THE EFFECT OF HIGH pH AND REDUCING REAGENTS ON α-LACTALBUMIN

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PREFACE

Prior to the research presented in this dissertation the author was involved in studies concerning cross-linking of proteins by means of the bifunctional reagent p,p'-difluro-m,m'-dinitrodiphenyl sulfone (FNPS) with bovine pancreatic ribonuclease, chicken egg white lysozyme and bovine α-lactalbumin. Internal cross-linking reagents are a means by which an enzyme can be stabilized and perhaps made less subject to changes by alterations of pH, temperature, and bacterial degradation. The reagent, FNPS, reacts with the phenolic hydroxyl group of tyrosines and the ε-amino group of lysine.

Bovine pancreatic ribonuclease was nitrated with tetranitromethane to produce nitrated tyrosines which were reduced to amino tyrosine with sodium dithionite. The pK of the amino group of amino tyrosine is 4.5 which is much lower than the pK (approximately 10) of the ε-amino group of lysine. Thus, the cross-linking reagent could then be used to react specifically with the amino tyrosines. Since the nitration and subsequent reduction to amino tyrosine did not produce a homogeneous product and the cross-linking reagent reacted with both the amino tyrosines and the unmodified tyrosines, this procedure was deemed of little value in producing a stabilized enzyme. Cross-linking of native ribonuclease produced an enzyme which in repeated experiments had variable activity and was composed of several species which were not separated on Bio-Gel P-100.

A precipitate formed when lysozyme was reacted with the cross-link-

ing reagent in a molar ratio of 1:1. The soluble fraction contained some modified enzyme that had no loss of activity.

α-Lactalbumin was reacted with the cross-linking reagent, FNPS. Three protein peaks were separated on Bio-Gel P-100 and ultracentrifuge experiments showed the presence of dimers and a heavy species which was not as heavy as a trimer. Activity was present in the NPS-α-lactalbumin but it was not determined if the activity resulted from unreacted α-lactalbumin or from a decrease in the overall activity of the cross-linked products.

During these studies, the author's advisor, Dr. P. E. Guire, left Oklahoma State University for another position. Since the author spent nearly three years on the previous work, it was agreed that the author would remain at this institution and that Dr. K. E. Ebner would supervise a new but abbreviated research project. The results of the work done on this research project are presented in this dissertation.

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CHAPTER I

INTRODUCTION

α-Lactalbumin is a soluble protein found in the milk of most species. In conjunction with a galactosyltransferase, α-lactalbumin is required for lactose biosynthesis to occur at meaningful rates. In essence, α-lactalbumin acts as a protein modifier of the galactosyltransferase and lowers the apparent Km of glucose so that it becomes an efficient substrate. Current research efforts are being focused on which amino acids in α-lactalbumin and which structural alterations change its modifier activity.

Previous work by Merriman (1) showed that there was inactivation of α-lactalbumin at high pH and that during exposure to pH between 10 to 12, sulfhydryls were formed. The extent of alkaline hydrolysis of the disulfide bonds in α-lactalbumin and its relationship to activity is examined in greater detail in this study. The possibility of a disulfide bond which is particularly susceptible to alkaline hydrolysis was considered likely. If one of the disulfide bonds in α-lactalbumin were more susceptible to alkaline hydrolysis than the other three disulfides it should also be more susceptible to reduction. Accordingly, the effects of reducing reagents on the rate of reduction of the disulfides of α-lactalbumin and its effect on the activity of α-lactalbumin were examined. The reducing agents chosen were 2-mercaptoethanol and dithiothreitol. One disulfide was reduced faster than the others and this was identified as

6-120. Reduction of this disulfide led to a loss of activity of α-lactalbumin but upon reformation there was complete regain of activity.

CHAPTER II

LITERATURE REVIEW

α-Lactalbumin Background

α-Lactalbumin is one of the major non-casein protein components in the milk of all mammalian species that are able to synthesize the milk carbohydrate lactose. Bovine milk contains from 70-150 mg per liter α-lactalbumin which is easily crystallized from milk. This was first accomplished in 1953 by Gordon and Semmett (2) who suggested the name for the protein. A review by Gordon (3) lists many of the properties of α-lactalbumin. The function of the protein was discovered by Brodbeck and Ebner in 1966 when they showed that α-lactalbumin was one of the two proteins involved in the biosynthesis of lactose (4-6).

The final step in the biosynthesis of lactose proceeds according to the following reaction:

$$\text{UDP-galactose} + \text{glucose} \rightarrow \text{lactose} + \text{UDP}$$

The enzyme catalyzing this reaction has been called lactose synthetase (E.C. 2.4.1.22) although the reaction requires two proteins, a galactosytransferase and α-lactalbumin. Actually, α-lactalbumin modifies kinetically the action of galactosytransferase so that lactose is formed at meaningful rates. The apparent Km of glucose for the galactosytransferase without α-lactalbumin is high (Km = 1.4 M) whereas, in the presence of α-lactalbumin, the apparent Km of glucose is lowered to the

millimolar range (7).

The reaction mechanism and steady state kinetics of lactose synthetase has been studied by Morrison and Ebner (8-10). The order of addition of reactants is as follows: Mn^{++}, UDPgalactose, the carbohydrate acceptor and α-lactalbumin. Mn^{++} reacts with the free enzyme under conditions of thermodynamic equilibrium and does not dissociate after each turn of the catalytic cycle. At high concentrations carbohydrate can add randomly to all enzyme forms but the active complex forms only if Mn^{++} and UDPgalactose have added previously.

The galactosytransferase readily reacts with N-acetylglucosamine as the carbohydrate substrate in the absence of α-lactalbumin as shown in the linear pathway of Figure 1 (8). When increasing concentrations of α-lactalbumin are added, the reaction flux increases in the direction of the branched pathway until a lower limiting maximum velocity is reached at infinite α-lactalbumin concentration. With glucose as the carbohydrated substrate, in the absence of α-lactalbumin, the reaction will occur along the linear path only if glucose is in the range of 1 to 2 molar. In the presence of α-lactalbumin, the reaction could theoretically occur along either pathway, but because of the great reduction of the apparent Km by α-lactalbumin, the reaction proceeds via the branched pathway.

The primary structure of bovine α-lactalbumin and hen egg white lysozyme have a large amount of homology (11). The disulfide bonds in α-lactalbumin were determined to be cysteine 6-120, 61-77, 73-91 and 28-111 as shown in Figure 2. These positions correspond to the disulfide bonds in lysozyme (12). The sequences of bovine (11), Human (13) and Guinea Pig (14) and the first 41 residues in Kangaroo (15) α-lactalbumin

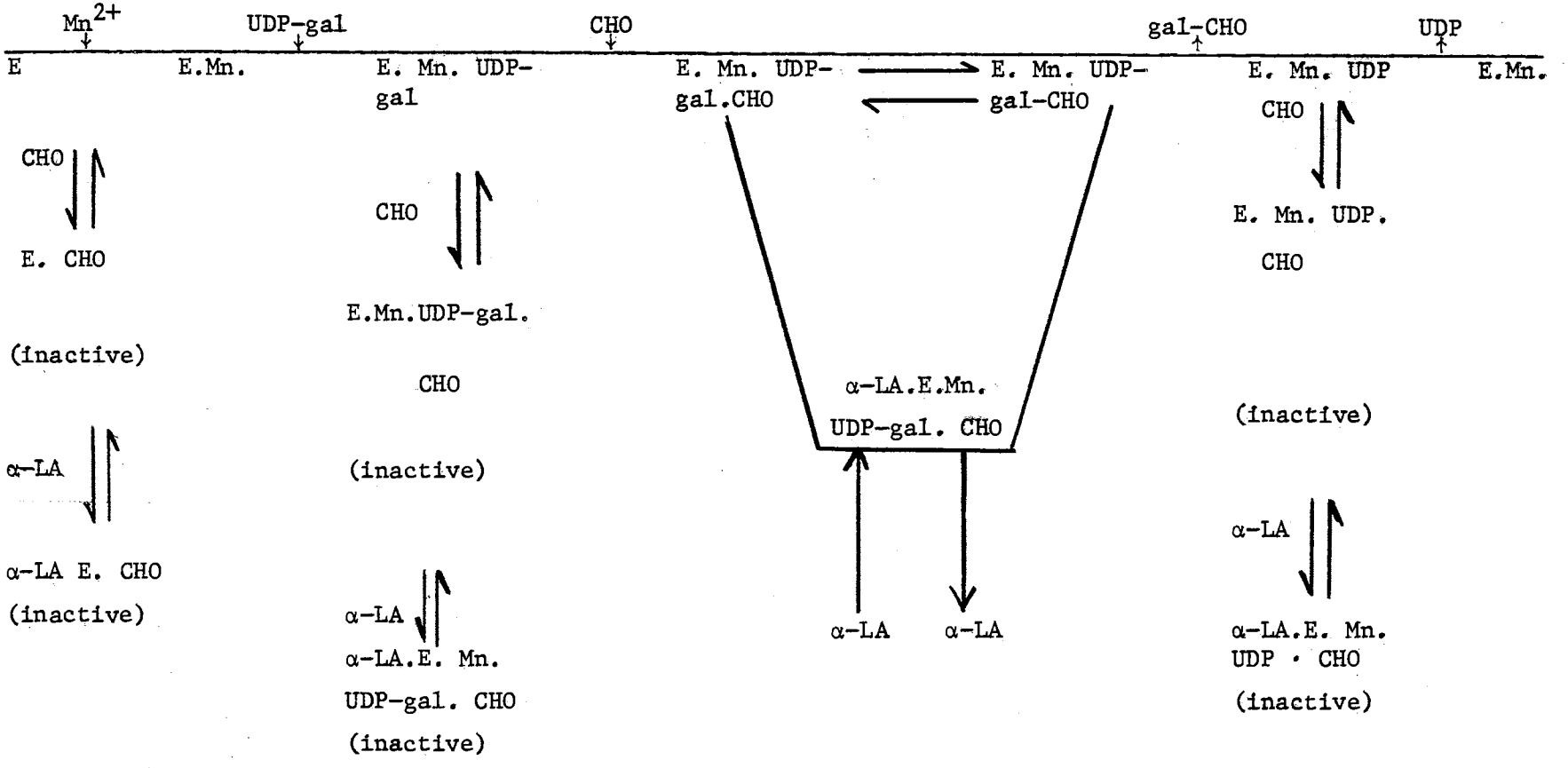


Figure 1. Schematic Representation of Order of Addition of Substrates and Release of Products of the Galactosyltransferase Reaction

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

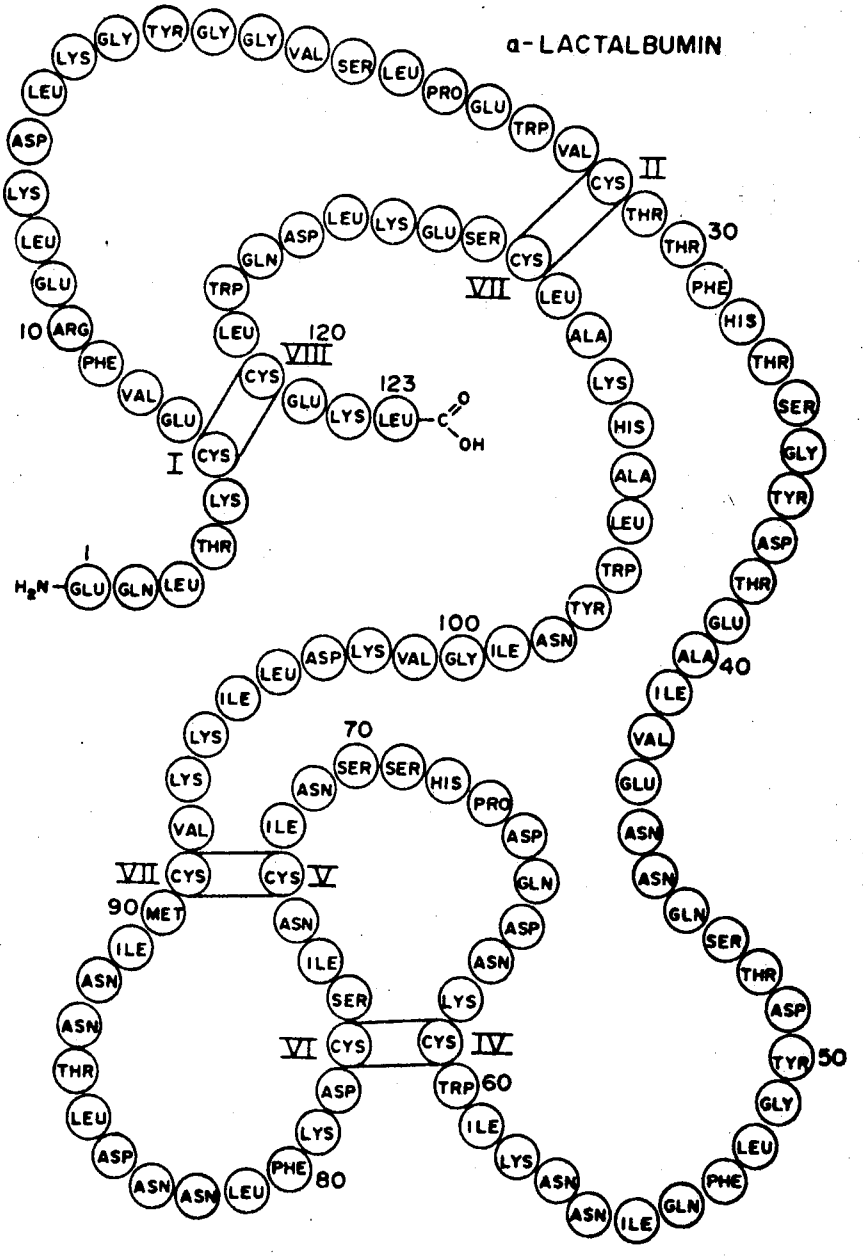


Figure 2. Covalent Structure of Bovine α-Lactalbumin

have been determined and are presented in Table I. Comparison of bovine α-lactalbumin with human α-lactalbumin shows that 72% of the residues and another 6% are chemically similar amino acids. The corresponding values for comparison of human α-lactalbumin to human lysozyme are 39% and 12% respectively.

