ENZYMATIC DEPHOSPHORYLATION OF OVALBUMIN AND PLAKALBUMIN

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In 1927, Sørensen and his collaborators showed that the phosphorus content of crystalline ovalbumin varies somewhat with the preparation and that this protein can be separated by "electrodialysis" into a phosphorus-poor and a phosphorus-rich fraction (1). Direct evidence of the inhomogeneity of this material was later reported by Longsworth (2) who found two electrophoretic components, A_1 and A_2, in the pH range of 5 to 10. In view of these facts Linderstrøm-Lang and Ottesen (3) suggested in 1949, that if the molecular weight of ovalbumin is taken as 44,000, the phosphorus content does not correspond to an integral number of phosphorus atoms per mole of protein and that this lack of stoichiometry might be explained by the assumption that A_1 contains 2 atoms of phosphorus per mole and A_2 1 atom. The work presented in this report was designed primarily to test this hypothesis by enzymatic dephosphorylation of ovalbumin and to compare the phosphorus content of the resulting proteins with their electrophoretic behavior. As will be shown, these experiments confirm the suggestion of Linderstrøm-Lang and Ottesen.

Materials and Methods

Materials.—Ovalbumin was prepared according to Sørensen and Høyrup (4) and the thrice recrystallized samples were stored as a paste under 80 per cent saturated ammonium sulfate at 5°C. Salt-free solutions were obtained by exhaustive dialysis of a suspension of the crystals against distilled water. These solutions, generally containing 5 to 6 gm. of protein per 100 ml., were filtered through a Coors bacteriological filter to remove insoluble protein and possible bacterial contaminants.

Plakalbumin was obtained as described by Linderstrøm-Lang and Ottesen (3) with the aid of the *B. subtilis* enzyme kindly furnished by Professor K. Linderstrøm-Lang. The protein was kept as crystals under saturated ammonium sulfate and the salt-free solutions were prepared in the same manner as described for ovalbumin.

The enzyme preparations used in this research, *i.e.* a prostate phosphatase and the alkaline phosphatase from calf intestine, were kindly supplied by Dr. Gerhard Schmidt of the Boston Dispensary. Their purification has been adequately described elsewhere (5, 6).

Methods.—The concentration of all protein solutions has been determined from

nitrogen analysis by the Pregl micro Kjeldahl method and the factor of 6.36 was used for the conversion of these measurements to a dry weight basis (4, 7).

The phosphorus content was measured according to Lohmann and Jendrassik (8).

Preliminary to the estimation of non-protein nitrogen and of inorganic phosphate released by the enzymatic action, the protein solutions were mixed with an equal volume of 20 per cent trichloroacetic acid. The protein precipitate was then separated by centrifugation and aliquots of the supernatant were used for analysis.

Electrophoresis.—The electrophoretic experiments were done in a single section cell of 11.0 ml. capacity at 0.5°C. in the apparatus described by Longsworth (9). Unless noted otherwise, a protein concentration of 1 per cent was chosen for these experiments, and prior to electrophoresis, the protein solutions were dialyzed for 2 to 3 days against liberal portions of the appropriate buffer. The concentrations of the electrophoretically separable components were calculated from the planimetric measurements of enlarged tracings. In the allocation of the area to each peak the method suggested by Pedersen (10) was used. The mobilities were then computed from the descending patterns, the boundary position being taken as that of the bisecting ordinate of the refractive index gradient curve, and refer to 0°C.

Enzymatic Experiments.—All dephosphorylation experiments were carried out in salt-free protein solutions which had been adjusted to the desired pH by the addition of a few drops of sodium hydroxide. Unless otherwise stated, prostate, or intestinal phosphatase, in concentrations of 0.2 per cent of the total protein was added and the mixture incubated at 37°C. for various lengths of time. A 5.0 ml. sample of the mixture was then deproteinized with an equal volume of 20 per cent trichloroacetic acid and after removal of the protein precipitate the inorganic phosphate and non-protein nitrogen determined. A second aliquot was diluted to 1 per cent with a sodium phosphate buffer of pH 6.8 and 0.1 ionic strength and analyzed electrophoretically. Since the presence of inorganic phosphate at this concentration completely inhibits the activity of the enzyme, the use of this buffer solvent in the electrophoretic analysis afforded a convenient means for stopping the dephosphorylation process.

RESULTS

The ovalbumin preparations used in this study are characterized by a marked constancy in the electrophoretic composition and in the phosphorus content. Thus, the patterns shown in Fig. 1, obtained after electrophoresis of a 1 per cent ovalbumin solution in a 0.1 ionic strength sodium phosphate buffer of pH 6.8 at a potential gradient of 6.2 volts per cm., are representative examples. In this buffer the A_1 component of all preparations investigated has a mobility of -5.9×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} and accounts for 80 to 85 per cent of the total protein, whereas A_2, with a mobility of -4.9×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} is present as 15 to 20 per cent. Occasionally, a small amount of a still slower moving component, A_3, is noticeable in the electrophoretic patterns. The phosphorus content of these preparations was found to be 8.0 to 8.2 mg. per gm. of protein nitrogen which corresponds to 1.8_0 to 1.8_5 atoms per mole.

If such preparations are stored as a paste under saturated ammonium sulfate at 3°C. for as long as 8 to 10 years, no change in the electrophoretic composition occurs and the phosphorus content remains constant. Likewise, if bacterial contamination of a salt-free isoelectric ovalbumin solution is prevented by filtration through a bacteriological filter, it can be kept unaltered with respect to its electrophoretic behavior and the phosphorus both at 3 and 37°C. At the higher temperature, however, marked surface denaturation occurs after several months.

