LXX. THE HEAT-COAGULATION OF PROTEINS.

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It has been long pointed out that the heat-coagulation of proteins is not so simple a phenomenon as it at first may seem to be. Schmidt and his pupils, Aronstein [1874], Heynsius [1874], Kieseritzky [1882] and Rosenberg [1883], showed that albumin solutions from which the electrolytes had been removed by dialysis did not coagulate on heating; heat-coagulation could only be produced after the addition of electrolytes to such solutions; it took place also if a small amount of any salt was added to the cold albumin solutions which had been preliminarily heated. These authors therefore suggested the hypothesis that heating may transform the albumin into an unknown body, the solutions of which coagulate on the addition of a very small quantity of salts [Rosenberg, 1883, p. 34].

Hardy [1899, 1900, 1906] showed later that in solutions of protein altered by heating ultramicros appear and that the protein can now be precipitated by adjusting the reaction so as to render the particles isoelectric with the solution. When the protein is made negative by adding alkali the valency of cations is of importance for the precipitation; on the contrary, the cations have no part in the precipitation if protein is made positive.

Then Michaelis came to the conclusion [Michaelis] that the properties of albumin solutions may be changed by heating from those of an "emulsoid" to those of a "suspensoid," whereas the high viscosity and the low surface-tension of solutions of albumin altered by heating point rather to their hydrophilic character. These solutions probably do not fall into either category of the colloidal state but form an intermediate group with some of the properties of both.

Chick and Martin [1913, 1] who investigated the heat-coagulation of proteins and the influence of various factors upon its course confirm the conclusion that this phenomenon consists of two distinct processes: (1) "denaturation" and (2) "agglutination" of the denaturated protein by electrolytes. They regard the former as a reaction between protein and hot water without determining this reaction more precisely.

Contradicting the results of the above investigations and of his own earlier experiments Pauli [1899, 1908] affirms that he has observed that protein solutions free from electrolytes ("amphoterer Eiweiss") coagulate on heating and that the addition of electrolytes to such solutions increases their coagulation temperature. Criticism and detailed consideration of this new opinion may be postponed till some experiments related to heat-coagulation have been described.

A number of scattered observations indicated that temperature is an accelerating factor in the process of heat-coagulation. Buglia [1909] was the first who precisely determined the dependence of the velocity of heat-coagulation upon the temperature, and found this dependence to be logarithmic.

Chick and Martin [1910] found that the coagulation-rate increases 1.3-1.9 fold per 1° temperature-rise. Such a high temperature coefficient bears only on the denaturation, while the agglutination-velocity does not increase so rapidly with the temperature as the denaturation-rate, its temperature coefficient being equal to 2.5 per temperature-rise of 10° [Chick and Martin, 1913, 2]. These authors showed moreover that, if precautions are taken to keep the concentration of hydroxyl or hydrogen-ions constant during the process, the denaturation may proceed as a reaction of the first order.

(1) EXPERIMENTAL METHODS.

My experiments were made before I was acquainted with the papers by Chick and Martin, and the method used by me differs from theirs. I find however that it is more convenient for observing slight, and sometimes short, alterations of velocity in both the processes that are comprised in heat-coagulation.

In order to estimate alterations in the velocity of heat-denaturation and in coagulation ("agglutination") of the denaturated protein under influence of various factors, in my experiments, the time required to produce a slight turbidity in the protein solutions like that of a ground glass (chosen for all the experiments) was noted. The solution to be tested was usually introduced by means of a capillary pipette into a small vessel made of two cover-glasses ($20 \times 10 \times 0.1$ mm.) cemented with oil-lacquer (linseed-oil and minium) on both sides of a rectangular brass plate (0.7 mm. thick, 22 mm. long, 13 mm. broad) the middle of which was rectangularly cut out, so that the rim-breadth (at three sides) was equal to 3 mm.

A drawing of this vessel is given in one of my previous papers devoted to the reactions between starch and water brought about by heating¹. There are also given a drawing and a photograph of the thermostat used.

