4. A few substituted benzyl chlorides have been measured. The results are not in accord with those obtained by other investigators with other reagents. The reactivities of the mononitro and dinitro compounds are in agreement with the other methods of evaluating the activating power of the substituted phenyl group but the reactivities of the chloro and bromo compounds are anomalous; these substances show a surprisingly great reactivity.

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[CONTRIBUTION FROM THE CHEMICAL LABORATORIES, COLUMBIA UNIVERSITY, No. 463]

THE "IRREGULAR SERIES" IN THE PRECIPITATION OF ALBUMIN[1]

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The "Irregular Series" or "Tolerance Zone" observed in the precipitation of albumin by salts of the heavier metals is accepted as a colloid-chemical phenomenon. Our knowledge concerning the precipitation of albumin by heavy metal metals and the dissolution of the coagulum upon addition of excess of the heavy metal salt is nearly a century old.[2] It has also been recorded[3] that an excess of ferric chloride causes dissolution of the precipitate first formed.

The first zone of precipitation (obtained with very dilute heavy metal solutions) has been ascribed by Bechhold[4] and by Pauli[5,6] to the formation of an insoluble complex between the albumin and the metallic oxide formed by hydrolysis of the salt. The zone of no precipitation or "Tolerance Zone" (obtained with increasing concentration of heavy metal salt) according to Bechhold[7] "results from ionization." Pauli[6] attributes it to the formation of soluble basic metallic compounds of albumin, while the second zone of coagulation is attributed by him to the salting out of the protein by the high concentration of heavy metal salt.

The existence of one soluble and two insoluble heavy metal compounds

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[1] Abstract of the dissertation submitted by Earl R. Norris to the Faculty of Pure Science, Columbia University, in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] For a review of the literature dating from 1831 see Harnack, *Z. physiol. Chem.*, **5**, 198 (1881), and O. Cohnheim, "Chemie der Eiweisskörper," F. Vieweg and Sohn, Braunschweig, **1911**.

[3] Sigfried, *Z. physiol. Chem.*, **21**, 360 (1895).

[4] Bechhold, *Z. physik. Chem.*, **48**, 385 (1904).

[5] Pauli, *Beitr. Chem. Physiol. Pathol.*, **6**, 233 (1905).

[6] Pauli and Flecker, *Biochem. Z.*, **41**, 461 (1912).

[7] Bechhold-Bullowa, "Colloids in Biology and Medicine," D. Van Nostrand Co., New York City, **1919**, p. 157.

of albumin, the formations of which are dependent upon the concentration of heavy metal salt in contact with the albumin has been postulated by Bonamartini and Lombardi[8] and by Lippich.[9]

While these explanations for the "Irregular Series" never were quite convincing they have become even less so in view of the development of the physical chemistry of proteins. Since it has been firmly established that proteins are amphoteric substances existing in solution as cations or anions according to whether the hydrogen-ion concentration of the solution is greater or less than that of the iso-electric point, a simple mechanism for heavy metal salt precipitation followed by dissolution upon acidification is apparent. When a dilute solution of such a salt is added to a solution of a protein on the alkaline side of its iso-electric point (a pure distilled water solution of albumin is actually in such a condition), combination between the anionic protein and the cation must take place, resulting in a precipitation if the complex or compound is insoluble. Due to the hydrolysis of a heavy metal salt, further addition of it increases the hydrogen-ion concentration of the solution. As soon as the solution becomes acid with respect to the iso-electric point of the protein, the precipitate must dissolve unless the anion of the added salt forms an insoluble compound or complex with the now positively charged protein ion.

The first zone of precipitation and the "Tolerance Zone" seem, therefore, to be readily explainable on a simple chemical basis. It was the purpose of this investigation to test this hypothesis. Incidentally it was found that the second zone of precipitation is due to the denaturing of the albumin.

Material

Two varieties of egg albumin were used. Fresh eggwhite was freed from globulin and crystallized according to the method of Sörensen[10] with the exception that instead of dialyzing with the diffusate under reduced pressure the albumin solution was subjected to an increased pressure. The dialysis was carried out at the iso-electric point of albumin (that is, *P*H 4.8).