In the patterns shown in Fig. 1, the A_1 component represents 85 per cent of the total protein and A_2 15 per cent. Assuming that A_1 contains 2 atoms of phosphorus and A_2 1 atom, the phosphorus content computed from the electrophoretic composition is $2 \times 0.85 + 0.15 = 1.8_5$. This is in good agreement with the chemically determined value of 1.8_2, and thus affords a confirmation

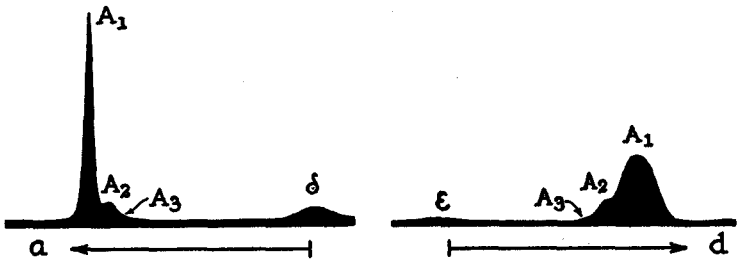


FIG. 1. Electrophoretic patterns of a 1 per cent ovalbumin solution in sodium phosphate buffer of pH 6.8 and ionic strength 0.1 after electrophoresis for 12,600 seconds at 6.2 volts per cm.

of the suggestion of Linderstrøm-Lang and Ottesen concerning the phosphorus content of ovalbumin (3).

Work on a direct test of this hypothesis, namely a study of the phosphorus content of purified A_1 and A_2 proved difficult. The similarity of the solubilities of these proteins has thus far prevented a fractionation by conventional chemical methods. However, a small amount of pure A_1 was separated electrophoretically in 0.1 N sodium bicarbonate as solvent and had a phosphorus content of 8.94 mg. per gm. of protein nitrogen, corresponding accurately to 2 atoms of phosphorus per mole. Because of the spreading of the descending boundaries, preparation of pure A_2 in this manner is not practicable. Consequently, the preparation of A_2 and also of A_3 by enzymatic dephosphorylation of ovalbumin was undertaken.

The Enzyme Reaction.—A prerequisite in the selection of the enzymes for this work is that the dephosphorylation process should not be accompanied by any other enzymatic reactions that might result from the presence of small amounts of impurities in even highly purified phosphatase preparations; in

particular, an extensive proteolysis has to be excluded. These considerations led to the choice of the prostate phosphatase active at pH 5.3 and the enzyme from calf intestine whose pH optimum has been reported to be at pH 9.0.

Thus far, it had not been possible to dephosphorylate phosphoproteins, such as casein (5), with these enzymes. However, they act readily on ovalbumin (11). The presence of buffer ions, *e.g.* acetate in the case of the prostate enzyme, has an inhibitory effect on the dephosphorylation process. The results listed in Table I illustrate clearly that the activity of the enzyme is reduced if acetate is added to the system and that this effect is dependent on the concentration of this ion. Similarly diethylbarbiturate inhibits slightly the dephosphorylation of ovalbumin with the intestinal phosphatase. Therefore, all experiments were carried out in salt-free solutions as described under Methods, advantage being taken of the buffer action of the protein.

TABLE I

Influence of Acetate Ions on the Dephosphorylation of Ovalbumin with Prostate Phosphatase

Each reaction mixture contained 48.2 mg. ovalbumin, 50 μg. prostate phosphatase, and sodium acetate buffer of pH 5.35. Incubation was carried out at 37°C. for 90 minutes.

Experiment No. (1)	Final concentration of acetate (2)	Phosphorus liberated (3)	Inhibition (4)
	molar	*μg.*	*per cent*
1	None	22.2	None
2	0.06	14.7	33.8
3	0.12	11.8	46.9
4	0.26	8.1	63.5

Dephosphorylation of Ovalbumin with Prostate Phosphatase.—As is shown in Fig. 2, in which the amount of phosphorus liberated in a given time interval is plotted as ordinate against the pH as abscissa, the prostate phosphatase dephosphorylates ovalbumin in the pH range of 4.6 to 6.6. However, dephosphorylation stops after about 46 per cent of the total phosphorus has been set free. Addition of fresh enzyme does not alter this result, thus excluding an inactivation or inhibition of the enzyme. In an ovalbumin preparation consisting of 85 per cent A_1, each of the 2 phosphorus atoms per mole of this component represents 46 per cent [$= 100 \times 85/(2 \times 85 + 15)$] of the total phosphorus. Therefore, the liberation of this amount suggests that the prostate phosphatase attacks only one of the two phosphate groups of the ovalbumin component, A_1, thus transforming it into a protein with 1 phosphorus per mole and with an electrophoretic mobility similar to the A_2 component of the starting material. That this is the actual case is borne out by the results of a typical experiment summarized in Fig. 3, in which ovalbumin was exposed to the prostate enzyme for various times.