This homology of guinea pig α-lactalbumin to human α-lactalbumin and human α-lactalbumin to human lysozyme support the idea that there is also structural homology. X-ray crystallography of α-lactalbumin has not clarified the structural question. Initial attempts on the X-ray crystallography of goat α-lactalbumin have been reported (16-17) but as yet it has been difficult to prepare adequate isomorphous crystals suitable for continuous study.

The four disulfide bonds in lysozyme and α-lactalbumin are in similar positions (18,12,19) as shown in Figure 2. However, the disulfide bonds of each of the proteins do not show the same degree of reductivity. The disulfides of α-lactalbumin are more easily reduced by mercaptoethanol in guanidine hydrochloride (20) and dithiothreitol in 4M urea (21) than those of lysozyme. Iyer and Klee (22) have shown that all four of the disulfide bonds of α-lactalbumin are readily reduced with dithiothreitol in aqueous buffers at room temperature in the absence of denaturing reagents but the disulfides of lysozyme reacts much more slowly. These data suggest that the conformation of α-lactalbumin and lysozyme differ in the region of the disulfides.

Reduction and Reoxidation of Disulfides

Disulfide bonds in proteins are reduced under mild conditions to sulfhydryl groups with reducing agents such as 2-mercaptoethanol,

TABLE I

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

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

	1	5	10	15

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

	20	25	30

TABLE I (Continued)

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

	35	40	45	50

	50	55	60	65
BαLA	Asp-Tyr-Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Ile-Trp-Cys-Lys-Asn-Asp-Gln-Asp-			
GPαLA	Glu-Tyr-Gly-Leu-Phe-Gln-Ile-Asn-Asn-Lys-Asp-Phe-Cys-Glu-Ser-Ser-Thr-Thr-			
HαLA	Glu-Tyr-Gly-Leu-Phe-Gln-Ile-Ser-Asn-Lys-Leu-Trp-Cys-Lys-Ser-Ser-Gln-Val-			
H Ly	Asp-Tyr-Gly-Ile-Phe-Gln-Ile-Asn-Ser-Arg-Tyr-Trp-Cys-Asn-Asp-Gly-Lys-Thr-			
C Ly	Asp-Tyr-Gly-Ile-Leu-Gln-Ile-Asn-Ser-Arg-Trp-Trp-Cys-Asn-Asp-Gly-Arg-Thr-			

	55	60	65

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

	70	75	80	85

TABLE I (Continued)

						90					95					
BαLA	Leu–	Thr–	Asn–	Asn–	Ile–	Met–	Cys–	Val–	Lys–	Lys–	Ile–	Leu–		–Asp–	Lys–	Val–
GPαLA	Leu–	Thr–	Asn–	Asn–	Ile–	Met–	Cys–	Val–	Lys–	Lys–	Ile–	Leu–		–Asp–	Ile–	Lys–
HαLA	Ile–	Thr–	Asn–	Asn–	Ile–	Met–	Cys–	Ala–	Lys–	Lys–	Ile–	Leu–		–Asp–	Ile–	Lys–
H Ly	Ile–	Ala–	Asp–	Ala–	Val–	Ala–	Cys–	Ala–	Lys–	Arg–	Val–	Arg–		–Asp–	Pro–	Gln–
CLy	Ile–	Thr–	Ala–	Ser–	Val–	Asn–	Cys–	Ala–	Lys–	Lys–	Ile–	Val–	Ser–	Asn–	Gly–	Asp–
			90					95					100			

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

							120			
BαLA	Gln–	Trp–	Leu–				–Cys–	Glu–	Lys–	Leu
GPαLA	Gln–	Trp–	Tyr–				–Cys–	Glu–	Ala–	Gln
HαLA	Gln–	Trp–	Leu–				–Cys–	Glu–	Lys–	Leu
H Ly	Arg–	Gln–	Tyr–	Val–	Gln–	Gly–	Cys–		–Gly–	Val
C Ly	Gln–	Ala–	Trp–	Ile–	Arg–	Gly–	Cys–		–Arg–	Leu
					125					

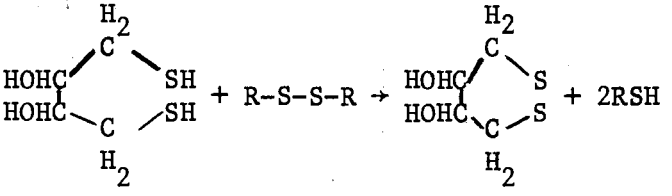
TABLE I (Continued)

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

cysteine, dithiothreitol, dithioerythritol, 5,5'-dithiobis-2-nitrobenzoic acid and sodium borohydride. For the reduction of the protein disulfides a large excess of the monothiol is required to force the reaction to completion according to the equation

$$-SX + R-S-S-R \rightarrow \underset{SX}{R-S-\overset{(-)}{S}-SR} \rightarrow RS-SX + -SR \qquad (1)$$

The dithiols such as dithiothreitol and dithioerythritol (Clelands' Reagent) (24) may be used at much lower concentrations because the stable cyclic disulfide formed drives the reaction to the right.



(2)

Since the reaction of the interchange is dependent upon the thiol anion, the rate of the reaction is strongly pH-dependent. The reduction, as well as the reoxidation, of the disulfides can be prevented by lowering the pH below 4. All of the disulfide bonds in a protein usually can be reduced in the presence of a reducing reagent accompanied by a denaturing reagent such as urea or guanidine hydrochloride. Some proteins can be completely reduced in the absence of denaturing reagents with dithioerythritol at pH 8.1 (i.e. lysozyme, prolactin, insulin, and human growth hormone) (25). In other proteins in the absence of denaturing reagents, only the more exposed or labile disulfide are reduced. In bovine serum albumin, 14 of the 18 disulfides are reduced, whereas in pancreatic ribonuclease, none of the disulfides are susceptible (26) and in bovine pancreatic trypsin inhibitor one of the three disulfides is reduced (27). Disulfide 179-203 of trypsin (5 mg/ml) can be selectively reduced by 0.5 mM dithioerythritol or 0.1 M sodium borohydride in 0.1 M Tris at pH 8.5

at 0^{o} (28). Iyer and Klee (22) have reported evidence that one of the disulfide bonds in α-lactalbumin is reduced by dithiothreitol at 0^{o} at pH 7.5.

Proteins in which the disulfides have been reduced completely or in part to sulfhydryls can be prevented from reoxidation by reacting the reduced protein with iodoacetate (29). Amino acid analysis for carboxymethyl cysteine gives a quantitative measurement of the extent of reduction. A partially reduced protein treated with iodoacetate can be assayed for activity to determine if the disulfides are necessary.

Reoxidation of reduced disulfides upon exposure to air in proteins to give an active form of the enzyme has been reported for bovine pancreatic ribonuclease (30). A review of other renatured proteins is given by White (31). In some instances, as in the reoxidation of chicken egg white lysozyme, mercaptoethanol is used to promote the reoxidation to the native form of the enzyme (32). Fully reduced α-lactalbumin has been reoxidized in the presence of Cu^{+2} ions with significant recovery of the physico-chemical properties of the native protein (33).

Alkaline Hydrolysis

Low molecular weight disulfides have been shown by Andersson (34) to cleave above pH 9.0 by the following reaction mechanism:

$$R\text{-}S\text{-}S\text{-}R + OH^{-} \rightleftarrows RS^{-} + RSOH \qquad I$$

$$2\ RSOH \rightarrow RSO_2H + RSH \qquad II$$

Above pH 12 β-elimination occurs according to the following mechanism:

$$OH^{-} + \rangle CH\text{-}CH_2\text{-}S\text{-}S\text{-}CH_2\text{-}CH\langle \rightarrow \rangle C{=}CH_2 + {}^{-}S\text{-}S\text{-}CH_2\text{-}CH\langle + H_2O \qquad III$$

The equilibrium of Equation I is far to the left and the concentration of RS^- is small even in 0.1 M NaOH. Mercuric chloride (35) and p-hydroxy-mercuribenzoate (34) have been used to drive the reaction to completion.

Alkaline hydrolysis of the disulfide bonds in proteins has been studied in insulin (36), ribonuclease (37), bovine serum albumin (38) and ovalbumin (38). Splitting occurs at pH 9-10 if the protein is denatured in urea solutions (38) or partially degraded as the result of proteolytic digestion (37).

Dimeric bovine serum albumin, prepared by oxidation of mercaptalbumin, was reported by Andersson (39) to cleave at pH 10. Spectrophotometric determination of free sulfhydryl in the monomer showed 0.47-0.58 -SH/mole. Treatment of the monomer with an excess of iodoacetate and subsequent amino acid analysis showed 0.4-0.5 carboxymethylcysteine/mole. No lysinoalanine or excess of alanine was detected as would be anticipated if the reaction occurred according to the mechanism in Equation III.

Donovan and White (40) studied the rate of alkaline hydrolysis of the disulfide bonds in ovomucoid. At pH 11.8, 30.4° they found a small amount of lysinoalanine after 15000 sec. This finding would indicate that some β-splitting occurred but the majority of the reaction was proceeding as shown in Equation I. The persulfite ion (RSS^-) was followed at 335 nm and no change in absorbance was noted whereas the absorbance at 240 nm, the absorbance maximum for the mercaptide ion (RS^-), paralleled the disulfide cleavage. Factors which determine the rate of alkaline cleavage of the disulfides are pH 9 (34); protein or disulfide concentration (37); presence of catalytic amounts of metal ions (41); ionic

strength (40) and steric strain (42). Molecular oxygen has been reported to have no effect on the rate of alkaline cleavage of disulfides (34, 38,39,40).

CHAPTER III

EXPERIMENTAL AND RESULTS

TPCK treated trypsin, pyruvate kinase (Type I from rabbit muscle, crystalline ammonium sulfate suspension containing lactic dehydrogenase), glycylglycine, phosphoenolpyruvate (PEP) K^+ salt, NADH, Tris (tris-hydroxy-methylaminomethane), bovine serum albumin, guanidine hydrochloride, lysozyme Grade I, and parachloromercuribenzoic acid were from Sigma Chemical Company. Iodoacetate, 5,5'-dithiobis-2-nitro-benzoic acid (DTNB) and dithiothreitol (DTT) were from Nutritional Biochemical Company. The iodoacetate was recrystallized from petroleum ether. α-Lactalbumin and galactosyltransferase were isolated by Dr. Kurt Ebner's laboratory.

Gel Filtration

Chromatographic columns were made by the glassblower, Mr. Wayne Atkins, from glass tubes of the desired length and diameter, fitted with fritted glass and Luer tips. Before packing the columns, they were silanized with a 1% solution of dichlorodimethyl silane in benzene. The gels were prepared as described in the Bio Rad Gel Filtration Manual (43). Before packing, the gels were equilibrated at the temperature they were to be used. The void volume and the salt peak were determined by applying 0.5 ml of Blue Dextran (10 mg/ml) and 0.1 M $(NH_4)_2SO_4$ in 5% sucrose. Fractions were read at 280 nm or 650 nm to determine the Blue

Dextran peak and the sulfate was detected by adding a drop of saturated $BaCl_2$ and observing the formation of the white precipatate of $BaSO_4$.

Peptic Digest

Peptic digestions of α-lactalbumin were done according to Vanaman et. al. (12) but on a smaller scale using 10-20 mg of α-lactalbumin. The ratio of α-lactalbumin to pepsin was kept at 40 to 1 (w/w). The progress of the reaction was followed by the ninhydrin assay (44). After 20 hours of incubation at 37° the mixture was lyophilized to dryness and dissolved in a buffer of choice.

Tryptic Digest

Tryptic digestions were done according to Brew et. al. (11) but on a smaller scale using 10-20 mg of S-carboxymethylated α-lactalbumin. The ratio of α-lactalbumin to trypsin was kept at 100 to 1 (w/w). During the incubation pH was maintained at 8.6 on the pH stat with 0.01 N NaOH.

α-Lactalbumin Assay

α-Lactalbumin was assayed in the presence of saturating amounts of galactosyltransferase (7). One unit of enzymic activity was the amount of enzyme which catalyzed the formation of one nanomole of UDP per minute, equivalent to an absorbance change of 0.0062/min at 340 nm in a 1 ml reaction volume. α-Lactalbumin activity was determined by the lactose synthetase assay spectrophotometrically at 340 nm by following the loss of NADH in a coupled assay. UDP, the product of the lactose synthetase reaction is coupled to NADH oxidation through pyruvate kinase

and lactic dehydrogenase. The rate of conversion of NADH to NAD was followed at 340 nm on a Cary 14 recording spectrophotometer or on a Gilford multiple sample absorbance recorder model 240 with a model 6040 recorder.

The final assay volume of 1.0 ml contained 5 mM $MgCl_2$, 100 mM KCl, 5 mM glycylglycine pH 8.5, 0.05 ml of 1 to 10 pyruvate kinase (Sigma Type I, 25 mg protein/ml with 2.4 I.U. pyruvate kinase/mg protein) in water, 0.25 mM UDP-galactose, 20 mM glucose, 0.15 units of galactosyltransferase from the HA_I step in purification (7). Standard curves from 0.25 to 2 μg of α-lactalbumin were determined for each set of assays and an endogenous rate for the galactosyltransferase was established for each assay. The components were added to the assay in the order listed with the following reagents made up in combination: $MgCl_2$ with KCl, UDP-galactose with glucose, and NADH with PEP.

Amino Acid Analysis

The amino acid analyses were performed on a Beckman Model 120C automated amino acid analyzer according to the method of Moore and Stein (45). α-Lactalbumin, 0.5-1 mg was prepared in 1 ml of 6 N HCl in hydrolysis tubes made from Kimax 16 x 2 cm test tubes, flushed twice with N_2 before the final evacuation to .1 mm Hg, sealed and placed in an oven at 110°C for 20 hours. After hydrolysis, the samples were placed on a Buckler shaker evaporator at 40° and taken to dryness. The samples were redissolved in 2 ml of water and evaporated to dryness again to assure complete removal of HCl and dissolved in pH 2.2 sample buffer for application to the amino acid analyzer. Each analysis required 0.3-0.5 mg of protein.