As a second source of albumin, a solution of commercial, dried egg albumin was dialyzed under pressure until ash-free, the precipitated globulin filtered off and the stock solution kept under toluene until used. The albumins prepared by these two methods exhibited practically identical properties for the purpose of this investigation when at the same hydrogen-ion concentration.

Procedure

The degrees of precipitation were measured by means of centrifuge tubes of 15-cc. volume, having conical bottoms and graduated in 0.1 cc. The desired concentration of heavy metal salt was obtained by adding from a buret or standardized Mohr pipet the required amount of analyzed

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[8] Bonamartini and Lombardi, *Z. physiol. Chem.*, **58**, 165 (1908).

[9] Lippich, *ibid.*, **74**, 360 (1911); **90**, 236 (1914).

[10] Sörensen, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1916).

stock solution of the salt, followed by distilled water to make the volume of electrolyte the same in each tube. The tubes were then shaken and to each was added from a buret an equal volume of an analyzed solution of ash-free albumin. The final volume in every case was 15 cc. The solutions were then well mixed by shaking and centrifuged at 1560 r.p.m.[11] for twenty minutes and the volume of precipitate measured by the graduations on the tube.

This method of procedure which permits making a large number of measurements in a short time was found to be quite accurate for the the purpose. In a series of 16 controls an error of 0.1 cc. occurred thrice and 0.05 cc. twice.

To obtain a 0.2 *N* acetate buffer solution in some cases, 3.0 cc. of a solution containing a 1 *N* concentration of the acetate ion and of the desired hydrogen-ion concentration was added.

The P_H of the supernatant liquid was determined by means of the hydrogen electrode with a saturated potassium chloride-calomel electrode. No correction was made for contact potential.

Experimental Results

Precipitation with Zinc and Thorium Chlorides.—In general the precipitation results (shown in Fig. 1) were similar to those obtained by previous workers; a zone of precipitation was obtained with dilute solutions disappearing upon further addition of heavy metal salt. A second zone of precipitation was obtained with concentrated solutions of heavy metal salt. The "Tolerance Zone" became narrower as the concentration of albumin present was increased.

It was found that a definite minimum concentration of heavy metal ion must be present before precipitation starts. Upon increasing the concentration above this minimum the amount of precipitate increases until, due to the hydrolysis of the heavy metal salt, the concomitant increasing hydrogen-ion concentration passes that of the iso-electric point of the protein. Then as the acidity further increases, there is a rapid decrease followed by disappearance of the precipitate.

The precipitate formed in dilute solutions of heavy metal salt is soluble in dilute acid solution or in solutions with a Sörensen value less than 4.8.

This is the behavior that would be expected if we had the equilibrium, for example, in the case of zinc chloride, of $ZnCl_2 + H_2R \rightleftharpoons 2HCl + ZnR$, in which case it would be necessary to have a minimum concentration of zinc ion to satisfy the solubility product, $C_{Zn^{++}} \times C_R = K_{S.P.}$, before precipitation would take place.[12] The precipitation would then increase

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[11] The effective radius was 24.5 cm.

[12] Pauli and Matula [*Biochem. Z.*, **80**, 187 (1917)] found quite a large solubility product for silver albuminate, by means of the silver electrode.

with increasing concentration of zinc ion. After passing the iso-electric point the acid ionization of the protein would be rapidly repressed and with the decrease and disappearance of the negatively charged protein ion there would also be a disappearance of the zinc-protein complex which depends upon the presence of the protein ion, and consequently the disappearance of the precipitate with increasing heavy metal salt concentration thus giving the zone of no precipitation (tolerance zone) in the so-called "irregular series."

