THE EFFECT OF TYROSYL REAGENTS UPON THE ACTIVITY OF ALPHA-LACTALBUMIN IN THE LACTOSE SYNTHETASE REACTION

By

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1965

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
May, 1971

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ACKNOWLEDGEMENTS

The author is most thankful to his major professor, Dr. Kurt E. Ebner, for his continual encouragement, guidance, and advice during the course of these investigations and the preparation of this dissertation. He sincerely thanks Drs. Patrick E. Guire, Ernest M. Hodnett, Eric C. Noller, and George V. Odell, for their contributions and their valuable time spent as members of the advisory committee. The author is grateful to Dr. Floyd L. Schanbacher and Dr. J. F. Morrison, NSF Visiting Professor from the Australian National University, Canberra, Australia, for their many helpful suggestions.

The author expresses a special thanks to Drs. I. Kiyosawa, B. M. Colvin, and R. B. Mawal for their generous expenditure of time and effort in supplying the A-protein.

The excellent technical assistance provided by Angela Tedesco is gratefully acknowledged.

A very special thanks goes to the author's wife, Rosemary, for her help, encouragement, and patience, during the years of graduate studies.

The financial assistance provided by the National Defense Education Association and the facilities provided by the Oklahoma State University Biochemistry Department and the Oklahoma Agriculture Experiment Station are gratefully acknowledged.

Finally, the author thanks his parents, Mr. and Mrs. Wayne L. Denton, Sr., for their sacrifices and their guidance which made this dissertation possible.

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CHAPTER I

INTRODUCTION

In 1964, no biological function for α-lactalbumin was known. Ebner and Brodbeck (1) first separated the soluble lactose synthetase from bovine milk into two components, the A and B proteins, by gel filtration, and the B protein component was subsequently identified as α-lactalbumin (2). Neither the A protein nor α-lactalbumin will catalyze separately the synthesis of lactose in significant amounts under normal biological conditions, but on recombination of the two proteins, lactose synthesis capability is fully restored.

α-Lactalbumin is an extensively studied and well characterized protein that is found in the skim milk of many species (3,4). An almost complete amino acid sequence of bovine α-lactalbumin has now been reported, and the molecular weight based on this sequence is 14,437 (5,6). Bovine α-lactalbumin is readily available and is a relatively stable protein. It is therefore an excellent protein for studying both chemical and physical modifications by reagents and conditions that would destroy the enzymatic activity of many enzymes.

α-Lactalbumin and lysozyme are structurally related proteins. Bovine α-lactalbumin and hen's egg-white lysozyme have similar molecular weights, homologous disulfide bonds, similar amino acid composition, and identical or similar $-NH_2$ and -COOH terminal residues. It is possible to actually fit the side chains of bovine α-lactalbumin to the

lysozyme polypeptide backbone, and thereby generate a structure which retains the major structural features of the lysozyme molecule (6). A related activity for the two proteins is suspected. It has been shown, however, that it is possible for the two molecules to differ in structure even in those regions where the primary sequences are similar. The pertinent questions evoked here can only be answered by a more complete understanding of the structure and the exact function of α-lactalbumin. Since there is a relation between the extent of exposure of a residue and its chemical reactivity; studying the reactivity of amino acid residues with specific reagents is a useful tool for probing their molecular environment. Therefore, the chemical modification of specific amino acid residues in α-lactalbumin and the study of the effects of these modifications on the ability of α-lactalbumin to activate lactose synthetase is an extremely important and most interesting area of investigation. Interest in this area is enhanced, since the X-ray crystallography of lysozyme has been completed, and that of α-lactalbumin is now in progress.

The chemical structure of proteins is primarily determined by the order of the amino acid residues along the peptide chains. These amino acid residues may be broadly classified into "essential" and "non-essential" groups with respect to their role in maintaining the biological activity of an enzyme. It is the customary approach to determine if certain of the amino acid groups in enzymes or biologically active proteins are essential by studying the effect of various chemical modifications on the specific biological activity of the protein. Interest then is directed toward determining the nature of the structures which are responsible for this distinctive biological action (7).

There are a large number of reagents available for modifying a given amino acid residue. However, most of the reagents presently used are nonspecific as judged by their reaction both with model compounds and proteins. Therefore, care must be taken in determining all the amino acid residues affected by a modification reaction and in addition the reaction mixture must be examined for side reactions such as polymerization.

The purpose of this study was to determine the role of the tyrosyl residues of α-lactalbumin as related to their activity in the lactose synthetase reaction. Such studies will provide further insight into the role of α-lactalbumin in the lactose synthetase reaction.

CHAPTER II

LITERATURE REVIEW

The Lactose Synthetase System

Up to one-half of the dry weight of milk consists of the carbohydrate lactose (8). No hypothesis, however, completely explains how the concentration of lactose is controlled in mammalian milk. It has been suggested that the rate of lactose synthesis in mammalian milk is proportional to the rate of protein synthesis. This suggestion, however, becomes paradoxical in view of the inverse relationship of fat and protein content to lactose content in milk from various mammals (8). It now appears that the control mechanism for the biosynthesis of lactose may be quite closely related to the functional control mechanism of the mammary gland itself (9,10,11).

Both the galactosyl and glucosyl moieties of lactose (4-O-β-D-galactosyl-α-D-glucose) are derived from blood glucose (12). The production of lactose in the mammary gland is accomplished through the utilization of three key enzymes. These three enzymes catalyze the following reactions:

I. UTP + glucose-1-P $\rightleftharpoons$ UDP-glucose + PP_i

II. UDP-glucose $\rightleftharpoons$ UDP-galactose

III. UDP-galactose + glucose $\longrightarrow$ lactose + UDP

Reaction I is catalyzed by the enzyme UDP-glucose pyrophosphorylase (UTP: α-D-glucose-1-phosphate uridyltransferase, E.C.2.7.7.9).

Reaction II is catalyzed by the enzyme UDP-galactose-4-epimerase (E.C.5.1.3.2), while the third and final reaction, which produces lactose, is catalyzed by the enzyme lactose synthetase (UDP-galactose: D-glucose 1-galactosyltransferase, E.C.2.4.1.22) (13).

In 1964, no biological function for α-lactalbumin was known (12). Ebner and Brodbeck (1) made a most significant discovery, when they first separated the soluble lactose synthetase from bovine milk into two components, the A and B proteins, by gel filtration. The A protein was shown to be associated mainly with the microsomal fraction, while the B protein was distributed between the microsomal and soluble fraction (14). Brodbeck et al. (2) subsequently identified the B protein component of lactose synthetase as α-lactalbumin. Neither the A protein nor α-lactalbumin will catalyze separately the synthesis of lactose in significant amounts under normal biological conditions, but on recombination of the two proteins, lactose synthesis capability is fully restored.

α-Lactalbumin, the B protein of lactose synthetase, is an extensively studied and well characterized protein that is found in the skim-milk of many species (3,4). Gordon and Ziegler (15) in 1955 reported its amino acid composition. Weil and Seibles (140) in 1965 reported that α-lactalbumin consisted of a single chain with the N-terminal as glutamic acid and the C-terminal as leucine. They later reported on the amino acid sequence of four peptides formed on peptic hydrolysis of the native protein. An almost complete amino acid sequence of bovine α-lactalbumin has now been reported, and the molecular weight based on this sequence is 14,437 (5,6). Bovine α-lactalbumin is readily available and is a relatively stable protein. It is therefore an excellent

protein for studying both chemical and physical properties, and allows treatments that would normally destroy the enzymatic activity of many enzymes.

Homogeneous preparations of bovine, buffalo, goat, sheep, pig, guinea pig, and human (Japanese) α-lactalbumin have been assayed with purified bovine A protein in the lactose synthetase reaction. The results showed that the enzymic capabilities of all these B proteins were qualitatively comparable (16,17).

The above preparations of α-lactalbumin were also assayed immunologically as described by Larson and Hageman (18). The results of these experiments (17), plus observations of Schmidt (19), indicate that ruminant α-lactalbumins react to antisera of bovine α-lactalbumin. Previous studies with antisera to bovine α-lactalbumin showed that the milks from ruminants reacted but nonruminant milks did not cross-react (20). These observations suggest that the catalytic or modifier site and the immunological site in α-lactalbumin are different and that there may be structural differences in these α-lactalbumins.

Hill et al. (6,9,10) have shown the A protein of lactose synthetase to be a potent UDP-galactose: N-acetylglucosamine galactosyl transferase. The A protein, in the absence of α-lactalbumin catalyzes the reaction:

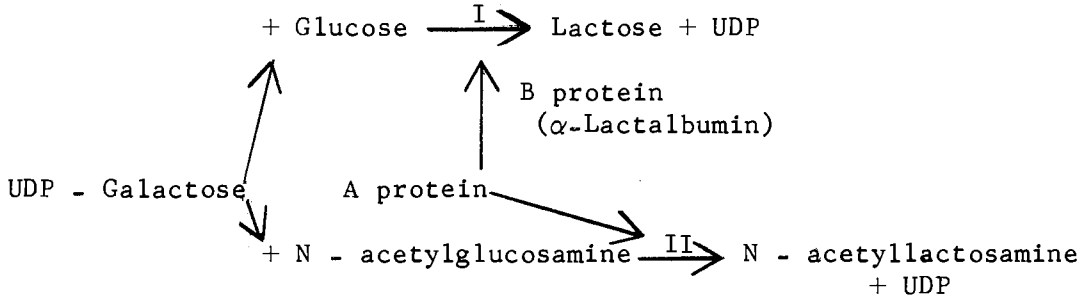
IV. UDP-D-galactose+N-acetyl-D-glucosamine $\longrightarrow$ N-acetyllactosamine+UDP

In the presence of α-lactalbumin, it catalyzes the reaction:

V. UDP-D-galactose+D-glucose $\longrightarrow$ lactose+UDP

but inhibits reaction IV. Recent work (21) has shown that reaction V is catalyzed by the A protein alone, when the glucose concentration is high

(K_m=1.4M). α-Lactalbumin lowers the K_m of glucose so that it becomes a good substrate. A schematic function of A and B proteins is shown below:



Brown et al. (6) have pointed out that it would be interesting if in the lactose synthetase reaction α-lactalbumin catalyzed a galactosyltransferase reaction:

VI. N-acetyllactosamine+D-glucose⟶ lactose+N-acetylglucosamine

This suggestion was based on the finding that α-lactalbumin is structurally similar to hen's egg-white lysozyme (5,22,23,24). The cleft region of lysozyme is directly involved in substrate binding and cleavage (25). The substrate N-acetyllactosamine would fit well into the proposed abbreviated cleft of α-lactalbumin formed from the structural model based on the X-ray crystallography model of lysozyme (6). This simple explanation, however, was clearly ruled out by the experimental observations of Brew et al. (9) who showed that α-lactalbumin has no activity toward N-acetyllactosamine.

Up to the present time, there has been no catalytic activity ascribed to α-lactalbumin, but instead it appears to act as a modifier or specifier of the A protein (9,10,22). The chief reasoning behind designating α-lactalbumin as a modifier or specifier protein is that under normal assay conditions, α-lactalbumin inhibits the A protein catalyzed galactosyltransferase to N-acetylglucosamine (IV), while in

the presence of glucose it allows the synthesis of lactose via the lactose synthetase reaction (V). Thus, α-lactalbumin must "modify" the substrate specificity of the galactosyltransferase from N-acetyl-D-glucosamine to glucose. The role "specifier" protein is one that has not been previously ascribed to a protein, and it is quite possible that this represents a new type of molecular control of a biological reaction (5).

From kinetic studies it appears that physical interaction between the A protein and α-lactalbumin is required for lactose synthetase activity (2,26). Coffey and Reithel (27,28) have reported that more than half of the lactose synthetase activity of the mammary gland can be recovered intact in subcellular particles co-sedimenting with lysosomes. These particles appeared to be related to the Golgi apparatus and suggest an in vivo particulate complex of A protein and α-lactalbumin. Palmiter (29) has proposed a third component, Z, which in crude preparations prevents the rapid dissociation of the A protein-α-lactalbumin complex. This component is supposedly lost during purification and the lactose synthetase subunits then obey the law of mass action and chemical equilibrium. The evidence for this Z component is not convincing at the present time.

Relationship Between Lysozyme and α-Lactalbumin

Lysozyme was the second protein and the first enzyme to have its molecular structure detailed by X-ray analysis (24,25,30,31,32,33,34). The enzyme attacks many bacteria by degrading the mucopolysaccharide structure of the cell wall. The catalytic mechanism of lysozyme was elucidated using the N-acetylglucosamine trimer to form a stable

inhibitory complex which was subjected to X-ray analysis (35). The molecule is egg-shaped and has a crevice or cleft running horizontally across the molecule which forms the active site.

Since the cleft region of lysozyme contains the active site, it is tempting to deduce that the proposed cleft of α-lactalbumin should likely be its active site. Since the reactions catalyzed by lysozyme and lactose synthetase are similar, it is reasonable to propose that the cleft region of lysozyme is designed for the cleavage of β(1→4) glucopyranosyl linkages, whereas the cleft region of α-lactalbumin might be involved in the synthesis of β (1→4) glucopyranosyl linkages if this were the catalytic function of α-lactalbumin.

Hill et al. (10) in the "Brookhaven Symposia in Biology" and Brown et al. (6) have thoroughly reviewed the structural relationship between lysozyme and α-lactalbumin. These findings are summarized and elaborated in the following section.

Yasunobu and Wilcox (23) observed that bovine α-lactalbumin and hen's egg-white lysozyme have similar molecular weights, the same number of disulfide bonds, similar amino acid compositions, and identical or similar $-NH_2$ and -COOH terminal residues. Brew, Vanaman, and Hill (5) found that the amino acid sequences of bovine α-lactalbumin and lysozyme are strikingly similar. This homology in primary structure suggests that the structural genes for lysozyme and α-lactalbumin have evolved from a relatively recent common ancestor and that α-lactalbumin may have a conformation quite similar to that reported for lysozyme (25). It is possible to actually fit the side chains of bovine α-lactalbumin to the lysozyme polypeptide backbone, and thereby generate a structure which retains the major structural features of the lysozyme molecule (6).

With only a small number of deletions, the amino acid sequences of the two proteins can be aligned so that at least 45, and possibly 54, residues in α-lactalbumin are identical to corresponding residues in lysozyme and at least 23 additional residues are structurally similar. Furthermore, the four disulfide bonds in α-lactalbumin have been found to be homologous to the corresponding bonds in lysozyme (6).

The most attractive explanation for the existance of this structural homology between α-lactalbumin and lysozyme can be given in terms of the evolutionary origins of the genes which control the primary structure of these proteins (10). It is possible that the structural genes for α-lactalbumin and lysozyme were derived from a common ancestor and by gene duplication two genes were formed, one giving rise to lysozyme and the other to α-lactalbumin (5).

Brew et al. (5) also reported the partial amino acid sequence of bovine α-lactalbumin in which 108 of the 123 residues of the molecule were in exact order. Hill et al. (10) have now reported a sequence in which the positioning of all but seven residues has been established and the positioning of all four disulfides is also established. It should be mentioned, however, in comparing α-lactalbumin and lysozyme that Perutz et al. (36) have shown that it is possible for the two molecules to differ in structure even in those regions where the primary sequences are similar.

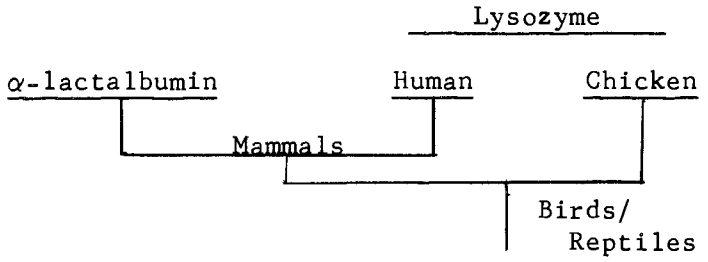
It is now suggested that the α-lactalbumins from other animal species possess structures very similar to that of bovine α-lactalbumin. Human, goat, sheep, dog, guinea pig, and kangaroo α-lactalbumin have amino acid compositions that differ only slightly from bovine α-lactalbumin (10). Human α-lactalbumin appears to be different in that

it contains one to two residues of glucosamine per molecule that are covalently bound in an unknown manner (10). It should be pointed out that bovine milk also contains an electrophoretically unique α-lactalbumin as a minor component (37). This minor component is indistinguishable from the major protein except that it contains about two residues of glucosamine (10). This difference in hexosamine content could possibly contribute to the rather low degree of immunological cross-reactivity reported recently for certain of the α-lactalbumins (17).

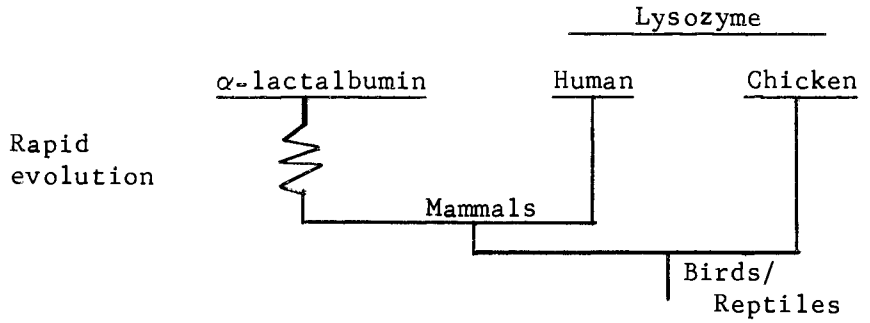
The data presented thus far certainly indicates that α-lactalbumin and lysozyme are structurally related proteins. A related activity for lysozyme and α-lactalbumin is suspected, since the two proteins may be involved in similar types of reactions (6). However, the pertinent questions evoked here can only be answered by a more complete understanding of the structure and the exact function of α-lactalbumin. Therefore, the chemical modification of specific amino acid residues in α-lactalbumin and the study of the effects of these modifications on the ability of α-lactalbumin to activate is an extremely important and most interesting area of investigation. Interest in this area is enhanced since the X-ray crystallography of lysozyme has been completed, and that of α-lactalbumin is now in progress.

Due to the marked similarity in the two proteins, it is predictable that during the evolution of mammals, α-lactalbumin diverged from lysozyme. Likely when a milk-producing system was being developed for terrestrial mammals, there was need for a polysaccharide-synthesizing enzyme, and it could be made by modifying the preexisting polysaccharide-cleaving enzyme, lysozyme. This adaptation of preexisting structures to new uses has been seen often in macroscopic anatomy, as when

fins evolve into feet. The evolution of lysozyme into α-lactalbumin would be one of the first documented examples of this same process at a molecular level (38). Such an evolutionary tree is diagrammed below:



However, if one assumes a constant rate of sequence change during evolutionary development, then bovine α-lactalbumin and human lysozyme, both being of mammalian origin, should be more similar to each other than either is to chicken lysozyme. This is not, however, the case since human and chicken lysozyme are more similar to each other than either is to bovine α-lactalbumin. One must assume then that the theory of a constant rate of sequence change is false and that during development there was a rapid evolution of α-lactalbumin as shown below (38).



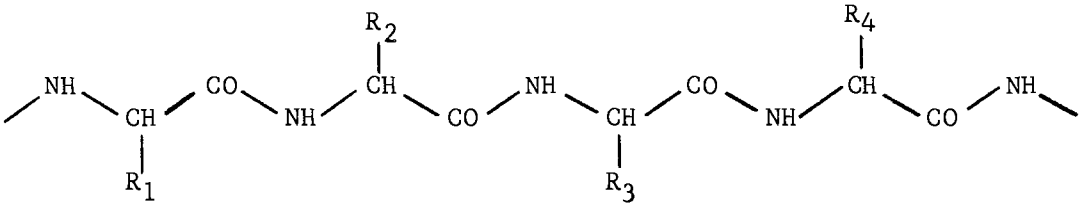
Hopefully it can soon be shown that proteins may be classified structurally according to families such as one to include α-lactalbumin, lysozyme, and possibly ribonuclease and others. These families of proteins could be structurally similar due to a relatively recent

common ancestral origin. If this were true then hopefully their mechanisms of action could be related.

Protein Modification Reactions

The chemical structure of proteins is primarily determined by the order of the amino acid residues along the peptide chains. These amino acid residues may be broadly classified into "essential" and "non-essential" groups with respect to their role in maintaining the biological activity of an enzyme. It is the customary approach when investigating if certain of the amino acid groups in enzymes or biologically active proteins are essential to study the effect of various chemical modifications on the specific biological activity of the protein. Interest then is directed toward determining the nature of the structures which are responsible for this distinctive biological action (7).

The side chains of proteins are those structures which extend outside the central peptide backbone. The side chains are often designated as R-groups as shown below:

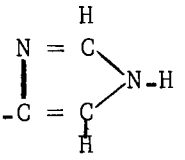


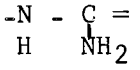
These side chains or R-groups are divided into two main categories, polar and nonpolar. The nonpolar hydrocarbon group consists of the amino acids alanine, isoleucine, leucine, phenylalanine, proline and valine. Due to the inertness of their hydrocarbon chain this nonpolar class is generally unsuitable to chemical modification study, and it is believed that their role is secondary to that of the polar amino

acid groups in enzymic catalysis (39).

It is the polar groups of amino acids upon which chemical modification is generally performed. These various polar groups and their sources within the protein structure are listed below (7,39,40).

1. Amino groups $-NH_2$ (lysine, N-terminal amino acid)

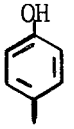
2. Imidazole groups  (histidine)

3. Guanidyl groups  (arginine)

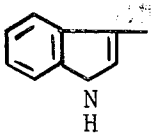
4. Carboxyl groups -COOH (aspartic, glutamic acid, C-terminal amino acid)

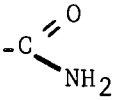
5. Sulfhydryl groups -SH (cysteine)

6. Disulfide groups -S-S- (cystine)

7. Phenol groups  (tyrosine)

8. Hydroxyl groups -OH (serine, threonine, hydroxyproline)

9. Indole groups  (tryptophan)

10. Amide groups  (glutamine, asparagine)

11. Thio ether groups CH_3-S-CH_2- (methionine)

These various groups vary greatly in their chemical reactivity and specificity toward modification.

There are a large number of reagents available to modify a given amino acid residue. However, most of the reagents presently employed are nonspecific as judged by their interaction both with model compounds

and with proteins (41). Modifying reagents may be designated as "selective" or "group-specific" reagents. Cohen (42) has defined group-specific reagents as chemical species which alter the covalent structure of a peptide or protein in a limited or controllable manner. True group-specific reagents are still in the early stage of development and in reality reagents that chemically modify a single type of functional group in a protein, to the exclusion of all other type groups, are by far the exception rather than the rule.

Within a single protein, one amino acid group such as the tyrosyl residues also often vary in their reactivity toward specific reagents. For example, when Kenner et al. (43) nitrated bovine trypsin and trypsinogen, they found that under normal conditions of nitration only six of the ten tyrosyl residues were nitrated. They also noted that three or four of these six nitrated residues were nitrated more readily than the others. This varying degree of reactivity toward specific reagents is a result of the location of the group within the three-dimensional structure of the protein. In the three-dimensional structure of proteins the individual amino acids may be present on the surface of the molecule and accessible to the solvent medium, or buried in the internal hydrophobic region and inaccessible to the solvent, or they may be in an intermediate class of partially buried and partially exposed (44). Surface or exposed groups resemble simple peptides in their reactivity toward specific reagents, or may also be super-active, while those groups buried in the interior of the protein have reactivities lower than those of simple peptides and are often not reactive at all. Those groups in the intermediate class of partially buried and partially exposed display a wide spectrum of reactivity. Since there is a relation

between the extent of exposure of a residue and its chemical reactivity, studying the reactivity of these residues with specific reagents is a tremendous tool for probing their molecular environment.

Protein Group-Specific Reagents

The term "Group-Specific Reagent" has been used repeatedly in the proceeding pages. Cohen (42) and Herriott et al. (7) have reviewed many of the protein group-specific reagents. Many of the useful group-specific reagents are listed below, with the advantages and disadvantages of each reagent's use in protein modification (7,39,41,42,44,45,46).

Acylating Agents

Acetylation of amino groups is one of the most common means used for chemical modification of enzymes. With simple acid anhydrides or acid halides the amino and the sulfhydryl groups of proteins are rapidly acylated. These reagents react also, but somewhat slower with both the phenolic and aliphatic hydroxyl groups present. It has been reported, however, that the modification can be limited to the amino groups by altering the reaction conditions. In most cases, however, simple acid anhydrides or acid halides have failed to provide the degree of selectivity or control needed for effective chemical modification of proteins (42). Acetic anhydride is the reagent of choice for the substitution of acetyl groups on the amino groups of proteins (47). By careful control of conditions only the amino groups are acylated and the extent of the reaction can be determined by relatively simple means. Acetyl chloride is as effective as acetic anhydride but results in greater protein denaturation. The cyclic anhydrides, such as succinic

anhydride, are milder reagents, and they seem to offer a greater degree of control for limited acylations (48). Thioltrifluroacetate may also be used for acylation. In the acylation of both ribonuclease (49) and cytochrome C (50) with thioltrifluroacetate, the acylation of amino groups was found to be more complete than with acetic anhydride. Trifluroacetic anhydride may also be used for acylation although it is not recommended, since its reaction is more vigorous and can cause considerable side reactions. Many other means of acetylation of proteins have been reported; these include ketene, N, S-diacetylthioethanolamine, acetic anhydride-ethyl acetate-formic acid, acetic acid-acetic anhydride, or hot 16% (v/v) acetic anhydride in acetic acid. These are in general, however, too vigorous for most enzymes.

The most useful type of acylating agent would be one that selectively acylated phenolic hydroxyls to the exclusion of amines and other nucleophiles (42). Although not perfect in this respect, N-acetylimidazole comes the closest of all present reagents to meeting these criteria. With N-acetylimidazole, the extent of acetylation of amino groups is usually considerably less than with acetic anhydride (51,52). In a number of proteins the reagent has been shown capable of distinguishing between reactive and nonreactive phenolic groups (53,54).

