219. Reinosuke Hara*: Studies on the Precipitation Reactions of Some Metallic Complex Salts towards Proteins.

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The reaction of metallic salts towards the protein has been the main subject of discussion which are of interest both from practical and theoretical points of view. Although various works have been carried out to clarify the reaction mechanisms, it has not yet been clearly demonstrated. Such systematic studies were recently given by Klotz, et al.¹⁾ through their long years of work dealing with the reaction of protein with various dyes and inorganic salts. The commonly used inorganic salts as protein precipitants are subject to certain limitations. A well-known fact is that some of them, such as hydrocyanic acid and metaphosphoric acid, cause the precipitation of protein in an acid solution, while various reagents are known, i.e. salts of heavy metals, which effect precipitation in neutral or weak alkaline solution. Owing to complicated factors, such as hydrolysis of metallic salts and possibilities of inner complex formation with amino acids or polypeptides in protein, the systematic studies become a difficult problem. To help prove the reaction of metallic salts with protein, attention was directed to the use of ammin-complex salts of cobalt and chromium which are stable and hardly cause hydrolysis, and some works have already been published^{2,3)}. In the present works, both qualitative and quantitative aspects are considered of the protein precipitation with metallic complex salts.

Purified casein and egg-albumin were chosen as the protein used. The metallic complex salts used were synthesized and purified with strict care.

Capacity of metallic complex salts for the precipitation of protein.

Using a buffer solution, 0.1 N ammonium hydroxide and ammonium chloride (pH 8.8) or 0.1 N tartaric acid and sodium tartrate (pH 2), protein was dissolved in this buffer solution to make the concentration of 0.5%. Five cc. of protein solution was taken in a test tube, then 5 cc. of the solution of the complex salts was added in various concentrations. Thirty minutes later, the evidence of precipitation was examined. The results are shown in Tables I and II.

Table I.

Complex salt	Casein	Concn. for ppt. (mol.)	Egg-al- bumin	Concn. for ppt. (mol.)	pH
$[\text{Co}-(\text{OH})_2\text{Co en}_3)_3](\text{NO}_3)_6 \cdot 3\text{H}_2\text{O}$	+++	1/100~1/5000	+	1/100~1/1000	8.8
$[\text{Cr}-(\text{OH})_2\text{Cr en}_3)_3]\text{Cl}_6 \cdot 6\text{H}_2\text{O}$	+++	1/100~1/5000	+	1/100~1/1000	"
$[(\text{NH}_3)_5\text{Cr}-\text{OH}-\text{Cr}(\text{NH}_3)_5]\text{Cl}_5 \cdot \text{H}_2\text{O}$	+++	1/300~1/2000	+	1/300~1/700	"
$[(\text{NH}_3)_4\text{Co}-(\text{OH})_2-\text{Co}(\text{NH}_3)_4]\text{Cl}_4 \cdot 4\text{H}_2\text{O}$	+++	1/100~1/1000	+	1/100~1/500	"
$[\text{en}_3\text{Co}-(\text{OH})_2-\text{Co}(\text{H}_2\text{O})_2-(\text{OH})_2-\text{Co en}_3]\text{Cl}_4$	+++	1/100~1/1000	+	1/100~1/500	"
$[\text{en}_3\text{Cr}-(\text{OH})_2-\text{Cr en}_3]\text{Cl}_4 \cdot 2\text{H}_2\text{O}$	+++	1/100~1/1000	+	1/100~1/500	"
$[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$	++	1/20~1/300	—		"
$[\text{Co en}_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$	++	1/40~1/300	—		"
$[\text{Co}(\text{NH}_3)_5\text{H}_2\text{O}](\text{NO}_3)_3$	++	1/80~1/250	—		"

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1) Klotz, Walkerd, Pivan: J. Am. Chem. Soc., **68**, 1486 (1946); Klotz, Curme: J. Am. Chem. Soc., **70**, 939 (1949); Klotz and Urquhart: J. Am. Chem. Soc., **71**, 1597 (1949).

2) Michael: Biochem. J., **33**, 924 (1939).

3) Hara: Bull. Chem. Soc. Japan, **22**, 109 (1949); **23**, 7 (1950).