Sulfhydryl Determination

Free sulfhydryl groups were determined by the method of Ellman using 5,5'dithiobis(2-nitrobenzoic acid) (DTNB) (46). To 1 ml of α-lactalbumin containing an estimated sulfhydryl content in the range of 0.7 to 2 x 10^{-5} M in 0.1 M Tris pH 8 was added 0.010 ml of 0.01 M DTNB (3.96 mg/ml) in 0.1 M Tris pH 8. After 30 minutes, the absorbance was measured at 412 nm and the molarity of the sulfhydryl was calculated by using an extinction coefficient of 11,400 according to Robyt et. al. (47). The ratio of sulfhydryl to α-lactalbumin on a mole per mole basis was calculated using A_{280} of 1 mg/ml α-lactalbumin = 2.037 (48) with a molecular weight of 14,437 (11).

Disulfide Determination

DTNB Methods

A 2.0 ml sample of protein containing approximately 0.25 - 1.0 x 10^{-5} M disulfides was heated at 90°C for 10 min in 50 mM Tris, 50 mM phosphate at pH 11.4 and 10 mM EDTA. After the pH was adjusted to pH 8.1 with 1.0 M HCl, 0.5 ml of 2 mM DTNB pH 8.1 and 0.1 ml of 2 x 10^{-4} M 2-mercaptoethanol were added. After sitting for 30 minutes at room temperature, the sample was centrifuged and the absorbance of the supernatant solution was read at 412 nm (47).

Dithiothreitol Methods

Two ml of α-lactalbumin, 1.18 x 10^{-4} M (1.6 mg/ml) in 0.05 M phosphate, 0.001 M EDTA pH 8.0 were treated with 1.0 mM dithiothreitol (DTT) and the absorbance read at 310 nm on a Coleman 124 spectrophotometer with

a Coleman No. 0319 thermostatted cell holder, No. 801 scale expander and No. 165 recorder set on 0 - 0.1 absorbance units on full scale. The disulfide content was calculated using a molar extinction coefficient of 110 (19). Determinations were made at 25° and 5°, the temperature of the solution being monitored with a Yellow Springs Instrument Co. thermistor.

Larger quantities (10-20 mg) of α-lactalbumin were treated with DDT for 30 min at room temperature or 0° (ice bath), and desalted on a Bio-Gel P-6 (1.2 x 27 cm) and sulfhydryl content determined by the method of Ellman.

Alkaline Hydrolysis of Disulfide Bonds in α-Lactalbumin

The observation that incubation of α-lactalbumin in the pH range of 11-12 causes loss of α-lactalbumin activity concomitant with sulfhydryl formation (1) led to the investigation of the relationship between the rate and extent of hydrolysis of disulfide bonds and the loss of activity. The ultimate goal would be to determine which disulfide(s) (6-120, 28-111, 61-77, 73-91) are cleaved and if there is a differential rate of cleavage. From previous work with alkaline hydrolysis of disulfide bonds in small molecules and proteins, it would be anticipated that the formation of the mercaptide ion is concomitant with disulfide cleavage according to the Equation (40,34,39)

$$RS\text{-}S\text{-}R + OH^- \rightarrow RS^- + RSOH \qquad I$$

Accordingly, the effect of pH 12 on the disulfides in α-lactalbumin was followed by determining the sulfhydryl formation as a function of in-

cubation conditions. α-Lactalbumin (1 mg/ml) at pH 12 in 0.15 M KCl was allowed to react in a 15 ml screw capped vial at room temperature. Aliquots were removed at various time intervals and the sulfhydryl content was determined with Ellman's reagent. Initial experiments at pH 12 showed an increase in sulfhydryl content with time (Figure 3). However, subsequent experiments, presented in Figure 4, showed that the rate of sulfhydryl formation under the conditions used in Figure 3 was not constant between experiments. Due to the large difference in the shape of the curve of Experiment III (Figure 4) the pH of the solution was measured after 12 hours of incubation, the pH was 11 instead of 12. This drop in pH was probably due to adjusting the pH of the 0.15 M KCl to 12 prior to dissolving the α-lactalbumin. Because of this finding, care was taken in Experiments IV and V to adjust the pH of the final solution containing α-lactalbumin to 12. The rate of sulfhydryl formation was essentially the same in Experiments IV and V.

The effect of pH 12 incubation on the activity of α-lactalbumin and formation of sulfhydryl is shown in Figure 5. There was a loss of activity of α-lactalbumin which was concomitant with sulfhydryl formation. About 60% of the α-lactalbumin activity was lost when 0.4 mole of sulfhydryl/mole of α-lactalbumin was formed. The possibility of β-elimination of the disulfide bond to give the persulfide ion and dehydroalanine

$$R\text{-}S\text{-}S\text{-}R + OH^- \rightarrow R\text{-}S\text{-}S^- + \rangle C{=}C\text{-}R$$

is small since there was no observed increase in the absorbance at 335 nm where this species has an absorbance maximum, but there was an increase in absorbance at 240 nm, where the mercaptide ion absorbs (Figure 6), which paralleled the increase in sulfhydryl.

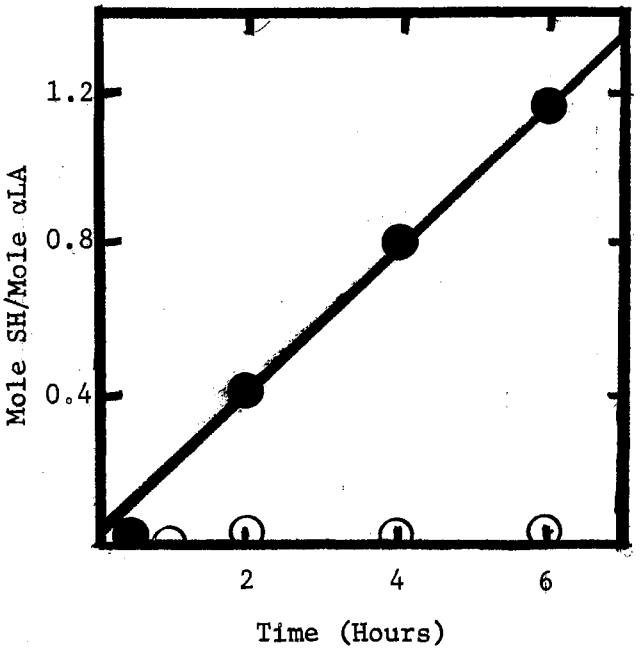


Figure 3. Sulfhydryl Formation During pH 12 Incubation of α-Lactalbumin

α-Lactalbumin, 0.78 mg/ml in 0.15 M KCl at room temperature was incubated at pH 12 (●) and pH 6.0 (○). Aliquots were removed at two hour intervals and assayed for sulfhydryl by DTNB.

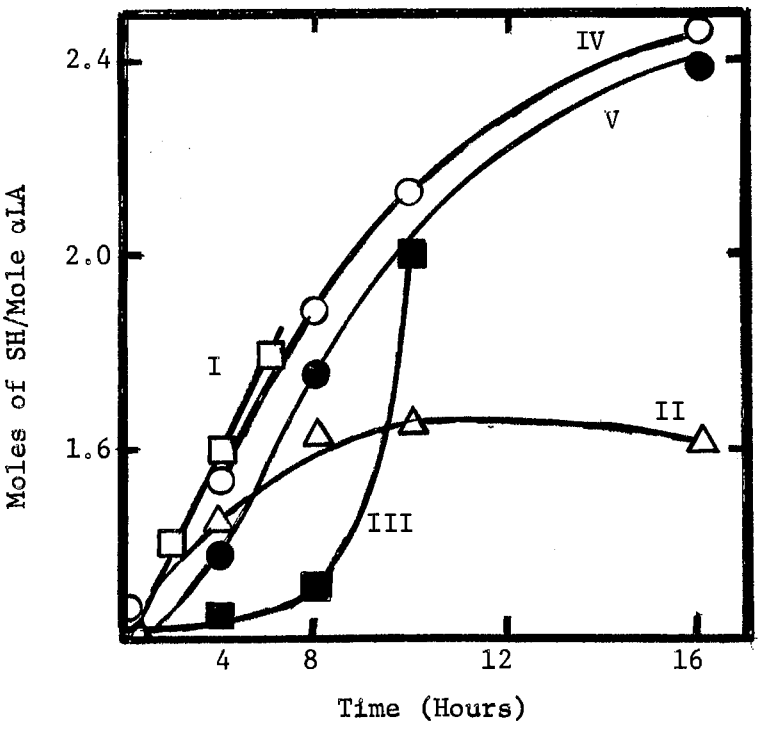


Figure 4. Sulfhydryl Formation During Incubation at Alkaline pH

α-Lactalbumin was incubated at room temperature as follows: I (□) 0.78 mg/ml protein, 0.15 M KCl, pH 12, II (Δ) 0.76 mg/ml 0.15 M KCl, pH 12, III (■) 0.81 mg/ml pH 11 at the end of the experiment, IV (○) 0.75 mg/ml 0.1 M (K) PO pH 12, V (●) 0.76 mg/ml 0.15 M KCl pH 12. α-Lactalbumin concentration was determined by absorption at 280 nm and sulfhydryl by DTNB.

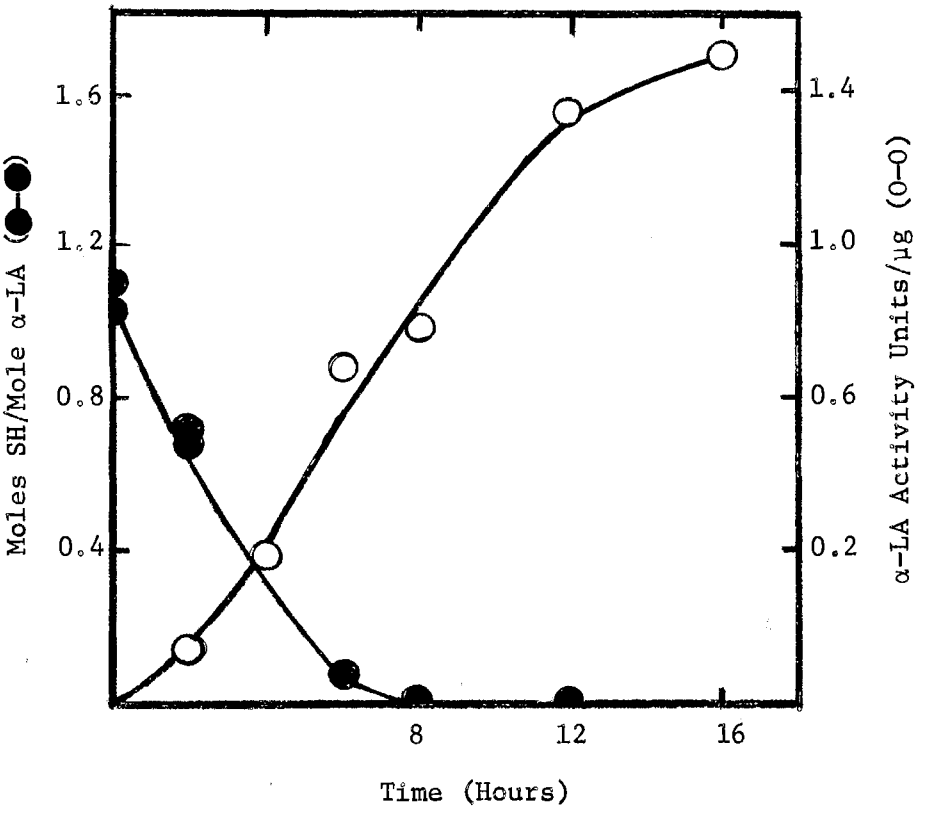


Figure 5. α-Lactalbumin Activity and Sulfhydryl Content After Incubation at pH 12

α-Lactalbumin, 0.77 mg/ml in 0.1 M (K) PO_4 was incubated at room temperature at pH 12. Aliquots were removed at varied time intervals to determine activity (●) and sulfhydryl content (○).

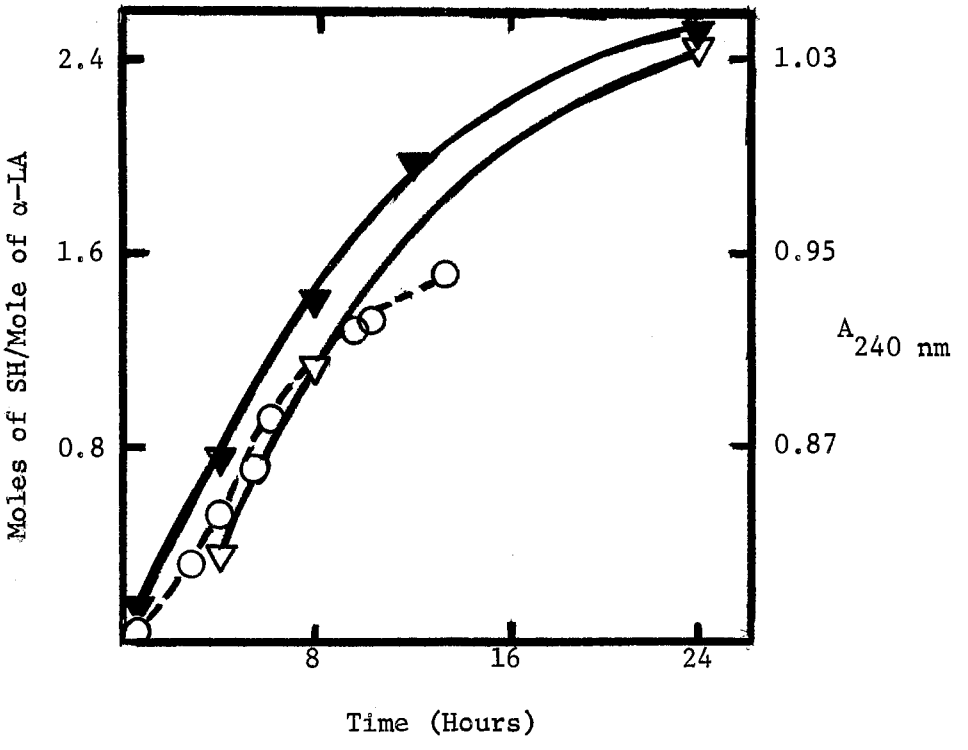


Figure 6. Sulfhydryl Formation in α-Lactalbumin Incubated at pH 12

α-Lactalbumin 0.76 mg/ml was incubated at pH 12, room temperature in 0.1 M PO_4 (▼) and 0.15 M KCl (▽). Sulfhydryl content at varied time intervals was determined with DTNB. The absorption of sulfhydryl was followed at 240 nm (○) which is the absorption maximum for the mercaptide ion.