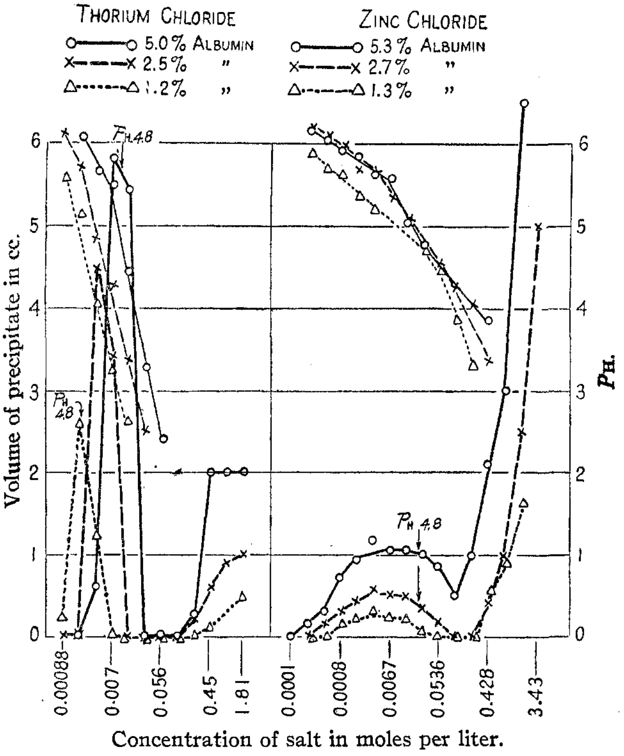

Fig. 1.

Precipitation in Solutions of Mixtures of Sodium Acetate and Acetic Acid.—An effort was made to precipitate the metal albumin complex in the presence of buffer solutions containing sodium acetate and acetic acid, thus holding the solution alkaline in relation to the iso-electric point of the albumin. In 0.2 *N* acetate mixtures it was found that in a solution originally at *P*H 5.1, precipitation did not take place until a zinc-salt concentration of 0.037 *M* was reached (Table I); with a solution originally at *P*H 5.7, a zinc-salt concentration of 0.01 *M* was necessary to produce precipitation, while in the least acid solution obtainable with the acetate mixture the first precipitation appeared at a zinc-salt concentration of 0.003 *M*. In a solution originally on the acid side of the iso-electric point of the protein no precipitation was obtained except in concentrated solutions of the zinc salt. In no case did the precipitation appear at as low a concentration of zinc salt as in the unbuffered solution, nor was the precipitation as great.

This may be explained on the basis of the solubility product of the zinc-protein complex. Addition of 0.2 *M* acetate ion to a zinc-salt solution of the order of concentration from 0.001 *M* to 0.01 *M* would repress the zinc-ion concentration in solution to such an extent that a higher concentration of zinc salt would be necessary to satisfy the solubility product of the heavy metal-protein complex. According to the work of Loeb the ionization of the protein is least at the iso-electric point. Therefore the solutions at *P*H 6 and 7 would have an increasingly greater protein-ion concentration than the solution of *P*H 5.1 which is very close to the iso-electric point. Consequently, it would be expected that the solution at *P*H 5.1 would require a greater concentration of zinc ion to cause precipitation.

The precipitates obtained in solutions containing the acetate mixture with low concentration of zinc chloride (up to 1.67 *M* zinc chloride) were soluble in dilute acid solution, while the precipitate formed in more concentrated solutions was insoluble upon dilution and in dil. acid solution.

TABLE I

PRECIPITATION OF ASH-FREE ALBUMIN IN ACETATE MIXTURES

Final concentration of albumin 1.8%

	No acetate		In 0.2 *N* acetate buffer mixtures									
			1		2		3		4		5	
Concn. $ZnCl_2$ M./l.	Vol.[a] Cc.	*P*H	Vol.[a] Cc.	*P*H	Vol.[a] Cc.	*P*H	Vol.[a] Cc.	*P*H	Vol.[a] Cc.	*P*H	Vol.[a] Cc.	*P*H
0.0008	0.5	7.07	0	7.2	0	5.74	0	5.17	...	4.8	...	4.0
.0017	.6	..	trace	..	0	..	0	..	...	..	..	..
.0034	.7	6.46	.3	6.97	0	..	0	..	...	..	..	..
.0067	.85	..	.35	..	trace	..	0	..	...	..	..	..
.0134	.85	6.09	.35	6.30	0.1	..	0	..	...	..	..	..
.0268	.75	5.71	.38	..	.2	5.53	0.1	5.10	...	..	..	..
.0536	.5	5.18	.38	6.15	.25	5.49	.2	5.04	...	..	..	..
.107	.25	4.61	.38	6.04	.3	5.43	.2	4.85	...	..	..	..
.214	.25	3.73	.38	5.56	.3	5.12	.25	4.60	...	..	0	3.51
.429	.3	2.71	.38	4.98	.3	4.67	.3	4.29	...	..	0.35	3.18
.858	.8	1.75	.38	4.44	.4	4.12	.4	3.72	...	..	.55	2.62
1.715	2.5	0.75	.65	3.75	.75	3.42	.85	3.07	1.0	2.75	2.0	1.35
3.430	2.5	..	2.5	1.91	2.5	1.91	2.5	1.81	2.5	1.21	2.5	0.1

