CXIII. THE PRECIPITATION OF PROTEINS WITH COMPLEX SALTS

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(Received 13 April 1939)

ATTENTION is directed to a group of protein precipitants which are of interest both from a practical and theoretical point of view. The commonly used protein precipitants are subject to certain limitations. Some of them, such as phosphotungstic, hydroferrocyanic and metaphosphoric acids, precipitate proteins in acid solution only, while only few reagents are known, e.g. salts of heavy metals, which effect precipitation in neutral or weakly alkaline solutions. These reagents suffer from the disadvantage that they tend to denature the proteins. Simple salts, such as Na_2SO_4 , work only in high concentrations.

It has now been found that complex salts can be used advantageously as protein precipitants, and from the numerous selection of complex salts available a variety of conditions are possible under which the precipitations can be carried out. Apart from the work of Bungenberg de Jong & Saubert [1936], the literature appears to contain no reference to the general use of complex salts as protein precipitants. These authors used complex salts in certain systems which produce what they term "coacervates", but the systems studied were of a more complicated nature than those in our investigations inasmuch as they comprised protein + complex salts + a third component which was usually an inorganic salt. With regard to the mechanism involved in such precipitations, the results of our quantitative experiments support the view that stoichiometric relations exist between protein and precipitant, as found by Chapman *et al.* [1927] for acid dyes, Meyer *et al.* [1937] for chondroitin sulphuric acid, and by Hermann & Perlmann [1937; 1938] for metaphosphoric acid. We have considered both the qualitative and quantitative aspects of the problem.

QUALITATIVE EXPERIMENTS

Methods. The native protein was dissolved in dilute hydrochloric acid to give a 1% solution having pH 3. A freshly prepared aqueous solution of the complex salt was then added in such amount that the proportion by weight of protein to complex salt was 5:1. The appearance or non-appearance of a precipitate was then noted. An analogous procedure was then followed at the other pH values tabulated in Table I. Solutions having a pH value more

Table I

Complex salt	Edestin at pH			Serum albumin at pH			Globin at pH		
	3	4-6	7-9	3	4-6	7-9	3-7.5	8-9	9-11
$[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$	-	+	+	-	+	+	-	+	+
$[\text{Co}(\text{NH}_3)_5(\text{NO}_2)]\text{Cl}_2$	-	+	-	-	+	-	-	+	-
$\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3]$	+	+	-	+	+	-	+	+	-

+ = precipitate. - = no precipitate.

alkaline than the isoelectric point of the protein were obtained by adding $N/10$ NaOH until the desired pH was attained. Precipitation, when it did occur, was instantaneous and the precipitates were flocculent and settled easily. Table I shows that edestin and serum albumin are precipitated in acid solution by the anionic complex salt $K_3[Cr(C_2O_4)_3]$ (these proteins are normally insoluble within their isoelectric range, pH 4–6); in more alkaline solutions, precipitation occurs with the kationic complex salt $[Co(NH_3)_6]Cl_3$. On the other hand, a basic protein, globin, is precipitated in the whole range from pH 3 to 7 by the anionic complex salt $K_3[Cr(C_2O_4)_3]$. Within its isoelectric range, pH 8–9, globin is insoluble, while in more alkaline solutions it is precipitated by the kationic complex salt $[Co(NH_3)_6]Cl_3$. The complex salt $[Co(NH_3)_5(NO_2)]Cl_3$, which is not ionized in aqueous solution, does not cause precipitation of any of the proteins studied, over the whole pH range. Precipitates of proteins with complex salts redissolve when the pH of the supernatant solution is altered; thus the precipitates formed by the addition of $K_3[Cr(C_2O_4)_3]$ to edestin and serum albumin were dissolved when the solutions were adjusted, by the addition of NaOH, to pH 7. The precipitates formed by the proteins with $[Co(NH_3)_6]Cl_3$ were readily soluble in dilute acid, and the precipitate of globin with $[Co(NH_3)_6]Cl_3$ was dissolved when the pH was adjusted to 7. Tests were carried out in the same way with a number of different complex salts. We quote as some typical representatives for the anionic complex salts:

and as typical representatives for kationic complex salts:

It has been found that the precipitation of native proteins can be carried out with all ionized complex salts, provided that anionic complex salts are used at a pH which is more acid than the isoelectric point of the protein, and kationic complex salts are used at a pH which is more alkaline than the isoelectric point of the protein. With a proper choice of complex salts precipitation of proteins can, therefore, be effected over a wide pH range.