The ϵ-amino groups of lysine can be guanidinated with O-methylisourea (55).

$$R\text{-}NH_2 + CH_3O\text{-}C(NH)NH_2 \longrightarrow R\text{-}NH\text{-}C(NH)NH_2 + CH_3OH$$

The reaction is specific for the ϵ-amino amino groups, when the α-amino group is properly masked, and little or no attack generally occurs at the α-amino terminals. The reaction requires a pH of 10-11

and 2 to 5 days which makes it an undesirable treatment for most enzymes. However, the effect of guanidination upon the biological activity of several enzymes has been studied. Histidine has been reported to disappear during guanidation (56), but conclusive evidence of this on more than one protein has not been demonstrated. S-methylisothiourea is a more reactive reagent, but shows little or no differentiation between α- and ϵ-amino groups while 1-guanyl-3, 5-dimethylpyrazone gives extensive guanidination of proteins at lower pH values and more rapidly than O-methylisourea (57,58). When performing protein modifications, this increased reactivity must be weighed against the decreased ability of α-amino groups to resist modification.

The sulfonyl halide, sulfonylation reagent, 5-dimethylaminonaphthalene-1-sulfonyl chloride (dansyl chloride) was originally developed for the identification of N-terminal residues (59). Dansyl chloride has been shown to react with a serine residue of chymotrypsin but does not act on histidine (60). The extent of dansylation can be conveniently determined spectrophotometrically. The sulfonyl halides are in general less reactive protein acylating agents than the acyl halides and anhydrides and also have a greater tendency to denature and insolubilize proteins.

Arylation

The arylation of proteins is in general a slower, and more selective modification than is acylation, and the reaction is often effective at significantly lower pH values than are needed for acylation. When the protein conformation does not restrict the accessibility of the various protein groups or alter reactivity, the general order of

nucleophilicity in arylation is SH>NH_2>phenol>imidazole. It has been demonstrated that arylation, can be limited to reaction with sulfhydryl groups (42).

Fluorodinitrobenzene (FDNB) has been studied extensively as a reagent for identification of N-terminal residues in proteins. The reagent seems to vary considerably in its reactivity toward amino groups from one protein to another. In many proteins it often causes rapid inactivation (61), while in rabbit muscle fructose-1,6-diphosphatase it can react with a specific cysteine residue, causing a substantial increase in enzymatic activity (62,63). Use of FDNB in protein modification is hampered by the insolubility of the reagent in aqueous media. Chlorodinitrobenzene is less reactive than FDNB and combines only with the sulfhydryl groups in rabbit muscle aldolase (64) and in glucose-6-phosphate dehydrogenase (65), and is generally unreactive toward simple amines. However, care must be taken in interpreting this since the reagent has been shown to react with two ϵ-amino groups of transaldolase (66).

In 1963, Cyanuric fluoride was introduced as a new tyrosine modifying reagent (67). The reagent is a very reactive aryl halide, and may because of its reaction mechanism actually belong to the acid halide class. It is suggested that the reagent is capable of detecting much more subtle gradation in the reactivity of tyrosine residues in proteins than is discernable with N-acetylimidazole or titration techniques (44). The chief advantage in using cyanuric fluoride for modification is that the cyanurate ester formed with tyrosine is stable at high pH and the absorption maximum of the phenolic chromophore is displaced sharply toward the blue in the derivative. Its usefulness in

modification is hindered by its lack of specificity in reacting with almost all nucleophiles, including tryptophan, in certain proteins.

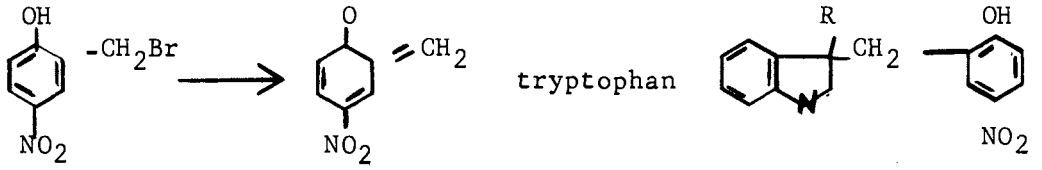
Alkylating Agents

Alkylation of proteins is normally performed with salts of α-haloacids such as iodoacetate or bromoacetate (68). Generally initial modification is observed on cysteine, methionine, or histidine. However, selective alkylations of ϵ-amino (69,70) and of carboxyl (71,72) groups have also been reported with these α-haloacids.

Carboxymethyl groups (carboxymethylation) can be reacted with the various side chains of cysteine, histidine, methionine, and lysine. The added group is identical to the side chain of aspartic acid. It is this similarity between the added carboxymethyl group and the structures already present that possibly leads to the usual small effect of carboxymethylation on the stability of the protein. The interpretation of the loss of biological activity after carboxymethylation, in terms of modification of a active center, is relatively secure (68). The reagent reacts most readily with sulfhydryl groups and the reaction can be limited to these groups.

Activated benzyl halides can be prepared by the introduction of hydroxyl groups into the ortho and para positions of the benzene ring. This modification greatly enhances the reactivity of the activated benzyl halide over that of the simple benzyl halides. The reagent 2-hydroxy-5-nitrobenzyl bromide is an excellent reagent of this type. It rapidly alkylates cysteine, methionine, and tryptophan in acidic or neutral media and in alkaline media it alkylates tyrosine (73). This is the only presently known reagent that will alkylate tryptophan

under mild conditions.



Unfortunately, the reagent is not very selective.

Electrophilic Reagents

Other than the classical iodination and diazotization protein modification methods, electrophilic substitution has been a relatively unexplored area (42). The selectivity achieved by this mechanism is quite different from that with nucleophilic reagents and appears very promising for future modification in introducing novel types of functional groups into proteins. In addition to the classical iodination of proteins, the recently developed technique for nitration of proteins has become one of the most used electrophilic reactions. These two techniques were used in this study and are therefore reviewed in greater detail.

Iodination of Proteins

Proteins in general rapidly decolorize iodine in neutral solution. This results from iodine being incorporated into the protein, and the iodination of protein phenolic groups is usually assumed upon decolorization. In most proteins the reaction is relatively specific for the tyrosyl residues, however, this is not always true and varies according to both the protein used and the method of iodination.

Schanbacher (74) has reviewed the three general methods employed for the iodination of proteins. The first method described, using iodine monochloride for radioiodination of proteins, was perfected by Reif (75). The iodine monochloride method has been adapted to permit labeling efficiencies of 70-90 percent for 1 to 1,000 mg of gamma globulin. There is no theoretical limit to the amount of protein that can be iodinated at one time by the ICl method. There are, however, significant disadvantages in the use of ICl for the iodination of protein. First, free SH groups tend to inactivate ICl. Therefore, proteins with relatively high amounts of free SH groups cannot be iodinated with high efficiencies by this method. The second significant disadvantage of this method of iodination is that it has been shown to inactivate enzymes such as papaya lysozyme (76), glyceraldyhyde-3-phosphate dehydrogenase (77), and egg white lysozyme (78). Inactivation of the latter two enzymes is due to tryptophan oxidation (79,80). This demonstrates that the method is not highly selective for tyrosyl residues and side reactions must be taken into account when attempting to relate loss of activity to extent of iodination.

The iodination method of Hunter and Greenwood (81,82) provides a simple and rapid method for preparing ^{131}I-labeled human growth hormone of high specific radioactivity (240-300μC/μg). Low amounts of carrier-free ^{131}I are used with chloramine-T as the oxidizing agent to produce a high yield of iodination and only μg quantities of protein are required. With human growth hormone, the ^{131}I-labeled hormone (up to 300 μC/μg) contained no detectable degradation products and was immunologically identical with the unlabeled hormone. The retention of immunological activity was attributed to: a low degree of iodination,

but with high specific activity; the use of carrier-free isotope in a high efficiency reaction; and rapid separation of the iodinated hormone from the reacted isotope. At specific activities above 300 $\mu C/\mu g$, protein denaturation is observed which increases with increasing specific activity.

The procedure of Covelli and Wolff (78,83) utilizes KI_3 to iodinate lysozyme. In addition to the iodination of two of the three tyrosyl residues, the histidyl residue of lysozyme is also readily iodinated (78,84). The third or buried tyrosyl residue required unfolding by 8M urea for iodination. It has been demonstrated that oxidative side reactions do occur during KI_3 iodination when the ratio of $[I_2]$/[lysozyme] exceeds two (78). This reaction results in the oxidation of tryptophan. No more than one tryptophanyl residue of lysozyme was oxidized, however, even on the addition of 14 moles of I_2 per mole of lysozyme (78). At levels of $[I_2]$/[lysozyme] below one, the ratio of [monoiodo-tyrosine]/[diiodo-tyrosine] is greater than three. When the ratio of $[I_2]$/[lysozyme] exceeds two, however, diiodo-tyrosine becomes the major species. It has been proposed (85) that the partly non-aqueous environment of buried tyrosyl residues directs their preferential conversion to diiodotyrosyl residues. KI_3 iodination itself seems to be a mild modification procedure, in that marked denaturation or gross conformation changes of the protein are not generally found upon iodination (85). One must carefully consider possible side reactions accompanying iodination when trying to relate iodination of tyrosyl residues to enzymatic activity.

The content of tyrosine, monoiodotyrosine, and diiodotyrosine in iodinated proteins is usually determined using labeled iodine. The

procedure usually involves enzymic digestion with pronase, separation by paper chromatography, and recording radioactivities in a strip scanner. Edelhoch (86) first presented evidence that monoiodotyrosine and diiodotyrosine of thyroglobulin could be detected by spectrophotometric titration. This spectrophotometric method was applied to lysozyme and compared well with the chromatographic and specific activity data determined on the same enzyme (78,83). Sherman and Kassell (87) have presented a method for the determination of mono- and diiodotyrosine, using an amino acid analyzer after alkaline hydrolysis of peptides containing these amino acids. Alkaline hydrolysis of standard solutions gave a recovery of 78 percent for MIT and 88 percent for DIT. In protein hydrolosates, however, an interfering substance was found which reportedly made recovery experiments not applicable to general proteins.

Nitration with Tetranitromethane

Tetranitromethane, $C(NO_2)_4$ may be prepared by allowing acetic anhydride and anhydrous nitric acid to stand in the cold, or by the reaction of ketene and nitric acid at 0° (88). Pure tetranitromethane is a stable compound. However, care must be taken in its usage, since mixtures with hydrocarbons, and especially aromatic hydrocarbons, are very sensitive to shock and detonate with considerable violence.

Herriott (7), in 1947 proposed tetranitromethane as a nitrating reagent which could be employed under mild conditions. Although a number of investigators have used the reagent for protein modification, it was not until 1966 that Sokolovsky *et al.* (89), attempted to identify which functional groups of proteins might react with tetranitromethane,

and what the nature of the products of the reaction might be. These investigators found the reagent to be a mild and specific reagent for the nitration of tyrosyl residues at pH 8.0. This mildness of reaction is supported by optical rotary dispersion work on ribonuclease which shows the protein is not substantially unfolded by extensive nitration (90). The product of the nitration reaction has been identified as 3-nitrotyrosine, and no evidence has been found for the formation of dinitrotyrosine (91). Sokolovosky et al. (89), also investigated the reaction of tetranitromethane with tryptophan and found both tryptophan and tryptophanyl peptides were unaffected by treatment with tetranitromethane as determined by increase in absorbance at 350nm or the release of protons.

We have observed in this laboratory that certain of the tryptophan residues of α-lactalbumin are destroyed during nitration under conditions similar to those used by Riordan (91). This determination was made by amino acid analysis using 4 percent thioglycolic acid to prevent tryptophan destruction during acid hydrolysis. Other investigators have also observed destruction of tryptophan by tetranitromethane. Cuatrecasas et al. (92) found the single tryptophanyl residue of staphylococal nuclease reacts with tetranitromethane to form a yellow derivative when this ordinarily buried residue is exposed to the reagent by urea. Robbins (93) has also reported tryptophan modification during nitration.

It has been reported recently that cross-linking occurs during the nitration of bovine insulin with tetranitromethane (94). Often upon nitration there is a good balance between the tyrosine content of the starting protein and the tyrosine plus nitrotyrosine content of the

nitrated product. This indicates that the tyrosine residues of the protein were either nitrated or remained unmodified (94,95,96,97). Upon nitration of collagen and γ-globulin a precipitate was formed which the authors attributed to cross-linking (98). With both trypsin (99) and insulin (94) the amount of tyrosine plus nitrotyrosine in the tetranitromethane treated protein is less than the amount of tyrosine in the untreated protein. This implies that some of the tyrosine is converted to something besides 3-nitrotyrosine on treatment with tetranitromethane. Even when essentially all the tyrosine molecules of insulin are modified at pH 8 as judged by the disappearance of tyrosine, only part of the molecules are accounted for by the appearance of 3-nitrotyrosine. The remaining residues are apparently involved in a cross-linked compound.

Bruice et al. (100) reported on the nitration of various phenols with tetranitromethane. In most cases their yields of nitrated phenols were only 20-30 percent, and polymer formation was indicated. Cross-linking is also reported on the nitration of glycyl-L-tyrosine with tetranitromethane (94). Therefore, cross-linking is likely a general phenomenon that should be investigated whenever tetranitromethane is used as a modifying reagent (94).

Oxidizing Agents

The oxidation of proteins can occur by a variety of different mechanisms. This restricts one from placing oxidizing agents into a general class with respect to their order of reactivity toward functional groups or even with respect to the groups that will be oxidized at all. There are presently a large number of oxidizing reagents or

conditions available for protein modification, but the degree of selectivity or usefulness of very few of these has been sufficiently investigated.

The use of hydrogen peroxide for protein modification was initially discouraged by the broad spectrum of functional groups with which it could react. Under alkaline conditions it has been shown to react with methionine, cysteine, cystine, tyrosine, tryptophan, histidine, and even the aliphatic amino acids such as alanine and leucine (42,101). The real breakthrough in the use of hydrogen peroxide came when it was demonstrated that in acidic medium the reagent oxidizes methionine faster than any other functional group (102,103). This has led to the development of conditions under which the reaction has been shown selective for only the methionine residues in some proteins. Both ACTH and parathyroid hormone, which lose their biological activity due to peroxide oxidation of methionine to its sulfoxide, regain their activity on mercaptan reduction (104,105). Difficulty in determining the extent of methionine oxidation is enhanced by the fact that methionine sulfoxide reverts almost completely to methionine under the acidic hydrolytic conditions generally used prior to amino acid analysis.

In the photochemical oxidation of proteins an aerated solution of a dye such as methylene blue acts as an oxidizing medium when exposed to visible light (106,107). The possible sites of photochemical oxidation within the protein are the side chains of histidine, methionine, cysteine, tryptophan, tyrosine, and cystine. However, conditions can be modified such that cystine and tyrosine can be oxidized very slowly relative to other members of the group (106). Also in the three proteins tested so far the photochemical oxidation of tryptophan is slow

relative to the oxidation of histidine and methionine. The products of the oxidation of these amino acids are:

methionine → methionine sulfoxide and methionine sulfone

cysteine → cystine and cysteic acid

cystine → cysteic acid

histidine → unknown products from ring cleavage

tyrosine → unknown products from ring cleavage

tryptophan → unknown products from ring cleavage

The photo-oxidation of cystine presents the greatest problem in detection of oxidized products, since any intermediates formed in its oxidation to cysteic acid would not survive acid or basic hydrolysis. However, studies on free amino acids indicate cystine is oxidized quite slowly relative to histidine, methionine, and cysteine.

N-bromosuccinimide was introduced by Patchornik et al. (108), as a reagent for use in the rapid and convenient spectrophotometric estimation of tryptophan in proteins. The reaction, under carefully controlled conditions, has in certain proteins been restricted to the modification of tryptophan residues. However, the oxidation of tyrosine, methionine, cysteine, cystine, histidine, lysine, and arginine residues has also been observed with N-bromosuccinimide (109). The reagent has shown selectivity for the tryptophan residues in lysozyme and loss of enzymatic activity has been related to modification of a single tryptophan residue (110).

Reduction

The reduction of protein disulfide bonds is one of the few protein modification reactions of guaranteed specificity (42). This is

simply due to the fact that very few functional groups in proteins are accessible to reduction.

Mercaptoethanol is generally used as the reducing agent for protein disulfides. The reagent has a long shelf-life, since the -SH group does not oxidize readily. Also, it has no other reactive groups which may cause side reactions in proteins (111,112). The extent of reduction can be measured in several ways, the most common being measuring the appearance of -SH groups.

From the data presented above, it is quite clear that there is considerable need for more selective chemical modifying reagents. In the future more selective reagents should be produced as well as more sophisticated approaches for the application of these reagents.

Structural Similarities Between Bovine α- Lactalbumin and Hen's Egg-White Lysozyme

As previously described, hen's egg-white lysozyme and bovine α-lactalbumin have quite different biological functions. However, there is considerable evidence for structural similarity between the two proteins (5,6,10,22,23,25). When the sequence of the two proteins is aligned, 42 residues are identical at corresponding positions in the sequence, with the possibility of eight other residues also being identical (10). In addition there are at least 23 corresponding residues which are structurally similar, and could be classified as conservative replacements (113,114). Another, and one of the most striking structural similarities, involves the location of the four disulfide bonds. The fact that the four disulfide bonds are formed by the corresponding homologous half-cystinyl residues in the two proteins along with the high degree of sequence homology certainly suggests

α-lactalbumin might possess a conformation quite similar to that of egg-white lysozyme (5,10,25).

Ingram (115) has shown 44 percent of the residues of the α and β chains of human hemoglobin are identical. This compares to 40 percent of the residues of α-lactalbumin and lysozyme being identical. Since the α- and β-hemoglobin chains have very similar tertiary structures, and since the four disulfide bonds in α-lactalbumin and lysozyme are formed in the same way, there is a possibility that these latter two proteins may also have very similar tertiary structures. Browne et al. (6) have used the amino acid sequence of lysozyme to build a hypothetical molecular model of α-lactalbumin by appropriate alteration of the known structure (24). To achieve maximum homology between the two structures it was necessary to make a few extra deletions other than those necessary to correct for the six fewer residues that α-lactalbumin contains. The deleted residues, however, occurred either in loops or at the ends of helical regions. This made it possible to build a molecular model of α-lactalbumin which retained essentially all of the features of both the secondary and tertiary structures of lysozyme. The crystal structure analysis will, when completed, reveal how well the predicted conformation of α-lactalbumin fits the true conformation. Until this analysis has been completed, the predicted conformation of α-lactalbumin can probably best be tested against studies of the physical and chemical properties of the protein in solution (6). In conformation studies, the two separate proteins, α-lactalbumin and lysozyme, can themselves be compared when studying overall conformation of the proteins with measurements such as optical rotary dispersion or small angle X-ray diffraction. However, since lysozyme has six

tryptophan residues as compared to four for α-lactalbumin and other potentially reactive groups are not in all cases in identical positions in the two proteins, it is not always meaningful to compare the proteins themselves with respect to their reactivity toward modification reagents. Therefore, where it is deemed more meaningful, the observed reactivity of modifying reagents toward α-lactalbumin will be compared to the predicted reactivity of the reagent used toward the hypothetical molecular model of α-lactalbumin.

The four tyrosyl residues of α-lactalbumin are located at positions 20, 38, 53 and 107. Inspection of the model of Browne et al. (6), shows that all four of these tyrosines are located near the surface of the molecule. The configuration of the phenolic group of tyrosine at position 38 may make this residue rather less accessible than the other three tyrosines. To test this predicted conformation, the results of different protein modification procedures that affect these tyrosine residues may be compared to determine if these reagents react with three to four of the tyrosines of α-lactalbumin as one would expect from the predicted conformation.

If these four tyrosine residues are actually located near the surface, they should ionize normally on H^+ titration. Robbins et al. (116), have shown these groups to ionize normally in agreement with the model. Gorbunoff (44) has shown all four tyrosyl groups to be readily available to reaction with acetylimidazole. However, in the reaction of α-lactalbumin with cyanuric fluoride five tyrosyl residues were assumed which makes the interpretation of her results difficult. She concludes that α-lactalbumin contains four reactive residues and one unreactive residue on the basis of spectrophotometric titration.

From interpretation of these results it appears that one of the four groups may have been unavailable to the reagent. This would not be unexplainable on the basis of the model since the tyrosine at position 38 may be less accessible than the others. Atassi et al. (117) have reported that 2.51±0.03 of the tyrosines of α-lactalbumin are modified upon nitration with tetranitromethane. In contrast Robbins et al. (93) have shown that all four tyrosines could be modified, but two were more reactive than the others. Care must be taken in trying to interpret these findings, since we have recently demonstrated in this laboratory that polymerization must be taken into consideration in the determination of the extent of nitration since this cross-linking destroys the enzymic activity and makes the tyrosines less accessible. This subject will be discussed in detail later in this dissertation.

The reaction of bovine α-lactalbumin with iodoacetate resulted in the extensive carboxymethylation of the side chains of methionine 90, histidine 32, histidine 68, and histidine 107. The relative rates of reaction of these residues were generally in accord with those expected on consideration of the predicted conformation of α-lactalbumin (118). Carboxymethylation of methionine at position 90 had little effect on the ability of α-lactalbumin to activate, but there was a progressive loss in activity as the histidyl residues reacted. About 40 percent of the activity of α-lactalbumin remained when all three histidyl residues were modified. However, according to the molecular model, histidine at position 32 in α-lactalbumin is equivalent to glutamic at position 35 of the homologous lysozyme sequence. Since glutamic at position 35 has been shown to be critical in the enzymic activity of lysozyme, it is expected that if lysozyme and α-lactalbumin do have the same

conformation, and if there is functional similarity in the two proteins, then the chemical modification of the histidine residues should completely destroy the activity of α-lactalbumin. As described above, this is not the case.

Lin (119) modified the carboxyl groups of α-lactalbumin with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. The carboxyl side chains in α-lactalbumin were found to be more accessible to the modification reagent than those in lysozyme. The modified lysozyme lost its cell-wall lytic activity, while the modified α-lactalbumin did not activate lactose synthetase nor inhibit uridinediphosphategalactose-N-acetyl-D-glucosamine galactosyl transferase activity. It appeared that partial protection toward modification of the carboxyl groups could be achieved by binding with the A protein. Lin (119) best describes his finding as he concludes his investigation with the following statement;

> While the result of this model study is consistent with the hypothesis that α-LA and lysozyme may have closely similar conformation, the model alone seems to be insufficient to provide an unequivocal explanation for the remarkable difference in the susceptability of the carboxyl groups in these two proteins toward the carbodiimide reagent.

The hypothetical model of α-lactalbumin shows that tryptophan at position 28 is completely buried and is unaccessible to solvent. Tryptophan at position 123 appears to be exposed and quite accessible to solvent as it is in lysozyme. Tryptophans at positions 63 and 108 are less well defined with respect to solvent accessibility. These two tryptophans are in the active cleft and are borderline cases with number 63, which is accessible in lysozyme likely inaccessible in α-lactalbumin, while number 108 is probably accessible. Solvent perturbation studies indicate that two of the four tryptophan residues of α-lactalbumin are buried and two are exposed at 25° (120). However, when

temperature is reduced to 1°, the two exposed tryptophan residues become inaccessible to all but the smallest solvent molecules. This suggests that these two residues lie in surface crevices which change shape as the result of a small conformational change in the molecule (6). The model can be interpreted as having two buried and two exposed groups, it is favorable toward the model that the two exposed groups become less accessible as result of a relatively small conformational change. Barman and Koshland (121) modified the tryptophan residues of α-lactalbumin with hydroxynitrobenzyl bromide, reportedly forming an inactive protein (119).

α-lactalbumin which undergoes a conformational change near pH 4 is not as stable as lysozyme toward acid denaturation (122,123). Several other recent findings seem to indicate that there may be appreciable conformational differences between α-lactalbumin and lysozyme. A lack of immunochemical cross-reaction was demonstrated by showing that none of five antisera to lysozyme reacted with α-lactalbumin, while full reactivity with lysozyme was maintained for each of these antisera after premixing with α-lactalbumin over a wide range of concentrations (117). The converse of this was also demonstrated with antisera to α-lactalbumin. Even though both proteins appear to possess tight folding, all four disulfides of α-lactalbumin are reducible in three hours at a concentration of about 2.5 M guanidine. These same conditions, however, result in the reduction of only 1,4 bonds in lysozyme (117). This is certainly strong evidence that there possibly are appreciable conformational differences between lysozyme and α-lactalbumin.

Evidence for a similar conformation of lysozyme and α-lactalbumin in solution was provided by Aune (124) and Kronman (120).

Aune (124) showed that the two proteins have indistinguishable optical rotary dispersion curves between 206 and 233 nm, and between 320 and 500 nm. This is, however, essentially a measure of short range interactions and it measure an overall average conformational parameter and does not give any insight concerning specific conformational details of various regions in the molecule. In addition, the ORD behavior of lysozyme is not highly sensitive to conformational changes (117). More direct information concerning the conformation of molecules in solution is provided by hydrodynamic or diffraction measurements, but of these two procedures, only diffraction permits independent evaluation of size and shape parameters (113). Krigbaum and Kugler (113) performed small angle diffraction measurements for hen's egg-white lysozyme and bovine α-lactalbumin. Lysozyme exhibits a radius of gyration, R, of 14.3 Å, and its equivalent scattering body is a prolate ellipsoid having dimensions 28 x 28 x 50 Å, while α-lactalbumin has an R value of 16.7 Å, and its equivalent ellipsoid is oblate with dimensions 22 x 44 x 57 Å. They concluded from these measurements that the two proteins have quite different molecular conformations in solution. Even though this shows that α-lactalbumin and lysozyme have grossly different sizes and shapes in solution, it is remotely possible that their conformations will show more resemblance in the crystalline state, but this is rather unlikely. In addition to these findings, one should keep in mind that the accommodation of a protein sequence by the conformation of another in model building does not prove identity, and the two molecules may even possess different conformations in regions that are very similar in sequence (6,36).

