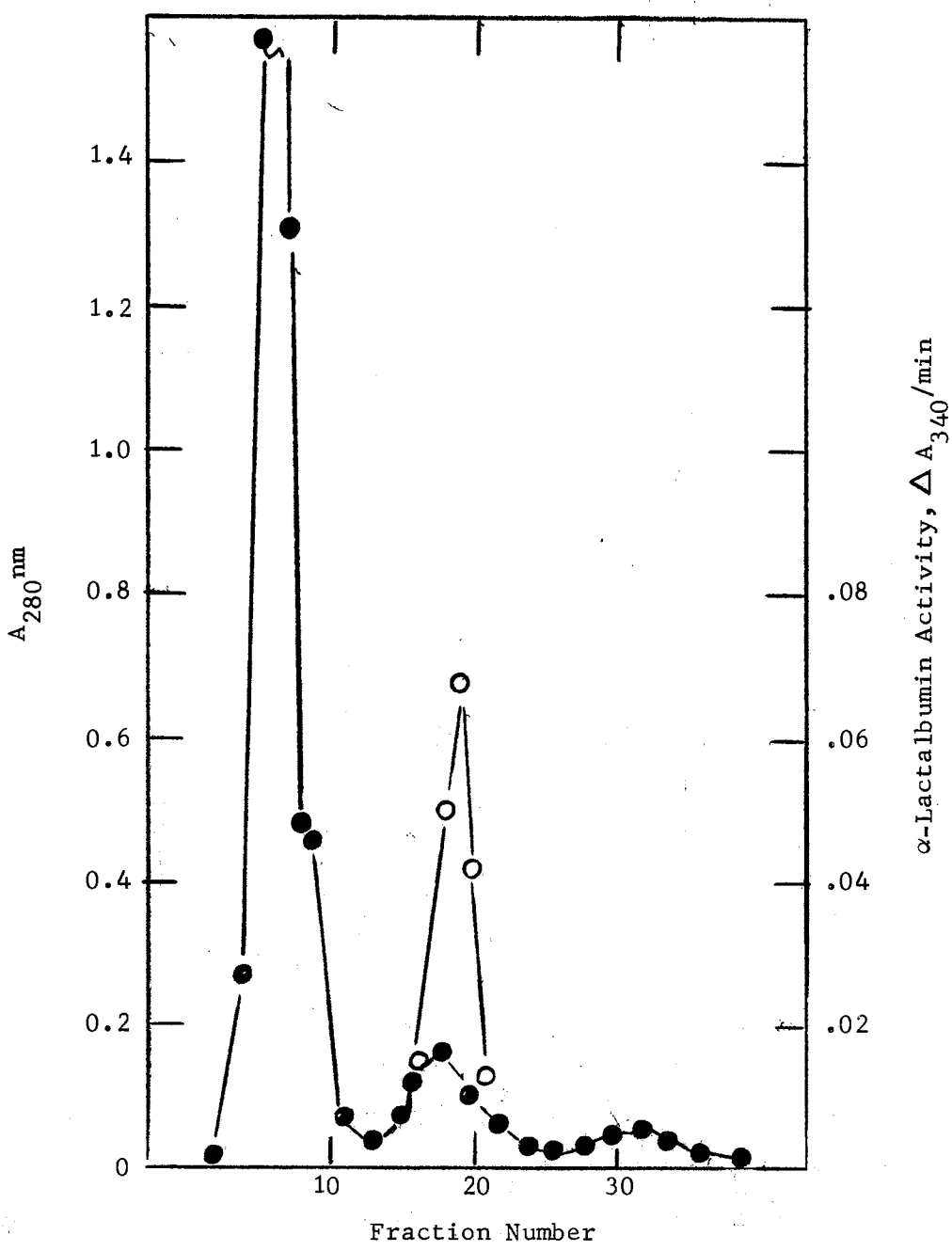


A Bio-Gel P-30 column (4.8 x 110 cm) was equilibrated and eluted at 4°C with 50 mM Tris, 0.1 M KCl pH 8.0 and 5 ml fractions were collected per tube after the first 200 mls passed through the column. Absorbance was read at 280 nm (●—●) and activity for α-lactalbumin was measured spectrophotometrically at 340 nm (○—○).

Figure 3. Typical Elution Pattern of Whey Proteins from Mouse Milk on Bio-Gel P-30

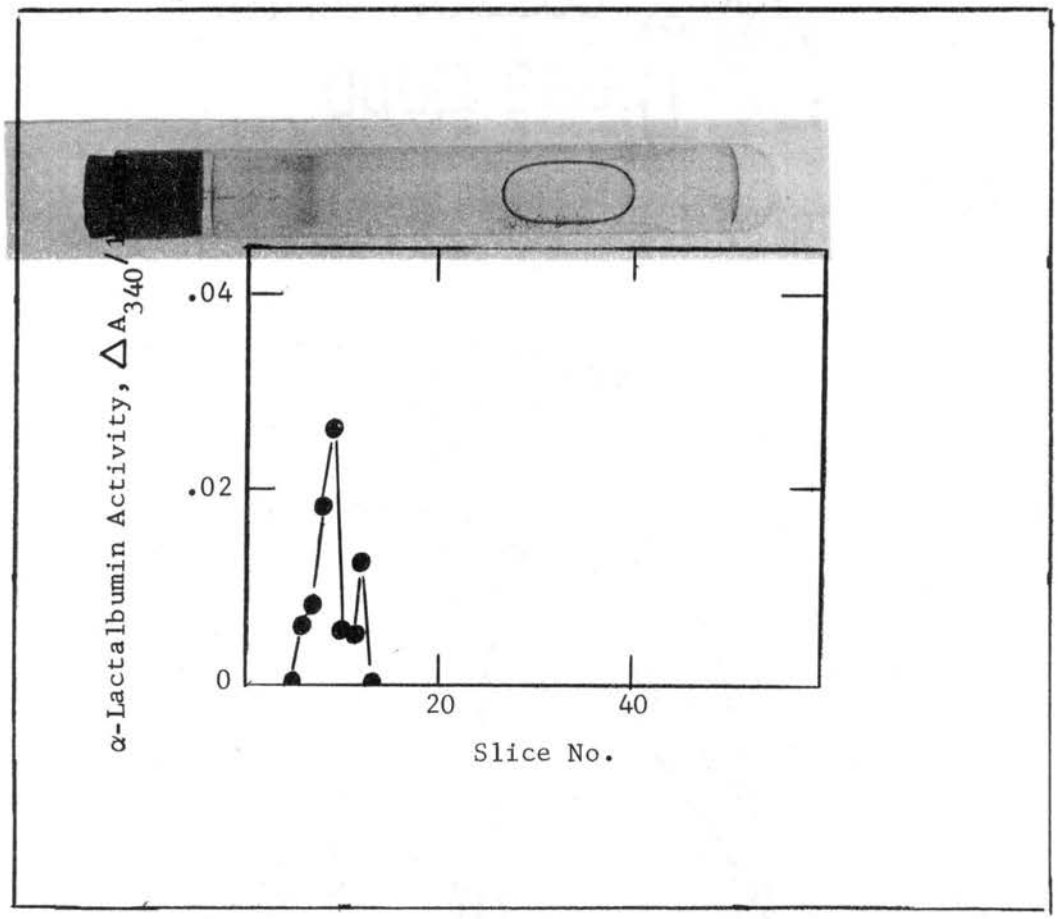
two rather large protein peaks and the second or lower molecular weight peak contains α-lactalbumin as determined by assay with bovine galactosyltransferase. The fractions in this peak were pooled, dialyzed against de-ionized water (overnight about 30 mls in 2 l) and fractionated on a DEAE-32 column and the elution profile is presented in Figure 4. Three protein peaks were observed but α-lactalbumin was found only in the second major peak. The fractions in this peak were dialyzed against de-ionized water (about 70 ml in 6 l H_2O) for two days with at least six changes, and then lyophilized. Figure 5 shows that two protein bands were present in this peak when separated by disc electrophoresis. Both major bands were found by enzymatic assays of gel slices to be α-lactalbumin (Figure 5). Duplicate disc gels were prepared and one was stained for protein while the other was sliced immediately for the α-lactalbumin assays. The gel to be sliced was placed in a gel slicer tube and 1 mm portions were forced out and sliced off with a razor blade. Each gel slice was placed in a small glass tube containing 0.2 ml 100 mM Tris, 0.02% NaAzide, pH 8.0 and macerated with a glass rod. Each tube was frozen and thawed several times and left at room temperature for 10 hours. Then 100 μl samples were assayed for α-lactalbumin activity and two active peaks were observed (Figure 5).

Generally the yields of mouse α-lactalbumin were low and approximately 0.08 mg of α-lactalbumin per ml of milk plus water were obtained. The mouse milk was always diluted about 2 fold with de-ionized water since the teats of the mouse had to be wiped with warm water in the milking procedure. The protein bands of mouse α-lactalbumin on the disc gels were stained for carbohydrate but no carbohydrate bands were observed.



Typical resolution of the pooled α-lactalbumin peak from Bio-Gel P-30 column. The DEAE-32 column (50 ml disposable syringe) was equilibrated with 10 mM Tris, 0.1 M KCl, ph 8.0 and the sample eluted with linear gradient from 10 mM to 150 mM Tris, 0.1 M KCl, pH 8.0, 200 ml each. Each 8 ml fraction was read at 280 nm for protein (●—●) and assays for α-lactalbumin activity (○—○).