QUANTITATIVE EXPERIMENTS

An analytical study of some precipitates obtained with protein and complex salts was now made. Both types of complex salts, anionic and kationic, were used in this investigation, the precipitations being carried out under the general conditions outlined above.

Experimental procedure. The proteins under examination were dissolved in dilute acid or alkali and the solutions then adjusted to a known pH value. The complex salts which served as precipitants were dissolved and, when necessary, the pH value of the solution was adjusted to that of the protein solution. In each case the two solutions were mixed and the precipitate which formed was immediately centrifuged off, washed 4–5 times with water until the washings contained no protein or complex salt, washed twice with alcohol, then twice with ether, and finally dried in a vacuum over $CaCl_2$. The relative amounts of complex ion and protein in the precipitate were then estimated by analysis. The metallic content of the precipitate was determined and in one case the N contents of the precipitate and the original protein were compared. The experiments were confined to complex salts which contained chromium as central atom, because the method of Smith & Sullivan [1935] for chromium determinations in organic compounds offered a reliable and rapid method for

the estimation of the metal. The nitrogen determinations were carried out using Pregl's micro-Kjeldahl method.

Exp. 1. Examination of a typical precipitate from a protein and a complex salt. A crude preparation of insulin¹ (200 mg.) was precipitated in *N*/20 HCl (20 ml.) with a 5% solution of $K_3[Cr(C_2O_4)_3]$ (1.2 ml.).

Table II

Protein	% N content	% Cr content	% $[Cr(C_2O_4)_3]$ in ppt. calc. from	
			Nitrogen	Chromium
Crude insulin	12.3	—	—	—
Insulin chromic oxalate	10.97	1.72	10.4	10.4

Table II shows that the amount of the complex anion $[Cr(C_2O_4)_3]$ in the precipitate (as estimated from the Cr content of the precipitate) is identical with the amount calculated from the difference in N content between the original protein and the precipitate. The Cr content of the precipitate may, in this instance, therefore, be taken as a true indication of the amount of complex ion present in the precipitate. A comparison between N and Cr contents could be carried out only in the case of chromic oxalate, because all other complex salts used in these experiments contained nitrogen.

Exp. 2. (a) Precipitation of protein in acid solution with an anionic complex salt. In this series both "crude" insulin and crystalline insulin were precipitated with varying amounts of the complex salt $K_3[Cr(CNS)_6]$. The precipitations were carried out either in *N*/20 HCl or in 2% acetic acid solution. The conditions were varied with respect to the relative amounts of protein and complex salt, to the concentrations of the protein and the complex salt and to the pH. The reactions were carried out in small centrifuge tubes, the final volume of the mixture always being adjusted to 10 ml.

Table III. *Precipitation of insulin with $K_3[Cr(CNS)_6]$ in acid solution*

Protein	Wt. of protein mg.	Wt. of $K_3[Cr(CNS)_6]$ mg.	% Cr in ppt.	% Cr in ppt. (average)	% $[Cr(CNS)_6]$ in ppt. calc. from % Cr
(a) Precipitation in <i>N</i> /20 HCl					
Crude insulin	200	60	1.62	1.62	12.45
"	200	60	1.74		
"	200	60	1.64		
"	200	60	1.55		
"	200	60	1.57		
"	200	80	1.69	1.69	13.0
"	200	90	1.64	1.61	12.4
"	200	90	1.57		
"	200	100	1.62	1.62	12.45
"	200	150	1.61	1.61	12.4
"	150	60	1.60	1.60	12.3
"	250	60	1.62	1.62	12.45
"	300	60	1.54	1.54	11.9
Crystalline insulin	200	60	1.70	1.70	13.1
(b) Precipitation in 2% acetic acid					
Crude insulin	250	75	1.57	1.57	12.1
"	250	115	1.59	1.59	12.2

Table III shows that the amounts of complex ion $[Cr(CNS)_6]$ combined with the protein (as determined by the chromium content of the precipitates) were constant within the limits of experimental error (*ca.* 10%).