$[\text{Co}(\text{NH}_3)_4(\text{H}_2\text{O})_2](\text{NO}_3)_3 \cdot \text{H}_2\text{O}$	++	1/40~1/250	—		8.8
$[\text{Co en}_2(\text{NH}_3)_2]\text{Br}_3 \cdot \text{H}_2\text{O}$ (<i>trans</i>)	++	1/40~1/300	—		"
$[\text{Cr en}_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$	++	1/40~1/300	—		"
$[\text{Co}(\text{NH}_3)_5\text{NO}_2]\text{Cl}_2$	+	1/40~1/80	—		"
$[\text{Co}(\text{NH}_3)_5\text{Cl}](\text{NO}_3)_2$	+	1/60~1/80	—		"
$[\text{Co}(\text{NH}_3)_5\text{NO}_2]\text{Cl}_2$	+	1/60~1/80	—		"
$[\text{Cr}(\text{NH}_3)_5\text{SCN}](\text{SCN})_2$	+	1/60	—		"
$[\text{Ni en}_3]\text{Cl}_2$	±	1/60	—		"
$[\text{Pt en}_2]\text{Cl}_2$	—		—		"
$[\text{Pt}(\text{SCNH}_2\text{NH}_2)_4]\text{Cl}_2$	—		—		"
$[\text{Co}(\text{NH}_3)_4(\text{NO}_2)_2]\text{Cl}$ (croseo)	—		—		"
$[\text{Co}(\text{NH}_3)_4\text{CO}_3]_2\text{SO}_4 \cdot 3\text{H}_2\text{O}$	—		—		"
$[\text{Co}(\text{NH}_3)_4\text{C}_2\text{O}_4]\text{Cl}_2$	—		—		"
$[\text{Cr en}_2\text{Cl}_2]\text{Cl} \cdot \text{H}_2\text{O}$ (<i>cis</i>)	—		—		"
$[\text{Cr en}_2(\text{SCN})_2] \cdot \text{SCN}2\text{H}_2\text{O}$ (<i>trans</i>)	—		—		"

Table II.

Complex salt	Casein	Concn. for ppt. (mol.)	Egg-al- bumin	Concn. for ppt. (mol.)	pH
$[\text{Co}(\text{NH}_3)(\text{NO}_2)_3]$	—		—		8.8, 2
$[\text{Pt}(\text{NH}_3)_2\text{Cl}_2]$ (<i>d-cis</i>)	—		—		"
$\text{K}[\text{Co}(\text{NH}_3)_2(\text{NO}_2)_4]$	+++	1/60	+++	1/60	2
$\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4] \cdot \text{H}_2\text{O}$	+++	1/30~1/60	+++	1/30~1/60	"
$\text{K}[\text{Ag}(\text{CN})_2]$	+++	1/20~1/100	+++	1/20~1/80	"
$\text{Na}_3[\text{Co}(\text{NO}_2)_6]$	+++	1/40~1/300	+++	1/40~1/350	"
$\text{K}_3[\text{Co}(\text{CN})_6]$	+++	1/100~1/300	+++	1/100~1/300	"
$\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 3\text{H}_2\text{O}$	+++	1/20~1/300	+++	1/20~1/350	"
$\text{K}_3[\text{Cr}(\text{SCN})_6] \cdot 4\text{H}_2\text{O}$	+++	1/30~1/300	+++	1/30~1/300	"
$\text{K}_3[\text{Fe}(\text{CN})_6]$	+++	1/30~1/300	+++	1/30~1/350	"
$\text{K}_4[\text{Fe}(\text{CN})_6]$	+++	1/30~1/500	+++	1/30~1/550	"
$(\text{NH}_4)_4[(\text{C}_2\text{O}_4)_2\text{Cr}-(\text{OH})_2-\text{Cr}(\text{C}_2\text{O}_4)_2]$	+++	1/20~1/500	+++	1/20~1/700	"

