

## Solubility Transformation of $\alpha$ -Lactalbumin

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### INTRODUCTION

A preparation of  $\alpha$ -lactalbumin, homogeneous in electrophoresis and by ultracentrifugation (1), was separated into two components by a solvent-gradient extraction procedure (2). The total preparation was precipitated at a concentration of 3.0 *M* ammonium sulfate; the "soluble" component was extracted at 2.5 *M* ammonium sulfate concentration; the "insoluble" component required 1.5 *M* ammonium sulfate (2). The "insoluble" component is not denatured  $\alpha$ -lactalbumin, since it precipitates when heated in solution as would be expected of the native protein.

The work reported here shows that transformation from one component to the other is determined by the presence of salts and is reversible. Dialysis produces the "insoluble" component; the addition of 0.1 *M* or less of NaCl or any of a variety of salts will bring about the transformation to the "soluble" component. Conditions for this transformation are reported, and also the effect of salts on solubility in the isoelectric region. The latter indicates that anions are bound to  $\alpha$ -lactalbumin which might account for the solubility transformation.

### MATERIALS AND METHODS

#### *Preparation of $\alpha$ -Lactalbumin Solutions*

The crystalline  $\alpha$ -lactalbumin was prepared (1, 4) by Dr. W. G. Gordon of this laboratory. Several different preparations were used. The solutions are prepared by dissolving  $\alpha$ -lactalbumin in water with the addition of 0.1 *N* NaOH to a pH of 6.5-7.0. The solutions are dialyzed for 5 days against a large volume of distilled water containing toluene to attain a minimal pH of 5.8-5.9. These solutions are

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not completely free of salts and hence are not equivalent to isoionic  $\alpha$ -lactalbumin. It has not been possible to obtain isoelectric precipitates by dialysis; the addition of a mixed-bed resin Amberlite MB-1, however, did lower the pH within the isoelectric region and give a precipitate. For the experiments at elevated pH values,  $N$  NaOH is added.

### *Solubility Test*

Solubility in  $2 M$  ammonium sulfate is used as a simple test to distinguish the two  $\alpha$ -lactalbumin components: the "insoluble" is precipitated, the "soluble" remains in solution. The test is performed by mixing 1.0 ml. of the protein solution with 1.0 ml. of  $4.0 M$  ammonium sulfate at pH 8.5 (2) in 15-ml. centrifuge tubes. This test was performed at pH values as low as 5.6 with similar results. After 5 min. the mixture is centrifuged for 5 min. at  $3000 \times g$ . The supernatant fluid is decanted and the precipitate is dissolved in 2.0 ml. of water. The protein in both solutions is determined by measuring the absorption at  $280 m\mu$  (2).

### *Determination of Precipitation in the Isoelectric Region*

For this purpose dilute solutions of  $\alpha$ -lactalbumin (1.0–5.0 mg./ml.) are used. The pH is lowered by the addition of HCl (0.01–0.04  $N$ ). The amount of precipitate is determined from the apparent absorbance at  $600 m\mu$  in 1-in. diameter test tubes in a Beckman model B spectrophotometer.

### *Separation by Solvent-Gradient Extraction*

The previous work (2) has shown that  $\alpha$ -lactalbumin that had been thoroughly dialyzed, precipitated at the isoelectric point, and dried from the frozen state could be separated into two components. With an undried crystalline preparation containing ammonium sulfate, only one component was shown (2). The present studies show that dialysis gives the two components—preponderantly the "insoluble" component, but not in excess of 75%—and that when the crystalline precipitate is dissolved in water the dilute ammonium sulfate brings about the transformation to approximately 95% of the "soluble" component.

Electrophoresis was performed by the usual free-flow technique at  $4^\circ$ .

Ultracentrifugal experiments were performed with the Spinco model E analytical ultracentrifuge, at 59,780 r.p.m.

## RESULTS

The pH changes observed (decrease above the isoelectric point, increase below) on the addition of neutral salts to  $\alpha$ -lactalbumin solutions are instantaneous. The change in solubility, however, takes some time as shown in Table I. In these experiments 0.4 ml. of  $4.0 M$  NaCl is added to 9.0 or 10.0 ml. of  $\alpha$ -lactalbumin solution. At intervals 1 ml. of the mixture is withdrawn, and its solubility in  $2 M$  ammonium sulfate is determined by mixing it with 1 ml. of  $4.0 M$  ammonium sulfate. The transformation and the solubility experiments were performed at  $25^\circ$ .