Figure 4. Chromatography of Mouse α-Lactalbumin from a Bio-Gel P-30 Column on DEAE-32



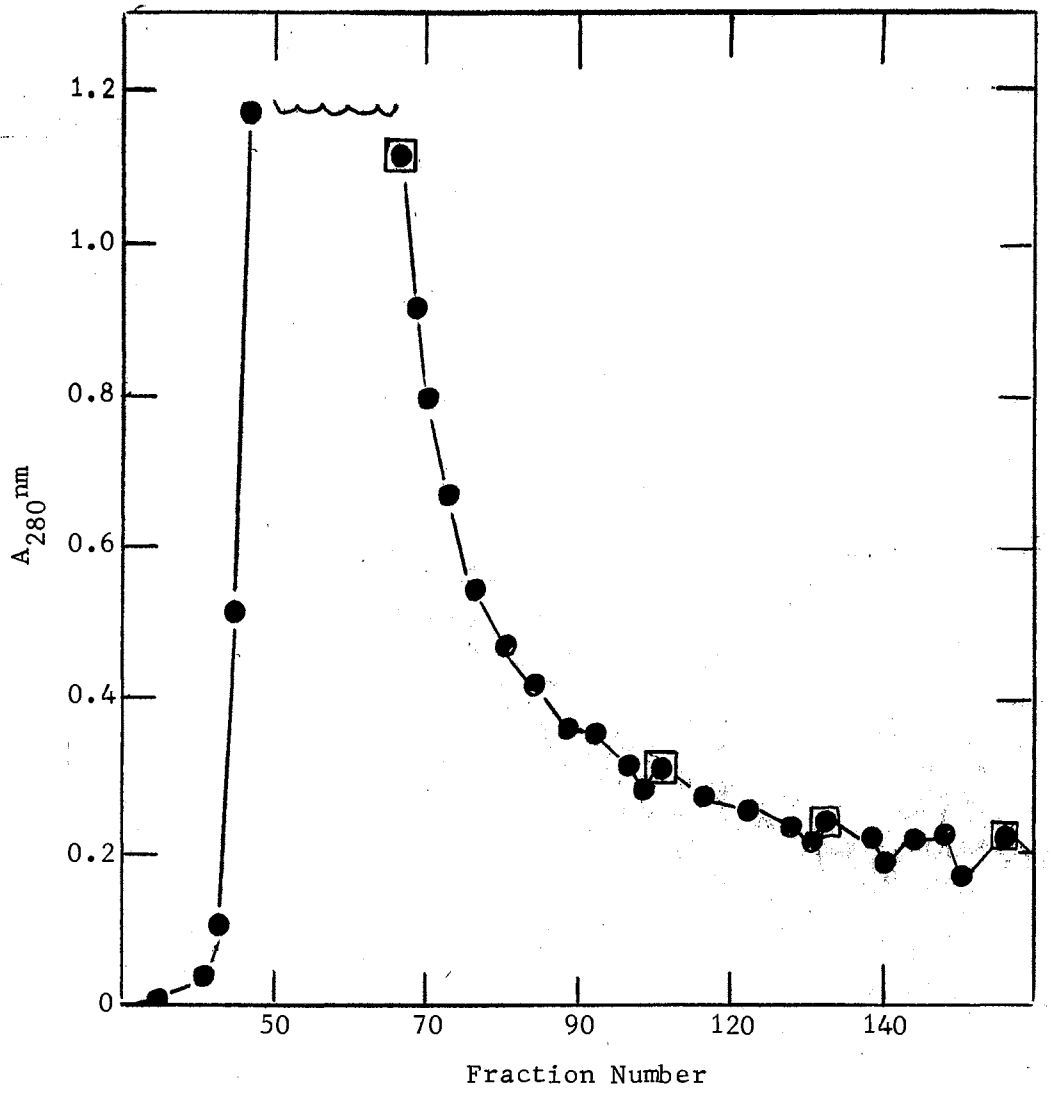
Mouse α-lactalbumin (65 μg) was separated on a 7% disc gel at pH 7.9. The gel was sliced into 1 mm slices and macerated in 0.2 ml 100 mM Tris, .02% Naazide, pH 8.0. The macerated slice was incubated for 10 hours at 25° C after freezing and thawing at least five times. One hundred μl of this solution was assayed for αlactalbumin activity by the spectrophotometric assay. The gel at the top of the graph is a duplicate gel at the same time and stained with Aniline Blue Black.

Figure 5. Assays for Mouse α-Lactalbumin Activity in Disc Gel Slices

Isolation of α-Lactalbumin from R3230 AC Rat Tumors

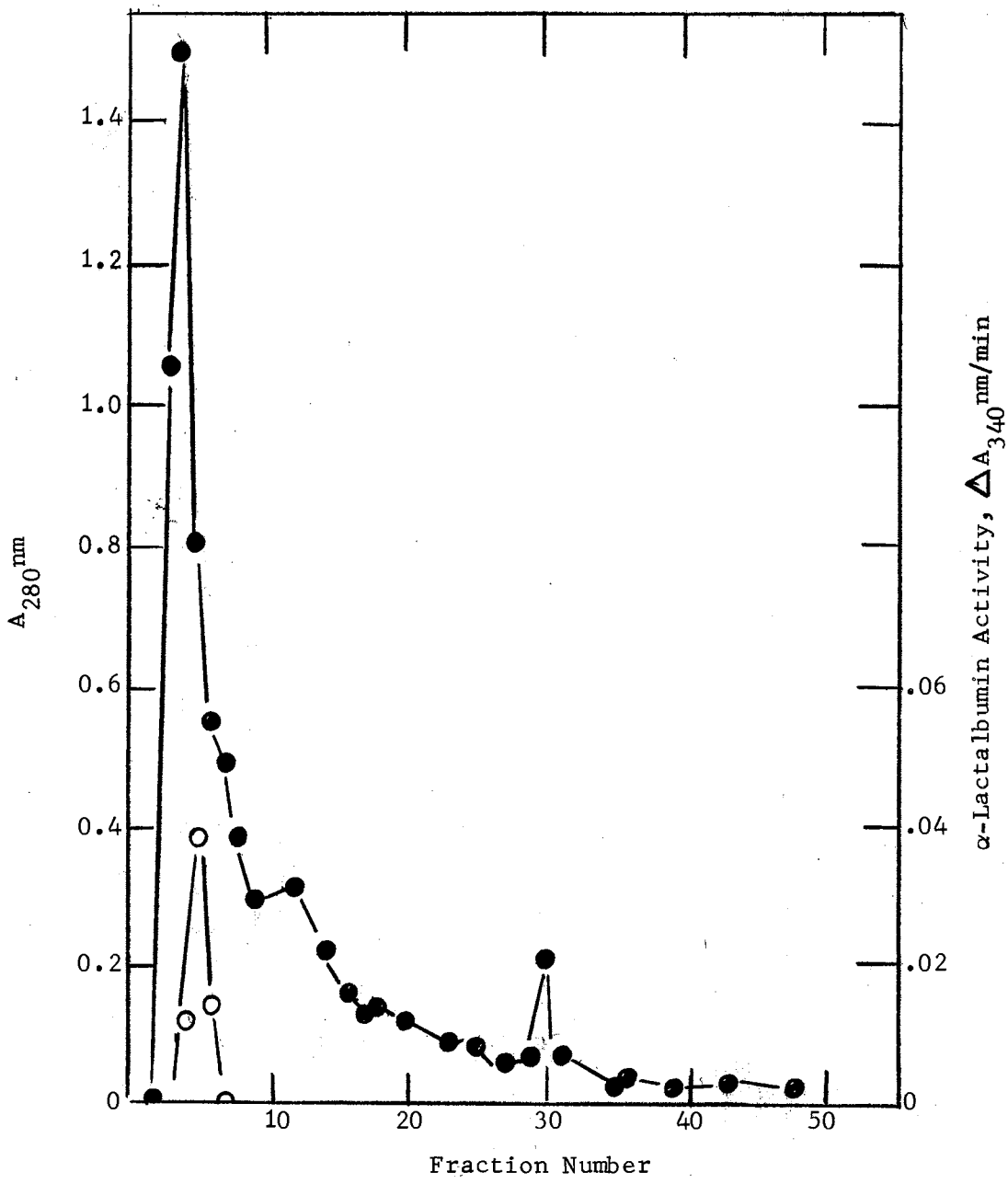
The isolation of α-lactalbumin from rat milk and tumor homogenates was a more complicated procedure.

Tumor homogenates after the 80% ammonium sulfate saturation step still contained too much protein when placed on the large P-30 column. The preparation was dialyzed against de-ionized water to remove all the ammonium sulfate and then refractionated between 40% (0.226 gm/ml) and 80% (0.516 gm/ml) ammonium sulfate. The last centrifugation was at 35,000 x g for 25 min. The precipitate was dissolved in 20-25 mls 50 mM phosphate buffer pH 8.0 and placed on the large P-30 column. This procedure removed a great deal of the higher molecular weight proteins. The resulting protein profile from this column is shown in Figure 6 and there was one major peak. Direct enzymatic assays for α-lactalbumin off this column which was in phosphate buffer were not possible since a precipitate was formed in the cuvettes when phosphate buffer was mixed with $MnCl_2$ required in the assays. Some of the fractions were dialyzed against de-ionized water for 24 hrs (20 mls in 6 l with 3 changes) and assayed. The results showed that the α-lactalbumin was spread throughout the protein peak and was not separated as a single low molecular weight component. However these assays may not be accurate because a NADH oxidase or PEP phosphatase in the tissue could give activity with these assay reagents. Therefore this entire fraction was loaded on a DEAE-32 column. However, before loading on the column the pooled fraction was dialyzed against water to remove the phosphate buffer and the DEAE-32 column (50 ml disposable syringe) was equilibrated in 20 mM Tris, 0.1 M KCl, pH 8.0. Figure 7 shows the results of chromatography of the pooled fractions from the Bio-Gel P-30 column on DEAE-32. There



Resolution of mammary tumor homogenate after precipitating a second time with ammonium sulfate. A Bio-Gel P-30 column (4.8 x 110 cm) was equilibrated and eluted with 50 mM phosphate buffer, 0.1 M KCl, pH 8.0 and 5 ml fractions were collected per tube after 200 ml passed through the column. Absorbance was read at 280 nm (●—●) and the α-lactalbumin activity measured spectrophotometrically at 340 nm (○-○). All fractions measured for activity were dialyzed against de-ionized water first.