CHAPTER III

CHEMICAL MODIFICATION OF α-LACTALBUMIN

Experimental Procedure

Materials and Reagents

Tyrosinase (Grade II from mushroom, 1050 units/mg), 1-fluoro-2, 4-dinitrobenzene, Pyruvate kinase (Type I from rabbit muscle, crystalline ammonium sulfate suspension containing lactic dehydrogenase), glycylglycine, Phosphoenolpyruvate, NADH, Tris (tris-hydroxymethyl-aminomethane), bovine serum albumin, egg albumin, and cytochrome C were purchased from Sigma Chemical Company. Potassium iodide and D-glucose were purchased from Baker Chemical Company. Bio-Gel P was obtained from Bio-Rad Laboratories and tetranitromethane (abbv. TNM) from Aldrich Chemical Company. Size 8 dialysis tubing was obtained from Union Carbide Corporation.

Bovine α-lactalbumin was prepared from skim milk according to the procedure of Brodbeck et al. (1), with the following modifications:

(a) The second ammonium sulfate step was done at 90 percent saturation (411 g/l).

(b) The Bio-Gel P-30 column was equilibrated with 0.1 M Tris-HCl, pH 7.4.

(c) The P-30 eluate was dialyzed against distilled water and placed on a 1.7 x 8.0 cm, DE-32 ion exchange column equilibrated with 5 mM KH_2PO_4, pH 8.0.

(d) The column was eluted with a linear gradient from 5 to 200 mM KH_2PO_4,pH 8.0, total volume 250 ml.

(e) The α-lactalbumin peak was dialyzed extensively against distilled water and then lyophilized.

The resultant α-lactalbumin was electrophoretically homogeneous on disc gel electrophoresis (see Methods).

Additional α-lactalbumin was purchased from Pentex Incorporated (Lot 21). The Pentex purchased α-lactalbumin is apparently a crude preparation and contains a high percentage of protein other than α-lactalbumin as evidenced in Figure 1. Further evidence for the impurity of Pentex α-lactalbumin was demonstrated by multiple bands on disc gel electrophoresis. Due to the high degree of impurity in the purchased α-lactalbumin, the α-lactalbumin used in these studies was prepared from skim milk according to the procedure previously described.

Lactose synthetase A-protein was prepared according to the procedure described by Fitzgerald et al. (21) and the product was obtained as an ammonium sulfate solution of partially purified A-protein from the HA_{II} step of the purification. UDP-galactose was synthesized by the method of Moffatt and Khorana (125,126) or purchased from California Biochemicals. All other materials were of reagent grade or equivalent.

Spectrophotometric Assay for Lactose Synthetase Activity

The lactose synthetase proteins were assayed in the presence of saturating amounts of the second protein (1). Enzymatic activity was

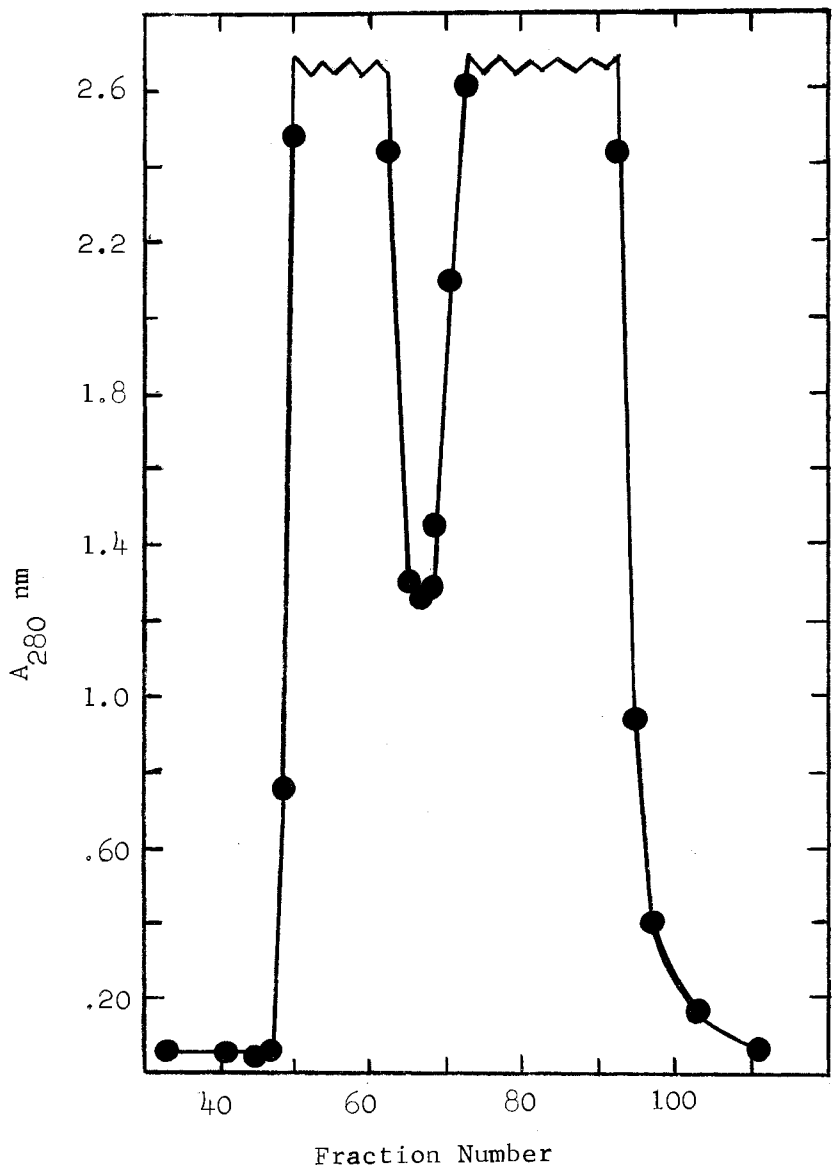


Figure 1. Chromatography of Pentex α-Lactalbumin on Bio-Gel P-30.

Bio-Gel P-30 columns (4.8 x 110 cm) were equilibrated and eluted at 25° with 0.1 M Tris-HCl, pH 7.4, and 5 ml samples were collected per fraction. The absorbance was determined at 280 nm.

assayed spectrophotometrically at 340 nm by coupling UDP formation of Reaction III to NADH oxidation by means of pyruvate kinase and lactic dehydrogenase. Assays were prepared for a final volume of 1.0 ml and contained 0.15 mM NADH, 1.0 mM PEP, 0.05 ml of a 1 to 10 dilution of pyruvate kinase (Sigma, Type I, 25 mg protein/ml, with 2.4 IU pyruvate kinase/mg protein), 5 mM glycylglycine, pH 8.5, 5 mM $MnCl_2$, 0.25 mM UDP-galactose, 20 mM glucose and approximately 100 units of A-protein. One unit of enzymatic activity is defined as that amount of enzyme which catalyzes the formation of one millimicromole of UDP per minute and is equivalent to a ΔA_{340}/min/ml of 0.0062. α-Lactalbumin was estimated by assuming an extinction coefficient of 2.0 at 1 mg/ml at A_{280}. Standard curves were prepared for each group of assays, and all assays were performed on a Beckman Model DB recording spectrophotometer with a water jacketed cell chamber thermoregulated at 25° (127).

Gel Filtration Methods

Gel filtration glass columns of desired length and diameter were silanized according to instruction in Bio-Rad, Price List T (128), using a 1 percent solution of dichlorodimethyl silane in benzene, prior to packing. Gels were swollen, deaerated, and packed in the columns according to the technical manuals (128,129). Sample density was increased to 10 percent by the addition of solid sucrose which permitted the layering of the sample on the top of the gel just below the eluant. Absence of zoning and general uniformity of packing was determined visually with a blue dextran sample (approximately 1 mg/ml) as was the void volume. The optimum flow rate determined for each column was the maximum flow rate which would allow migration of a blue dextran band

without local band distortion.

Disc Electrophoresis Methods

A standard 7 percent, pH 9.5, separating gel solution was prepared according to Canalco specifications (130). The poured gel solutions were layered with distilled water and allowed to polymerize in the dark for 30 minutes at room temperature. Approximately 0.2 ml of stacking gel was layered above the separating gel and photopolymerized. Gels were preelectrophoresed for 30 minutes at a constant current of 5 milliamps per gel. Protein solutions were 10 percent with respect to sucrose and were layered directly on top of the stacking gel. No sample gel solution was used. Separations were accomplished with the Canalco Model 6 system according to the Canalco Model 6 System Instructions. The current was maintained at 2 milliamps per gel until the tracking dye had visibly migrated into the separating gel. The current was then increased to and maintained at 5 milliamps per gel. Size and charge isomers also were distinguished using disc gel electrophoresis by varying the acrylamide gel concentration according to the method of Hedrick et al. (131).

Sample Preparation for Amino Acid Analysis

Acid hydrolyses of α-lactalbumin were performed in thick walled, Pyrex, 16 x 125 mm, ignition tubes. In addition to α-lactalbumin, each sample contained 1 μ mole of nor-leucine as an internal standard and 4 percent thioglycolic acid to decrease tryptophan destruction (132). The sample volume of each tube was adjusted to 1 ml with distilled water and an equal volume of concentrated reagent grade HCl (Fisher

Scientific Company) was added. The neck of each ignition tube was partially closed with an oxygen-gas flame. The samples were frozen and the tubes were evacuated to 20 microns Hg or less and then sealed. The sealed, evacuated tubes were placed in an oven maintained at 110° for 20 hours. After hydrolysis the tubes were opened and the samples were evaporated at 40° to dryness on a rotary evaporator at 1 mm Hg. Two milliliters of distilled water were added and each sample was evaporated to dryness.

Basic hydrolyses were performed in sealed pyrex tubes 0.5 x 6.0 cm. The samples were 5 N in NaOH with a final volume of 0.1 ml. After 16 hours hydrolysis at 110°, the tubes were opened, and 0.1 ml. of 5N HCl was added to each sample to neutralize the NaOH. The hydrolysate was usually dissolved in 4.8 ml of pH 2.2 buffer and centrifuged before placing on the column.

Nitration Method

α-Lactalbumin was nitrated with tetranitromethane (TNM) according to the method of Sokolovsky and Vallee (133). The reaction mixture contained a 64 molar excess of TNM. Aliquots of the reaction were removed at specific time intervals, and the nitration reaction was stopped by the addition of sodium acetate buffer pH 4.6. The α-lactalbumin was extracted from the reaction mixture with tributylphosphate and ether (132) and then dialyzed extensively against distilled water. In later experiments the extraction step was omitted and the α-lactalbumin was either placed on a Bio-Gel P-10 column or dialyzed exhaustively against distilled water. The best product was obtained from the Bio-Gel column as it shows the sharpest bands on disc

electrophoresis. The extent of nitration of tyrosine and the side reactions occurring during nitration were determined by amino acid analyses.

Iodination Method

α-Lactalbumin was iodinated by a method similar to that described as the "slow iodination" method by Covelli and Wolff (82). α-Lactalbumin (10 mg) was placed in each of eight one dram reaction vials containing 0.5 ml of 0.2 M Tris-HCl buffer pH 8.5. The protein was iodinated with potassium triiodide solution (0.046 M I_2 in 0.14 M KI). The iodinating solution was added to the magnetically stirred α-lactalbumin solution with a motor driven syringe and a capillary glass catheter immersed in the protein solution. The amount of iodinating solution needed to attain the desired I_2/α-lactalbumin ratio was added over a 30 minute period. After iodination, the solutions were kept at 4° for 30 minutes, after which 15 μl of 1.0 M $K_2S_2O_3$ were added to stop iodination and to eliminate any reversible interactions between the iodine and the protein. The iodinated samples were dialyzed at 4° for 24 hours with frequent changes of distilled water. Both acidic and basic hydrolyses were performed on each iodinated sample in preparation for amino acid analyses.

Modification of α-Lactalbumin by FDNB

Reaction of α-Lactalbumin with FDNB

The compound 1-fluoro-2, 4-dinitrobenzene (FDNB) reacts with the N-terminal and ε-amino (lysine) groups, the phenolic hydroxyl (tyrosine)

groups, and the imidazole (histidine) groups in proteins to form the dinitrophenyl derivatives (134). The dinitrophenyl derivative of α-lactalbumin was prepared by the method of Sanger (135). After one hour incubation at room temperature the yellow reaction mixture was dialyzed exhaustively against distilled water. The modified α-lactalbumin had no activity in the lactose synthetase reaction.

Modification of α-Lactalbumin by Tyrosinase

Reaction of α-Lactalbumin with Tyrosinase

The oxidation of tyrosine and many phenolic derivatives is catalyzed by tyrosinase (136). α-Lactalbumin was reacted with tyrosinase in order to assess the function of its tyrosyl residues in the lactose synthetase system. The extent of reactivity of tyrosyl residues, in chemical modification reactions, often indicates their degree of exposure in the molecule. However, urea treatment of lysozyme did not change the resistance of lysozyme to the action of tyrosinase, and Yasunobu and Wilcox (23) suggested that the primary structure of the protein is a determining factor in susceptibility or nonreactivity of a protein to enzymatic oxidation.

Previous studies suggested that the intact tyrosyl residues of proteins are oxidized by tyrosinase. For example, ribonuclease appears to be readily oxidized by tyrosinase (137,138), but other proteins such as β-lactoglobulin and lysozyme do not appear to be substrates for tyrosinase. Yasunobu and Dandliker (137) reported in 1957 that α-lactalbumin is readily oxidized by tyrosinase and, as determined by amino acid analysis, about 28 percent of the tyrosine originally present in the native protein was still present after oxidation by tyrosinase.

However, the present work suggests that tyrosinase does not oxidize the tyrosyl residues of α-lactalbumin. Rather, it is suggested that the oxygen uptake recorded during the incubation of tyrosinase with α-lactalbumin is mainly due to the oxidationof 1.1 to 1.5 of the tryptophanyl residues of α-lactalbumin.

In order to determine the oxygen uptake during the reaction of tyrosinase with α-lactalbumin, 12 mg of α-lactalbumin were incubated at 37° with 8 mg tyrosinase. The total volume of the reaction mixture was 2.0 ml and was 50 mM in Tris, pH 7.3. Oxygen consumption was determined on a Gilson respirometer and by conventional manometric techniques (139). The oxygen uptake as a function of time is shown in Figure 2.

If it is assumed that the oxidation of one tyrosyl residue consumes one molecule of oxygen, then from 12 mg of α-lactalbumin, a total of 74 μl of oxygen could be consumed, if all four tyrosines were oxidized. Since α-lactalbumin contains 4 tyrosyl residues, 18.5 μl of oxygen utilized corresponds to the oxidation of one tyrosyl residue of α-lactalbumin. Figure 2 shows the uptake of 28 μl of oxygen which corresponds to the oxidation of 1.5 tyrosyl residues. However, as determined by amino acid analysis, no tyrosyl residues were lost after tyrosinase treatment for 300 minutes at 37°.

A 0.15 ml sample of the α-lactalbumin, which was incubated with tyrosinase for 300 minutes, was chromatographed on a Bio-Gel P-100 column and the elution pattern is shown in Figure 3. The α-lactalbumin eluted as a relatively homogenous peak as is evidenced by the good correspondence of the lactose synthetase activity plot to the absorbance plot in Figure 3. The small peak at fraction 38 is probably

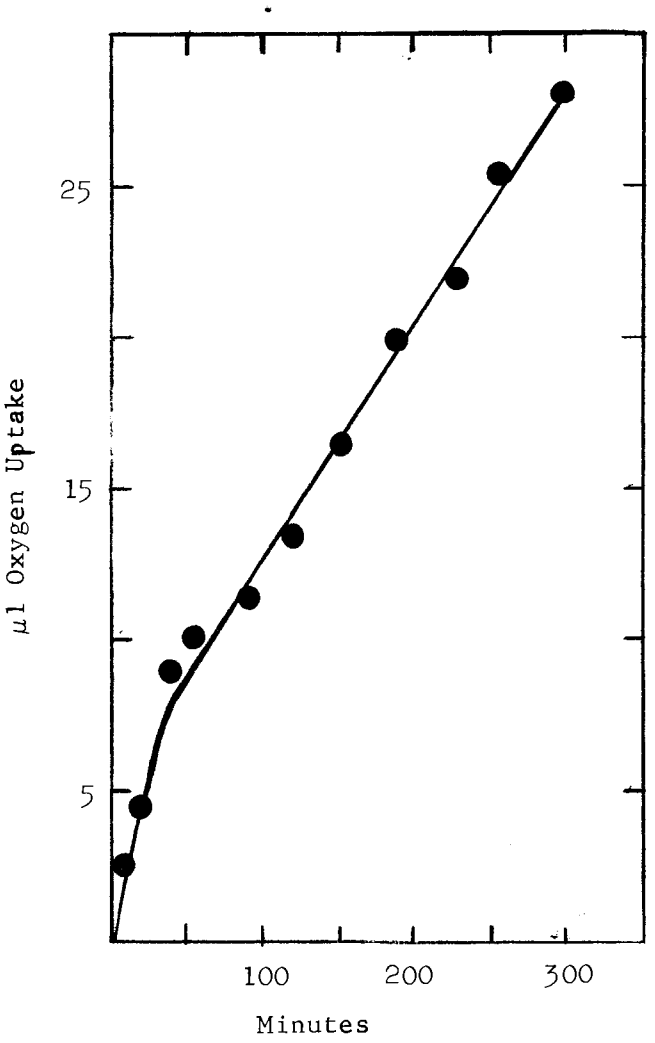


Figure 2. Oxygen Uptake from the Reaction of Tyrosinase with α-Lactalbumin.

The side-arm of the Warburg flask contained 12 mg of α-lactalbumin in 0.5 ml of 50 mM Tris, pH 7.3, and the main vessel contained 8 mg of tyrosinase in 1.5 ml of 50 mM Tris, pH 7.3. The center well contained 0.2 ml of 6 M KOH. The reaction was performed at 37° and the oxygen uptake was determined on a Gilson respirometer.

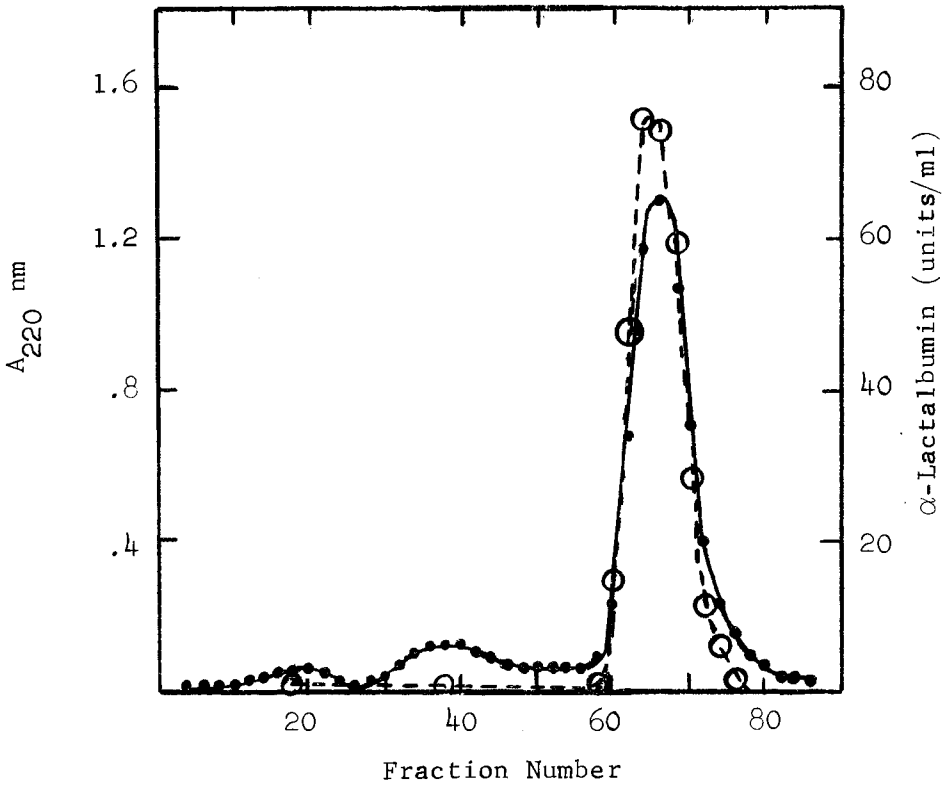


Figure 3. Bio-Gel P-100 Chromatography of α-Lactalbumin Reacted with Tyrosinase for 300 Minutes.

A Bio-Gel P-100 column (0.6 x 95 cm) was equilibrated and eluted at 25° with 50 mM Tris, 0.1 M KCl, pH 7.5 and 0.2 ml fractions were collected per tube. The tubes were diluted to 0.8 ml with distilled water and the absorbance was read at 220 nm. ●—● A_{220} nm; O—O , α-lactalbumin, lactose synthetase activity (units/ml). Fraction 31 corresponds to the void volume and an α-lactalbumin dimer should theoretically elute around fraction 51.

due to tyrosinase. Fractions 61 through 72, collected from the Bio-Gel P-100 column in Figure 3, were pooled and dialyzed at 4° for 24 hours against distilled water. The dialyzed solution was lyophilized to dryness and the total product was analyzed for amino acid composition (see Methods). The result of amino acid analysis of the α-lactalbumin incubated at 37° for 300 minutes with tyrosinase is summarized in Table I. The amino acid analysis data suggests the total oxidation of about 2 amino acid residues per molecule of α-lactalbumin after 300 minutes incubation with tyrosinase at 37°. This compares reasonably well to the oxidation of 1.5 residues as indicated by oxygen utilization. The amino acid analysis data indicated that tyrosinase did not oxidize the tyrosyl residues of α-lactalbumin, but instead oxidized tryptophan and possibly histidine. Control experiments were performed with the free amino acids tyrosine, histidine, and tryptophan in order to determine if they were oxidized by tyrosinase.

Oxygen utilization was recorded during the incubation at 37° of L-tyrosine with (a) native tyrosinase, (b) tyrosinase inactivated by boiling at 100° for 10 minutes, and (c) 2 ml of 50 mM Tris, pH 7.3. The result of this experiment is shown in Figure 4. Essentially no oxygen uptake occurred during the 37° incubation of L-tyrosine with tyrosinase boiled for 10 minutes or during the 37° incubation of L-tyrosine in 50 mM Tris, pH 7.3 (Figure 4). The L-tyrosine is readily oxidized by native tyrosinase as demonstrated in Figure 4.

Control experiments, similar to those previously described for tyrosine, were performed with the amino acid histidine. Histidine was incubated at 37° with (a) native tyrosinase, (b) tyrosinase previously boiled at 100° for 10 minutes, and (c) with 50 mM Tris, pH 7.3. The

TABLE I

SUMMARY OF AMINO ACID ANALYSIS OF α-LACTALBUMIN
TREATED 300 MINUTES BY TYROSINASE

AMINO ACID	μ MOLES	TOTAL RESIDUES IN NATIVE α-LA.	RESIDUES RECOVERED	% RECOVERY
Histidine	.026	3	2.7	91
Tryptophan	.021	4	2.5	64
Tyrosine	.037	4	3.8	95
Phenylalanine	.039	4	4.0	100
59 min. Unknown	.011	-	---	---

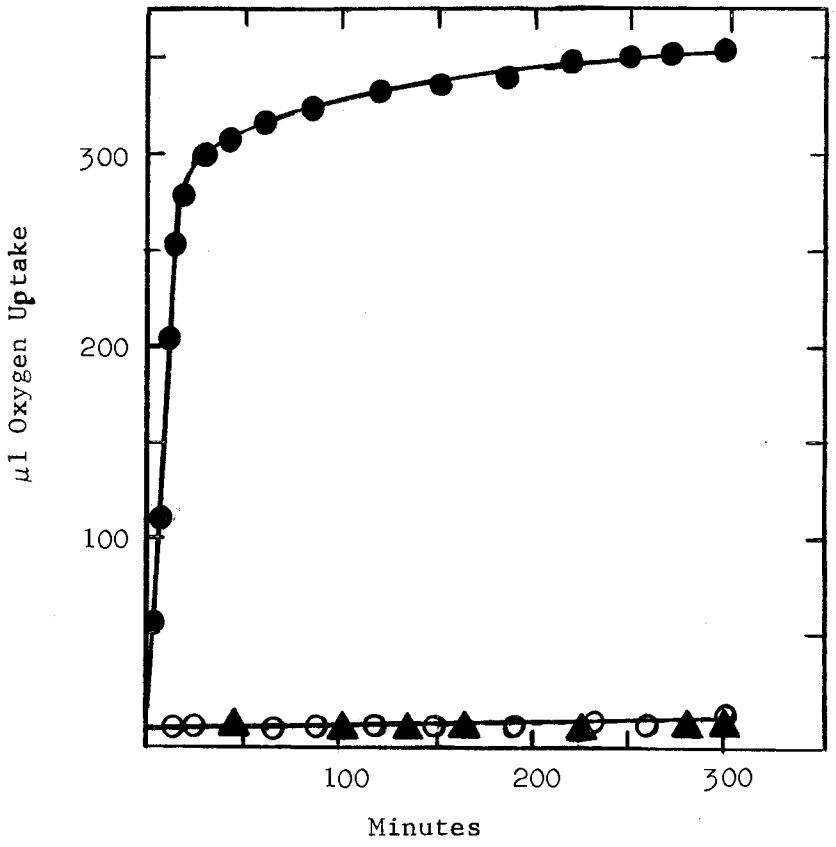


Figure 4. Oxygen Uptake of Tyrosine by Tyrosinase.

Oxygen uptake was recorded at 37° on a Gilson respirometer using single arm Warburg flasks. ●—●, the side-arm contained 5 µmoles of L-tyrosine in 0.5 ml of 50 mM Tris, pH 7.3, and the main vessel contained 8 mg of tyrosinase in 1.5 ml of 50 mM Tris, pH 7.3; ○—○, the sidearm contained 10 µmoles of L-tyrosine in 0.5 ml of 50 mM Tris, pH 7.3 and the main vessel contained 8 mg of tyrosinase boiled previously for 10 minutes in 1.5 ml of 50 mM Tris, pH 7.3; ▲—▲, the sidearm contained 10 µmoles of L-tyrosine in 0.5 ml of 50 mM Tris, pH 7.3, and the main vessel contained 1.5 ml of 50 mM Tris, pH 7.3.