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The addition of NaCl to  $\alpha$ -lactalbumin at pH 6.6 produced close to the maximum transformation in solubility in 45 minutes, as shown in Table I. The rate and extent of the transformation was also about the same when the NaCl was added to the protein at pH 8.8, but at pH 9.9 the transformation attained only 63% in 90 min. With the pH constant there appeared to be little dependence of the transformation on NaCl concentration in the range 0.1–0.5 M.

The new solubility properties imparted by the addition of NaCl are, for the most part, retained on repetition of the ammonium sulfate precipitation. When the precipitate, or "insoluble" fraction, was quickly redissolved and then tested with 2.0 M ammonium sulfate, it was 87% precipitated. When the solution, or "soluble" fraction, was precipitated with 3.0 M ammonium sulfate, filtered, dissolved, and tested with 2.0 M ammonium sulfate, it was 30% precipitated.

Not only ammonium sulfate, but also sodium acetate, sodium potassium tartrate, and sodium citrate are capable of effecting this transformation with  $\alpha$ -lactalbumin. Ethanolamine chloride, however, is apparently incapable of it, since a salt-free solution diluted with 0.1 M concentration of this substance as a buffer, pH 9.6 (3), did not effect the transformation.

The transformation does not occur in 2.0 M ammonium sulfate, presumably because the "insoluble" component is completely insoluble at this concentration. At 1.3 M concentration of ammonium sulfate, however, a large amount of precipitate will be obtained if no salt is present,

TABLE I  
Transformation in Solubility of  $\alpha$ -Lactalbumin by Action of Sodium Chloride

NaCl concn. M	$\alpha$ -Lactalbumin %	pH	Time after NaCl min.	Solubility in 2 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> %
0.17	1.9	6.60	(no NaCl)	25.7
		6.10	1	59.8
		6.10	10	73.2
		6.10	20	77.5
		6.10	45	84.0
		6.10	1,080	88.6
		6.10	(no NaCl)	32.4
0.17	1.9	8.83	30	79.8
		8.74	120	85.2
		8.74	(no NaCl)	42
0.15	2.0	9.88	30	61
		9.88	90	63
		9.88		

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but the precipitate will slowly redissolve. At this concentration of ammonium sulfate presumably the "insoluble" component is partly soluble, and the soluble part is transformed gradually to the "soluble" component. This transformation, by a shift in the equilibrium, gradually brings the "insoluble" precipitate into solution.

Solutions of  $\alpha$ -lactalbumin in 0.1 *M* ethanolamine buffer, pH 9.6 (25% "soluble" component), and in Veronal buffer, pH 8.6, after solubilization with ammonium sulfate (85% "soluble" component) have been subjected to ultracentrifugation. No detectable difference could be observed between the two, as both solutions resulted in a single sedimenting peak with  $S_{20,w} = 1.55$  at 1.0% protein. These and other solutions were subjected to electrophoresis; in ethanolamine buffer at pH 9.8 and an ionic strength of 0.1 the ascending peak was sharp but the descending peak was very broad; at pH 8.6 in Veronal buffer at ionic strength of 0.1 both peaks were normal and there was no evidence of inhomogeneity. The electrophoretic homogeneity of  $\alpha$ -lactalbumin has been reported previously (1). When the ionic strength was reduced to 0.01 several peaks were observed in Veronal (pH 8.6), phosphate (pH 6.6), and cacodylic acid (pH 6.0) buffers on the ascending side with very broad descending peaks. The amount of these apparent components did not conform to the composition based on solubility.

The electrophoretic mobility ( $10^{-5}$  sq. cm./v./sec., calculated from the descending boundary) was determined in various buffers to be as follows:

Buffer	pH	Ionic strengths			
		0.1	0.05	0.02	0.01
Veronal	8.6	-4.2 (1)	-5.1		-7.2
Cacodylic acid	6.0	-2.4			-3.7
Glycine	3.5	4.8		6.6	

It is apparent that the mobility curves for high and low ionic strength cross somewhere in the isoelectric region. Since the salt environment influences the mobility directly through binding, and also through the ionic atmosphere (6), the extent of salt binding cannot be determined from these data.

