CXXIII. ON THE REACTION BETWEEN METAPHOSPHORIC ACID AND EGG ALBUMIN

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METAPHOSPHORIC acid is considered to be a protein-precipitating reagent. Pohl [1889], Lorenz [1890], and Fuld [1902] analysed precipitates obtained from proteins with metaphosphoric acid. Their results did not lead to any important interpretation because at that time the knowledge of protein chemistry was not yet sufficiently developed.

In 1935 Schofield gave theoretical reasons for assuming a chemical combination between metaphosphoric acid and the positively charged amino-groups of the protein molecules. Since no experimental work on the question has been published hitherto, we decided to attack this problem with improved analytical methods.

We were well aware of the difficulty that solutions of metaphosphoric acid contain various association-products and certain amounts of sodium metaphosphate and orthophosphoric acid which might complicate the reaction to be studied. During our investigations we did not, however, observe any indication that other forms of metaphosphoric acid than the simple PO_3^- ion take part in the reaction. The same opinion has already been expressed by Pascal [1924] on the basis of his studies of the inorganic reactions of metaphosphoric acid.

Experimental

In our first experiments we investigated the influence of the concentration of the metaphosphoric acid on the amount and composition of the precipitate.

The protein used was egg albumin, thrice crystallized from ammonium sulphate [Sørensen & Høyrup, 1917]. The product so obtained was dissolved in water and dialysed at 0° till no ammonium sulphate could be detected in the solution with Nessler's reagent and barium chloride. The solution of metaphosphoric acid was freshly prepared for each experiment from a sample Schering-Kahlbaum *pro analysi*.

5 ml. samples of egg albumin solution were mixed with increasing amounts of 0.1N metaphosphoric acid and the resulting precipitates centrifuged and washed carefully with distilled water. The washings were tested for protein with ammonium sulphate and with metaphosphoric acid and for metaphosphoric acid with a protein solution and by the method mentioned below. When the filtrates were found free from both, the precipitates were dissolved in 10 ml. concentrated sulphuric acid in a boiling water bath and analysed. Nitrogen was determined by the micro-Kjeldahl method, phosphorus as described by Lohmann & Jendrassik [1926].

In the preceding experiments the variation of the PO_3^- concentration caused a shift of the hydrogen ion concentration. To avoid this complication we carried out a series of experiments at constant pH.

Table I. *Dependence of the amount and the phosphorus content of the precipitate from egg albumin and metaphosphoric acid on the concentration of metaphosphoric acid.*

Protein solution %	mM HPO_3 added per protein solution	pH of the filtrate	Precipitate		
			mg. total N per ml. of protein solution	mg. bound P per ml. of protein solution	P/N ratio $\times 100$
1.6	1.5×10^{-2}	—	0.72	0.075	10.4
	2.0	—	1.10	0.145	12.6
	2.5	—	2.06	0.19	9.25
	3.0	—	2.11	0.27	12.8
	4.0	—	2.20	0.35	15.9
	5.0	—	2.20	0.35	15.9
1.8	1.5×10^{-2}	4.4	0.90	0.075	8.35
	2.0	4.2	1.12	0.145	12.9
	2.5	3.9	2.17	0.20	9.20
	3.0	3.7	2.38	0.28	11.7
	4.0	3.1	2.88	0.46	15.9
	5.0	2.7	2.90	0.47	15.7

Fig. 1.

Fig. 2.

Fig. 3.

Table II. *Dependence of the amount and the phosphorus content of the precipitate from egg albumin and metaphosphoric acid on the metaphosphate ion concentration at constant pH.*

Protein solution %	mM HPO_3 added per ml. protein solution	pH of the filtrate	Precipitate		
			mg. total N per ml. of protein solution	mg. bound P per ml. of protein solution	P/N ratio $\times 100$
3.32	1.0×10^{-1}	3.88	1.7	0.148	10.8
	1.5	3.89	2.1	0.225	10.7
	2.0	3.88	2.2	0.230	10.7
	4.0	3.91	2.15	0.230	10.7
	6.0	3.90	2.1	0.230	11.0
2.9	0.5×10^{-1}	3.69	2.58	0.303	11.5
	1.0	3.69	3.94	0.455	11.5
	2.0	3.69	4.07	0.455	11.4
	5.0	3.70	3.47	0.457	13.1
3.32	1.0×10^{-1}	3.30	1.66	0.210	12.6
	1.5	3.31	3.30	0.430	13.0
	2.0	3.29	4.80	0.660	13.7
	5.0	3.15	5.40	0.832	15.4

5 ml. samples of egg albumin solution were mixed with various amounts of a neutralized solution of metaphosphoric acid (pH 7.0). The solution remained perfectly clear. On adding N acetic acid the egg albumin was precipitated. The concentrations of acetic acid in the protein solution were 0.6, 0.8 and 2.0 % and these corresponded respectively to final pH of 3.9, 3.7 and 3.3. In this way the pH was kept constant throughout each series as shown in Table II. The pH of the filtrate was controlled by measurements with a glass electrode.