[a] Volume of precipitate after centrifuging.

The Second Zone of Precipitation

The precipitate formed by the action of concentrated solutions of heavy metal salts upon albumin is irreversible,[13] that is, does not redissolve upon

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[13] Thomas and Norris, *Proc. Soc. Exptl. Biol. Med.*, **21**, 173 (1924).

dilution with water. When it is washed with a solution of approximately the same hydrogen-ion concentration as that in which it was coagulated, until the washings no longer show a test for the heavy metal ion, an insoluble, ashless protein residue is obtained with the appearance and properties of heat-coagulated albumin. This would indicate that the denaturing of the albumin which will take place if this protein is heated in solution (the temperature depending upon the hydrogen-ion concentration of the solution and the salt content) will take place at room temperature in the presence of heavy metal salts.

Although the heat coagulation or denaturing of albumin has never been thoroughly understood, a great deal of work has been done by various investigators to determine the factors which influence it. Scheele[14] and others among the early biochemists state that when the white of egg is mixed with exactly ten parts of water and boiled the solution remains unchanged, but upon addition of acid it coagulates with the appearance of milk.

Schmidt and his students, Aronstein[15] and Heinsius,[16] found that albumin solutions which had been dialyzed until free from salt did not coagulate when heated, but that after heating and cooling, coagulation took place when an electrolyte was added.

Hardy[17] concluded that heat coagulation takes place in two processes, first, denaturing and second, agglutination of the denatured albumin, to which Chick and Martin[18] added that heat coagulation consists of denaturing followed by agglutination by the electrolyte. They considered the first reaction to be one between albumin and water.

That denaturing is a chemical reaction follows from the fact that it does not take place at a definite temperature, but as with other chemical reactions requires a period of time, which is dependent upon the temperature. Chick and Martin obtained an acceleration of the speed of heat denaturing of 1.9 times for every degree rise in temperature.

Lepeschkin[19] concluded that denaturing must be an hydrolysis, since it depends upon the hydrogen-ion concentration and temperature and is affected by the presence of salts in a manner similar to the hydrolysis of sugar. Chick and Martin obtained no evidence of hydrolysis when solutions were heated to 100° for two hours in the presence of just sufficient alkali to prevent precipitation, using the Sörensen formalin titration method as a test for hydrolytic products.

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[14] Scheele, "Mémoires de Chimie de W. Scheele," Dijon (1785), from Grimaux, *Bull. soc. chim.*, **42**, 78 (1884).

[15] Aronstein, *Pflüger's Arch. ges. Physiol.*, **8**, 75 (1874).

[16] Heinsius, *ibid.*, **9**, 514 (1874).

[17] Hardy, *J. Physiol.*, **24**, 158 (1899).

[18] Chick and Martin, *ibid.*, **40**, 404 (1910); **43**, 1 (1911); **45**, 61, 261 (1912); **46**, 104 (1913).

[19] Lepeschkin, *Kolloid. Z.*, **31**, 342 (1922).

Wo. Ostwald[20] found that certain neutral salts cause a lowering of the temperature of coagulation.

Wagner[21] discovered that serum albumin is irreversibly precipitated by an excess of acid, and that the temperature of coagulation depends upon the acidity of the solution.

Werigo[22] and Bülow[23] working with Harnack's ash-free albumin, which is formed by treating albumin with a concentrated solution of copper sulfate, dissolving the precipitate in strong alkali and re-precipitating with acid, concluded that the copper acts upon the albumin changing it to an insoluble compound, which they erroneously called an albuminate.