Since optical rotation of proteins is sensitive to structural changes, the rotation of a dialyzed 4.2% solution of  $\alpha$ -lactalbumin was measured at pH's of 6.3 and 7.1. The specific rotation was found to be  $-60^\circ$  as reported by Gordon *et al.* (1); the change in rotation on adding NaCl to give a concentration of 0.1 *M*, however, was less than 10%.

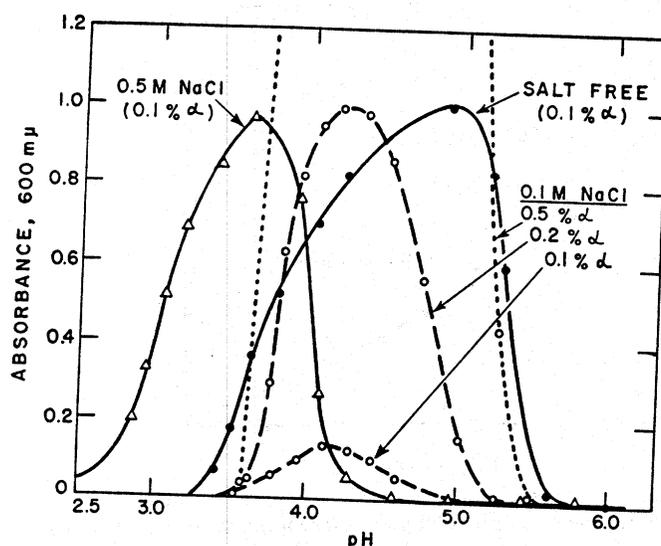


FIG. 1. The influence of NaCl concentration and pH on the precipitation of  $\alpha$ -lactalbumin in the isoelectric region. ●—● 0.1%  $\alpha$ -lactalbumin, dialyzed ("salt-free");  $\Delta$ — $\Delta$  0.1%, 0.5 M NaCl; ○---○ 0.1%, 0.1 M NaCl; ○---○ 0.2%, 0.1 M NaCl; ○·····○ 0.5%, 0.1 M NaCl. For the last curve the absorbance maximum is 2.04 at pH 4.5. The pH readings were made immediately after adding the NaCl; subsequent readings were unchanged. The absorbance readings were made within 3-5 min.

Sodium chloride has a considerable effect on the solubility of  $\alpha$ -lactalbumin in the isoelectric region. A dialyzed solution of  $\alpha$ -lactalbumin (about 1.5% concentration) at pH 5.9 precipitated to the extent of 20-30% on the addition of NaCl to a concentration of 0.1 M. The pH dropped immediately to about 5.4, but the precipitate formed slowly. Because of the time lag it was first thought that this precipitation was related to the solubility transformation. A study of precipitation in the isoelectric region, however, showed that the addition of NaCl had lowered the pH into the region of precipitation. A detailed study of the effect of NaCl and pH on the isoelectric precipitation is shown in Fig. 1. Curves are shown for 0.1%  $\alpha$ -lactalbumin dialyzed, and with 0.5 M NaCl, and for 0.1, 0.2, and 0.5%  $\alpha$ -lactalbumin with 0.1 M NaCl. The shift of the apparent isoelectric point to more acidic solutions is evidence for the strong binding of anions. It has previously been noted that the apparent isoelectric point in the presence of dilute ammonium sulfate is at pH 4.0 (4).

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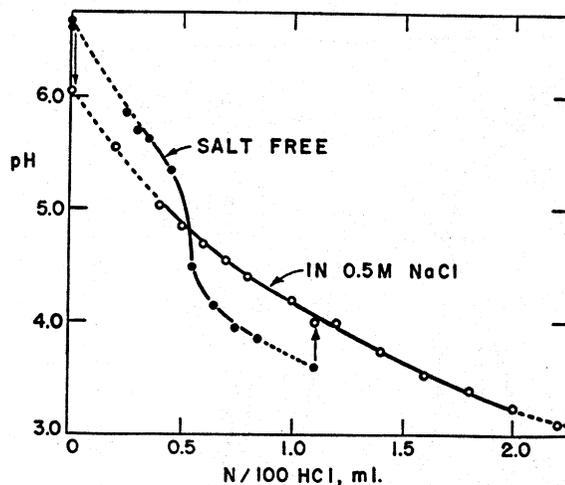


FIG. 2. HCl titration of dialyzed  $\alpha$ -lactalbumin ("salt-free"), and in the presence of 0.5 *M* NaCl. The  $\alpha$ -lactalbumin, although thoroughly dialyzed, still contains traces of salts and is not equivalent to isoionic  $\alpha$ -lactalbumin.