Properties of the precipitate. The compound of egg albumin and metaphosphoric acid is easily soluble in salt solutions (sodium chloride, half-saturated ammonium sulphate and buffer solutions from pH 4.5 to the alkaline side). This observation was astonishing because it has often been supposed that the precipitation of proteins with precipitants such as salicylsulphonic acid, trichloroacetic acid and picric acid causes denaturation. The solubilities found in our experiments seem to contradict, in our case at least, this opinion. Neither the older conception of irreversible denaturation, founded on a simple decrease in solubility at the isoelectric point, nor the newer conception of reversible denaturation where reversion is never instantaneously complete, are compatible with the phenomenon described above. Furthermore we carried out the following test to prove the existence of unchanged protein in our redissolved precipitates.

To 50 ml. of egg albumin solution was added an equal volume of 0.1 N metaphosphoric acid and the liquid centrifuged. The precipitate was washed with distilled water, suspended in a small amount of water and dissolved by the addition of half-saturated ammonium sulphate solution. More ammonium sulphate was added until the solution was saturated to about 80 % and the precipitation of the protein completed. After filtration the egg albumin was dissolved in as little water as possible and crystallized according to Sørensen & Høyrup [1917].

The crystals had the same appearance as normal egg-albumin and were free from phosphorus. In a dialysed solution the amount of amino-N was determined by the acetone titration of Linderstrøm-Lang [1927]. We found the same amount of amino-N as in freshly prepared solution of egg albumin.

To 1 ml. egg albumin solution were added 15 ml. acetone and 2 ml. water. The solution then contained 83.5 % of acetone, this being the highest concentration at which the liquid remained perfectly clear. The mixture was titrated with 0.025 N alcoholic HCl using naphthyl red as indicator.

Table III. *Acetone titration of native egg albumin (control) and egg albumin prepared from an egg albumin precipitate (experiment)*

Protein solution	mg. total N per ml. protein solution	ml. 0.025 N HCl used for titration	mg. amino-N per ml. protein solution	% amino-N
Blank	0	0.825	—	—
Con rol	3.1	1.71	0.253	8.15
		1.715	0.254	8.2
		1.719	0.255	8.2
Experiment	1.6	1.276	0.131	8.2
		1.276	0.131	8.2
		1.272	0.128	8.0

DISCUSSION

The results of our experiments show that adding increasing amounts of metaphosphoric acid to the solution of egg albumin causes the precipitate to increase, both in quantity and in phosphorus content, to a limiting value, which is not influenced by changing the concentration or the reaction time.

In a recent paper [Herrmann & Perlmann, 1937] we report that the number of bound phosphorus atoms corresponding to this limiting value is nearly equal to the number of positively charged groups in the protein molecules. In the case of egg albumin the number of positively charged groups calculated in this way agreed with that found in other ways by different authors [Bugarsky & Liebermann, 1898; Hitchcock, 1922-23]. There is however a small difference between this number and the number which we determined by titrating egg albumin in acetone solution by the method of Linderstrøm-Lang [1927]. As the positively charged groups in proteins differ in their basicity we assume that only the stronger basic groups (e.g. free amino-groups, guanidino-groups) react with metaphosphoric acid, forming complexes in stoichiometric proportions. The acid-binding in acetone solution, however, gives the total amount of positive groups, including amino-acid residues like proline, hydroxyproline and histidine which perhaps do not react with metaphosphoric acid.

Table IV. *Number of basic groups in the egg albumin molecule calculated from the results of different methods*

Author	Method	Total N/amino-N	Number of basic groups calculated for a molecular weight of 34,500
Bugarsky-Liebermann	Acid-binding	14.0	27
Hitchcock	Acid-binding	14.0	27
Herrmann-Perlmann	Precipitation with metaphos- phoric acid	13.9	27
Perlmann-Herrmann	Acid-binding in acetone solution	12.2	31

Consideration of Table I and the figures (1, 2, 3) shows that the ratio of P to N in the precipitate depends on the concentration of metaphosphoric acid. At high concentration we found a constant limiting value, but the ratio does not increase continuously with diminishing pH . On the contrary it exhibits a sharp break at a pH of 3.9. An increase of the P/N ratio could be explained by assuming that the shift of the hydrogen ion concentration on addition of metaphosphoric acid causes a rise in the dissociation of amino-groups. The sharp turn in our curves could be explained by the diminishing effect of a low pH on the dissociation of carboxyl groups. Dissociation of these groups keeps protein molecules in solution but at lower pH the ionization of the carboxyls is less, causing precipitation of the egg albumin molecules without affecting their P content.

We were led to the same conclusion by a series of experiments at a constant pH and increasing metaphosphoric acid concentration. From Table II it is seen that the P/N ratio remains nearly constant at a fixed pH of 3.9, 3.7. At the pH of 3.2, however, the P/N ratio rises steadily to a limiting value.

We shall postpone drawing definite conclusions about the origin of the above-mentioned discontinuity until the behaviour of a greater number of proteins towards different precipitants has been studied under similar conditions.

SUMMARY

1. Egg albumin is precipitated by metaphosphoric acid and the amount of the precipitate as well as the phosphorus content of the precipitate are estimated under various conditions.

2. The limiting, constant phosphorus content of the precipitates is equivalent to 27 positively charged groups per egg albumin molecule.

3. The precipitates are easily soluble in salt solutions and from these solutions the phosphorus-free, crystallized egg albumin can be regained.

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