CHARACTERIZATION OF PHYSICAL AND CHEMICAL PROPERTIES OF α-LACTALBUMIN ISOLATED FROM RAT MILK

By

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CHAPTER I

INTRODUCTION

Enzymes, proteins, carbohydrates, lipids, cations and anions comprise the many constituents of milk and they make it a good source of nourishment for the young of many species. For a short period of time milk can even serve as the sole source of nourishment for the young.

Lactose is the major carbohydrate constituent of most milks, and lactose synthetase is the enzyme system responsible for the biosynthesis of lactose. Lactose synthesis requires two proteins, a galactosyltransferase and α-lactalbumin (1,2,3,4). α-Lactalbumin was suggested by Hill et al. (5) to act as a specifier protein because it could alter the galactosyl acceptor specificity of the galactosyltransferase from N-acetylglucosamine to glucose (6). However, it was shown later that α-lactalbumin is best described as a modifier protein because it lowers the apparent Km for glucose in the galactosyltransferase reaction so that glucose becomes a good substrate.

Studies made on the chemical and physical properties of α-lactalbumin have shown that α-lactalbumin's from different species are closely related. For example, the amino acid composition, molecular weights, and structure of α-lactalbumins are similar (7,8,9,10). There are apparent differences between various α-lactalbumins which can be detected by immunological methods. α-Lactalbumin from ruminant species will not cross react with antibodies made to α-lactalbumin from non-ruminant species.

However, bovine galactosyltransferase will react with α-lactalbumin from non-ruminants to form lactose suggesting that the modifier regions on α-lactalbumins are similar.

The amino acid sequence of bovine α-lactalbumin and hen's egg lysozyme are very similar as shown by Hill et al. (7). However, antibodies to bovine lactalbumin will not react with lysozyme (11). It appears that the antibody determinant groups involved in antibody binding are different between the α-lactalbumin's from various species and bovine α-lactalbumin and lysozyme.

To date α-lactalbumin from rat milk has not been isolated in pure form although McFarland (12) has made some initial studies. The purpose of this study was to isolate α-lactalbumin from rat milk and to characterize some of its chemical and physical properties. Rat α-lactalbumin would be useful for making antibodies to be used in future studies on the biosynthesis of α-lactalbumin in isolated normal and tumor cell suspensions.

CHAPTER II

LITERATURE REVIEW

Lactose Biosynthesis in Mammary Tissue

α-Lactalbumin has received a great deal of attention because of its fundamental role in lactose biosynthesis. α-Lactalbumin found in bovine skim milk contains approximately 70-150 mg per 100 mls of skim milk, and is considered a major component of the whey proteins of milk (13).

The biological function of α-lactalbumin was first described by Ebner and Brodbeck (1,3,4,14,15). Lactose synthetase (E.C. 2.4.1.22.) is the enzyme system responsible for the biosynthesis of lactose, a carbohydrate that makes up half the dry weight of milk. The sequence of reactions of the enzymes (16) that catalyzes the formation of lactose from glucose-1-phosphate are listed below.

1. UTP + glucose-1-P $\rightleftarrows$ UDP - glucose + PPi
2. UDP - glucose $\rightleftarrows$ UDP - galactose
3. UDP - galactose + glucosee $\rightarrow$ lactose + UDP

Reaction 1. is catalyzed by UDP-glucose pyrophosphaylase (UTP: α-D-glucose-1-phosphate uridyl transferase, E.C. 2.7.7.9), reaction 2, by UDP-galactose-4-epimerase (E.C. 5.1.3.2) and the last reaction by lactose synthetase (UDP-galactose; D-glucose-1-galactosyltransferase). Lactose synthetase was first shown to exist in lactating mammary glands of cows and guinea pigs (17) and in bovine milk (18). Lactose synthetase was separated from bovine milk into two protein fractions by Ebner

and Brodbeck (1,2,3). They designated the two protein fractions as A and B proteins according to their separation by gel filtration. The A and B proteins did not have lactose synthesis activity when assayed separately, but when the two proteins were combined, lactose synthesis occurred. It was shown by Hill et al. (19) that the A protein catalyzes the transfer of galactose to N-acetylglucosamine to form N-acetyllactosamine, and that α-lactalbumin inhibited this reaction. They proposed that α-lactalbumin changed the galactosyl acceptor specificity of the A protein from N-acetylglucosamine in its absence to glucose in its presence. They reported no separate enzymatic activity for α-lactalbumin.

Fitzgerald et al. (20) showed that galactosyltransferase catalyzed slowly the formation of lactose (reaction 3) in the absence of α-lactalbumin. α-Lactalbumin reduced the apparent Km for glucose from 1.5 M in its absence to 1 mM in its presence. Klee and Klee (21) reported similar results, and have stated that α-lactalbumin also slightly lowered the Km for N-acetylglucosamine. It should be noted that galactosyltransferase activity is important in the formation of secreted glycoproteins in that it transfers N-acetylglucosamine to galactosyl residues (22).

The steady state kinetics of the lactose synthetase reaction has been studied in detail by Morrison and Ebner (3,4,6,23,24) and they demonstrated that α-lactalbumin participates in the reaction. Morrison and Ebner demonstrated that Mn++, UDP-galactose, and the carbohydrate acceptor of the galactosyl group add to the enzyme in an ordered manner, and α-lactalbumin adds after the substrates and dissociates before product is released. Mn++ reacts with the free enzyme under conditions of thermodynamic equilibrium and does not dissociate after each turn of the

catalytic cycle. At high concentrations, carbohydrate can add randomly to all enzyme forms, but an active complex is not formed unless Mn++ and UDP-galactose have been added previously.

The galactosyltransferase readily reacts with N-acetylglucosamine as the carbohydrate acceptor in the absence of α-lactalbumin as shown in the linear pathway (Figure 1). Where α-lactalbumin is added in increasing amounts, the reaction will proceed along the branched pathway until a lower limiting maximum velocity is reached at an indefinite α-lactalbumin concentration. If glucose is the substrate, and α-lactalbumin is not present, the reaction will proceed along the linear pathway if the glucose concentration is in the range of 1 to 2 molar. If α-lactalbumin is present, the reaction can proceed more rapidly along the branched pathway and results in a reduction of the apparent Km of glucose. It is postulated that the release of UDP is the rate limiting step.

Under maximum conditions of product formation, Schanbacher and Ebner (25) were not able to demonstrate the formation of the α-lactalbumin-galactosyltransferase complex by sucrose gradient centrifugation, equilibrium dialysis, fluorescence quenching, and gel filtration techniques. However, Morrison and Ebner (3,4,24) have shown that α-lactalbumin dissociates from the enzyme prior to product release and suggested that this may be the reason why Schanbacher and Ebner (25) could not demonstrate the α-lactalbumin-galactosyltransferase complex.

Brodbeck and Ebner (4,26) have determined the subcellular distribution of galactosyltransferase and α-lactalbumin. These studies indicated that the B protein (α-lactalbuminn) was found in both the microsomal and soluble fraction, whereas the A protein (galactosyltransferase) was primarily associated with a crude microsomal fraction

(3,4,26). α-Lactalbumin is synthesized on the ribosomes of the rough endoplasmic reticulum (22) and travels through the lumen of the endoplasmic reticulum to the golgi region where it may come in contact with galactosyltransferase to synthesize lactose (4,26). α-Lactalbumin is eventually secreted from the golgi into the milk after its reaction with galactosyltransferase (3).

Brew (27) has proposed that α-lactalbumin may be involved in the control of lactose synthesis because it is released into milk. The rate of α-lactalbumin synthesis could therefore regulate the synthesis of lactose. For example, Schmidt et al. (28) have shown in northern fur seal milk where lactose and α-lactalbumin levels are low that α-lactalbumin and lactose synthetase activity are directly related to the levels of lactose in milk.

Physical and Chemical Properties of α-Lactalbumin from Various Species

Bovine α-lactalbumin is the most thoroughly studied α-lactalbumin. Studies of α-lactalbumin isolated from other species have been completed to varying degrees. Bovine α-lactalbumin is a folded globular protein and possesses about 40% helical structure (29,30) and contains four tryptophanyl residues (31).

It was shown by Robbins et al. (32) that the four tyrosyl residues will ionize under normal conditions, and it has been reported by Gorbunoff (33) that they will react with N-acetylimadazole and cyanofluoride. Denton and Ebner (34) demonstrated that all the tyrosyls are modified by iodination and nitration in a random manner.

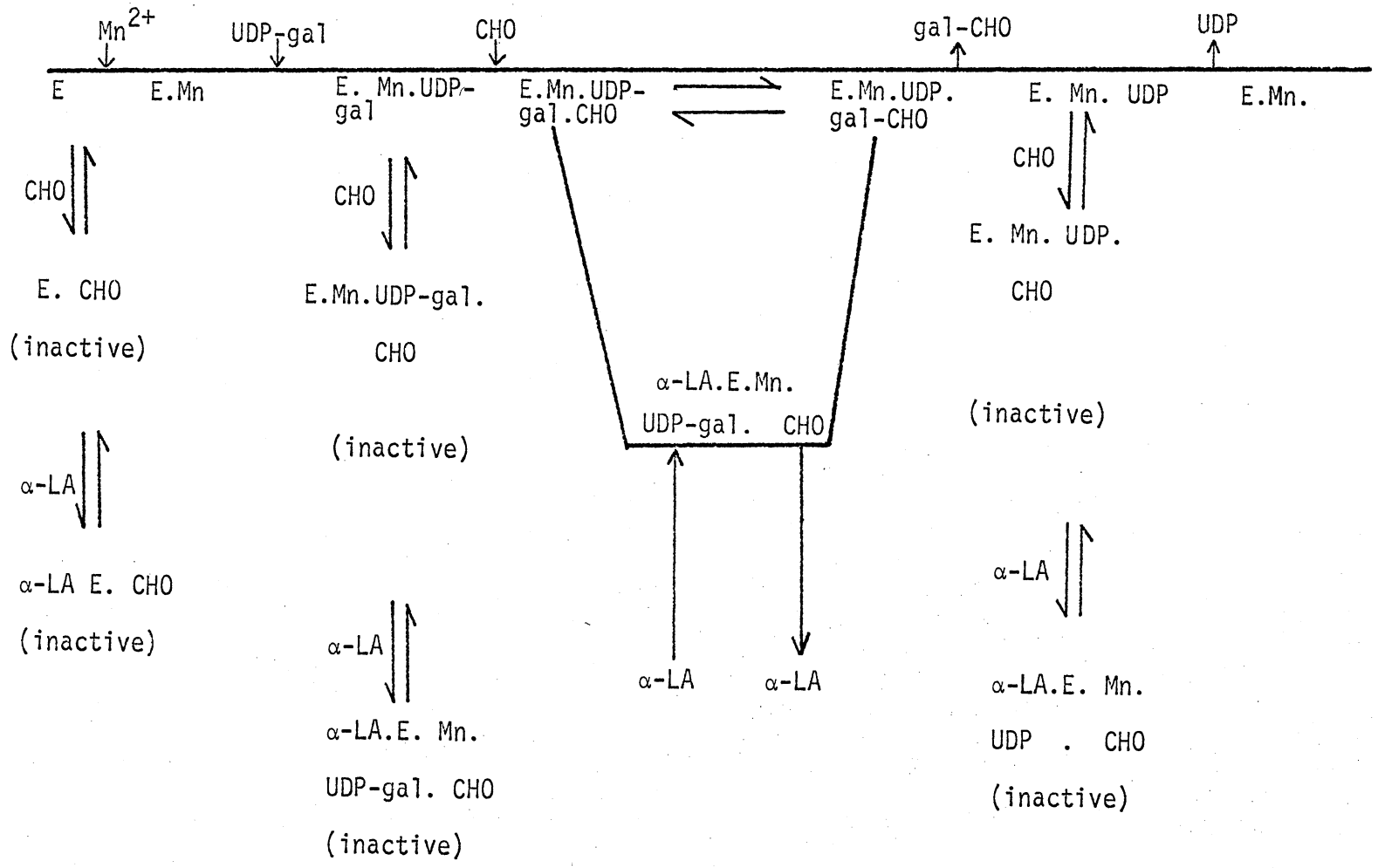

Figure 1. Schematic Representation of Order of Addition of Substrated and Release of Products of the Galactosyltransferase Reaction.

α-Lactalbumin is represented by α-LA, UDP-galactose by UDP-gal, and carbohydrate by CHO.

Zittle (35) and Kronman (36) have shown that bovine α-lactalbumin has an absorbtivity of ($E^{1\%}_{280}$) of 20.9 and 20.1 respectively.

Heterogenity of α-Lactalbumin

Several investigators have reported that α-lactalbumin is heterogenious. A soluble and insoluble form of α-lactalbumin was reported by Zittle and Della-Monica (35) using Gordon and Semmett's (36) procedure for isolation. Kronman et al. (36,37,38,39-43) has studied extensively the heterogeneity of bovine α-lactalbumin at acid and basic pH's. The acid denatured form of α-lactalbumin shows alteration of the tryptophan residues which was reflected as changes in emission (36) and absorbtion spectra (41,42). Also, acid denatured α-lactalbumin shows a marked tendency to associate and aggregate (42,43).

Titration (32) and optical rotary dispersion (38) measurements indicate a comparable structural change above pH 10. The denaturation process at the alkaline pH results in a swollen molecule, but appears less complete when denatured by the acid process. α-Lactalbumin at the alkaline pH shows only a tendency to associate (43), whereas the acid denatured molecule shows low solubility, exhibits time dependent aggregation, and an enhanced tendency to associate (42,43). Differences between the two forms in hydrophobic and hydrophilic groups on the molecular surface may account for the absence of aggregation at the alkaline pH. Optical rotary dispersion spectra indicate the molecule is less disrupted at a higher pH than the lower pH values.

Barman (44) reported that bovine milk contains two α-lactalbumin components that can be separated by anion exchange chromatography. The major α-lactalbumin peak has a molecular weight of 14,437 (5). The minor peak is glyco-α-lactalbumin, a glycoprotein with a molecular

weight of 16,800 and contains 11 to 12 sugar residues per mole of protein. The glyco-α-lactalbumin represents about 15% of the total and it is as active with galactosyltransferase as the α-lactalbumin found in the major peak (44).

Two genetic types of bovine α-lactalbumin have been identified, α-lactalbumin A and α-lactalbumin B. These two types of α-lactalbumins have been classified as genetic variants A and B respectively (45). The A gene is responsible for α-lactalbumin A synthesis and the B gene synthesizes α-lactalbumin B. Blumberg and Tombs (46) have shown by paper electrophoresis that two types of α-lactalbumin occur in Fulani cattle. α-A is the fastest moving band and α-B is the slower moving band. These two types of α-lactalbumins are found in a number of other cattle and Gordon et al. (10) have shown that the two variants are identical in amino acid composition, except for the absence of an arginine residue of α-B which is replaced by an arginine residue or by a glutamic acid in α-A. The two genetic variants are indistinguishable in their enzymatic activity with respect to the lactose synthetase reaction (47).

Schmidt and Ebner (8) have made a detailed study of goat, pig, human and sheep α-lactalbumins. They found that the α-lactalbumins of pig, sheep, and goat separate into two bands of 7% disc gels at pH 7.5, whereas human α-lactalbumin did not.

Structural Similarities Between Bovine and Other α-Lactalbumins

The structure of bovine α-lactalbumin is known to a great extent, and it is possible to compare it with other species. It was found from

the detailed study made by Schmidt and Ebner (8) that goat, pig, sheep and human α-lactalbumins have similar ultraviolet spectra, as well as similar molecular weights of 14,500 ± 500. Hill and his co-workers (7) have completed the amino acid sequence of bovine α-lactalbumin as well as the location of the disulfide bonds (7,48). Figure 2 shows the location of the disulfide bonds and covalent structure of bovine α-lactalbumin. Yasunabu and Wilcox (9) demonstrated that bovine α-lactalbumin was a single peptide chain, and the N-terminal residue was glutamic acid and the C-terminal residue was leucine.

Brew, et al. (19) suggested that the α-lactalbumins from other 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 (Table 1). Gordon (10) has analyzed the amino acid composition of α-lactalbumin A prepared from milks of Indian Zebu cows and α-lactalbumins from water buffalo and found only minor differences.

The various α-lactalbumins have similar amino compositions, locations of disulfide bonds, molecular weights (7,8,9,10,48), and similar activities with the same galactosyltransferase (47) which indicates very little gross structural differences. However, peptide mapping (49) and immunological cross reactivity (47,50,51) have indicated some differences in tertiary structure.

Purification of α-Lactalbumin

In 1936 Pederson (52) described a slow moving peak in the sedimentation velocity experiments of whey proteins as the "α-peak," and in the same year Kekwick (53) isolated and crystallized the protein that

appeared to be responsible for this peak. Svedberg and Pederson (52) stated that the slow moving "α-peak" was α-lactalbumin.

Zwieg and Block (54) isolated α-lactalbumin by ferric chloride precipitation of the whey proteins and subsequently isolated α-lactalbumin from this precipitate (36). This method was not used frequently because it required that the solution must be adjusted to pH 1.3. Gordon and Ziegler (36,55,56) used several methods for isolating α-lactalbumin that are the basis for some of the current procedures. Their procedure requires that the casein be precipitated from skim milk by lowering the pH of the solution to 4.6. The whey proteins are precipitated with ammonium sulfate, and then α-lactalbumin, β-lactoglobulin and other proteins beside the crude globulins are precipitated at 80% saturation. The crude α-lactalbumin is precipitated from a 30% ammonium sulfate fractionation and subsequently crystallized by ammonium sulfate.

Brodbeck and Ebner (1,2) used column chromatography such as DEAE Cellulose and Bio-gel P-30 to remove minor protein contaminants and were able to prepare highly purified α-lactalbumin.

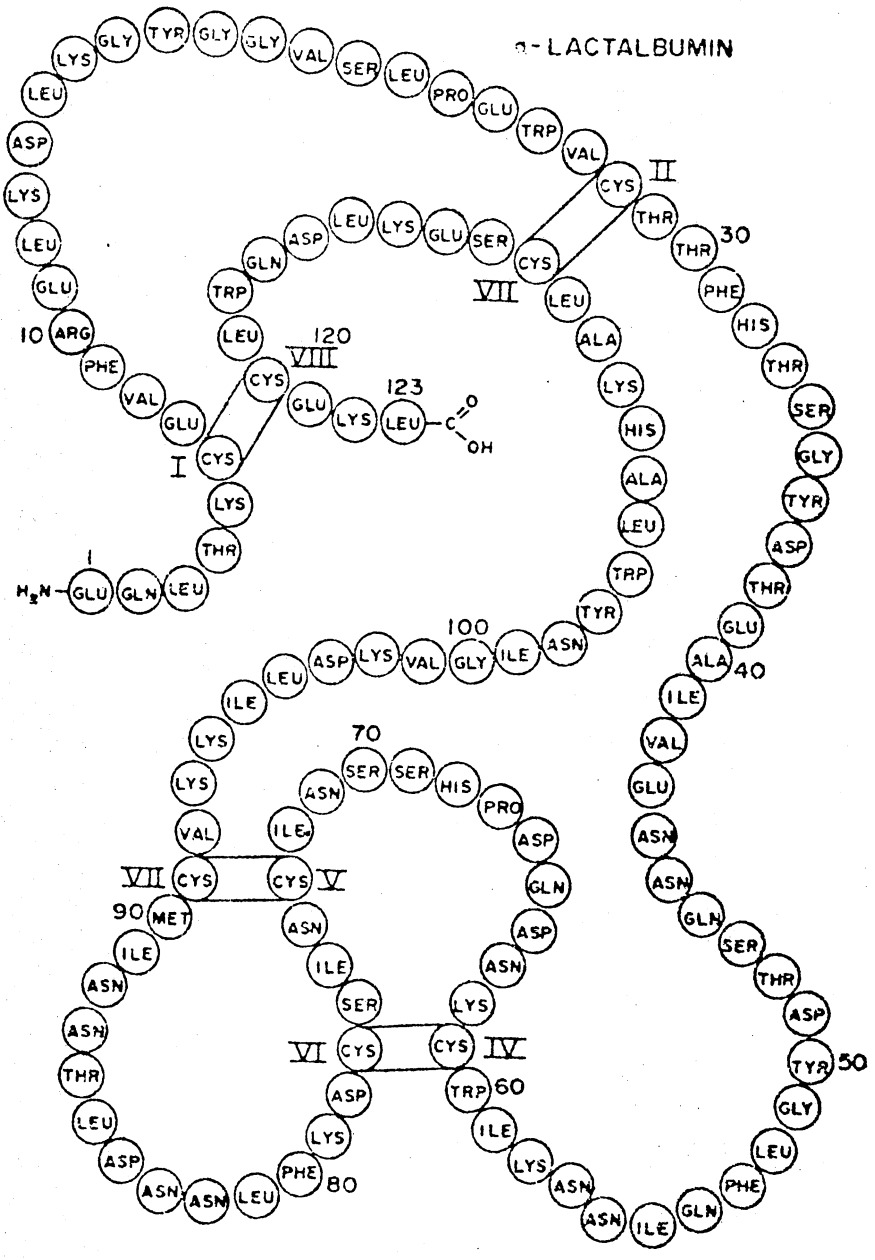

Figure 2. Covalent Structure of Bovine α-Lactalbumin

TABLE I

AMINO ACID SEQUENCE OF BOVINE, GUINEA PIG, HUMAN AND KANGAROO (PARTIAL)
α-LACTALBUMINS COMPARED TO HUMAN LEUKEMIC AND CHICKEN LYSOZYMES

	1	5	10	15
BαLA	Glu-Gln-Leu-Thr-Lys-Cys-Glu-Val-Phe-Arg-Glu-Leu-Lys-			-Asp-Leu-
GPαLA	Lys-Gln-Leu-Thr-Lys-Cys-Ala-Leu-Ser-His-Glu-Leu-Asn-			-Asp-Leu-
HαLA	Lys-Gln-Phe-Thr-Lys-Cys-Glu-Leu-Ser-Gln-Leu-Leu-Lys-			-Asp-Ile-
KαLA	Ile-Asp-Tyr-Arg-Lys-Cys-Gln-Ala-Ser-Gln-Ile-Leu-Lys-Glu-His-Gly-Met-			
H Ly	Lys-Val-Phe-Glu-Arg-Cys-Glu-Leu-Ala-Arg-Thr-Leu-Lys-Arg-Leu-Gly-Met-			
C Ly	Lys-Val-Phe-Gly-Arg-Cys-Glu-Leu-Ala-Ala-Ala-Met-Lys-Arg-His-Gly-Leu-			

	1	5	10	15

	20	25	30
BαLA	Lys-Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Thr-Phe-His-		
GPαLA	Ala-Gly-Tyr-Arg-Asp-Ile-Thr-Leu-Pro-Glu-Trp-Leu-Cys-Ile-Ile-Phe-His-		
HαLA	Asp-Gly-Tyr-Gly-Gly-Ile-Ala-Leu-Pro-Glu-Leu-Ile-Cys-Thr-Met-Phe-His-		
KαLA	Asp-Lys-Val-	-Ile-Pro-Leu-Pro-Glu-Leu-Val-Cys-Thr-Met-Phe-His-	
H Ly	Asp-Gly-Tyr-Arg-Gly-Ile-Ser-Leu-Ala-Asn-Trp-Met-Cys-Leu-Ala-Lys-Trp-		
C Ly	Asp-Asn-Tyr-Arg-Gly-Tyr-Ser-Leu-Gly-Asn-Trp-Val-Cys-Ala-Ala-Lys-Phe-		

	20	25	30

TABLE I (Continued)

```
                    35                  40                  45
BαLA    Thr-Ser-Gly-Tyr-Asp-Thr-Glu-Ala-Ile-Val-Glu-Asn-        -Asn-Gln-Ser-Thr-
GPαLA   Ile-Ser-Gly-Tyr-Asp-Thr-Gln-Ala-Ile-Val-Lys-Asn-        -Ser-Asn-His-Lys-
HαLA    Thr-Ser-Gly-Tyr-Asp-Thr-Gln-Ala-Ile-Val-Glu-Asn-        -Asn-Gln-Ser-Thr-
KαLA    Ile-Ser-Gly-Leu-Ser-Pro-Gln-Ala-Glu-Val-
H Ly    Glu-Ser-Gly-Tyr-Asn-Thr-Arg-Ala-Thr-Asn-Tyr-Asn-Ala-Gly-Asp-Arg-Ser-Thr-
C Ly    Glu-Ser-Asn-Phe-Asn-Thr-Gln-Ala-Thr-Asn-Arg-Asn-Tyr-    -Asp-Gly-Ser-Thr-

          35                  40                  45                  50

            50                  55                  60                  65

BαLA    Asp-Tyr-Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Ile-Trp-Cys-Lys-Asn-Asp-Gln-Asp-
GPαLA   Glu-Tyr-Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Asp-Phe-Cys-Glu-Ser-Ser-Thr-Thr-
HαLA    Glu-Tyr-Gly-Leu-Phe-Gln-Ile-Ser-Asn-Lys-Leu-Trp-Cys-Lys-Ser-Ser-Gln-Val-
H Ly    Asp-Tyr-Gly-Ile-Phe-Gln-Ile-Asn-Ser-Arg-Tyr-Trp-Cys-Asn-Asp-Gly-Lys-Thr-
C Ly    Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg-Trp-Trp-Cys-Asn-Asp-Gly-Arg-Thr-

                    55                  60                  65

                    70                  75                  80

BαLA    Pro-His-Ser-Ser-Asn-Ile-Cys-Asn-Ile-Ser-Cys-Asp-Lys-Phe-Leu-Asn-Asn-Asp-
GPαLA   Val-Gln-Ser-Arg-Asp-Ile-Cys-Asp-Ile-Ser-Cys-Asp-Lys-Leu-Leu-Asn-Asp-Asn-
HαLA    Pro-Gln-Ser-Arg-Asn-Ile-Cys-Asp-Ile-Ser-Cys-Asp-Lys-Phe-Leu-Asn-Asp-Asn-
H Ly    Pro-Gly-Ala-Val-Asn-Ala-Cys-His-Leu-Ser-Cys-Ser-Ala-Leu-Leu-Gln-Asp-Asn-
C Ly    Pro-Gly-Ser-Arg-Asn-Leu-Cys-Asn-Ile-Pro-Cys-Ser-Ala-Leu-Leu-Ser-Ser-Asp-
          70                  75                  80                  85
```

TABLE I (Continued)

	90 95
BαLA	Leu-Thr-Asn-Asn-Ile-Met-Cys-Val-Lys-Lys-Ile-Leu- -Asp-Lys-Val-
GPαLA	Leu-Thr-Asn-Asn-Ile-Met-Cys-Val-Lys-Lys-Ile-Leu- -Asp-Ile-Lys-
HαLA	Ile-Thr-Asn-Asn-Ile-Met-Cys-Ala-Lys-Lys-Ile-Leu- -Asp-Ile-Lys-
H Ly	Ile-Ala-Asp-Ala-Val-Ala-Cys-Ala-Lys-Arg-Val-Arg- -Asp-Pro-Gln-
C Ly	Ile-Thr-Ala-Ser-Val-Asn-Cys-Ala-Lys-Lys-Ile-Val-Ser-Asn-Gly-Asp-
	90 95 100
	100 105 110 115
BαLA	Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Ala-Leu-Cys-Ser-Glu-Lys-Leu-Asp-
GPαLA	Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Pro-Leu-Cys-Ser-Asp-Lys-Leu-Glu-
HαLA	Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys-Ala-Leu-Cys-Thr-Glu-Lys-Leu-Glu-
H Ly	Gly-Ile-Arg-Ala-Trp-Val-Ala-Trp-Arg-Asn-Arg-Cys-Gln-Asn-Arg-Asp-Val-
C Ly	Gly-Met-Asn-Ala-Trp-Val-Ala-Trp-Arg-Asn-Arg-Cys-Lys-Gly-Thr-Asp-Val-
	105 110 115 120
	120
BαLA	Gln-Trp-Leu- -Cys-Glu-Lys-Leu
GPαLA	Gln-Trp-Tyr- -Cys-Glu-Ala-Gln
HαLA	Gln-Trp-Leu- -Cys-Glu-Lys-Leu
H Ly	Arg-Gln-Tyr-Val-Gln-Gly-Cys- -Gly-Val
C Ly	Gln-Ala-Trp-Ile-Arg-Gly-Cys- -Arg-Leu
	125

TABLE I (Continued)

BαLA (bovine α-lactalbumin) (11); GPαLA (guinea pig α-lactalbumin) (14); HαLA (human α-lactalbumin) (13); KαLA (Kangaroo α-lactalbumin) (15); H Ly (human Leukemic lysozyme) (13) and C Ly (chicken lysozyme) (13). The top numbers refer to the sequence of amino acids in bovine α-lactalbumin and the bottom numbers are the sequence of amino acids in chicken lysozyme. The sequences are aligned to give the highest degree of homology.