¹ Lepeschkin, "Etude sur les réactions chimiques pendant le gonflement de l'amidon dans l'eau chaude." Le manuscrit est renvoyé à M. le professeur Chodat (Genève, Suisse) pour la publication dans l'*Archives des sciences naturelles*.

The above vessel with the protein solution to be tested was immersed in the water of the thermostat, in which the temperature variation did not surpass 0.03° . Close by the vessel, a similar rectangular brass plate (cut out in the middle) was placed; instead of the cover-glasses (see above) a piece of ground glass of the same size was cemented on one side of this plate. Both (viz. the vessel and the plate with ground glass) were observed through the front glass-wall of the thermostat box by means of a horizontal microscope of which the eye-piece was removed and the objective was substituted by a black plate having two rectangular apertures corresponding respectively to the front cover-glass of the vessel and to the ground glass. The protein solution and the ground glass were illuminated by an electric lamp (50 candles) through the back glass-wall of the thermostat, and the microscope-tube was situated obliquely to the front glass-wall (in order to observe most easily the turbidity produced in the protein solution by heating).

The water of the thermostat was uninterruptedly stirred by means of an electric stirrer.

The time-intervals were defined by means of a seconds pendulum.

The preliminary experiments showed that the liquid contained in the above vessel takes up the temperature of the thermostat water in about 20 seconds. This interval of time was always deducted from the time required to produce the above turbidity of the solution to be tested.

The material used was filtered egg-white, egg-albumin Kahlbaum from which electrolytes had been removed by dialysis, serum-albumin Kahlbaum similarly treated or purest recrystallised egg-albumin.

The first experiments are concerned with denaturation-velocity at various temperatures.

(2) THE TEMPERATURE COEFFICIENT OF DENATURATION.

Preliminary experiments showed that the coagulation-velocity of the denaturated proteins in solutions containing a sufficiently great quantity of electrolytes is extraordinarily great in comparison with the denaturation-velocity at the same temperature. It could thus be assumed that the coagulation of denaturated protein proceeds instantaneously at the temperatures the influence of which upon the denaturation was investigated in my experiments. The appearance of the turbidity in the protein solutions containing a sufficient quantity of electrolytes marked, therefore, the denaturation produced by heating (or is it truer to say the degree of denaturation corresponding with the turbidity of the solution like that of the ground glass). The results were the following.

Filtered egg-white. The temperature of water in the thermostat was in the experiments: 63.01° , 62.01° , 61.02° , 60.03° , 59.03° , 58.01° , 57.04° and 56.00° . The average (from six experiments) times (in seconds) passed from the moment of immersing the vessel with egg-white into the water to the appearing of the required turbidity of the solution T were found respectively:

40 secs., 97 s., 230 s., 595 s., 1535 s., 3720 s., 9520 s., and 22,600 s. The temperature coefficients per rise of 1° thus were found to be: 2.4, 2.4, 2.5, 2.6, 2.4, 2.6, 2.4 or on the average 2.5.

3 % solution of *egg-albumin Kahlbaum*. This had been dialysed during 5 days and no longer coagulated on heating; in order to make it coagulable 0.4 % ammonium sulphate was added. Temperatures of the thermostat: 70.04° , 69.00° , 68.00° , 67.04° , 66.04° , 65.06° . The average (from five experiments) times T : 91 secs., 136 s., 207 s., 306 s., 452 s., 690 s. The average temperature coefficient per rise of $1^\circ = 1.5$.

2.5 % solution of *serum-albumin Kahlbaum*. This had been dialysed during 20 hours and no longer coagulated on heating. 0.7 % potassium chlorate was added. The temperatures were: 75.00° , 72.00° , 70.00° , 68.00° . The average times T : 59 secs., 205 s., 470 s., 1298 s. The average temperature coefficient = 1.6.

1 % solution of *recrystallised egg-albumin* (not coagulating on heating). 1.5 % ammonium sulphate was added. The temperatures were: 70.00° , 69.00° , 68.00° , 67.00° . The average times T : 154 secs., 331 s., 784 s., 1860 s. The average temperature coefficient = 2.3.