The results in Tables I and II show that the complexes containing cation complex radical precipitate the protein in a more alkaline range than the isoelectric point of proteins, while, in an acid range, the precipitation is caused with complexes having complex anion. In an acid range, casein and egg-albumin are, without any exception, precipitated by complexes of anion radical, in an alkaline range, they are not always precipitated by complex salts containing complex cations. Some of the complex of low valency, such as $[\text{Co}(\text{NH}_3)_4(\text{NO}_2)_2]\text{Cl}$, $[\text{Co}(\text{NH}_3)_4\text{CO}_3]_2\text{SO}_4 \cdot 3\text{H}_2\text{O}$, $[\text{Co}(\text{NH}_3)_4\text{C}_2\text{O}_4]\text{Cl}$, $[\text{Ni en}_3]\text{Cl}_2$, $[\text{Cr en}_2\text{Cl}_2]\text{Cl} \cdot \text{H}_2\text{O}$ (*cis*) and $[\text{Cr en}_2(\text{SCN})_2]\text{SCN} \cdot 2\text{H}_2\text{O}$ (*trans*), and non-electric complex, such as $[\text{Co}(\text{NH}_3)_3(\text{NO}_2)_3]$, $[\text{Pt}(\text{NH}_3)_2\text{Cl}_2]$ (*α-cis*), cause no precipitation in the whole pH range.

Considering the results, it is shown that the capacity of precipitating protein mainly depends on the valency, i.e. the complex salt, $[\text{Co}-((\text{OH})_2\text{Co en}_2)_3](\text{NO}_3)_6$, causes the precipitation of casein even in a concentration of 1/5000 mole, while a limit concentration of complex salt, $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$, is approximately 1/300 mol.

Determination of equivalence of complex ion combined with a given protein.

Fifty cc. of 0.5% protein solution were taken (pH 8.8 or 2), then 50 cc. of a solution containing complex salts in various amounts was added. Thirty minutes later, the

protein precipitated was centrifuged, washed with distilled water until the washing contained no protein or complex salts. Then it was dried in a vacuum desiccator. The equivalence of complex ion combined with 1 g. of the given protein was calculated from the metal content of the precipitated protein. After the decomposition of the protein, the metal content was determined as follows: Cobalt content...colorimetric determination using nitroso-R-salt. Chromium content...colorimetric determination by chromate, using sodium peroxide as the oxidizing agent. Iron content...colorimetric determination by thiocyanate method.

The results obtained are shown in Tables III to XIII.

The results in Tables III to XIII show that, in the range of concentrations under examination, the equivalence of complex ion combined with 1 g. of a given protein remains almost constant independent of the variety and the amounts added of the complex salts. For example, in casein $[\text{Co}((\text{OH})_2\text{Co en}_2)_3](\text{NO}_3)_6 \cdot 3\text{H}_2\text{O}$, whereas the weight of complex salt added was varied by as much as 250%, the maximum variation in the equivalence was only 11%. No marked change in equivalence was observed whether the precipitation is caused by 1/500 mole, 1/300 mole or 1/200 mole of the com-

Table III.

Cation complex equivalence of $[\text{Co}(\text{NH}_3)_5\text{NO}_2]\text{Cl}_2$ towards casein

Concn. of compl. salt (mol.)	Co% in ppt.	Compl.ion % in ppt.	Equiv. $\times 10^{-5}$
1/50	1.96	6.31	71
	1.71	5.51	61
	2.03	6.54	74
	1.78	5.74	64
	2.09	6.71	76
1/80	1.85	5.96	67
	2.20	7.08	80
	1.81	5.83	65
	2.14	6.89	78
	2.26	7.28	83

Table IV.

Cation complex equivalence of $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$ towards casein

Concn. of compl. salt (mol.)	Co% in ppt.	Compl.ion % in ppt.	Equiv. $\times 10^{-5}$
1/200	1.52	4.15	81
	1.64	4.48	87
	1.47	4.01	78
	1.58	4.31	84
	1.61	4.39	86
1/150	1.40	3.82	74
	1.70	4.64	91
	1.54	4.20	82
1/100	1.65	4.50	88
	1.66	4.53	89

Table V.