Comparison of α-Lactalbumin and Hen's Eggwhite Lysozyme

α-Lactalbumin and lysozyme have many characteristics that are similar. It has been reported by Yasunobu and Wilcox (9) that the two proteins have similar molecular weights, the same number of disulfide bonds, similar amino acid composition, and identical or similar amino and carboxy terminal residues. They also reported that α-lactalbumin is readily oxidized by tyrosinase while lysozyme is not oxidized even after treatment with urea. Table 1 presents the amino acid sequences of bovine α-lactalbumin and hens egg lysozyme. Forty-nine residues are the same at corresponding positions and twenty-three are conservative replacements. It has been shown when a α-lactalbumin is theoretically folded into a three dimensional structure, its structure is similar to that of lysozyme (3,4,31).

Although α-lactalbumin does not act upon lysozyme substrates and lysozyme does not participate in lactose biosynthesis; they do act on the same carbohydrate linkage (3,4,19,31). Lysozyme cleaves β-1-4-glucopyranosyl linkages, whereas α-lactalbumin acts with galactosyl-transferase which forms these linkages.

α-Lactalbumin and lysozyme have many physical properties in common. Kronman (36) has shown that the circular dichroic spectra and optical rotary dispersion spectra (57,58) were similar for both proteins. However, the overall charge of the two proteins differs markedly since lysozyme has an isoelectric point at pH 10.5 as compared to α-lactalbumin's isoelectric point at pH 4.8.

The Evolution of α-Lactalbumin and Lysozyme

Due to the fact that α-lactalbumin and lysozyme have similar structures, a hypothesis has been made that they arose from a common ancestral origin. After millions of years of mutational changes in genes, natural selection has produced enzymes with most amino acids unchanged. Two reasons for this phenomena must be the requirement to define the overall shape and dynamic properties of the molecule, and the requirement for the enzyme to be resistant to denaturation under the range of normal conditions for that organism (59).

The α-lactalbumin gene and lysozyme gene could have evolved by convergence of two separate origins, or they could have evolved from a common ancestral gene (3-5). It is currently believed that they evolved from a common ancestral gene. A possibility would be when the milk producing system evolved, a gene mutation occurred. The gene responsible for the carbohydrate degrading enzyme, lysozyme, could have mutated to form the gene which produces α-lactalbumin.

Immunological Studies

Only limited studies have been made on the immunological properties of α-lactalbumin. It was shown by Tanahashi *et al.* (47,50,51) and others that no cross reaction occurred between antisera to α-lactalbumin of ruminant and non-ruminant species. Antisera from bovine α-lactalbumin does not react with pig, guinea pig, and human α-lactalbumins. However, the ruminant α-lactalbumins (bovine, buffalo, sheep, and goat) all reacted with antibodies to bovine α-lactalbumin (47). Sen *et al.* (60) reported similar results with bovine A and B, water buffalo, and goat α-lactalbumin.

The α-lactalbumin antibody binding site is not known. Tanahashi et al. (47) suggested that the immunological sites and active sites are different because ruminant and non-ruminant α-lactalbumin both react with galactosyltransferase in the lactose synthetase reaction but they do not immunologically cross react. Atassi and Habeeb (61) reported a decrease in antibody and enzymatic activity when tyrosine 20 and 23 of lysozyme were nitrated with tetranitromethane. The antigencity returned when the $(NO_2)_2$ - lysozyme was reduced to $(NH_2)_2$ - lysozyme by sodium hydrosulfite. However, the enzymatic activity did not return even though $(NO_2)_2$ - lysozyme and $(NH_2)_2$ - lysozyme appear to have similar conformation states. Atassi and Habeeb (61) postulated that the antibody binds at tyrosines 20 and 23, and these tyrosyl residues are not located at the active site. It also has shown with lysozyme that N-acetyl-glucosamine, an inhibitor of lysozyme, does not bind at the tyrosines 20 and 23 (62). However, Denton and Ebner (34) have shown in bovine α-lactalbumin that trytophan and histidine residues may also be destroyed by nitration and if this occurs in lysozyme, then the antibodies may be binding at these residues. Antibodies to α-lactalbumin do not cross react with lysozyme, nor do antibodies to lysozyme cross react with α-lactalbumin (11,63).

Disulfide Bonds in α-Lactalbumin

α-Lactalbumin has four disulfide bonds as shown in Figure 2 (31,50, 64). Iyer and Klee (65) have reported evidence that all four disulfide bonds of α-lactalbumin are readily reduced with dithiothreitol in aqueous buffer at room temperature in the absence of denaturing reagents, but the disulfides of lysozymes react much more slowly. Dalrymple (66)

has reported that the disulfide bonds in α-lactalbumin under go alkaline hydrolysis upon incubation above pH 10, and the rate of hydrolysis increases as the pH increases with a concomitant loss of activity. Dalrymple (66) also reported that peptic and tryptic digests of the reduced and alkylated α-lactalbumins gave evidence that disulfide 6-120 was the only disulfide reduced with 10 molar excess of dithiothreitol at 0°C in 0.1 M tris pH 7.5. Schechter et al. (67) reported that disulfide 6-120 was reduced by dethioerethreitol at room temperature faster than the other disulfides in α-lactalbumin.

CHAPTER III

ISOLATION AND CHARACTERISTICS OF α-LACTALBUMIN FROM RAT MILK

Experimental Procedure

Materials and Reagents

Rat milk was obtained from Fischer Rats. Oxytocin, Tris (trishydroxymethylaminomethane), glycine, glycylglycine, pyruvate kinase (rabbit muscle type I), SDS (sodium dodecylsulfate) and mercaptoethanol were obtained from Sigma.

Sodium pentobarbital was purchased from Haver-Lockhart Laboratories. Glucose was obtained from Fisher Scientific Products. Temed, acrylamide, bis-acrylanide, and ammonium persulfate used for gel electrophoresis and the Bio-gel P molecular sieves were purchased from Bio-Rad Laboratories. DEAE cellulose 32 was obtained from Whatman. Sucrose was purchased from Mallinkrodt, and dialysis tubing was obtained from National Scientific (Spectrophor Number 3). Ammonium sulfate was obtained from Schwarz/Mann.

Methods

Enzymatic Assays for α-Lactalbumin. The assay used for the detection of α-lactalbumin from rat milk was the same as the method described by Fitzgerald et al. (20) for the determination of bovine α-lactalbumin. α-Lactalbumin was assayed in the presence of saturating amounts of bovine

galactosyltransferase provided by Dr. S. Magee or Dr. C. Geren. One unit of enzymatic activity was defined as the amount of enzyme required to form one nanomole of UTP per minute which was an A_{340} change/min/ml of .0062 under the conditions of the assay. The reaction rate was followed by coupling the formation of UDP to NADH oxidation by adding PEP and pyruvate kinase to the reaction mixture. The rates of reaction were measured on a Gilford 240 spectrophotometer at room temperature. Assay mixtures contained 1.0 mM PEP, 60 µl 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 bovine galactosyltransferase. One hundred microliters of effluents off columns were added to the assay, and the final volume of the assay solution was brought to one ml with water. An endogeneous rate was determined for each assay and subtracted from the total rate to give the true rate of reaction.

Milking Procedures. Female Fischer rats were milked at approximately 14 days after parturition. Before milking, the animal was first injected intraperitoneally with sodium pentobarbital (.05 mg/gm wt). After the rat had become unconscious, 0.2 ml of oxytocin was injected intraperitoneally. Approximately four or five minutes later the teats were rubbed with warm water (70°C) and the suction apparatus was applied to the teats. The collection apparatus consisted of a small vaccine bottle with two lines of PE-200 tubing running out of the top. One top was connected to a small soft latex rubber hose, and the other tubing piece was connected to a water aspirator. Low levels of suction were used to prevent hemorrhaging the tissues.

Approximately 5 ml of milk were obtained from each rat. The milk was kept at 4°C during milk, and frozen after collection.

Extinction Coefficient of α-Lactalbumin Isolated from Rat Milk. α-Lactalbumin isolated from rat milk by Scheme VI was used to determine the extinction coefficient. The purified α-lactalbumin was dialyzed exhaustively against water, lyophilized and placed in a 5 ml volumetric flask which had been dried overnight and weighed to four decimal places. The volumetric flask containing the lyophilized α-lactalbumin was placed in a vacuum oven and dried under vacuum at 40°C at 1 mm Hg for 24 hours. The volumetric flask containing the dried α-lactalbumin was placed in a dried dessicator containing drierite and ultimately weighed to determine the weight of the α-lactalbumin. De-ionized water was added to the volumetric flask to make a final volume of 5 ml. A 0.5 ml aliquoit from the flask was diluted with a solution containing 200 mM KCl and 40 mM Tris, pH 7.5 and the absorbance was read at A_{280} on a Cary 14 spectrophotometer. The final salt concentration of the aliquot was 20 mM Tris and 100 mM KCl. All subsequent concentration calculations of rat α-lactalbumin were based on this absorbance reading.

Discontinuous Gel Electrophoresis. The procedure used for 7.5 % disc gels was the same as that described by Brewer and Ashworth (70). Proteins in the gel were stained with 0.007 % coomassie blue in 40 % methanol and 7.5 % acetic acid.

Disc gels were also stained for carbohydrate. Twelve percent disc gels were prepared by the method of Hedrick and Smith (69) and 100 to 300 μg of sample were applied per gel. After the tracking dye had migrated to the end of the gel; it was marked with a stainless steel pin, and

the gel was stained for glycoproteins by the method of Neveille (70) with the following modifications. The gels did not contain SDS and therefore did not require the 3-4 day wash. Instead, the gels were fixed in 40 % methanol and 7.5 % acetic acid for 24 hours.

To check for the possibilities of impurities, aggregates, and charged isomers, the procedure by Hedrick and Smith (69) was followed. No stacking gel was employed and the gels were stained with coomassie blue.

In all the electrophoresis procedures, the gels were poured in 0.5 cm x 9.5 cm tubes. The tracking dye used was always bromophenol blue, and the gels were ran on a Canalco electrophoresis apparatus. All Disc gels were run in a Tris-glycine buffer at pH 8.9.

Sodium Dodecyl Sulfate Gel Electrophoresis. The ten percent acrylamide gels and samples were prepared and run by the procedure described by Weber and Osborn (71). Both the gel buffer and the reservoir buffer were 55 mM in sodium phosphate pH 7.2-7.4.

Electrophoresis was performed on a Canalco apparatus at 10 milliamperes per gel and the proteins were stained with 0.007 % coomassie blue. The standards used for molecular weight determinations were bovine serum albumin, bovine α-lactalbumin, catalase, human α-globulin, glyceraldehyde dehydrogenase, myoglobin and lysozyme. SDS gels were also stained for carbohydrate by the method of Neville (70).

Enzymatic Activity Measured from Gel Slice. Protein samples were run on 7.5 % acrylamide disc gels as described by Brewer and Ashworth (68). Following electrophoresis, the gel was removed from the gel tube and sliced either in one or two millimeter slices by a Bio-Rad Model 190 gel slicer. The slices were placed in test tubes and frozen at -20°C.

Before thawing the frozen acrylamide gel slices, 100 µl of a solution consisting of 100 mM KCl and 20 mM Tris, pH 7.5, were added to the test tubes containing the gel slices. The acrylamide gel slices were allowed to thaw at room temperature, and 100 µl samples were assayed for α-lactalbumin activity.

Gel Filtration Chromatography. Gel filtration chromatography was accomplished with various acrylamide sizes from the Bio-Rad (P) series. The gels were swollen, deaerated and packed in columns as described in the Bio-Rad gel filtration manual (72). Two methods were used to apply the sample on to the column. The first was by adding ten percent sucrose to the sample, and the second method utilized draining the buffer down to the top of the gel and then carefully layering on the sample (72). Blue dextran (10 mg/ml) was used to determine the void volume and uniformity of packing of the columns. All molecular sieve columns were run at 4°C.

Ion Exchange Chromatography. All ion exchange chromatography was performed with microgranular DEAE 32 purchased from Whatman. The resins were pre-cycled, packed and equilibrated according to the manual published by Reeve Angel and Co. (73). Samples were also equilibrated to the same pH and ionic strength as the starting buffer. Flow rates were obtained by utilizing a peristaltic pump. All ion-exchange columns were run at 4°C.

Spectral Determinations of Rat α-Lactalbumin. Circular dichroism spectra of α-lactalbumin from rat milk were obtained at 24°C with a Cary model 61 spectropolarimeter. The final concentration of the sample solution was calculated from the extinction coefficient of α-lactalbumin from rat milk. All samples were dissolved in a solution consisting of 100 mM KCl and 20 mM Tris, pH 7.5. The far circular dichroism spectra were

scanned from 200 nm to 250 nm at pathlength of 1 mm. The near circular dichroism spectra were run from 245 nm to 330 nm at a pathlength of 2 cm. The data are expressed as mean residue molar ellipticity [θ]. A mean residue weight of 118 was used for calculating the percentage of α-helix, according to the method described by Chen and Yang (74).

Ultraviolet spectra of α-lactalbumin isolated from rat milk were obtained with a Cary 14 spectrophotometer and appropriate base line corrections were made. A sample concentration was used to give an A_{280} reading of approximately 0.8. The spectra were run from 320 nm to 240 nm. The sample was placed in a solution containing 20 mM Tris and 100 mM KCl at pH 7.5.

Amino Acid Composition. Amino acid analyses were determined on α-lactalbumin isolated from rat milk as purified by Scheme V. The amino acid analyses of the rat α-lactalbumin were determined by hydrolyzing the samples in 6 N HCl containing 4 percent thioglycollic acid under reduced pressure for 24 and 48 hours by the method of Matsubara and Saski (75). The analyses were performed on a Beckman 120 C amino acid analyzer by the method of Spackman et al. (76). The data are reported as the average of two 24 and 48 hour hydrolyzates, respectively. These analyses were conducted under the direction of Dr. B. G. Hudson at Oklahoma State University.

Carbohydrate Analysis. The total neutral sugars were determined on the intact protein with the antrone reagent of Roe (77). The standard sugars were a mixture of galactose and mannose in a molar ration of 1:1.

The neutral sugars and hexosamines were released from the protein by hydrolysis in 1 and 2 N sulfuric acid for 4 hours at 100°C in sealed

tubes. The hydrolysate was passed through a column of Dowex 50-X4 (H^+) (200-400 mesh) following the procedure of Spiro (78). Lyophilzation was used to take the effluent and wash to dryness. The Technicon automatic sugar chromatography system described by Lee et al. (79) was used to quantitate the amount of neutral sugars present in the effluent.

The thiobarbituric assay method of Warren (80) was used to determine sialic acid following the hydrolysis of protein with 0.1 N HCl for 1 hr. The carbohydrate analysis was done under the direction of Dr. B. G. Hudson at Oklahoma State University.

Molecular Weight Determination. Sodium dodecyl sulfate electrophoresis described by Weber and Osborn (71) was used to determine molecular weights. The standards used were bovine serum albumin, catalase, human γ-globulin, ovalbumin, glyceraldehyde dehydrogenase, myoglobin, bovine α-lactalbumin and lysozyme. It should be noted that the leading edge of the tracking dye and protein were marked for determination of relative mobilities.

A molecular weight for rat α-lactalbumin was also determined by gel filtration on Bio-Rad 0.5 m agarose. A 1 cm x 96 cm column was packed with 0.5 m agarose gel in 5 mM phosphate, 1 mM mercaptoethanol, pH 7.6. Three mg each of β-lactoglobulin, bovine serum albumin, myoglobin, and ovalbumin were used to standardize the column and blue dextran was used to determine the void volume. A plot of elution volume versus molecular weight of these proteins gave a straight line. Fractions, 1.5 ml, were collected at a flow rate of 13.6 ml/hr.

Two mg of rat α-lactalbumin purified by Scheme VI was chromatographed on this column to determine its molecular weight.

Isolation of Rat α-Lactalbumin from Rat Milk

General Scheme

Disc gel electrophoresis of whole rat milk, Figure 3, shows a number of proteins. Two general methods were used to remove the caseins from rat milk. The first method generally followed the procedure outlined by Schmidt et al. (28) and in the second method, acid precipitation was used to remove the caseins.

In the first method, the cream was separated by centrifugation for 20 minutes at 15,000 x g. The casein fraction was precipitated by centrifugation at 1000,000 x g for 1 hour at 4° C. A typical standard disc gel pattern of the 100,000 x g supernant solution is shown in Figure 4. The supernatant solution was saved and passed through a 0.4 μ millipore filter and clarified further by passage through a 0.22 μ millipore. An 80 % ammonium sulfate precipitation (561 g/1000 ml) was performed on the supernatant solution from the last step. The salt solution was centrifuged at 40,000 x g for 20 minutes. The pellet was redissolved in a minimum volume of 20 mM Tris and 100 mM KCl at pH 7.5. The disc gel patterns are shown by the gel scans in Figure 5. The redissolved pellet of whey proteins was used for subsequent column chromatrography.

In the second method, a 15,000 x g centrifugation for 20 minutes was used to remove the cream. The cream was removed with a spatula and the pH of the rat skim milk was lowered to approximately 4.6. The acidified rat skim milk was centrifuged at 20,000 x g for 20 minutes to remove the precipitated caseins. The pH of the supernatant solution was raised to 7.4 with 0.5 M KOH. The supernatant solution was decanted,

and a scan of a typical disc gel of the decanted supernatant solution is shown in Figure 4. An 80% ammonium precipitation was performed on the supernatant solution. The solution was centrifuged at 40,000 x g for 20 minutes, and the pellet was redissolved in a minimum volume of 20 mM Tris and 100 mM KCl at pH 7.5. The redissolved pellet had enough protein removed so that it could be used for column chromatography.

There were several observations that were made from the patterns of the disc acrylamide gels. The first was that the casein fraction of proteins found in whole rat milk could be removed by either centrifugation at 100,000 x g or by acid precipitation as shown in Figure 3 and 4. The second observation was that a single major protein was observed after the removal of the caseins by a 100,000 x g centrifugation or acid precipitation of the whey proteins as shown by disc electrophoresis (Figure 4). The third and most important observation was that the disc gel patterns of the 100,000 x g supernatant and the supernatant of the acid precipitation (Figure 4) were very similar. This meant that either method could be used to remove the casein fraction in order to prepare the rat milk for column chromatography.

Scheme I

The first purification scheme is outlined as follows. Sixty-five mls of whole rat milk and water were diluted with normal saline (0.89% NaCl in water) to a final volume of 85 mls. The general purification scheme utilizing the 100,000 x g centrifugation was used with the diluted rat milk except that cheese cloth was used instead of millipores for filtering the rat milk after the removal of the caseins. Following the 100,000 x g centrifugation, the amount of protein remaining in the super-

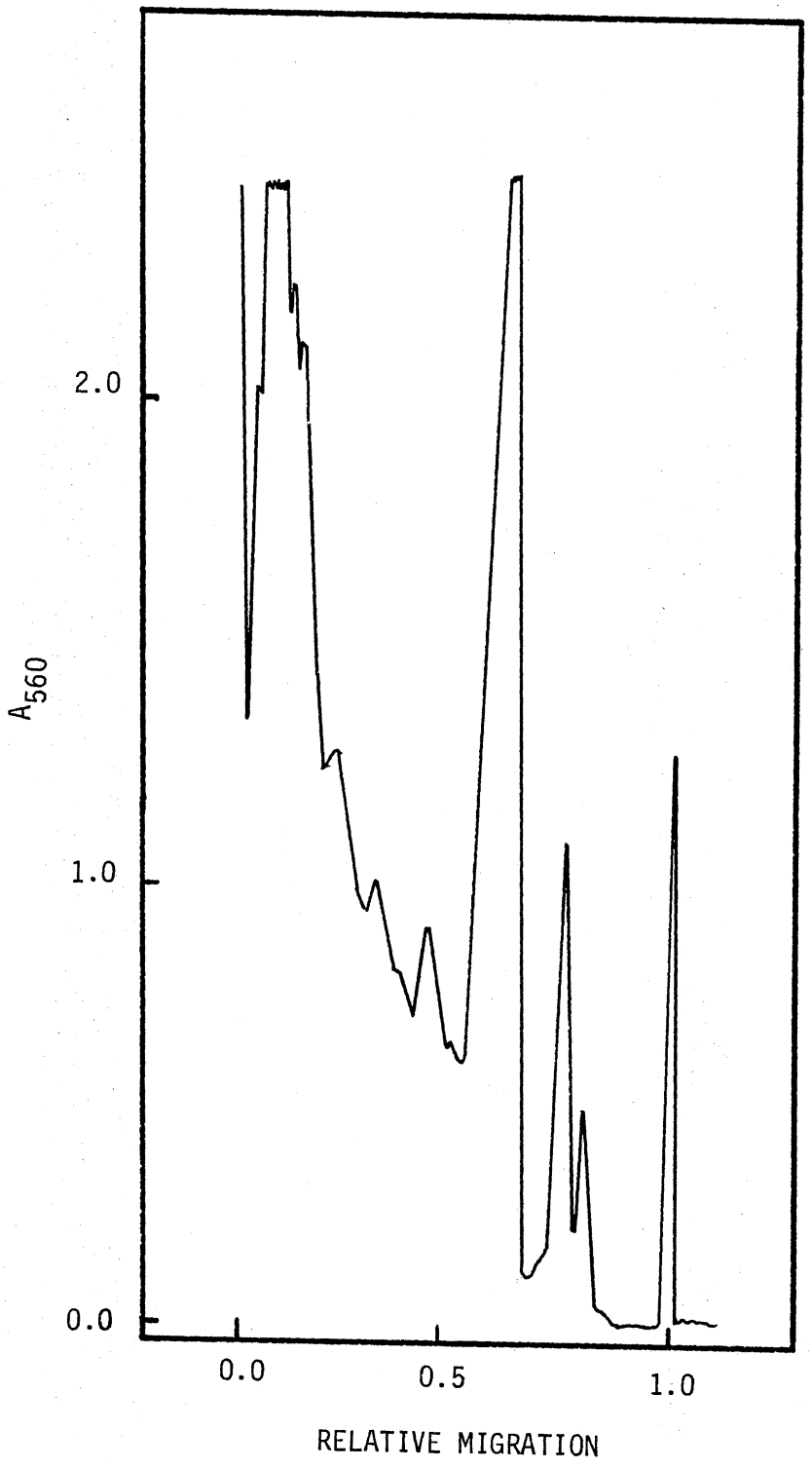

Figure 3. Disc Gel Electrophoresis of Whole Rat Milk at pH 8.9.

Scans of 7.5 percent polyacrylamide disc gels.
The scan of the gel is from whole rat milk (5 µl)
The number 1.0 is the tracking dye.