From the electrophoretic patterns shown in column 1 of Fig. 3, it is ap-

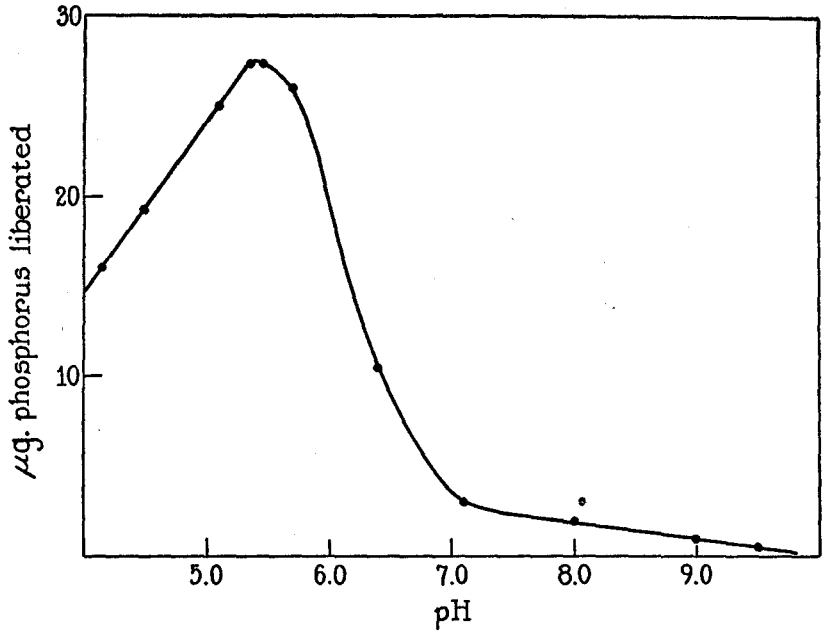


FIG. 2. Dephosphorylation of ovalbumin with prostate phosphatase as function of pH. Each reaction mixture contained 46 mg. ovalbumin and 0.1 mg. enzyme. Incubation was carried out at 37°C. for 6 hours.

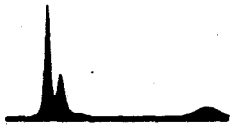
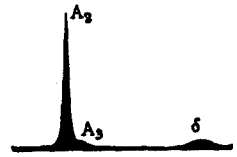
	Time in hours	Electrophoretic composition	Atoms phosphorus per mole protein	
			Computed	Observed
	0	85% A_1 14% A_2 trace A_3	1.8_4	1.8_2
	1/2	58% A_1 40% A_2 trace A_3	1.5_6	1.4_8
	3/4	47% A_1 49% A_2 4% A_3	1.4_3	1.3_5
	1 3/4	36% A_1 58% A_2 6% A_3	1.3_0	1.2_0
	7	94% A_2 6% A_3	0.9_4	0.9_7

FIG. 3. Dephosphorylation of ovalbumin with prostate phosphatase as function of time.

parent that the A_2 component progressively increases at the expense of A_1 as the enzyme reaction proceeds. Simultaneously, the protein phosphorus of each sample, a value derived from the difference between the total and inorganic phosphorus, decreases. If the phosphorus content is then computed from the electrophoretic composition of each mixture, (column 3), in the manner indicated above, the agreement between the chemically determined values, (column 5), and those derived from the electrophoretic analyses is excellent. After 7 hours A_1 has been converted into a protein with an electro-

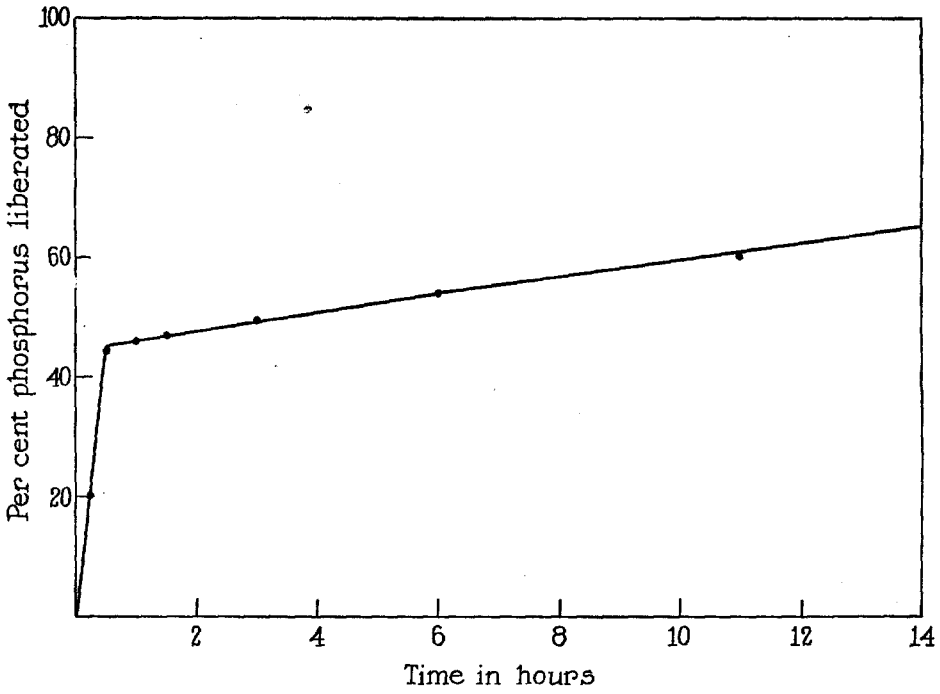


FIG. 4. Dephosphorylation of ovalbumin with intestinal phosphatase as function of time. Each reaction mixture contained 46 mg. ovalbumin and 0.06 mg. enzyme. Incubation was carried out at 37°C. for 6 hours.

phoretic behavior similar to that of the A_2 component of the starting material and which contains 1 atom of phosphorus per mole of protein. Exposure to the enzyme for 24 or 48 hours did not alter the result. Moreover, the absence of non-protein nitrogen in the trichloroacetic acid filtrate shows that this transformation involves only the removal of one phosphate group from A_1 and thus represents a highly specific reaction.

Dephosphorylation of Ovalbumin with the Intestinal Phosphatase.—In an attempt to obtain complete dephosphorylation of ovalbumin the intestinal enzyme was then tested; but as is shown in Fig. 4, this reaction is characterized by a greater complexity.