To insure that the reaction mixture was maintained at the desired pH throughout the incubation period, the pH stat was used along with a water saturated nitrogen atmosphere to exclude oxygen and carbon dioxide. A comparison of the effect on activity of α-lactalbumin and sulfhydryl formation at pH 10, 11, 11.5, and 12 was carried out in the pH stat. The α-lactalbumin (1 mg/ml) in 0.15 M KCl was adjusted to the desired pH with 1 N KOH and the pH was maintained with 0.1 N KOH. Aliquots were removed at various time intervals and α-lactalbumin activity and sulfhydryl formation were determined. The results of these experiments are shown in Figures 7-10. At pH 10, Figure 7, there was no sulfhydryl formation, with little or no activity loss as compared to the control. The rate of sulfhydryl production, hence disulfide cleavage, increases from pH 11 to pH 12.

The experiments presented in Figures 7-10 showed that as the pH was increased there was a concomitant increase in the rate of sulfhydryl formation paralleled by a loss of activity of α-lactalbumin. The logistics of these experiments are somewhat difficult and as a result, the controls gave somewhat variable results, as the data presented in Figure 7. Accordingly, the experiments whose data are presented in Figures 7-10 were performed in a single experiment using septum covered vials.

Ten ml of α-lactalbumin, 0.75 mg/ml in 0.15 M KCl, were placed in a pH stat cuvette and as the pH was brought to each successive level, (i.e., 10, 11, 11.5 and 12), a 2 ml aliquot was taken and placed in a 6 ml Pierce reaction vial, flushed with nitrogen and capped with a septum. At 8 hr. intervals 0.4 ml samples were removed with a syringe fitted with a 22 gauge needle and 0.1 ml was diluted 1:10 for activity measurements and 0.3 ml was diluted 1:1 for sulfhydryl determinations with 0.1 M Tris pH

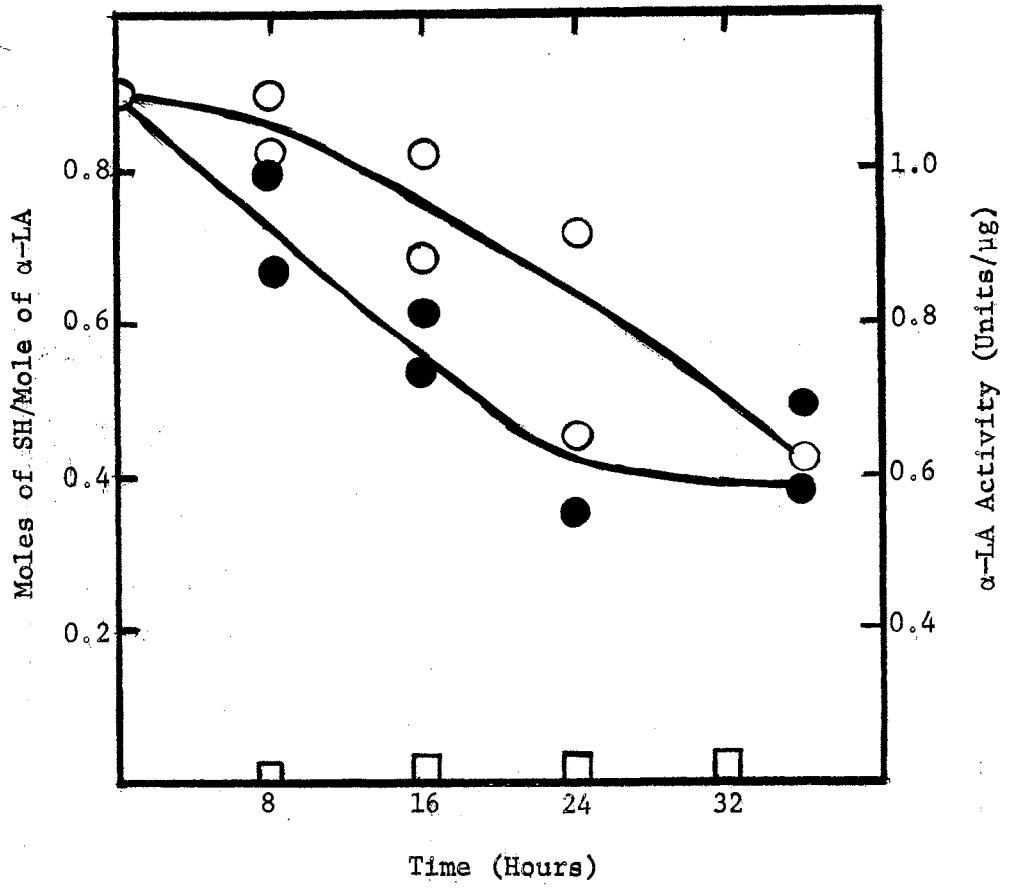


Figure 7. The Effect of Incubation at pH 10 (pH Stat.) on the Activity and Sulfhydryl Formation in α-Lactalbumin

α-Lactalbumin, 0.76 mg/ml in 0.15 M KCl, was adjusted to pH 10 in a pH Stat. Aliquots were taken at 8 hour intervals for activity measurements of the control (○) and pH 10 (●) activity and for sulfhydryl determination at pH 10 (□).

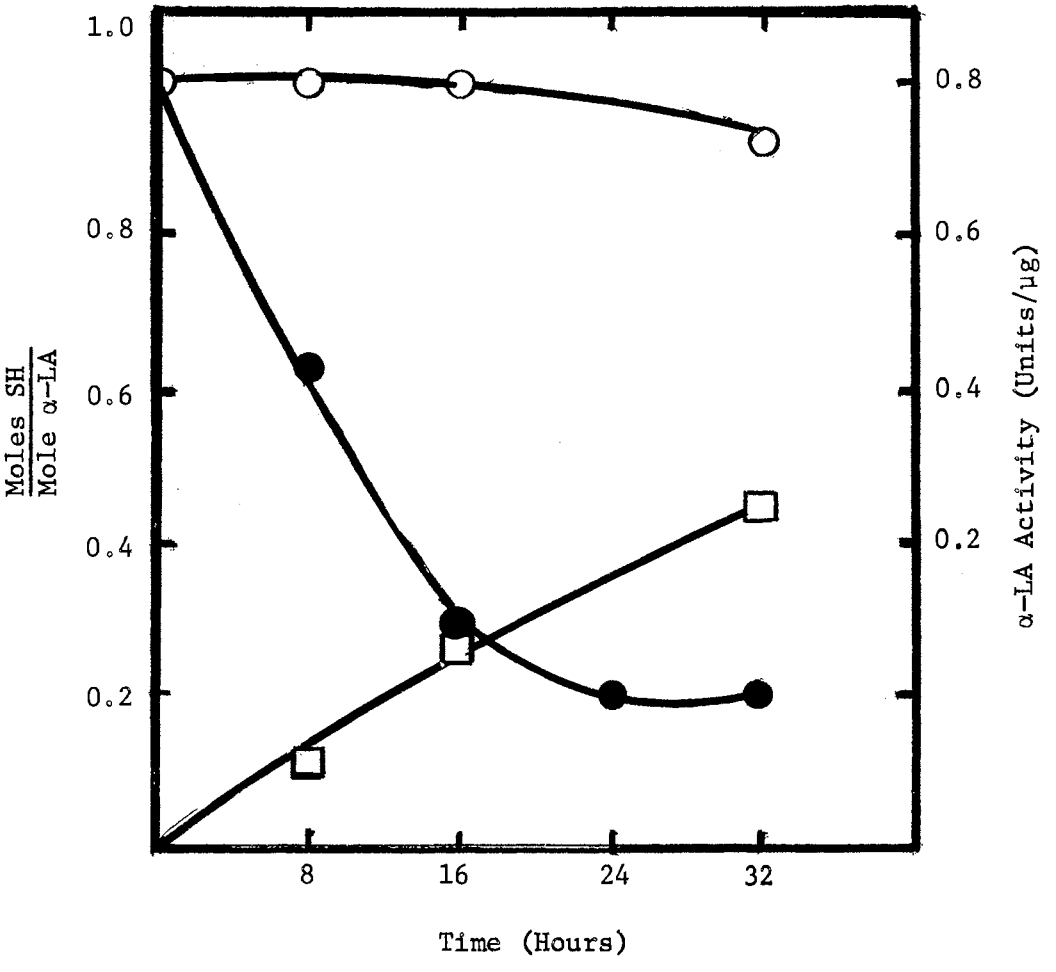


Figure 8. The Effect of Incubation at pH 11 (pH Stat.) on the Activity and Sulfhydryl Formation in α-Lactalbumin

α-Lactalbumin 0.76 mg/ml in 0.15 M KCl was adjusted to pH 11 in pH stat. Aliquots were taken at 8 hour intervals for activity measurements of the control (○) and pH 11 (●) activity and for sulfhydryl determination at pH 11 (□).

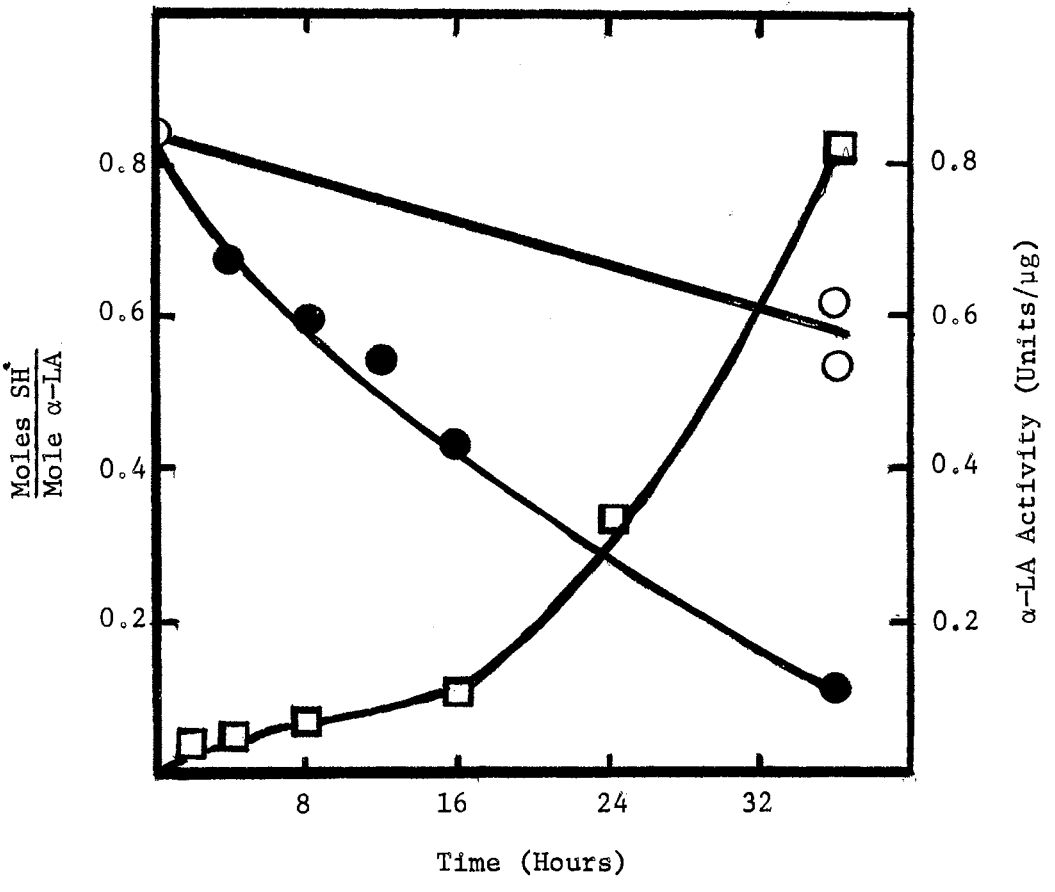


Figure 9. The Effect of Incubation at pH 11.5 (pH Stat.) on the Activity and Sulfhydryl Formation in α-Lactalbumin

α-Lactalbumin 0.76 mg/ml in 0.15 M KCl was adjusted to pH 11.5 in a pH stat. Aliquots were taken at 8 hour intervals for activity measurements of the control (○) and pH 11.5 (●) activity and for sulfhydryl determination at pH 11.5 (□).