Experimental Part.—Upon precipitating acetate buffer solutions of albumin of various hydrogen-ion concentrations at room temperature, a marked difference in the amount of precipitate with a zinc-salt concentration of 1.72 *M* was noticed (Table I), the precipitate apparently increasing with increasing hydrogen-ion concentration.

In this experiment all tubes were allowed to stand the same length of time and are therefore comparable. It was found that the precipitate continued to form for a considerable period of time in the tubes in which it was not complete initially.

An identical series was prepared and centrifuged for 20 minutes at time intervals up to 72 hours. The increases in volume are recorded in Table II.

TABLE II

RATE OF DENATURING OF ALBUMIN AT ROOM TEMPERATURE

Final concentration of albumin, 1.8%; $ZnCl_2$ = 1.72 *M*

	Volume of precipitate in cc.					
*P*H	Upon mixing	1 hour	8 hours	24 hours	48 hours	72 hours
2.75[a]	1.0	1.1	1.4	1.8	1.85	1.9
3.07	0.6	0.7	0.8	1.1	1.3	1.4
3.42	.5	.6	.75	0.95	1.15	1.2
3.73	.3	.45	.6	.75	0.9	0.9

[a] At *P*H = 1.35 and lower, the denaturing was complete instantaneously.

In order to study the denaturing over shorter periods of time a series of tubes was set up, each holding 15 cc. of solution containing 1.8% of crystallized egg albumin and 1.72 *M* zinc chloride. The solutions in the tubes were allowed to stand at room temperature for various lengths of time from ten seconds to one hour, upon the expiration of which they were quickly poured into 400 cc. of distilled water and the mixture was filtered by suction and thoroughly washed. Kjeldahl determinations of nitrogen were made upon the precipitates (Table III).

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[20] Wo. Ostwald, *Kolloid. Z.*, **2,** 108, 138 (1907).

[21] Wagner, *Biochem. Z.*, **104,** 190 (1920).

[22] Werigo, *Arch. ges. Physiol.* (*Pflüger's*), **48,** 127 (1891).

[23] Bülow, *ibid.*, **58,** 207 (1894).

If the albumin coagulum in the second zone were reversible upon dilution as most previous workers have stated, it would be dissolved by this method, but these experiments show that the precipitate formed in concentrated solutions of heavy metal salts is not reversible even after very short periods of time of contact with the heavy metal ion.

TABLE III

PRECIPITATION OF ALBUMIN OVER SHORT PERIODS OF TIME IN CONCENTRATED SOLUTIONS OF ZINC CHLORIDE

Final concentration of albumin, 1.5%[a]

Time	Amount of coagulum in terms of milligrams of nitrogen				
	1.72 M $ZnCl_2$			0.86 M $ZnCl_2$	0.43 M $ZnCl_2$
	PH 0.7	PH 1.4	PH 2.7	PH 1.7	PH 2.7
10 seconds	6	..	..	..	..
30	10	14	..	0	..
1 minute	..	..	0	..	..
5	27	26	2	trace	..
10	30	..	..	3	..
15	..	34	..	13	..
30	32	..	7	18	..
1 hour	32	34	11	25	3
5	..	..	..	31	..
20	..	..	..	..	16

[a] Total nitrogen in 15 cc. of solution is 34 mg. ± 1 mg.

The effect of both temperature and hydrogen-ion concentration upon denaturing of crystalline egg albumin in the presence of a heavy metal salt was next studied. Three series of experiments were prepared in which the hydrogen-ion concentrations were varied by means of 0.2 N mixtures of sodium acetate and acetic acid, in solutions containing a final concentration of 1.5% of egg albumin and 1.72 M zinc chloride. Two of the series of tubes were placed in 40° and 25° water thermostats, respectively, which were regulated to within 0.01°. The third series was placed in an ice box, the temperature of which varied between 10° and 11°. When at the desired temperature, the solutions were mixed and centrifuged for 20 minutes and then at intervals of one, five, ten, 24 and 48 hours, meanwhile standing at the temperature noted. At the end of the 48-hour period the coagulum was filtered off, washed and nitrogen determined by the Kjeldahl method. Then, assuming that the partial volumes obtained at one, five, ten and 24 hours contained a proportionate amount of the precipitate of the final volume, the corresponding amounts of nitrogen were calculated (Table IV).