¹ A specimen of insulin which was not fully purified and not biologically standardized.

(b) *Precipitation of a protein with a kationic complex salt.* A series of experiments, analogous to those described above under (a), was carried out using the kationic complex salt $[\text{Cr}(\text{CO}(\text{NH}_2)_2)_6]\text{Cl}_3$. Casein was chosen as the protein since it is readily soluble at pH 7, and this pH is sufficiently removed from the isoelectric point of the protein. The casein was dissolved in dilute NaOH to give a 1% solution having pH 7. An aqueous solution of the complex salt was added. The relative amounts of protein and complex salt were varied.

Table IV. *Precipitation of casein with $[\text{Cr}(\text{CO}(\text{NH}_2)_2)_6]\text{Cl}_3$ at pH 7*

Protein	Wt. of protein mg.	Wt. of $[\text{Cr}(\text{CO}(\text{NH}_2)_2)_6]\text{Cl}_3$ mg.	% Cr in ppt.	% Cr in ppt. (average)	% $[\text{Cr}(\text{urea})_6]$ in ppt.
Casein	200	40	1.05	1.05	8.35
"	200	60	1.07	1.07	8.5
"	200	60	1.07		
"	250	25	0.93	0.95	7.55
"	250	25	0.87		
"	250	25	1.06		
"	250	80	1.00	1.00	7.95
"	250	100	1.06	1.08	8.65
"	250	100	1.11		

Table IV shows that whereas the weight of complex salt added was varied by as much as 400%, the maximum variation in the amount of complex ion in the precipitate was only 12%. It appears from the results shown in Tables III and IV that a given protein, when precipitated with a complex salt, combines with it in stoichiometric proportions, which are independent of concentration and relative amounts of protein and complex salt present.

Exp. 3. Determination of equivalent of complex ion combined with protein in the precipitate. (a) *Anionic complex ions.* In the experiments recorded in Table V each of the proteins was precipitated with three different complex salts, two of which had trivalent complex anions, while the third (Reinecke salt) had a monovalent complex anion. The figures in column 3 show the percentage of complex ion in the precipitates. It will be seen that for a given protein these values vary considerably for different precipitants. When, however, these figures are recalculated with reference to the equivalent weights of the complex ions and expressed for 1 g. protein (shown in column 4) it will be seen that the

Table V. *Precipitations with anionic complex salts in acid solution*

1 Protein	2 Complex ion*	3 % Complex ion in ppt.	4 M.-equiv. of complex ion combined with 1 g. protein	5 Average
Crude insulin	$[\text{Cr}(\text{CNS})_6]'''$	12.4	1.06	—
"	$[\text{Cr}(\text{C}_2\text{O}_4)_3]'''$	10.6	1.13	1.04
"	$[\text{Cr}(\text{CNS})_4(\text{NH}_3)_2]'$	22.6	0.92	—
Crystalline insulin	$[\text{Cr}(\text{CNS})_6]'''$	13.1	1.12	1.12
Egg albumin	$[\text{Cr}(\text{CNS})_6]'''$	9.4	0.78	—
"	$[\text{Cr}(\text{C}_2\text{O}_4)_3]'''$	7.0	0.715	0.75
"	$[\text{Cr}(\text{CNS})_4(\text{NH}_3)_2]'$	19.4	0.76	—
Clupein	$[\text{Cr}(\text{CNS})_6]'''$	34.1	3.90	—
"	$[\text{Cr}(\text{C}_2\text{O}_4)_3]'''$	30.4	4.15	4.1
"	$[\text{Cr}(\text{CNS})_4(\text{NH}_3)_2]'$	58.0	4.35	—

* The salts used were the potassium salts.

equivalents of the complex ions combined with a given protein are essentially the same (within the limits of experimental error), and moreover are independent of the nature and the valency of the complex ion used. It is also evident from these figures that the trivalent complexes combine with the proteins as "tribasic acids", while the monovalent complex combines as a "monobasic acid".