Nineteen milligrams of  $\alpha$ -lactalbumin in a volume of 3.0 ml. titrated with 0.01 *N* HCl. The pH changes on the addition of NaCl (90 mg.) are shown by arrows. The solid parts of the curves indicate the pH range in which a precipitate is apparent.

The isoelectric point of  $\alpha$ -lactalbumin is also evident from the sharp inflection at pH 4.8 in the titration curve as shown in Fig. 2. The pH changes on the addition of NaCl to a concentration of 0.5 *M*, and the titration in this environment are also shown, as well as the precipitation range.

### DISCUSSION

A number of observations (5) suggests that proteins change their physical configuration to some degree when salts are bound to them. The strong binding of anions by  $\alpha$ -lactalbumin, shown by shifts in the isoelectric precipitation, suggests that some change in physical configuration might be a responsible factor in the solubility transformation of  $\alpha$ -lactalbumin. It is not the mere binding of anions, which is probably rapid, that alters the solubility, but the relatively slower configuration change which follows. The solubility test in 2.0 *M* ammonium sulfate, an ionic environment, is possible because the "insoluble" component is completely precipitated and has no opportunity to be transformed to the "soluble." A change in the optical rotation would be expected for a change in physical

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configuration. The constancy of the optical rotation, salt-free and with NaCl, may reflect compensatory effects within the  $\alpha$ -lactalbumin molecule.

A significant observation in the isoelectric studies (Fig. 1), in relation to the solubility transformation, is the decrease in the isoelectric point from pH 4.8, salt-free, to 4.2 in 0.1 *M* NaCl, to 3.6 in 0.5 *M* NaCl. This change indicates a strong binding of the chloride ion. The experiments in 0.1 *M* NaCl with several concentrations of protein show that as the NaCl to protein ratio decreases, the precipitation zone broadens to the alkaline side with a consequent shift of the isoelectric point toward that of the salt-free. This is to be expected of a property that is determined by the number of ions bound.

The changes in the isoelectric point with salt concentration lead to some interesting precipitation effects. The ability of 0.1 *M* NaCl to precipitate  $\alpha$ -lactalbumin at pH 5.9 can be explained by the pH shift to 5.4, within the isoelectric precipitation range. The addition of 0.5 *M* NaCl will give much less precipitate or none at all; this occurs because the shift in the isoelectric point is much greater (to pH 3.6) than the shift in pH (to pH 5.3). Salt-free  $\alpha$ -lactalbumin is soluble at pH 3.0. The addition of 0.1 *M* NaCl will give no precipitate; the pH will rise but not to within the isoelectric zone, and there will be salting-in effects. The addition of 0.5 *M* NaCl, however, will precipitate the bulk of the  $\alpha$ -lactalbumin since the resulting pH (about 3.5) falls at the maximum precipitation point for this concentration of NaCl. Effects of this type may have promise in fractionating protein mixtures. For example, the addition of 0.5 *M* NaCl to dialyzed whey at pH 3.0 gives a precipitate that is preponderantly  $\alpha$ -lactalbumin. The experiments in Fig. 1 also show that 0.1 *M* NaCl salts-in  $\alpha$ -lactalbumin slightly (about 1 mg. per 1 ml.), an effect that is no longer apparent in 0.5 *M* NaCl.

#### ACKNOWLEDGMENTS

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#### SUMMARY

The ability of  $\alpha$ -lactalbumin to occur in two forms differing in solubility appears to depend on the binding of anions. In dialyzed solutions  $\alpha$ -lactalbumin exists preponderantly (75%) in a form insoluble in 2 *M*

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ammonium sulfate. In the presence of 0.1 *M* NaCl or a variety of other salts it is transformed largely (89 %) to a form soluble in 2 *M* ammonium sulfate. These two forms do not differ in the ultracentrifuge; in electrophoresis at 0.1 ionic strength  $\alpha$ -lactalbumin is homogeneous.

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

The study of precipitation in the isoelectric region has shown also that 0.1 *M* NaCl salts-in the  $\alpha$ -lactalbumin to some extent (about 1 mg./ml.); this effect is no longer apparent at 0.5 *M* NaCl concentration.

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