In the first attempt to obtain phosphorus-free ovalbumin, intestinal phosphatase was added to a salt-free ovalbumin solution of pH 9.0. As illustrated in Fig. 4, the protein is rapidly dephosphorylated until 46 per cent of the phosphorus is liberated. At this point, electrophoretic analysis reveals that A_1 is again converted into a protein with the electrophoretic properties of A_2. Dephosphorylation continues but at a much slower rate. This further loss of phosphorus is reflected by the appearance of a new component, A_3, which in the sodium phosphate buffer of pH 6.8 and 0.1 ionic strength, migrates with a mobility of -4.4×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. Comparison of the phosphorus analyses of several such mixtures with the electrophoretic patterns indicates that A_3 does not contain phosphorus. Dephosphorylation invariably stopped, however, after 70 to 75 per cent of the phosphorus had been liberated. This is due to the fact that at pH 9.0 and 37°C. a slow but measurable denaturation of the protein occurs that apparently counteracts the dephosphorylation process. The enzyme itself remains active.

In a further search to establish experimental conditions under which this reaction could be carried to completion, it was next observed that although intestinal phosphatase acts on low molecular weight phosphate esters with -O-P- linkages at an alkaline pH only, this enzyme will liberate phosphorus from ovalbumin at pH 5.3 as well as at pH 9.0. As is shown in Fig. 5, two pH maxima exist and under the experimental conditions chosen the amount of phosphorus liberated at pH 5.3 is only slightly smaller than that obtained at the alkaline pH.

Although ovalbumin is stable at pH 5.3 and 37°C., it has been reported that alkaline phosphatases are slowly inactivated at this pH (12). This observation was confirmed, but it also was noted that in the presence of an inert protein, *e.g.* bovine serum albumin, the loss of activity in a given time is smaller. The stabilizing effect on the enzyme by such a protein may explain why a complete dephosphorylation of ovalbumin is obtained if the pH of the reaction mixture is controlled by the buffer action of the protein.

This observation led to the following procedure for the preparation of phosphorus-free ovalbumin, A_3: Ovalbumin is dephosphorylated to the phosphorus-poor protein, A_2, with the prostate phosphatase and crystallized from ammonium sulfate. Subsequently, a salt-free solution of this protein is incubated with the intestinal enzyme at pH 5.3. Even in this salt-free solution a slow inactivation of the enzyme occurs, and a relatively high phosphatase concentration of 1 per cent of the total protein was necessary to effect complete dephosphorylation before the enzyme activity was lost. Moreover, since both Mg^{++} (5) and Mn^{++} activate this enzyme slightly, either 1×10^{-3} M Mg^{++} or 1×10^{-4} M Mn^{++} was added to the solution. Under these conditions, the reaction goes to completion within 12 hours and a phosphorus-free ovalbumin is obtained.

It was noticed that the dephosphorylation with the intestinal enzyme was

accompanied by the formation of 0.2 to 0.8 per cent non-protein nitrogen. Since, however, the amount of the trichloroacetic acid–soluble nitrogen varies (*a*) from one enzyme preparation to another, (*b*) with the time of incubation, and (*c*) with the pH of the reaction mixture, it originates most likely from

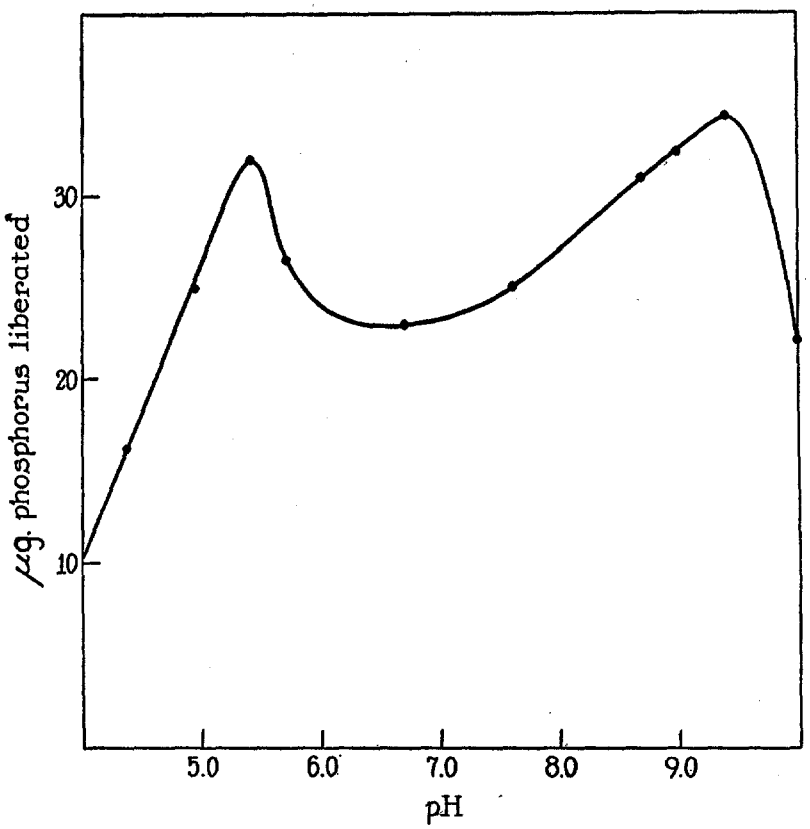


FIG. 5. Dephosphorylation of ovalbumin with intestinal phosphatase as function of pH. Each reaction mixture contained 46 mg. ovalbumin and 0.06 mg. enzyme. Incubation was carried out at 37°C. for 6 hours.

traces of proteolytic enzymes present as impurity in the phosphatase preparation.

Properties of the Dephosphorylated Ovalbumins.—Except for the phosphorus content and the mobility, all three proteins, *i.e.* ovalbumin which is the naturally occurring mixture of A_1, A_2, and A_3, the monophosphorus A_2, and the phosphorus-free A_3 have similar properties. They crystallize readily as *needles* from 60 per cent saturated ammonium sulfate in the pH range of 4.6 to 5.6. No significant changes have thus far been observed in their stability towards acid and alkali; and their electrophoretic behavior is not altered on

successive crystallizations. However, it has not been possible to remove from the A_3 preparations the 3 to 4 per cent of a slower moving component.