Cation complex equivalence of $[\text{Cr en}_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$ towards casein

Concn. of compl. salt (mol.)	Cr% in ppt.	Compl.ion % in ppt.	Equiv. $\times 10^{-5}$
1/200	1.30	5.80	80
	1.42	6.34	88
	1.37	6.12	85
	1.28	5.71	79
	1.26	5.62	77
1/150	1.47	6.55	91
	1.32	5.99	81
	1.45	6.48	90
1/100	1.47	6.55	91
	1.41	6.29	87

Table VI.

Cation complex equivalence of $[(\text{NH}_3)_4\text{Co}-(\text{OH})_2-\text{Co}(\text{NH}_3)_4]\text{Cl}_4 \cdot 4\text{H}_2\text{O}$ towards casein

Concn. of compl. salt (mol.)	Co% in ppt.	Compl.ion % in ppt.	Equiv. $\times 10^{-5}$
1/400	2.40	5.85	86
	2.53	6.17	91
	2.58	6.30	93
	2.63	6.41	95
	2.45	5.98	88
1/200	2.55	6.22	92
	2.47	6.02	89
1/100	2.43	5.93	87
	2.62	6.40	95

Table VII.

Cation complex equivalence of $[\text{en}_2\text{Cr}-(\text{OH})_2\text{Cr en}_2]\text{Cl}_4 \cdot 2\text{H}_2\text{O}$ towards casein

Concn. of compl. salt (mol.)	Cr% in ppt.	Compl. ion % in ppt.	Equiv. $\times 10^{-5}$
1/400	2.03	7.37	84
	1.98	7.20	82
	2.15	7.82	90
	2.10	7.64	87
	2.21	8.02	93
1/200	2.20	7.99	92
	2.00	7.26	83
1/100	2.25	8.17	94
	2.13	7.74	89

Table VIII.

Cation complex equivalence of $[\text{Co}-((\text{OH})_2\text{Co en}_2)_3](\text{NO}_3)_6 \cdot 3\text{H}_2\text{O}$ towards casein

Concn. of compl. salt (mol.)	Co% in ppt.	Compl. ion % in ppt.	Equiv. $\times 10^{-5}$
1/500	3.61	10.69	103
	3.74	11.08	107
	3.43	10.15	97
	3.60	10.60	102
	3.38	10.00	95
1/300	3.40	10.05	97
	3.50	10.30	99
	3.57	10.50	101
1/200	3.32	9.80	94
	3.77	11.15	108
	3.63	10.71	103

Table IX. Cation complex equivalence of $[\text{Cr}-((\text{OH})_2\text{Cr en}_2)_3]\text{Cl}_6 \cdot 6\text{H}_2\text{O}$ towards casein.

Complex salt	Protein	Concn. of compl. salt (mol.)	Cr% in ppt.	Compl. ion % in ppt.	Equiv. $\times 10^{-5}$
$[\text{Cr}-((\text{OH})_2\text{Cr en}_2)_3]\text{Cl}_6 \cdot 6\text{H}_2\text{O}$	Casein	1/500	2.98	9.49	94
			2.83	9.12	90
			2.87	9.24	91
			2.98	9.60	95
			3.08	9.91	98
		1/400	3.03	9.81	97
			3.12	10.05	100
			2.80	9.02	89
		1/200	3.18	10.25	102
			2.90	9.34	92
			2.98	9.60	95

Table X. Anion complex equivalence of $\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4] \cdot \text{H}_2\text{O}$ towards casein and egg-albumin.

Complex salt	Protein	Concn. of compl. salt (mol.)	Cr% in ppt.	Compl. ion % in ppt.	Equiv. $\times 10^{-5}$
$\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4] \cdot \text{H}_2\text{O}$	Casein	1/60	3.61	21.02	84
			3.67	22.19	90
			3.70	22.64	92
			3.65	21.70	87
		1/40	3.76	22.99	94
			3.72	22.76	93
			3.63	21.78	88
	Egg-albumin	1/60	2.75	16.82	64
			2.83	17.30	66
			2.88	17.61	67
			2.97	18.32	71
		1/40	3.05	18.64	72
			2.78	17.01	65
			2.80	17.12	65

Table XI. Anion complex equivalence of $\text{Na}_3[\text{Co}(\text{NO}_2)_6]$ towards casein and egg-albumin.