The coefficient of denaturation thus is equal to 1.5–2.5 per 1° , viz. 58–9540 per 10° . A similar coefficient was found by me for the chemical reaction between starch and water during the swelling of starch in hot water. See footnote p. 679.

(3) ON THE NATURE OF DENATURATION. THE INFLUENCE OF HYDROGEN- AND HYDROXYL-IONS UPON THE DENATURATION-RATE.

Chick and Martin's researches [1910] show that water is of great significance in the denaturation process of proteins, and reasoning from analogy with the above mentioned reaction between starch and water, I should incline, in accordance with Chick and Martin, to regard this process as a chemical reaction between protein and water. Further we shall see indeed, that the chemical properties of denaturated protein differ considerably from those of native protein.

In my cited paper it was suggested that during the swelling of starch in hot water no hydrolysis takes place but the starch forms a compound with water. Indeed, the velocity of the reaction between starch and water increases not at all proportionally to the hydrogen-ion concentration but depends upon the nature of the acid added, *e.g.* sulphuric acid, in weak concentrations, even lowers the reaction-rate. So then the hypothesis, that starch preliminarily forms a compound with acid before it reacts with water, must be adopted. The preliminary formation of compounds with potassium and sodium hydroxide which react more easily with water than free starch is also certain.

The fact that denaturation proceeds as a reaction of the first order (molecular reaction) indicates that the protein reacts with the solvent, but leaves it uncertain whether the denaturation is a hydrolysis, like the inversion

of sugar, or is accompanied by the formation of a compound between protein and water. It is important in this connection to know the effect of acids and alkalies upon the denaturation-velocity.

Chick and Martin [1913] who found the denaturation-rate to be increased with increasing concentration of hydrogen- or hydroxyl-ions in protein solutions suggest that the cause of this phenomenon lies in the fact that protein forms salts with acids and alkalies, and that these salts are in a more intimate association with water.

The researches of Hoffmann [1889, 1890], however, had already shown that slight quantities of acid which are added to a pure protein solution form no salts with protein, and this result is in accordance with the recently found weakly acid nature of proteins (about 0.00001 *N* hydrogen-ion concentration). Nevertheless my experiments showed that a very remarkable increase of denaturation-rate is observed even when albumin solutions contain 0.00016 mol. nitric acid per litre. The increasing effect of such small concentrations of acid upon the denaturation-rate cannot be explained except by an acceleratory influence of hydrogen-ions like that observed in the case of hydrolysis of carbohydrates, viz. by their catalytic properties. The denaturation-velocity was however found in my experiments not to be proportional to the concentration of hydrogen-ions present in the solution when strongly different concentrations of the same acid had been tested. It increased more slowly than the hydrogen-ion concentration when the latter was not greater than 0.002 mol. per litre, but it began to increase more rapidly when the hydrogen-ion concentration attained 0.01 mol. per litre.

However, if various acids were tested at about the same molecular concentrations, the denaturation-velocity was found to depend upon the hydrogen-ion concentration, so that contrary to the case of the reaction between starch and water, the nature of the acid has here no influence upon the denaturation-velocity.

The material used in my experiments was a solution of egg-albumin Kahlbaum which had been dialysed during many days. The solution was diluted partly with water, partly with nitric acid so that the albumin-content in all solutions was 0.66 %. In order to make the solutions obtained coagulable 0.8 % $(\text{NH}_4)_2\text{SO}_4$ was added. When the concentration of nitric acid was 0 *N*, 0.00016 *N* mol., 0.00032 *N*, 0.002 *N*, 0.016 *N*, the average time of coagulation at 63° was found respectively to be 2440 secs., 1550 s., 1220 s., 315 s., 11 s. In the case of different acids the average coagulation-time at 53° was found to be equal to 167 secs. in the presence of 0.016 *N* HNO_3 (viz. hydrogen-ion concentration = 0.015 *N*), 161 secs. in the presence of 0.015 *N* HCl (hydrogen-ion concentration = 0.014 *N*), 86 secs. in the presence of 0.018 *N* H_2SO_4 (hydrogen-ion concentration = 0.024 *N*) and 1275 secs. in the presence of 0.016 *N* acetic acid (hydrogen-ion concentration = 0.0016 *N*).