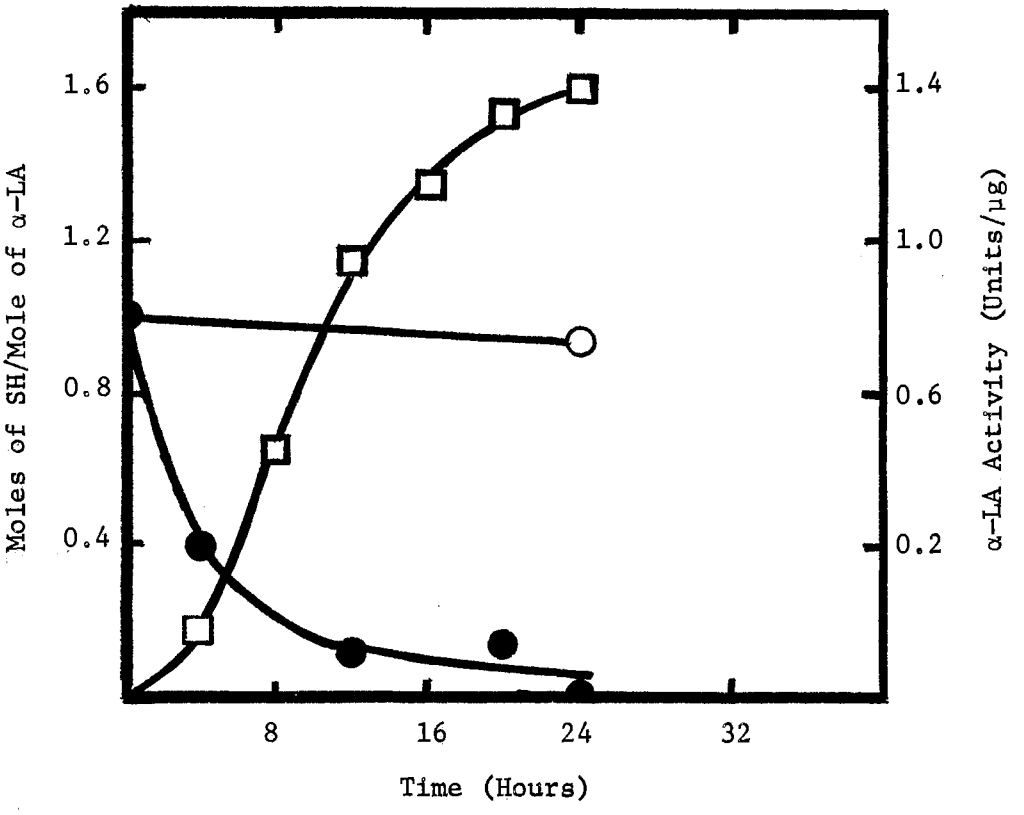


Figure 10. The Effect of Incubation at pH 12 (pH Stat.) on the Activity and Sulfhydryl Formation in α-Lactalbumin

α-Lactalbumin 0.76 mg/ml in 0.15 M KCl was adjusted to pH 12 in a pH stat. Aliquots were removed at timed intervals for activity measurements of the control (○) and pH 12 (●) and for sulfhydryl determination at pH 12 (□).

7.5 as the dilution buffer. The results as shown in Figures 11 and 12 are in good agreement with those presented in Figures 7-10.

Further comparison of the alkaline treated α-lactalbumin with native α-lactalbumin with respect to amino acid analysis showed no significant difference between the analyses except that anticipated in the half cystine region. An increase in absorbance at 250 nm was observed when α-lactalbumin, incubated at alkaline pH, was reacted with p-chloromercuribenzoate (PCMB) which paralleled the increase in sulfhydryl content as shown by DTNB. Since the molar extinction coefficient and the absorption maximum of the PCMB-treated protein varies with the pH and the protein used (49), no quantitative data were obtained from the PCMB experiments.

The alkaline disulfide cleavage of α-lactalbumin and lysozyme was compared. At pH 12 after 24 hours at room temperature, lysozyme had 0.55 moles of sulfhydryl per mole, whereas α-lactalbumin had 1.95 moles of sulfhydryl per mole of indicating that the disulfides in α-lactalbumin are more susceptible to high pH than those in lysozyme.

Summary

These studies have confirmed the initial observations by Merriman (1) that at a pH above 11, the sulfhydryl content of α-lactalbumin increases and that α-lactalbumin activity decreases. There was no sulfhydryl formation nor activity loss when α-lactalbumin was held at pH 10 for 32 hours. However, as the pH was increased, there was an increase in the rate and amount of sulfhydryl formation. When α-lactalbumin was held at pH 12 for 24 hours, the amount of sulfhydryl leveled off at 2.0-2.5 moles of sulfhydryl per mole of α-lactalbumin. The activity of

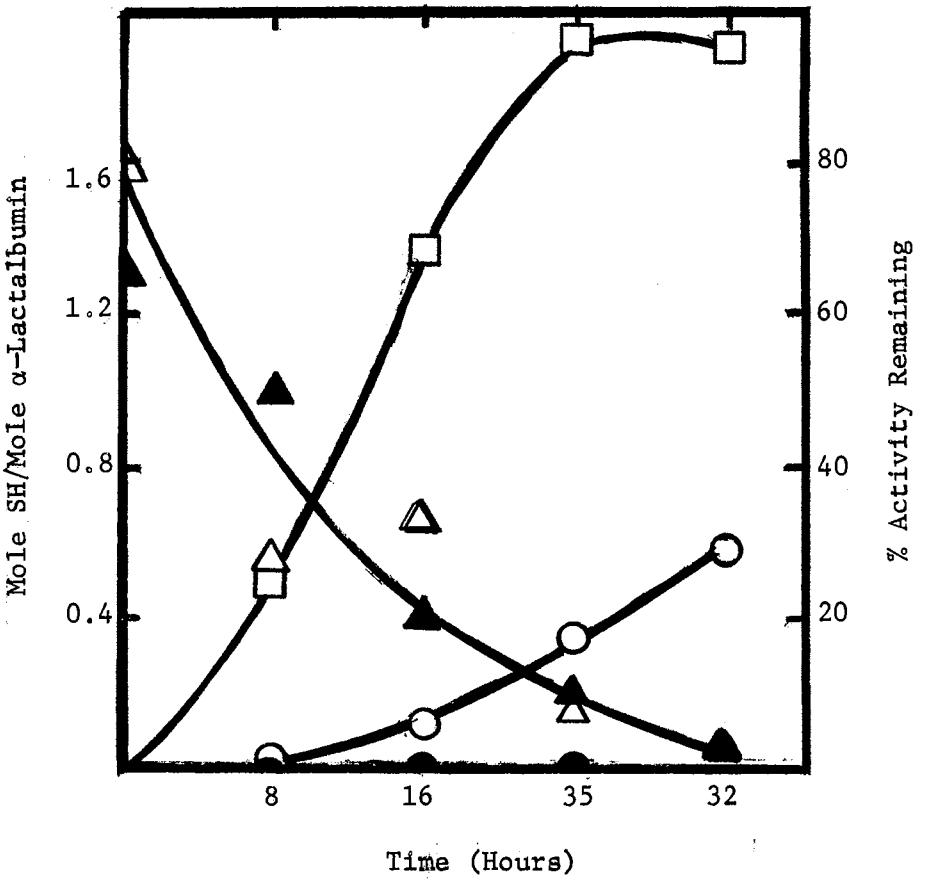


Figure 11. The Effect of Incubation of α-Lactalbumin at pH 10, 11, 11.5 and 12, and Activity Loss at pH 11.5 and 12 in Septum Vials

α-Lactalbumin 0.72 mg/ml in 0.15 M KCl was adjusted to pH 10, 11, 11.5, and 12. Samples were taken at each pH and placed under nitrogen in septum covered vials. At 8 hour intervals, aliquots were removed from the control (pH 6) (●), pH 10 (●) pH 11 (●) pH 11.5 (○) and pH 12 (□) and the sulfhydryl content was determined. The result of control, pH 10 and pH 11 are the same except at 32 hours where pH 11 showed 0.04 moles of sulfhydryl per mole of α-lactalbumin. The activity of α-lactalbumin at pH 11.5 (△) and 12 (▲) are shown. Figure 12 compares all the activities.

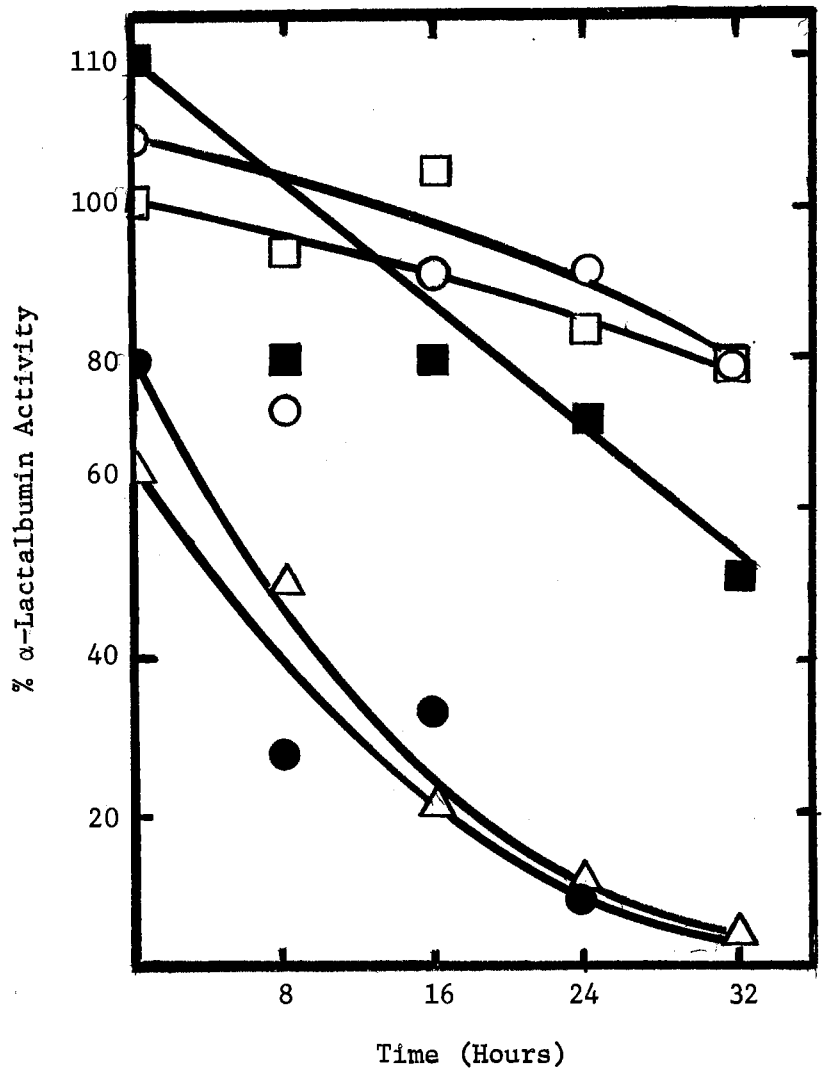


Figure 12. Activity of α-Lactalbumin Incubated at pH 10, 11, 11.5, and 12 in Septum Covered Vials

α-Lactalbumin 0.76 mg/ml in 0.15 M KCl was incubated at pH 10, 11, 11.5 and 12 and activity was measured, control (□), pH 10 (○), pH 11 (■), pH 11.5 (●), pH 12 (Δ) at 8 hour time intervals.

α-lactalbumin decreased more rapidly than the sulfhydryl formation increased. When α-lactalbumin had lost 60-70% of the original activity, the sulfhydryl content was 0.3-0.4 moles per mole of α-lactalbumin, suggesting that one of the disulfides may be more susceptible to alkaline cleavage than the others.

The rate of alkaline cleavage of the disulfide bonds in lysozyme was one fourth in α-lactalbumin. At alkaline pH, α-lactalbumin is in an expanded conformation whereas lysozyme does not undergo this transition (4). This may cause increased exposure or strain of the disulfide bonds, thereby increasing the susceptibility to alkaline cleavage.

Equating sulfhydryls to disulfides hydrolyzed on a mole per mole basis, the α-lactalbumin has two disulfide bonds cleaved after incubation at pH 12 for 24 hours. Since there is a rapid initial rate of sulfhydryl formation followed by a slower rate (Figure 8), these results would indicate one disulfide was hydrolyzed preferentially and a second disulfide was hydrolyzed more slowly.

Reduction and Reformation of Disulfides in α-Lactalbumin

The results from the experiments on the alkaline hydrolysis of α-lactalbumin indicated that one of the disulfide bonds was being cleaved more readily than the other three. Hence, the effect of reducing agents on the rate and extent of reduction of the disulfide bonds in α-lactalbumin as correlated with the activity loss of α-lactalbumin was examined.

α-Lactalbumin, 5 mg/ml in 0.1 M Tris pH 7.5 at room temperature, was made 0.2 M in 2-mercaptoethanol which is a 143 molar excess for each disulfide bond in α-lactalbumin. After five hours, the control and the

sample treated with 2-mercaptoethanol were assayed for α-lactalbumin activity. The reduced material lost 75% of the α-lactalbumin activity when compared to the control. The control and reduced material, 0.5 ml of each, were dialyzed against 0.1 M Tris pH 7.5 at 4° for 36 hours with four buffer changes of 500 mls each. After dialysis the α-lactalbumin activities were not changed. The sample was kept frozen at -20° overnight and thawed at room temperature. In the bottom of the tube there was a gelled mass which could be partially dissolved by the addition of 0.005 ml of stock (14.3M)2-mercaptoethanol. This experiment was repeated and the incubation with 2-mercaptoethanol was shortened from five hours to 30 minutes. The α-lactalbumin treated with 2-mercaptoethanol lost 80% of its original activity in that time period.

Dithiothreitol was reported to break all four disulfide bonds in α-lactalbumin at 25° and only one at 1° (22). α-Lactalbumin, 1.18×10^{-4} M (i.e., 1.6 mg/ml) in 0.05 M (K) PO_4 0.001 M EDTA pH 8.0, was made 0.001 M with respect to dithiothreitol with 0.1 M DTT in 0.001 M EDTA, pH 7.0 and the change in absorbance at 310 nm was recorded on a 0-0.1 slide wire in a spectrophotometer equipped with temperature control and a thermistor monitor. The results at 25° and 5° agreed with the reported values (22) and are shown in Figure 13. That is, four disulfides were reduced at 25° and, initially, one at 5°.