Table IV and Fig. 2 show that the rate of denaturing is a function of the hydrogen-ion concentration and of temperature of the solution.

To determine whether the denaturing effects observed were due to the action of the acid of hydrolysis upon the albumin or due to the heavy metal salt, three series of tubes containing 3.8% of albumin in hydrochloric acid solution were set up at hydrogen-ion concentrations varying from *P*H 0.8 to 4.0 and placed in thermostats at 10°, 25° and 40°. Immediately upon mixing, the contents of the tubes at *P*H = 0.8 became turbid and after one hour they had set to an opaque jelly. The solutions at *P*H 1 set to a transparent jelly in ten hours at 40° and in 48 hours at 25°. The other solutions remained clear and liquid after several days; however, upon titration to a Sörensen value of approximately 5 a heavy precipitate was obtained in all the solutions which had remained in the thermostat at 40°. There was only a slight precipitate at *P*H 3.7 and no precipitate at *P*H 4.0 and 4.4 in the solutions which were kept at 25°.

TABLE IV

EFFECT OF TEMPERATURE AND HYDROGEN-ION CONCENTRATION UPON THE PRECIPITATION OF ALBUMIN IN 1.72 *M* ZINC CHLORIDE SOLUTION

Final concentration of albumin, 1.5%

*P*H	Amount of coagulum in terms of milligrams of nitrogen Immediately upon mixing					
		1 hour	5 hours	10 hours	24 hours	48 hours [a]
			TEMPERATURE 10–11°			
1.11	33	33	33	33	33	33
2.63	6	10	14	19	24	27
..	3	4	5	7	13	16
3.03	2	3	4	5	7	9
3.20	1	2	2	4	4	7
			TEMPERATURE 25°			
1.05	33	33	33	33	33	33
2.58	11	18	27	33	34	34
2.94	2	6	9	13	18	26
3.25	2	3	6	8	13	20
3.69	1	3	4	6	10	18
			TEMPERATURE 40°			
0.99	33	33	33	33	33	33
2.80	4	20	28	29	31	32
3.25	3	15	24	26	30	32
3.69	2	13	23	25	30	33

[a] These values were obtained from Kjeldahl determinations. The other columns of data are calculated from the 48-hour data from the precipitate volume proportions.

Total nitrogen in 15 cc. of solution = 34 mg. ± 1 mg.

These experiments show that albumin is denatured in acid solution, even though no precipitate is obtained while the solution remains acid.

In order to determine whether the precipitates obtained in concentrated solutions of heavy metal salt were due to an accelerating effect of the heavy metal or merely to a neutral salt precipitation of the albumin denatured by the high hydrogen-ion concentration of the solutions, similar experiments were performed using an alkali and alkaline earth salt. Such experiments were arranged at 25° with 1.9% of albumin in the presence of 0.83 *M* and 1.66 *M* sodium chloride and 1.48 *M* magnesium chloride.

The results shown in Table V indicate that while the albumin is denatured and precipitated in the presence of sodium and magnesium chlorides, the denaturing is not started at so low a hydrogen-ion concentration as in the case of heavy metal salts. No marked difference was observed between 0.8 *M* and 1.66 *M* sodium chloride nor between sodium chloride and magnesium chloride. At a Sörensen value of approximately 2.5 the albumin is completely denatured in the presence of 1.72 *M* zinc chloride while it is only about 20% denatured in the presence of sodium and magnesium chlorides. Consequently, the theory of Chick and Martin, namely, that the salt present merely acts as a precipitant for denatured albumin present in the solution (as a hydrophobic colloid), is shown to be untenable for a heavy-metal salt. The heavy-metal cation is an albumin denaturant, as is