The center well contained 0.2 ml of 6 M KOH.

oxygen uptake was determined on a Gilson respirometer and the results are shown in Figure 5. The results in Figure 5 suggest that the oxidation of histidine is slow when compared to the oxidation of tyrosine in Figure 4, but does occur at about the same rate in the presence of both native and boiled tyrosinase. Oxidation of histidine does not occur in the absence of tyrosinase.

Control experiments on oxygen uptake resulting from the incubation of tryptophan with: (a) native tyrosinase, (b) tyrosinase boiled for 10 minutes and (c) 50 mM Tris, pH 7.3, are shown in Figure 6. The incubation of tryptophan with native tyrosinase at 37° resulted in a linear uptake of oxygen (Figure 6). Oxygen uptake did not occur when tryptophan was incubated either with tyrosinase boiled for 10 minutes or with 50 mM Tris, pH 7.3. This suggests that tryptophan is oxidized by the enzyme tyrosinase. The oxidation of the free amino acid tryptophan (Figure 6) is about 20 times slower than the oxidation of the free amino acid tyrosine as shown in Figure 4.

Native tyrosinase was incubated at 37° in 2 ml of 50 mM Tris, pH 7.3 and the oxygen uptake was recorded (Figure 7). The rapid oxygen uptake after 300 minutes incubation suggests the uptake becomes autocatalytic after approximately 300 minutes incubation at 37°. The above experiment will be repeated using boiled tyrosinase under identical conditions to further support or discredit the above suggestion.

It was previously shown in Figure 2 that the reaction of tyrosinase with α-lactalbumin for 300 minutes resulted in the uptake of 28 μl of oxygen. The amino acid analysis data suggested that the oxygen uptake was due to the oxidation of tryptophan and possibly histidine, and tyrosinase had no significant effect upon the tyrosyl residues of

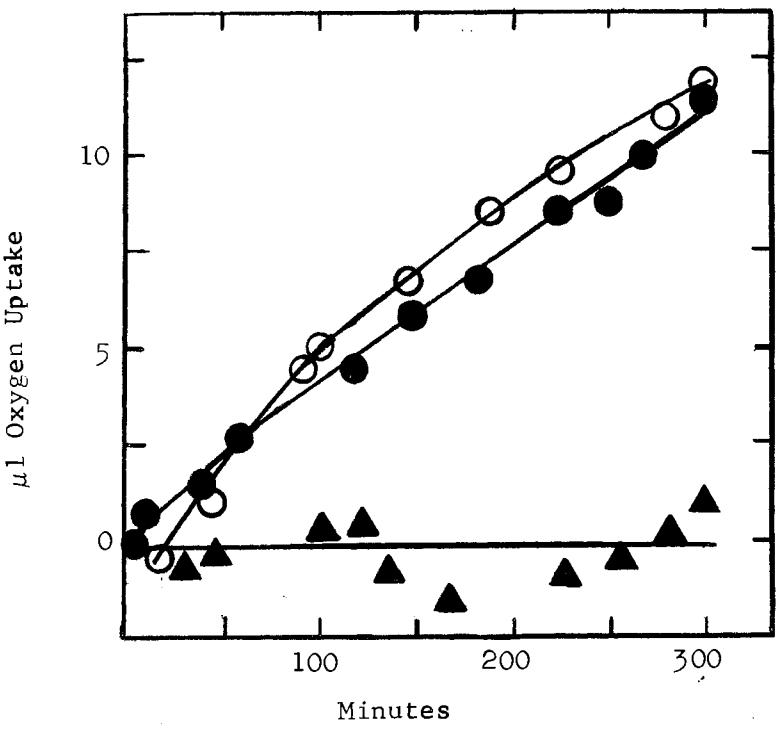


Figure 5. Oxygen Uptake of Histidine by Tyrosinase.

Oxygen uptake was recorded at 37° on a Gilson respirometer using single arm Warburg flasks. In each experiment the side-arm of the Warburg flask contained 10 µmoles of histidine in 0.5 ml of 50 mM Tris, pH 7.3, and the center well contained 0.2 ml of 6 M KOH.

●—●, The main vessel contained 8 mg of tyrosinase in 1.5 ml of 50 mM Tris, pH 7.3.

○—○, The main vessel contained 8 mg of tyrosinase boiled previously for 10 minutes in 1.5 ml of 50 mM Tris, pH 7.3.

▲—▲, The main vessel contained 1.5 ml of 50 mM Tris, pH 7.3.

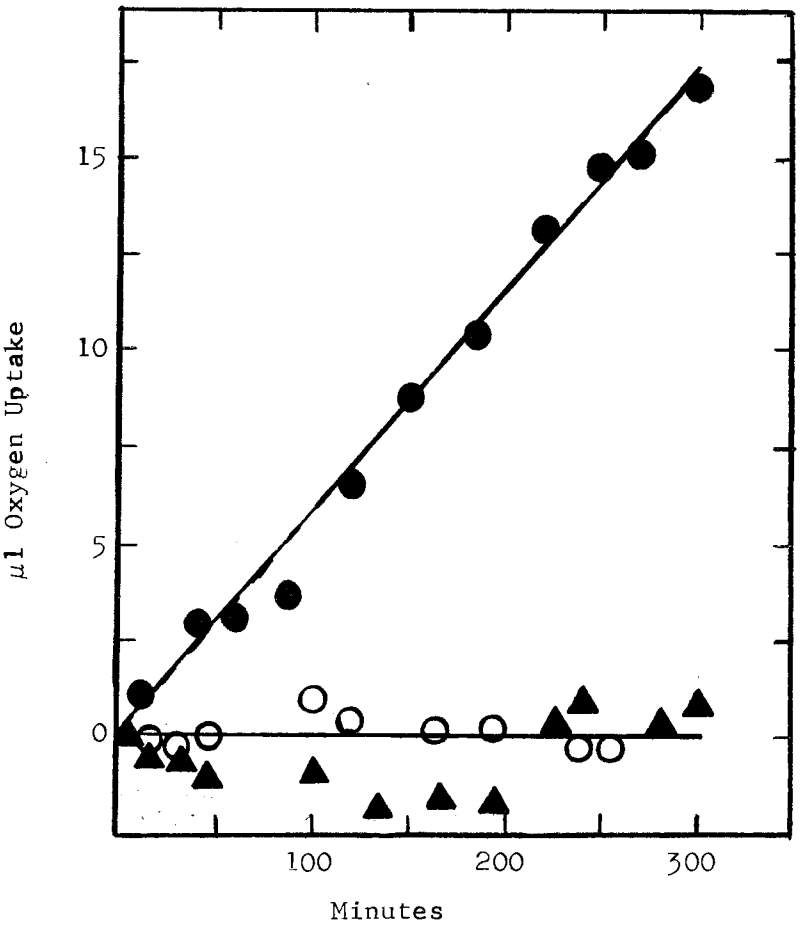


Figure 6. Oxygen Uptake of Tryptophan by Tyrosinase.

Oxygen uptake was recorded at 37° on a Gilson respirometer using single arm Warburg flasks. In each experiment the side-arm of the Warburg flask contained 10 µmoles of tryptophan in 0.5 ml of 50 mM Tris, pH 7.3, and the center well contained 0.2 ml of 6 M KOH.

●—●, The main vessel contained 8 mg of tyrosinase in 1.5 ml of 50 mM Tris, pH 7.3.

○—○, The main vessel contained 8 mg of tyrosinase boiled previously for 10 minutes in 1.5 ml of 50 mM Tris, pH 7.3.

▲—▲, The main vessel contained 1.5 ml of 50 mM Tris, pH 7.3.

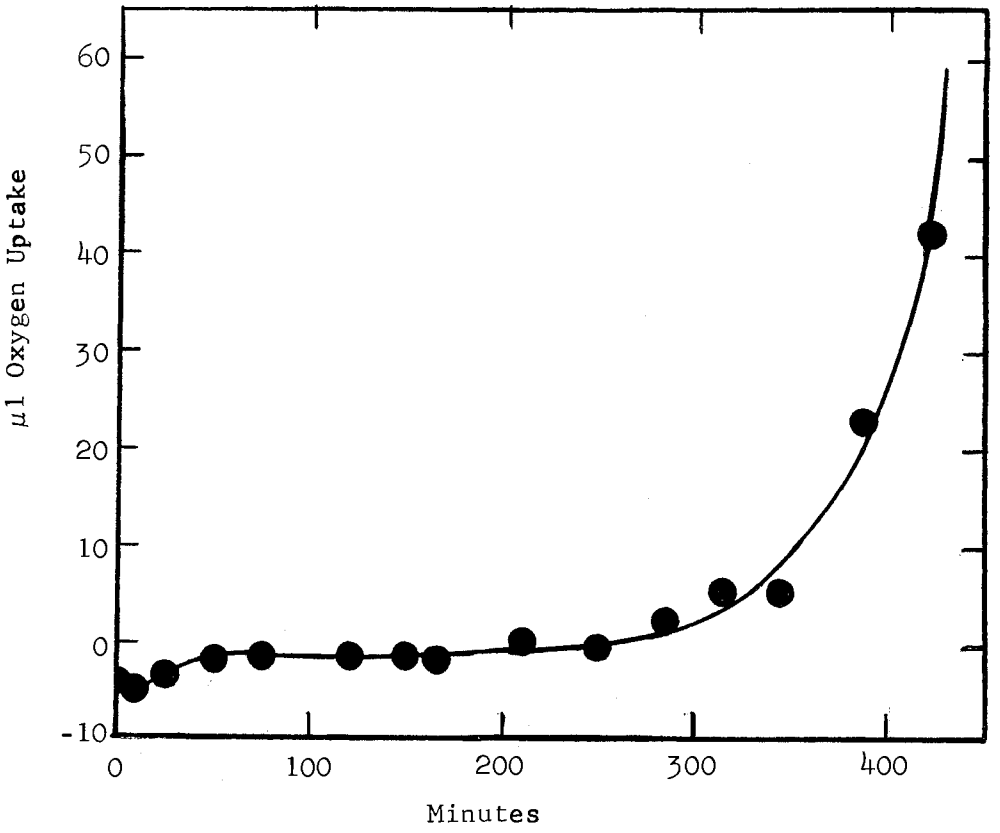


Figure 7. Oxygen Release and Uptake on Mixing Tyrosinase with Buffer Solution.

The side-arm of the Warburg flask contained 0.5 ml of 50 mM Tris, pH 7.3, and the main vessel contained 8 mg of tyrosinase in 1.5 ml of 50 mM Tris, pH 7.3. The center well contained 0.2 ml of 6 M KOH. The temperature was maintained at 37° and oxygen uptake was measured by conventional manometric techniques (see Methods).

α-lactalbumin. The oxidative effect of tyrosinase upon α-lactalbumin was again determined by repeating the experiment with additional controls. The results of the 37° incubation of α-lactalbumin in 50 mM Tris, pH 7.3, and the incubation of α-lactalbumin with tyrosinase boiled for 10 minutes are shown in Figure 8; these results are compared to the uptake from incubation of α-lactalbumin with native tyrosinase. No oxygen uptake was recorded when α-lactalbumin was incubated at 37° for 300 minutes in 50 mM Tris, pH 7.3. The incubation of α-lactalbumin with tyrosinase boiled for 10 minutes resulted in the uptake of a small amount (approximately 6 μl) of oxygen. This is possibly due to incomplete inactivation of the tyrosinase by boiling at 100° for 10 minutes. If it is assumed that the oxidation of one amino acid residue results in the uptake of one molecule of oxygen, then the oxygen uptake for α-lactalbumin incubated with native tyrosinase in Figure 8 corresponds to the oxidation of 1.5 amino acid residues per α-lactalbumin molecule.

The α-lactalbumin treated with tyrosinase for 300 minutes was chromatographed on a 1.5 x 50 cm Bio-Gel P-60 column to separate the α-lactalbumin from the tyrosinase and the fractions corresponding to the α-lactalbumin peak were pooled and dialyzed against distilled water at 4° for 24 hours. The pooled and dialyzed fractions were lyophilized to dryness and duplicate amino acid analyses of the α-lactalbumin, 300 minute tyrosinase treated, were performed with native α-lactalbumin analyzed as a control. The results of these analyses are shown in Table II. The amino acid analyses indicate the oxidation of 1.1 residues of tryptophan, 0.2 residues of tyrosine and no histidine oxidation. This is a total of 1.3 residues of α-lactalbumin oxidized, compared

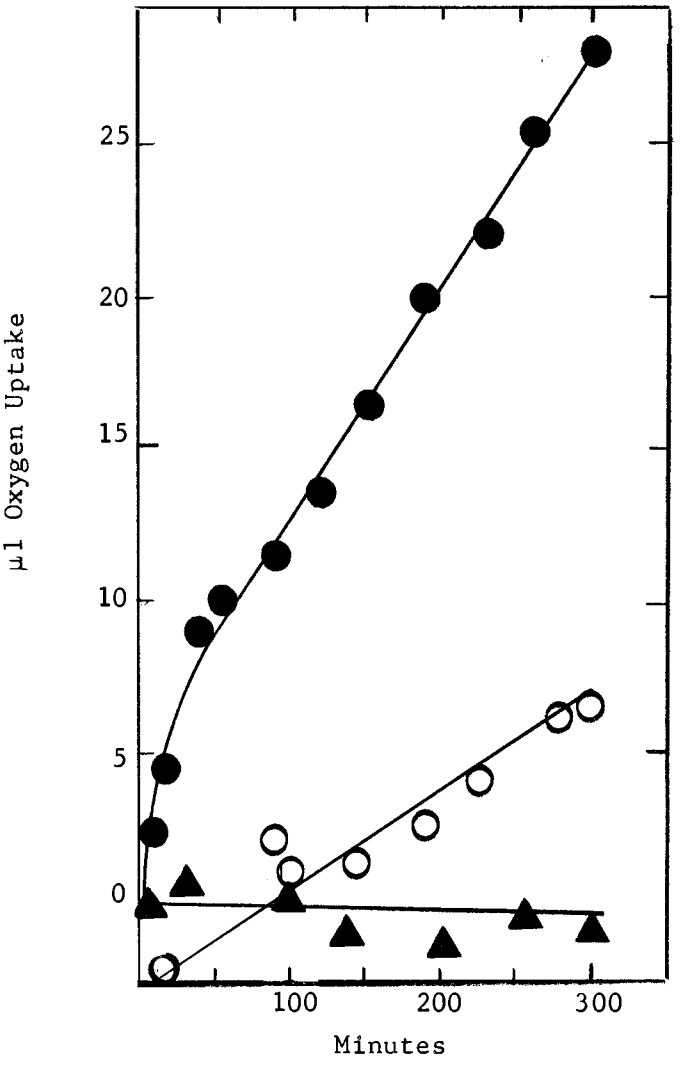


Figure 8. Oxygen Uptake of α-Lactalbumin by Tyrosinase.

Oxygen uptake was recorded at 37° on a Gilson respirometer using single arm Warburg flasks. In each experiment the side-arm of the Warburg flask contained 12 mg of α-lactalbumin in 0.5 ml of 50 mM Tris, pH 7.3 and the center well contained 0.2 ml of 6 M KOH.

●—●, The main vessel contained 8 mg of tyrosinase in 1.5 ml of 50 mM Tris, pH 7.3.

○—○ ,The main vessel contained 8 mg of tyrosinase boiled previously for 10 minutes in 1.5 ml of 50 mM Tris, pH 7.3.

▲—▲ ,The main vessel contained 1.5 ml of 50 mM Tris, pH 7.3.

TABLE II

SUMMARY OF AMINO ACID ANALYSIS OF NATIVE α-LACTALBUMIN AND α-LACTALBUMIN INCUBATED WITH TYROSINASE FOR 300 MINUTES.

AMINO ACID	Native α-LA.		300 minute tyrosinase treated α-LA. #1		300 minute tyrosinase treated α-LA. #2	
	Residues Recovered	% Recovery	Residues Recovered	% Recovery	Residues Recovered	% Recovery
Histidine	3.0	101	3.2	107	3.2	107
Tryptophan	4.0	100	2.9	72	2.9	72
Tyrosine	3.0	101	3.9	97	3.8	94
Phenylalanine	4.0	100	4.0	100	4.0	100

*An unknown peak occurred at 58 minutes in the tyrosinase treated α-lactalbumin. This peak is possibly a tryptophan degradation product.

to 1.5 residues oxidized as determined by oxygen uptake.

At various time intervals during the reaction at 37° of tyrosinase with α-lactalbumin aliquots of the reaction mixture were removed and the α-lactalbumin was separated from tyrosinase by gel filtration on a 1.5 x 50 cm Bio-Gel P-30 column. After 300 minutes reaction, the modified α-lactalbumin retained 95 percent activity in the lactose synthetase reaction, and 29 hours of reaction at 37° were required before α-lactalbumin lost all activity in the lactose synthetase reaction.

These results suggest that tyrosinase does not oxidize the tyrosyl residues of α-lactalbumin. The uptake of 28 μl of oxygen during the reaction of tyrosinase with α-lactalbumin is due to the oxidation of 1.1 to 1.5 tryptophanyl residues, and only 0.2 tyrosyl residues are oxidized. The α-lactalbumin with 1.1 to 1.5 tryptophanyl residues modified retains 95% of its activity in the lactose synthetase reaction. After 29 hours of reaction at 37° no enzymatic activity is detected.

Modification of α-Lactalbumin by Nitration

Nitration of α-Lactalbumin

α-Lactalbumin was nitrated in order to assess the function of the tyrosyl residues in α-lactalbumin as related to their role in the lactose synthetase system. The extent of reactivity of the tyrosyl residues toward modification indicates their degree of exposure in the molecule. The degree of reactivity toward nitration when compared to the expected accessibility of the tyrosines should indicate the relationship between the true molecular structure of α-lactalbumin and the hypothetical molecular model of α-lactalbumin based on the known structure of lysozyme (6).

The extent of nitration was determined by amino acid analyses. Both the appearance of 3-nitrotyrosine and the disappearance of tyrosine are shown in Figure 9. It has been reported (89,91) that the product of the reaction of tetranitromethane with proteins is 3-nitrotyrosine, and as expected, no evidence for dinitrotyrosine was found from the amino acid analyses. Upon nitration there is often stoichiometry between the tyrosine content of the native protein and the 3-nitrotyrosine plus tyrosine content of the nitrated product. For α-lactalbumin this balance would indicate that the tyrosyl residues were either nitrated or remained unmodified. The sum of the tyrosine plus 3-nitrotyrosine content of the nitrated α-lactalbumin is not constant nor equal to the tyrosine content of the native protein, but decreases with time as shown in Figure 10.

There are several studies which report that the tyrosine plus 3-nitrotyrosine content of the nitrated product equaled the tyrosine content of the starting material (95,96,97). This indicates that the tyrosyl residues of these proteins were either nitrated or remained unmodified. Recent reports, however, have shown that both with trypsin and insulin the sum of the tyrosine plus 3-nitrotyrosine content of the tetranitromethane treated protein is less than the amount of tyrosine in the untreated protein (95,99). With insulin, essentially all four of the tyrosyl residues in the molecule were modified as judged by the disappearance of tyrosine; however, only 2.5 of the 4 residues were nitrated. The remaining 1.5 tyrosyl residues are apparently involved in a cross-linked compound. However, it is not clear why they are not detected after acid hydrolysis. Amino acid analysis of the nitrated α-lactalbumin shows two unidentified peaks which elute from

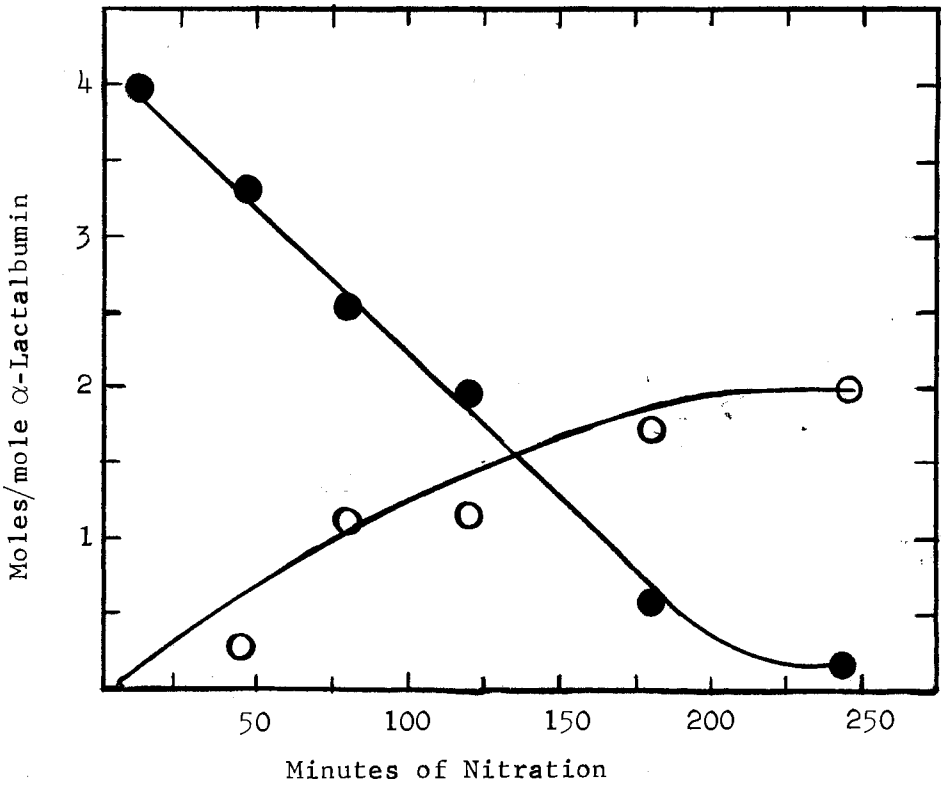


Figure 9. Nitration of α-Lactalbumin.

Time course of nitration of α-lactalbumin as described previously in Methods. (●) Disappearance of tyrosyl residues. (O) Appearance of 3-nitrotyrosine. Residues were determined by amino acid analyses.

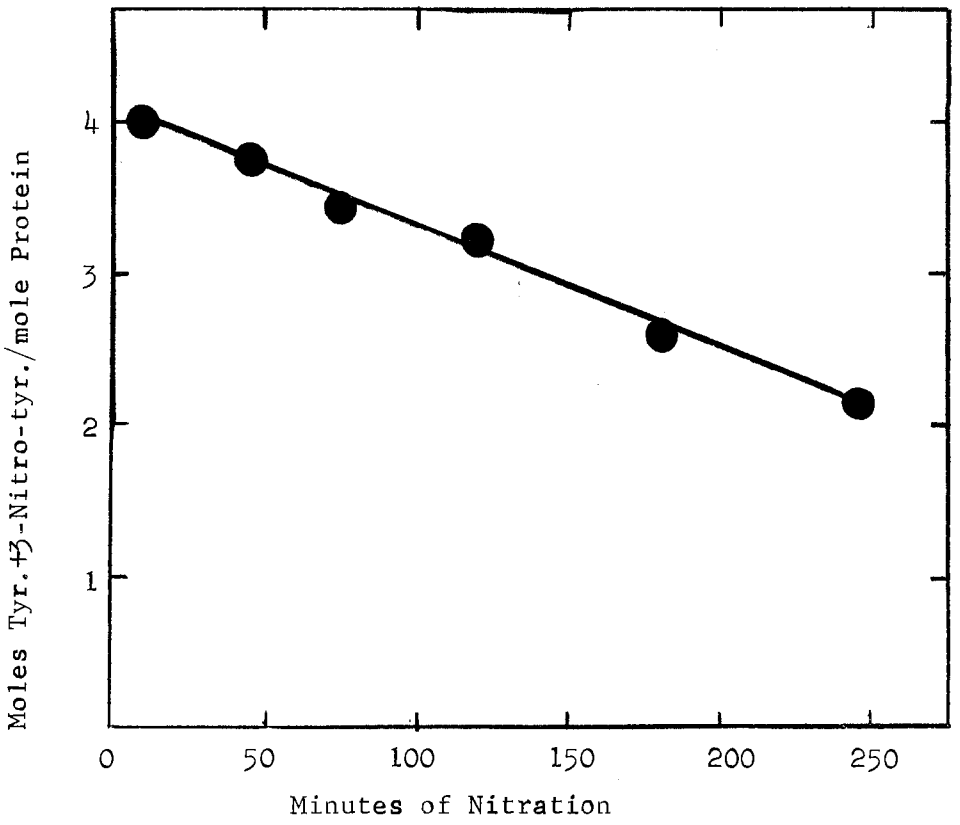


Figure 10. Sum of the Tyrosine Plus 3-Nitrotyrosine Content of Nitrated α-Lactalbumin.

The value for the tyrosine content remaining as shown in Figure 9 was added to the corresponding value for 3-nitrotyrosine at each specified time.

the basics column at 58 and 120 minutes. These peaks are not present on analysis of native α-lactalbumin. The area of the peak at 58 minutes increases as the time of nitration increases, while the area of the 120 minute peak does not.

Samples of nitrated α-lactalbumin were placed on a Bio-Gel P-60 column to determine if cross-linking had occurred. The results of this experiment are shown in Figure 11. Apparently there is polymer formation upon nitration of α-lactalbumin and the amount of polymer (Peak I) with respect to monomer (Peak II) increases as the nitration time increases (A-D, Figure 11). The molecular weight of the polymeric species formed during the nitration reaction was determined on the Bio-Gel P-60 column as shown in Figure 12.