The absence of proportionality between the denaturation-velocity and the concentration of hydrogen-ions in the case of strongly different concen-

trations of the same acid, is not unexpected. Indeed a strict proportionality between the hydrogen-ion concentration and the reaction-rate also does not exist in the case of the hydrolysis of sugar by acids, particularly when salts are present in the sugar solution.

Arrhenius [1889] who investigated the influence of salts upon the inversion-velocity showed that the latter departs from its normal value almost proportionally to the salt-content of the solution; moreover, the weaker the acid concentration, the more considerable is the influence of salt; so for instance, the inversion-rate increased by 35–40 % on adding KBr to the concentration of 0.4 *N*, when hydrobromic acid was 0.002 *N*, and it increased by 200–240 % when the acid was 0.0005 *N*.

At the same time, it was necessary, in my experiments, to add ammonium sulphate to the albumin solutions to bring about the coagulation of denaturated albumin. No doubt the salt increased the catalytic power of the hydrogen-ions normally contained in the albumin solution, the presence of which is due to the electrolytic dissociation of the protein molecules (hydrogen-ion concentration of about 0.00001 *N*); then, the salt induced thereby an extraordinarily strong increase of the denaturation-rate¹. However, this increasing effect of salt upon the catalytic power of hydrogen-ions strongly diminished on the further increase of the concentration of these ions by adding nitric acid so that the denaturation-rate increased only by 1.6 fold, while the hydrogen-ion concentration increased by 15 fold. By virtue of the same cause the denaturation-velocity continued to increase more slowly than the concentration of hydrogen-ions on the addition of acid. When, however, this concentration became more than 0.002 *N* the denaturation-rate began to increase more rapidly than the hydrogen-ion concentration (an increase of the latter of 8.3 fold was accompanied by an increase of the former of 28 fold). The same was found by Chick and Martin [1912].

The more rapid increase of the denaturation-rate by stronger concentrations of acid could not be explained except by the formation of compounds between albumin and acid which react more easily with water than the unaltered albumin, the acid concentration 0.016 *N* being evidently sufficient for the formation of such compounds.

As the increasing effect of salts upon the catalytic power of hydrogen-ions varies only with a very considerable alteration of concentration of these ions (Arrhenius, see above), it is very comprehensible that the denaturation-rate remained in my experiments almost proportional to the hydrogen-ion concentration in the case where denaturation proceeded in solutions containing similar concentrations of various acids.

In view of the importance of hydrogen-ions in the reaction between protein and water, it seems to be very probable that the denaturation is a slight hydrolysis of protein; such hydrolysis cannot be discovered by chemical

¹ It is uncertain whether this increase of denaturation-rate is due to the formation of an active albumin, analogically to the active sugar of Arrhenius.

analysis, the protein molecule being too large, and it is not surprising that Chick and Martin could obtain no evidence of hydrolysis by denaturation of pure proteins in the chemical way.

We go on now to consider the influence of alkali upon denaturation.

From Chick and Martin's experiments [1913] it follows that in the presence of very weak alkali concentration (hydroxyl-ion concentration equal to $10^{-3.8} N$) the denaturation proceeds too slowly and only on addition of more alkali (hydroxyl-ion concentration $10^{-2.7} N$) does the denaturation-rate increase so as to be comparable with that in the original, slightly acid solution. These experiments confirm therefore the above hypothesis that the denaturation of albumin, free from electrolytes, is accelerated by its own hydrogen-ions present in the solution in concentration of about $10^{-5} N$: after the neutralisation of albumin by a small amount of alkali the accelerating effect of these ions is abrogated and the denaturation-rate strongly diminished. The strong increase of the latter on the addition of more alkali could then be explained by the formation of albumin salts with alkali (alkali albuminate) which react more easily with water than the unaltered albumin.