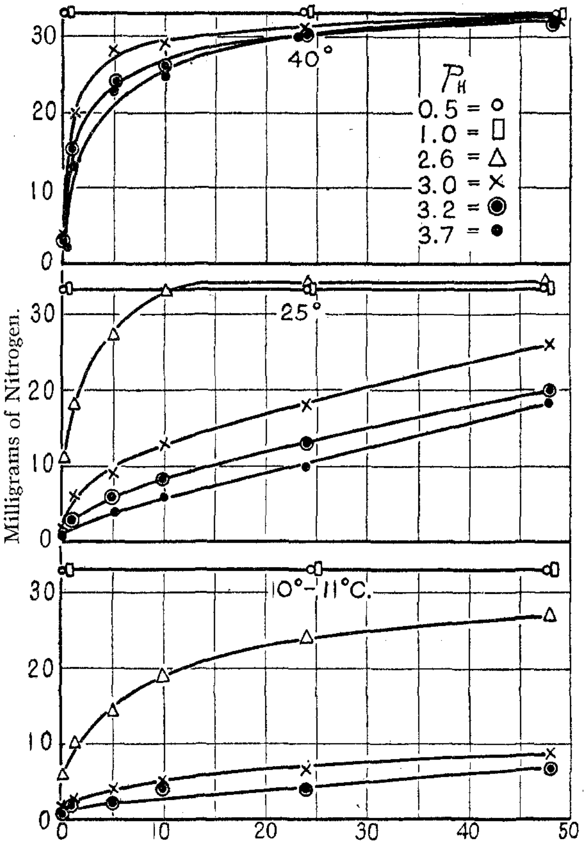

Fig. 2.

TABLE V

PRECIPITATION OF EGG ALBUMIN IN THE PRESENCE OF SODIUM AND MAGNESIUM CHLORIDES AT 25°

Final concentration of albumin, 1.9%

*P*H	Amount of coagulum in terms of milligrams of nitrogen 1 hour	2 hours	5 hours	24 hours	48 hours	56 hours [a]
CONCENTRATION OF NaCl, 0.83 *M*						
0.68	..	36	38	42	44	**44**
1.17	..	22	33	44	44	**44**
1.67	..	11	18	42	42	**42**
2.41	..	3	4	11	16	19
3.20	..	1	1	2	2	3
CONCENTRATION OF NaCl, 1.66 *M*						
0.69	14	..	27	40	43	44
1.04	9	..	21	38	42	43
1.53	4	..	10	33	36	38
2.30	1	..	3	8	14	14
3.22	0	..	0	1	1.5	2
CONCENTRATION OF $MgCl_2$, 1.48 *M*						
0.07	24	..	41	43	43	43
0.56	18	..	35	42	42	42
1.10	13	..	30	42	42	42
1.99	4	..	13	27	31	34
2.70	1	..	2	3	3	4
3.79	0	..	0	0	0	0

[a] These values were obtained from Kjeldahl determinations. The other columns of data are calculated from the 56-hour data from the precipitate volume proportions.

Total nitrogen in 15 cc. of solution is 43 mg. ± 1 mg.

hydrogen ion, and at a given hydrogen-ion concentration the rate of denaturing is catalyzed by the presence of a heavy-metal ion.

Precipitation by Other Metallic Salts

Thorium nitrate, cupric and ferric chlorides all showed the "irregular series" effect just as did thorium and zinc chloride but for obvious reasons they could not be studied as a function of the hydrogen-ion concentration. A 0.16 *M* final concentration of ferric chloride converted a 7.6% solution of albumin to a solid coagulum.

Previous workers have reported that silver salts acted differently from other heavy-metal salts in that no irregular series was observed, the silver salt precipitating the albumin in dilute, intermediate and concentrated solutions forming only one zone of precipitation, but the results given in Table VI indicate that the difference between silver and other heavy-metal salts is not so much a difference in kind as it is a difference in degree. An effort was made to estimate the hydrogen-ion concentration of the solution within the range between *P*H 4.5 and *P*H 6.0 by means of the indicator methyl red. Comparison was made with acetate-acetic acid buffer solutions which were standardized by the hydrogen electrode. The results were not satisfactory because many of the solutions were turbid, the high protein and salt content also interfering with the color of the indicator, but indicating that albumin is denatured in a solution of silver nitrate at a Sörensen value as low as 4 to 5 and at very low concentrations of the salt. The irregular series is obtained if the original albumin solution is sufficiently dilute.