The lower molecular weight peak (Peak II, Figure 11-D) corresponds to a molecular weight of 16,000 as compared to a weight of 14,500 for native α-lactalbumin. The higher molecular weight peak (Peak I, Figure 11-D) corresponds to a molecular weight of 37,500 and represents a molecular weight greater than a dimer but less than a trimer of α-lactalbumin (Figure 12). This polymer peak, however, is broad and appears to be skewed in a direction which indicates that in addition to dimer, there may be larger polymeric units. The nitrated α-lactalbumin was chromatographed on a (0.6 x 95 cm) Bio-Gel P-100 column (Figure 13), in order to increase the resolution of the nitrated α-lactalbumin in the higher molecular weight range. Figure 13-B shows that there is a larger increase in polymer as compared to monomer after 600 minutes nitration than after 120 minutes nitration (Figures 13-A). The P-100 column was marked for molecular weight determination as described in Methods, and the molecular weights of various fractions from Figure 13

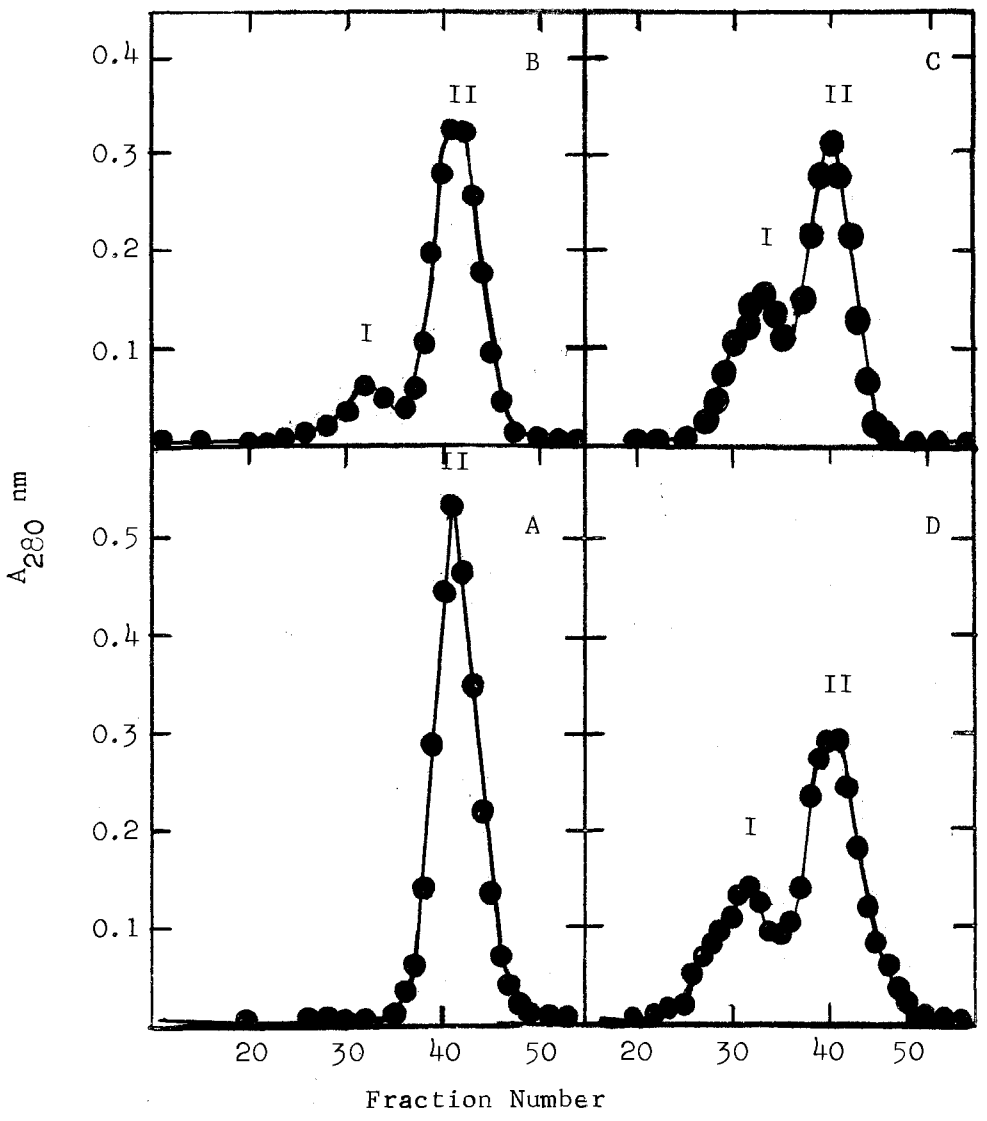


Figure 11. Bio-Gel P-60 Chromatography of Nitrated α-Lactalbumin.

Bio-Gel P-60 columns (1.4 cm x 45 cm) were equilibrated and eluted at 25° with 0.1 M Tris, pH 7.4, and 0.9 ml samples were collected per fraction. Absorbance was determined at 280 nm on a Beckman Model DB recording spectrophotometer. (A) Native α-Lactalbumin; (B) α-Lactalbumin nitrated for 110 minutes; (C) α-Lactalbumin nitrated for 180 minutes; (D) α-Lactalbumin nitrated for 240 minutes.

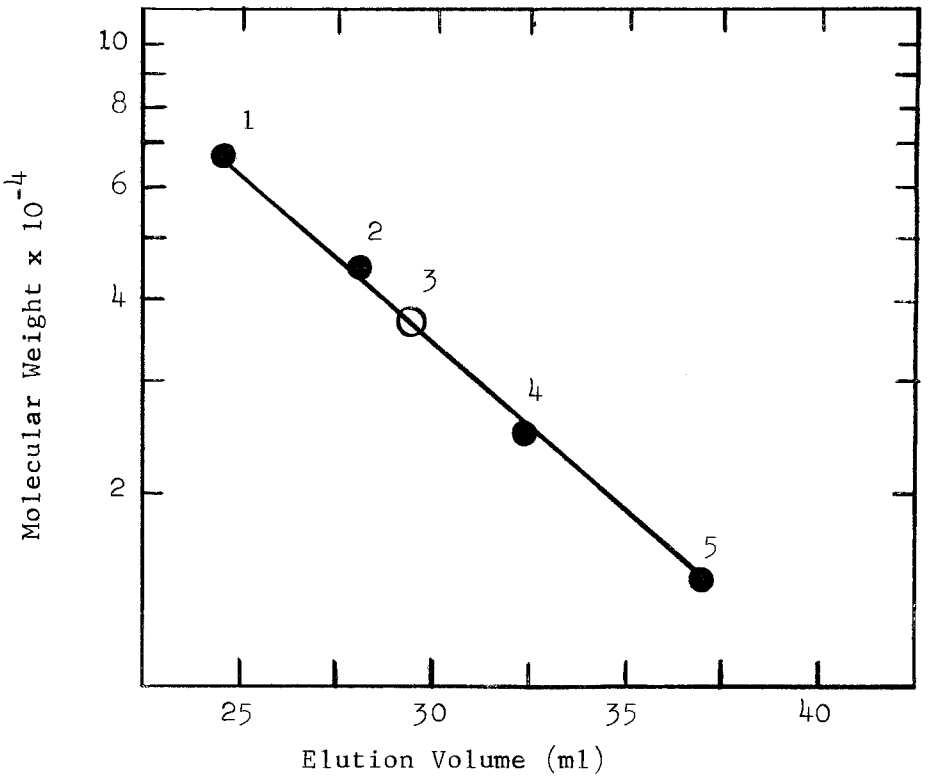


Figure 12. Molecular Weight Determination of Nitrated α-Lactalbumin.

Bio-Gel P-60 column (1.4 x 45 cm) equilibrated and eluted with 0.1 M Tris, pH 7.4. The standard proteins were: 1, 1 mg serum albumin; 2, 1 mg ovalbumin; 4, 1 mg α-chymotrypsinogen; 5, 1 mg α-lactalbumin. Number three is the nitrated α-lactalbumin polymer (Peak I, Figure 11). Blue dextran was used to determine the void volume.

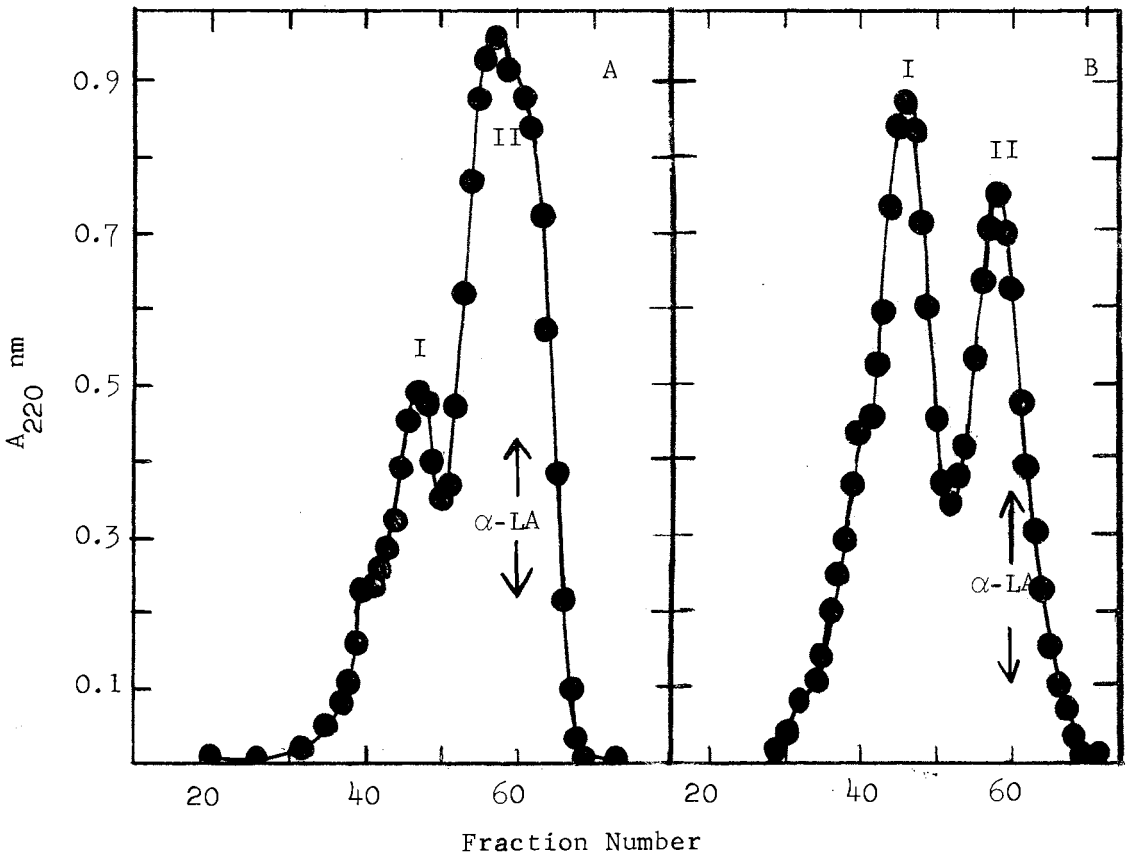


Figure 13. Bio-Gel P-100 Chromatography of Nitrated α-Lactalbumin

Bio-Gel P-100 column (0.6 x 95 cm) was equilibrated and eluted at 25° with 50 mM Tris, 0.1 M KCl, pH 7.5 and 0.2 ml fractions were collected per tube. The tubes were diluted to 0.8 ml with distilled water. The absorbance was determined at 220 nm on a Beckman Model DB Recording Spectrophotometer. (A) α-Lactalbumin nitrated for 120 minutes; (B) α-Lactalbumin nitrated for 600 minutes.

were determined as shown in Figure 14.

The first shoulder (Fraction 33, Figure 13-B) corresponds to the void volume of the P-100 column as determined by blue dextran. This indicates the presence of a polymer of α-lactalbumin with a molecular weight greater than 67,000. The second shoulder (Fraction 40, Figure 13-A and 13-B) correspond to a molecular weight of 53,000, and the peak (Fraction 47) to a molecular weight of 34,000.

The second peak (Peak II, Figure 13B) is symmetrical and corresponds to a molecular weight of 17,000. The corresponding Peak II in Figure 13-A, however, is not symmetrical but is skewed in the direction that corresponds more closely to the true molecular weight of α-lactalbumin, 14,437. This is apparent from the position of the shoulder (Fraction 60, Figure 13-A) which corresponds to a molecular weight of 14,500.

It was shown (Figure 9) that at 120 minutes nitration, approximately 2 moles of tyrosine per mole of protein are nitrated. This suggests that the monomer peak (Peak II, Figure 13-A, 120 minutes nitration) consists of a mixture of native and nitrated α-lactalbumin. The corresponding Peak II, Figure 13-B, contains nitrated but no native α-lactalbumin, which accounts for its symmetry. The above proposals as to the components of Peak II, Figure 13-A and 13-B are further supported by gel studies which are presented later in this dissertation. It is suggested that the nitration of α-lactalbumin, which reportedly results in a conformational change (93), effects a change of shape which causes an increase in the apparent molecular weight of α-lactalbumin as determined by gel filtration. This change in shape may result in broadening of the monomer peak (Peak II, Figure 13-A). Additional support for this hypothesis will be presented later.

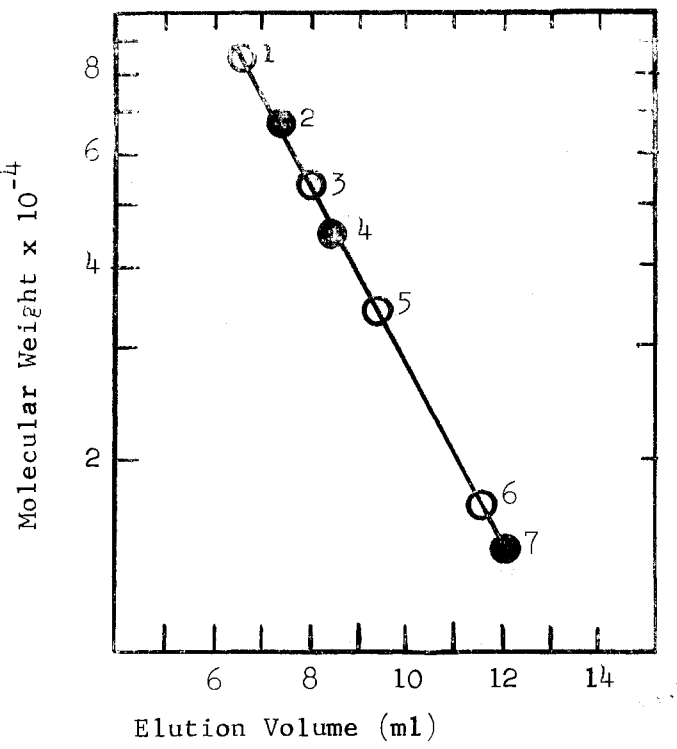


Figure 14. Molecular Weight Determination of Nitrated α-Lactalbumin.

Bio-Gel P-100 column (0.6 x 95 cm) equilibrated and eluted at 25° with 50 mM Tris, 0.1 M KCl, pH 7.5. The standard proteins were: 2, 1 mg Serum albumin; 4, 1 mg Ovalbumin; 7, 1 mg Native α-Lactalbumin. The (0) corresponds to: 1, Fraction 33, Figure 13-B; 3, Fraction 40, Figure 13-A and 13-B; 5, Fraction 47, Figure 13-A; 6, Fraction 58, Figure 13-B. Blue dextran was used to determine the void volume.

Tetranitromethane has been reported to be a mild and specific reagent for the nitration of tyrosyl residues at pH 8.0 (89), and the product of the nitration reaction has been reported as 3-nitrotyrosine (91). However, in this laboratory it has been observed that in addition to the loss of tyrosyl residues certain of the tryptophanyl residues of α-lactalbumin are also destroyed during nitration. The determination for tryptophan was made using 4 percent thioglycolic acid to prevent tryptophan destruction during acid hydrolysis (see Methods).

Due to the observed tryptophan destruction in the nitrated α-lactalbumin it was necessary to determine the specificity of nitration by tetranitromethane toward α-lactalbumin. This was accomplished by complete amino acid analyses (see Methods) after nitration. Following nitration, amino acid analyses of the reaction mixtures, when compared to control analyses on native α-lactalbumin, showed that all the component amino acids of α-lactalbumin, except tyrosine and tryptophan, were recovered in essentially quantitative yield. An unknown product of the nitration reaction present in the amino acid mixture was identified as 3-nitrotyrosine, using standard 3-nitrotyrosine as a marker. No peak was found corresponding to the 3,5-dinitrotyrosine standard. An unidentified asymmetrical peak was detected in both the controls and the nitrated samples which eluted from the basics column at 120 minutes.

The effect of nitration of α-lactalbumin upon the tyrosyl, histidyl, and tryptophanyl residues was determined by amino acid analyses, and the status of these residues is related to the loss of activity of α-lactalbumin in the lactose synthetase reaction (Figure 15). The 3 histidyl residues per mole of α-lactalbumin are not modified after 240 minutes nitration (Figure 15-A). The histidines are mentioned,

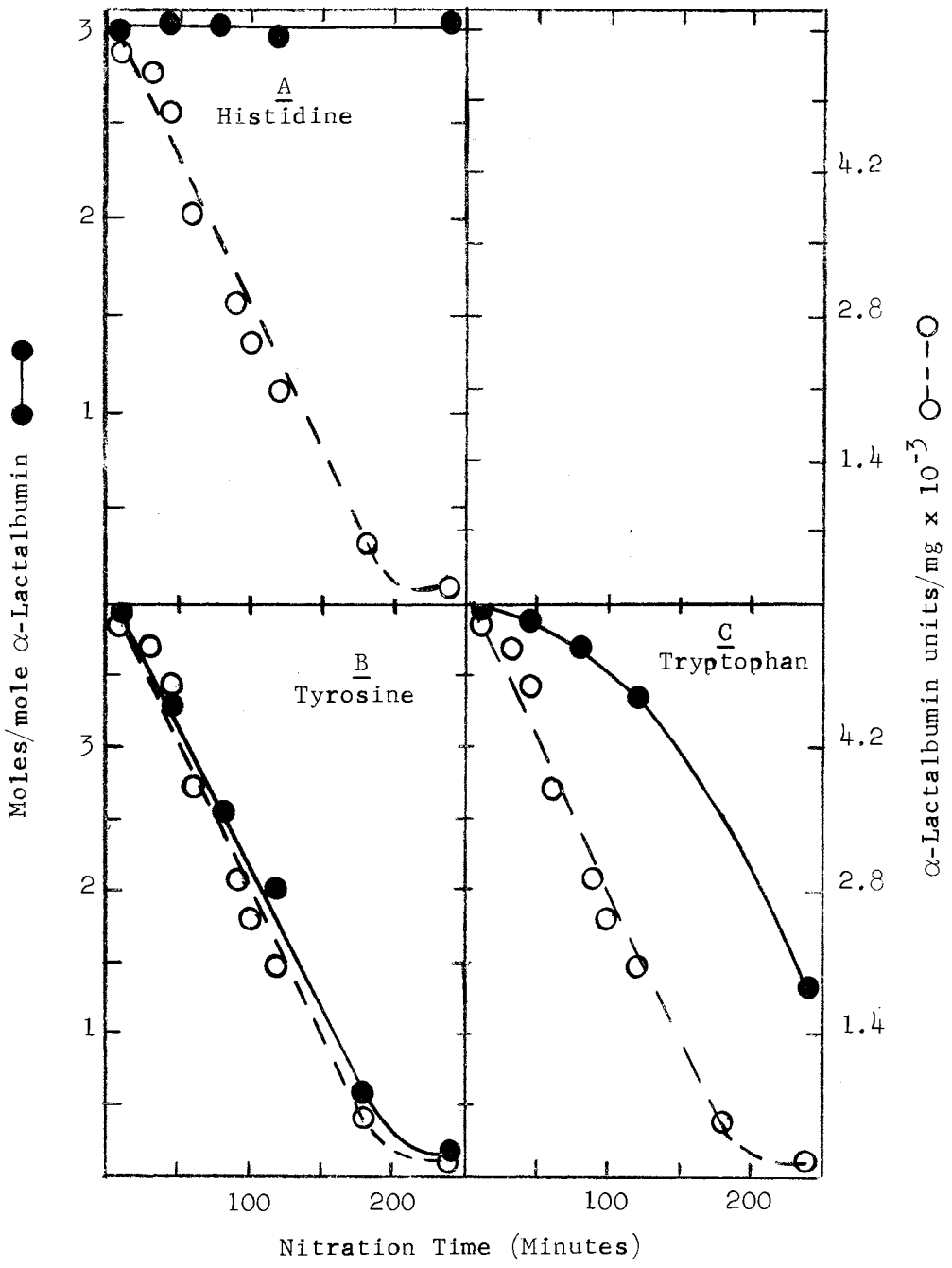


Figure 15. Modification of Amino Acid Residues as Related to Lactose Synthetase Activity.

Effect of nitration of α-lactalbumin upon tyrosine, histidine, and tryptophan and the relationship of these amino acids to the loss of lactose synthetase activity. (A) Histidine modification; (B) Tyrosine modification; (C) Tryptophan modification.

since they are modified upon iodination. The activity of nitrated and iodinated α-lactalbumin will be considered later in this dissertation. The 4 tryptophanyl residues of α-lactalbumin were affected by the nitration reaction. After 240 minutes nitration, an average of 2.7 of the tryptophanyl residues were modified. The rate of modification of tryptophan is relatively slow when compared to the rate of modification of tyrosine. The rate of modification of tryptophan does not parallel the loss of lactose synthetase activity (Figure 15-C). The modification of the tyrosyl residues of α-lactalbumin, resulting from nitration with tetranitromethane, is rapid relative to tryptophan destruction, and essentially all 4 tyrosyl residues are lost after 240 minutes nitration (Figure 15-B). The rate of modification of tyrosine very closely parallels the loss of lactose synthetase activity which suggests a functional role for the tyrosyl residues of α-lactalbumin in the lactose synthetase system.

A 64 molar excess of tetranitromethane was used in the nitration reaction. Therefore the nitration of α-lactalbumin is a pseudo first-order reaction (see Methods). The slope of the log plot (Figure 16) indicates the "rate constant" of $k_1 = 0.33$, and $k_2 = 1.74$ for tyrosine loss. There is, however, a gradual increase in the slope (Figure 16), until approximately 2 tyrosyl residues are lost. After this there is a marked increase in the overall disappearance of tyrosine during the nitration reaction. The rate of total tyrosyl loss was compared to the rate of 3-nitrotyrosine formation (Figure 17). The formation of 3-nitrotyrosine is also first order, $k_3 = 0.30$, and the two curves (Figure 17) correspond only until 1 tyrosyl residue is modified. Figure 17 suggests that after the nitration of one tyrosyl residue, a

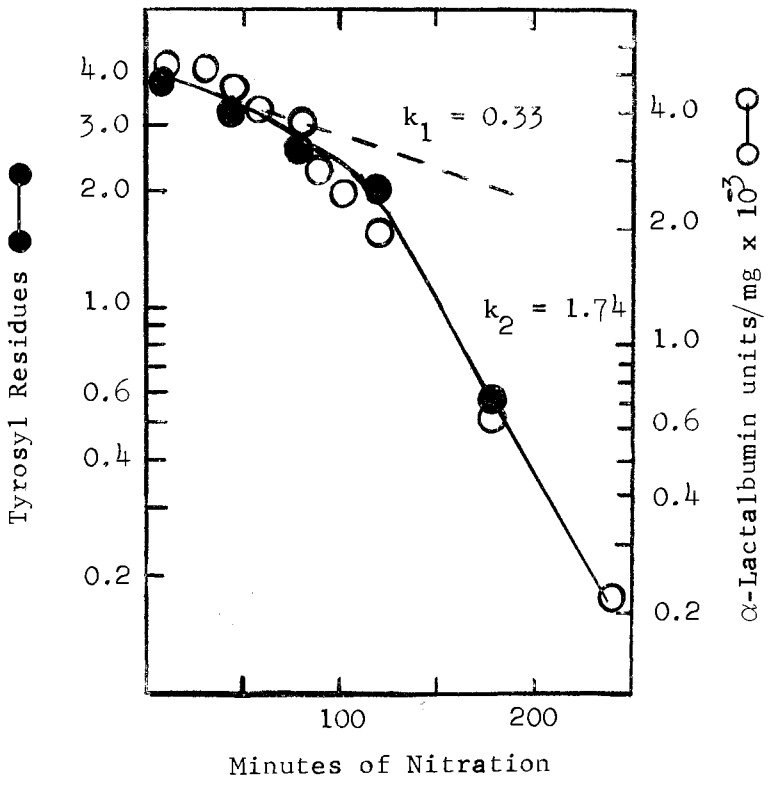


Figure 16. Lactose Synthetase Inactivation by Nitration of α-Lactalbumin's Tyrosyl Residues.

Effect of nitration of α-lactalbumin upon the tyrosyl residues and upon lactose synthetase activity. The slope of the line indicates the rate constant for tyrosyl modification and for lactose synthetase inactivation (k_1 = 0.33, k_2 = 1.74).

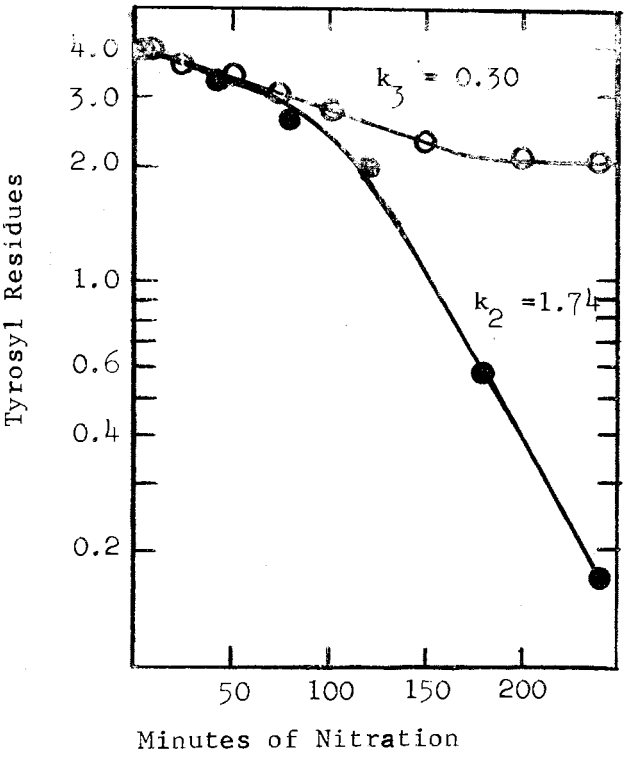


Figure 17. Tyrosyl Loss as A Function of the Formation of 3-Nitrotyrosine During the Nitration of α-Lactalbumin.