Figure 6. Rechromatography of Tumor Mammary Homogenates on Bio-Gel P-30



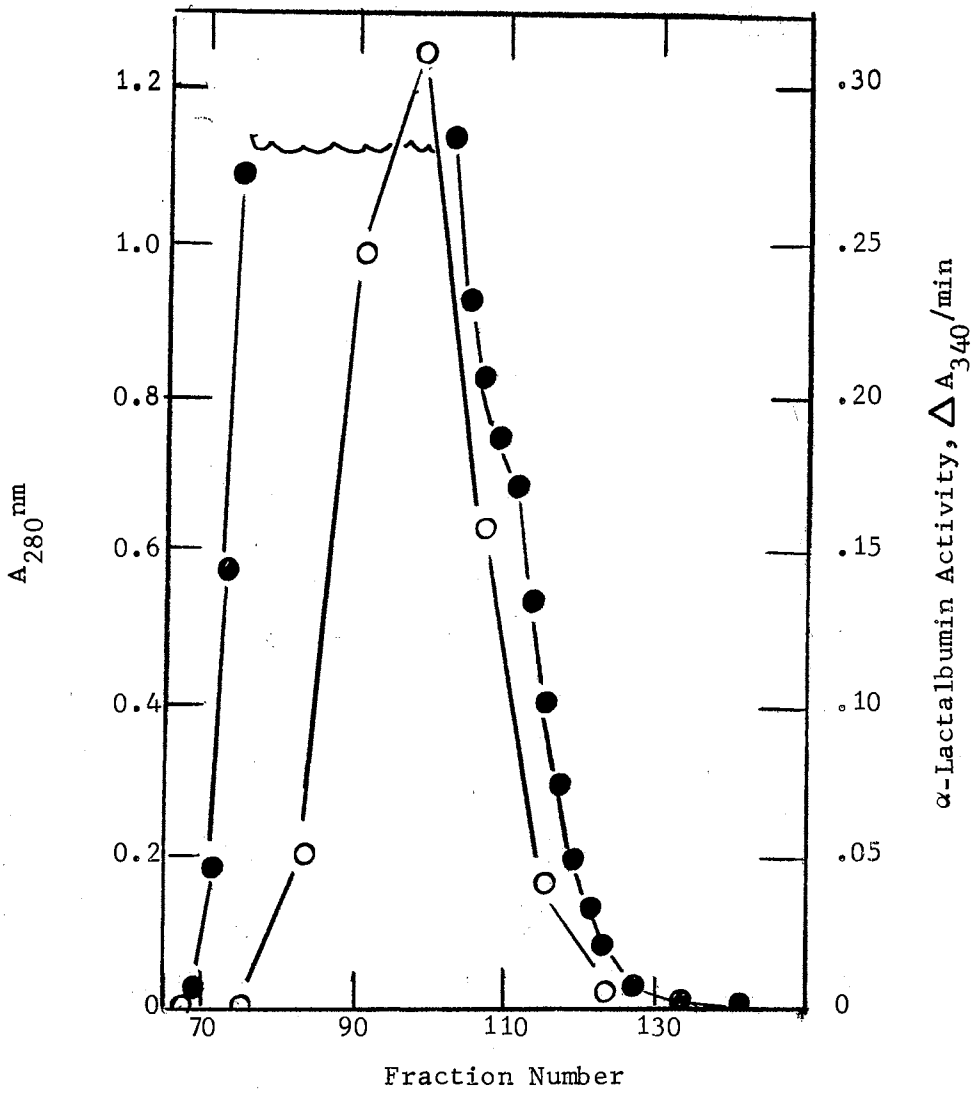
Typical resolution of a pooled rat tumor α-lactalbumin peak from a Bio-Gel P-30 column. The DEAE-32 column (50 ml disposable syringe) was equilibrated with 20 mM Tris, 0.1 M KCl, pH 8.0 and eluted with a linear gradient of 20 mM to 150 mM Tris, 0.1 M KCl, pH 8.0 (300 ml each) at 4°C. Eight ml fractions were collected and measured for absorbance at 280 nm, (●—●) and activity was measured spectrophotometrically at 340 nm (○—○).

Figure 7. Typical DEAE-32 Chromotography of a Rat Mammary Tumor α-Lactalbumin Peak from a P-30 Column

was primarily one protein peak and the activity profile showed that α-lactalbumin was in this major protein peak which indicated that it had a low negative charge, or that the ionic strength of the buffer was too high. The α-lactalbumin peak was pooled, extensively dialyzed against de-ionized water (70 mls sample to 6 l H_2O) for two days with at least five changes of water and lyophilized. The movement on disc gel electroporesis showed that it migrated slower than bovine α-lactalbumin. Yields were about 0.2 mg/gm tumor tissue. Disc gel electrophoresis of 65 μg of protein stained with aniline blue black and a gel scan of the disc at 600 nm showed only one protein band. The protein band did not contain carbohydrate by staining on the disc gels.

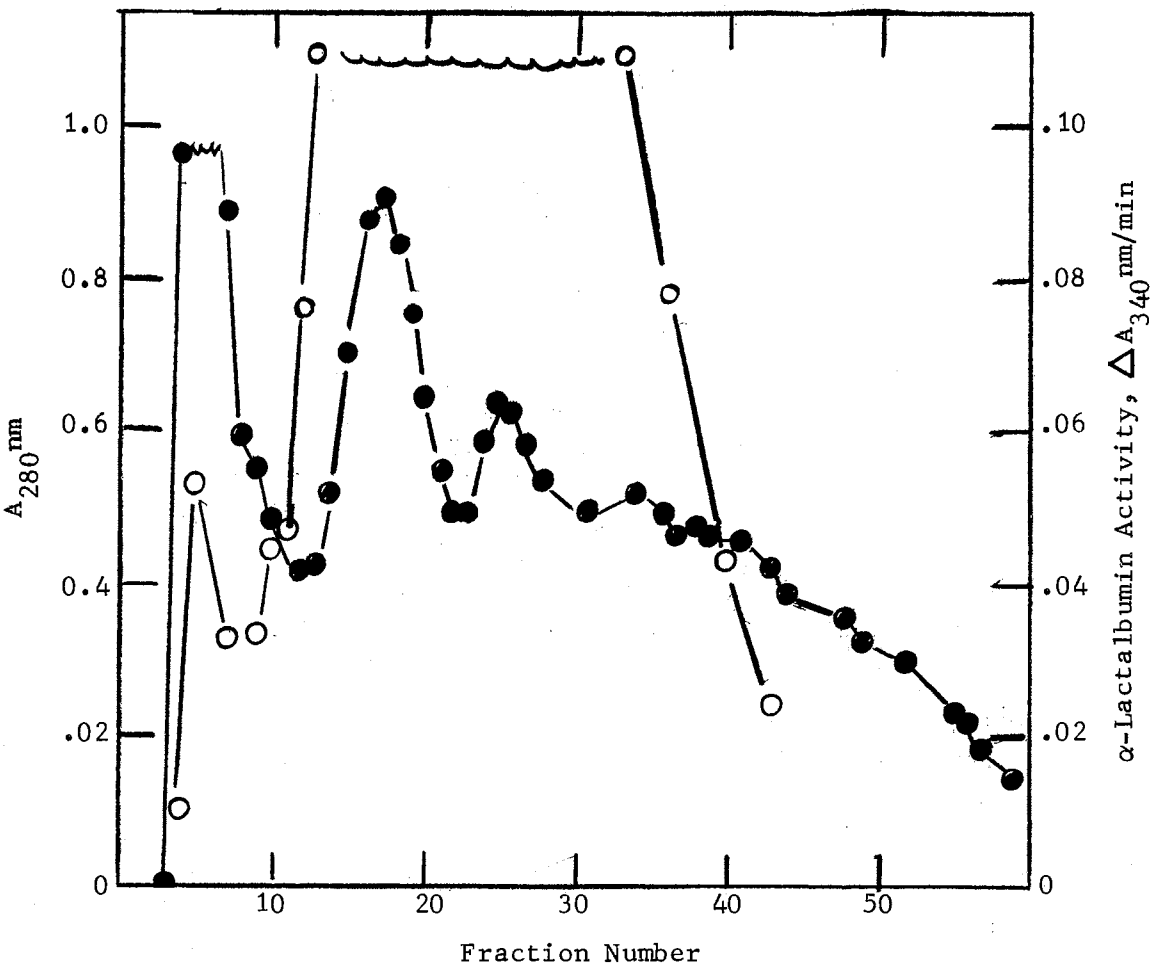
Isolation of α-Lactalbumin from Fischer Rat Milk

Isolation of α-lactalbumin from the milk of normal Fischer rats gave results that were very similar to those obtained in the tumor experiments. Figure 8 shows a typical elution profile of a rat milk preparation (precipitate from 80% ammonium sulfate step, 0.516 gm/ml) from the large P-30 column. There was only one major protein peak which was similar to the results obtained in the chromotography of the tumor homogenate (Figure 6). This major protein fraction contained α-lactalbumin and the fractions of several separation were pooled and dialyzed against 6 l of de-ionized water overnight with two changes. The dialyzed sample was separated on a DEAE-32 column and the elution pattern is presented in Figure 9. The α-lactalbumin activity profile shows two peaks which suggest that there may be two forms of α-lactalbumin in rat milk. The first peak was designated as Peak-1 (tube 2-7) and the other as Peak-2 (tubes 8-45). The irregular A_{280} and



A Bio-Gel P-30 column (4.8 x 110 cm) was equilibrated and eluted with 20 mM Tris, 0.1 M KCl, pH 8.0. Five ml fractions were collected per tube and the absorbance was read at 280 nm (●—●) while α-lactalbumin activity was measured spectrophotometrically at 340 nm (○—○). Two hundred mls of buffer were passed through the column before collection of fractions.

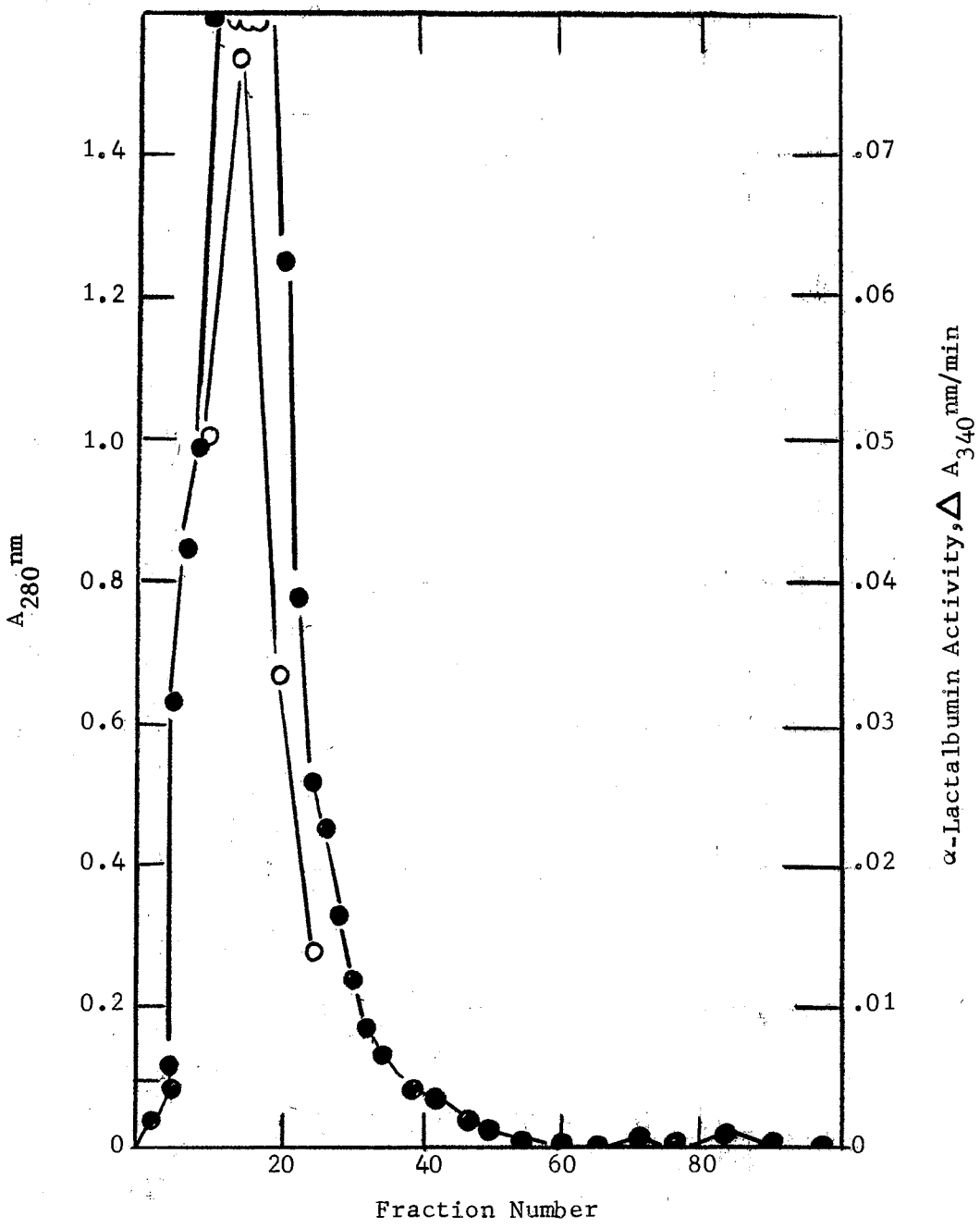
Figure 8. Typical Chromatography of Whey Proteins in Rat Milk on Bio-Gel P-30