TABLE VI

PRECIPITATION OF ALBUMIN IN THE PRESENCE OF SILVER NITRATE

Concn. $AgNO_3$, M./l.	0.0025	0.005	0.01	0.02	0.04	0.08	0.17
Ppt. in 1.9% of albumin, cc.	.3	0.25	0.0	0.0	0.0	3.7	2.4
3.8% of albumin, cc.	.15	1.1	4.3	.25	5.8	3.8	..

Chromium sulfate acted differently from the other metal salts studied in that no precipitate was obtained immediately after mixing the solutions at any concentration. There was however, an increasing grayish appearance and opacity of the solution reaching a maximum at the iso-electric point of the protein, which disappeared progressively with increasing chromium and hydrogen-ion concentration, giving place to the clear green of the chromium solution. After the solutions had stood overnight at room temperature the viscosities had changed considerably, augmenting with increasing chromium salt concentration to a maximum at the iso-electric point, where the albumin had set to a jelly and then decreased with further increasing salt concentration. Portions of the jelly which had formed at the iso-electric point dissolved when placed in *M* chromium sulfate solution and in 0.1 *N* sulfuric acid.

It has been stated by Friedman[24] and by Pauli[6] that the precipitate formed, when albumin solution is mixed with ferric oxide hydrosol, is soluble in excess of ferric oxide hydrosol. Our experiments showed this to be true provided that the hydrosol has not been thoroughly dialyzed and consequently is still slightly acid in reaction. However, with a thoroughly dialyzed sol no tendency for the precipitate to dissolve was observed. The supernatant liquid from the precipitated albumin was found to have a Sörensen value of approximately 6. Thomas and Frieden[25] found the hydrogen-ion concentration of a pure sol to be approximately 10^{-5}; therefore it would be impossible by further addition of ferric oxide sol to pass to the acid side of the iso-electric point of the albumin. The precipitate formed was readily soluble in dilute acid solution, however, as was to be expected.

The literature contains many data concerning the amounts of heavy-metal ions which combine with proteins to form coagula. A few of these

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[24] Friedman, *Arch. Hyg.*, **55**, 361 (1906).

[25] Thomas and Frieden, THIS JOURNAL, **45**, 2522 (1923).

precipitates may have formed on the alkaline side of the iso-electric point, but most of them were made with concentrated solutions of heavy-metal salts. In the latter instance the coagulum of denatured albumin with its entrained heavy-metal salt was washed with distilled water, resulting in the hydrogen-ion concentration approaching that of the wash water.

When the hydrogen-ion concentration of the protein being washed, reached and passed that of the iso-electric point of the protein, the entrained heavy-metal ion still present then combined with the protein. This would account for the great variation in results which have been published; for example, Bonamartini and Lombardi[8] found that upon treating albumin with a large excess of copper sulfate in varying proportions and washing the precipitate with water, so-called compounds containing from 5 to 8% of copper were obtained while, when they treated the precipitate with various concentrations of alkali before washing and then washed with water the resulting residue contained from 19 to 31% of copper.

In view of these facts and because heavy-metal proteinates are not absolutely insoluble, it is evident that existing data on the composition of heavy-metal protein coagula are of no value.

Conclusions

1. The first precipitation zone in the "irregular series" of protein precipitation by heavy metals has been shown to be dependent upon the hydrogen-ion concentration of the solution.
2. The precipitate in the first zone is probably a definite chemical compound, which is only relatively insoluble and formed by interaction of heavy-metal ion and the protein anion.
3. The zone of no precipitation ("tolerance zone")is due to the passage of the solution from the alkaline to the acid side of the iso-electric point of the protein, the protein changing from anion to cation.
4. The second precipitation zone is shown to be due to the denaturing of the albumin.
5. The denaturing of albumin in the presence of heavy-metal salt is shown to be a function of the temperature, hydrogen-ion concentration of the solution, concentration of heavy-metal salt and the time of reaction, increasing in rate as any or all of these factors are increased.
6. A heavy-metal ion is intrinsically an albumin denaturant.

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