Effect of nitration of α-lactalbumin upon the tyrosyl residues and upon the formation of 3-nitrotyrosine. (●), loss of tyrosyl residues due to total tyrosine modification; (○), loss of tyrosyl residues due to the formation of 3-nitrotyrosine. The rate constants are; $k_2 = 1.74$, $k_3 = 0.30$.

secondary polymerization reaction is initiated. This secondary reaction, $k_2 = 1.74$, also appears to be a first order process (Figure 17).

The rate of inactivation of lactose synthetase as a function of time (Figure 16) is quite similar to the total loss of tyrosyl residues (Figure 16). This suggests a correlation between the tyrosyl residues of α-lactalbumin and α-lactalbumin's lactose synthetase activity.

A 0.03 ml sample (approximately 6 mg/ml) of α-lactalbumin nitrated for 120 minutes was lyophilized to dryness and the residue was suspended in 0.1 ml of 50 mM Tris, 0.1 M KCl, pH 7.5. The resultant solution was placed on the Bio-Gel P-100 column as previously described in the Methods section. The elution pattern from the Bio-Gel P-100 is shown in Figure 18. The plot of the lactose synthetase activity in total units per tube (Figure 18) is a symmetrical peak but covers only a small area of the entire lower molecular weight peak (Peak II). It was previously suggested that the comparative broadness of Peak II is due to a change in shape in the native α-lactalbumin upon nitration. Native α-lactalbumin placed on the same P-100 column coincides with the plot for total units (Figure 18). It is also shown (Figure 18) that native α-lactalbumin which has undergone this change in shape during nitration does not retain lactose synthetase activity. More evidence for a change in shape in α-lactalbumin upon nitration and for a loss of lactose synthetase activity in the nitrated product which has undergone this change is indicated by the sharp increase in the specific activity. The positive slope of the specific activity plot (Figure 18) indicates that the purest native α-lactalbumin or the α-lactalbumin with highest specific activity is at the lower molecular weight edge of Peak II. These

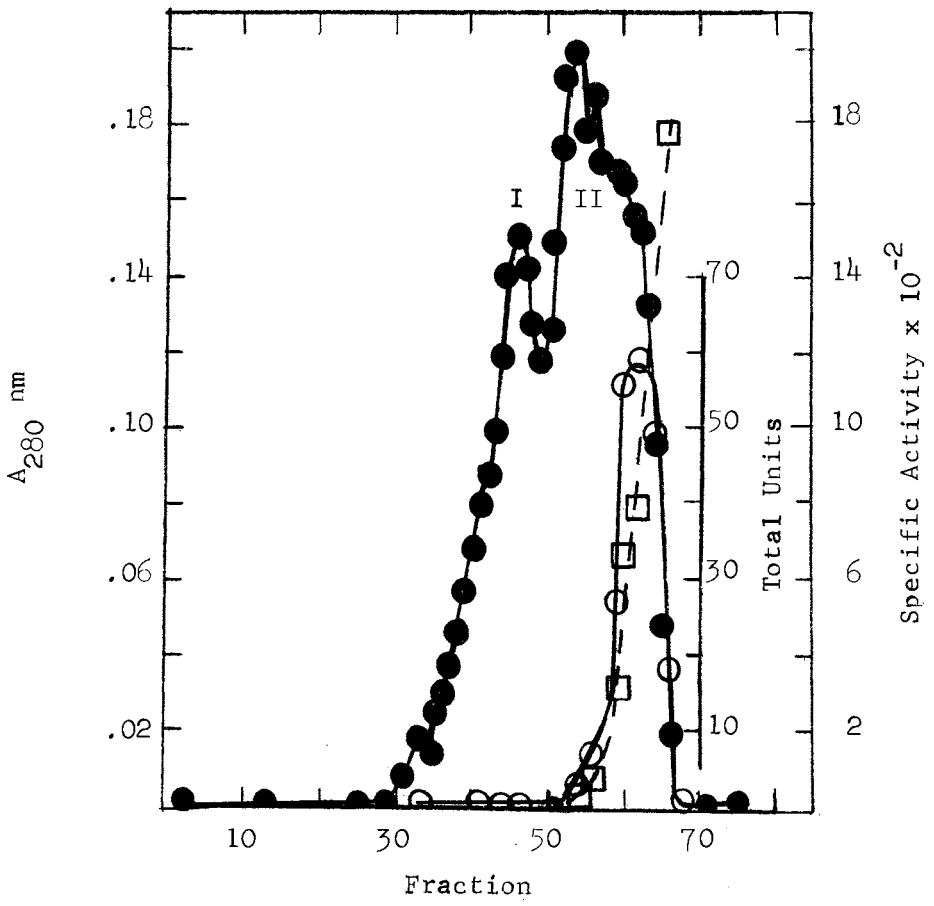


Figure 18. Lactose Synthetase Activity of Nitrated α-Lactalbumin Eluted from a Bio-Gel P-100 Column.

A 0.3 ml sample of 120 minute nitrated α-lactalbumin was lyophilized, dissolved in 0.1 ml of eluant buffer, and eluted at 25° from a Bio-Gel (0.6 x 95 cm) P-100 column in 0.2 ml fractions. The column was equilibrated and eluted with 50 mM Tris, 0.1 M KCl, pH 7.5. ●—●, Absorbance at 280 nM; O—O, Lactose synthetase activity (Total units), □—□ Lactose synthetase specific activity (Units/mg Protein x 10^{-2}).

results further support the proposal that nitrated α-lactalbumin which has undergone this shape change has no lactose synthetase activity.

A standard 7 percent disc gel electrophoretic separation of nitrated α-lactalbumin and control samples were performed (see Methods). The disc gel electrophoretic patterns as a function of nitration time are shown in Figure 19. Tube 1 (Figure 19) contains native α-lactalbumin. Tube 2 is a zero time control and was subjected to all the conditions of the nitration reaction except no tetranitromethane was added to the sample, while Tube 3 is a 300 minute time control subjected to the same conditions as Tube 2. Aliquots from the control samples (Tubes 1, 2, and 3, Figure 19) remained as a single band throughout the time span of the nitration reaction. The nitrated samples, however, form 2 to 5 distinct bands on disc gel electrophoresis (Figure 19). Tube 4 (10 minutes nitration) contains a dark band which corresponds to the mobility of native α-lactalbumin and a second minor band which increases in intensity with respect to the native α-lactalbumin band as the time of nitration increases (Tubes 4-10). In Tube 7 a third band appears, in Tube 8 a fourth, and in Tube 9 (Figure 19) five bands are clearly visible. It is suggested from previous studies on the gel filtration column that the diffuse bands (Tube 11) may be due to the high percentage of polymeric α-lactalbumin present at this time period.

Attempts were made to separate the 5 electrophoretic bands seen on disc gel electrophoresis by a preparative procedure. The goal was to isolate the bands in a relatively pure form so they could be further characterized. Partial separation of the bands was achieved by Preparative Disc Gel Electrophoresis, but no pure material could be obtained since the bands overlapped. The bands did <u>not</u> separate during

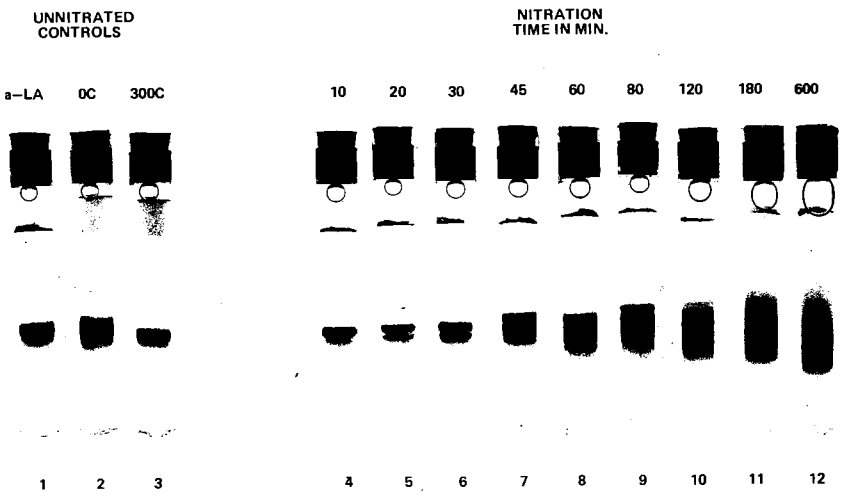


Figure 19. Disc Gel Electrophoresis of α-Lactalbumin Nitrated for Varying Times.

Disc Gel Electrophoresis studies were performed on standard 7 percent gels (see Methods). Tube 1, native α-lactalbumin; Tube 2, Zero time control (see text); Tube 3, 300 minute control; Tube 4, 10 minute nitrated α-lactalbumin; Tubes 5 through 12, 20 through 600 minute nitrated α-lactalbumin.

Isoelectric Focusing in normal pH gradients. The nitrated α-lactalbumin (120 minute) was separated into two peaks on a DE-32 ion exchange column (Figure 20). A (1.5 x 7 cm) DE-32 column was equilibrated with 5 mM glycine buffer pH 9.5 and a 250 ml linear gradient from 5 to 700 mM glycine, pH 9.5 was used to elute the protein. Disc gel electrophoresis was performed on Tubes 92, 99, 106 from Figure 20. The result of the study is shown in Figure 21. Each of the two peak tubes (Figure 20) form single bands on electrophoresis (Figure 21) and the band corresponding to Peak II appears below the band corresponding to Peak I. It is suggested that in Figure 20, Peak I is native α-lactalbumin and Peak II is α-lactalbumin with 1 tyrosyl residue nitrated. Amino acid analysis studies are presently being performed to determine the composition of the two peaks.

The plot of lactose synthetase activity in Figure 20 shows that both Peaks I and II have lactose synthetase activity and the specific activity of Peak I (1300 units/mg) is greater than the specific activity of Peak II (935 units/mg). If the studies now in progress do indicate that Peak II (Figure 20) is mono-nitrated α-lactalbumin, this would suggest that the mono-nitrated α-lactalbumin retains approximately 75 percent of its ability to activate lactose synthetase.

Of the several methods attempted, the five bands which are clearly visible in Tubes 9 and 10 (Figure 19) could only be separated by disc gel electrophoresis. The method of Hedrick and Smith (131) was used to determine which of the 5 bands were charge or size isomers. When the logarithm of protein mobility relative to the dye front is plotted versus acrylamide gel concentration, size isomeric proteins give a family of nonparallel lines extrapolating to a common point in the

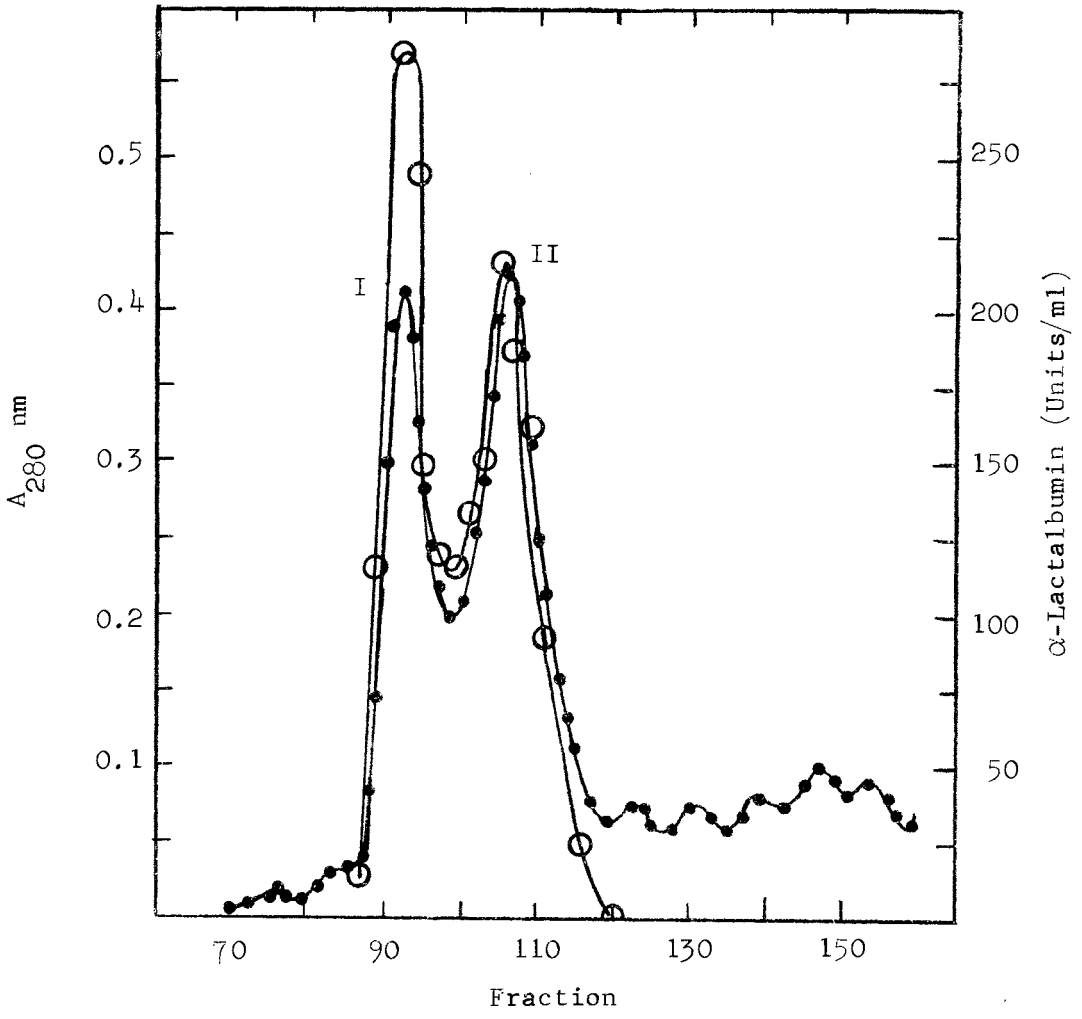


Figure 20. Separation of Nitrated α-Lactalbumin on a DE-32 Column.

A sample of 120 minute nitrated α-lactalbumin on a (1.5 x 7 cm) DE-32 column equilibrated with 5 mM glycine, pH 9.5. The absorbance was determined at 280 nm on a Beckman Model DB spectrophotometer. The column was eluted at 25° with a linear, 250 ml gradient from 5 to 700 mM glycine, pH 9.5, and 2.5 ml were collected per fraction. The specific activities in units/mg are: Tube 92, 1300; Tube 106, 935. ●—●, A_{280} nm; O—O, α-lactalbumin, lactose synthetase activity (units/ml).

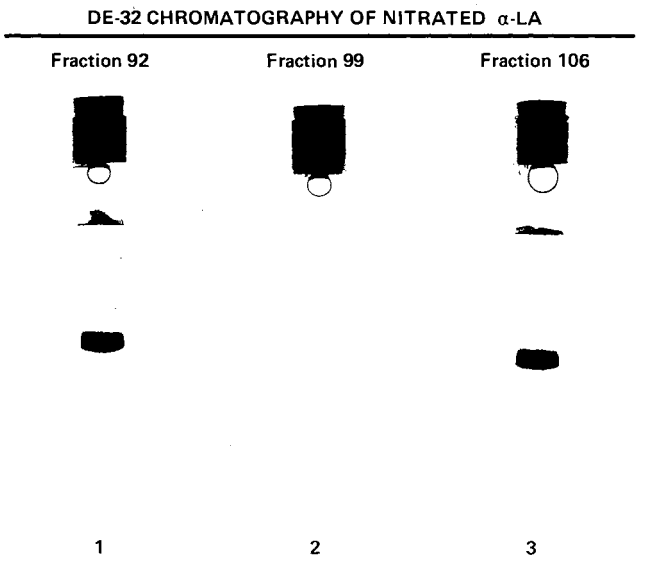


Figure 21. Disc Gel Electrophoresis of Selected Fractions from a DE-32 Column (Figure 20).

Fractions 92, 99, and 106 (Figure 20) were run on standard 7 percent disc gel electrophoresis.

vicinity of zero percent gel concentration; charge isomeric proteins give a parallel family of lines. Proteins differing in both charge and size show nonparallel lines intersecting at gel concentrations other than zero percent gel concentration. Samples of 120 minute nitrated α-lactalbumin as shown (Tube 10, Figure 19) were run on different percent gels, varying from 4 to 10 percent (Figure 22). This study shows that the five bands formed by nitrated α-lactalbumin on disc gel electrophoresis gave parallel lines (Figure 23) and therefore they are charge isomers. The delayed appearance of the 2nd, 3rd, 4th and 5th bands requiring increased nitration time (Figure 19), coupled with the identification of the five bands as charge isomers, suggests that the 5 bands (Tube 10, Figure 19) may correspond to:

Band I - native α-lactalbumin

Band II - α-lactalbumin with 1 tyrosyl residue nitrated

Band III- α-lactalbumin with 2 tyrosyl residues nitrated

Band IV - α-lactalbumin with 3 tyrosyl residues nitrated

Band V - α-lactalbumin with 4 tyrosyl residues nitrated

There was variation among the different preparations in the amount of polymer formed during the nitration of α-lactalbumin. Preparation with low amounts of polymer were selected for the previous gel study. Therefore the polymers formed are in too low a concentration to appear as weight isomers in the gels.

In order to further investigate the 5 bands formed on disc gel electrophoresis, a procedure was developed for extracting the protein bands from the gel and performing amino acid analyses on the isolated protein. Samples of 120 minute nitrated α-lactalbumin were run on 50 standard 7 percent gels (see Methods). The gels were sliced into 5

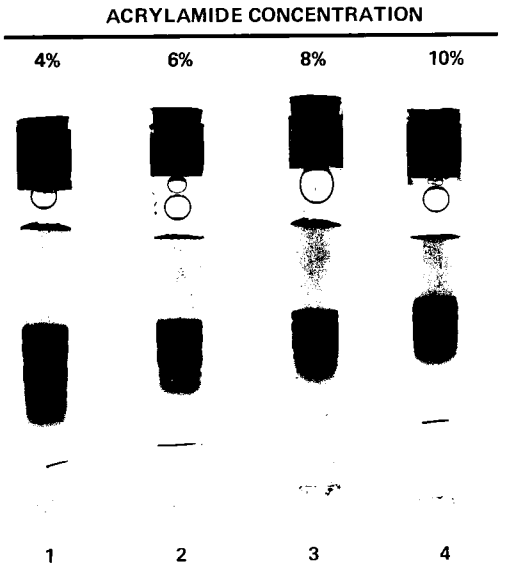


Figure 22. Disc Gel Electrophoresis of 120 Minute Nitrated α-Lactalbumin on Gels Varying From 4 to 10 Percent Acrylamide.

Varying percent gel studies were run to distinguish between charge and size isomers in the 5 bands formed on disc gel electrophoresis of 120 minute nitrated α-lactalbumin. Tube 1, 4 percent gel; Tube 2, 6 percent; Tube 3, 8 percent; Tube 4, 10 percent.

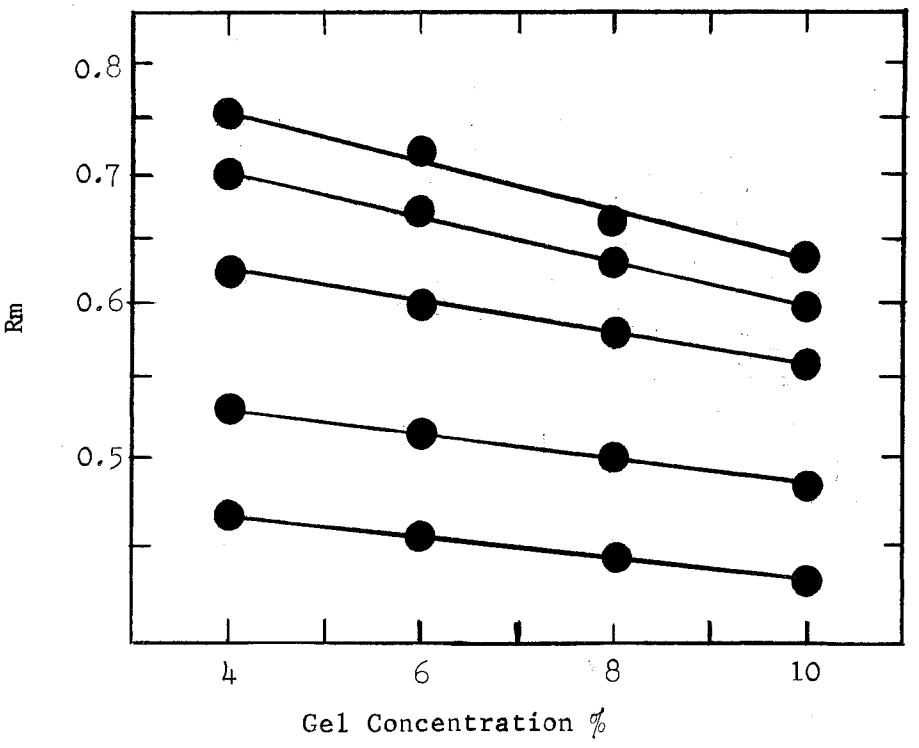


Figure 23. Percent Gel Study of 120 Minute Nitrated α-Lactalbumin on Disc Gel Electrophoresis.

The effect of different gel concentrations on the mobility of the 5 bands formed on disc gel electrophoresis of 120 minute nitrated α-lactalbumin. The parallel lines indicate that all 5 bands are charge isomers. The relative amount of polymer varied with different preparations of 120 minute nitrated α-lactalbumin, and samples with a low polymer content were specifically selected for the above study. Rm is the ratio of protein migration to dye migration.

small sections in such a way that each section contained a single protein band. The protein was extracted from the gel and the separated proteins were hydrolyzed and studied by amino acid analysis. The result of the amino acid analysis data is shown below:

Band I contains 4.3 moles of tyrosine per mole of α-lactalbumin

Band II contains 3.1 moles of tyrosine per mole of α-lactalbumin

Band III contains 2.2 moles of tyrosine per mole of α-lactalbumin

Bands IV and V were too low in concentration to be determined.

This data shows that the first three bands obtained upon disc gel electrophoresis of nitrated α-lactalbumin are native α-lactalbumin, mononitrated and dinitrated α-lactalbumin respectively and implies that the fourth and fifth bands are the tri and tetra-nitrated α-lactalbumin.

Summary of Nitration Studies on α-Lactalbumin

The nitration of α-lactalbumin results in the formation of 2 moles of nitrotyrosine per mole of α-lactalbumin. The two remaining tyrosyl residues, however, are unaccountable and may be involved in the formation of polymers. However, it is not clear why they are not detected after acid hydrolysis. It is suggested that after the nitration of one tyrosyl residue, α-lactalbumin undergoes a change in shape which increases the apparent molecular weight as determined by gel filtration. Essentially all 4 tyrosyl residues are lost after 240 minutes nitration.

This indicates that either all 4 tyrosyl residues are exposed to the solvent as predicted by the hypothetical molecular model of α-lactalbumin based on the known structure of lysozyme, or that all 4 tyrosyl residues become exposed after α-lactalbumin undergoes a conformational change during nitration. In addition to tyrosine, the tryptophanyl residues are modified during the nitration reaction, but at a slower rate than the tyrosines. The loss of lactose synthetase activity closely parallels the loss of the tyrosyl residues and it is suggested that the tyrosines are critical for lactose synthetase activity. After the nitration of one tyrosyl residue there is a secondary reaction initiated which is likely polymerization of the α-lactalbumin. The 120 minute nitrated α-lactalbumin is separated into 5 bands on disc gel electrophoresis and the bands represent native α-lactalbumin plus mono-, di-, tri-, and tetra-nitrated α-lactalbumin.

Modification of α-Lactalbumin by Iodination

Iodination of α-Lactalbumin

The effects of tyrosinase and nitration on α-lactalbumin were described previously. The modification of α-lactalbumin with tyrosinase resulted in the oxidation of 1.1 to 1.5 tryptophanyl residues, though 95 percent of the lactose synthetase activity remained. The results of the nitration experiments showed that in addition to the tyrosyl residues, the tryptophanyl residues were modified during the nitration reaction, but at a slower rate than the tyrosyl residues. The loss of lactose synthetase activity upon nitration closely parallels the loss of tyrosyl residues, and it is suggested that the tyrosyl residues of α-lactalbumin are critical for lactose synthetase activity.

α-Lactalbumin was iodinated in order to further assess the function of the tyrosyl and tryptophanyl residues in α-lactalbumin as related to their role in the lactose synthetase system. The results from the iodination of α-lactalbumin, when compared to the results from the previous modification reactions, further suggest the importance of the tyrosyl and tryptophanyl residues of α-lactalbumin in the lactose synthetase reaction. In addition, the degree of reactivity of the tyrosyls toward iodination, when compared to the expected accessibility of the tyrosyls should indicate the relationship between the true molecular structure of α-lactalbumin and the hypothetical molecular model of α-lactalbumin based on the known structure of lysozyme (6).