Resolution of the pooled fractions from a Bio-Gel P-30 column on a DEAE-32 column (50 ml disposable syringe) equilibrated in 5 mM Tris, 0.1 M KCl, pH 8.0 and eluted with a linear gradient of 5 mM to 150 mM Tris, 0.1 M KCl, pH 8.0 (250 ml each). Each fraction collected contained 6 ml and its absorbance was read at 280 nm (●—●) and activity was measured spectrophotometrically at 340 nm (○—○). Activity was measured beyond that shown in the graph for Peak-2 and was symmetrical with the major peak (tubes 11-22).

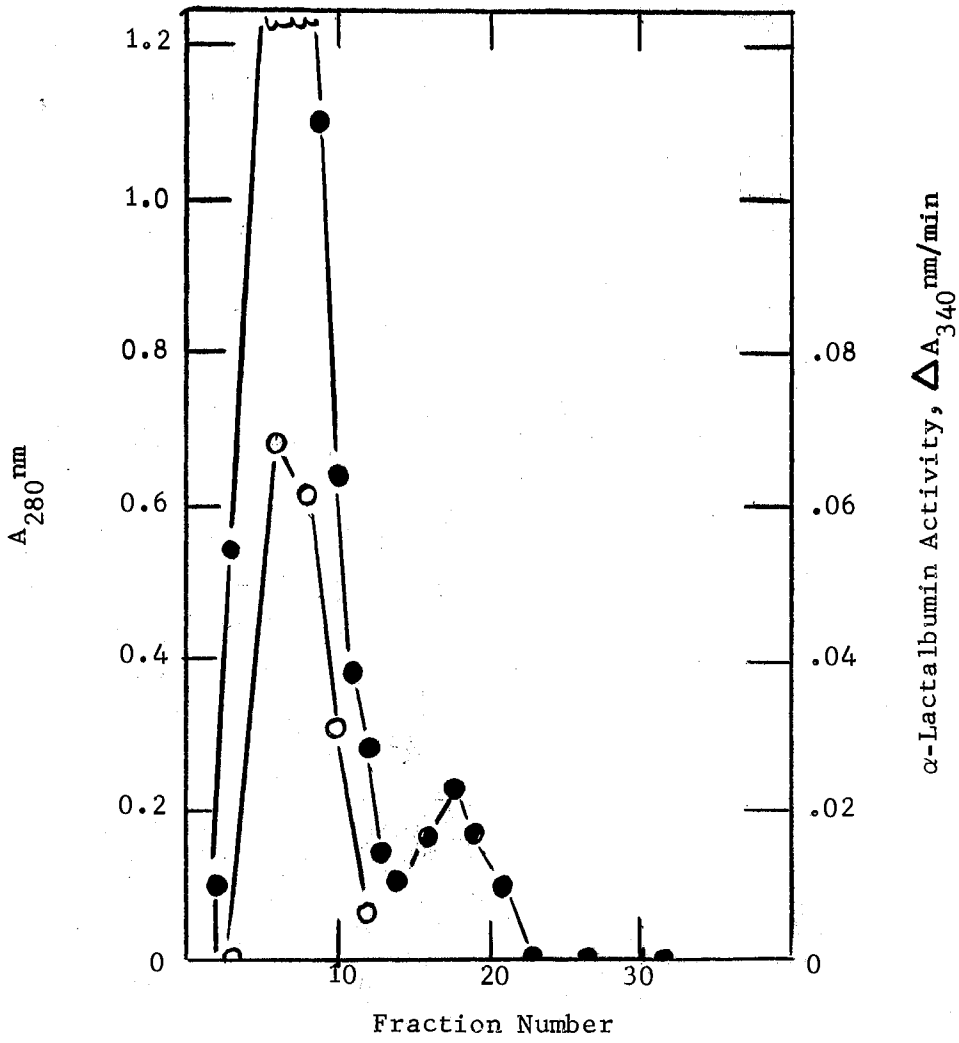
Figure 9. DEAE-32 Chromatography of the Active Rat Milk α-Lactalbumin Peak Off a Bio-Gel P-30 Column

assay profile of the second peak was hard to explain and may be due to a chromatography problem. Therefore, Peak-2 was rechromatographed two additional times on DEAE-32 (10 ml disposable syringe) once with a gradient of 5 mM to 150 mM Tris, 0.1 M KCl, pH 8.0 (65 mls each) and second with a gradient of 2 mM Tris, 0.1 M KCl, pH 8.0 (180 mls each). Figure 10 shows a typical elution profile that was produced from both gradients. Disc gel electrophoresis on various fractions in the α-lactalbumin peak showed that there were eight protein bands present. The disc gel also showed that a majority of the proteins were of low mobility so the active peak was dialyzed, lyophilized and rechromatographed on a long and narrow P-30 column (1.0 x 56 cm) that was equilibrated in 50 mM Tris, 0.1 M CKl, pH 8.0. This gave separation into two protein peaks only one of which contained α-lactalbumin (Figure 11). Disc gels of various fractions in the active peak still showed that approximately seven proteins were present. The active peak was chromatographed on a Sephadex G-100 column (1.0 x 103 cm) equilibrated and eluted with 50 mM Tris, 0.1 M KCl, pH 8.0. The elution profile is presented in Figure 12. There was better separation of the protein peaks but disc gels on the activity peak still showed that more than one protein was present in the α-lactalbumin peak. The active peak was pooled, dialyzed extensively against de-ionized water and lyophilized. The sample was stored at -15^{o}C in a jar containing Drierite. Disc gel electrophoresis of this sample (Figure 13) showed that three major proteins were present. Measurement of α-lactalbumin in slices of the disc gels showed that two of the proteins contained most of the activity. Also a carbohydrate stain of the same sample subject to disc gel electrophoresis showed that the two active bands (slice 23-28) were



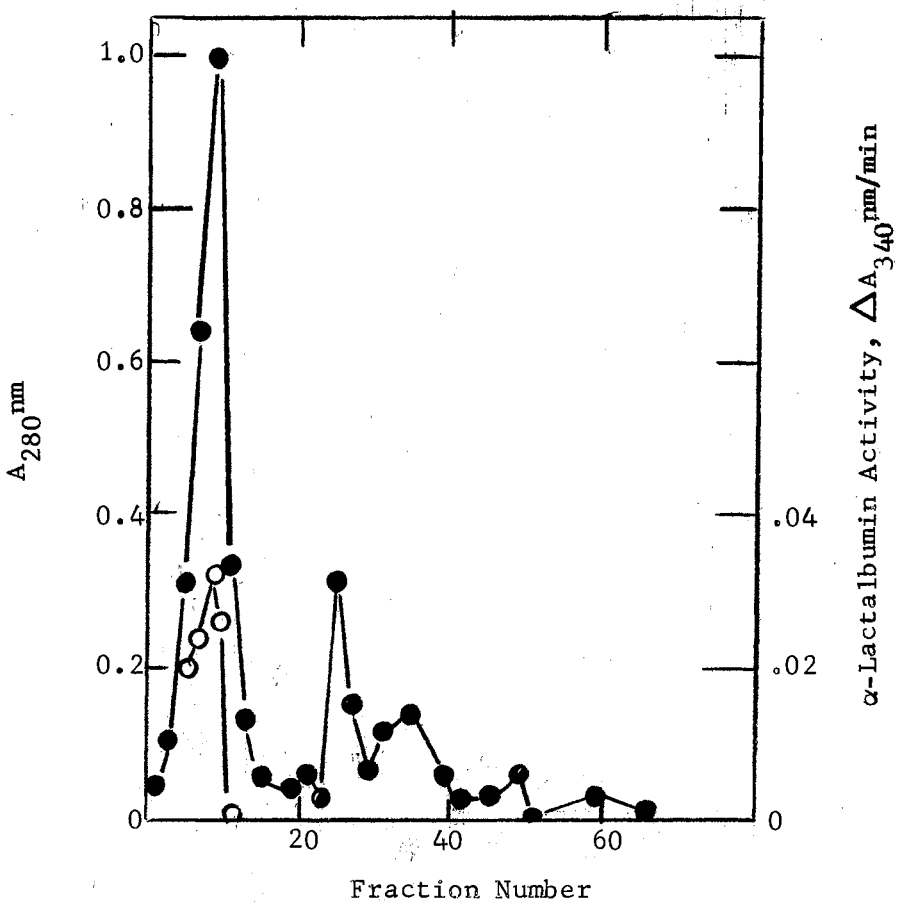
Typical resolution of Peak-2 (rat milk) on a DEAE-32 (10 ml disposable syringe) equilibrated with 2 mM Tris, 0.1 M KCl, pH 8.0 and eluted with a linear gradient of 2 mM to 150 mM Tris, 0.1 M KCl pH 8.0 (180 mls each). Two ml fractions were collected per tube and the absorbance was read at 280 nm (●—●) and α-lactalbumin activity was measured spectrophotometrically at 340 nm (○—○).