Complex salt	Protein	Concn. of compl. salt (mol.)	Co% in ppt.	Compl. ion % in ppt.	Equiv. $\times 10^{-5}$
$\text{Na}_3[\text{Co}(\text{NO}_2)_6]$	Casein	1/150	1.52	8.63	85
			1.62	9.20	91
			1.65	9.49	93
		1/100	1.50	8.52	83
			1.57	8.92	88
			1.63	9.25	91
		1/75	1.60	9.09	90
			1.55	8.80	87
			1.63	9.25	91
	Egg-albumine	1/150	1.30	7.38	71
			1.28	7.27	70
			1.35	7.67	74
		1/75	1.37	7.78	76
			1.31	7.44	72
			1.25	7.09	68
			1.39	7.89	78

Table XII. Anion complex equivalence of $\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 3\text{H}_2\text{O}$ towards casein and egg-albumin.

Complex salt	Protein	Concn. of compl. salt (mol.)	Cr% in ppt.	Compl. ion % in ppt.	Equiv. $\times 10^{-5}$
$\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 3\text{H}_2\text{O}$	Casein	1/150	1.37	8.34	86
			1.43	8.79	91
			1.48	8.99	94
		1/75	1.40	8.51	88
			1.51	9.18	95
			1.45	8.81	92
			1.35	8.21	85
	Egg-albumin	1/150	1.15	6.99	72
			1.23	7.47	77
			1.20	7.29	75
		1/75	1.10	6.69	68
			1.18	7.17	73
			1.21	7.35	76
			1.12	6.81	69

Table XIII. Anion complex equivalence of $\text{K}_4[\text{Fe}(\text{CN})_6]$ towards casein and egg-albumin.

Complex salt	Protein	Concn. of compl. salt (mol.)	Fe% in ppt.	Compl. ion % in ppt.	Equiv. × 10 ⁻⁵
K ₄ [Fe (CN) ₆]	Casein	1/200	1.20	4.54	90
			1.24	4.69	93
			1.14	4.32	85
			1.16	4.39	87
		1/100	1.19	4.51	89
			1.26	4.77	94
			1.22	4.58	91
	Egg-albumin	1/200	1.00	3.79	74
			0.92	3.48	68
			1.02	3.83	76
			1.08	4.09	81
		1/100	0.96	3.64	71
			1.06	4.01	79
			1.02	3.83	76

plex salt. These facts show that, in the given concentration, complex ion combines with protein in stoichiometric proportions, which are independent of the concentration and of the relative amount of protein and complex salts present. However, some irregularities are observed in the equivalences of $[\text{Co}(\text{NH}_3)_5\text{NO}_2]\text{Cl}_2$. In Tables XIV and XV, the equivalence values for casein and egg-albumin are given of various complex salts.

Of some cation complexes, such as $[\text{Co}(\text{NH}_3)_5\text{H}_2\text{O}](\text{NO}_3)_3$, $[\text{Co}(\text{NH}_3)_4(\text{H}_2\text{O})_2](\text{NO}_3)_3 \cdot \text{H}_2\text{O}$, $[\text{Co}(\text{NH}_3)_5\text{Cl}](\text{NO}_3)_2$, and $[\text{Co}(\text{NH}_3)\text{NO}_3]\text{Cl}_2$, it seems that their equivalences are slightly less than that of other complexes. This might be partly due to the slight change of pH due to the acidity of these complex salts. Of anion complexes, the values found for each complex salts combined with a given protein are in a fairly good agreement, being $65 \sim 80 \times 10^{-5}$ for egg-albumin and $85 \sim 100 \times 10^{-5}$ for casein.

These results show that the trivalent complexes combine with protein as "tribasic acid or triacidic base", while monovalent complexes combine as "monobasic acid or monoacidic base." Of the combination of serum albumin with some inorganic salts, such as CuCl_2 and KCl , similar facts were found by Tsubomura and Hosoya⁴⁾. The precipitation of protein with complex salts appears to take place in such a manner that each complex displays a definit capacity for combining with a given protein.

Effect of time on standing.