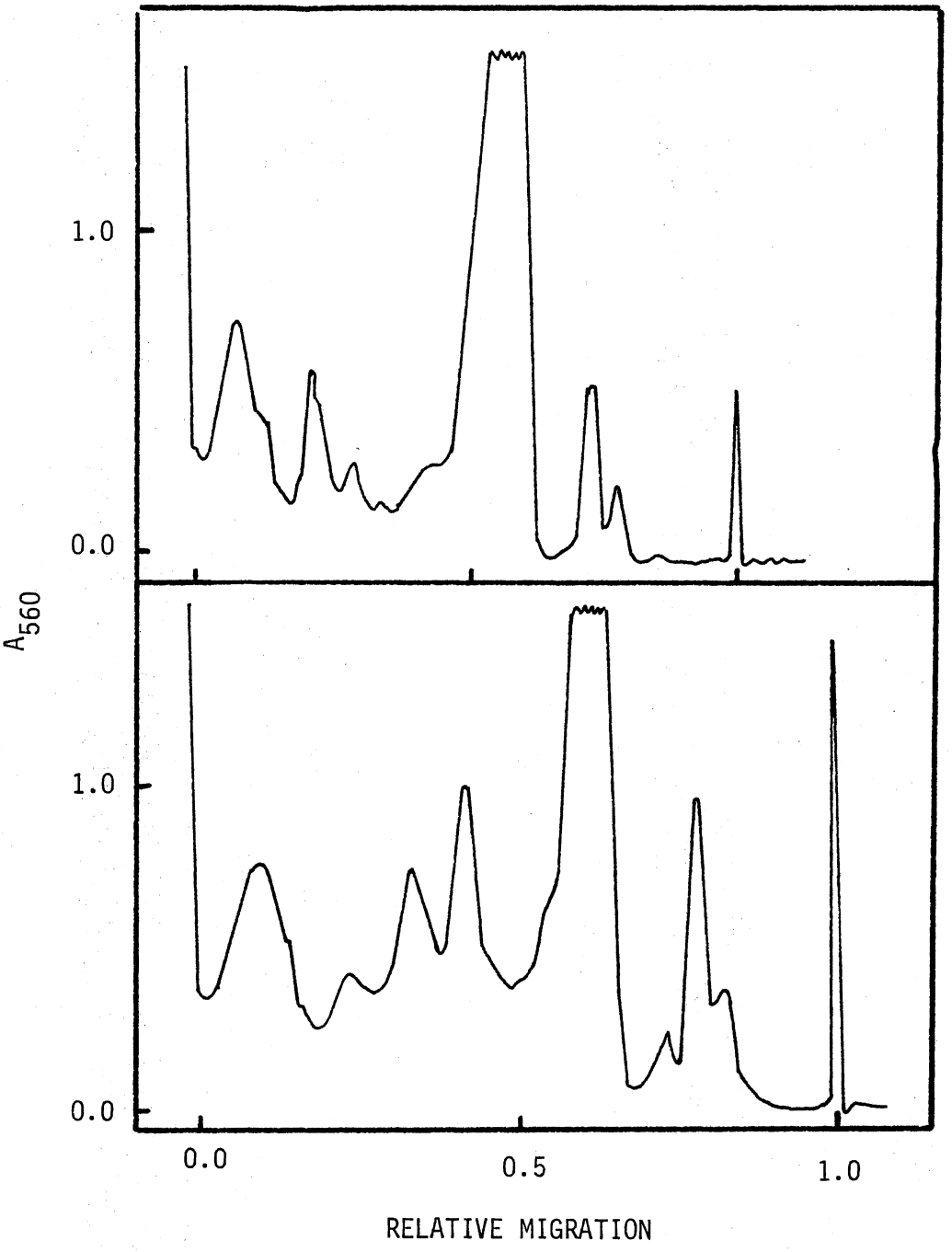

Figure 4. Disc Gel Electrophoresis of Whey Proteins from Rat Milk.

Gel scans of 7.5 percent polyacrylamide disc gels. The gel scans are from (bottom), 100,000 x g centrifugation of rat skim milk, and (top), acid precipitation of rat skim milk.

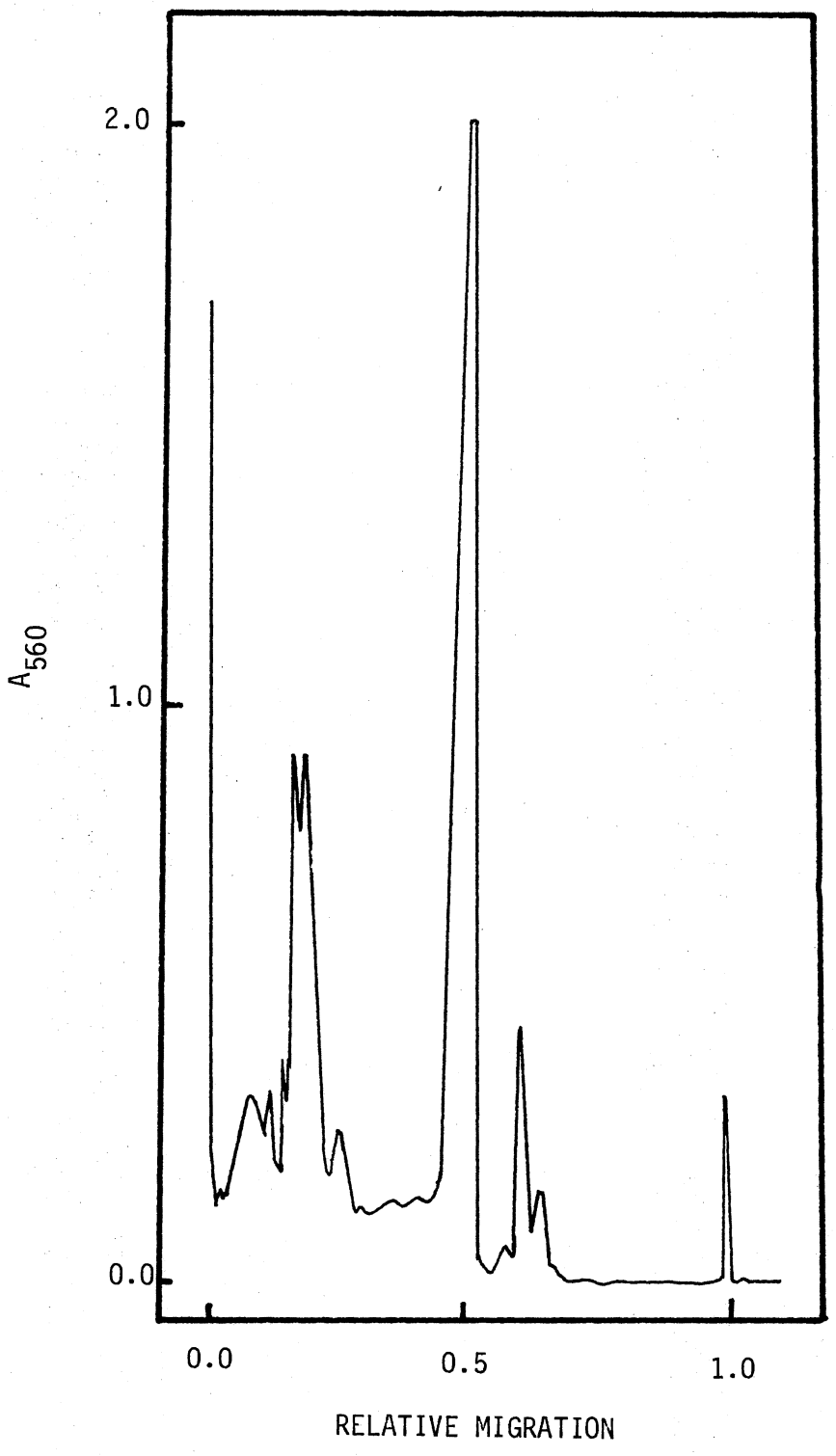

Figure 5. Disc Gel Electrophoresis of a Redissolved Ammonium Sulfate Pellet from Rat Whey Milk.

Scan of 7.5 percent polyacrylamide disc gels. The gel scan is from a redissolved 80 % ammonium sulfate pellet (100 µgs).

natant solution as measured by absorbance at 280 nm (assuming one absorbance unit equals one mg of protein) was 471 mg. Approximately 250 mg of protein from the original 471 mg of protein remained in the 80% ammonium sulfate pellet. The pellet was redissolved in 9.3 ml of 50 mM potassium phosphate and 100 mM KCl at pH 7.4. The redissolved pellet was layered on a 5.0 x 100 cm Bio-Gel P-30 (100-200 mesh) column equilibrated with potassium phosphate and 100 mM KCl, pH 7.4. Ten ml fractions were collected at a flow rate of 60 ml per hour, and the A_{280} was read for each tube. One protein peak was observed, (Figure 6), and various tubes were assayed spectrophotometrically for α-lactalbumin activity. It was observed that the α-lactalbumin activity was slightly shifted toward the trailing edge of the void peak. This result was unusual since with most milks, two major whey proteins peaks are observed and α-lactalbumin is in the lower molecular weight peak. Disc and sodium dodecyl sulfate acrylamide gels of the trailing middle and leading edge of the void protein peak of the P-30 column in Figure 6 are shown in Figures 7 and 8 respectively. It is clear that a number of proteins were present and that the P-30 column was not separating α-lactalbumin from other proteins.

Scheme II

In this scheme a Bio-Gel P-60 column was used in an attempt to separate α-lactalbumin from other proteins. Approximately 60 ml of rat milk were diluted to 12 ml with normal saline and purified as outlined in Scheme I. Following the 80% ammonium sulfate precipitation and redissolving the pellet in two ml of column buffer, approximately 67 mg of protein were obtained. Twenty-eight mg of the redissolved pellet in one

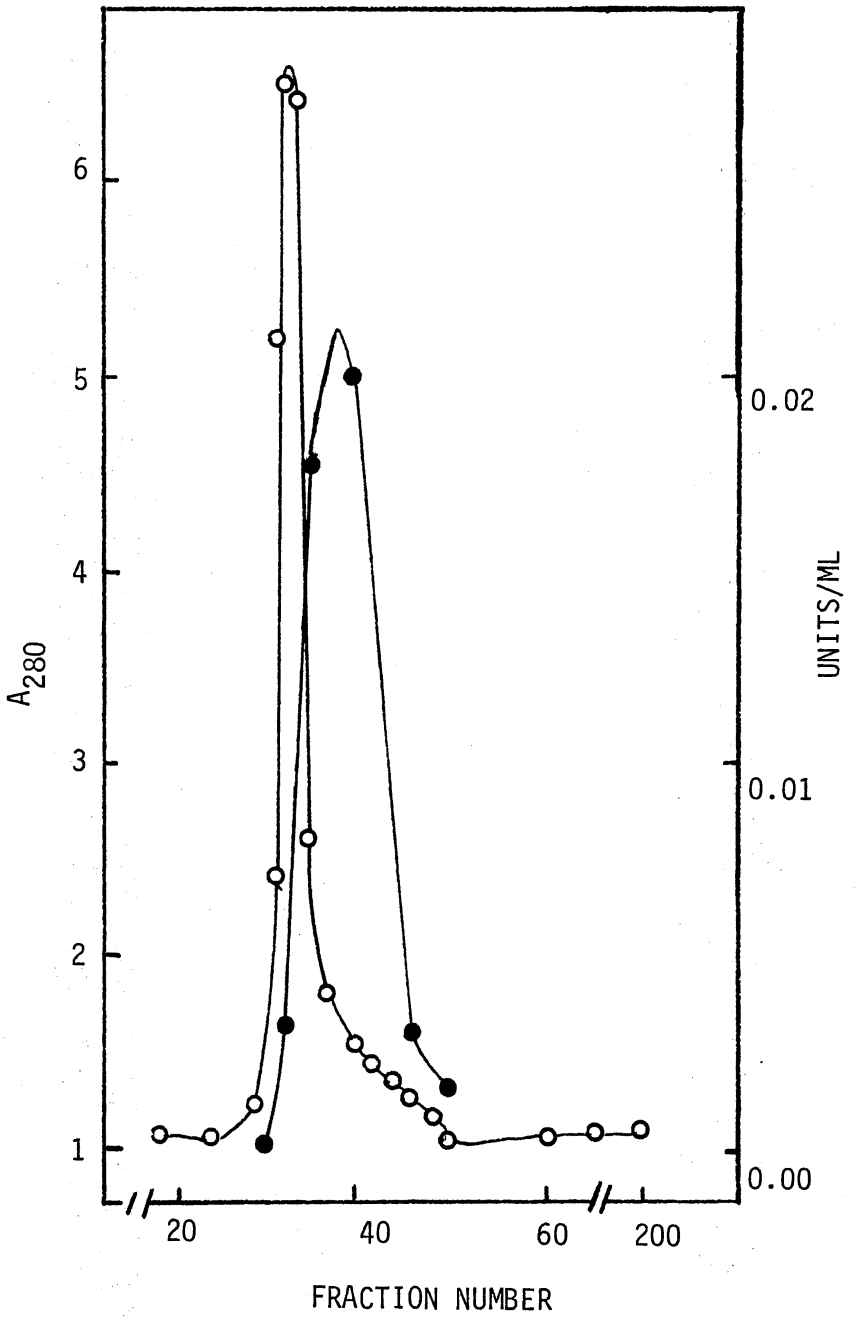

Figure 6. Typical P-30 Molecular Sieve Chromatography of Whey Proteins in Rat Milk

Resolution of rat whey proteins over a 3.15 x 90 cm Bio-Gel (100-200 mesh) column equilibrated with 20 mM Tris, 100 mM KCl, pH 7.5. Ten ml fractions were collected and measured at 280 nm (o—o). α-Lactalbumin activity was measured spectrophotometrically at 340 nm (●—●).

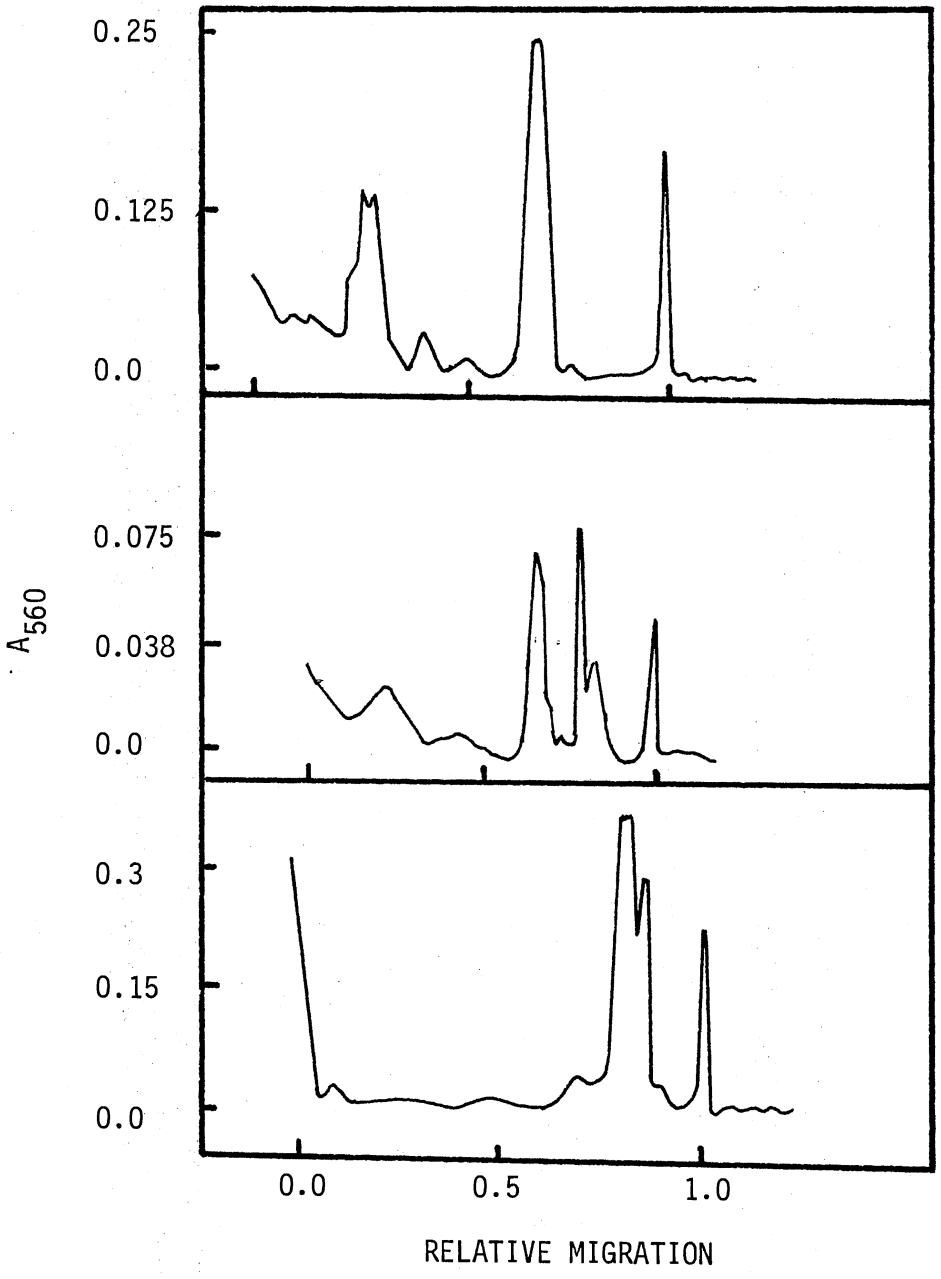

Figure 7. Disc Gel Electrophoresis of Rat Whey Milk Proteins Separated on P-30 Column

The disc gels were operated at 1.5 ma per 7.5 % gel and the tracking dye marked with a pin. The gel scans are labeled from bottom to top as (bottom) leading edge of P-30 peak (41 µg) (middle) trailing edge of P-30 peak (50 µg) (top) middle area of P-30 peak (50 µg).

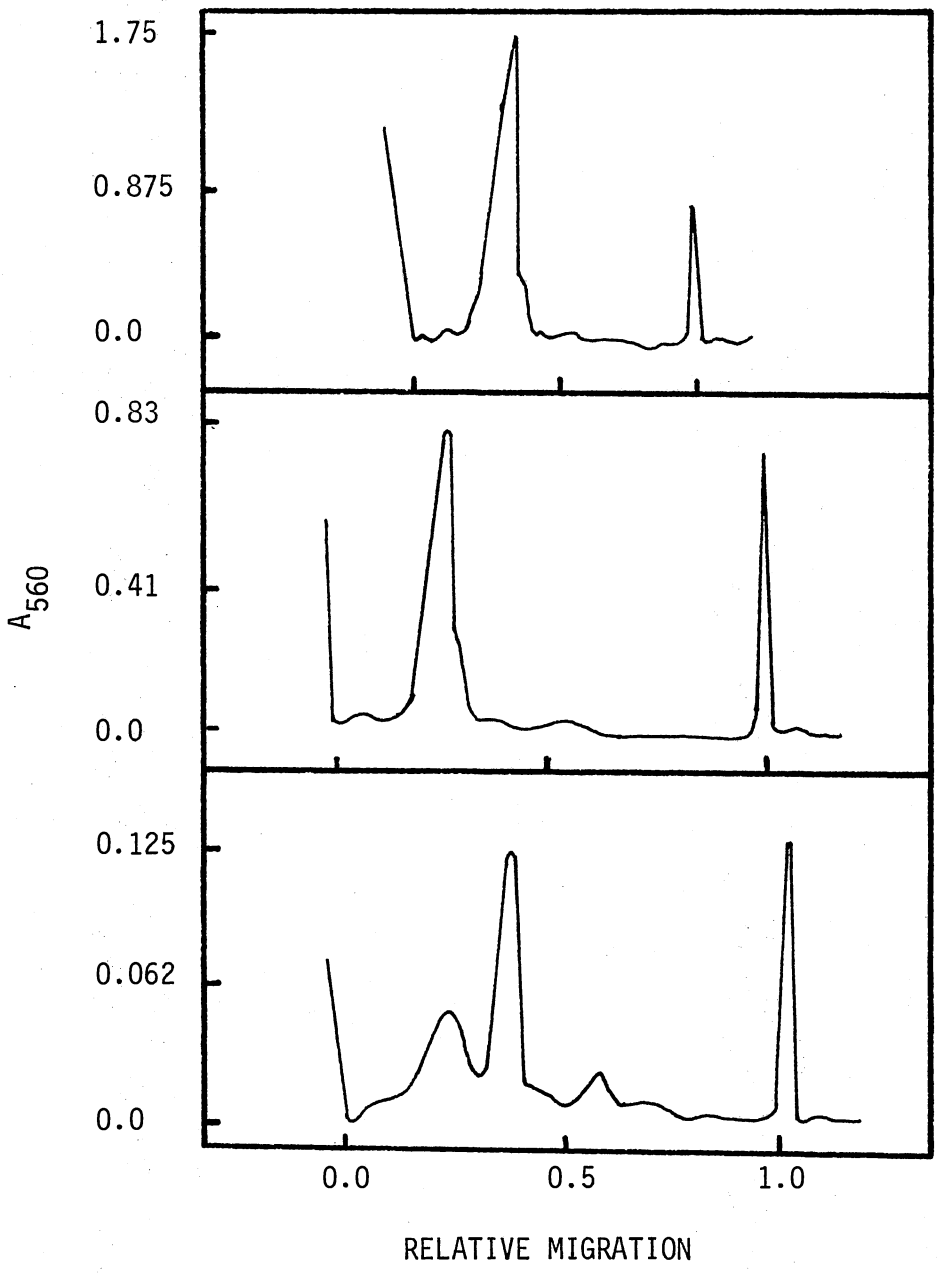

Figure 8. Sodium Dodecyl Sulfate Electrophoresis of Rat Whey Milk over a P-30 Column.

The 11 percent sodium dodecyl sulfate acrylamide gels were operated at 10 ma per gel. The gel scans are labeled from top to bottom as (top) leading edge of P-30 peak (middle) middle area of P-30 peak (bottom) trailing edge of P-30 peak.

ml of column buffer was layered on a 1.0 x 100 cm Bio-Gel P-60 (100-200 mesh) column equilibrated with 20 mM Tris and 100 mM KCl at pH 7.5. 0.96 ml fractions were collected and each tube was read at 280 nm and measured for α-lactalbumin activity. These results are presented in Figure 9. Three protein peaks were observed but only the second peak contained α-lactalbumin activity. Samples from the peak tubes were prepared and run on 7.5 % acrylamide disc gels and 11 % phosphate sodium dodecyl sulfate gels as shown in Figure 10 and 11 respectively.

It should be noted that the first or void peak had a very large band which migrated at a molecular weight of approximately 68,000 on sodium dodecyl sulfate gels. The second peak which contained the α-lactalbumin activity had a large protein band which migrated at about 24,000 on sodium dodecyl sulfate acrylamide gels, and no band was observed at 14,500 which is the molecular weight of α-lactalbumin from other species. The molecular weight standardization curve is shown in Figure 12.

Scheme III

This scheme followed the general procedure which utilized acid precipitation instead of centrifugation to remove the caseins. Following the removal of the caseins, an 80 % ammonium sulfate precipitation was performed on the supernatant solution. After centrifugation at 40,000 x g for 20 minutes, the pellet was redissolved in 20 nm and assayed for α-lactalbumin activity.

It was observed that rat whey milk which had been prepared by the acid precipitation method had a similar protein and α-lactalbumin activity profile on a Bio-Gel P-60 column as rat whey milk prepared by the 100,000 x g centrifugation method and eluted on a Bio-Gel P-60 column

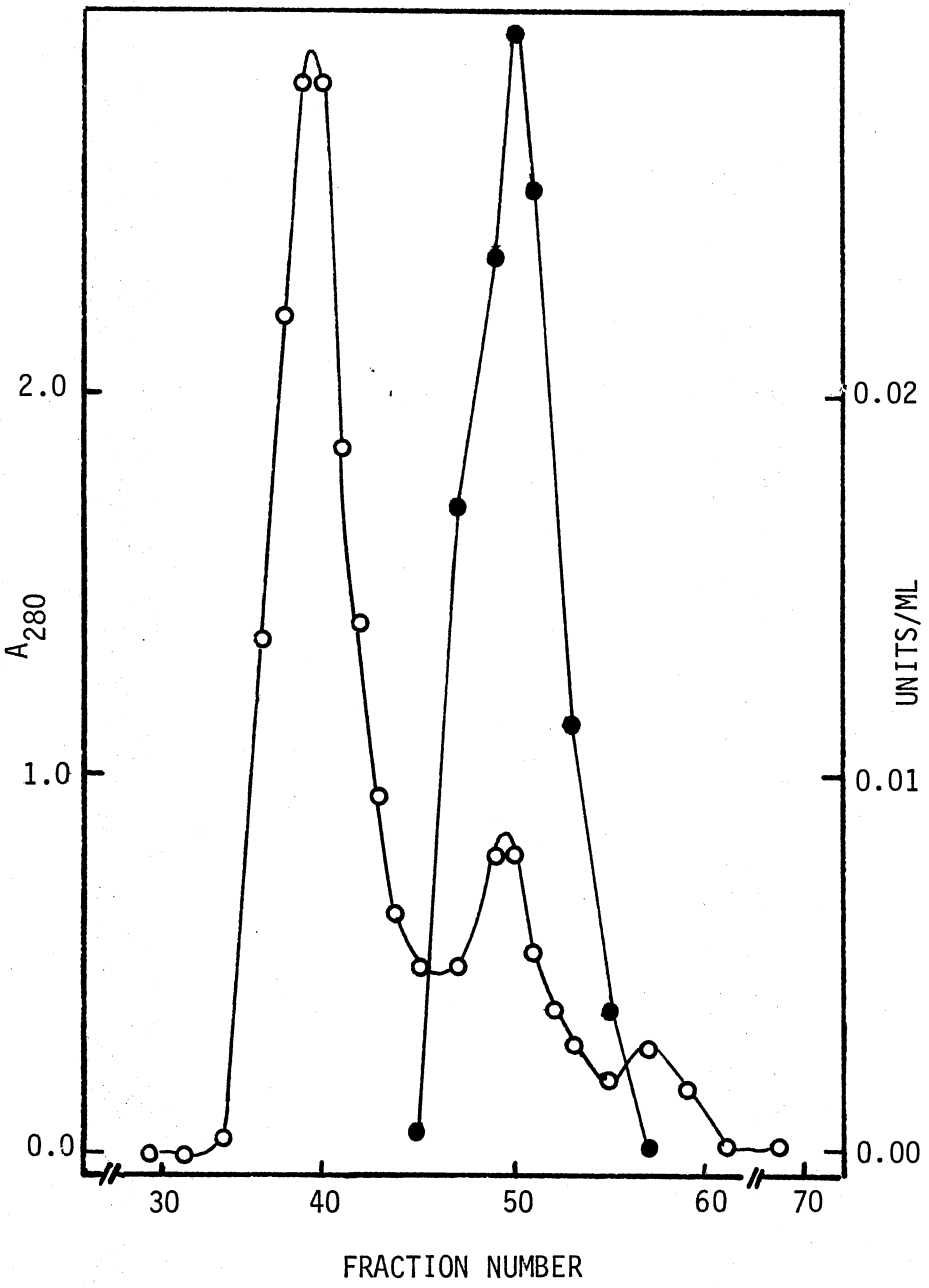

Figure 9. Molecular Sieve Chromatography (Bio-Gel P-60) of Whey Proteins in Rat Milk.