The rate of activity loss was determined with α-lactalbumin, 1.6 mg/ml in 0.1 M Tris pH 7.5, made 0.001 M in DTT at 0°. At 2, 4, 7 and 10 min after addition of the DTT, aliquots were removed, diluted to 0.1 mg/ml and assayed immediately for α-lactalbumin activity. The results (Figure 14) show a loss of 50% of the original activity during the first 3 minutes, followed by a slower rate of loss. To correlate α-lactalbumin

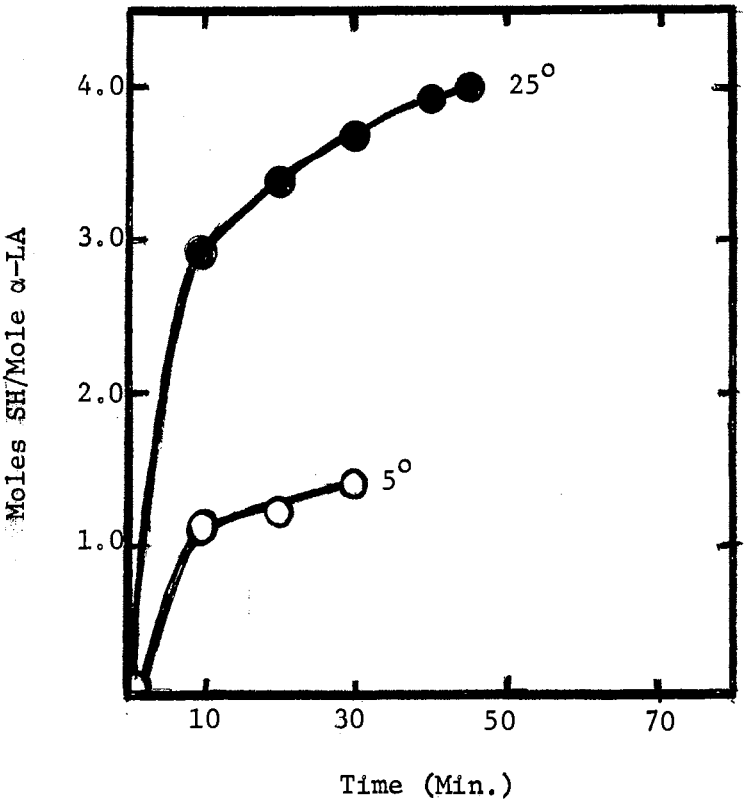


Figure 13. The Rate of Reduction of α-Lactalbumin With Dithiothreitol at 25° and 5°

α-Lactalbumin, 1.67 mg/ml in 0.05 M (R) PO_4, 0.001 M EDTA pH 8.0, was made 0.001 M in DTT with 0.1 M DTT in 0.001 MEDTA, pH 7.0. The change in absorbance at 310 nm was recorded on a 0-0.1 slide wire in a spectrophotometer equipped with temperature control. The rate of change was followed at 25° (●) and 5° (○).

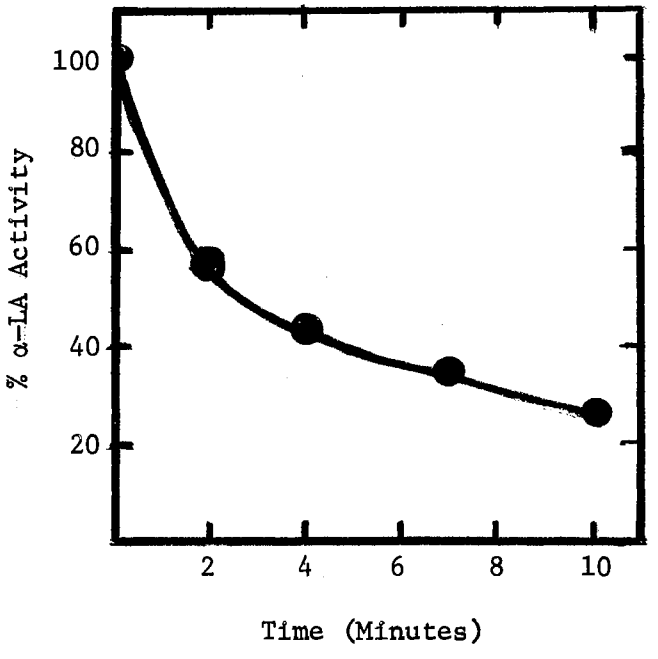


Figure 14. α-Lactalbumin Activity Loss After Incubation With Dithiothreitol at 0°

α-Lactalbumin, 1.28 mg/ml in 0.1 M Tris pH 7.5 was incubated with 0.001 M DTT. An aliquot was removed at timed intervals, diluted 1:10 with 0.1 M Tris pH 75 and assayed immediately for α-lactalbumin activity.

activity loss with the reduction of one disulfide bond, α-lactalbumin, 2.5 mg in 0.5 ml of 0.1 M Tris pH 7.5 was incubated with 0.02 ml of 0.1 M DTT for 30 minutes at 0° in an ice-bath. The sample was applied to a Sephadex G-25 (coarse) column (0.9 x 12 cm), eluted at 4° with 0.1 M Tris pH 7.5 and 0.45 ml fractions were collected. By analysis there were 1.96 moles of sulfhydryl per mole of α-lactalbumin which means 0.98 moles of disulfide were reduced per mole of α-lactalbumin at the time of analysis. The pooled protein peak was treated with a 10 molar excess of iodoacetic acid and the mixture was stirred at room temperature for 30 minutes. The presumably alkylated α-lactalbumin was analyzed for carboxymethyl cysteine. No carboxymethyl cysteine was detected suggesting that no free sulfhydryls were present.

In another experiment, 10 mg/ml of α-lactalbumin in 0.5 ml of 0.1 M Tris pH 7.5 was reacted with 0.05 ml of 0.1 M DTT in 0.001 M EDTA, 0.1 M Tris pH 7.5 at 0° for 30 minutes. The sample was desalted at 4° on the Sephadex G-25 column as described in the previous experiment. The protein peak was pooled and the number of disulfide bonds reduced per mole of α-lactalbumin was found to be 0.94. The pooled fractions were then passed over a PCMB-Sepharose column (7 x 0.7 cm) and the eluant was tested for sulfhydryls but none were found. Aliquots from the α-lactalbumin before incubation with DTT (control), from the Sephadex G-25 column eluant, and from the PCMB Sepharose column eluant were assayed for α-lactalbumin activity. The Sephadex G-25 eluant with 0.94 mole of disulfide reduced had lost 72% of the original activity, the PCMB-Sepharose eluant with no sulfhydryls had 98% of the α-lactalbumin activity remaining. From the protein determination by absorbance at 280 nm, only 75% of the protein applied to the PCMB-Sepharose column was recovered. This

result, along with the α-lactalbumin activity results from the PCMB Sepharose raised the question whether one fourth of the α-lactalbumin was being completely reduced or if all of the α-lactalbumin had one disulfide bond reduced. Combining the α-lactalbumin activity results from the Sephadex G-25 eluant and the results from the PCMB-Sepharose eluant, the conclusion was made that one disulfide bond was being reduced and then reforming during the course of the experiment.

To test this conclusion, 2 mg α-lactalbumin in 0.2 ml 0.1 M Tris pH 7.5 were incubated with 0.01 ml of 0.1 M DTT for 10 min at 0° and desalted on the Sephadex G-25 as described previously. The fractions from the Sephadex G-25 were assayed for sulfhydryl content and the results are reported in terms of disulfide bonds reduced per mole of α-lactalbumin. The absorbance at 280 nm was read (Figure 15) and fractions 20, 18 and 16 were assayed for sulfhydryl. Fraction 18 was assayed a second time after 16 had been assayed. Activity of α-lactalbumin was determined on α-lactalbumin treated with DTNB. Table II shows the results of these experiments. Figure 15 also shows conductivity measurements of the odd numbered fractions. The resolution of the protein from the salt on the Sephadex G-25 column is not entirely satisfactory. However, one further experiment was conducted using this column for desalting purposes.

α-Lactalbumin was incubated with DTT as previously described and passed through the Sephadex G-25 column. The fractions were collected at room temperature. The odd numbered fractions had 0.01 ml of 0.01 M DTNB placed in the tube prior to collection in order to trap any sulfhydryls formed. As soon as the 70 fractions were collected, a time period of 15 minutes, the absorbance at 280 nm of the even numbered fractions was recorded. Thirty minutes after the collection of the fractions,

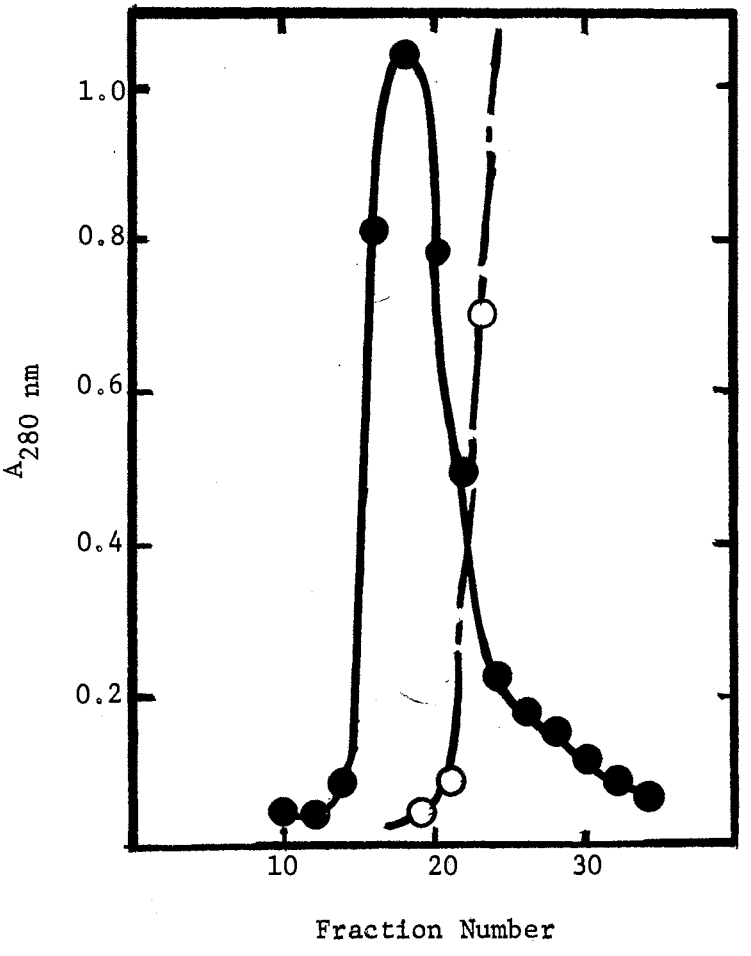


Figure 15. Separation of α-Lactalbumin and DTT on Sephadex G-25

α-Lactalbumin, 2 mg in 0.2 ml of 0.1 M Tris pH 7.5 was incubated with 0.01 ml of 0.1 M DTT for 30 minutes and applied to a Sephadex G-25 (coarse) column (0.9 x 12 cm) and eluted at 4° with 0.1 M Tris pH 7.5. Fractions of 0.9 ml were collected and the absorbance at 280 nm (●) and conductivity was measured (○).

TABLE II

COMPARISON OF DISULFIDES REDUCED AND ACTIVITY OF α-LACTALBUMIN

Fraction #	Disulfide Bonds Reduced / Mole of α-Lactalbumin	α-Lactalbumin Activity
Control	0.00	100
20	0.83	61
18a	0.41	---
16	0.35	---
18b	0.33	82

the absorbance at 412 nm of the odd numbered fractions was read. The number of disulfide bonds reduced was determined for fractions 33 and 36 on either side of the protein peak and they contained 1.05 and 0.99 disulfides reduced per mole of α-lactalbumin respectively. After sitting at room temperature for 1.5 hours, 0.01 ml of 0.01 M DTNB was added to the even numbered fractions and after 30 minutes the absorbance at 412 nm was recorded. Fraction 36 contained 0.27 moles of disulfide per mole of α-lactalbumin, a 75% reformation of the reduced disulfide. Figure 16 shows the protein peak at 280 nm and absorbance at 412 nm initially and after 1.5 hours.

Since the resolution was not entirely satisfactory on the Sephadex G-25 column, a Bio-Gel P-6 (100-200 mesh) column (1.2 x 27 cm) in 0.1 M Tris pH 7.5 was prepared and run at 4°. The Bio-Gel P-6 column gave a separation of 5 mls between the protein and salt peaks (Figure 17). At this point an ultraviolet column monitor was used to collect the protein peak in one pool in order to minimize handling the reduced protein, hence removing one possible factor in the reforming of the disulfide. Also, the tip of the eluant tube, plastic tubing, was placed at the bottom of a 10 ml graduated cylinder for collection to minimize surface area and exposure to oxygen.

To follow the reformation of the reduced disulfide in conjunction with α-lactalbumin activity, α-lactalbumin, 3 mg in 0.2 ml of 0.1 M Tris pH 7.5 was incubated at 0° with 0.02 ml of 0.1 M DTT for 30 minutes, desalted at 4° on the Bio-Gel P-6 desalting column and the protein peak was collected as described above. The protein peak was divided at 4° into 0.7 ml portions in 5 ml beakers. The beakers were brought to room temperature and at various timed intervals, samples were assayed for

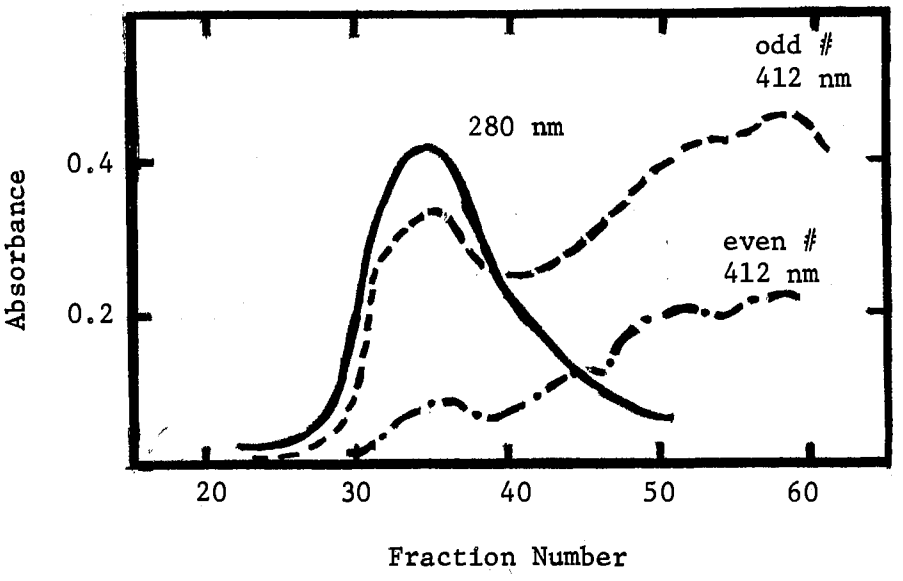


Figure 16. Reformation of Disulfide Bonds in α-Lactalbumin at Room Temperature

α-Lactalbumin, 2 mg in 0.2 ml of 0.1 M Tris pH 7.5 was incubated with 0.01 ml of 0.1 M DTT for 30 minutes at 0° applied to a Sephadex G-25 coarse (0.9 x 12 cm) column, and eluted with 0.1 M Tris pH 7.5. Fractions of 0.22 ml were collected at room temperature. The odd numbered fractions were collected in tubes to which 0.01 ml of 0.01 M DTNB had been placed prior to collection. To all fractions were added 0.4 ml of 0.1 M Tris pH 7.5. The even numbered fractions were read at 280 nm (——) and the odd numbered fractions at 412 (----). After 15 hr. 0.01 ml of 0.01 M DTNB was added to the even numbered fractions and the absorbance at 412 nm was read after 30 minutes (- ◦ -).