Thirty cc. of complex salt solution was added to 50 cc. of 0.5% protein solution (pH 8.8 or 2), and the centrifugation was carried out at various intervals, after the addition, of 30 minutes to a maximum of 24 hours. Casein was used as the protein. The same experiments were repeated twice. The results are shown in the Table XVI. The results seem to show that no marked change occurs in the equivalence, whether centrifugation was carried out after 30 min., 1 hr., 4 hrs., 8 hrs. or 24 hrs.

Effect of pH.

Sodium hydroxide of 0.1 N or 0.01 N HCl was added to the protein solution (0.5%) until the desired pH was attained. Complex salt which serves as a precipitant was

Table XIV.

Complex salt	Protein	Equivalence $\times 10^{-5}$	pH
$[\text{Co}(\text{NH}_3)_5\text{Cl}](\text{NO}_3)_2$	Casein	65~80	8.8
$[\text{Co}(\text{NH}_3)_5\text{NO}_3]\text{Cl}_2$	"	60~75	"
$[\text{Co}(\text{NH}_3)_5\text{NO}_2]\text{Cl}_2$	"	60~85	"
$[\text{Cr}(\text{NH}_3)_5\text{SCN}](\text{SCN})_2$	"	70~85	"
$[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$	"	75~90	"
$[\text{Co en}_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$	"	75~90	"
$[\text{Co}(\text{NH}_3)_5\text{H}_2\text{O}](\text{NO}_3)_3$	"	70~85	"
$[\text{Co}(\text{NH}_3)_4(\text{H}_2\text{O})_2](\text{NO}_3)_3 \cdot \text{H}_2\text{O}$	"	70~85	"
$[\text{Co en}_2(\text{NH}_3)_2]\text{Br}_3 \cdot \text{H}_2\text{O}$ (<i>trans</i>)	"	75~90	"
$[\text{Cr en}_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$	"	85~95	"
$[(\text{NH}_3)_4\text{Co}-(\text{OH})_2-\text{Co}(\text{NH}_3)_4]\text{Cl}_4 \cdot 4\text{H}_2\text{O}$	"	85~95	"
$[\text{en}_2 \text{Co}-(\text{OH})_2-\text{CO}(\text{H}_2\text{O})_2-(\text{OH})_2-\text{Co en}_2]\text{Cl}_4$	"	85~95	"
$[\text{en}_2 \text{Cr}-(\text{OH})_2-\text{Cr en}_2]\text{Cl}_4$	"	80~95	"
$[(\text{NH}_3)_5\text{Cr}-\text{OH}-\text{Cr}(\text{NH}_3)_5]\text{Cl}_5 \cdot \text{H}_2\text{O}$	"	85~95	"
$[\text{Co}-(\text{OH})_2\text{Co en}_2)_3](\text{NO}_3)_6 \cdot 3\text{H}_2\text{O}$	"	90~110	"
$[\text{Cr}-(\text{OH})_2\text{Cr en}_2)_3]\text{Cl}_6 \cdot 6\text{H}_2\text{O}$	"	90~100	"

4) Private communication.

Table XV.

Complex salt	Equivalence for casein $\times 10^{-5}$	Equivalence for egg-albumin $\times 10^{-5}$	pH
$K[Co(NH_3)_2(NO_2)_4]$	90~95	70~85	2
$NH_4[Cr(NH_3)_2(SCN)_4] \cdot H_2O$	85~95	65~75	"
$Na_3[Co(NO_2)_6]$	85~95	65~75	"
$K_3[Co(CN)_6]$	90~95	70~80	"
$K_3[Cr(C_2O_4)_3] \cdot 3H_2O$	85~95	65~75	"
$K_3[Cr(SCN)_6] \cdot 4H_2O$	90~100	65~75	"
$K_3[Fe(CN)_6]$	90~100	65~75	"
$K_4[Fe(CN)_6]$	85~95	70~80	"
$(NH_4)_4[(C_2O_4)_2Cr-(OH)_2-Cr(C_2O_4)_2]$	90~100	70~80	"

Table XVI.