Resolution of rat whey proteins over a 1.0 x 100 cm P-60 column equilibrated with 20 mM Tris and 100 mM KCl, pH 7.5. 0.96 ml fractions were collected and measured at 280 nm (o—o). α-Lactalbumin activity was measured at 340 nm (●—●).

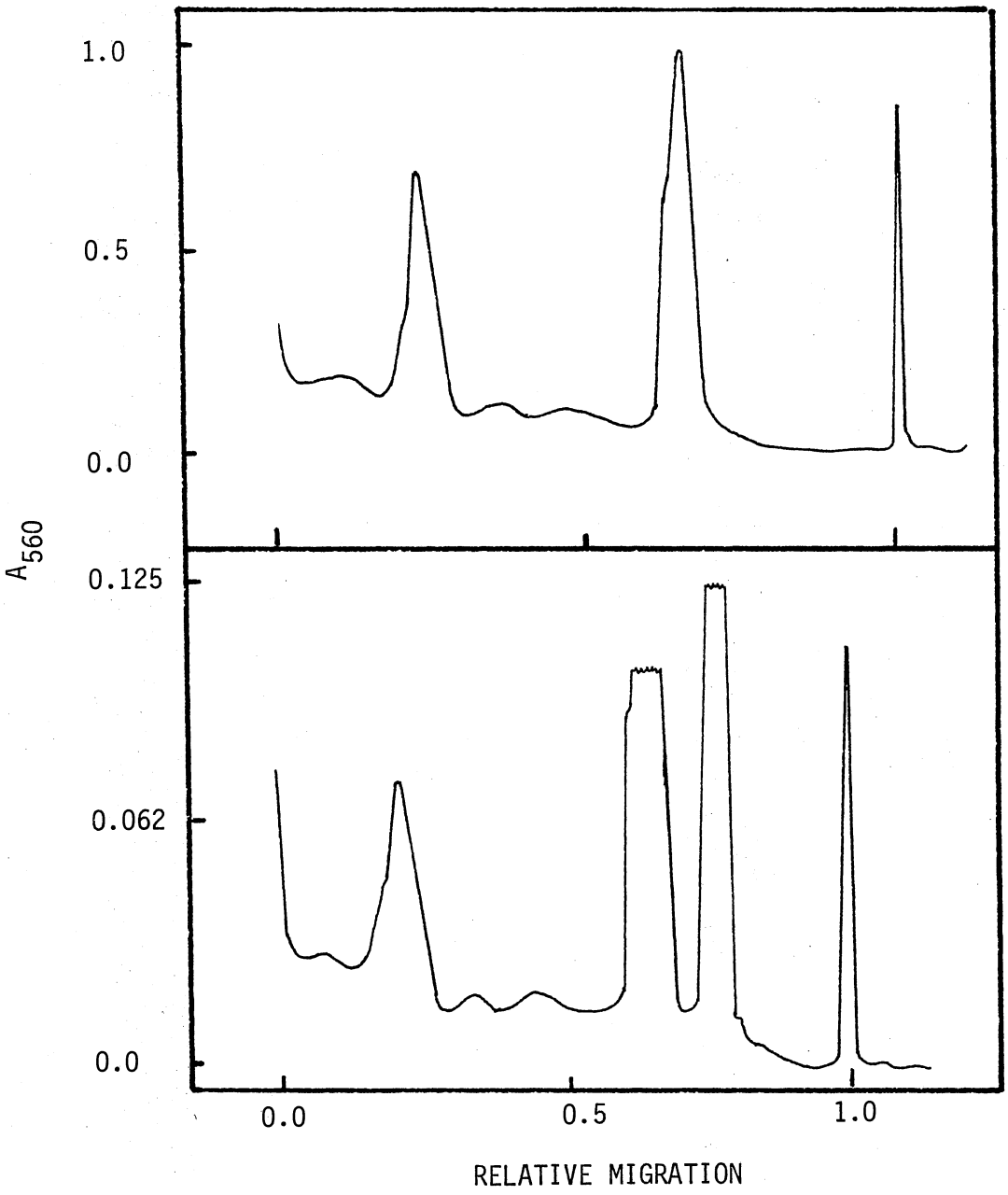

Figure 10. Disc Electrophoresis of Rat Whey Proteins Separated on a P-60 Column.

Scans of 7.5 percent polyacrylamide disc gels. The samples are from rat whey proteins passed over a P-60 column. The gel scans are (top) Void peak of rat whey proteins off P-60 column (bottom) α-Lactalbumin peak from skimmed rat milk off P-60 column.

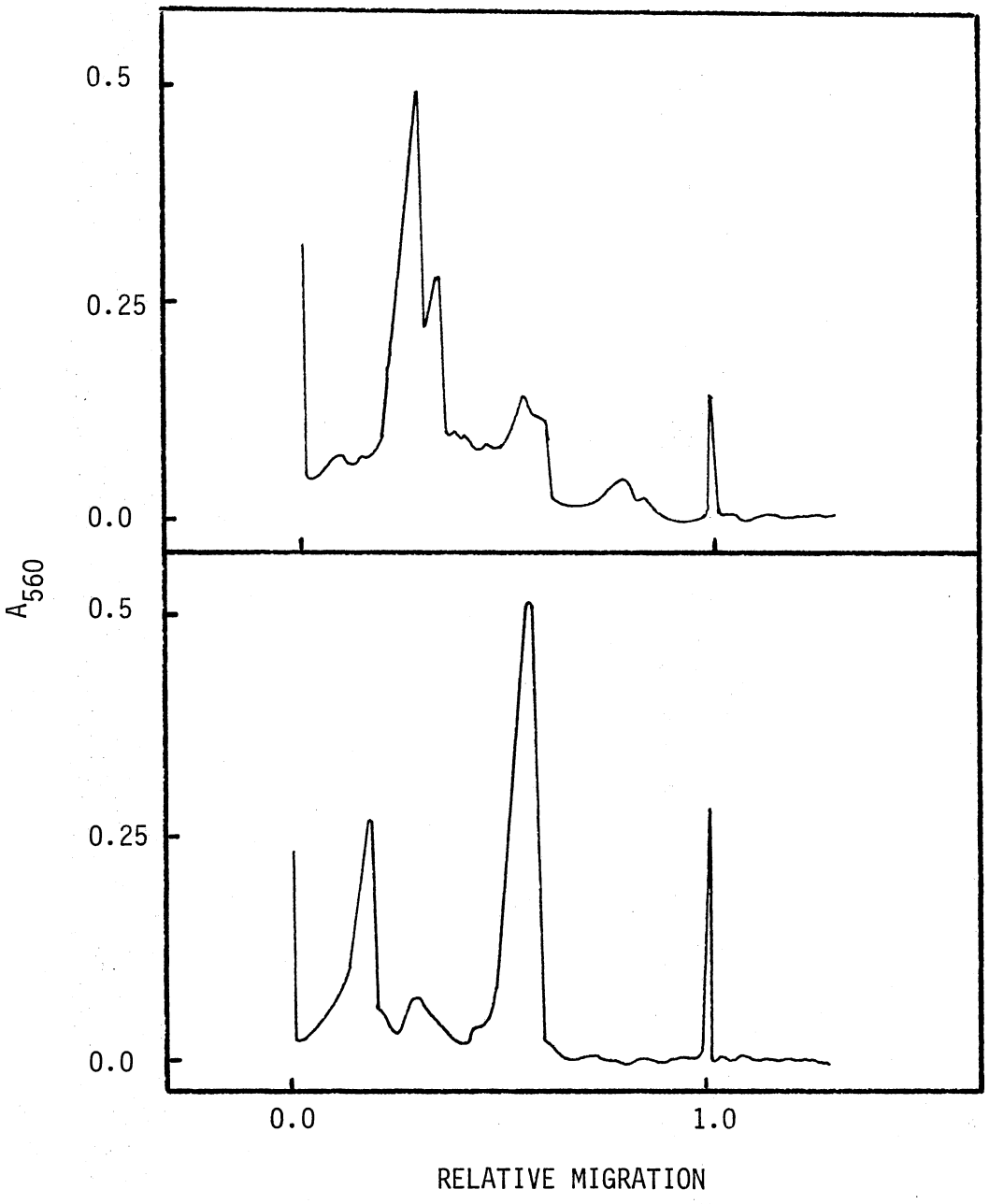

Figure 11. Sodium Dodecyl Sulfate Electrophoresis of Rat Whey Off P-60 Column.

Scans of 11 percent polyacrylamide sodium dodecyl sulfate gels of rat whey proteins. The gel scans are from (top) void peak of skim rat milk off P-60 column (50 μg) (bottom) α-lactalbumin peak of skim rat milk off P-60 column (50 μg).

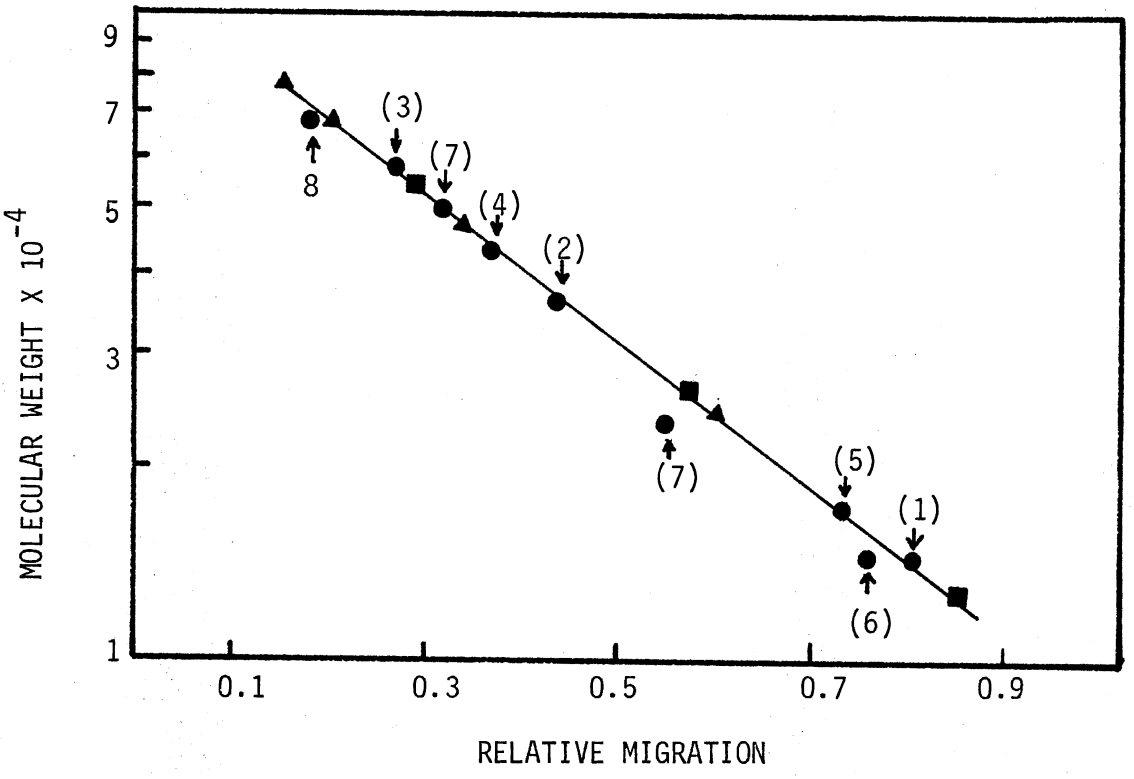

Figure 12. Molecular Weight Standardization Curve of Proteins on Sodium Dodecyl Sulfate Acrylamide Gels.

Protein standardization curve for sodium dodecyl sulfate acrylamide gels of whey proteins chromatographed on a P-60 column. Standards (●) were bovine α-lactalbumin (1), glyceraldehyde dehydrogenase (2), catalase (3), ovalbumin (4), myoglobin (5), lysozyme (6), and human γ-globulin (7). The protein bands from the void peak are denoted by (■), and the α-lactalbumin peak is denoted by (▲). Bovine serum albumin was denoted by (8).

(Figure 9). Gel scans from the first and second peaks of rat whey milk prepared by acid precipitation and eluted on a Bio-Gel P-60 column were similar to gel scan of the first and second peaks of rat whey milk prepared by centrifugation at 100,000 x g and eluted on a Bio-Gel P-60 column (Figures 10 and 11). From these observations it was concluded that either method of casein removal gave the same results. However, the 100,000 x g centrifugation was the preferred method of casein removal because it reduced the possibility of denaturation of protein by lowering the pH.

Scheme IV

The previous purification schemes did not separate α-lactalbumin from the other low molecular weight proteins and accordingly the whey proteins were separated on a Bio-Gel P-150 column. Approximately 6.0 ml of rat milk were diluted to 12 ml with a normal saline solution (0.89 % NaCl in H_2O). The rat milk-normal saline solution was centrifuged at 15,000 x g for 20 minutes. 8.4 ml of the supernatant solution remained after removal of the cream. The supernatant solution was diluted to a total volumn of 10 ml with normal saline. The supernatant solution was centrifuged at 114,000 x g to remove the caseins. After decanting and passing the supernatant solution through a millipore filter, approximately 5.8 ml of the supernatant solution were recovered and it contained 133.1 mg of protein. The supernatant solution was precipitated with 80 % ammonium sulfate, (56.1 g/100 ml), and the precipitate was centrifuged at 40,000 x g for 20 minutes. The pellet was redissolved in 2.5 ml of 20 mM Tris and 100 mM KCl at pH 7.5 and contained 67.2 mg (28 mg/ml) of protein (A_{280}). 0.5 ml (14 mg) were layered on a 0.6 x 110 cm Bio-Gel P-150

(100-200 mesh) column equilibrated with 100 mM KCl and 20 mM Tris at pH 7.5. Approximately 0.48 ml fractions were collected. The tubes were measured at 280 nm and assayed spectrophotometrically for α-lactalbumin activity.

The elution profile of the column is shown in Figure 13. Four protein peaks were present but only the third peak contained α-lactalbumin activity. Sodium dodecyl sulfate acrylamide gels of the first three peaks are shown in Figure 14. The first peak contained numerous large molecular weight proteins. The second peak contained one major protein which marked on sodium dodecyl sulfate gels at a molecular weight of 68,000. It was thought this protein was serum albumin since it appears to be the prominant protein in whey (81). The peak containing the α-lactalbumin activity also contained a small amount of the 68,000 band, but the predominant band had a molecular weight of 22,500 as determined by sodium dodecyl sulfate electrophoresis.

Scheme V

From the results of previous column elution profiles of the whey proteins from rat milk, it was concluded that the best molecular sieve column was Bio-Gel P-150 (100-200 mesh). The tubes (34-48) which contained the α-lactalbumin activity from rat whey milk which had been previously eluted on the P-30 column (Figure 6) were pooled, precipitated with 80 % $(NH_4)_2SO_4$ (56.1 g/100 ml) at 4° C, and the pellet redissolved in 5 ml of 20 mM Tris, and 100 mM KCl at pH 7.5. The redissolved pellet contained 100 mg of protein and 10 % (w/v) sucrose was added to the solution. The sucrose-redissolved pellet solution was layered on a 3.15 x 90 cm Bio-Gel P-150 column (100-200 mesh) equilibrated with 20 mM Tris

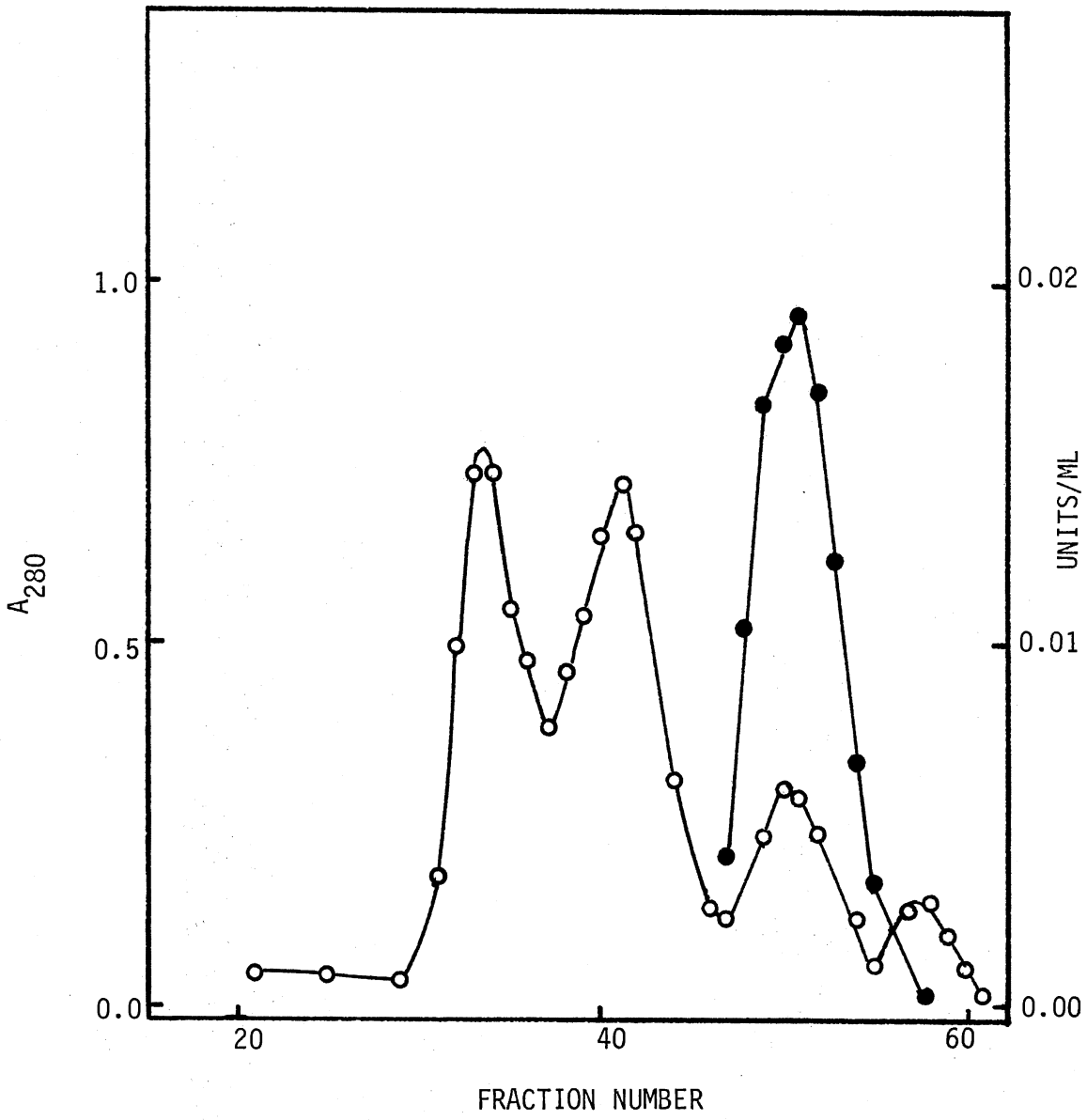

Figure 13. Typical P-150 Molecular Sieve Chromatography of Whey Proteins of Rat Milk.

Resolution of whey proteins of rat milk by a 0.6 x 110 cm P-150 column equilibrated with 20 mM Tris and 100 mM KCl, pH 7.5. 0.48 ml fractions were collected and measured at 280 nm (o—o). α-Lactalbumin activity was measured spectrophotometrically at 340 nm (●—●).

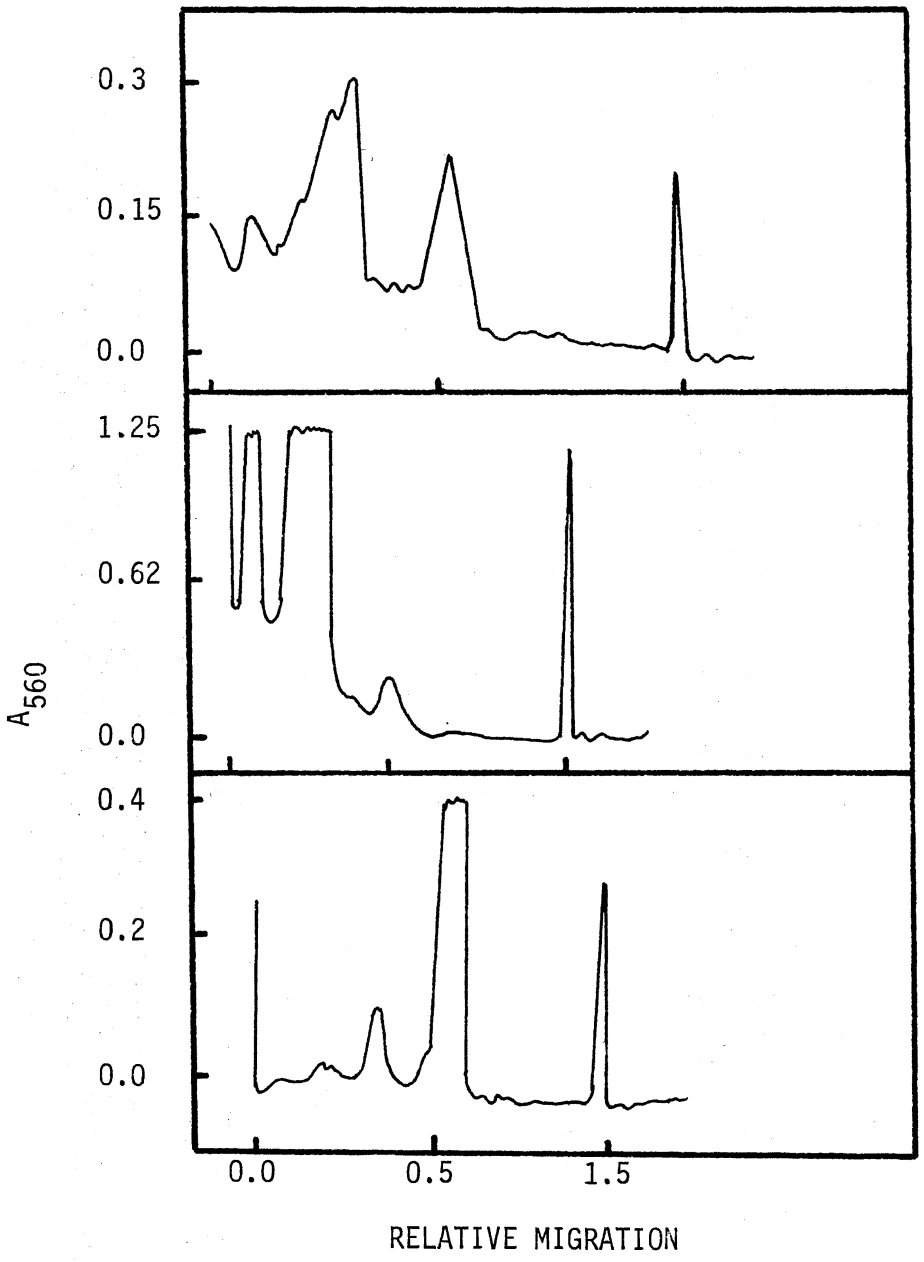

Figure 14. Sodium Dodecyl Sulfate Electrophoresis of Whey Proteins from Rat Milk Off P-150 Column.

Scans of eleven percent polyacrylamide sodium dodecyl sulfate gels. The samples are from skim rat milk passed over a P-150 column with the caseins removed by centrifugation. The gel scans are from (top) void peak from skim rat milk off P-150 column (50 μg) (middle) 60 K peak from skim rat milk off P-150 column (50 μg) (bottom) α-lactalbumin peak from skimmed rat milk off P-150 column (30 μg).

and 100 mM KCl, pH 7.5. Approximately 4 ml fractions were collected, and the elution profile of the column was similar to the P-150 column shown in Figure 13 as were the sodium dodecyl sulfate gel patterns.

It should be noted that the major protein band from the α-lactalbumin peak migrated on sodium dodecyl sulfate acrylamide gels at an approximate molecular weight of 23,000. Rat whey milk passed over the P-30 column and then over the Bio-Gel P-150 column had the same elution profile as the rat whey milk passed only over the P-150 column.

A sample taken from the fourth peak or lowest molecular weight peak (see Figure 13) gave two predominant protein bands in sodium dodecyl sulfate electrophoresis (Figure 15). One protein had a molecular weight of 22,500, presumably α-lactalbumin, and the second protein band migrated at 17,500.