The most striking difference between the three proteins is in their electrophoretic properties. Thus in the sodium phosphate buffer of pH 6.8 and 0.1 ionic strength, after electrophoresis for 12,600 seconds at a potential gradient

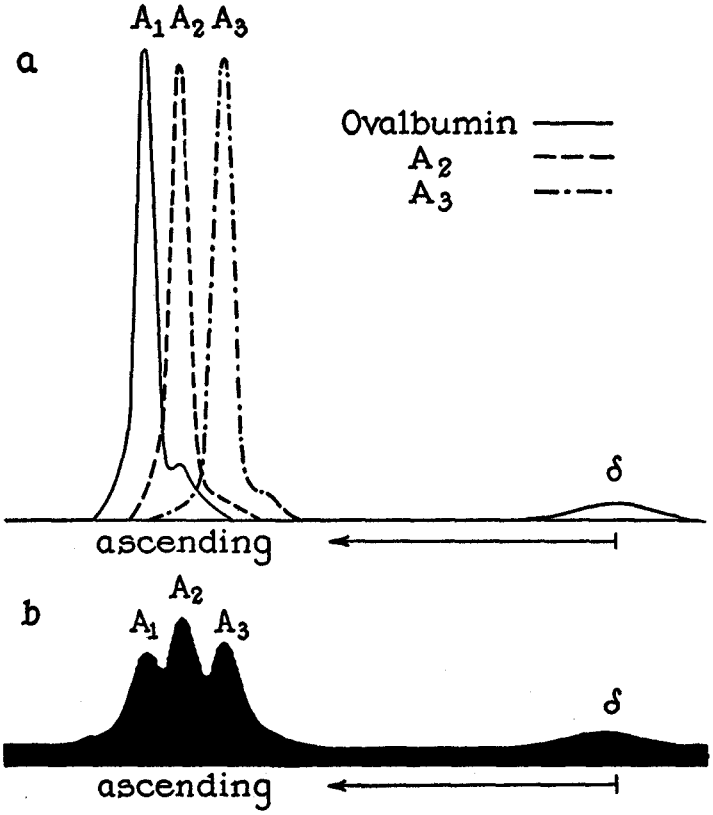


FIG. 6. (*a*) Superimposed tracings of the patterns of ovalbumin, A_2 and A_3. (*b*) Pattern of a mixture of equal quantities of ovalbumin, A_2 and A_3. Electrophoresis was carried out in sodium phosphate buffer of pH 6.8 and ionic strengh 0.1 for 12,600 seconds at 6.1 volts per cm.

of 6.1 volts per cm., the monophosphorus A_2 and the phosphorus-free A_3 give patterns as presented in the superimposed tracings of Fig. 6 *a*, which for comparison also shows that of ovalbumin. The mobilities of $u_{A_2} = -5.2 \times 10^{-5}$ and $u_{A_3} = -4.5 \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1} are but slightly more negative than the values reported above for the measurements on the A_2 and the A_3 components of ovalbumin. The different mobilities of A_1, A_2, and A_3 are further illustrated with the patterns of Fig. 6 *b* which was obtained with a mixture of equal quantities of A_1, A_2, and A_3 at a total concentration of 0.75 per cent.

Because of these electrophoretic characteristics in the phosphate buffer, it seemed of interest to compare the mobilities of the three proteins at other pH values. As is indicated in the first column of Table II, these measurements were done in a series of monovalent buffers whose pH values, at room temperature, are given in the second column. The mobilities of A_1, listed in column 3, refer to the fast moving component of ovalbumin; those for A_2 and A_3, columns 4 and 5, are the ones obtained after treatment with the prostate and intestinal enzyme, respectively. The differences accompanying the dephosphorylation steps, $A_1 \rightarrow A_2$ and $A_2 \rightarrow A_3$, are shown in columns 6 and 7. As previously noted (13), below pH 4 the ovalbumin components A_1 and A_2 have

TABLE II

Mobilities of the Ovalbumin Component, A_1, and the Dephosphorylated Ovalbumins A_2 and A_3 in Buffer Solutions of Ionic Strength 0.1

Buffer (1)	pH (2)	$u \times 10^5$			Δu	
		A_1 (3)	A_2 (4)	A_3 (5)	$A_1 \rightarrow A_2$ (6)	$A_2 \rightarrow A_3$ (7)
0.1 N HCl-0.5 N glycine	3.05	6.2_5	6.2_5	6.4_0		
0.02 N NaAc-0.1 N HAc-0.08 N NaCl	3.91	2.7_8	2.8_0	2.9_0		
0.1 N NaAc-0.14 N HAc	4.50		0.7_0			
0.1 N NaAc-0.1 N HAc	4.64	-0.3_0	0.0	0.4_8	0.3	0.48
0.1 N NaAc-0.08 N HAc	4.76		-0.5_0	-0.2_0		0.3
0.1 N NaAc-0.01 N HAc	5.65	-3.5_6	-3.1_6	-2.7_4	0.4	0.4
0.02 N NaCac-0.004 N HCac-0.08 N NaCl	6.79	-5.1_6	-4.6_0	-4.1_8	0.56	0.42
0.02 N NaV-0.02 N HV-0.08 N NaCl	7.82	-5.7_6	-5.2_0	-4.6_9	0.56	0.51
0.1 N NaV-0.02 N HV	8.60	-5.8_2	-5.2_3	-4.6_0	0.59	0.63
0.1 N NaV-0.005 N HV	9.10	-5.9_3	-5.4_0	-4.9_0	0.53	0.50

Ac = acetate; Cac = cacodylate; V = diethylbarbiturate.

the same mobility. The present work indicates that this is also true for the dephosphorylated proteins, A_2 and A_3. Above that pH the mobilities diverge until a constant difference of about 0.6×10^{-5} is reached in the interval of pH 7 to 9.