Figure 10. Typical DEAE-32 Rechromatograph of Peak-2 from a DEAE-32 Column



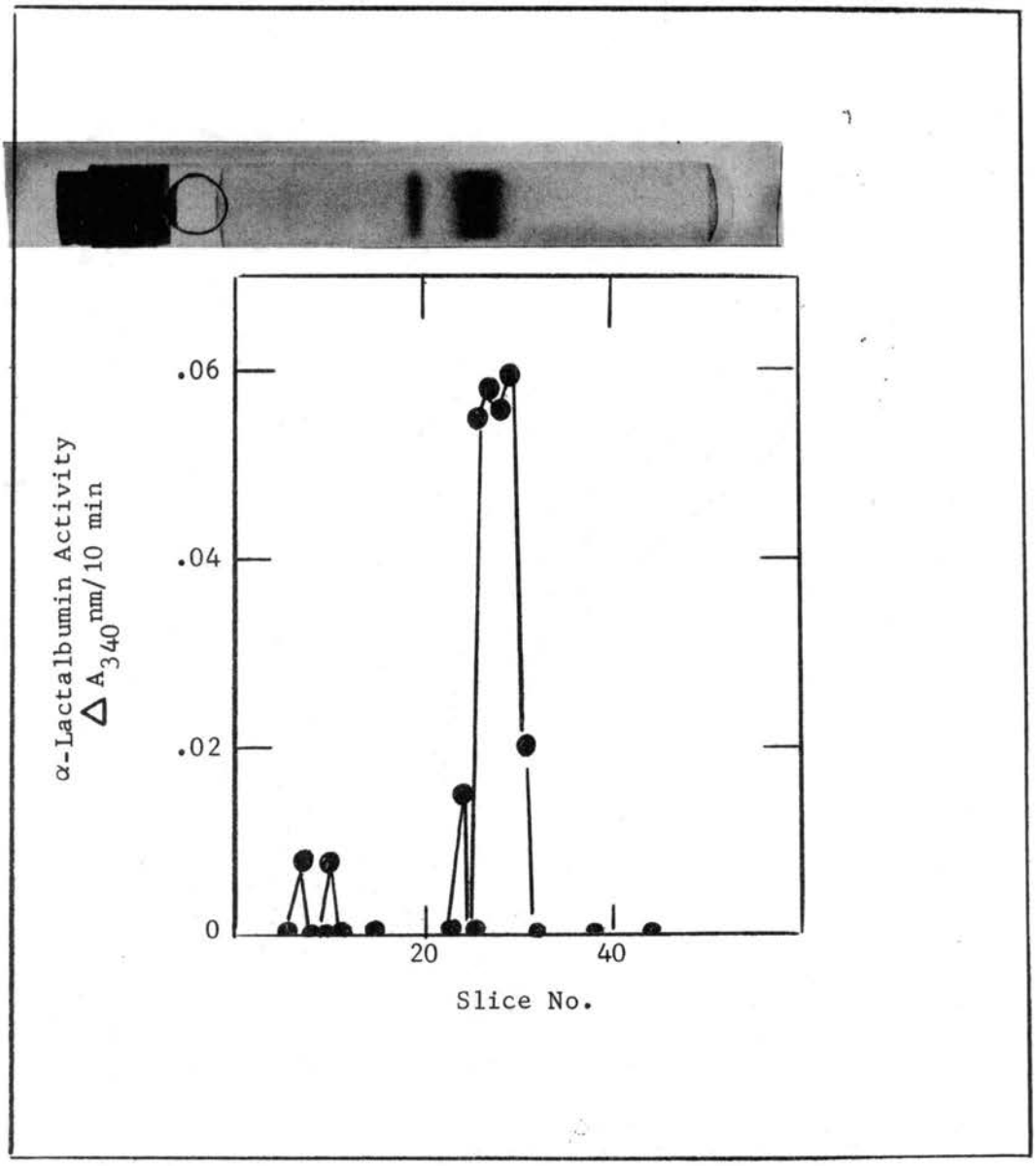
Resolution of Peak-2 from rat milk on a Bio-Gel P-30 column (1.0 x 56 cm) equilibrated and eluted with 50 mM Tris, 0.1 M KCl, pH 8.0. Three ml fractions per tube were collected and the absorbance was read at 280 nm (●–●) and α-lactalbumin activity was followed spectrophotometrically at 340 nm (○–○).

Figure 11. Chromatography of Peak-2 from Rat Milk on a Small Bio-Gel P-30 Column



Resolution of rat milk α-lactalbumin on a Sephadex G-100 column (1.0 x 103 cm) equilibrated and eluted with 50 mM Tris, 0.1 M KCl, pH 8.0. Three ml fractions were collected per tube. Absorbance was read at 280 nm (●—●) and α-lactalbumin activity was followed spectrophotometrically at 340 nm (○—○).

Figure 12. Sephadex G-100 Chromatography of the Active Peak from the Small P-30 Column



Rat milk α-lactalbumin (65 μg) from Peak-2 was run on a 7% disc gel at pH 7.9. The gel was cut into 1 mm slices and macerated in 0.2 ml 100 mM Tris, 0.02% NaAzide, pH 8.0. The macerated slice was then incubated for 10 hrs at room temperature after freezing and thawing at least five times. One hundred μl of each sample were assayed for α-lactalbumin activity at 340 nm. The gel at the top is a duplicate gel run at the same time and stained with Aniline Blue Black.

Figure 13. Assays for Rat Milk α-Lactalbumin from Peak-2 in Disc Gel Slices

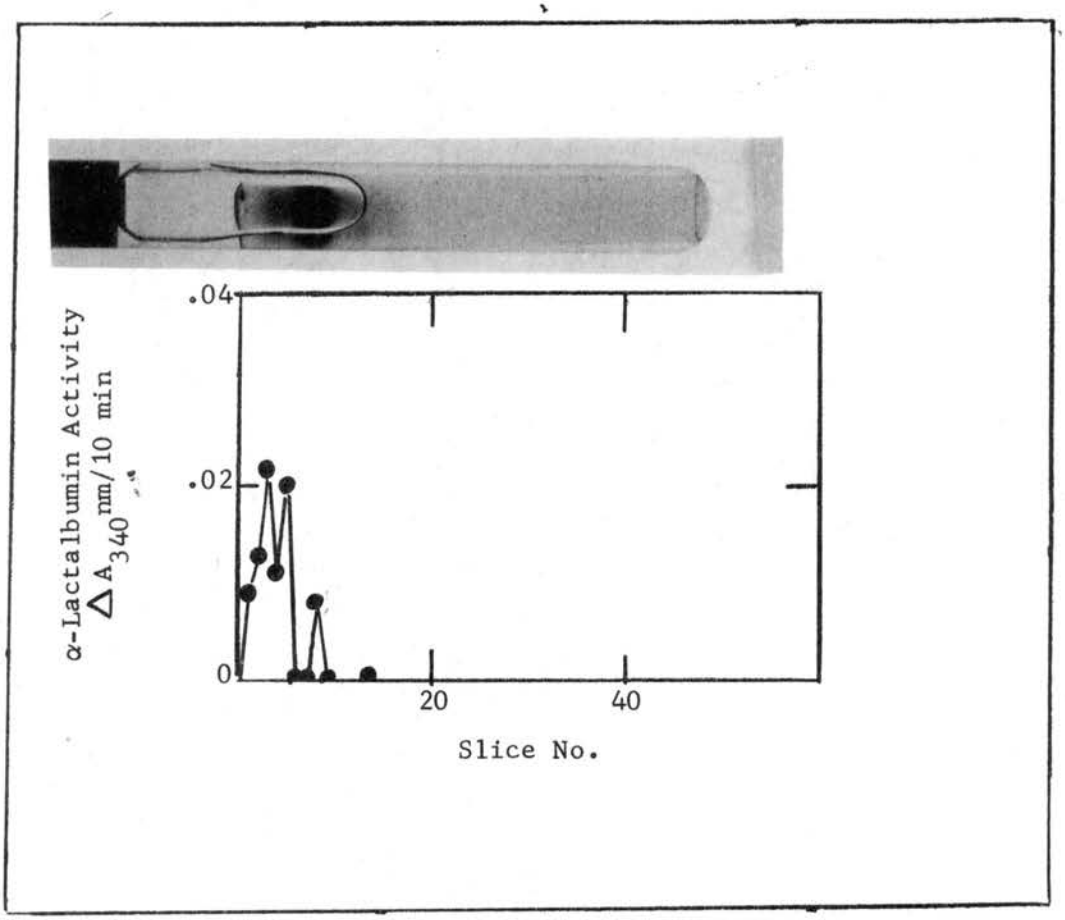
stained and therefore probably contains carbohydrate.

Peak-1 off the first DEAE-32 column (Figure 9) was also extensively dialyzed against de-ionized water and lyophilized. The disc gel pattern showed that the proteins in this peak have a low negative charge because they migrated very slowly (Figure 14) and also the major protein band contained most of the α-lactalbumin activity. The carbohydrate stain of this disc gel showed that all the protein bands contained carbohydrate.

Isolation of Bovine α-Lactalbumin from Disc Gels

The isolation of bovine α-lactalbumin from the large disc gels was effective. After staining the disc's with 0.25% Coomasie Brilliant Blue in 7% acetic acid the bands were cut out and washed in 50 mM NH_4HCO_3 containing 0.1 M KCl three times four hours each at 37°C. The wash was dialyzed against de-ionized water (40 mls sample in 6 l) overnight with at least two changes, lyophilized and placed on a G-25 column (1.0 x 24 cm) that was equilibrated and eluted with 50 mM NH_4HCO_3, pH 7.7. Separation on this column was satisfactory because at pH 7.7 the dye adhered to the column and α-lactalbumin came through in the void volume. A_{280} readings showed only one protein peak and this was in the first twenty mls eluted. The fractions in this peak were dialyzed against de-ionized water for two days with at least four changes and lyophilized. Yields were higher than expected. Eight mgs of α-lactalbumin were added to the four gels but the final lyophilized material weighed 10.2 mgs. The excess weight may be due to acrylamide obtained from the gels during the elution procedure.