Tubes containing the α-lactalbumin activity were pooled and dialyzed against 4 l of 20 mM Tris, pH 7.65 at 4° C for 48 hours. The absorbance was read at 280 nm and 18.5 mg in 55 ml of 20 mM Tris, pH 7.65 at 4° C (calculated from the correct extinction coefficient of rat α-lactalbumin, 1.624 absorbance units equals 1 mg of rat α-lactalbumin) was placed over a 1 x 24 cm DEAE 32 cellulose ion-exchange column equilibrated with 20 mM Tris at pH 7.65 at 4° C. A linear gradient was run from 0 M KCl and 20 mM Tris, pH 7.68 (75 mls) 4° C to 0.3 M KCl and 20 mM Tris pH 7.68 (75 mls). The second gradient was run from 0.3 M KCl and 20 mM Tris, pH 7.68 (75 mls) at 4° C to 1.0 M KCl and 20 mM Tris pH 7.68 (75 mls) at 4° C. Approximately, 1.5 ml fractions were collected. The elution profile of the column is shown in Figure 16. Samples were taken for disc and sodium dodecyl sulfate acrylamide electrophoresis from the peak tube containing the α-lactalbumin activity and lyophilized after the samples had been

dialyzed against 4 l of water and changed every 6 hours for a total of 3 changes. The scans of the disc and acrylamide gels are shown in Figure 17 and 18 respectively. The major protein band marked at 23,000 and a minor band marked at 68,000 on sodium dodecyl sulfate acrylamide gels. The peak areas of both proteins from the gel scan were cut out and weighed to four decimal places, and the minor band (68,000) represented less than 2 % of the total area of the large band.

Two 100 μg samples of rat α-lactalbumin from the peak tubes off the DEAE 32 cellulose were prepared for disc electrophoresis by the procedure of Brewer and Ashworth (68). Two 100 μg samples of bovine α-lactalbumin were prepared by the same procedure. 7.5 % disc acrylamide gels with 100 μg of the four samples were run. One gel of the bovine α-lactalbumin and one gel of the rat α-lactalbumin were cut into 1 mm slices and assayed spectrophotometrically for α-lactalbumin activity. The results are shown in Figures 19 and 20 respectively. It was evident that the major protein contained almost all the α-lactalbumin activity. The molecular weight of rat α-lactalbumin was determined to be 23,000 by sodium dodecyl sulfate acrylamide gel electrophoresis.

The rat α-lactalbumin was stained for carbohydrate as described by Neville (70). Samples of rat α-lactalbumin were from the peak tubes of the DEAE 32 cellulose column and were dialyzed against 18 l of H_2O, lyophilyzed and then prepared for electrophoresis by following the general scheme of Weber and Osborn (71). Bovine α-lactalbumin and ovalbumin were used as controls. Two hundred μg samples of each protein were run on 11 % sodium dodecyl sulfate acrylamide gels and stained for carbohydrate by Neville's procedure (70). The gels were scanned at 550 nm and the results are shown in Figure 21. The results indicated that the rat α-lactalbumin contained carbohydrate.

Approximately 10 mg of rat α-lactalbumin isolated utilizing this isolation scheme was given to Dr. B. G. Hudson at Oklahoma State University for amino acid carbohydrate analysis. It should be noted that this material contained a 68,000 molecular weight protein contaminant less than 2 %).

Scheme VI

Based on the results of Scheme V it was concluded that a Bio-Gel P-30 molecular sieve column was unsatisfactory and unnecessary. Scheme VI utilized the general purification scheme involving the removal of the caseins by a 100,000 x g centrifugation. Forty-one mls of whole rat were diluted with 41 ml of normal saline (0.89 gm of NaCl in 100 ml of deionized water) to make a total volume of 82 ml. A 15,000 x g centrifugation for 20 minutes was used to remove the cream. The caseins were removed by centrifuging at 100,000 x g for 1 hour. The supernatant solution was decanted and passed through a 0.45 μ millipore filter followed by a 0.22 μ filtration. Approximately 56 ml of the supernatant solution remained after the last filtration and contained 740.5 mg of protein (A_{280}). The proteins from the filtrate were precipitated by 80 % ammonium sulfate (56.1 gm/100 ml) and the solution centrifuged for 20 minutes at 40,000 x g, and the supernatant solution was decanted. The pellet was redissolved in 9.9 ml of 20 mM Tris and 100 mM KCl at pH 7.76 at 4° C, and contained 591 mg of protein. The solution was divided into three parts, the first contained 200 mg of protein, the second contained 269 mg of protein, and the third part contained the remainder. Both the first and second part were processed separately by the procedure described below. The first part was dissolved in 3.34 ml of 20 mM Tris and 100 mM KCl, pH 7.5 at 4° C, and the second part was dissolved in 8 ml of the same solution.

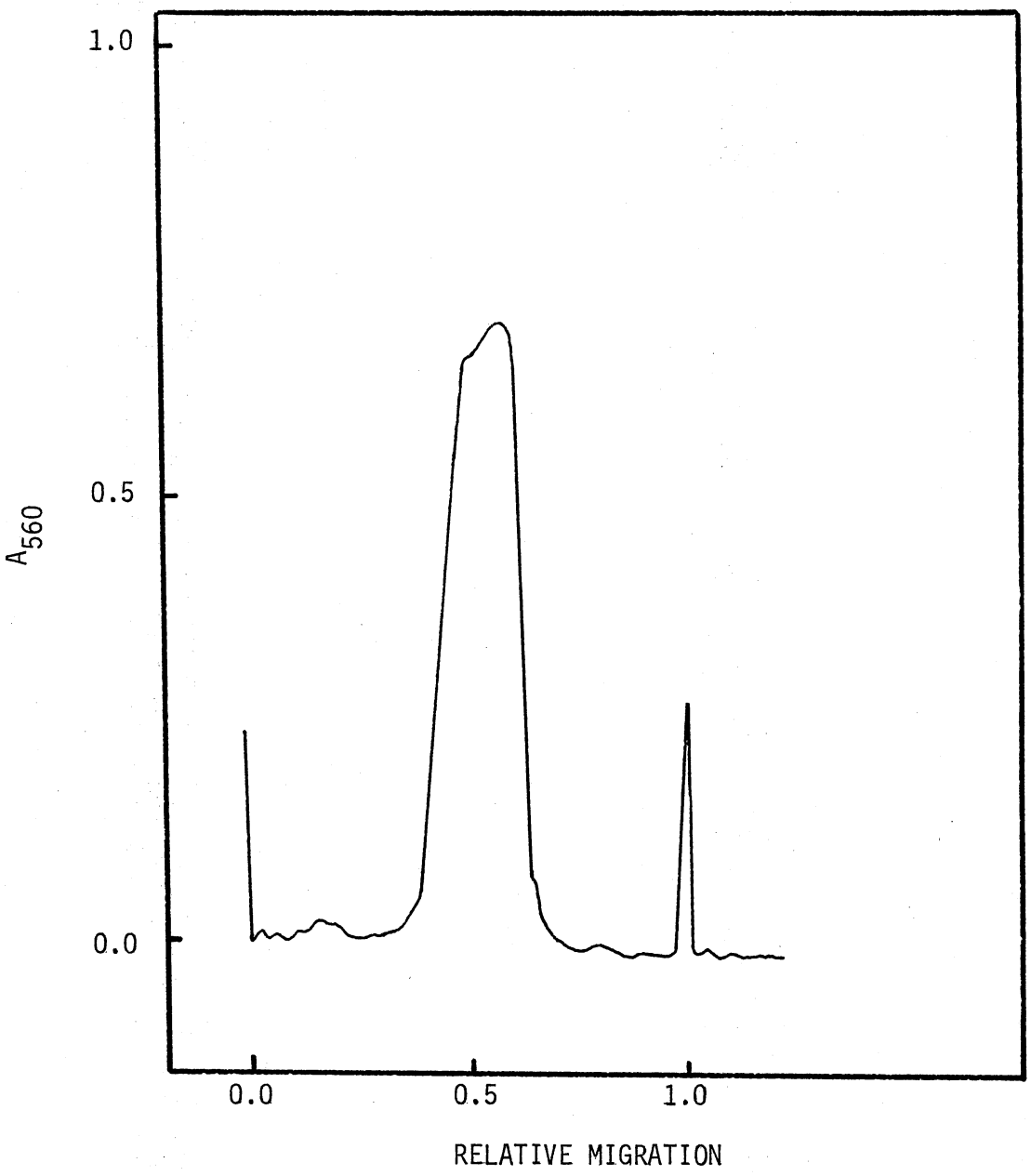

Figure 15. Sodium Dodecyl Sulfate Electrophoresis of the Fourth Peak of a Pooled α-Lactalbumin Peak from a Bio-Gel P-30 Column Rechromatographed on a Bio-Gel P-150 Column.

Scan of an eleven percent polyacrylamide sodium dodecyl sulfate gel. The sample (100 μg) was from a pooled α-lactalbumin peak from a Bio-Gel P-30 column which was rechromatographed on a Bio-Gel P-150 column. The caseins were removed by centrifugation. The scan is from the gel run on the fourth peak of the P-150 column.

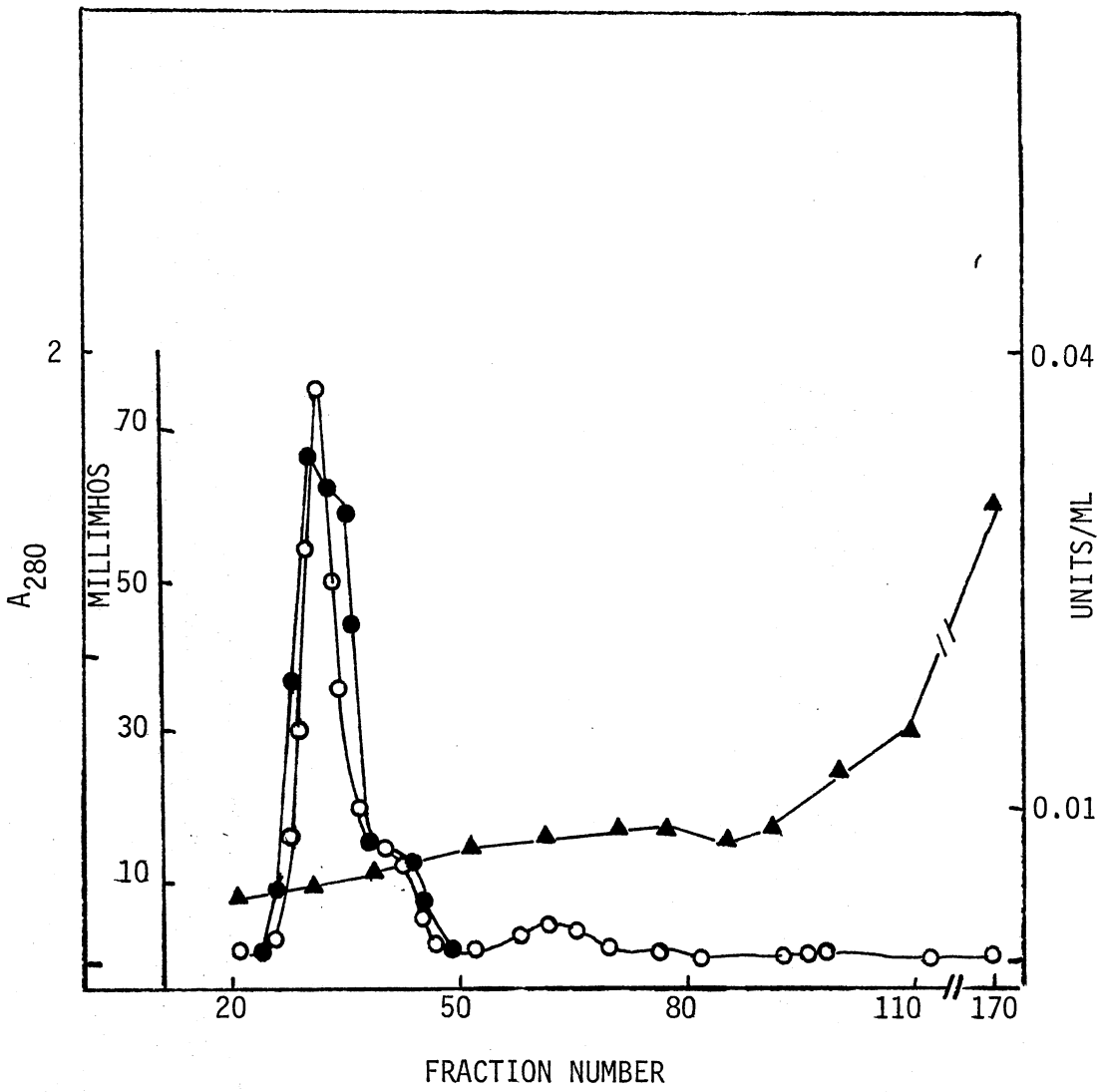

Figure 16. Typical DEAE 32 Cellulose Chromatography Profile of Rat α-Lactalbumin Isolated Utilizing Scheme V.

Typical resolution of a pooled rat α-lactalbumin peak from a Bio-Gel P-30 column followed by chromatography on a Bio-Gel P-150 column. The α-lactalbumin peak was pooled and rechromatographed on a 1 x 24 cm DEAE 32 Cellulose ion exchange column (see text). 1.5 ml fractions were collected and measured at 280 nm (o—o). The α-lactalbumin activity was measured spectrophotometrically at 340 nm (●—●). The gradient was measured by conductivity (▲—▲).

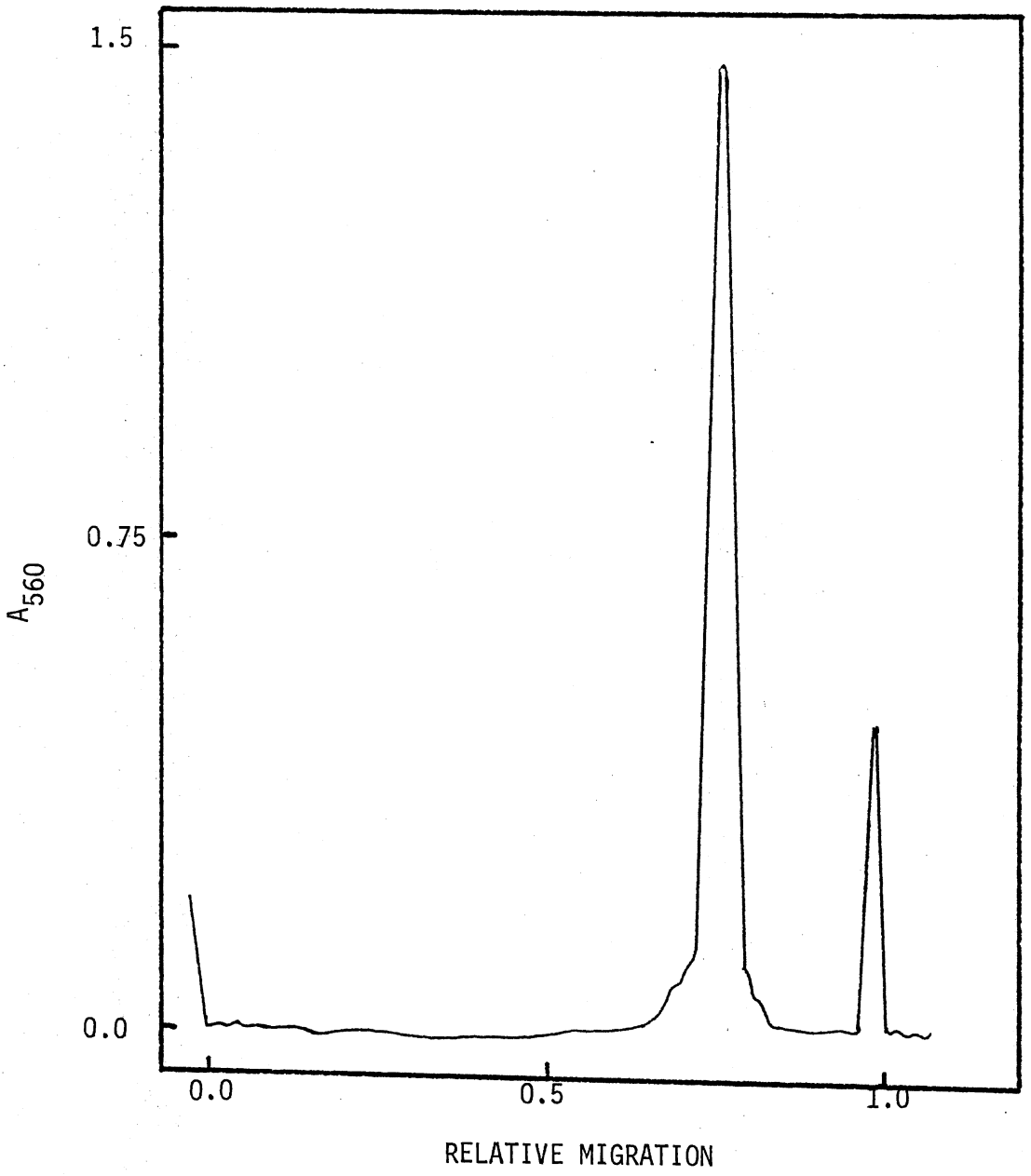

Figure 17. Disc Electrophoresis of Pooled Rat α-Lactalbumin from a Typical DEAE 32 Cellulose Column Utilized in Isolation Scheme V.

Scan of a 7.5 percent polyacrylamide disc gel. The sample was from a lyophilized pooled rat α-lactalbumin peak from a DEAE 32 cellulose column used in isolation Scheme V. Approximately 50 μg of sample were applied to the gel.

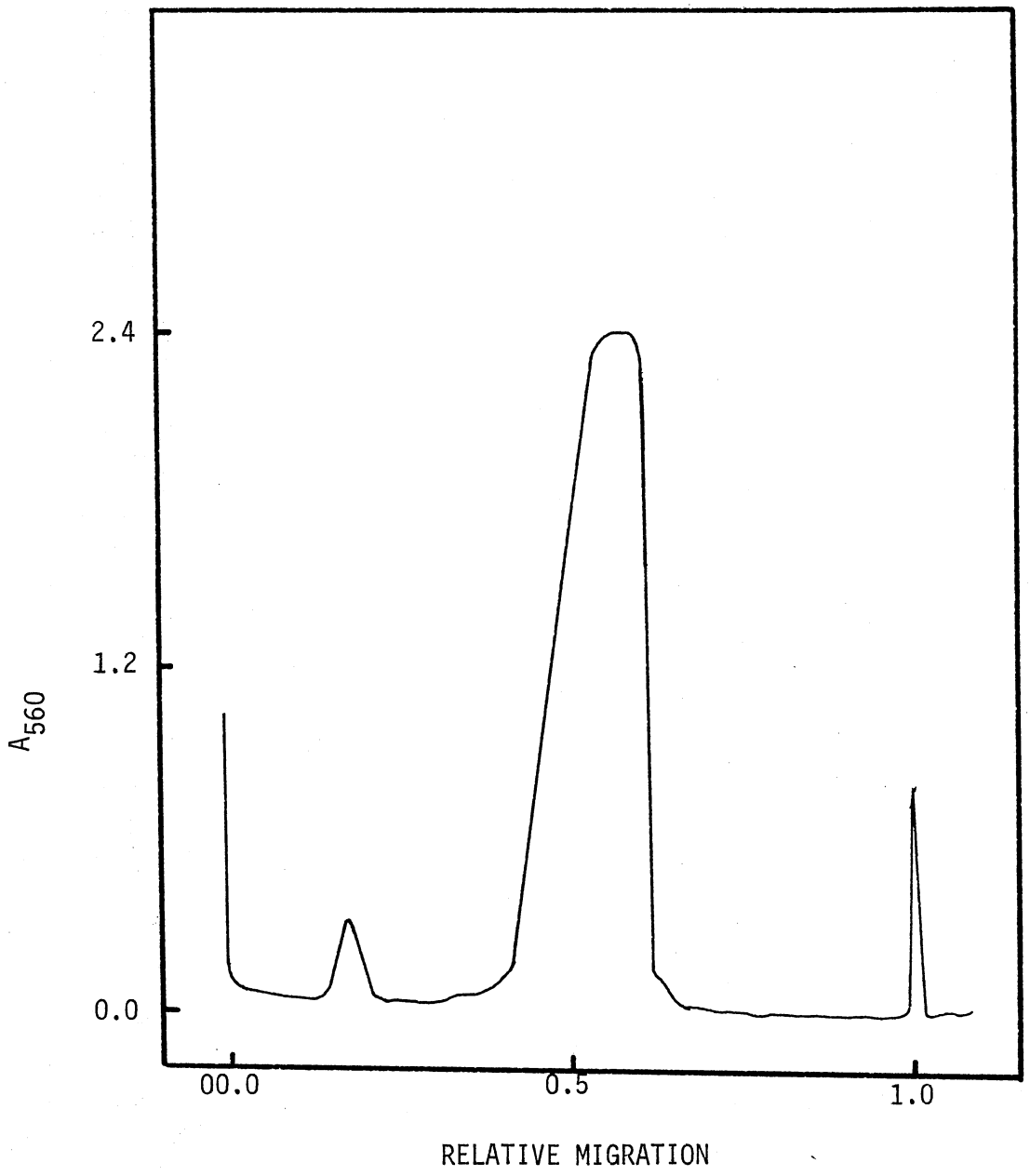

Figure 18. Sodium Dodecyl Sulfate Electrophoresis of Rat α-Lactalbumin from a Typical DEAE 32 Cellulose Column Utilized in Isolation Scheme V.

Scan of an eleven percent polyacrylamide sodium dodecyl sulfate gel. The sample is from a lyophilized pooled rat α-lactalbumin peak off a DEAE 32 cellulose column used in isolation Scheme V. Approximately 200 μg of sample were applied to the gel.

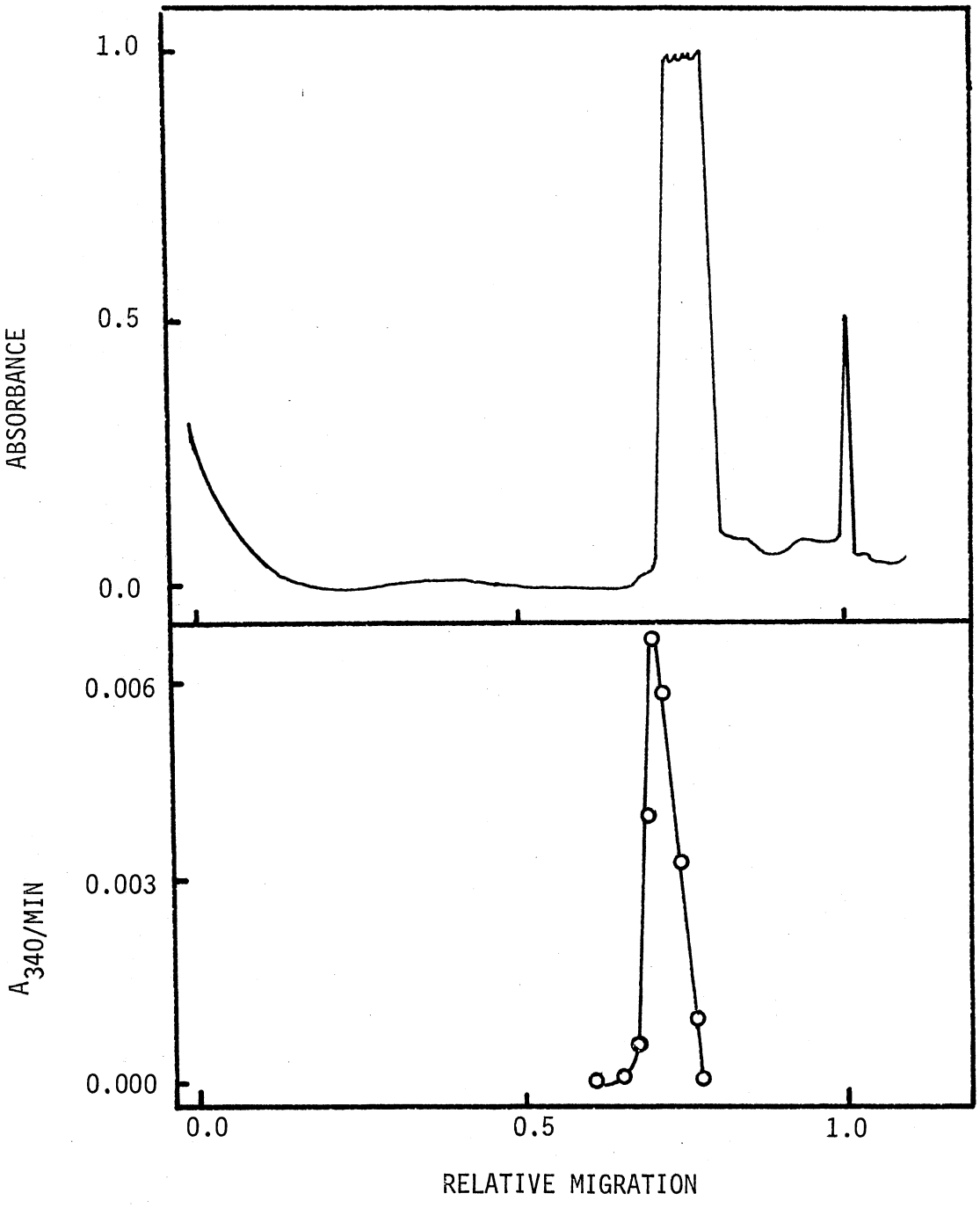

Figure 19. Activity of Bovine α-Lactalbumin Extracted from a Disc Gel.

Bovine α-lactalbumin (100 μg) was run on a 7.5 % disc gel. The gel was sliced into a 1 mm sections and the α-lactalbumin was extracted (see methods) and assayed spectrophotometrically at 340 nm for α-lactalbumin activity.