As in the ovalbumin → plakalbumin transformation (14), the mobility shifts which accompany the dephosphorylation process can be used to estimate a change in the net charge of the proteins. If the removal of the phosphorus is not accompanied by an alteration in (*a*) the friction coefficient of the protein, (*b*) the binding of ions other than hydrogen ions, and (*c*) the value of du/de, the difference of 0.6×10^{-5} corresponds to a change in the net charge of -2. Here, du/de is the slope of the line obtained by plotting the mobility against the titration increment, and for ovalbumin (15) at an ionic strength of 0.1 has a value of 0.3×10^{-5} (14). If this interpretation is correct, it may

be inferred that two ionizable hydrogens are involved in the removal of each phosphorus and that these are dissociated at neutral pH.

In another series of experiments the isoelectric pH values, pI, of A_2 and A_3 were obtained at several ionic strengths from mobility measurements in the range from pH 4.5 to 5.0. The results are given in Table III, together with those reported by Tiselius and Svensson for ovalbumin (column 6) (16).

For these experiments, a protein concentration of 0.5 to 0.6 per cent was chosen. It is of interest that both the A_2 and A_3 preparations, although 96 to 97 per cent homogeneous at other pH values, showed a complex behavior on electrophoresis at low ionic strengths just below the isoelectric pH similar

TABLE III

Dependence of the Isoelectric pH on Ionic Strength

(1)	A_2		A_3		pI			ΔpI	
	pH* (2)	$u \times 10^5$ (3)	pH* (4)	$u \times 10^5$ (5)	A‡ (6)	A_2 (7)	A_3 (8)	$A \rightarrow A_2$ (9)	$A_2 \rightarrow A_3$ (10)
0.1	4.52	0.6_9	4.64	0.4_8	4.58	4.65	4.74	0.07	0.09
	4.76	-0.5_6	4.76	-0.1_0					
0.05	4.66	0.3	4.74	0.2_0	4.63	4.70	4.80	0.07	0.10
	4.88	-1.0_9	4.80	0.0					
0.02	4.75	0.0	4.75	0.6	4.68	4.75	4.85	0.07	0.10
	4.87	-0.8_0	4.87	-0.1_2					
0.01	4.80	0.0	4.88	0.2	4.71	4.80	4.90	0.09	0.10
	4.92	-1.0_7	4.96	-0.9_0					

* pH values refer to 0°C. by applying a correction of 0.02 to the values measured at 25°C. (16).

‡ Taken from Tiselius and Svensson (16).

to that reported for bovine serum albumin (17). Consequently, the first moment of the entire gradient curve was used for the computation of the mobilities reported in Table III. The isoelectric pH at each ionic strength was then obtained as the point at which the straight line through the two mobility values intersected the pH axis.

As is shown by the data of this table, the shift of the isoelectric pH values of A_2 and A_3 with the ionic strength parallels that observed by Tiselius and Svensson for ovalbumin. It will also be noted that a constant difference of 0.07 pH unit was found in the $A_1 \rightarrow A_2$ reaction, and of 0.1 in the $A_2 \rightarrow A_3$ transformation (columns 9 and 10).

Dephosphorylation of Plakalbumin.—In 1947 Linderstrøm-Lang and Ottesen showed that a proteolytic enzyme from *B. subtilis* transforms ovalbumin into

a new protein that crystallizes as *plates* and was named plakalbumin (3). As shown by the Danish workers, the formation of plakalbumin involves the liberation of several amino acids, including aspartic acid (18). It was found in this laboratory that the electrophoretic components of plakalbumin, P_1 and P_2, exist in the same ratio as the A_1 and A_2 constituents of the parent substance ovalbumin, but that the mobilities of P_1 and P_2 are lower (14). In the sodium phosphate buffer of pH 6.8 and 0.1 ionic strength, P_1 has a mobility of -5.5×10^{-5} and P_2 -4.3×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}, respectively. As discussed elsewhere this change in the mobility may be explained by the loss, during the A $\rightarrow$ P transformation, of two negatively charged groups (14).

Inasmuch as plakalbumin has the same phosphorus content as ovalbumin (3, 19), it can be assumed that P_1 contains 2 atoms of phosphorus per mole and P_2 1 atom. Thus, it also should be possible to transform enzymatically

TABLE IV

Comparison of the Properties of Plakalbumins and Ovalbumins

Protein	Plakalbumins			Ovalbumins		
	P_1	P_2	P_3	A_1	A_2	A_3
Crystal form	Plates	Plates	Plates	Needles	Needles	Needles
Atoms phosphorus per mole of protein*	2	1	0	2	1	0
$u \times 10^5$‡	−5.5	−4.6	−3.8	−6.1	−5.2	−4.5
Isoelectric pH at 0.1 μ	4.72	4.8		4.58	4.65	4.74

* Assuming a molecular weight of 44,000.

‡ Electrophoresis was carried out in sodium phosphate buffer of pH 6.8 and 0.1 ionic strength.

P_1 into P_2. In preliminary experiments *B. subtilis* was grown in salt-free plakalbumin solutions and the bacterial degradation of the protein was followed with phosphate determinations and electrophoretic analyses. Although the phosphorus content, if calculated from the electrophoretic composition of each mixture, correlated closely with the actual protein-phosphorus, an appreciable formation of non-protein nitrogen accompanied the dephosphorylation process and obscured the significance of these results (19).