(b) *Kationic complex ions.* In the experiments recorded in Table VI various proteins were precipitated in approx. neutral solution with kationic complex salts. The two complex salts used have trivalent complex kations, but their

Table VI. *Precipitations with kationic complex salts at pH 7-8*

1 Protein	2 Complex ion*	3 % Complex ion in ppt.	4 Complex combined m.-equiv./g. protein	5 Average
Casein	$[\text{Cr}(\text{urea})_6]^{+++}$	8.30	0.66	0.68
"	$[\text{Cr}(\text{NH}_3)_6]^{+++}$	3.52	0.71	
Amorphous insulin	$[\text{Cr}(\text{urea})_6]^{+++}$	5.3	0.41	0.415
"	$[\text{Cr}(\text{NH}_3)_6]^{+++}$	2.15	0.42	
Crystalline insulin	$[\text{Cr}(\text{urea})_6]^{+++}$	4.8	0.37	0.37
Egg albumin	$[\text{Cr}(\text{NH}_3)_6]^{+++}$	1.95	0.39	0.39

* The salts used were the chlorides.

molecular weights (412 and 154 respectively) are far apart. Thus the combining weights of these complex ions differ considerably, and this difference is significant, inasmuch as it excludes any accidental agreement of results. The values found for the two complex ions combined with a given protein are in fairly good agreement. Thus casein combined with 0.66 and 0.71 m.-equiv./g. of protein in the case of $[\text{Cr}(\text{urea})_6]\text{Cl}_3$ and $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$ respectively. The agreement between the values of the two complex ions is even better in the case of amorphous insulin (0.41 and 0.42 m.-equiv./g. of protein respectively). The corresponding figure for crystalline insulin is slightly lower (0.37). These results show that a protein combines on the alkaline side of its isoelectric point with a definite equivalent of complex kations, independently of the nature of the complex salt used.

The precipitation of proteins with complex salts appears, therefore, to take place in such a manner that each protein displays a definite capacity for combining with acid or basic complex ions.

The averaged values of acid- and base-binding capacities shown in column 5 of Tables V and VI are compared in Table VII with values which have been found by other methods.

The values for insulin found by precipitation with complex anions are slightly higher than the values obtained by Harington & Neuberger [1936] by means of electrometric titration. The figure (0.75), calculated from our experiments for egg albumin, is higher than the value (0.61) which Meyer *et al.* [1937] found from precipitation experiments with chondroitin sulphuric acid, but is in good agreement with the figure (0.78) obtained by Perlmann & Herrmann [1938] from precipitation experiments with metaphosphoric acid. There is good agreement between our value for clupein and that of Felix & Mager [1937] calculated from the combination of clupein with various acids.

Our values for the base-combining capacities of proteins are of the same order as those found by other methods in the case of insulin and casein. Our value for egg albumin, on the other hand, is much smaller than the value of the "maximal base-combining capacity" given by Cohn *et al.* [1925].

Table VII. *Acid- and base-combining capacities of some proteins determined by different methods*

Protein	Acid-combining capacities m.-equiv./g. protein		Method	Author
	Complex ppt.	Other methods		
Amorphous insulin	1.04	—	—	—
Crystalline insulin	1.12	1.01	Electrometric titration	Harington & Neuberger
Egg albumin	0.75	0.61	Precipitated with chondroitin sulphuric acid	Meyer <i>et al.</i>
	—	0.78	Precipitated with HPO_3	Perlmann & Hermann
Clupein	4.1	4.1	Combination with acids	Felix & Mager
Protein	Base-combining capacities m.-equiv./g. protein		Method	Author
	Complex ppt.	Other methods		
Amorphous insulin	0.41	0.45	Electrometric titration	Harington & Neuberger
Crystalline insulin	0.37	—	—	—
Casein	0.68	0.55	Ca-salts	Van Slyke [1915]
Egg albumin	0.39	0.8	Maximal base-combining capacity, electrometric titration	Cohn <i>et al.</i>

DISCUSSION

Attention is drawn to the fact that all ionized complex salts possess the capacity of precipitating soluble proteins from their solutions. Salts which possess a complex anion precipitate proteins only when the latter are in kationic state, i.e. at a *pH* more acid than the isoelectric points of the proteins; salts which possess complex kations precipitate proteins only when the protein is present in its anionic state, i.e. at a *pH* more alkaline than the isoelectric point of the protein. This rule applies to all complex salts and all native proteins which have been examined. The precipitation appears to be, therefore, the effect of a salt-like combination of the complex ion with the protein carrying the opposite charge.