Complex salt	Concn. of complex (mol.)	pH	Metal % in ppt.	Comp. ion % in ppt.	Equiv. $\times 10^{-5}$	Time
$[Co(NH_3)_6]Cl_3$	1/200	8.8	1.56	4.26	83	30 min.
			1.42	3.88	75	
			1.48	4.04	78	
			1.63	4.45	87	1 hr.
			1.54	4.20	82	
			1.61	4.64	91	4 hrs.
			1.47	4.01	78	
			1.51	4.12	80	8 hrs.
			1.58	4.31	84	
			1.67	4.57	89	24 hrs.
$[Co-(OH)_2Co(en)_3](NO_3)_6 \cdot 3H_2O$	1/500	8.8	3.36	9.94	95	30 min.
			3.51	10.41	100	
			3.42	10.11	97	
			3.55	10.49	101	1 hr.
			3.33	9.78	93	
			3.50	10.30	99	4 hrs.
			3.48	10.29	99	
			3.33	9.85	94	8 hrs.
			3.61	10.69	103	
			3.56	10.51	101	24 hrs.
$K_3[Cr(C_2O_4)_3] \cdot 3H_2O$	1/150	2	1.34	8.14	84	30 min.
			1.42	8.63	90	
			1.38	8.39	87	
			1.45	8.81	91	4 hrs.
			1.40	8.51	88	
			1.46	8.87	92	8 hrs.
			1.50	9.12	95	
			1.45	8.81	91	24 hrs.

dissolved and, when necessary, the pH value of complex solution was adjusted to that of the protein solution. Precipitation occurred by adding 30 cc. of the complex solution to 50 cc. of the protein solution. The results are shown in Tables XVII and XVIII.

Of cation complexes, their equivalences increase with the increase of pH. In the range of pH 8~9, the increase being slight in each complex salt. Of anion complexes, in the acid range of pH 1~3, they gradually increase. These facts suggest, in an alkaline range, the complex cations combine with the acidic radicals of protein, such as the carboxylic and the phosphoric groups, while in an acid range, they react with basic radicals, such as the amino and the imino groups. The precipitation appears to be, therefore, the effect of a salt-like combination of the complex ion with the protein carrying an opposite charge. With regard to the mechanism involved in such precipitation the results

Table XVII.
Protein Casein

Complex salt	Concn. of complex (mol.)	pH					
		6	7	8	9	10	11
$[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$	1/150	47,66,53	57,72,75	74,78,82	78, 85, 91	87, 94, 96	97,103,106
$[\text{Cr en}_3]\text{Cl}_3 \cdot 3\text{H}_2\text{O}$	1/150	52,63,65	68,74,81	78,86,91	87, 92, 96	97,102, 93	106,110,103
$[(\text{NH}_3)_4\text{CO}(\text{OH})_2\text{Co}(\text{NH}_3)_4]\text{Cl}_4 \cdot 4\text{H}_2\text{O}$	1/300	64,58,67	77,74,82	80,85,91	91, 97, 86	97, 95,103	107,101,105
$[\text{en}_2\text{Cr}(\text{OH})_2\text{Cr en}_2]\text{Cl}_4 \cdot 2\text{H}_2\text{O}$	1/300	72,77,78	84,82,78	83,87,91	87, 97, 94	98,101, 92	98,107,110
$[\text{Co}((\text{OH})_2\text{Coen}_2)_3](\text{NO}_3)_6 \cdot 3\text{H}_2\text{O}$	1/500	81,72,74	82,87,88	93,90,94	96,102, 98	101,106,100	102,108,107

Table XVIII

Complex salt	Protein	Concn. of complex (mol.)	pH		
			3	2	1
$\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4] \cdot \text{H}_2\text{O}$	Casein	1/60	82, 86, 83	87, 92, 93	98, 102, 107
$\text{Na}_3[\text{Co}(\text{NO}_2)_6]$	"	1/150	78, 84, 83	82, 90, 91	95, 105, 97
$\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 3\text{H}_2\text{O}$	"	1/150	86, 75, 81	86, 92, 90	107, 111, 101
$\text{NH}_4[\text{Cr}(\text{NH}_3)_2(\text{SCN})_4] \cdot \text{H}_2\text{O}$	Egg-albumin	1/60	57, 61, 64	67, 72, 73	81, 86, 84
$\text{Na}_3[\text{Co}(\text{NO}_2)_6]$	"	1/150	62, 58, 66	70, 68, 75	82, 87, 80
$\text{K}_3[\text{Cr}(\text{C}_2\text{O}_4)_3] \cdot 3\text{H}_2\text{O}$	"	1/150	58, 61, 63	68, 74, 76	81, 83, 90

obtained support the view that stoichiometric relations exist between the protein and the precipitant, as found by Chapman, et al.⁵⁾ for acid dyes, by Meyer, et al.⁶⁾ for chondroitin sulfuric acid, and by Herman and Perman⁷⁾ for metaphosphoric acid.