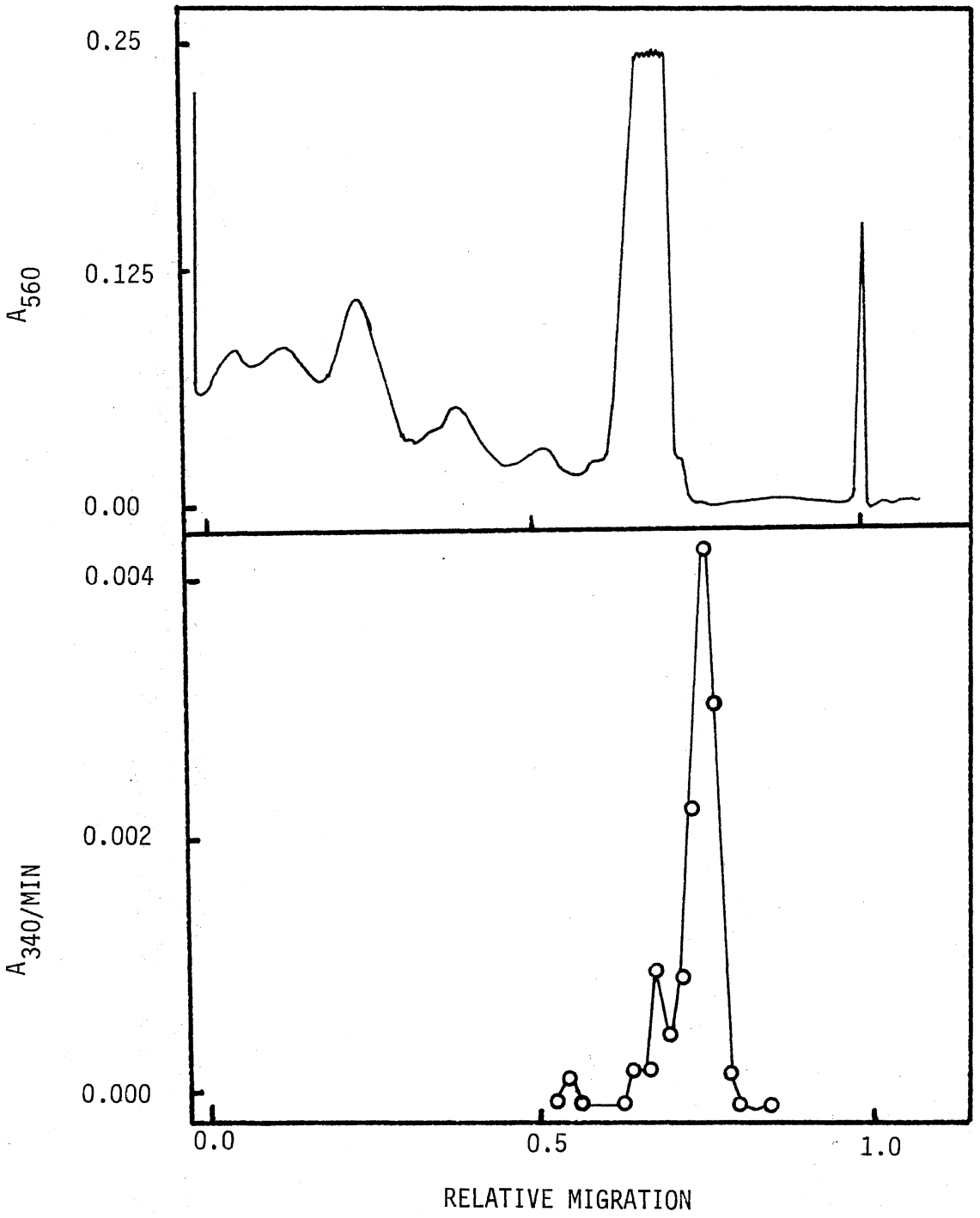

Figure 20. Activity of Rat α-Lactalbumin Extracted from a Disc Gel.

Rat α-lactalbumin (100 μg) isolated by Scheme V was run on a 7.5 % disc gel. The gel was sliced into 1 mm sections, and the protein was extracted (see methods) and assayed spectrophotometrically at 340 nm for α-lactalbumin activity. At the top of the figure is a scan of a duplicate gel, but stained with commassie blue.

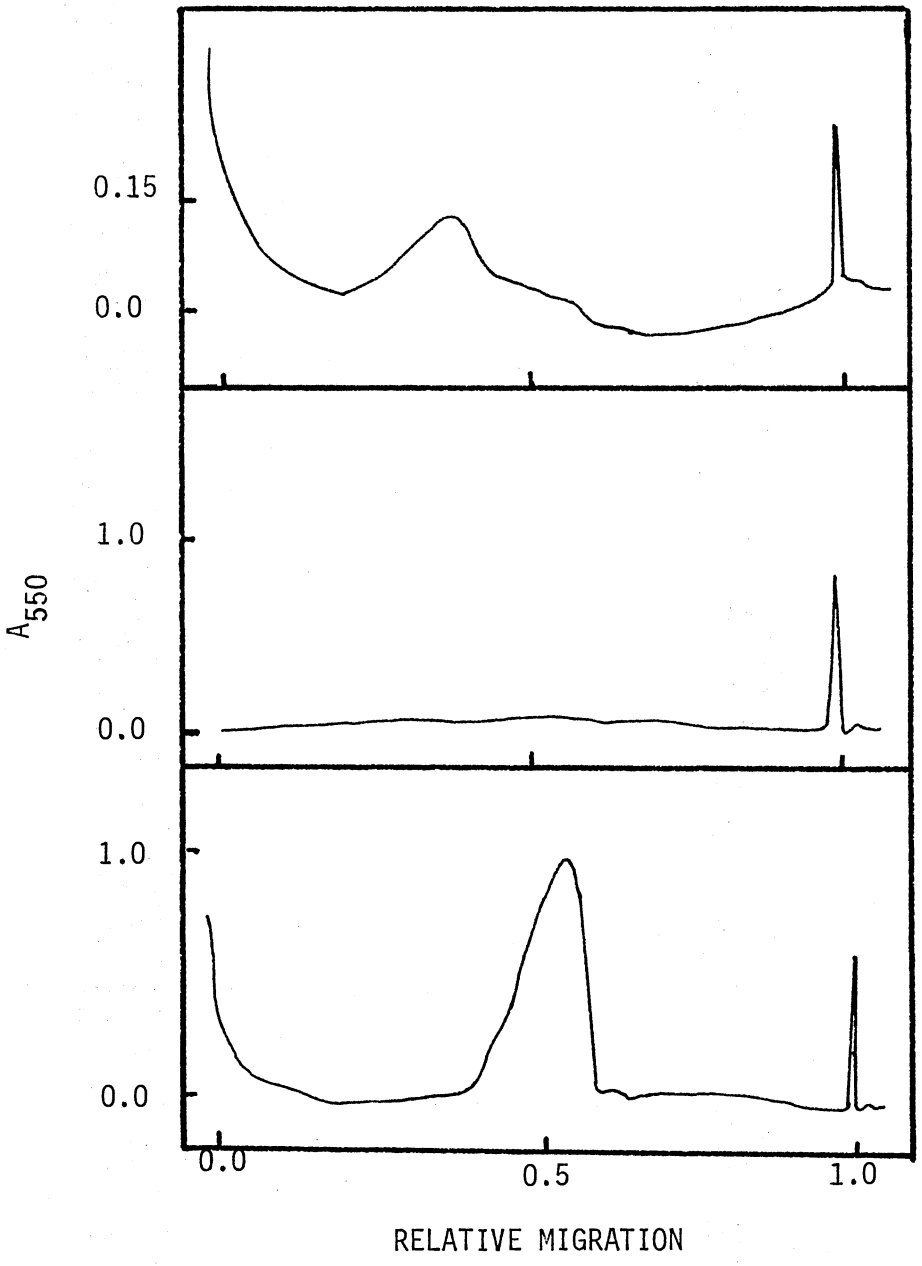

Figure 21. Carbohydrate Stain of Rat α-Lactalbumin Isolated from Scheme V.

Scans of eleven percent sodium dodecyl sulfate polyacrylamide gels. The samples were stained for carbohydrate by the procedure described by Neville (70). The gel scans are (top) ovalbumin positive control standard (middle) bovine α-lactalbumin negative control standard (bottom) rat α-lactalbumin isolated from Scheme V. 200 μg of sample were applied to each gel.

Both the first and second part were layered separately on a 3.5 x 90 cm Bio-Gel P-150 (100-200 mesh) molecular sieve column equilibrated with 20 mM Tris and 100 mM KCl, pH 7.76 at 4° C. Approximately 3.65 ml fractions were collected. The tubes were read at 280 nm for proteins and α-lactalbumin activity was assayed spectrophotometrically. All the α-lactalbumin activity was in the third peak. The elution profile of the first part passed over the P-150 column is shown in Figure 22. Galactosyltransferase was also assayed spectrophotometrically. It should be noted that the galactosyltransferase activity marked ahead of the second peak on the P-150 column. The predominant protein of the second peak marked at 68,000 on sodium dodecyl sulfate gels. This is unusual because galactosyltransferase from bovine milk has an apparent molecular weight of 55,000, whereas the galactosyltransferase from rat milk has an apparent molecular weight of greater than 80,000 on a P-150 column.

The tubes containing the α-lactalbumin activity (90-180 and 90-110 respectively) were pooled from both columns and dialyzed against 4 l of 20 mM Tris, pH 7.25 at 4° C and changed every six hours for a total of 4 changes. The absorbance at 280 nm was read and 120 mg of protein (by 1 mg/ml of protein equals 1 absorbance unit at 280 nm) were present in 138.7 ml of solution. The dialyzed solution containing the α-lactalbumin activity was layered on a 1.0 x 24 cm DEAE 32 cellulose ion exchange column equilibrated with 20 mM Tris at pH 7.1. The linear gradient was run from 0 M KCl and 20 mM Tris, pH 7.1. Each portion of the gradient contained 150 ml for a total of 300 ml. Approximately 1.4 ml fractions were collected and the absorbance at 280 nm was read. The fractions were assayed spectrophotometrically for α-lactalbumin activity. The elution profile of the column is shown in Figure 23. The major peak contained

most of the α-lactalbumin activity, but a small side peak also contained some α-lactalbumin activity. It should also be noted that the activity profile of the major peak was not symmetrical, indicating that charge forms may be present.

The tubes (189-211) under the major peak containing the α-lactalbumin activity were pooled and lyophilized. The lyophilized material was redissolved in 5 ml of deionized water and the absorbance at 280 nm was determined. Approximately 52.3 mg of protein in 5 ml of 20 mM Tris and 100 mM KCl at pH 7.5 at 4° C were layered on a 3.15 x 90 cm Bio-Gel P-150 column equilibrated with 20 mM Tris and 100 mM KCl at pH 7.5. Approximately 5.1 ml fractions were collected, and the peak tube from the major peak was assayed spectrophotometrically for α-lactalbumin. It was found that the peak tube contained α-lactalbumin activity. The elution profile of the column is shown in Figure 24. Tubes 68-90 were pooled and exhaustively dialyzed against 6 l of deionized water with changes every twelve hours for a total of 4 changes. The exhaustively dialyzed solution was lyophilized and used for circular dichroism studies, ultraviolet spectroscopy studies and the determination of the extinction coefficient of α-lactalbumin isolated from rat milk. These results are presented in the section of the properties of rat α-lactalbumin.

The purity of the rat α-lactlalbumin was examined by disc and sodium dodecyl sulfate acrylamide gel electrophoresis. The gel patterns from twelve percent disc acrylamide gels with 25 μg of rat α-lactalbumin off the last P-150 column gave 4 protein bands (Figure 25). On 11 % phosphate sodium dodecyl sulfate acrylamide gels only one band was observed as shown by the gel scan in Figure 26. These results indicated that charge forms were present in rat α-lactalbumin.

Varying percent disc acrylamide gels prepared by the procedure of Hedrick and Smith (69) were used to determine if the four bands present on 12 % disc electrophoresis were charge forms, aggregates, or impurities. Approximately 25 μg of rat α-lactalbumin isolated off the last P-150 column was layered on each gel. Duplicate gels of seven, eight, nine, ten, and eleven percent acrylamide gels were run. The results are shown in Figure 27. The results indicate that the four bands observed on twelve percent disc gels were probably charge forms of rat α-lactalbumin (Figure 27).

Two 100 μg samples of rat α-lactalbumin were prepared for electrophoresis by the procedure of Brew and Ashworth (68). Both 100 μg samples were layered on twelve percent disc gels and run at 3 milliamperes per gel. Following electrophoresis, one gel was cut into 2 mm slices and assayed for α-lactalbumin activity spectrophotometrically and the second gel was stained with 0.007 % coomassie blue. The results are shown in Figure 28. The results indicate that all four protein bands observed on twelve percent disc gels possess α-lactalbumin activity.

Properties of α-Lactalbumin Isolated from Rat Milk

Amino Acid Analysis

The amino acid analysis was carried out on rat α-lactalbumin isolated from Scheme V. The method of analysis has been previously described in the Methods section. The results of the amino acid analyses are shown in Table 2. Duplicate runs were made on both the 24 hour and 48 hour hydrolysates and 0.4772 mg samples were used for each hydrolysates. A minimum molecular weight was calculated based on methionine.

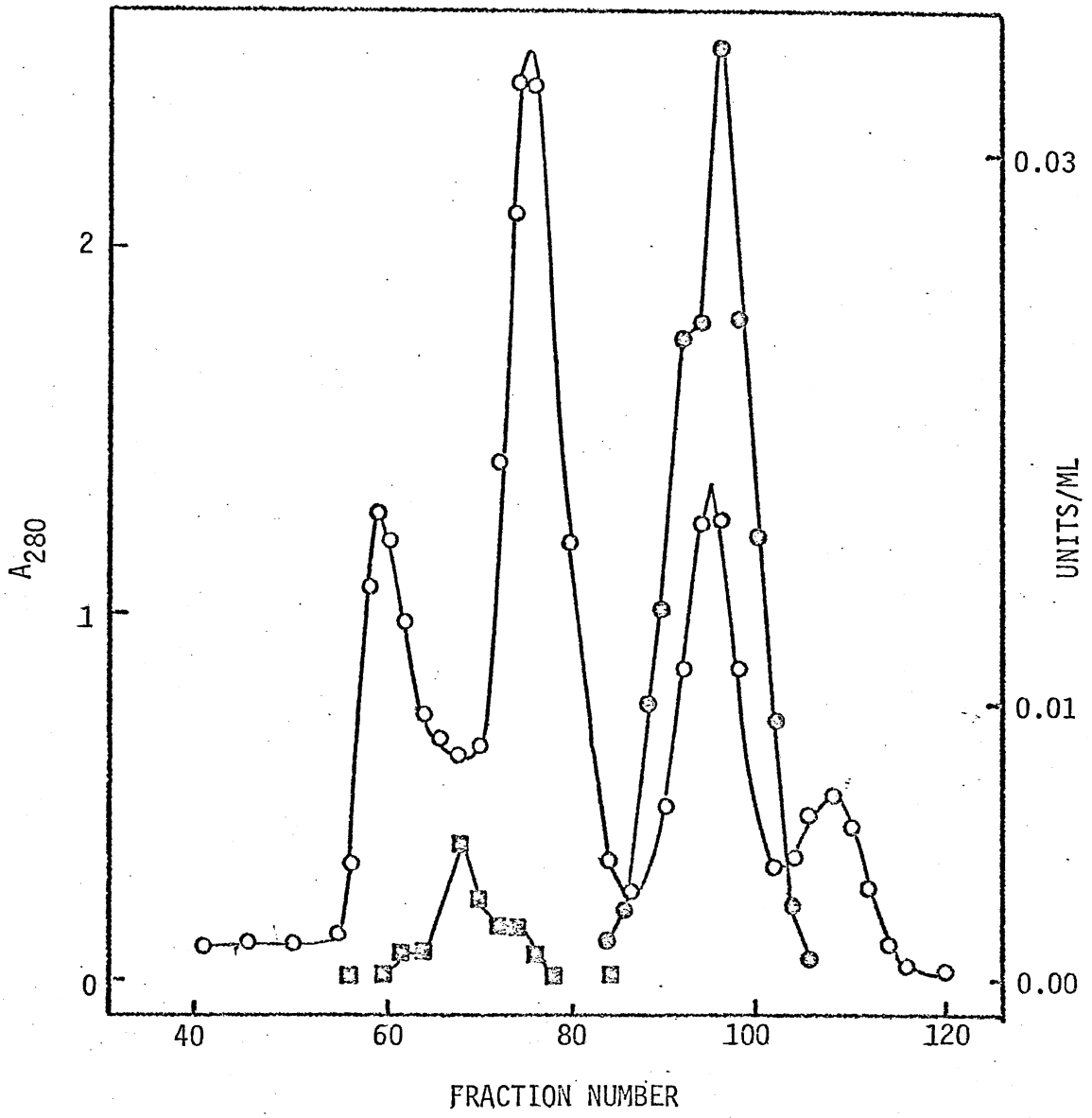

Figure 22. Typical Molecular Sieve Chromatography Profile of Whey Proteins from Rat Milk Chromatographed on a Bio-Gel P-150 Column.

Resolution of the whey proteins of skimmed rat milk chromatographed on a 3.15 x 90 cm Bio-Gel P-150 (100-200 mesh) column equilibrated with 20 mM Tris and 100 mM KCl, pH 7.5. The sample contained 200 mg in 3.34 ml of 20 mM Tris and 100 mM KCl, pH 7.5 (first part). Approximately 3.65 ml fractions were collected and measured for absorbance at 280 nm (o—o). α-Lactalbumin activity (●—●) and galactosyltransferase activity (■—■) were measured spectrophotometrically at 340 nm.

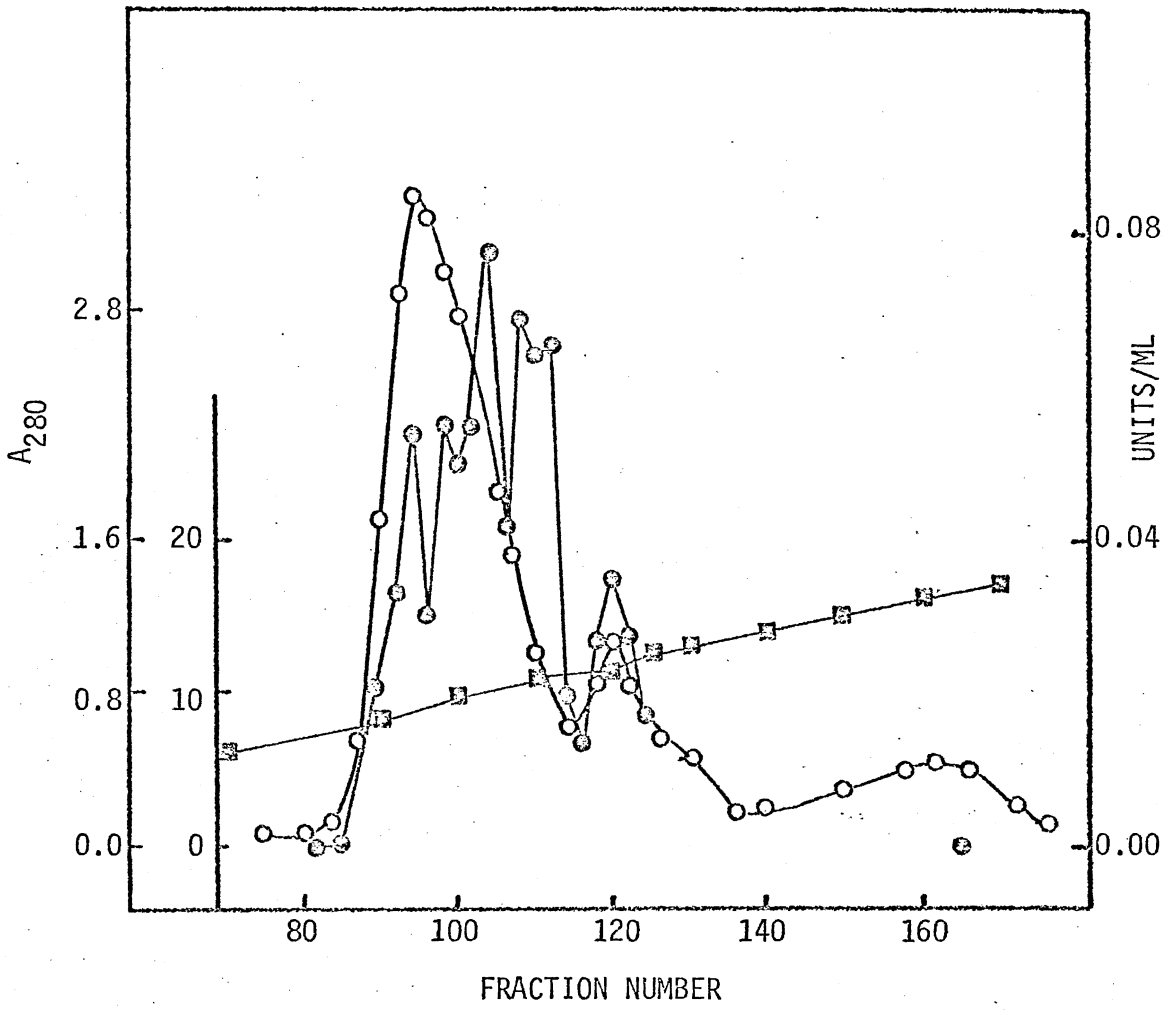

Figure 23. Typical DEAE 32 Cellulose Chromatography Profile of Rat α-Lactalbumin Utilizing Isolation Scheme VI.

Resolution of pooled rat α-lactalbumin peaks from two Bio-Gel P-150 columns rechromatographed on a 1 x 24 cm DEAE 32 Cellulose ion exchange column equilibrated with 20 mM Tris, pH 7.68 at 4° C. The protein was eluted with a linear gradient of 0 mM KCl to 300 mM KCl, 20 mM Tris, pH 7.68 at 4° C (150 ml each). Approximately 1.4 ml fractions were collected and measured for absorbance at 280 nm (o—o). α-Lactalbumin activity was measured spectrophotometrically at 340 nm (●—●). The gradient was measured by conductivity (■—■).

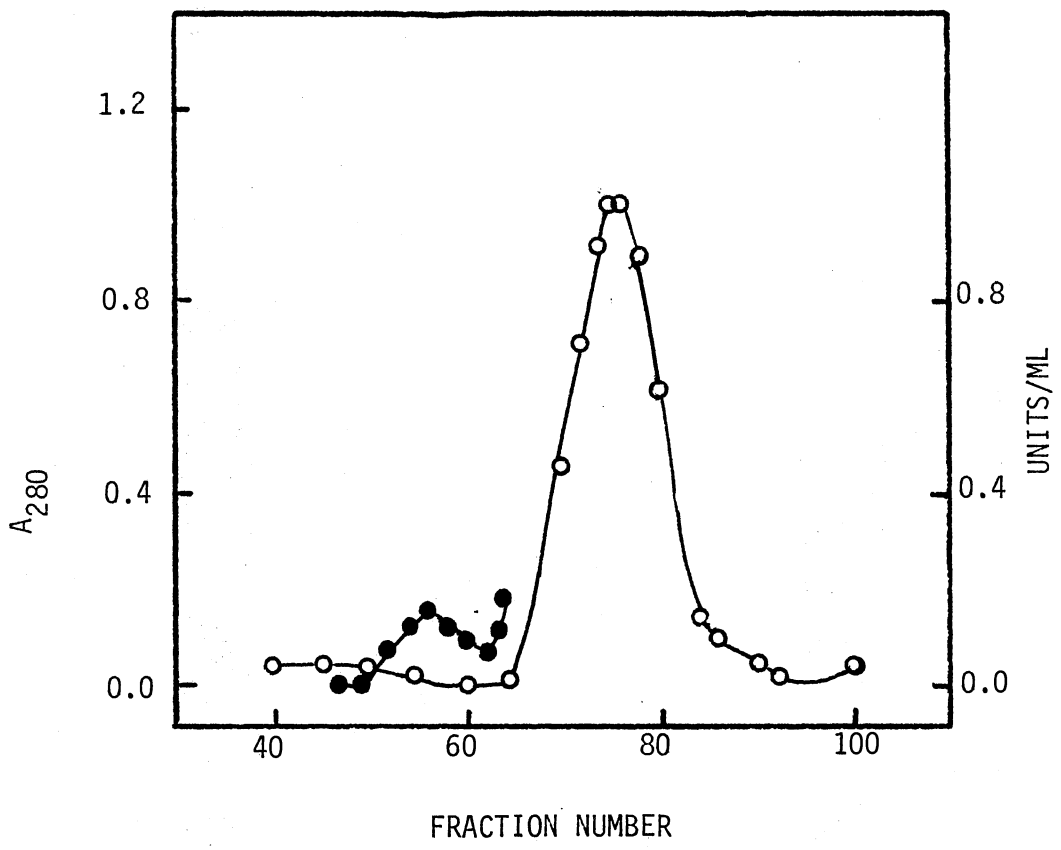

Figure 24. Molecular Sieve Chromatography Profile of Rat α-Lactalbumin over Final P-150 Column Utilizing Isolation Scheme VI.

Resolution of pooled rat α-lactalbumin peaks from two Bio-Gel P-150 columns followed by chromatography on a DEAE 32 ion exchanged column. The α-lactalbumin peak was pooled and rechromatographed on a 3.15 x 90 cm Bio-Gel P-150 (100-200 mesh) equilibrated with 20 mM Tris and 100 mM KCl, pH 7.5 at 4° C. Approximately 5.1 ml fractions were collected and measured for absorbance at 280 nm (o—o) and 220 nm (●—●).