Separate 10 mg samples of α-lactalbumin were iodinated while varying the iodine to α-lactalbumin ratio from 1 to 40 as is described in the Methods Section. The effect of iodination of α-lactalbumin upon the loss of tryptophanyl residues and the relation of tryptophan loss to loss of lactose synthetase activity is shown in Figure 24. One tryptophanyl residue is oxidized by an iodine to α-lactalbumin molar ratio of 40 to 1 and the loss of lactose synthetase activity is approximately 6 times faster than the loss of tryptophanyl residues. Since the lactose synthetase activity decreases rapidly before any significant tryptophan loss occurs, and since previous studies showed 95 percent of the lactose synthetase activity remains after the loss of 1.1 to 1.5 trytophanyl residues upon tyrosinase treatment of α-lactalbumin, it is suggested that the tryptophanyl residues of α-lactalbumin are not critical for lactose synthetase activity.

Previous studies indicated that all 4 tyrosyl residues of α-lactalbumin were lost during the nitration of α-lactalbumin as

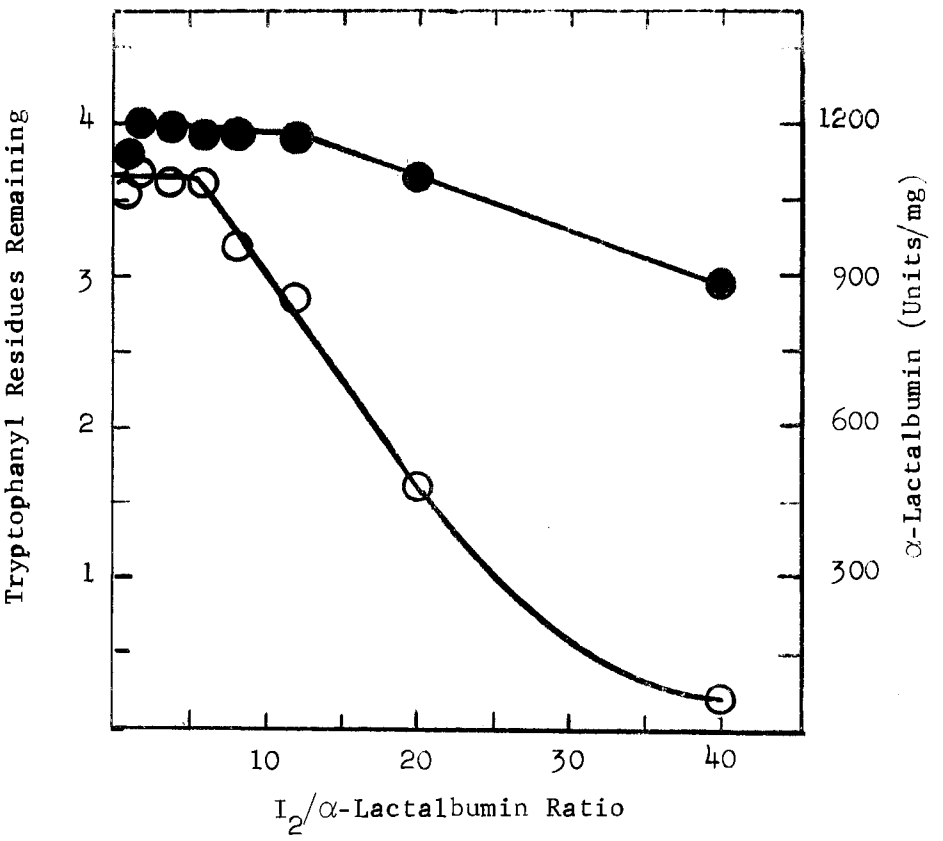


Figure 24. Effect of Iodination of α-Lactalbumin Upon Loss of Tryptophan and Lactose Synthetase Activity.

The effect of iodination of α-lactalbumin upon the loss of tryptophanyl residues and the relationship of tryptophan loss to the loss of lactose synthetase activity. The loss of tryptophanyl residues was determined by amino acid analysis after acid hydrolysis using 4 percent thioglycolic acid to prevent tryptophan destruction as described in the Methods Section. ●—●, tryptophanyl residues remaining unmodified, O—O, α-lactalbumin lactose synthetase activity (units/mg).

determined after acid hydrolysis. The effect of iodination of α-lactalbumin upon the tyrosyl residues as determined after acid hydrolysis is shown in Figure 25. It appears (Figure 25) that about 2 of the 4 tyrosyl residues were modified by the iodination of α-lactalbumin with an I_2/α-lactalbumin ratio of 40/1. However, control acid hydrolysis experiments show that both monoiodotyrosine (MIT) and diiodotyrosine (DIT) are destroyed upon acid hydrolysis, and it appears that about 50 percent of the MIT and DIT is recovered as tyrosine, after acid hydrolysis. This suggests that the tyrosine loss (Figure 25) is incorrect, since much of tyrosine which is converted to MIT and DIT upon iodination is converted back to tyrosine on acid hydrolysis.

Alkaline hydrolysis of MIT and DIT resulted in less than 1 percent of the MIT and DIT being converted to tyrosine. This suggests that amino acid analysis after alkaline hydrolysis should result in an accurate estimation of tyrosyl loss during iodination. The effect of iodination of α-lactalbumin upon the loss of tyrosyl residues as determined after alkaline hydrolysis as related to the loss of lactose synthetase activity is shown in Figure 26. All 4 tyrosyl residues appear to be available for modification, and the loss of tyrosyl residues upon iodination of α-lactalbumin closely parallels the loss of lactose synthetase activity. A similar relationship between tyrosyl loss and loss of lactose synthetase activity was previously described upon nitration of α-lactalbumin and supports the view that the tyrosyl residues maintain a critical functional role in the activity of α-lactalbumin in the lactose synthetase reaction.

The first detectable DIT appeared after the iodination of α-lactalbumin at an I_2/α-lactalbumin ratio of 12/1, and 2.5 residues of DIT

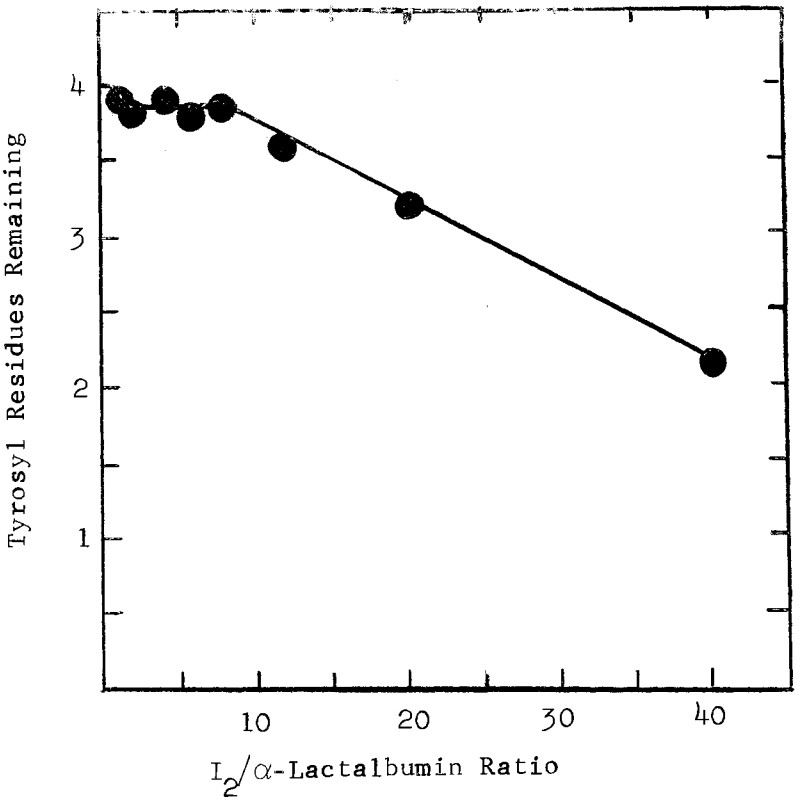


Figure 25. Effect of Iodination of α-Lactalbumin Upon the Loss of Tyrosyl Residues as Determined After Acid Hyrolysis.

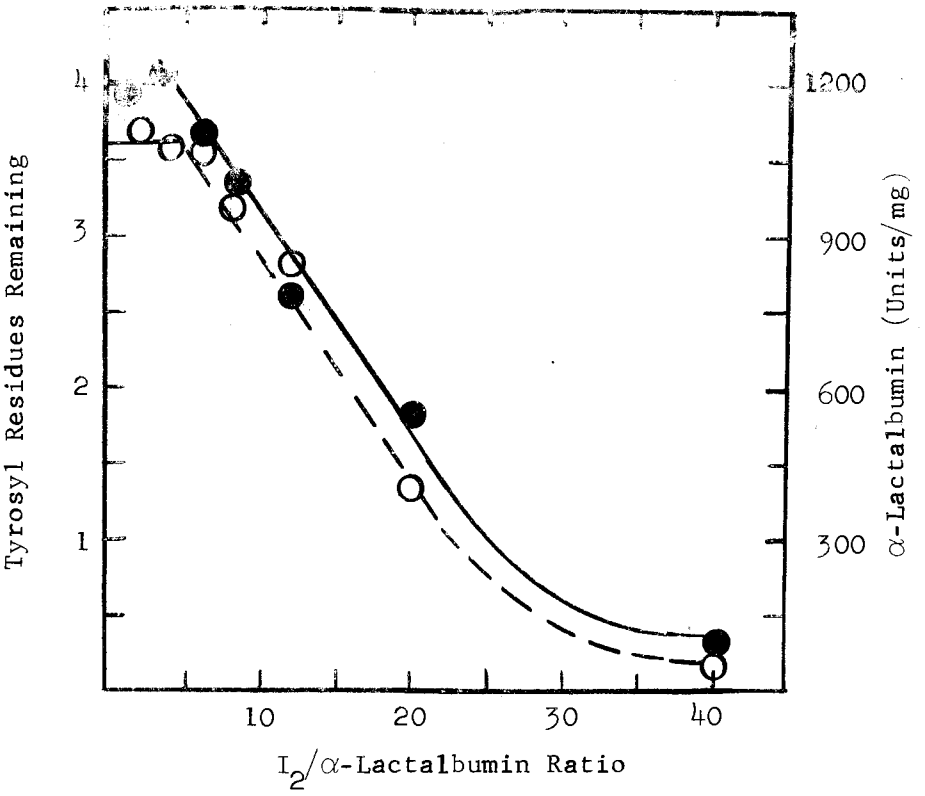


Figure 26. The Relationship Between Lactose Synthetase Activity and Tyrosyl Residues as Determined After Alkaline Hydrolysis.

The effect of iodination of α-lactalbumin upon the loss of tyrosyl residues as determined by amino acid analysis after alkaline hydrolysis and the relation of tyrosyl loss to the loss of lactose synthetase activity. ●—●, tyrosyl residues remaining unmodified; ○—○ , α-lactalbumin lactose synthetase activity (units/mg).

per molecule of α-lactalbumin were formed upon iodination at an I_2/α-lactalbumin ratio of 40/1. Sherman and Kassell (87) presented a method for the determination of mono- and diiodotyrosine, using alkaline hydrolysis of peptides containing these amino acids. However, they did not determine MIT in the presence of protein because of an interfering substance eluting with MIT upon amino acid analysis. Present amino acid analysis studies indicated that the interfering substance is tryptophan, since it and MIT elute at the same position. A method for determination of MIT in proteins is presently being developed. Additional control experiments are needed before publication of the method but a brief outline is given in the following procedure:

(A) Alkaline hydrolysis and amino acid analysis results in an eluent peak which gives an estimation of the MIT plus tryptophan content of the sample.

(B) Acid hydrolysis using 4 percent thioglycolic acid destroys the MIT but gives from 87 to 92 percent recovery of the tryptophan.

(C) Subtraction of the tryptophan content (B) from the total tryptophan plus MIT content (A) gives an estimation of the proteins MIT content.

Preliminary results indicate the procedure is feasible for the determination of MIT content in proteins.

In comparing the amino acid analyses of native α-lactalbumin to the analyses of iodinated α-lactalbumin, the only amino acid other than tyrosine and tryptophan which appeared to be affected was histidine. The effect of iodination of α-lactalbumin upon loss of histidine is related to the loss of lactose synthetase activity as shown in Figure 27.

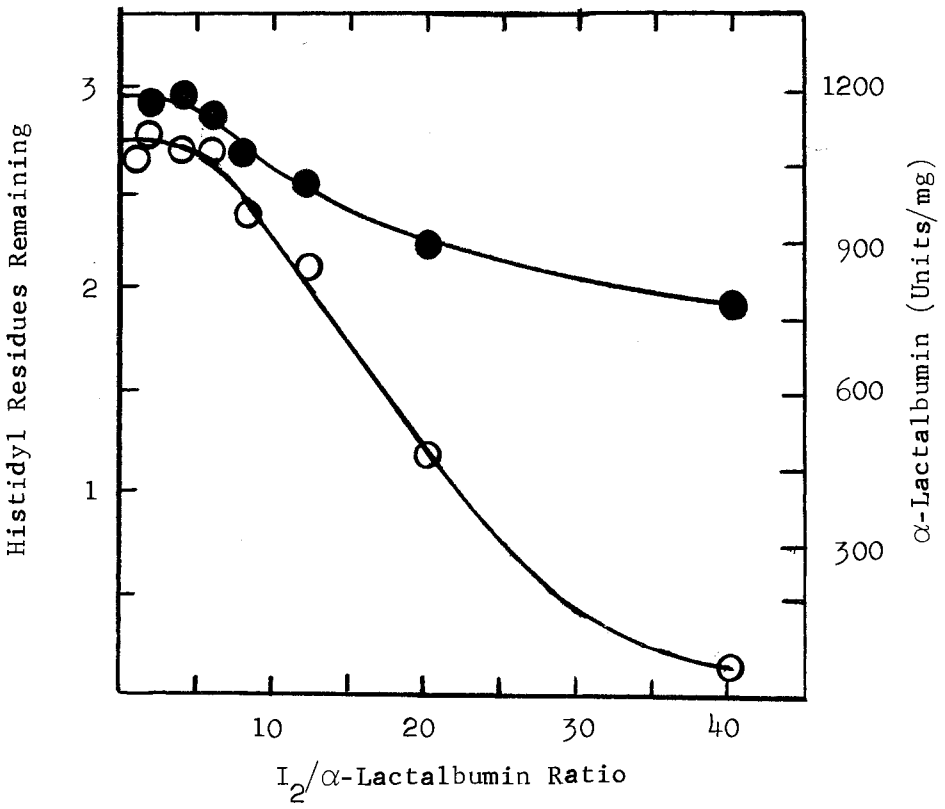


Figure 27. Effect of Iodination of α-Lactalbumin Upon Loss of Histidines and Lactose Synthetase Activity.

The loss of histidyl residues was determined by amino acid analysis after acid hydrolysis as described in the Methods Section. ●—●, histidyl residues remaining unmodified. ○—○, α-lactalbumin lactose synthetase activity (units/mg).

The loss of lactose synthetase activity upon iodination is about 3 times faster than the loss of histidyl residues. In previous studies on the nitration of α-lactalbumin the histidyl residues were unmodified and the loss of lactose synthetase activity closely paralleled the loss of tyrosyl residues. The loss of tyrosyl residues on iodination also parallels the loss of lactose synthetase activity (Figure 26), and it appears that the modification of histidine has no additive effect upon the loss of lactose synthetase activity. Castellino and Hill (118) reported that when all three histidyl residues of α-lactalbumin are carboxymethylated about 40 percent of the activity of α-lactalbumin remains. They did not check for polymer formation after carboxymethylation which could possibly account for the loss in activity. Polymer formation which often occurs upon modification of α-lactalbumin is discussed below.

Previous studies showed that the nitration of α-lactalbumin resulted in the formation of polymers. To determine if the iodination of α-lactalbumin resulted in polymer formation, the iodinated α-lactalbumin was chromatographed on a (0.6 x 95 cm.) Bio-Gel P-100 column (Figure 28). Figure 28-A shows the elution pattern obtained from α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 12/1 as described in Methods. The same elution pattern shown in Figure 28-A was obtained for native α-lactalbumin, and for α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 1, 2, 4, 8 and 12. At an I_2/α-lactalbumin ratio of 12/1, 1.2 of the 4.0 tyrosyl residues have been modified and no polymer formation is detectable. Iodination at an I_2/α-lactalbumin ratio of 20/1 resulted in the modification of 2.2 tyrosyl residues and polymer formation does occur (Figure 28-B). The ratio of monomer to

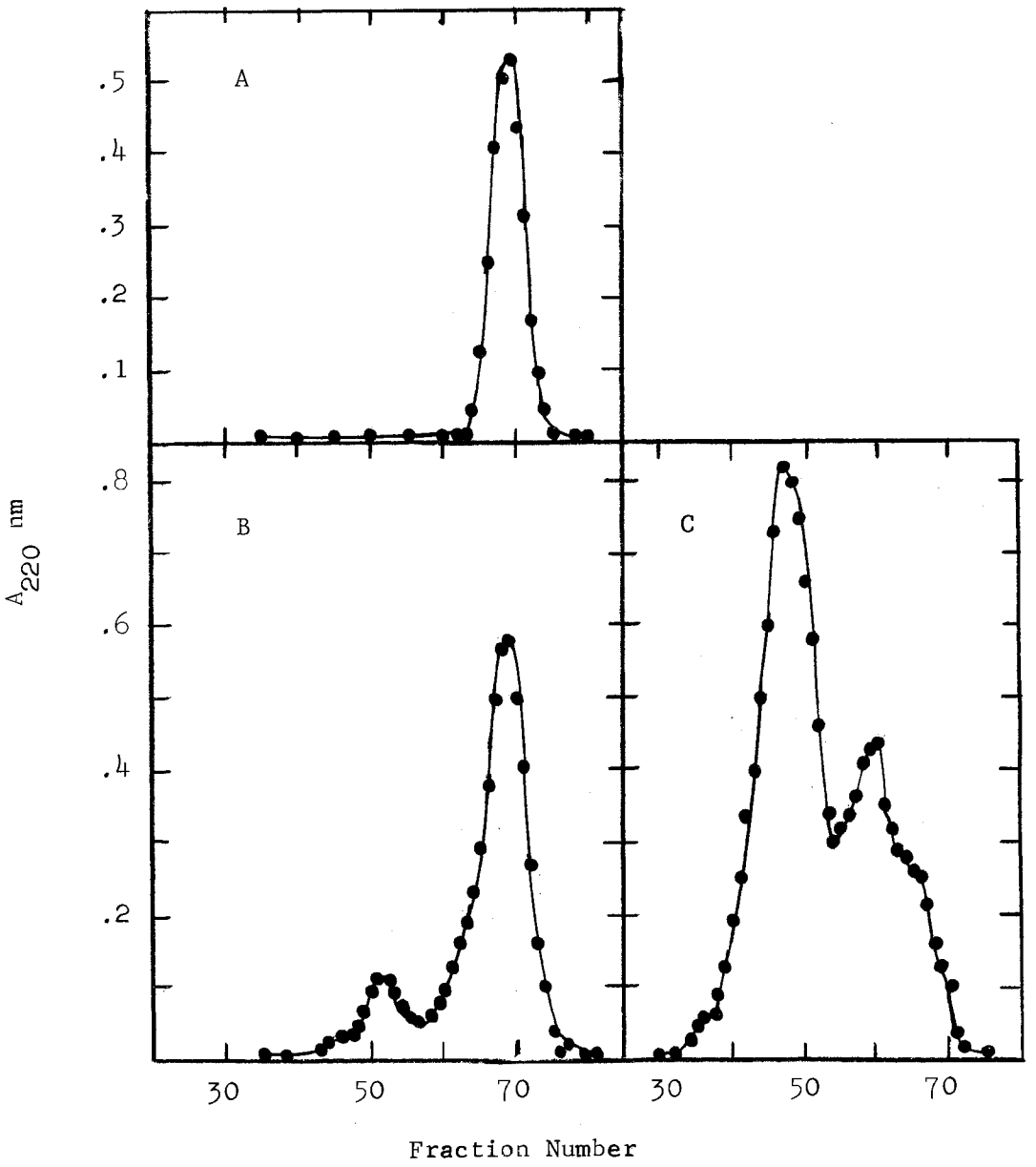


Figure 28. Bio-Gel P-100 Chromatography of Iodinated α-Lactalbumin

Bio-Gel P-100 column (0.6 x 95 cm) was equilibrated and eluted at 25° with 50 mM Tris, 0.1 M KCl, pH 7.5 and 0.2 ml fractions were collected per tube. The tubes were diluted to 0.8 ml with distilled water and the absorbance was read at 220 nm. (A) is α-lactalbumin iodinated at I_2/α-LA ratio of 12/1; (B), I_2/α-LA ratio is 20/1; (C) I_2/α-LA ratio is 40/1.

polymer in Figure 28-B is apparent [illegible] elution pattern, after the iodination of [illegible] bumin ratio of 40/1 (Figure 28-C), appears [illegible] Bio-Gel P-100 elution pattern previously obtained for [illegible] nitrated α-lactalbumin shown in Figure 18. Native α-lactalbumin elutes from the Bio-Gel P-100 column (Figure 28-C) at fraction number 68, and the α-lactalbumin dimer and trimer should theoretically elute at fraction 54 and 45 respectively. The eluent peak from the iodination at an I_2/α-lactalbumin ratio of 20/1 (Tube 52, Figure 28-B) corresponds to an α-lactalbumin dimer. The peak (Tube 69, Figure 28-B) is a symmetrical peak and corresponds to monomer α-lactalbumin. The eluent peaks from the iodination at an I_2/α-lactalbumin ratio of 40/1 (Figure 28-C) are shifted in comparison to the peaks in Figure 28-B. It is suggested, however, that the iodination of α-lactalbumin is similar to the nitration of α-lactalbumin in that upon nitration and iodination, α-lactalbumin undergoes a change in shape which increases the apparent molecular weight as determined by gel filtration. In the diffusion experiments on α-lactalbumin, fractions containing lactose synthetase activity eluted in the same position as native α-lactalbumin, which would be around fraction 68 from the P-100 column (Figure 28-C). The lactose synthetase activity of α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 40/1 is related to the Bio-Gel P-100 column shown in Figure 28. As previously suggested, α-lactalbumin containing lactose synthetase activity (Figure [illegible]) eluted in the same position as native α-lactalbumin, and the iodinated α-lactalbumin, which has undergone an apparent change in shape, has no lactose synthetase activity. The polymer formed (Tube 36) upon iodination of α-lactalbumin (Figure 29) also contained no lactose synthetase activity.

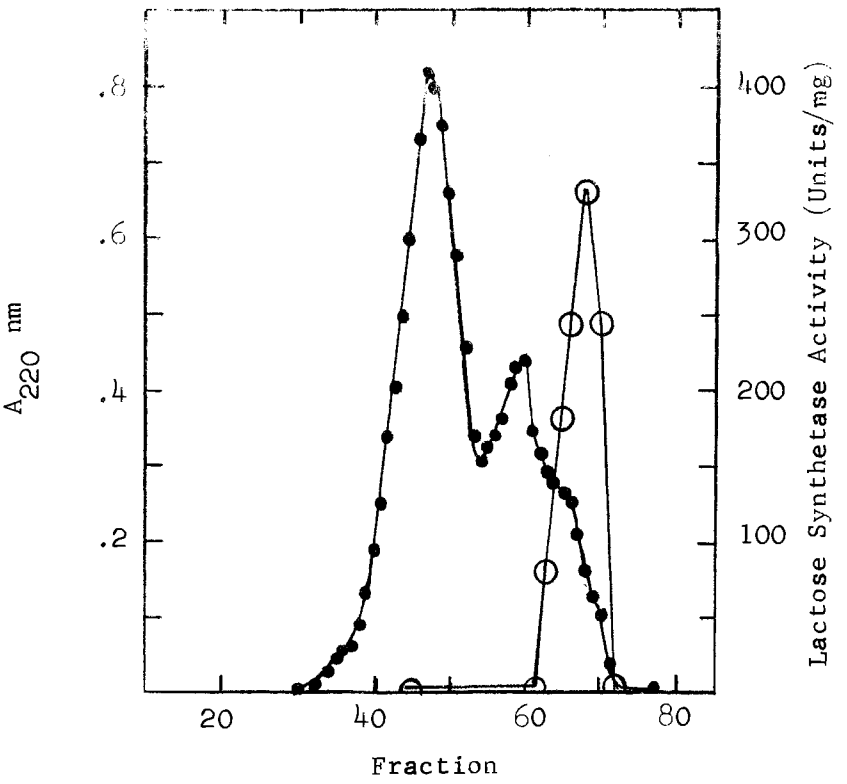


Figure 29. Lactose Synthetase Activity of Iodinated α-Lactalbumin Eluted From A Bio-Gel P-100 Column.

A 0.3 ml sample of α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 40/1 was lyophilized, dissolved in 0.1 ml of eluent buffer, and eluted at 25° from a Bio-Gel (0.6 x 95 cm) P-100 column in 0.2 ml fractions. The column was equilibrated and eluted with 50 mM Tris, 0.1 M KCl, pH 7.5 and the absorbance was determined at 220 nm. ●—●, Absorbance at 220 nm; O—O, Lactose synthetase, specific activity (units/mg protein).

Separation of the various forms of native and iodinated α-lactalbumin was performed on standard 7 percent disc gel electrophoresis as described in Methods. The disc gel electropheretic patterns as a function of the I_2/α-lactalbumin ratio used for iodination are shown in Figure 30. Tube 1 (Figure 30) contains native α-lactalbumin and appears as a single band. Tubes 2-6 also appear as single bands and Tube 7, iodinated at an I_2/α-lactalbumin ratio of 12/1, is the first tube to show a second component. The sample placed on Tube 7 has 1.2 tyrosyl residues modified and was the first I_2/α-lactalbumin ratio which showed significant tyrosyl modification as determined by amino acid analysis. The ratio of I_2/α-lactalbumin is increased to 20/1 in Tube 8 and two minor bands appear below the major band corresponding to native α-lactalbumin. The sample (Tube 8) had 2.2 tyrosyl residues modified as determined by amino acid analysis. Tube 9 (Figure 30) also contains 3 bands, however, the lower band is the band of major intensity and is diffuse. It is suggested from previous studies on gel filtration that this may be due to the high percentage of polymeric α-lactalbumin produced at this level of iodination. The upper band (Tube 8) which corresponds in migration distance to native α-lactalbumin is quite faint and amino acid analysis shows 3.6 tyrosyl residues modified by iodination at the I_2/α-lactalbumin ratio of 40/1 shown in Tube 8.