We can conclude that the complex ions remain intact in the precipitates from the fact that precipitation is instantaneous and that the colour of the complex ion is retained in the precipitate: e.g. protein precipitates with Reinecke salt are red, with $[\text{Cr}(\text{urea})_6]\text{Cl}_3$ green, with $[\text{Cr}(\text{NH}_3)_6]\text{Cl}_3$ orange-yellow. The precipitations described here are, therefore, different in character from those which Elöd & Schachousky [1935] observed when gelatin was heated with certain unstable complex salts.

The results of our analytical determinations indicate that proteins, when precipitated with a complex salt, combine in stoichiometric proportions with the complex kation or anion respectively. This observation finds a parallel in the combination of certain amino-acids with complex salts [Bergmann, 1935], e.g. *l*-proline rhodanilate, $[\text{Cr}(\text{CNS})_4(\text{C}_6\text{H}_5\text{NH}_2)_2] \cdot (\text{C}_5\text{H}_{10}\text{O}_2\text{N})$, in which case the monovalent complex anion is combined with the monovalent amino-acid, the proline reacting in its kationic form.

We can conclude from our figures that proteins combine in acid solution with the complex anion with their "maximal acid-combining capacity", i.e. that all basic groups of the protein are able to react with the precipitant. This is shown in the case of insulin by the agreement of our figure with the highest value of acid-combining capacity found by electrometric titration; it is also in agreement

with Chapman's observation that acid dyes combine with proteins at pH 2-2.5 in constant proportions and in amounts which are to be expected when all basic groups are ionized. Our figures for the base-combining capacities of proteins indicate, on the other hand, that the proteins, when precipitated with kationic complex salts at or near pH 7, do not combine with their "maximal base-combining capacity". A possible explanation is that the phenolic hydroxyl groups are not ionized and therefore do not react.

That precipitation of proteins with complex salts is not accompanied by coagulation or denaturation of the protein is shown by the ease with which the precipitates are redissolved on appropriate change of pH , and by the fact that the precipitations are often reversed by certain neutral salts, such as acetates, phosphates and ammonium salts.

The degree of precipitation of a given protein varies considerably with different complex salts, but we have not detected any considerable differences in the degree of precipitation of different proteins by one and the same complex salt.

SUMMARY

1. It has been shown that ionized complex salts precipitate proteins from their aqueous solutions.
2. Anionic complex salts precipitate proteins at a pH more acid than the isoelectric point of the protein, whereas kationic complex salts precipitate at a pH more alkaline than the isoelectric point.
3. These precipitations are not accompanied by denaturation or coagulation of the protein.
4. Quantitative estimations of the N and metal contents of such precipitates have shown that the proteins combine with a definite equivalent of complex ion, independently of the relative proportions and concentrations of the reagents, i.e. in stoichiometric proportions.
5. The amounts of complex ions combined in the precipitates with various proteins represent, with some reservations, the acid- and base-combining capacities of the proteins; they were in several cases found to be in reasonable agreement with values calculated from other determinations.
6. It appears that proteins, when precipitated in acid solution with complex anions, combine with their maximal acid-combining capacity, whereas proteins, when precipitated with complex kations in approximately neutral solution, combine with only a fraction of their maximal base-combining capacity.

The author wishes to express grateful thanks to Prof. W. N. Haworth for his encouragement and interest in this work, and to the University of Birmingham for the award of a Research Fellowship. Gratitude is also expressed to the British Drug Houses Ltd. for the gift of insulin which was used in this work.

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