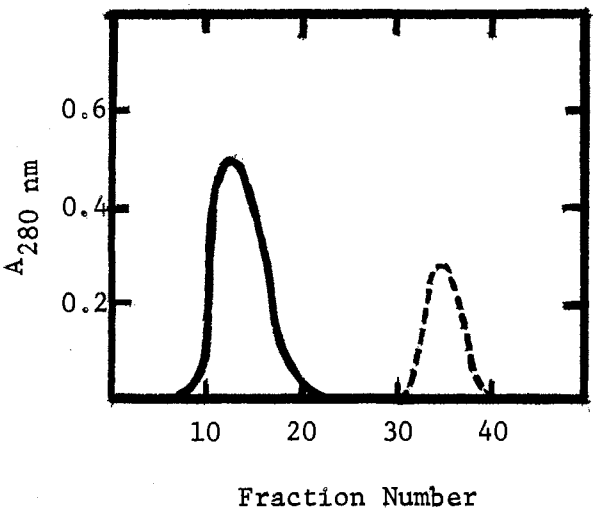


Figure 17. Separation of α-Lactalbumin and Salt Peak on Bio-Gel P-6 (1.2 x 27)

α-Lactalbumin 1 mg in 0.3 ml of 0.1 M Tris pH 7.5 made 0.1 M in $(NH_4)_2\ SO_4$ was applied to a Bio-Gel P-6 (100-200 mesh) (27 x 1.2 cm) column at 4°. Fractions of 1 ml were collected. Protein was detected at 280 nm (——) and the salt peak detected by adding $BaCl_2$ and observing the formation of the white precipatate $BaSO_4$ (----).

α-lactalbumin activity and sulfhydryl content. As shown in Figure 18, there is good correlation between the α-lactalbumin activity regained and the reformation of the disulfide bond.

The rate of reoxidation of the disulfide bond is dependent upon temperature and the amount of oxygen in the solution. A sample kept under nitrogen at 0° showed slower regain than a sample under nitrogen at room temperature as shown in Figure 19. A portion of the same sample when aerated at room temperature with a disposable pipette gave immediate and complete regain of α-lactalbumin activity and complete loss of sulfhydryls.

Summary

Dithiothreitol reduces one disulfide bond at 0° and at a molar ratio of DTT to α-lactalbumin of 5-10:1. There is a 75% loss of activity of α-lactalbumin with the reduction of one disulfide bond. The reduced disulfide reforms rapidly upon exposure to air and the reformation of the disulfide is accompanied with full regain of activity. These results indicate that one disulfide bond is being reduced by DTT. At 0° only one disulfide was reduced whereas at 25° all four disulfides were reduced. At 5° one disulfide was reduced rapidly which is followed by a slow reduction of the remaining disulfide(s).

Peptic and Tryptic Digests of Reduced and Alkylated α-Lactalbumin

The reduction of α-lactalbumin by DTT at 0° as given in the previous section resulted in one disulfide reduced. To determine which one of the four disulfides in α-lactalbumin was reduced, the reduced protein was

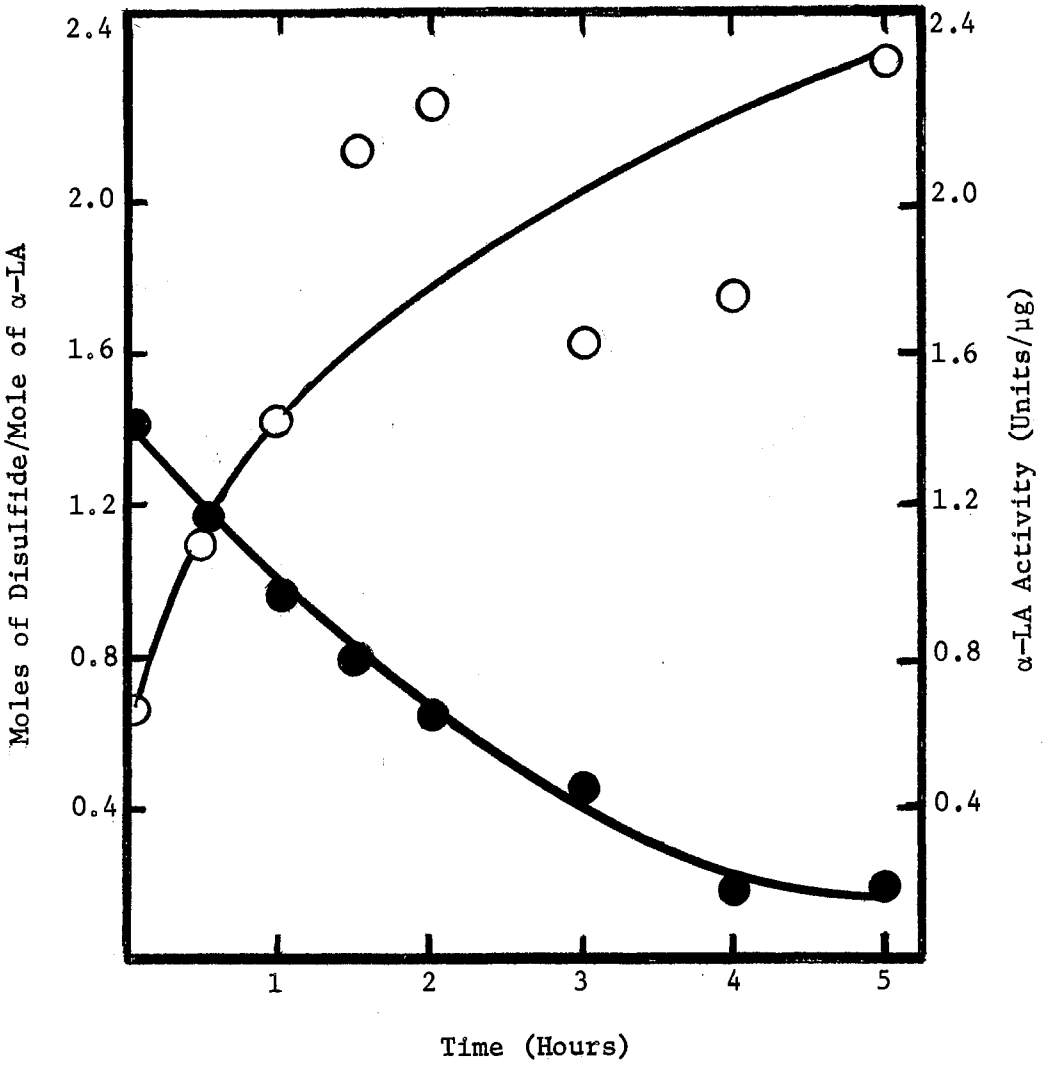


Figure 18. Regain of α-Lactalbumin Activity After Reduction With Dithiothreitol at 0°

α-Lactalbumin, 3 mg in 0.2 ml of 0.1 M Tris pH 7.5 was incubated with 0.02 ml of 0.1 M DTT for 30 minutes at 0°, desalted on a Bio-Gel P-6 (100-200 mesh). (1.2 x 27 cm) column. Aliquots of 0.7 ml of the protein containing fractions were placed in 5 ml beakers and brought to room temperature. At varied timed intervals α-lactalbumin activity was assayed (○) and sulfhydryl content determined with DTNB (●).

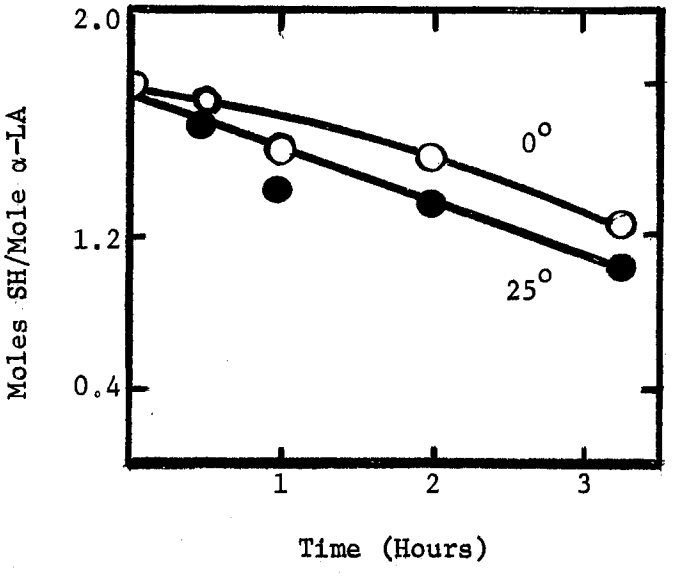


Figure 19. Reformation of the Disulfide Bond of α-Lactalbumin at 0° and 25°

α-Lactalbumin, 3 mg in 0.2 ml 0.1 M Tris pH 7.5 was incubated with 0.01 ml of 0.1 M DTT for 30 minutes at 0° and desalted at 4° on a Bio-Gel P-6 column. The protein pool was divided into two fractions and sealed under a nitrogen atmosphere. One fraction was placed at 25° (●) and the other at 0° (○). The sulfhydryl content was determined at timed intervals.

alkylated with 1-^{14}C iodoacetic acid at a four molar excess over the sulfhydryl content of the α-lactalbumin under nitrogen at pH 8.0 for 30 minutes. Twenty mg of reduced α-lactalbumin (Methods) in 8 ml of 0.1 M Tris pH 8.0 were alkylated with ^{14}C-iodoacetic acid (1-2 μc) and dialyzed against deionized water at 4° in a 4 liter vessel with the water changed three times over a 24 hour period. The solution containing α-lactalbumin was lyophilized and dissolved in 1 ml of the buffer of choice and an aliquot of 10 μℓ was counted in 10 ml of Bray's scintillation fluid.

Peptic digestion of native α-lactalbumin results in fragments containing intact disulfide bonds (12). The four disulfides were contained in three fragments with one fragment containing two disulfides. These fragments are from residues 4-9 with 119-123 containing cysteines 6 and 120; 27-31 with 105-117 containing cysteines 28 and 111; and 59-80 with 91-94 containing cysteines 61 and 77, and 73 and 91. The largest fragment is 59-80 bridged to 91-94 and contains one intra peptide disulfide, 61-77, and one inter peptide disulfide, 73-91. Figure 20 shows the size and disulfides of these fragments. These fragments were separated by gel filtration on Sephadex G-50. If one of the disulfides were reduced before digestion, the elution pattern would be altered from that of native α-lactalbumin. By introducing a radioactive label, identification of the position of the reduced disulfide on the fragments could be determined.

The tryptic digest of fully reduced and 5-carboxymethylated α-lactalbumin has been reported by Brew et al. (11). This digestion contains a large fragment, 17-58, which is insoluble at pH 3. Cysteine 28 is in this fragment. By reducing one disulfide in α-lactalbumin with DTT at

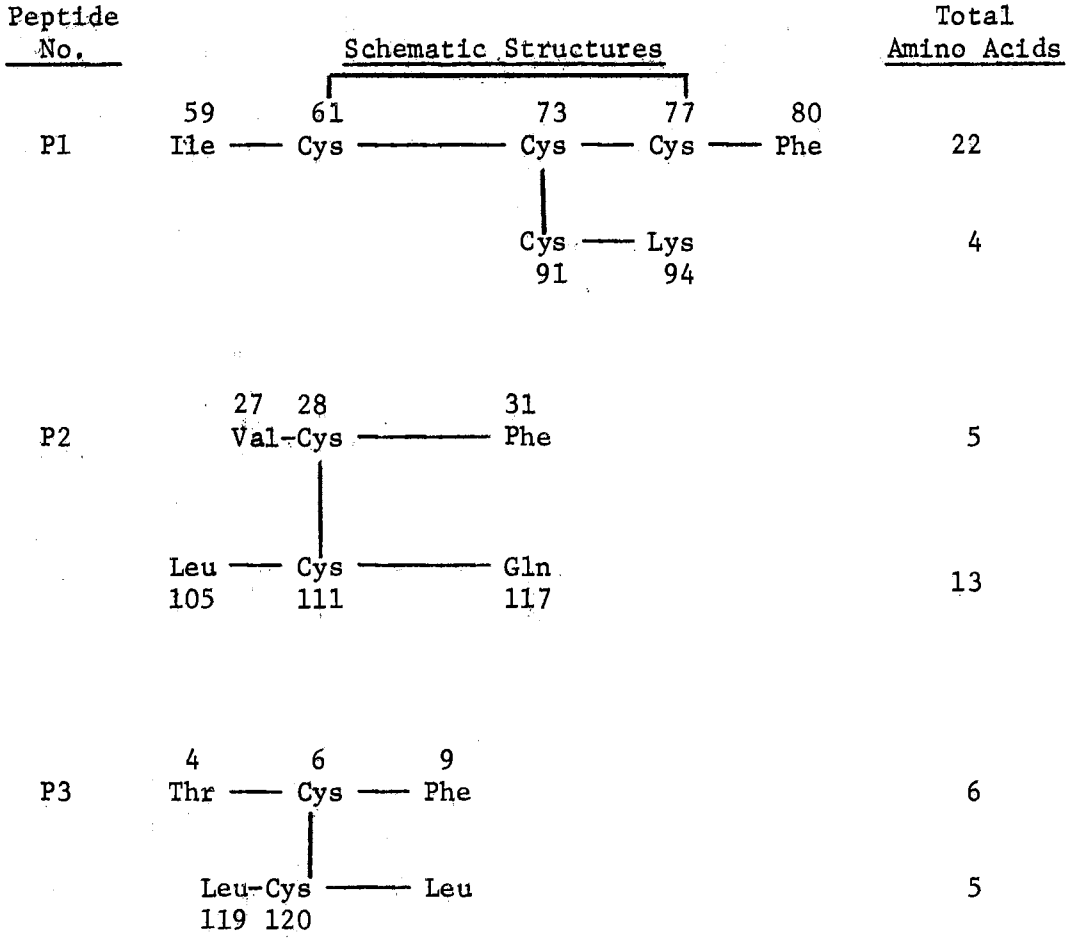


Figure 20. Peptic Fragments of α-Lactalbumin Containing Intact Disulfides

0° and labeling with ^{14}C-iodoacetic acid, then fully reducing the labeled protein and reacting it with unlabeled iodoacetic acid, one can determine if cysteine 28, of the disulfide 28-111, was labeled.