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Summary

Using various metallic complex salts, the precipitation of protein was studied of casein and egg-albumin. The results obtained are summarized as follows:

1. In an acid range of the isoelectric point of protein, the precipitation occurs by complex salts containing complex anion, while in an alkaline range, most of the complex salts containing complex cation cause the precipitation. Neither complex of low valency, such as $[\text{Co}(\text{NH}_3)_4\text{C}_2\text{O}_4]\text{Cl}$, $[\text{Co}(\text{NH}_3)_4(\text{NO}_2)_2]\text{Cl}$ and $[\text{Co}(\text{NH}_3)_4\text{CO}_3]\text{SO}_4 \cdot 3\text{H}_2\text{O}$, nor non-electric complex, such as $[\text{Co}(\text{NH}_3)(\text{NO}_2)_3]$, cause any precipitation in the whole pH range.

2. Of complex salts used, the capacity of precipitating protein depends on the valency.

3. In the range of concentration under examination, the equivalence of complex ion combined with a given protein remains almost constant independent of the variety, valency and the concentrations of complex salt added.

4. The effect of the time of standing hardly influences the equivalence of complex ion.

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5. While the equivalence of cation complex salt increases with the increase of pH, that of anion complex increases with the decrease of pH.

6. These results support the facts that the precipitation appears in stoichiometric ratio and that each complex salts have the capacity for combining with acid or basic radicals in protein.

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220. Yosoji Ito, Bun-ichi Tamaoki and Hideharu Sakamoto : Studies on Sexual Hormones. V. On the Synergisms between Hormones and Other Compounds. (2). The Interaction between Androgen and Estrogen in the Castrated Male Hamster.

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In male sexual sources, some remarkable quantities of estrogen were reported to be found. Ruzicka and Prelog¹⁾ obtained an extract from hog testis which contained 370 R.U. per kg. of estrogenic activity, and referred to the previously reported quantity of estrogen in ovaries, 240 R.U. per kg.²⁾ as a comparison, though, strictly speaking, results of both these experiments cannot be compared with each other because of the difference between the methods used. On the other hand, Beall succeeded in obtaining estrone and α -estradiol in crystalline forms from equine testis³⁾. In our preliminary experiment on the extract of whale testis⁴⁾, we confirmed the existence of estrogen in the extract, by the qualitative bioassay based on the uterine weight and vaginal smear test, using mice. Zondek⁵⁾ reported that stallion urine was found to contain 170,000 M.U. per liter of estrogenic activity, which was much more estrogenic than the one in pregnant mare urine, of 100,000 M.U. per liter. This remarkable quantity of estrogen in stallion urine was not likely to be metabolites of androgen in the testis or corticoid, but its origin seems to be gonadal estrogen^{5,6)}. The endocrinological significance of these estrogens in male sources, however, has not been satisfactorily clarified yet, though Δ^5 -pregnen-ol-(3 β)-on-(20) was explained as the inhibitor of estrogen in the testis¹⁾.

Many works⁷⁾ have been reported on the interaction between androgen and estrogen, but from these reports, it is not possible to ascertain any synergistic action between them clearly. In the review of their work, Emmens and Parkes⁶⁾ wrote on the test of interaction that "it seems to be the usual practice to describe the action of two substances as synergistic when the increase in weight of the test-object, resulting from the simultaneous administration of given amounts of each, is significantly greater than the added increases in weight produced by the separate administration of the same amounts to different animals of the same species."

We agree with their idea on the definition of a synergism between two different substances, and it seems to us that this idea can be statistically transformed into our

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