Assays for α-lactalbumin showed that the protein was still active and immunodiffusion experiments showed that it would still react with

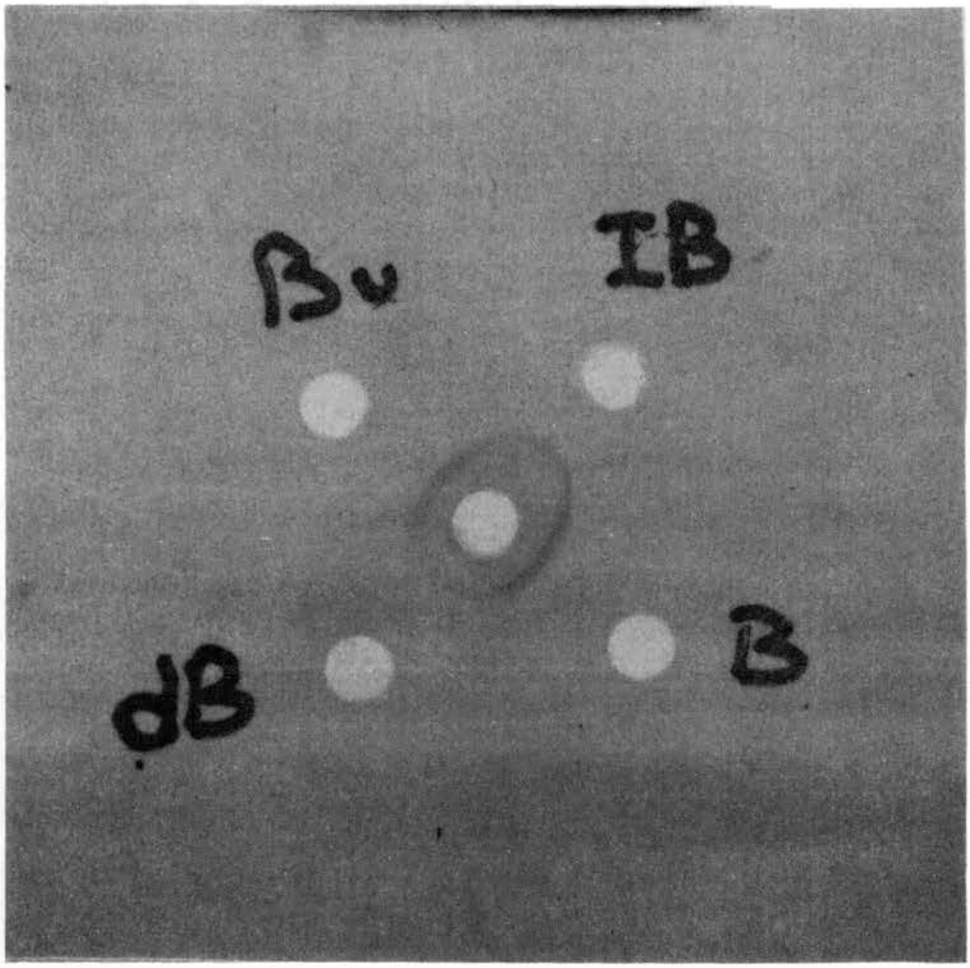


Rat α-lactalbumin (65 μg) from Peak-1 was run on a 7% disc gel at pH 7.9. The gel was cut into 1 mm slices and macerated in 0.2 ml 100 mM Tris, 0.02% NaAzide, pH 8.0. The macerated slices were incubated at room temperature for 10 hrs after freezing and thawing at least five times and then 100 μl were assayed for α-lactalbumin activity. The gel at the top is a duplicate gel run at the same time and stained with Aniline Blue Black.

Figure 14. Assays for Rat Milk α-Lactalbumin from Peak-1 in Disc Gel Slices

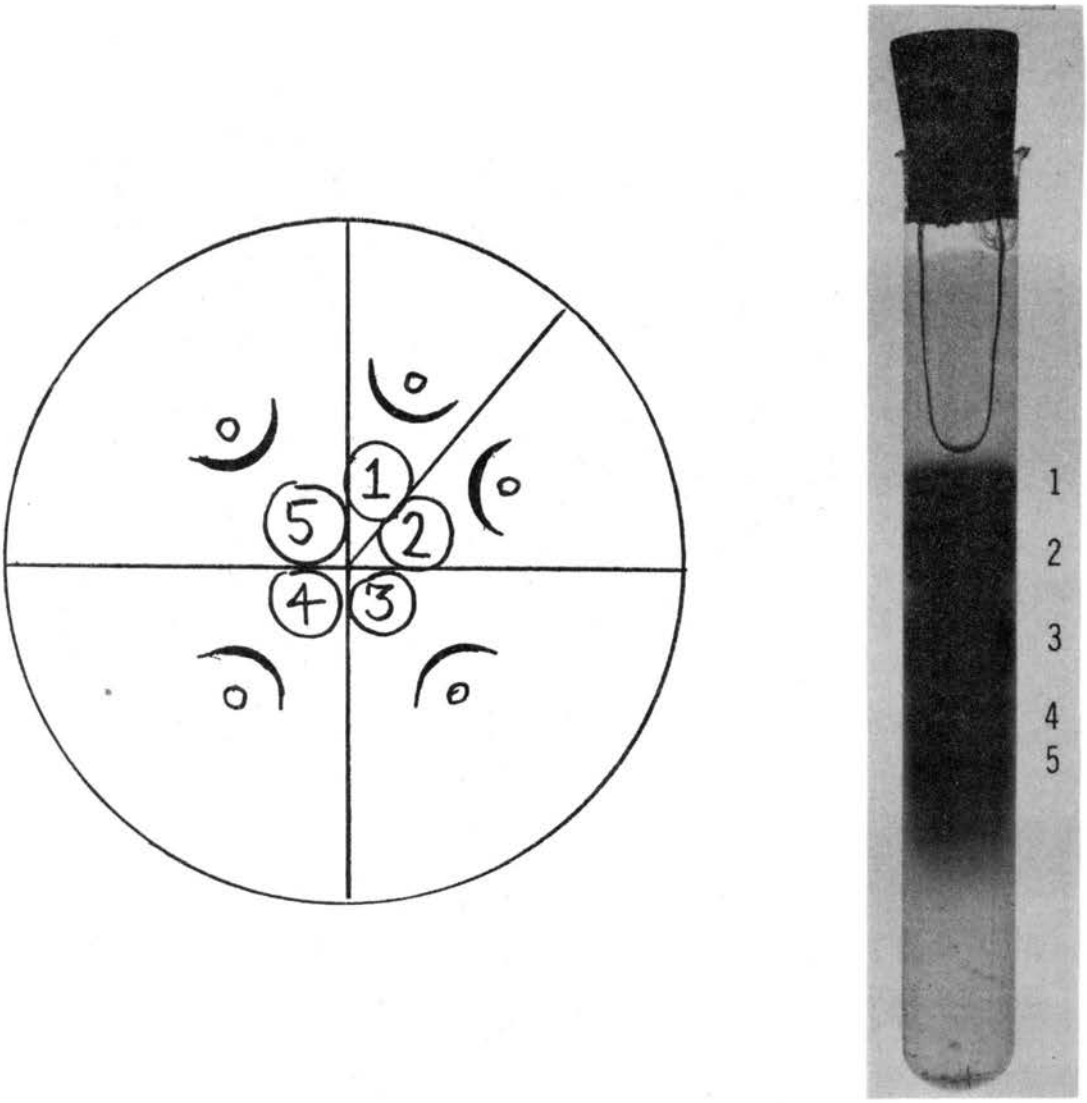
antibodies to bovine α-lactalbumin in the double diffusion precipitin test (Figure 15).

Nitrated bovine α-lactalbumin was also isolated from the disc gels but in a somewhat different manner. In this case the objective was to see if nitrated α-lactalbumin would react with antibodies to native bovine α-lactalbumin. One hundred μl of a 20 mg/ml solution of 110 min nitrated bovine α-lactalbumin made to 10% in sucrose was layered on the large gels and electrophoresis was run until the tracking dye just ran off the bottom. The gels were stained with freshly prepared 0.25% Coomasie Brilliant Blue in 7% acetic acid until the bands could be barely detected (about 15-20 min). The bands were removed by sectioning and placed on the top of freshly poured solid agar plates (petri dish containing 8 mls Noble agar + Trypan Blue). The dye was arranged to provide the correct number of wells and placed on the plate to make holes about 10 mm from each disc. Eight mls of the same agar solution were added to surround the disc pieces and form the antibody wells. After the agar had solidified, antibodies were added to each well and the plates were incubated at 37°C overnight. There are four tyrosyl residues in bovine α-lactalbumin and therefore one should see five bands, i.e. one for native, mono, di, tri, and tetra nitrated α-lactalbumin. All five bands were observed on the disc gels and were cut out for the immunodiffusion assays. The results of the immunodiffusion test are shown in Figure 16. Five bands were seen between the disc antibody well, one for each disc representing the different forms of α-lactalbumin. This experiment showed that all the nitrated forms of α-lactalbumin reacted with antisera to native bovine α-lactalbumin. De-O-acetylated bovine α-lactalbumin also reacted with antibodies to bovine



The center well contains anti-bovine α-lactalbumin and the outside wells have (B) bovine α-lactalbumin, (IB) bovine α-lactalbumin isolated from the large disc gels, and (Bu) buffalo α-lactalbumin. Diffusion was done on 1% Noble agar in borate buffer plus Trypan Blue, at 37° for 18 hrs (see Methods p 24).

Figure 15. Immunodiffusion of Bovine α-Lactalbumin Isolated from Disc Gels and Anti-Bovine α-Lactalbumin



Two mgs of 110 min nitrated bovine α-lactalbumin were added to large disc gels. After electrophoresis the gels were stained in Coomasie Brilliant Blue for 15-20 min and each band was cut out and placed in a agar filled perti dish (see text p 24). Wells were then cut out of the plates about 10 mm from each gel slice and filled with 10 μl of anti-bovine α-lactalbumin antisera. After ten hours the reaction was completed. Native bovine α-lactalbumin is band (1), mono nitrated (2), di (3), tri (4), and tetra (5).