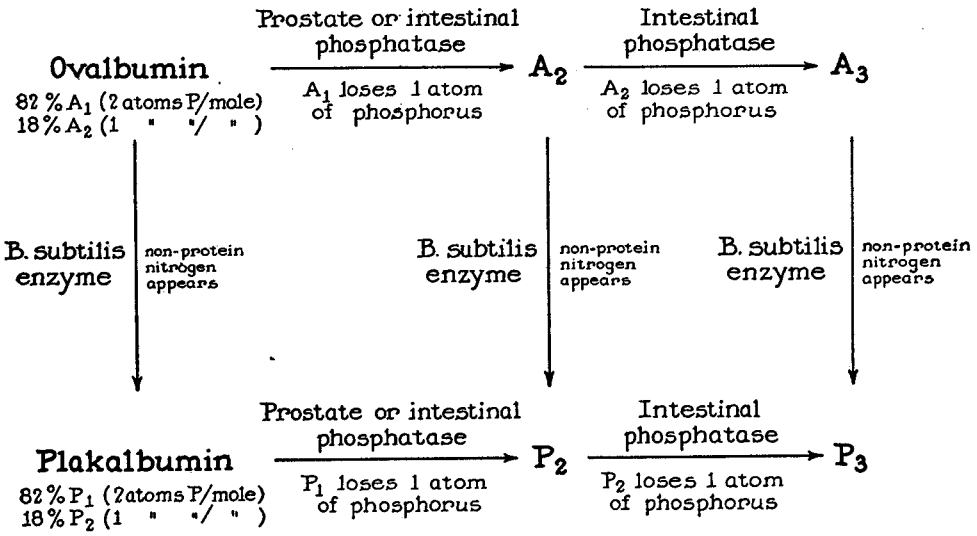
Subsequently it was found that both the prostate and the intestinal phosphatase dephosphorylate plakalbumin in a manner similar to their action on ovalbumin (11). Both the monophosphorus plakalbumin, P_2, and the phosphorus-free P_3 crystallize as *plates*. They differ from the corresponding ovalbumins in their electrophoretic behavior and also in their solubility (3). The properties of these proteins upon which their identification is based are summarized in Table IV together with the corresponding data for the three ovalbumins.

In contrast with the ovalbumins, storage of P_2 or P_3 as salt-free solutions leads to the loss of crystallizability. No detectable changes, however, are observed if the proteins are kept as crystals under saturated ammonium sulfate. Moreover, it was observed that the plakalbumins are more readily denatured by acid than the corresponding ovalbumins.

In the preparation of P_2 and P_3 described above, proteolysis preceded dephosphorylation. If this order is reversed and A_2 or A_3 is treated with the proteolytic enzyme from *B. subtilis*, 1.2 to 1.5 per cent non-protein nitrogen is liberated. The resulting proteins crystallize as *plates* and have the same electrophoretic behavior as P_2 and P_3 prepared by dephosphorylation of plakalbumin.

DISCUSSION

The results of the studies on the stepwise dephosphorylation of ovalbumin and plakalbumin and of the ovalbumin → plakalbumin transformation can be summarized by the following scheme:



Here it is apparent that by the proper selection of three enzymes, five different modifications of ovalbumin are obtained, all of which are crystallizable. These are the monophosphorus ovalbumin, A_2, the phosphorus-free ovalbumin, A_3, both derived from the naturally occurring mixture of A_1 and A_2 by selective dephosphorylation, and the corresponding three plakalbumins. Although these proteins are well defined chemically and have a marked stability, they represent successive steps in a mild degradation, perhaps also in the synthesis of ovalbumin.

In this research, the modified ovalbumins have been identified on the basis of only three characteristics: (*a*) the electrophoretic behavior, (*b*) the crystal form, and (*c*) the phosphorus content. No analytical methods capable of detecting other differences have been used. It therefore should be borne in mind that such may exist; for instance, the partially dephosphorylated ovalbumin, designated here as A_2 may differ from the original A_2 component of ovalbumin by small variations in the amino acid content. Consequently, until such information is available the enzymatically modified ovalbumins cannot be looked upon as pure substances. The mobility increments between A_1, A_2, A_3 and P_1, P_2, P_3, can, however, be interpreted as due to the loss of the charged phosphate groups.

Several observations reported in the literature can now be explained with the aid of this reaction scheme. Mâcheboeuf, Sørensen, and Sørensen (1) reported that the phosphorus content, and in certain cases also the solubility of some of their ovalbumin preparations, changed if the protein was stored as a salt-free solution. In 1944, MacPherson, Moore, and Longsworth described an $A_1 \rightarrow A_2$ transformation which had occurred in an aged salt-free ovalbumin solution (20). A phosphorus-free plakalbumin was recovered by Linderstrøm-Lang and Ottesen from a 20 year old ovalbumin,[1] and in 1948 a plakalbumin with the electrophoretic properties of P_2 was found in this laboratory (21). Since this P_2 preparation was contaminated with *B. subtilis*, it is quite clear that the changes observed by these authors at different times are the result of enzymatic action due to bacterial contamination and that the proteins are similar to those reported in this paper.[2]

A few additional features of the work presented here merit comment. These experiments are the first successful attempts to dephosphorylate a protein without the simultaneous liberation of a considerable amount of non-protein nitrogen, using enzymes of the "classical" phosphatase type from mammalian tissue (12). The only work of similar nature is the dephosphorylation of phosphovitin (23, 24) and casein (24) with an enzyme from citrus fruit.