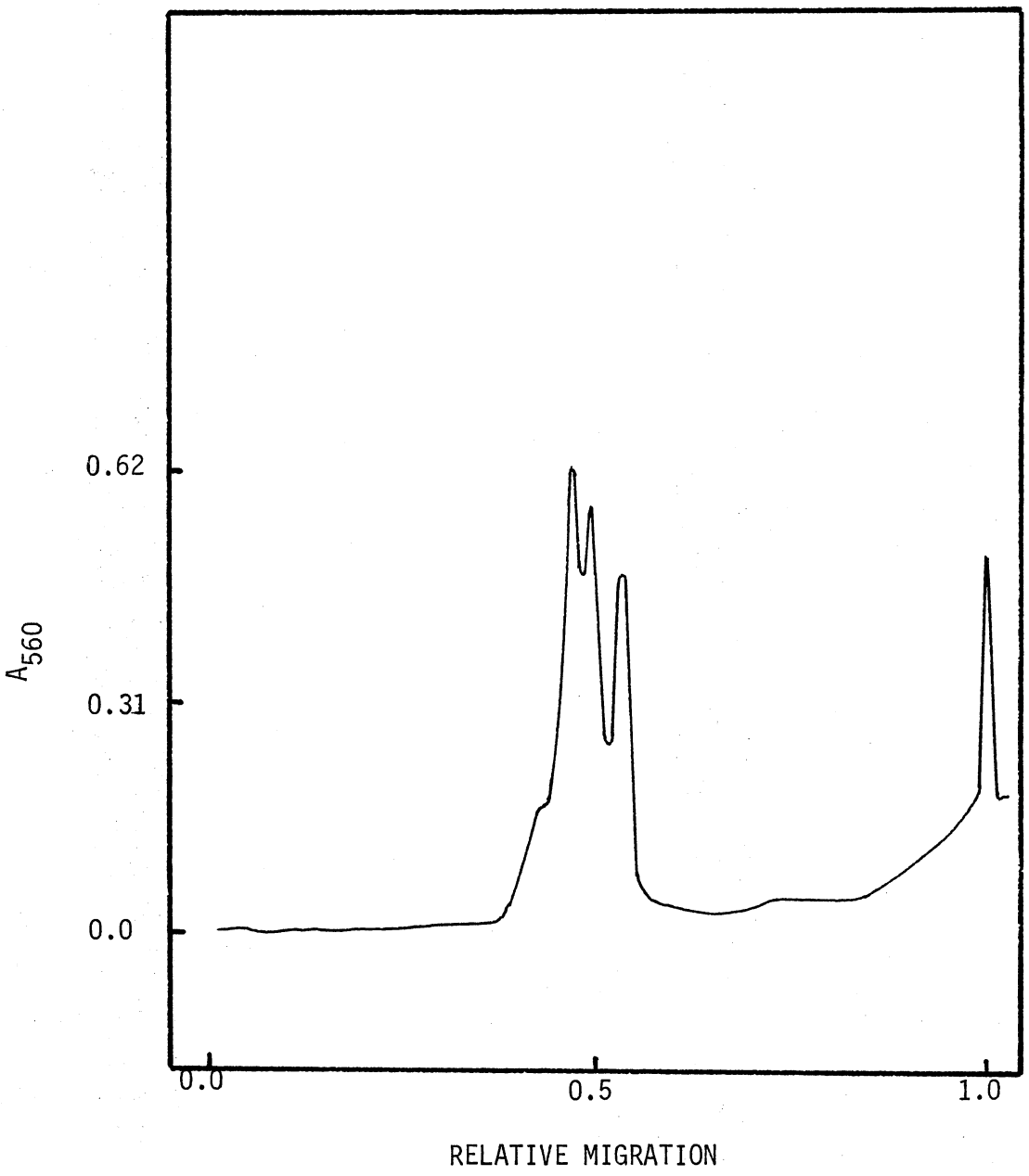

Figure 25. Disc Electrophoresis of Pooled α-Lactalbumin from the Final P-150 Column Utilized in Isolation Scheme VI.

Scan of a 12 percent polyacrylamide disc gel is shown in the figure. The samples are from the pooled rat α-lactalbumin peak from the final P-150 column utilized in isolation Scheme VI. A 25 μg sample was applied to the gel.

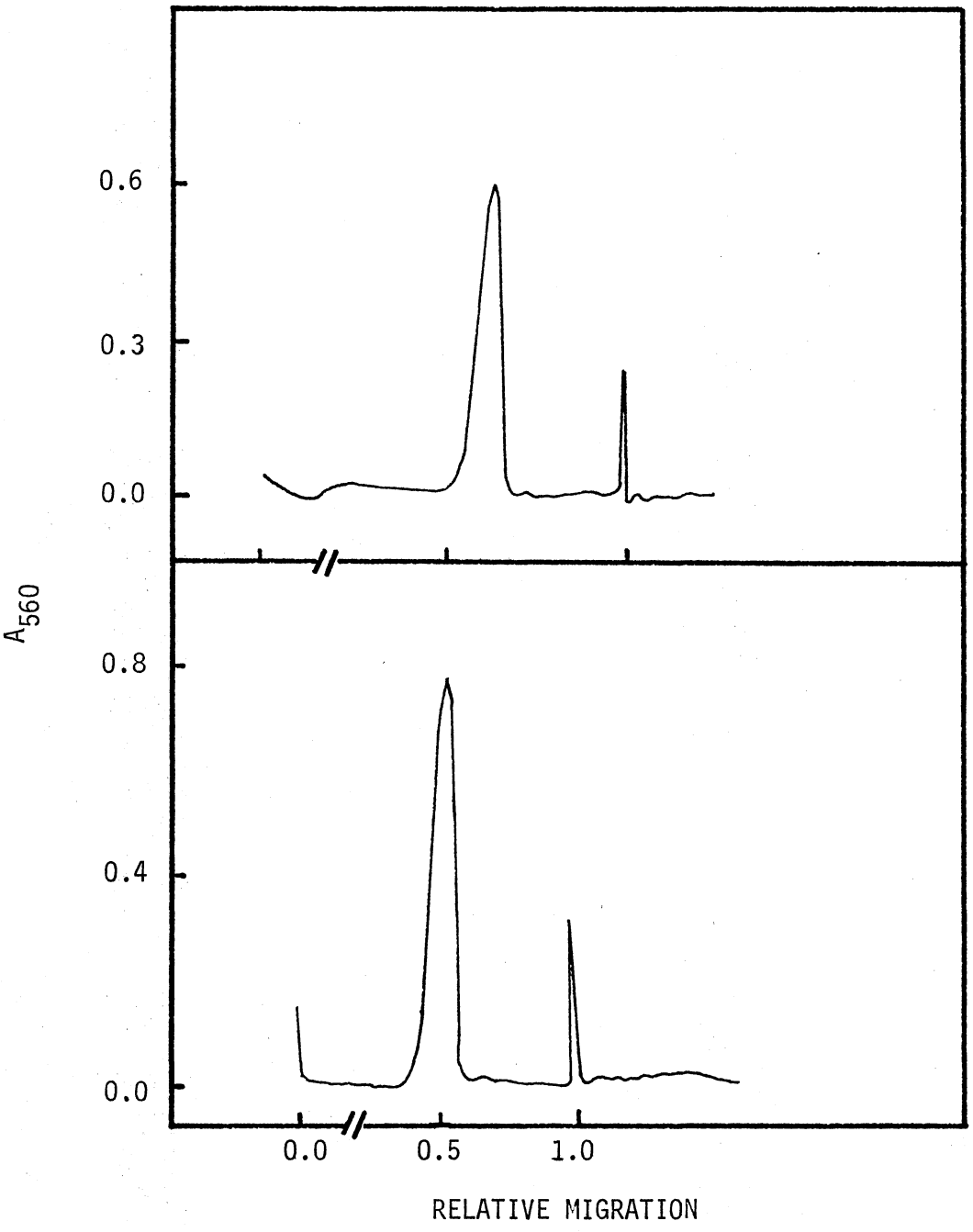

Figure 26. Eleven Percent Sodium Dodecyl Sulfate Acrylamide Electrophoresis of Pooled α-Lactalbumin from the Final P-150 Column Utilized in Isolation Scheme VI.

Scans of 11 percent polyacrylamide sodium dodecyl sulfate gels are shown in the figure. The samples are from the pooled rat α-lactalbumin peak from the final P-150 column utilized in isolation Scheme VI. The scans of the gels are (top) 25 μg of rat α-lactalbumin (bottom) 50 μg of rat α-lactalbumin.

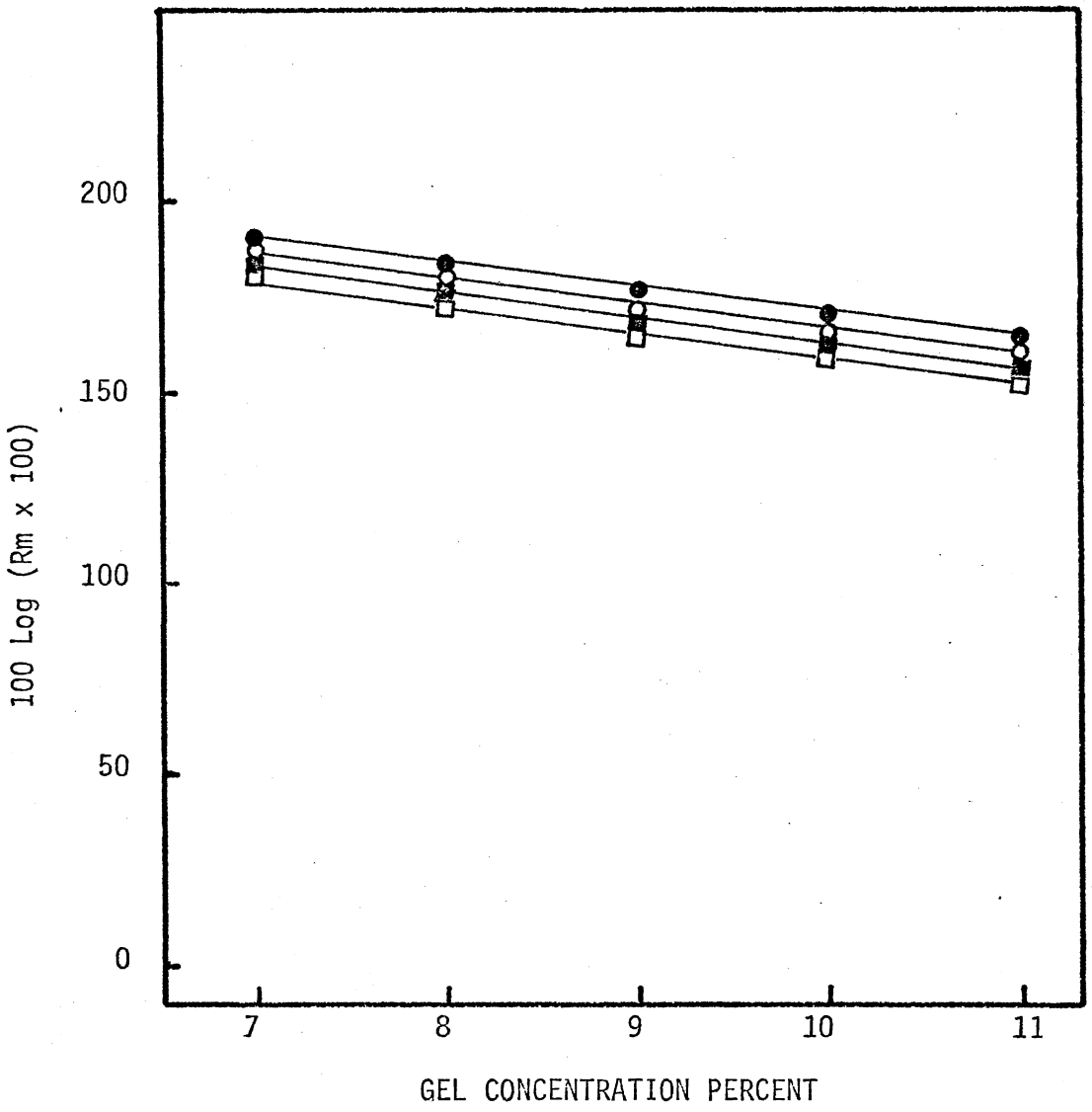

Figure 27. Graph of Various Percent Disc Gels of Rat α-Lactalbumins.

A graph of the various percent disc gel electrophoresis of rat α-lactalbumin. From top to bottom the protein bands are listed as (1) the fastest moving band (●) (2) the second or next to fastest moving band (○) (3) the third band (■) (4) the slowest protein band (□).

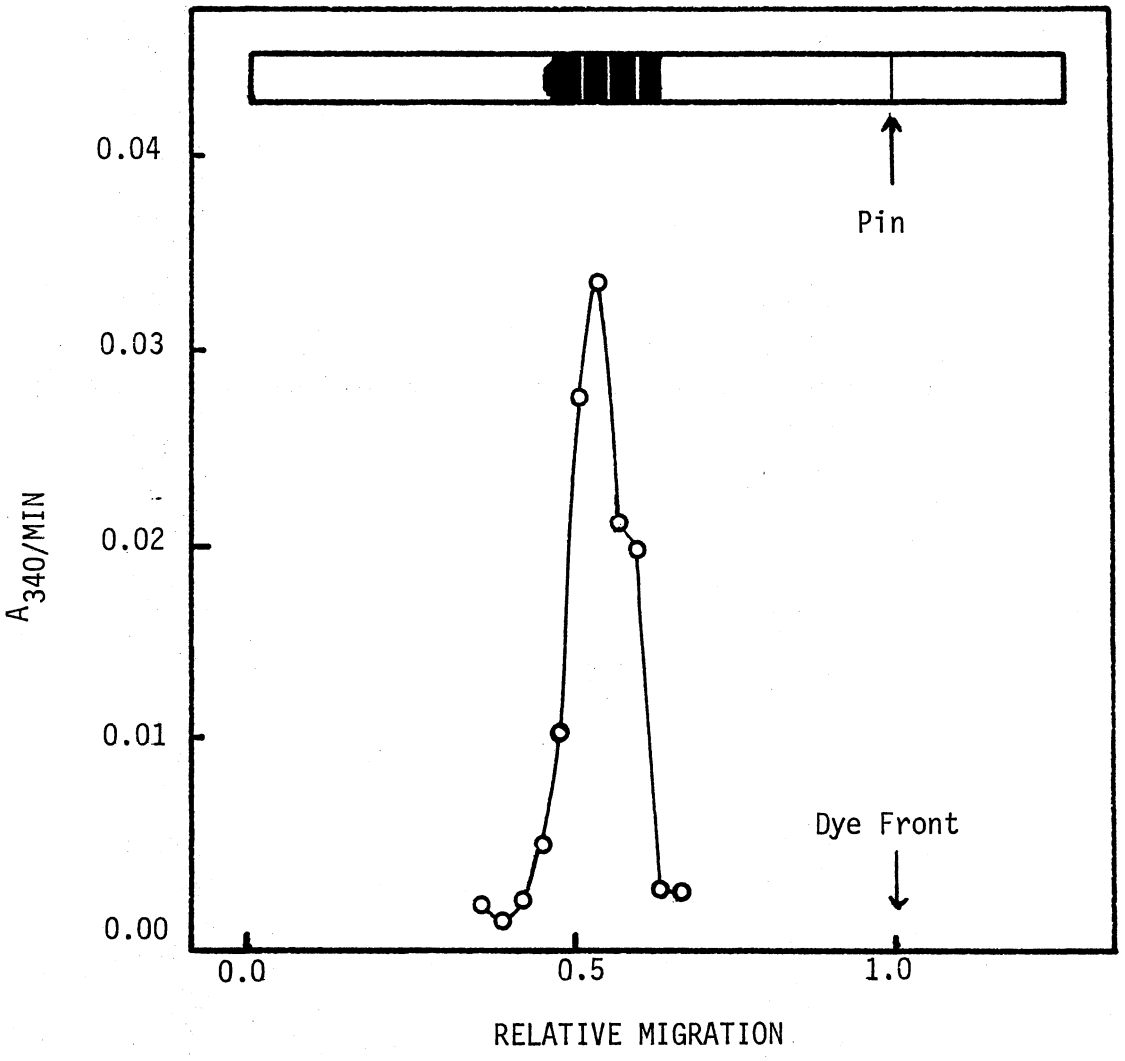

Figure 28. Activity of Rat α-Lactalbumin Extracted from Disc Gels.

Rat α-lactalbumin (100 μg) isolated by Scheme VI was run on 12 percent disc gels. One gel was sliced into 1 mm sections, and the protein extracted and assayed spectrophotometrically at 340 nm for α-lactalbumin activity. At the top of the figure is a drawing of a duplicate gel run simultaneously and stained with commassie blue.

TABLE II

AMINO ACID ANALYSIS OF RAT α-LACTALBUMIN
ISOLATED FROM RAT MILK

Amino Acid	μmoles	μmole 100 mg	Mg/100 mg	Residues/1000	Minimum Molecular Weight	Minimum Number of Residues
Lysine	0.3061	64.14	8.222	84.2	769.2	6
Histidine	0.08585	17.99	2.468	23.6	274.4	2
Arginine	0.09415	19.73	3.082	25.9	312.4	2
Aspartic Acid	0.4230	88.64	10.202	116.9	920.8	8
Threonine	0.1836	38.47	3.889	50.5	404.4	4
Serine	0.3177	66.51	5.798	87.4	522.6	6
Glutamic Acid	0.4875	102.15	13.188	134.1	1161.9	9
Proline	0.1944	40.73	3.955	53.5	388.4	4
Glycine	0.1989	41.68	2.380	54.7	228.4	4
Alanine	0.2413	50.56	3.595	66.4	355.5	5
Half Cystine	0.1547	32.41	3.345	42.5	309.6	3
Valine	0.1759	36.86	3.653	48.4	297.3	3
Methionine	0.05171	10.84	1.422	14.2	131.2	1
Isoleucine	0.2337	48.97	5.543	64.3	566.00	5
Leucine	0.2373	49.72	5.628	65.3	566.0	5
Tyrosine	0.1013	21.22	3.463	27.8	326.4	2
Phenylalanine	0.1461	30.61	4.506	40.2	441.60	3
Total			84.3391		7976.1	72
Carbohydrate (mg/100 mg)			10.61			
Recovery			94.949 %			

24 hour hydrolysis of rat α-lactalbumin. Each amino acid analysis represents an average of duplicate analysis.

TABLE III

AMINO ACID ANALYSIS OF RAT α-LACTALBUMIN
ISOLATED FROM RAT MILK

Amino Acid	μmoles	μmole 100 mg	Mg/100 mg	Residues/1000	Minimum Molecular Weight	Minimum Number of Residues
Lysine	0.2985	62.55	8.019	86.10	769.2	6
Histidine	0.08369	17.54	2.406	24.1	274.40	2
Arginine	0.08949	18.73	2.926	25.86	312.40	2
Aspartic Acid	0.3801	79.65	9.168	109.7	808.8	8
Threonine	0.1578	33.06	3.342	45.5	310.10	3
Serine	0.2693	56.43	4.915	77.7	609.7	6
Glutamic Acid	0.4765	99.85	12.891	137.5	1291.00	10
Proline	0.2064	43.25	4.200	59.5	388.40	4
Glycine	0.1911	40.04	2.286	55.1	228.40	4
Alanine	0.2333	48.88	3.475	67.3	355.50	5
Half Cystine	0.1343	28.14	2.904	38.7	309.60	3
Valine	0.1927	40.38	4.002	55.6	396.40	4
Methionine	0.4738	9.928	1.303	13.6	131.2	1
Isoleucine	0.2427	50.85	5.756	70.0	566.2	5
Leucine	0.2252	47.19	5.342	65.0	566.2	5
Tyrosine	0.09172	19.22	3.137	26.4	326.40	2
Phenylalanine	0.1443	30.23	4.450	41.6	441.60	3
Total			80.522		8085.50	73
Carbohydrate (mg/100 mg)			10.61			
Recovery			91.132 %			

48 hour hydrolysis of rat α-lactalbumin. Each determination represents an average of duplicate analysis.

Carbohydrate Analysis

The total neutral sugars of rat α-lactalbumin were determined by the procedure described by Roe (77). Free sialic acid content of rat α-lactalbumin was determined by the thiobarbituric assay method of Warren (80). Neutral sugars and hexosamines were determined after being released from the protein by hydrolysis in 1 and 2 sulfuric acid for 4 hours in sealed tubes. The hydrolysate was passed first over a column of Dowex 50- x 4(H^+)(200-400 mesh) as described by Spiro (78). Neutral sugars in the effluent were determined by the method described by Lee et al. (79). The results of the carbohydrate analysis are shown in Table III.

Spectroscopic Studies of α-Lactalbumin Isolated from Rat Milk

The rat α-lactalbumin used for spectroscopic studies was obtained from isolation Scheme VI. The results of ultraviolet circular dichroism studies of both the near and far ultraviolet spectra of rat α-lactalbumin are shown in Figures 29 and 30 respectively. The present α-helic of rat α-lactalbumin calculated by the method of Chen and Yang (74) at 221 nm was 12.36 %. An ultraviolet scan of rat α-lactalbumin from 290 nm to 320 nm is shown in Figure 31. An extinction coefficient for rat α-lactalbumin was determined and the 1 % E_{280} was 16.24.

Quantitation of α-Lactalbumin Isolated from Rat Milk

The yield of α-lactalbumin isolated from rat milk utilizing Scheme VI was calculated. The yield of rat α-lactalbumin was based on the initial volume of whole rat milk and the α-lactalbumin recovered off the last

TABLE IV

CARBOHYDRATE DETERMINATION OF RAT α-LACTALBUMIN

Hexose	moles	moles Corrected	moles	moles Corrected	Average	mg of Carbohydrate	% of α-Lactalbumin
Mannose	.0593	0.0599	0.0646	0.672	0.0635	0.0103	2.16
Fucose	.0173	0.0174	0.0136	0.0141	0.0159	0.00232	0.49
Galactose	.0708	0.0715	0.0675	0.0702	0.0708	0.0115	2.40
Glucose	.0228	0.0231	0.0157	0.0160	0.0197	0.00319	0.67
Total Hexose							5.72
Total Sialic Acid							4.89
Total Carbohydrate (%)							10.61 %

*Total hexose of protein determined by anthrone (77) (6.89)

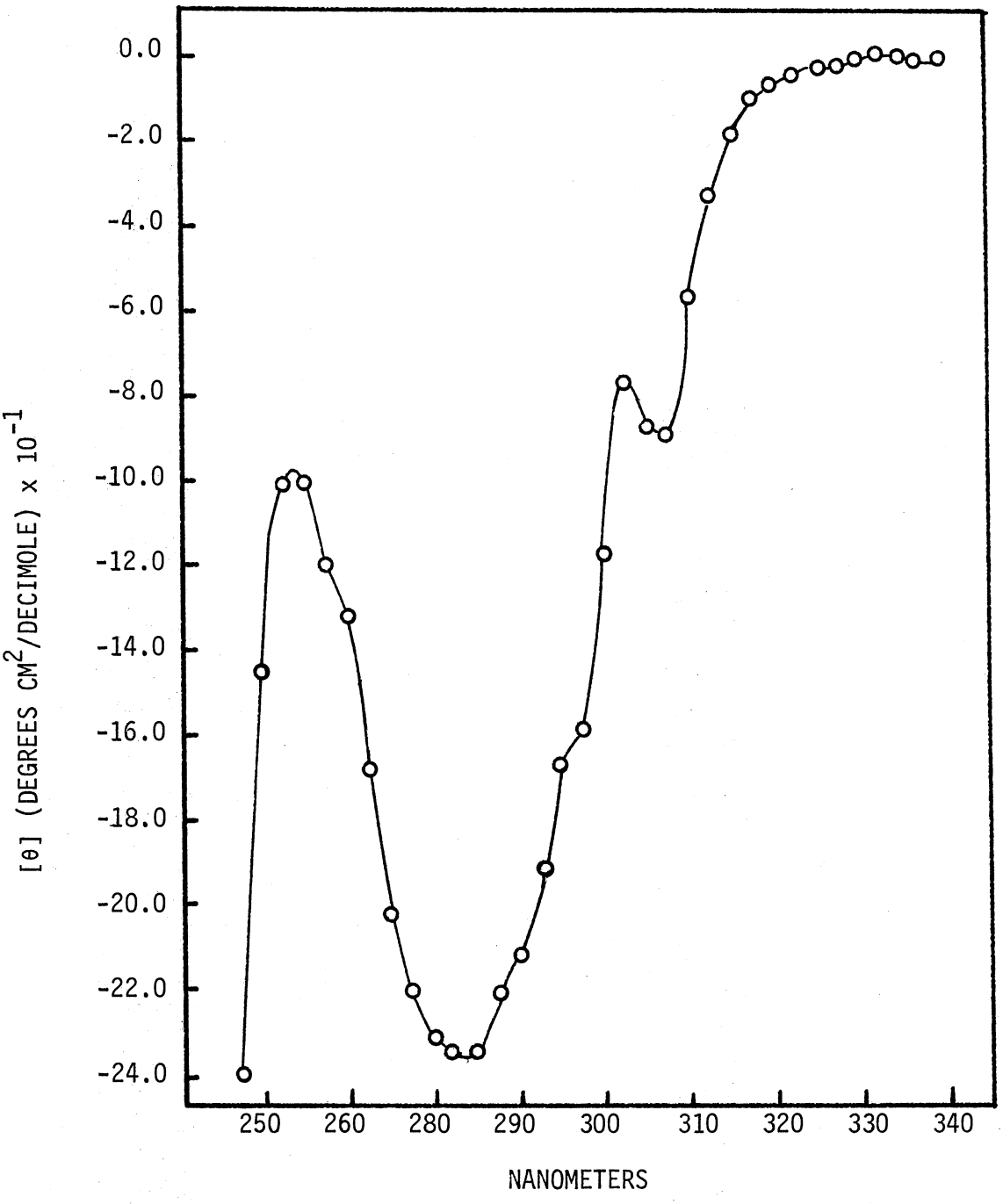

Figure 29. Near Ultraviolet Circular Dichroism Spectrum of Rat α-Lactalbumin.

Ultraviolet circular dichroism spectrum from 350 nm to 247.5 nm (near spectrum) of rat α-lactalbumin from isolation Scheme VI. 0.463 mg of rat α-lactalbumin was placed in 20 mM Tris and 100 mM KCl, pH 7.5. The scan was run using a 2 mm pathlength with a range of 0.05 at a scanning rate of 0.2 nm/second.