α-Lactalbumin 6 mg in 1 ml 0.01 N HCl, reduced and reacted with ^{14}C-iodoacetic acid was treated with pepsin at a ratio of 40 to 1 (w/w) α-lactalbumin to pepsin at pH 2 at 37° for 20 hours. The optimum incubation time was determined on native α-lactalbumin by assaying aliquots with ninhydrin at various time intervals. When there was no further increase in absorbance in the ninhydrin reaction, the digestion was considered complete. The peptic digest was lyophilized and 5 mg were dissolved in 0.2 ml of 0.1 M Tris pH 7.5. This solution was made 5% in sucrose (w/v) and was applied to a Bio-Gel P-6 (100-200 mesh) (0.9 x 116 cm) column, developed with 0.1 M Tris pH 7.5, and 0.55 ml fractions were collected. Native α-lactalbumin was prepared in the same manner. The patterns of absorbance at 280 nm were compared and the radioactivity of the reduced α-lactalbumin was plotted against the absorbance, Figures 21 and 22. The radioactive pattern showed that the heavy peptic fragment containing disulfides 61-77 and 73-91 was not reduced and labeled. The separation of the two radioactive peaks was poor and would indicate that 6-120 or 28-111 had been reduced. The peptides from the reduced 6-120 disulfide would be 6 and 5 amino acid residues and the peptides from 28-111 would be 13 and 5 residues Figure 20.

Distinguishing, by size difference, between these peptides is not reliable with this P-6 column. Therefore, the tryptic digest was employed to determine if 28-111 was the reduced disulfide.

α-Lactalbumin with one disulfide reduced and alkylated with

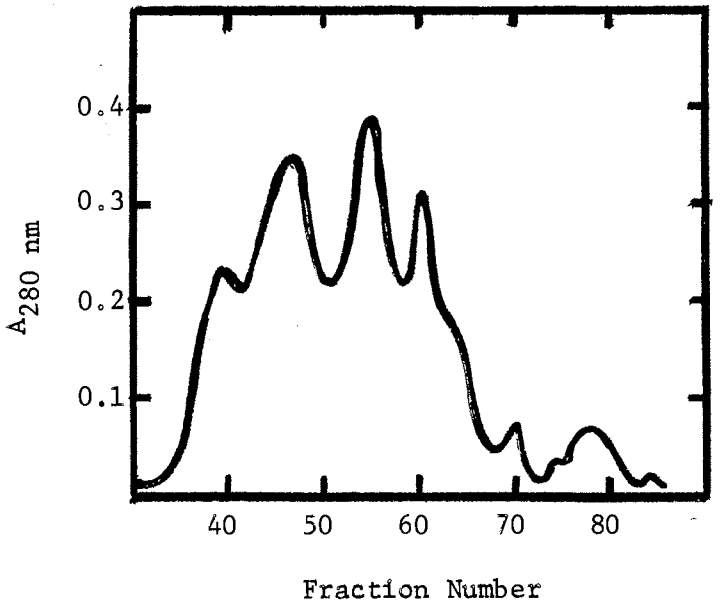


Figure 21. Separation of Peptic Digest of Native α-Lactalbumin on Bio-Gel P-6

Peptic digest of α-lactalbumin, 5 mg in 0.2 ml of 0.1 M Tris pH 7.5 were applied to a Bio-Gel P-6 (100-200) (116 x 0.9 cm) column. Fraction of 0.55 mls were collected and absorbance read at 280 nm.

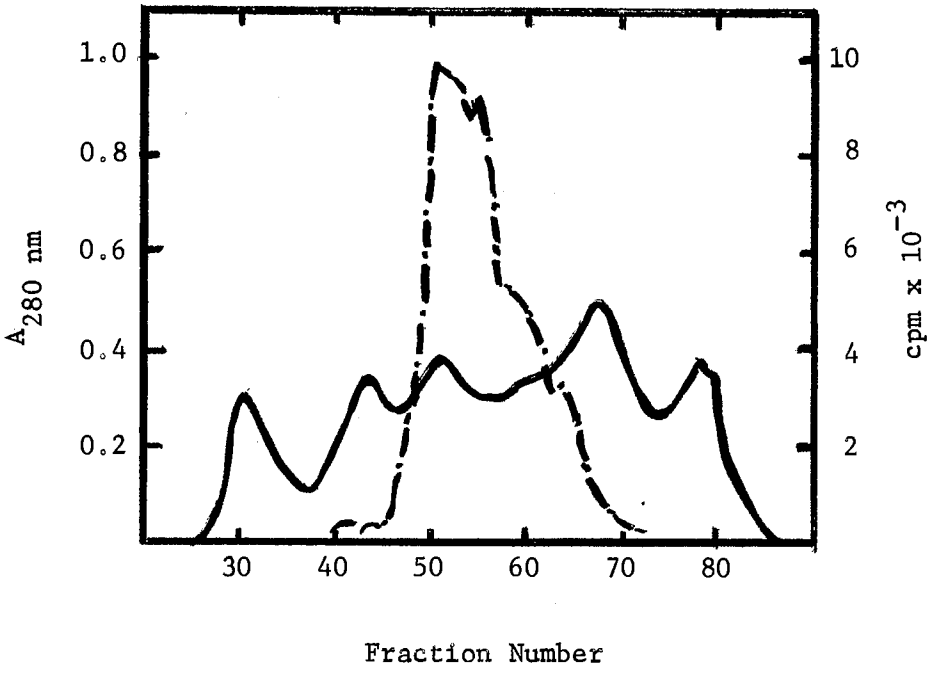


Figure 22. Separation of Peptic Digest of Partially Reduced and ^{14}C-S-carboxymethylated α-Lactalbumin on Bio-Gel P-6

Peptic digest of partially reduced and ^{14}C-S-carboxymethylated α-lactalbumin, 5 mg in 0.2 ml of 0.1 M Tris pH 7.5 was applied to a Bio-Gel P-6 (100-200) (116 x 0.9 cm) column. Fractions of 0.55 mls were collected and absorbance read at 280 nm (——). Radioactive peaks were determined by liquid scintillation (---).

^{14}C-iodoacetic acid was fully reduced with a 10 molar excess of DTT at room temperature and then reacted with a four molar excess of iodoacetic acid over the sulfhydryls produced. The S-carboxymethylated α-lactalbumin 10 mg in 1 ml was dialyzed against 2 liters of deionized water at 4° with four changes of water in 36 hours. The dialyzed sample was made up to 3 mls with water and the pH was adjusted on the pH-stat to 8.6 at 37°. Trypsin was added in a ratio of 1:100 (w/w) trypsin to α-lactalbumin and the pH was maintained with 0.1 N NaOH. After 4 hours trypsin was added in the same amount as initially and the incubation was continued for 17 hours. When pH was adjusted to 3, a precipatate formed. The suspension was centrifuged in a clinical centrifuge, the supernatant solution was decanted and the pellet, amino acids 17-58, was resuspended in pH 3 HCl, washed and recentrifuged three times. The supernatant solution, washings and suspended pellet were assayed for ^{14}C and compared to the total count of the protein before the pH 3 step. All the counts were in the supernatant solution and the first two washings. The elution pattern, comparing absorbance at 220 nm with radioactivity, shows that two peaks are labeled which confirms the elimination of disulfide 28-111 as the disulfide reduced the precipatate contained no significant amount of radioactivity (Figure 23).

The results showed that peptide 28-111 was not labeled with ^{14}C-iodoacetic acid after reduction of one disulfide of α-lactalbumin with DTT. Results of the tryptic and peptic digestion experiments are consistent with disulfide 6-120 being reduced with DTT at 0°. While these studies were completed Schechter *et al.* (51) showed by isolation and subsequent amino acid sequencing that disulfide 6-120 is readily reduced with dithioerythritol at room temperature.

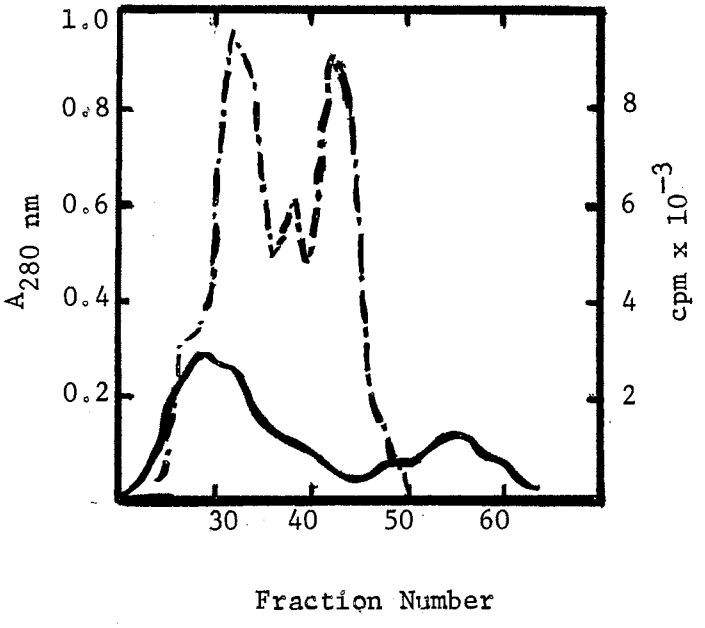


Figure 23. Separation of the Soluble Tryptic Digest of Partially Reduced and ^{14}C-S-carboxymethylated α-Lactalbumin on Bio-Gel P-6

Tryptic digest, of partially reduced, ^{14}C-S-carboxymethylated α-lactalbumin soluble fraction, 5 mg in 0.2 ml of 0.1 M Tris pH 7.5 was applied to a Bio-Gel P-6 (100-200) (116 x 0.9 cm) column. Fractions of 0.55 mls were collected and absorbance read at 280 nm (——). Radioactive peaks were determined by liquid scintillation (—◦—).

CHAPTER IV

SUMMARY AND DISCUSSION

α-Lactalbumin has been under investigation since it was established as the B protein in the synthesis of lactose. The interaction of α-lactalbumin with galactosyltransferase as a modifier protein has led to studies designed to determine the region in α-lactalbumin responsible for its function.

Alkaline hydrolysis of the disulfide bonds in α-lactalbumin occurs upon incubation above pH 10. The rate of the hydrolysis increases as the pH increases to 12 with a concomitant loss of activity of α-lactalbumin, 60-70% activity loss occurs at the point of 0.3-0.4 moles of sulfhydryl per mole of α-lactalbumin. At pH 12, the initial rate of sulfhydryl formation is followed after 12 hours by a slower rate. This indicates that one of the disulfide bonds is more readily cleaved than the other three disulfides. Amino acid analysis and treatment of α-lactalbumin with DTNB showed that the cleavage is a sulfur-sulfur scission, producing a sulfhydryl and not a β-elimination as reported for several proteins when hydrolyzed above pH 12. Reduction of α-lactalbumin with 2-mercaptoethanol resulted in an 80% loss in activity in a 30 min incubation at room temperature at a molar ratio of 143 to 1, mercaptoethanol to disulfide in α-lactalbumin.

Incubation of α-lactalbumin with a 10 molar excess of DTT at 0° in 0.1 M Tris pH 7.5 resulted in the reduction of one disulfide bond as de-

termined spectrophotometrically with DTT and DTNB. The partially reduced α-lactalbumin completely regained activity on exposure to air at room temperature with the reformation of the reduced disulfide.

Peptic and tryptic digests of the reduced and alkylated α-lactalbumin gave evidence that disulfide 6-120 was the disulfide reduced under these conditions. Schechter et al. (51) reported that disulfide 6-120 was reduced by dithioerithritol at room temperature faster than the other three disulfides in α-lactalbumin. Their findings agree with those found in this study.

The results of the reduction experiments which show that one disulfide is readily reduced indicate that disulfide 6-120 is more exposed or in a more strained configuration. It is probable that the same disulfide, 6-120, is cleaved readily by alkaline hydrolysis. Further experiments are required to show if this is the case. Peptic and tryptic digestion experiments should provisionally identify the disulfide as was done in the reduction experiments. Radioactive peptides may be isolated after carboxymethylation with ^{14}C-iodoacetic acid and their amino acid composition may be compared to the known sequence of such fragments. In addition, it may be possible to cleave α-lactalbumin at the disulfides with DTNB and KCN (52) to produce unique fragments useful for identification.

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