Figure 16. Immunodiffusion of Nitrated Bovine α-Lactalbumin

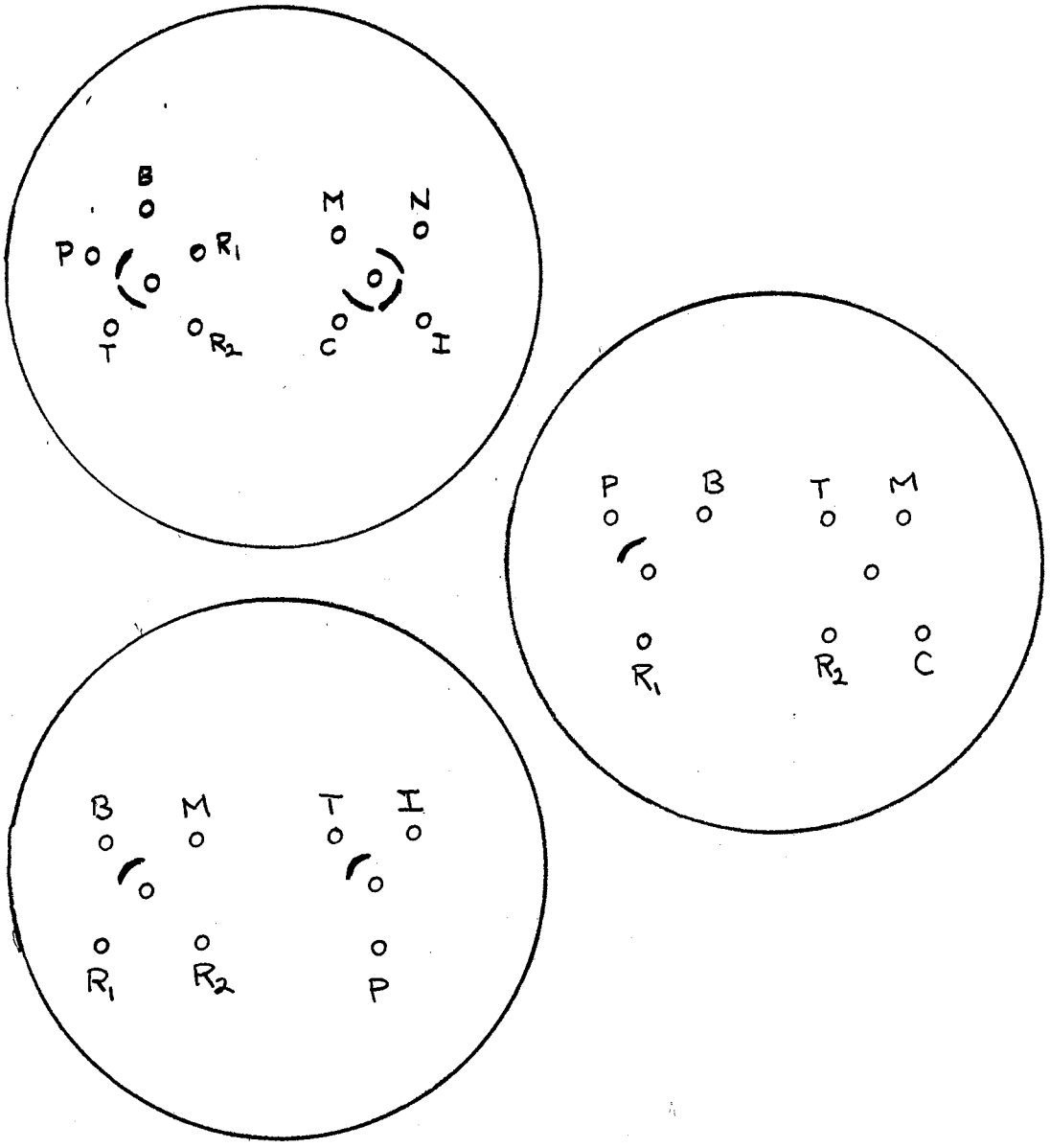
α-lactalbumin and formed a precipitin band. The α-lactalbumin used in this experiment was acetylated as a 2 mg/ml solution of bovine α-lactalbumin in 50 mM Tris, 0.1 M KCl, pH 8.0 by adding dry N-acetylimidazole in a 500-1 ratio and stirring at room temperature for one hour. The reaction was stopped by passing the solution through a Sephadex G-25 column (1 x 24 cm, 50 mM NH_4HCO_3, 0.1 M KCl). The tyrosyl residues were de-O-acetylated by mixing equal volumes of 0.4 M hydroxylamine in 50 mM Tris buffer, pH 7.5 with the acetylated sample for two hours at room temperature. The reaction was stopped by passing through a G-25 column as above. The product contained α-lactalbumin acetylated in the ε-NH_2 group of lysine. Fully acetylated α-lactalbumin was not used because the tyrosyl residues became de-O-acetylated at room temperature.

Immunological Cross Reactions

All the α-lactalbumin's isolated were reacted with antisera to bovine pig, and human α-lactalbumin. Figure 17 describes the results of these experiments which showed that antibodies to human α-lactalbumin reacted with pig, tumor, Negro, Indian and Caucasian α-lactalbumins, antibodies to pig α-lactalbumin reacted with pig α-lactalbumin only and antibodies to bovine α-lactalbumin reacted with bovine and rat tumor α-lactalbumin. There was no cross reaction between the ruminant and non-ruminant species other than rat tumor α-lactalbumin.

Electrophoretic and Spectral Comparisons of Various α-Lactalbumins

The electrophoretic migration of bovine, mouse, rat (Peak-1), rat (Peak-2) and rat tumor α-lactalbumin on disc gels run at the same time

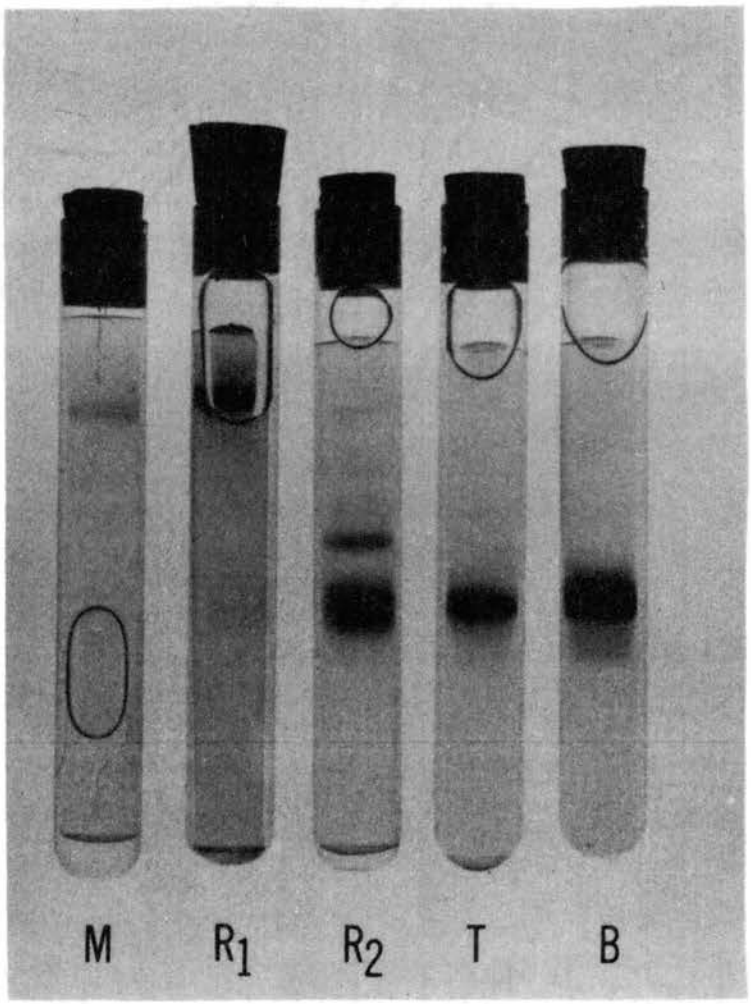


Wells were filled with 10 μl protein solution (0.01 mg/ml to 1.0 mg/ml) or antisera and incubated for 10-18 hrs at 37°C (see text p 24). The α-lactalbumins are (B) bovine, (T) tumor, (R_1) rat milk, Peak-1, (R_2) rat milk, Peak-2, (M) mouse milk, (N) Negro, (I) Indian, (C) Caucasian, and (P) pig.

Figure 17. Immunodiffusion Experiments with α-Lactalbumins from Different Species and Antibodies to Pig, Human and Bovine α-Lactalbumin.

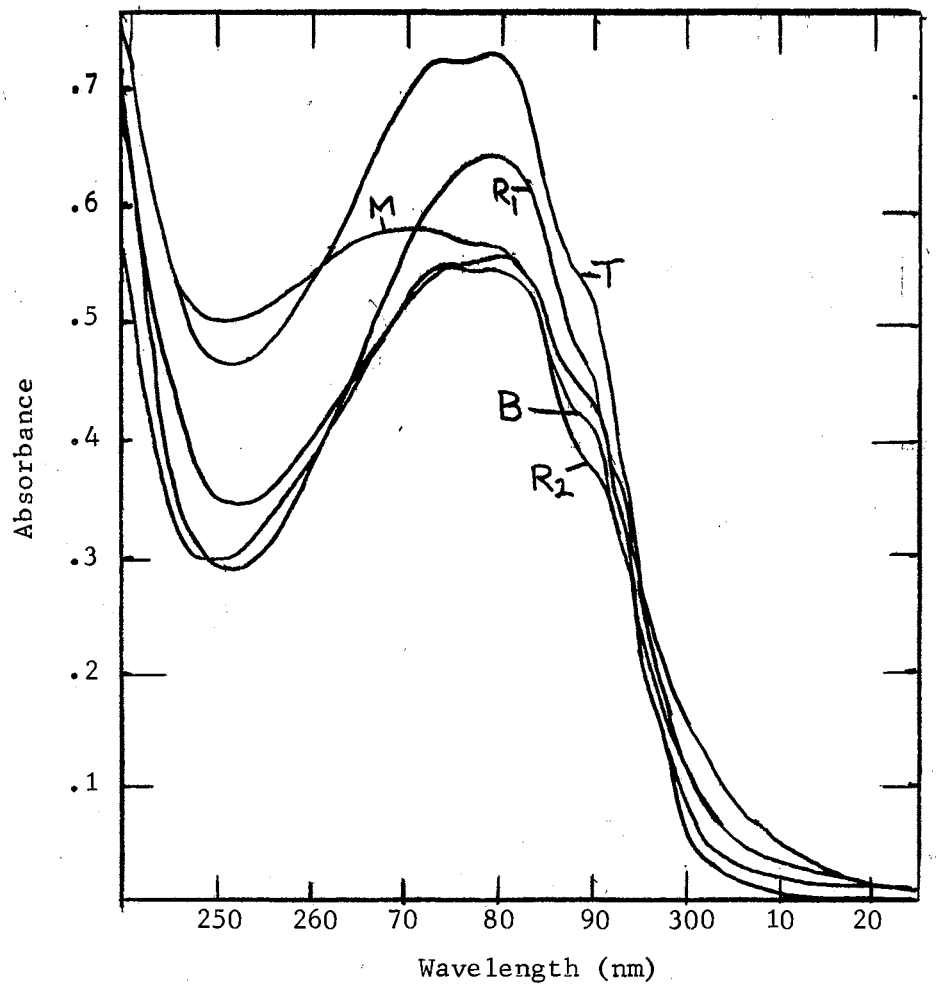
are shown in Figure 18. All of the α-lactalbumins isolated move at a slower rate than bovine α-lactalbumin except for rat milk (Peak-2) and tumor α-lactalbumin. This indicates that they probably have a less negative charge than bovine α-lactalbumin.

The ultra-violet spectra shown in Figure 19 for the various α-lactalbumins are quite similar. They all have peaks at 280 nm and a shoulder at 290 nm. Also all but rat Peak-1 have small peaks at 275 nm.