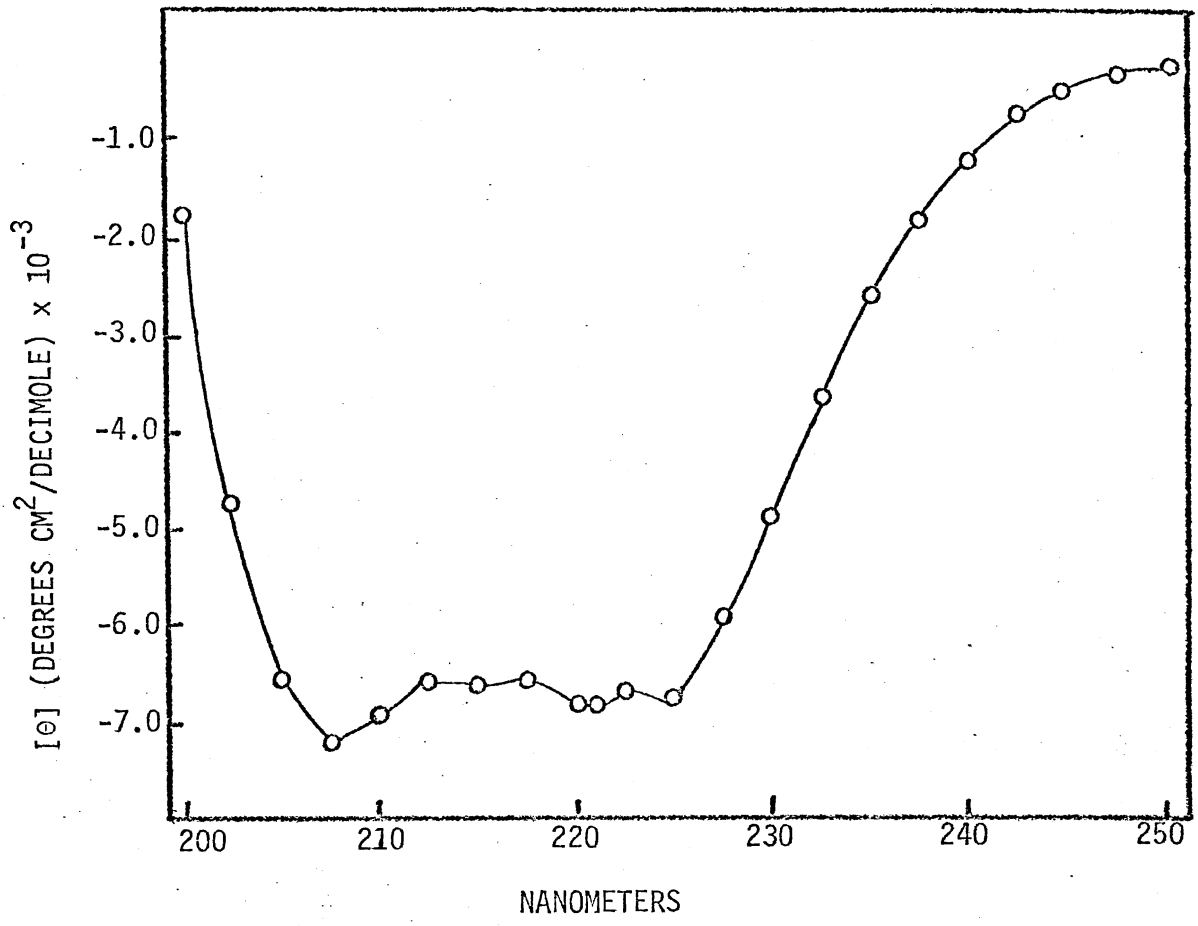

Figure 30. Far Ultraviolet Circular Dichroism Spectrum Scanned from 250 nm to 200 nm of Rat α-Lactalbumin.

Ultraviolet circular dichroism spectrum scanned from 250 nm to 200 nm (far spectrum) of rat α-lactalbumin from isolation Scheme VI. 0.2315 mg of rat α-lactalbumin was placed in 20 mM Tris and 100 mM KCl, pH 7.5. The scan was run utilizing a 1 mm pathlength with a range of 0.5 at a scanning rat of 0.2 nm/second.

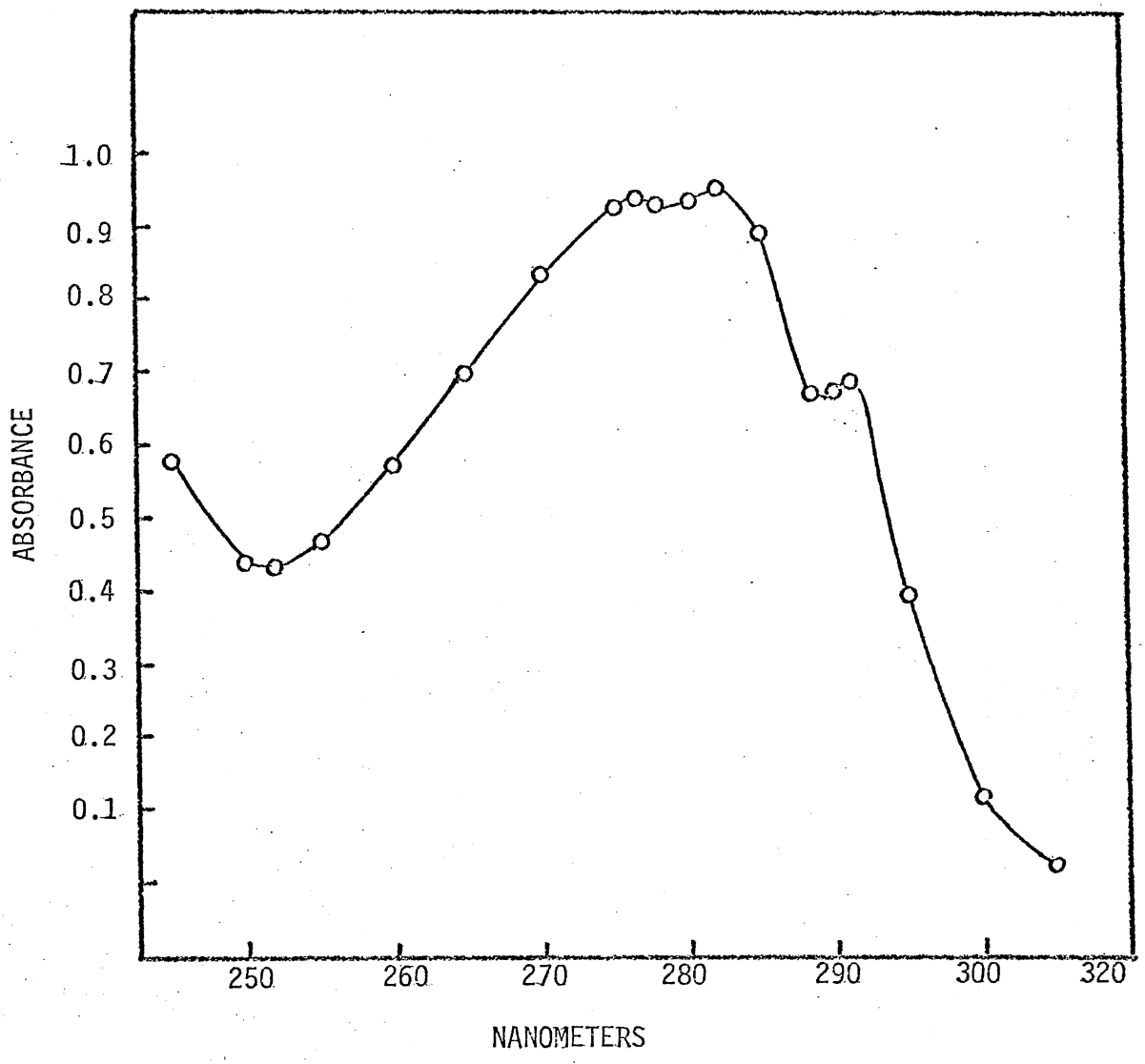

Figure 31. Ultraviolet Spectrum of Rat α-Lactalbumin.

An ultraviolet scan of rat α-lactalbumin isolated from rat milk utilizing Scheme VI. 0.541 mg was placed in one ml of 20 mM Tris and 100 mM KCl, pH 7.5.

rat milk contained low amounts of water obtained during the milking process. Approximately 0.94 mg of pure rat α-lactalbumin was obtained per ml of whole rat milk.

Molecular Weight Determinations of Rat α-Lactalbumin

The molecular weight of rat α-lactalbumin was determined on 10 percent sodium dodecyl sulfate acrylamide gels. The rat α-lactalbumin was isolated utilizing Scheme VI. The procedure used was outlined in the experimental section. Both a 25 μg and 50 μg sample of rat α-lactalbumin was used to determine its molecular weight. The α-lactalbumin marked at 25,500 and 26,000, giving an average molecular weight of 25,750.

The molecular weight of rat α-lactalbumin was also determined by gel filtration using a 2.0 x 90 cm Bio-Gel A0.5m. The procedure used is outlined in the experimental section. The molecular weight of the rat α-lactalbumin was determined to be 28,500 by gel filtration.

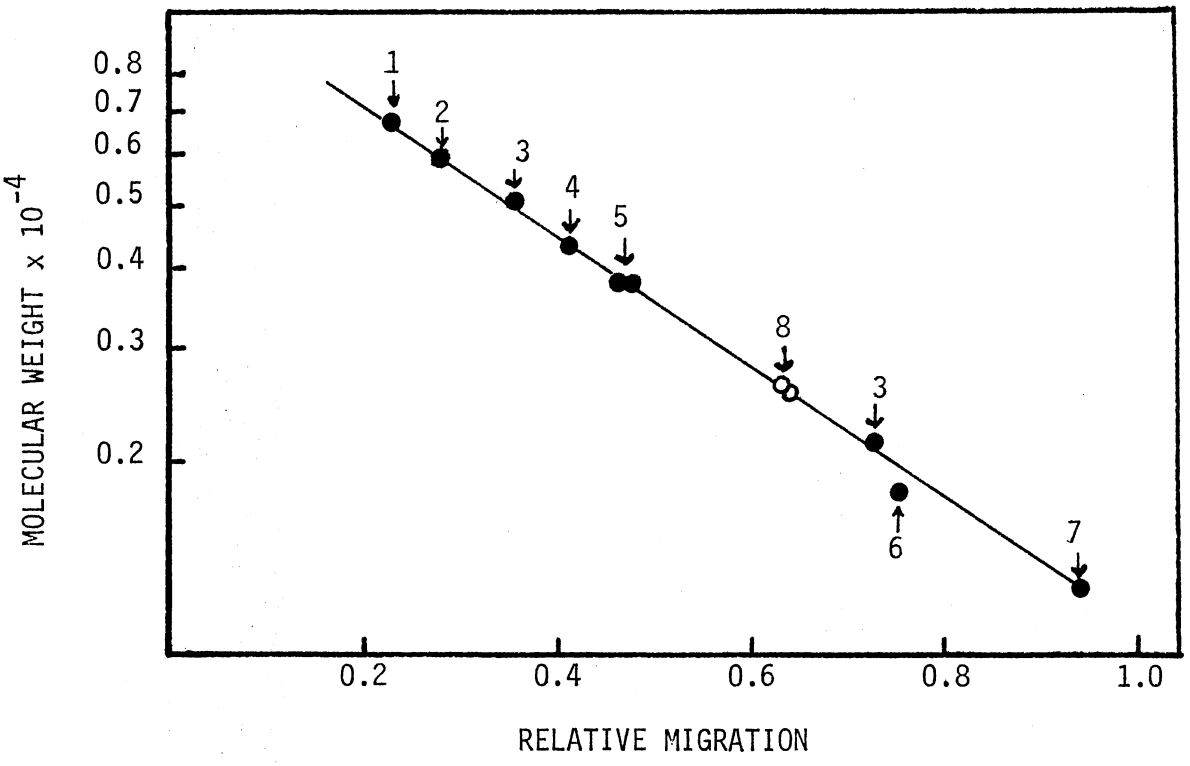

Figure 32. Molecular Weight Standardization Curve of Rat α-Lactalbumin in Sodium Dodecyl Sulfate Acrylamide Gels.

A protein standardization curve for sodium dodecyl sulfate acrylamide gels of rat α-lactalbumin. The standards proteins were: 1, bovine serum albumin; 2, catalase; 3, γ-globulin; 4, ovalbumin; 5, glyceraldehyde dehydrogenase; 6, myoglobin; 7, bovine α-lactalbumin. Duplicate samples of rat α-lactalbumin; 8, were run, 25 and 50 μg of protein respectively. Ten μg of each standard protein was used.

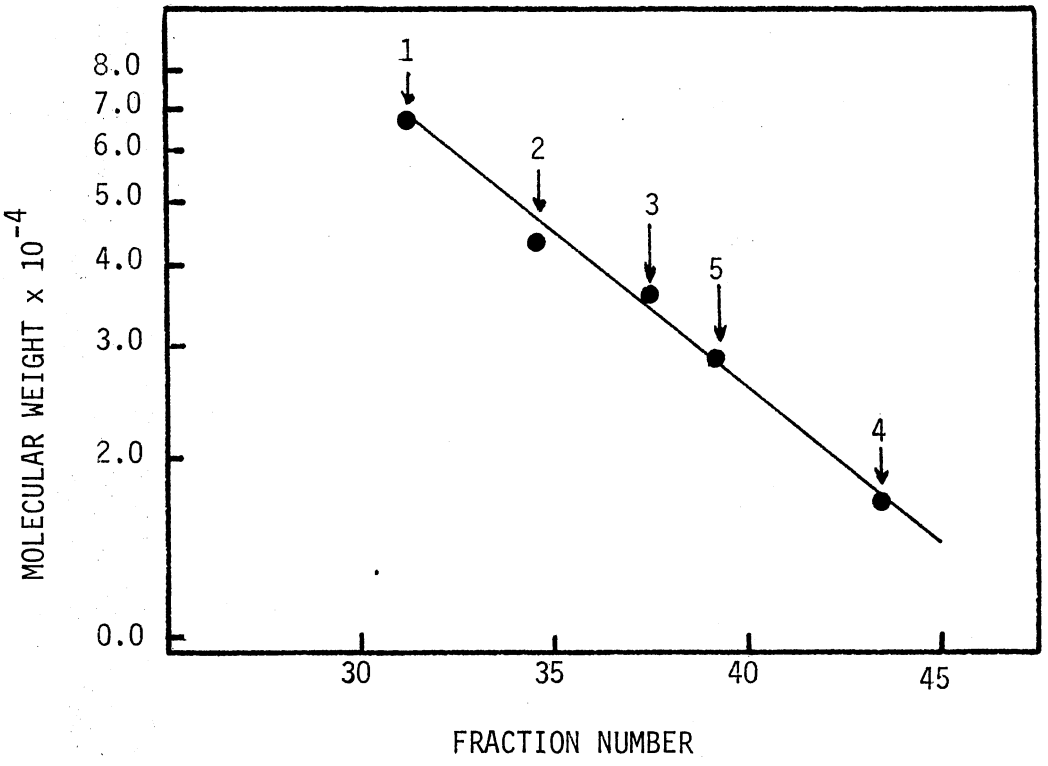

Figure 33. Molecular Weight Determination of Rat α-Lactalbumin.

A Bio-Gel 0.5 m agarose (2 cm x 90 cm) column was equilibrated and eluted with 20 mM Tris, 100 mM KCl, 0.02 percent sodium azide at pH 7.5. 1.5 ml fractions were collected. The standard proteins were: 1, serum albumin (5mg); 2, ovalbumin (5mg); 3, β-lactoglobulin (5mg); 4, myoglobin (5mg). Blue dextran with ten percent sucrose was used with the standard proteins for determination of the void volume. The rat α-lactalbumin (5) was placed on the column separately and run as an individual experiment.

CHAPTER IV

DISCUSSION AND SUMMARY

Caseins from whole rat milk could be removed by either centrifugation or acid precipitation (Figure 4), and material obtained by both methods gave identical elution profiles on a Bio-Gel P-60 column (Figure 9). However, the centrifugation method is preferred because it does not involve a drastic pH change, though there was no evidence in this study to indicate differences in α-lactalbumin due to isolation method. Rat α-lactalbumin did not separate from the larger protein on a Bio-Gel P-30 column as does bovine and all other α-lactalbumin known to date. These results suggested that rat α-lactalbumin may have different properties or a different molecular weight than other α-lactalbumins. Therefore, a different isolation scheme was necessary to obtain highly purified α-lactalbumin from rat milk. The column of choice was a Bio-Gel P-150 column equilibrated and eluted with 20 mM Tris, 100 mM KCl, at pH 7.5 (Figure 22). This procedure was particularly useful in separating a protein with a molecular weight of 68,000, and this protein is most likely rat serum albumin (81). It was also noted that the galactosyltransferase in rat milk eluted ahead of this protein on the P-150 column indicating a molecular weight greater than 68,000. Another protein chromatographed on the P-150 column near that of rat α-lactalbumin activity peak. On sodium dodecyl sulfate electrophoresis this protein had a molecular weight of 18,000, but it did not have any α-lactalbumin activity.

A Whatman DEAE 32 column separated the 18,000 molecular weight protein from the rat α-lactalbumin. However, an additional P-150 column was needed to completely remove the remainder of the 68,000 molecular weight protein which usually was 2 percent of the total α-lactalbumin after chromatography on DEAE 32.

The material off the DEAE 32 column gave a positive Schiff-Periodate (Figure 21), indicating it contained carbohydrate. This observation was unique since the only carbohydrate reported in α-lactalbumin to date was glyco-α-lactalbumin found to a small extent in bovine milk (44). Further carbohydrate analysis was done on rat α-lactalbumin isolated by Scheme V utilizing the auto-technicon to determine free hexoses (78), the anthrone assay (77) to determine total hexoses on the intact protein, and the thiobartituric assay (80) to determine the free sialic acid. The total free hexose was 5.72 %. Mannose, frucose, galactose, and glucose, comprised 2.16 %, 0.49 %, 2.40 %, and 0.67 % respectively of the total hexose content of rat α-lactalbumin. The total hexose by the anthrone assay was 6.89 % in the intact protein. The sialic acid content was 4.89 %. The total carbohydrate content of rat α-lactalbumin was 10.61 % based on free hexose and sialic acid content.

Rat α-lactalbumin isolated by Scheme V on the DEAE 32 column utilized a steeper gradient (Figure 16) than the rat α-lactalbumin isolated on the DEAE 32 column in Scheme VI (Figure 23). However, the DEAE 32 column utilized in Scheme V did show an unsymmetrical α-lactalbumin activity which coincided with the A_{280} profile. This indicated the possibility of multiple forms of α-lactalbumin which was verified by Scheme VI. Four α-lactalbumin activity peaks were observed. Electrophoresis of this α-lactalbumin on 7 % polyacrylamide gels yielded 3

active bands, whereas electrophoresis of 12 % polyacrylamide disc gels yielded 4 bands. On 10 percent polyacrylamide sodium dodecyl sulfate gels (Figure 26) the rat α-lactalbumin isolated by Scheme VI migrated as one band. It also should be noted that the α-lactalbumin active peak off the DEAE 32 column had a symmetrical A_{280} profile while the α-lactalbumin activity was unsymmetrical. This was probably due to the inability of the DEAE cellulose to resolve completely the charge forms. By varying the percent acrylamide of the disc gels by the method of Hedrick and Smith (69), it was ascertained that all four bands gave parallel lines on varying the percent of acrylamide (Figure 27) indicating that they were charge isomers. Upon cutting the bands out of a 12 % disc gel and assaying the extracted slices for α-lactalbumin activity (Figure 28) all four bands were found to be active. Therefore, it was concluded that all four bands observed on twelve percent polyacrylamide disc gels were charge forms of α-lactalbumin. The charge forms could be due to varying amounts of sialic acid content or differences in amino acid composition.

Circular dichroism experiments were performed on the rat α-lactalbumin and the results are shown in Figures 29 and 30. The near ultraviolet spectra (250 nm to 340 nm) of rat α-lactalbumin (Figure 29) is almost identical to the near ultraviolet spectra of bovine α-lactalbumin (82). The far ultraviolet spectra (200 nm to 250 nm) of rat α-lactalbumin (Figure 30) is slightly different from the far ultraviolet spectra of bovine α-lactalbumin (82) in the region of 210 to 221 nm. This difference at 221 nm is reflective of the percent α-helix. The percent α-helix calculated by the method of Chen and Yang (74) at 221 nm was found to be 12.4 % in rat α-lactalbumin as compared to 40 % in bovine α-lactalbumin (29, 30). These results indicate that rat α-lactalbumin

may be different than the tightly folded globular bovine α-lactalbumin. However, it is realized that calculation of α-helical content from circular dichroism measurements is at times uncertain.

The ultraviolet spectrum of rat α-lactalbumin is shown in Figure 31. It should be noted that the spectrum shows a pronounced shoulder at 290 nm which is characteristic of an exposed tryptophanyl residue, as found in goat, sheep, pig, guinea pig, human and bovine α-lactalbumin (6). $E^{1\%}_{280}$ of rat α-lactalbumin was determined to be 16.24 which is similar to other α-lactalbumins.

The amino acid composition reported by Schmidt (8) was used to compare goat, sheep, pig, bovine and human (caucasian) α-lactalbumins to the amino acid composition of rat α-lactalbumin. The amino acid composition of rat α-lactalbumin is difficult to compare to other α-lactalbumins because the molecular weight of rat α-lactalbumin is considerably higher than the molecular weights of other α-lactalbumins. The amino acid composition of rat α-lactalbumin was compared to a composite α-lactalbumin amino acid composition obtained by averaging the residues/1000 residues of goat, human (caucasian), pig, sheep, and bovine α-lactalbumins (TABLE V). Rat α-lactalbumin has a higher basic amino acid composition (arginine, lysine and histidine) than found in the composite α-lactalbumin. Rat α-lactalbumin contains 85.1 lysine residues/1000 residues, 23.8 histidine residues/1000 residues and 25.8 arginine residues/1000 residues compared to 87.9 lysine residues/1000 residues, 16.8 histidine residues/1000 residues and 8.5 arginine residues/1000 residues. Rat α-lactalbumin contains more hydrophobic amino acids (proline, alanine, valine, leucine, isoleucine, phenylalanine and methionine) than found in composite α-lactalbumin. Rat α-lactalbumin contains 56.5 pro-

line residues/1000 residues, 66.8 alanine residues/1000 residues, 52.0 valine residues/1000 residues 65.2 leucine/residues/1000 residues, 67.2 isoleucine residues/1000 residues, 40.9 phenylalanine residues/1000 residues and 13.9 methionine residues/1000 residues as compared to 22.0 proline residues/1000 residues, 35.8 residues/1000 residues, 30.3 valine residues/1000 residues, 98.1 leucine residues/1000 residues, 67.6 isoleucine residues/1000 residues, 27.0 phenylalanine residues/1000 residues and 10.1 methionine residues/1000 residues found in the composite α-lactalbumin. The high proline content may account for the low percentage of α-helix in rat α-lactalbumin (12.4 %) as compared to 40 % found in bovine α-lactalbumin. Rat α-lactalbumin has slightly less acidic amino acids than composite α-lactalbumin. Rat α-lactalbumin contains 113.3 aspartic acid residues/1000 residues and 135.8 glutamic acid residues/1000 residues compared to 152.0 aspartic acid residues/1000 residues and 103.2 glutamic acid residues/1000 residues, found in composite α-lactalbumin. Rat α-lactalbumin contains more serine than composite α-lactalbumin, but approximately the same number of threonine residues as found in composite α-lactalbumin. Rat α-lactalbumin contains 82.6 serine residues/1000 residues and 48.0 threonine residues/1000 residues compared to 47.3 serine residues/1000 and 47.3 threonine residues/1000 residues found in composite α-lactalbumin. The tyrosine content of both rat α-lactalbumin and composite α-lactalbumin are approximately equal. Composite α-lactalbumins contains 27.0 tyrosine residues/1000 residues compared to 27.1 tyrosine residues/1000 residues found in composite α-lactalbumin. Composite α-lactalbumin has higher half cystine content than found in rat α-lactalbumin. Composite α-lactalbumin contains 67.8 half cystine residues/1000 residues compared to

40.6 half cystine residues/1000 residues found in rat α-lactalbumin. The recovery from the 24 hour hydrolysis was good (94.95 %) while the 48 hour hydrolysis gave fair recovery (91.13 %).

The molecular weight of rat α-lactalbumin isolated from Scheme VI was 26,000 as determined by sodium dodecyl sulfate electrophoresis (Figure 32). One gel filtration using 0.5 m agarose, the molecular weight was determined to be 28,500 (Figure 33). From amino acid analysis (allowing for 93.04 % recovery) and carbohydrate analysis (10.61 % total carbohydrate) the molecular weight was calculated to be 28,965.

It is apparent from the amino acid analysis, molecular weight determinations, and circular dichroism studies that rat α-lactalbumin has a different molecular weight, composition and structure and has properties that differ from other α-lactalbumins (goat, pig, sheep, human and bovine).

TABLE V

AMINO ACID COMPARISON BETWEEN RAT α-LACTALBUMIN AND GOAT, PIG, SHEEP, HUMAN (CAUCASIAN) AND BOVINE α-LACTALBUMIN

Amino Acid	*Rat α-Lactalbumin Residues/1000 Residues	**Composite α-Lactalbumin	Range
Lysine	85.1	87.9	75.0 - 97.6
Histidine	23.8	16.8	8.6 - 24.4
Arginine	25.8	8.5	8.1 - 9.0
Aspartic Acid	113.3	152.0	120.7 - 170.3
Threonine	48.0	47.3	41.3 - 56.9
Serine	82.6	47.3	33.1 - 56.9
Glutamic Acid	135.8	103.2	105.7 - 112.1
Proline	56.5	22.0	18.0 - 25.9
Glycine	54.9	42.2	33.1 - 48.8
Alanine	66.85	35.8	24.4 - 45.0
Half Cystine	40.6	67.8	66.1 - 72.0
Valine	52.0	30.3	8.6 - 41.3
Methionine	13.9	10.1	0 - 25.0
Isoleucine	67.2	67.6	54.0 - 86.2
Leucine	65.2	98.1	83.3 - 105.7
Tyrosine	27.1	27.0	24.8 - 32.5
Phenylalanine	40.9	27.0	24.8 - 32.5

*Represents an average of the 24 hour and 48 hour hydrolysates (not including tryptophan).

**Represents an average of residues/1000 residues for sheep, pig, goat, human (caucasian) and bovine α-lactalbumin (including tryptophan).

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