In order to further investigate the nature of the bands formed upon iodination of α-lactalbumin with an I_2/α-lactalbumin ratio of 40/1, the method of Hedrick and Smith (131) was used to distinguish between charge and size isomers. When the logarithm of protein mobility relative to dye front is plotted versus acrylamide gel concentration, size isomeric proteins give a family of nonparallel lines extrapolating to a

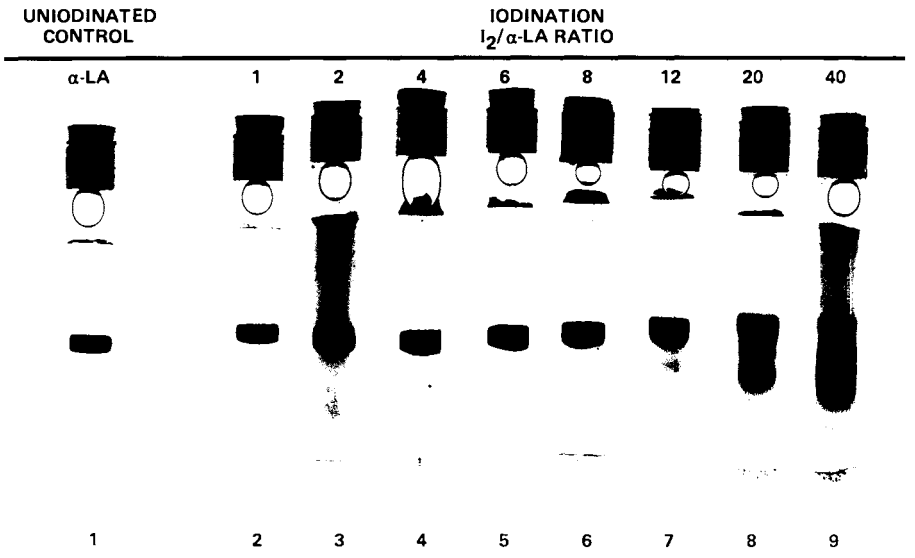


Figure 30. Disc Gel Electrophoresis of α-Lactalbumin Iodinated at Varying I_2/α-Lactalbumin Ratios.

Disc gel electrophoresis studies were performed on standard 7 percent gels (see Methods), Tube 1, native α-lactalbumin; Tubes 2 through 9, α-lactalbumin iodinated at I_2/α-lactalbumin ratios from 1 through 40.

common point in the vicinity of zero percent gel concentration; charge isomeric proteins give a parallel family of lines. Proteins differing in both charge and size show nonparallel lines intersecting at gel concentrations other than zero percent gel concentration. Samples of α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 40/1 as shown (Tube 9, Figure 30) were run in gels, varying from 4 to 10 percent acrylamide (Figure 31). The ratio of protein migration to dye migration (Rm) is plotted as a function of the percent gel concentration in Figure 32. The parallel lines labeled (A) and (B) in Figure 32 suggest that there is a charge isomer formed upon iodination of α-lactalbumin. The diffuse lower band shown in Figure 31 corresponds to line C in Figure 32 and as previously suggested this diffuse band is a size isomer and contains in addition to dimer what appears to be larger polymeric units whose migration is retarded by the higher percent gels in Figure 32.

Summary of Iodination of α-Lactalbumin

The iodination of α-lactalbumin at an I_2/α-lactalbumin ratio of 40/1 results in the modification of 1.1 of the tryptophanyl residues, 1 histidyl residue, and 3.6 of the tyrosyl residues. The loss of lactose synthetase activity is more than 5 times faster than the loss of tryptophanyl residues and more than twice as fast as the loss of histidine. The loss of tyrosyl residues closely parallels the loss of lactose synthetase activity and it is suggested that the tyrosyl residues maintain a functional role in α-lactalbumin's lactose synthetase activity. The iodination of α-lactalbumin results in the formation of inactive polymers. It is suggested that after the iodination of one tyrosyl residue, α-lactalbumin undergoes a change in shape which

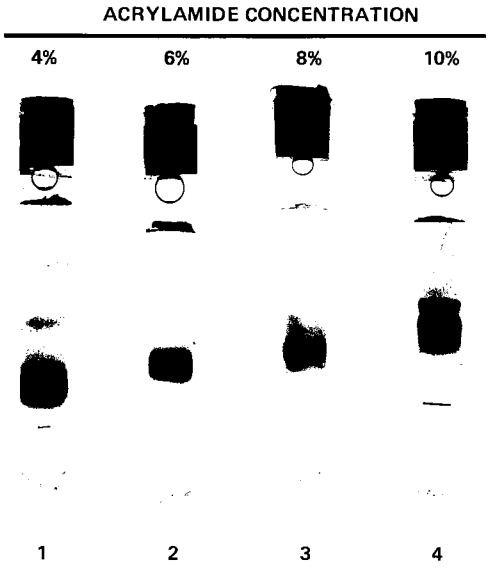


Figure 31. Disc Gel Electrophoresis of Iodinated α-Lactalbumin on Gels Varying from 4 to 10 Percent Acrylamide.

Varying percent gels were run to distinguish between charge and size isomers in the iodinated α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 40/1. Tube 1, 4 percent gel; Tube 2, 6 percent; Tube 3, 8 percent; Tube 4, 10 percent.

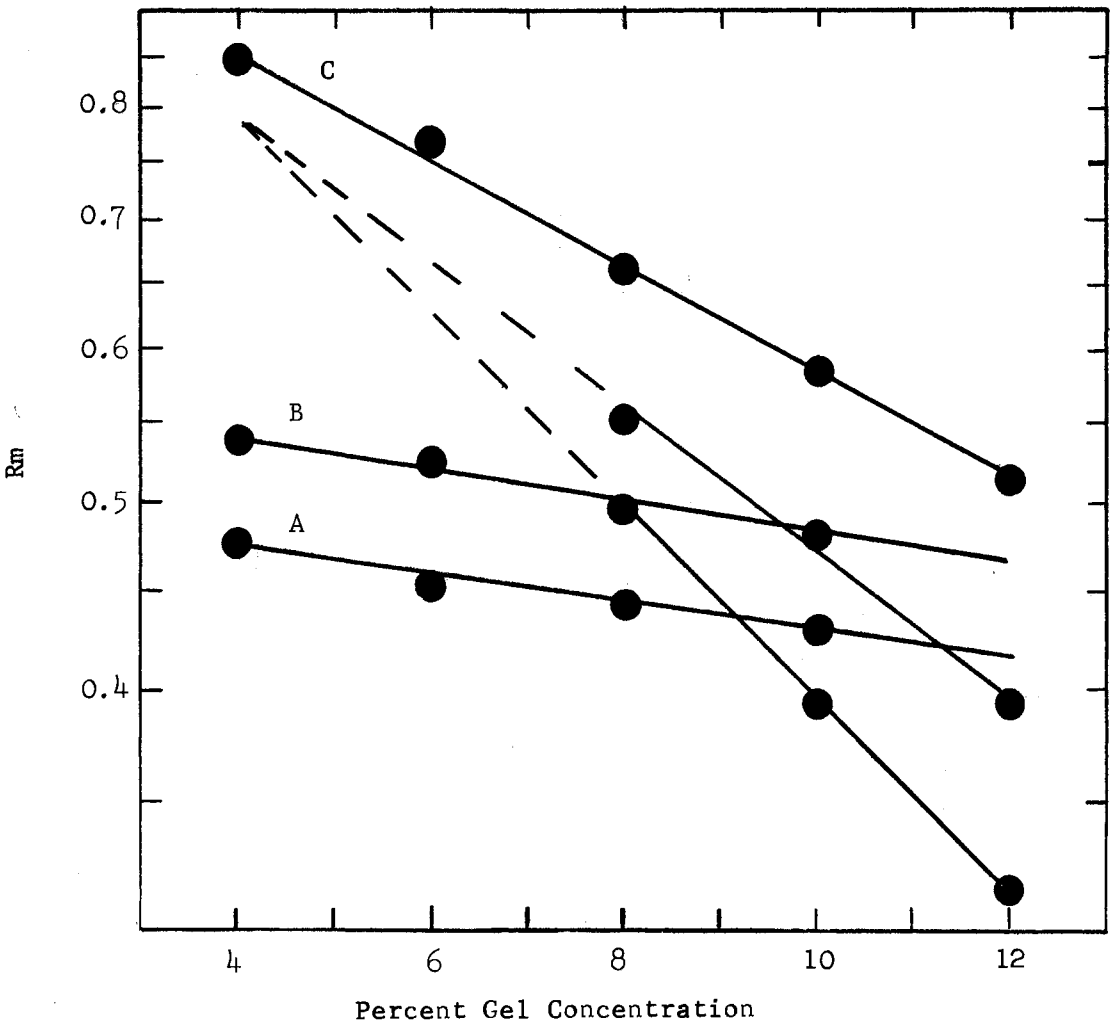


Figure 32. Effects of Varying Percent Gel Concentrations Upon Iodinated α-Lactalbumin.

The effect of 4 to 12 percent acrylamide gel concentration on disc gel electrophoresis of α-lactalbumin iodinated at an I_2/α-lactalbumin ratio of 40/1. Rm is the ratio of protein migration to dye migration. A is native α-lactalbumin; B corresponds to the upper band (Figure 31); C corresponds to the heavy lower band (Figure 31).

increases the apparent molecular weight as determined by gel filtration. Essentially all the tyrosyl residues are lost upon iodination at an I_2/ α-lactalbumin ratio of 40/1. This indicates that either all 4 tyrosyl residues are exposed to the solvent as predicted by the hypothetical molecular model of α-lactalbumin based on the known structure of lysozyme, or that all 4 tyrosyl residues become exposed after α-lactalbumin undergoes a change in shape during iodination. The iodinated α-lactalbumin is separated into 3 bands on disc gel electrophoresis and the bands appear to represent (1) native α-lactalbumin, (2) an α-lactalbumin charge isomer and (3) size isomers of α-lactalbumin.

CHAPTER IV

DISCUSSION

As previously described, hen's egg-white lysozyme and bovine α-lactalbumin have different known biological functions. However, the fact that the four disulfide bonds are formed by the corresponding homologous half-cystinyl residues in the two proteins along with the high degree of sequence homology suggests that bovine α-lactalbumin might possess a conformation quite similar to that of hen's egg-white lysozyme. Browne et al. (6) built a speculative molecular model of α-lactalbumin based on the coordinates of lysozyme, and it had essentially all of the features of both the secondary and tertiary structures of lysozyme. The x-ray crystallographic analysis will, when completed, reveal how well the predicted conformation of α-lactalbumin fits its true conformation. Until this analysis is completed, the predicted conformation of α-lactalbumin can best be tested by studies of the physical and chemical properties of the protein in solution (6). The two proteins, α-lactalbumin and lysozyme, can be compared by studying the overall conformation of the proteins with measurements such as optical rotary dispersion or small angle x-ray diffraction. However, since lysozyme has six tryptophanyl residues as compared to four for α-lactalbumin and other potentially reactive groups are not, in all cases, in identical positions in the two proteins, it may not be always meaningful to compare the two proteins with respect to their

reactivity toward modifying reagents. Therefore, the observed reactivity of modifying reagents toward α-lactalbumin is perhaps better compared to the predicted reactivity of the reagent as determined from the hypothetical molecular model of α-lactalbumin.

The four tyrosyl residues of α-lactalbumin are located at residues 20, 38, 53, and 107. Inspection of the model of Browne et al. (6) shows that all four of these tyrosines are located near the surface of the molecule. The position of the phenolic group of tyrosine number 38 could possibly make this residue less accessable than the other three tyrosines. From the predicted conformation one would expect tyrosyl modifying reagents to react readily with at least three and probably all four tyrosyl residues. Robbins et al. (116) have shown that all four tyrosyl groups ionize normally on H^+ titration. Gorbunoff (46) has shown that all four tyrosyl residues are readily available to reaction with acetylimidazole and three to four tyrosyls are available for reaction with cyanuric fluoride. Atassi et al. (117) have reported that 2.51 ± 0.03 of the tyrosines of α-lactalbumin are modified upon nitration with tetranitromethane. In contrast, Robbins et al. (93) have shown that all four tyrosines could be modified, but two tyrosyl residues appeared more reactive than the others. Care must be taken in trying to interpret these findings, since it is shown in this study that polymerization occurs during both the nitration and iodination of α-lactalbumin and it is possible that polymerization could affect the accessibility of certain amino acid residues. In addition to the preceeding modification reactions, present studies on the nitration and the iodination of α-lactalbumin suggest that all four tyrosyl residues are quite reactive. This is in agreement with the model of

Browne et al. (6) showing that all four tyrosines are located near the surface of the molecule.

The reagent, tetranitromethane, is not selective for the modification of the tyrosyl residues of α-lactalbumin. In addition to tyrosine, the tryptophanyl residues are modified during the nitration reaction, but at a slower rate than the tyrosines. After 240 minutes of nitration, essentially all 4 tyrosyl residues and 2.7 of the tryptophanyl residues are lost as determined by amino acid analysis. The initial rate of tyrosine loss (Figure 16) was equal to the rate of formation of nitrotyrosine (Figure 17). The psuedo first order constant for both processes was 0.3 up to one residue modified. The rate of tyrosine loss increased (k_2 = 1.74) dramatically as the reaction proceded. No dimer is visable after 60 minutes of nitration and about 10% is formed at 110 minutes, 30% at 180 minutes, and about 40% at 240 minutes (Figure 11). There appears to be no direct relationship between dimer formation and loss of activity. However, this is difficult to ascertain, since shape changes occur in the monomer region at higher nitration levels. Monomer with a shape change is also not active (Figure 18). It appears that polymer and shape changes occur, after one residue is modified by nitration or iodination. On nitration 4 moles of tyrosine are lost per mole of α-lactalbumin, but only 2 moles of nitrotyrosine are formed per mole of α-lactalbumin. The two remaining tyrosyl residues are unaccountable as determined by amino acid analysis. An unidentified peak at 58 minutes is correlated with the extent of nitration. Similar results on the nitration of insulin (94) showed that all 4 of the tyrosyl residues were modified but only 2.5 residues were nitrated. Boesel and Carpenter (94) suggested that the remaining 1.5 residues

were apparently involved in a crosslinked compound but presented no evidence. The nitration of glycyl-L-tyrosine resulted in polymers which were separated from the monomer on a Sephadex G-10 column (94). The hydrolysate of the monomer peak contained equal amounts of glycine and 3-nitrotyrosine, indicating it was glycyl-3-nitro-L-tyrosine. The amino acid analysis of the hydrolysate of the polymer peak eluting at the void volume indicated the presence of glycine and ammonia but no tyrosine or 3-nitrotyrosine. It is not clear why the tyrosyl residues which are apparently involved in a crosslinked compound are not detected after acid hydrolysis.

If lysozyme and α-lactalbumin do have the same conformation and if there is functional similarity in the two proteins, then the chemical modification of an amino acid residue in α-lactalbumin which corresponds to a critical residue in lysozyme should completely destroy the activity of α-lactalbumin. Inspection of the model (38) shows that tyrosines 20 and 53 are not located in the area of the active site of lysozyme. Tyrosines 38 and 107 in bovine α-lactalbumin are replaced by phenylalanine and alanine in chicken lysozyme. Residues 38 and 107 in chicken lysozyme appear to be involved in hydrogen bonding with the substrate and modification of the substituted tyrosyl residues in these positions could result in loss of lactose synthetase activity. Present studies show that a rapid loss of lactose synthetase activity closely parallels tyrosyl loss during both the nitration and iodination of α-lactalbumin.

The iodination of α-lactalbumin is not selective for tyrosyl modification as determined by amino acid analysis. Iodination of α-lactalbumin at an I_2/α-lactalbumin molar ratio of 40/1 results in the modification of 1.1 of the tryptophanyl residues, 1 histidyl residue, and

3.6 of the tyrosyl residues. As was previously shown on nitration, the iodination of α-lactalbumin also results in the formation of inactive polymers. Upon iodination, the loss of lactose synthetase activity is more than 5 times faster than the loss of tryptophanyl residues and more than twice as fast as the loss of histidine. The loss of tyrosyl residues closely parallels the loss of lactose synthetase activity, and it is suggested that the tyrosyl residues maintain a functional role in α-lactalbumin's lactose synthetase activity. In both nitration and iodination it is suggested that after the modification of one tyrosyl residue, α-lactalbumin undergoes a change in shape which increases the apparent molecular weight as determined by gel filtration. Acetylation of the tyrosyl residues of α-lactalbumin reportedly resulted in a change in environment of the tryptophan side chains (93). This is possibly due to a change in shape similar to that found upon nitration and iodination of α-lactalbumin.

Sizer (138) demonstrated by chemical and spectroscopic studies the ability of tyrosinase to oxidize the tyrosyl groups of crystalline pepsin, trypsin, chymotrypsin, and insulin. Yasunobu and Wilcox (23) showed that lysozyme is not attacked by tyrosinase. However, tyrosinase is reported to oxidize the tyrosyl residues of α-lactalbumin (137). The present study indicates that the incubation of tyrosinase with α-lactalbumin results in oxygen uptake corresponding to the oxidation of 1.5 amino acid residues. Amino acid analysis of α-lactalbumin previously incubated with tyrosinase for 300 minutes revealed the loss of 1.1 to 1.5 tryptophanyl residues, and only 0.2 tyrosyl residues. Other amino acid residues did not appear to be effected by treatment with tyrosinase. These results suggest that tyrosinase does not oxidize the

tyrosyl residues of α-lactalbumin but instead oxidizes 1.1 to 1.5 tryptophanyl residues. These results do not agree with those of Sizer (138). The α-lactalbumin with 1.1 to 1.5 tryptophanyl residues modified retains 95 percent of its activity in the lactose synthetase reaction. After 29 hours of reaction at 37° no enzymatic activity is detected.

The findings on modification of the tyrosyl residues of α-lactalbumin, although not conclusive, are in agreement with expected results predicted from the hypothetical molecular model of α-lactalbumin.

Solvent perturbation studies indicate that two of the four tryptophanyl residues of α-lactalbumin are buried and two are exposed at 25° (120). However, when the temperature is reduced to 1°, the two exposed tryptophan residues become inaccessible to all but the smallest solvent molecules. This suggests that these two residues lie in surface crevices which change shape as the result of a small conformational change in the molecule (6). The model can be interpreted with two buried and two exposed groups and would predict that the two exposed groups become less accessible as the result of a relatively small conformational change. Barman and Koshland (121) modified the tryptophanyl residues of α-lactalbumin with hydroxynitrobenzyl bromide, and this modification has recently been reported to produce an inactive protein (119). Our finding, although not conclusive, do not implicate the tryptophanyl residues of α-lactalbumin as critical for lactose synthetase activity. Since inactive polymers form under a variety of conditions, it is possible that other reagents may cause polymer formation with a resulting loss of activity.

Histidine 32 in α-lactalbumin is equivalent to glutamic 35 of the homologous lysozyme sequence. Since glutamic 35 is critical in the

enzymic activity of lysozyme, it is expected that if lysozyme and α-lactalbumin do have the same conformation and if there is functional similarity in the two proteins, then chemical modification of the histidyl residues should completely destroy the activity of α-lactalbumin. The reaction of bovine α-lactalbumin with iodoacetate (118) resulted in the carboxymethylation of the side chains of methionine 90, histidine 32, histidine 68, and histidine 107. The relative rates of reaction of these residues were generally in accord with those expected on consideration of the predicted conformation of α-lactalbumin. However, about 40 percent of the activity of α-lactalbumin remained when all three histidines were carboxymethylated. As described above, this is not the expected result based on the model of α-lactalbumin. It seems more reasonable that if the histidines are directly involved in the lactose synthetase reaction, the modification of all three residues should result in the complete loss of activity rather than only a partial loss. It is possible that the observed loss of activity on modification of the histidines could be due to the formation of an inactive polymer. No experiments to check for the presence of polymer were reported.

The findings presented above, in addition to our present findings on nitration and iodination of α-lactalbumin, appear in general to be consistant with the predicted conformation of α-lactalbumin. However, several other recent findings seem to indicate that there may be appreciable conformational differences between α-lactalbumin and lysozyme. α-Lactalbumin which undergoes a conformational change near pH 4 is not as stable as lysozyme toward acid denaturation (122, 123). Lin (119) found that the carboxyl side chains in α-lactalbumin were

more accessible to modification with carbodiimide than those in lysozyme. A lack of immunochemical cross-reaction was demonstrated by showing that none of the antisera to five different lysozymes reacted with α-lactalbumin, while full reactivity with lysozyme was maintained for each of these antisera after premixing with α-lactalbumin over a wide range of concentrations (117). The converse of this was also demonstrated with antisera to α-lactalbumin. Even though both proteins appear to possess tight folding, all four disulfides of α-lactalbumin are reducible in three hours at a concentration of about 2.5 M guanidine. These same conditions, however, result in the reduction of only 1.4 bonds in lysozyme (117). This strongly suggests that there possibly are appreciable conformational differences between lysozyme and α-lactalbumin.

Evidence for a similar conformation of lysozyme and α-lactalbumin in solution was provided by Aune (124) and Kronman (120). Aune (124) showed that the two proteins have indistinguishable optical rotary dispersion curves between 206 and 233 nm and between 320 and 500 nm. This is, however, essentially a measure of short range interactions, and it measures an overall average conformational parameter and does not give any insight concerning specific conformational details of various regions in the molecule. In addition, the ORD behavior of lysozyme is not highly sensitive to conformational changes (117). More direct information concerning the conformation of molecules in solution is provided by hydrodynamic or diffraction measurements, but of these two procedures, only diffraction permits independent evaluation of size and shape parameters (113). Krigbaum and Kugler (113) performed small angle diffraction measurements for hen's egg-white lysozyme and bovine

α-lactalbumin. Lysozyme exhibits a radius of gyration, R, of 14.3 Å, and its equivalent scattering body is a prolate ellipsoid having dimensions 28 x 28 x 50 Å, while α-lactalbumin has an R value of 16.7 Å, and its equivalent ellipsoid is oblate with dimensions 22 x 44 x 57 Å. They concluded from these measurements that the two proteins have quite different molecular conformations in solution. Even though this suggests that α-lactalbumin and lysozyme have grossly different sizes and shapes in solution, it is remotely possible that their conformations will show more resemblance in the crystalline state, but this is rather unlikely. In addition to these findings one should keep in mind that the accomodation of a protein sequence by the conformation of another in model building does not prove identity, and the two molecules may even possess different conformations in regions that are very similar in sequence (6,36).

SUMMARY

The nitration of α-lactalbumin results in the formation of 2 moles of nitrotyrosine per mole of α-lactalbumin. The two remaining tyrosyl residues, however, are unaccountable and may be involved in the formation of polymers. However, it is not clear why they are not detected after acid hydrolysis. It is suggested that after the nitration of one tyrosyl residue, α-lactalbumin undergoes a change in shape which increases the apparent molecular weight as determined by gel filtration. Essentially all 4 tyrosyl residues are lost after 240 minutes of nitration. This indicates that either all 4 tyrosyl residues are exposed to the solvent as predicted by the hypothetical molecular model of α-lactalbumin based on the known structure of lysozyme, or that all 4 tyrosyl residues become exposed after α-lactalbumin undergoes a conformational change during nitration. In addition to tyrosine, the tryptophanyl residues are modified during the nitration reaction, but at a slower rate than the tyrosines. The loss of lactose synthetase activity closely parallels the loss of the tyrosyl residues and it is suggested that the tyrosines are critical for lactose synthetase activity. After the nitration of one tyrosyl residue there is a secondary reaction initiated which is likely polymerization of the α-lactalbumin. The 120 minute nitrated α-lactalbumin is separated into 5 bands on disc gel electrophoresis and the bands represent native α-lactalbumin plus mono-, di-, tri-, and tetra-nitrated α-lactalbumin.

The iodination of α-lactalbumin at an I_2/α-lactalbumin ratio of 40/1 results in the modification of 1.1 of the tryptophanyl residues, 1 histidyl residue, and 3.6 of the tyrosyl residues. The loss of lactose synthetase activity is more than 5 times faster than the loss of tryptophanyl residues and more than twice as fast as the loss of histidine. The loss of tyrosyl residues closely parallels the loss of lactose synthetase activity and it is suggested that the tyrosyl residues maintain a functional role in α-lactalbumin's lactose synthetase activity. The iodination of α-lactalbumin results in the formation of inactive polymers. It is suggested that after the iodination of one tyrosyl residue, α-lactalbumin undergoes a change in shape which increases the apparent molecular weight as determined by gel filtration. Essentially all of the tyrosyl residues are lost upon iodination at an I_2/α-lactalbumin ratio of 40/1. This indicates that either all 4 tyrosyl residues are exposed to the solvent as predicted by the hypothetical molecular model of α-lactalbumin based on the known structure of lysozyme, or that all 4 tyrosyl residues become exposed after α-lactalbumin undergoes a change in shape during iodination. The iodinated α-lactalbumin is separated into 3 bands on disc gel electrophoresis and the bands appear to represent (1) native α-lactalbumin, (2) an α-lactalbumin charge isomer and (3) size isomers of α-lactalbumin.

The treatment of α-lactalbumin by tyrosinase does not oxidize the tyrosyl residues of α-lactalbumin. The uptake of 28 μl of oxygen during the reaction of tyrosinase with α-lactalbumin is due to the oxidation of 1.1 to 1.5 tryptophanyl residues, and only 0.2 tyrosyl residues are oxidized. The α-lactalbumin with 1.1 to 1.5 tryptophanyl residues modified retains 95% of its activity in the lactose synthetase

reaction. After 29 hours of reaction at 37° no enzymatic activity is detected.

This study strongly implicates that the tyrosyl residues of α-lactalbumin are critical for activity in the lactose synthetase reaction.

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