The disc gels were operated at 5 ma per 7% gel until the tracking dye ran to the bottom. The α-lactalbumins used were labled as: (M) mouse, (R_1) rat milk Peak-1, (R_2) rat milk Peak-2, (T) tumor, and (B) bovine.

Figure 18. Disc Gel Electrophoresis of Various α-Lactalbumins at pH 7.9.



Ultraviolet spectras were run on 0.5 mg/ml solutions in 50 mM Tris pH 8.0 from 320 to 240 nm (M) mouse α-lactalbumin, (R_1) rat milk Peak-1, (R_2) rat milk Peak-2 (T) tumor and (B) bovine α-lactalbumin.

Figure 19. Ultraviolet Spectra of the Purified α-lactalbumins

CHAPTER IV

DISCUSSION

The procedure used for the isolation of mouse, rat and tumor α-lactalbumin was similar to the one described by Schmidt et al. (30) for the isolation of α-lactalbumin from various sources. In general the procedures worked very well, and only a few parts of the procedure were changed for the isolation of α-lactalbumin from the rat milk and tumor.

Some of the problems that did occur in the rat and tumor isolation were probably due to the presence of other proteins that were apparently closely related to α-lactalbumin in molecular weight and overall charge. This was especially true for the isolation of α-lactalbumin from rat milk as it was difficult to purify this α-lactalbumin because the gel filtration and DEAE-cellulose columns did not remove all of the contaminating proteins. This research indicates it would probably be best to use a lower ionic strength elution system on the DEAE-cellulose column so that the α-lactalbumin would be retained on the column.

Yields of α-lactalbumin from all of these procedures were not high per gram tissue or ml of milk plus water. Also it was difficult to obtain large amounts of milk from the rats and mice in order to isolate the α-lactalbumin and as a result the amounts of α-lactalbumin available for these studies was limiting.

Isolating native bovine α-lactalbumin from the large disc gels proceeded well and is a useful technique. The yields were good and the

isolated protein was still active with galactosyltransferase as well as with specific antibodies. Rat (Peak-2), tumor and bovine α-lactalbumins all migrate at about the same rates in the disc gel electrophoresis experiments. However the mouse and rat (Peak-1) α-lactalbumin migrated slower than the other proteins which indicates that they may have a lower negative charge than bovine, rat (Peak-2) and tumor α-lactalbumins.

Only small differences were observed in the ultraviolet spectra of the α-lactalbumins. They all had the typical peaks at 290 nm and 280 nm though there was a small difference in the 280 nm peak for rat (Peak-1) α-lactalbumin.

The α-lactalbumin isolated from Fischer rat R3230 AC tumors was different in several ways from Fischer rat milk α-lactalbumin. The differences are listed in Table 1. These results are interesting because the rat milk was from the same strain in which the tumors were propogated. The fact that rat tumor α-lactalbumin, for example, will react with antibodies to human and bovine α-lactalbumin and rat milk α-lactalbumin will not is also an interesting observation. In general the antigen-antibody reaction in the α-lactalbumin system has been shown to be very specific and usually the α-lactalbumins only react with their homologous proteins (9), though not all the various possibilities have been tested to date. It therefore seems, based on the present evidence, that the α-lactalbumin isolated from the rat mammary tumors is structurally different than that isolated in normal Fischer rat milk.

The immunodiffusion experiments have shown also that α-lactalbumin from the ruminant species will not react with the non-ruminant antisera. However one difference was shown where antibodies to bovine α-lactalbumin will cross react with rat tumor α-lactalbumin, a

non-ruminant. Antibodies to pig α-lactalbumin will not react with bovine α-lactalbumin, antibodies to bovine α-lactalbumin will not react with mouse, rat (Peak-1 and Peak-2), human (Indian) or pig α-lactalbumin and antibodies to human α-lactalbumin will not react with bovine α-lactalbumin. These results are similar to those observed by Tanahashi et al. (9).

TABLE I

SUMMARY OF PROPERTIES OF ISOLATED α-LACTALBUMINS COMPARED TO BOVINE α-LACTALBUMIN

Source	Relative Migration On Disc Gels	No. Of Protein Bands	No. Of Active Bands	CHO Stain	Reactive Antibody
Bovine	Fast	1	1	-	Bovine
Mouse Milk	Slow	3	2	-	None
Rat Tumor	Fast Same As Bovine	1	1	-	Human Bovine
Rat Milk					
Peak-1	Slow	4	2 Major 1 Minor	+	None
Peak-2	Fast Same As Bovine	6	2 Major 2 Minor	+	None

It has been reported that α-lactalbumins of certain ruminant species will cross react with each other (9) and similar results were

observed with buffalo α-lactalbumin in this study. To date, it is more unusual for α-lactalbumins within the non-ruminant species to cross react with antisera from another non-ruminant. However in this study it was observed that rat tumor α-lactalbumin and pig α-lactalbumin will cross react with antibodies to human α-lactalbumin. The immumodiffusion experiments with nitrated bovine α-lactalbumin gave results that may provide some explanation why lysozyme and α-lactalbumin will not cross react with each others antisera. The immunodiffusion experiments have shown that de-O-acetylated bovine α-lactalbumin where nearly all lysines are acetlyated and nitrated bovine α-lactalbumin (mono, di, tri, and tetra) will still react with the antibodies to α-lactalbumin, which would indicate that antibodies to bovine α-lactalbumin do not necessarily bind at the tyrosyl or lysine residues in bovine α-lactalbumin. It has been reported in a study with lysozyme that antibodies to hen's egg-white lysozyme bind at tyrosyl residues 20 and 23 (71). This indicates that some of the difference between the antibodies to hen's egg-white lysozyme and antibodies to bovine α-lactalbumin is that lysozyme antibodies bind at tyrosyls and bovine antibodies do not. These results represent only one small difference in the two antibodies, since there are probably several other binding sites to which the antibodies can bind.

SUMMARY

Isolation of mouse α-lactalbumin was accomplished with very few problems. Yields were rather low and only an average of two mls of milk plus water (25%) could be obtained per mouse. Disc gel electrophoresis of the mouse α-lactalbumin showed that there were three minor impurities present and assays for α-lactalbumin in gel slices show that the major protein band was mouse α-lactalbumin. Also no carbohydrate form of mouse α-lactalbumin could be detected by the carbohydrate stain of the disc gels.

The migration of mouse α-lactalbumin on disc gels was very slow when compared to bovine α-lactalbumin and also it elutes from the DEAE-32 column sooner than bovine α-lactalbumin which indicated that its charge was less negative than bovine α-lactalbumin. The ultraviolet spectrum of mouse α-lactalbumin was similar to that of bovine α-lactalbumin. Immunodiffusion with antisera to bovine, human, and pig α-lactalbumin showed that there was no cross reaction between these species and mouse α-lactalbumin.

Isolation of rat tumor α-lactalbumin required a few more ammonium sulfate fractionations in order to remove more higher molecular weight proteins in the tumor tissue homogenate. Yields per gram of tissue were again low (0.2 mg/gm tissue). From the results of the disc gel electrophoresis experiments it appeared that there were no protein contaminants in the isolated rat tumor α-lactalbumin. Also, rat tumor α-lactalbumin did not appear to contain any carbohydrate by staining of the disc gels.

The migration of rat tumor α-lactalbumin on the disc gels was very similar to that of bovine α-lactalbumin, as was its ultraviolet spectra.

There was no cross reaction with antibodies to pig α-lactalbumin but pig α-lactalbumin did form a pricipitin band with antibodies to human and bovine α-lactalbumin in double diffusion experiments.

Rat milk α-lactalbumin was the most difficult of the α-lactalbumins to isolate. The Bio-Gel P-30 column gave only one protein peak and when this peak was fractionated on the DEAE—cellulose column two active α-lactalbumin peaks were obtained. However, some of this activity may be due to an NADH oxidase or PEP phosphatase. One small active peak (Peak-1) came off the column in the first ten fractions and a larger active peak (Peak-2) came off immediately following the first peak. Peak-2 was chromatographed on several DEAE-32 columns and several gel filtration columns to try to remove the six to eight protein impurities. However all these efforts only removed one or two of these impurities. The major α-lactalbumin activity was always in the first protein peak from the gel filtration experiments and at the end of the one protein peak obtained by ion exchange chromotography on DEAE-32. Peak-1 was lyophilized after the first DEAE-32 column (Figure 7) and disc gel patterns showed only two protein impurities.

Assays for α-lactalbumin on the disc gels showed that the major protein band in each case (Peak-1 and Peak-2) was active. Both forms of α-lactalbumin (Peak-1 and Peak-2) stain for carbohydrate. α-Lactalbumin from Peak-1 migrates slower than bovine α-lactalbumin whereas the α-lactalbumin from Peak-2 migrates at about the same rate. The ultraviolet spectra of both forms were similar to bovine α-lactalbumin although it was possible to observe some small differences

between Peak-1 α-lactalbumin and bovine α-lactalbumin. The immunodiffusion experiments with the rat milk α-lactalbumin showed that there were no cross reactions with the three antibodies tested.

Isolation of bovine α-lactalbumin from the large disc gels gave protein which was still active with bovine galactosyltransferase and it also reacted with the antibodies to native bovine α-lactalbumin. Apparently, some acrylamide from the disc gels was also isolated with the α-lactalbumin, during the isolation procedure, but it could probably be easily separated from the α-lactalbumin by chromatography on a Bio-Gel P-30 column.

Bovine α-lactalbumin nitrated with tetranitromethane for 110 min gave five bands when separated by disc gel electrophoresis. All of these bands when cut out of lightly stained large disc gels and placed in agar filled petri dishes reacted with antibodies to native bovine α-lactalbumin.

The ultraviolet spectra of the mouse, rat (Peak-1 and Peak-2), rat tumor and bovine α-lactalbumin are in general similar. They all have peaks at 280 nm and a shoulder at 290 nm. The spectral curve of rat (Peak-1) α-lactalbumin is a little different than the others. Table 1 summarizes the results found in studies of α-lactalbumins from rat, mouse and bovine milk, and rat tumors.

Immunodiffusion experiments showed that antibodies to pig α-lactalbumin would only react with pig α-lactalbumin. Antibodies to bovine α-lactalbumin would react with bovine, de-O-acetylated bovine, isolated bovine from disc gels, all the nitrated forms of bovine (mono, di, tri, and tetra), buffalo and rat tumor α-lactalbumin. Anti-human α-lactalbumin sera reacted with Caucasian, Negro and Indian

α-lactalbumins. It also reacted with the tumor and pig α-lactalbumins though the tumor α-lactalbumin was slower to react.

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