Of equal interest is the failure of the prostate phosphatase to attack the second phosphorus of A_1. It is known that a phosphate group present as a diester resists the action of the prostate enzyme (6) and thus might account for the difficulty in removing the phosphorus from A_2 and P_2. The mobility changes observed in the $A_1 \rightarrow A_2$ and $A_2 \rightarrow A_3$ transformations are hardly consistent with this possibility. On the other hand, esterification of the two phos-

[1] This protein, in the sodium phosphate buffer of pH 6.8 and ionic strength 0.1, however, had a mobility of -4.3×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} which is more negative than that given in Table IV for P_3.

[2] Toluene added to a protein solution does not necessarily prevent bacterial contamination, but may be the factor determining whether proteolysis or a dephosphorylation process prevails (22).

phate groups to different amino acid residues might explain the specificity observed with this enzyme.

In preliminary experiments directed toward learning the points of attachment of the phosphorus, a phosphorus-containing peptide was isolated from a partial proteolytic hydrolysate of ovalbumin by starch column chromatography (25). This peptide fraction contained glutamic acid, aspartic acid, alanine, and serine.[3] Since serinephosphate has been isolated from hydrolysates of vitellinic acid (26) and casein (27), the simultaneous occurrence of phosphorus and serine in one fraction makes it reasonable to suppose that part of the phosphorus in ovalbumin is bound to this amino acid. Moreover, it has been found that intestinal phosphatase dephosphorylates low molecular weight esters with an amide-phosphorus linkage, both at pH 5.3 and 9.0, as in the case of ovalbumin, but that such a substrate is not attacked by the prostate enzyme. This observation may perhaps be taken as an indication of differences in the chemical nature of the phosphate linkages in ovalbumin.

SUMMARY

It has been shown by the work presented in this paper that it is possible to carry out a stepwise dephosphorylation of ovalbumin. With the aid of a prostate phosphatase that attacks only one of the two phosphorus-containing groups in the major component, A_1, of ovalbumin, a protein, A_2, containing 1 atom of phosphorus per mole has been prepared. Further dephosphorylation with an enzyme of intestinal origin gives a phosphorus-free ovalbumin, A_3. Plakalbumin has been similarly dephosphorylated to give P_2 and P_3. Significant changes in the electrophoretic mobility accompany each dephosphorylation step. This, together with the phosphorus content of the proteins and the crystal form, has been used to characterize and study the five modifications of ovalbumin thus produced.

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BIBLIOGRAPHY

1. Mâcheboeuf, M., Sørensen, M., and Sørensen, S. P. L., *Compt.-rend. trav. Lab. Carlsberg*, 1927, **16**, No. 12.
2. Longsworth, L. G., *J. Am. Chem. Soc.*, 1939, **61**, 529.

[3] These experiments were carried out at the Carlsberg Laboratory, Copenhagen. It is a great pleasure to thank Professor K. Linderstrøm-Lang for the privilege of working in his Laboratory.

3. Linderstrøm-Lang, K., and Ottesen, M., *Compt.-rend. trav. Lab. Carlsberg*, 1949, **26,** No. 16.
4. Sørensen, S. P. L., and Høyrup, M., *Compt.-rend. trav. Lab. Carlsberg*, 1915–1917, **12.**
5. Schmidt, G., and Thannhauser, S. J., *J. Biol. Chem.*, 1943, **149,** 369.
6. Schmidt, G., Cubiles, R., and Thannhauser, S. J., *Cold Spring Harbor Symp. Quant. Biol.*, 1947, **12,** 161.
7. Perlmann, G. E., and Longsworth, L. G., *J. Am. Chem. Soc.*, 1948, **70,** 2719.
8. Lohmann, K., and Jendrassik, L., *Biochem. Z.*, 1926, **178,** 419.
9. Longsworth, L. G., *Chem. Rev.*, 1942, **30,** 323; *Ind. and Eng. Chem., Analytical Edition*, 1946, **18,** 219.
10. Pedersen, K. O., *in* The Ultracentrifuge, (T. Svedberg and K. O. Pedersen, editors), London, Oxford University Press, 1940, 296.
11. Perlmann, G. E., *Nature*, 1950, **166,** 870.
12. Roche, J., and Thoai, N., *Advances Enzymol.*, 1950, **10,** 83.
13. Longsworth, L. G., Cannan, R. K., and MacInnes, D. A., *J. Am. Chem. Soc.*, 1940, **62,** 2580.
14. Perlmann, G. E., *J. Am. Chem. Soc.*, 1949, **71,** 1146.
15. Cannan, K., Kibrick, A. C., and Palmer, A. H., *Ann. New York Acad. Sc.*, 1941, **41,** 243.
16. Tiselius, A., and Svensson, H., *Tr. Faraday Soc.*, 1940, **36,** 16.
17. Longsworth, L. G., and Jacobsen, C. F., *J. Physic. and Colloid Chem.*, 1949, **53,** 126.
18. Eeg-Larsen, N., Linderstrøm-Lang, K., and Ottesen, M., *Arch. Biochem.*, 1948, **19,** 340.
19. Perlmann, G. E., *Nature*, 1949, **164,** 961.
20. MacPherson, C. E. C., Moore, D. M., and Longsworth, L. G., *J. Biol. Chem.*, 1944, **156,** 381.
21. Perlmann, G. E., *Nature*, 1948, **161,** 720.
22. Perlmann, G. E., *Tr. Conf. Connective Tissues*, Josiah Macy, Jr. Foundation, New York, 1950, 101.
23. Axelrod, B., *J. Biol. Chem.*, 1947, **167,** 57.
24. Mecham, D. K., and Olcott, H. S., *J. Am. Chem. Soc.*, 1949, **71,** 3670.
25. Perlmann, G. E., Abstracts, 119th Meeting, American Chemical Society, April, 1951.
26. Lipmann, F., and Levene, A., *J. Biol. Chem.*, 1932, **98,** 109.
27. Lipmann, F., *Biochem Z.*, 1933, **262,** 1.