Unfortunately it was only possible for Chick and Martin to institute a comparison over a small portion of the curves expressing the dependence of the denaturation-rate upon the hydroxyl-ion concentration (namely, from a concentration equal to $10^{-3.8}$ to $10^{-2.7}$). Nevertheless it seemed to me to be desirable to investigate the effect of stronger concentrations of alkali upon the denaturation-velocity. My experiments made for this purpose showed that an increase of the hydroxyl-ion concentration beginning from $10^{-2.2} N$ only influences the denaturation-rate very little. Thus an increase of alkalinity of over sixty-fold was accompanied by a decrease in denaturation-rate of 27 %.

In my experiments a solution of serum-albumin Kahlbaum dialysed during 6 weeks and containing 0.49 % albumin was used. This solution was diluted with an equal volume of a solution of potassium hydroxide of known concentration and in order to keep it coagulable ammonium sulphate (concentration of 0.2 N , or 0.4 N when the alkali concentration was great) was added to it. When the hydroxyl-ion concentrations were 0.006 N ($=10^{-2.2}$), 0.012 N , 0.067 N , 0.150 N , 0.400 N , the average denaturation-times at 70° were found to be equal respectively to 270 secs., 285 s., 300 s., 320 s., 370 s.

The study of the influence of alkalies and acids upon the denaturation showed therefore that the above hypothesis concerning the nature of this reaction is true: the denaturation is a weak hydrolysis of protein.

This hydrolysis can be accompanied also by the splitting off of any amino-acids containing sulphur from the protein molecule [Moll, 1904] and by an increase in the amount of carboxyl group, for, as will be shown below, the acid properties of albumin increase after the denaturation.

Concerning the known increase of alkalinity of a solution of albumin,

after denaturation, Chick and Martin showed that the diminution of acidity during heat-coagulation takes place only in acid solutions, while in alkaline solutions a diminution of alkalinity is observed [Chick and Martin, 1913, 1]. It has already been pointed out that protein can unite with acids and alkalies before denaturation; the coagulation following the latter would then evidently remove not only protein from the solution but also a quantity of acid or alkali so that the acidity or the alkalinity of solution would be diminished during the heat-coagulation.

(4) INFLUENCE OF SALTS UPON THE DENATURATION-VELOCITY OF PROTEIN.

As mentioned above, salts increase the catalytic power of hydrogen-ions and produce thereby an increase of inversion-rate of sugar. Salts probably also accelerate the denaturation of protein for they increase the catalytic power of hydrogen-ions whose presence in the electrolyte-free protein solution is due to the electrolytic dissociation of the protein molecules which are more acid than alkaline (see above, hydrogen-ion concentration of about $10^{-5} N$). Moreover, Arrhenius found the accelerating effect of salts upon inversion to increase with the increasing salt concentration. On the other hand, as is known, salts diminish the electrolytic dissociation of electrolytes, and they may thus diminish that of protein; moreover the researches of Lillie [1907] showed that salts considerably diminish the osmotic pressure of albumin solutions; that is to say they lower their degree of dispersion and, therefore, the particle-surface of the protein; so that the reaction between protein and water may be retarded.

We should therefore expect that an increase of salt concentration would accelerate the denaturation of albumin when the salt concentration is small, it would however diminish it when the salt concentration is great and it would leave it unaltered if the salt concentration is intermediate.

My experiments showed that this suggestion is right. But, unfortunately, they could not render clear the effect of very small concentration of salts upon the denaturation-velocity, the method employed having required the presence in the protein solution of an amount of salt sufficiently great to produce the coagulation of the denaturated albumin (see above).

The material used in my experiments was egg-albumin Kahlbaum, which had been dialysed many days (content 0.66 %). When the concentration of added ammonium sulphate was 0.03 *N*, 0.06 *N*, 0.12 *N*, 1.1 *N* and 1.32 *N*, the average times of denaturation at 70° were found respectively to be 932 secs., 185 s., 185 s., 270 s. and 605 s. These results are in accordance with the results of Chick and Martin, who investigated only the concentrations from 0.1 to 1 *N* and above.

As is known, salts accelerate the inversion of sugar by acid in different degree, according to their lyotropic properties, which are chiefly due to the effect of anions: $SO_4 < Cl < Br < SCN$ [Spohr, 1888]. It was therefore to be

expected that salts would accelerate the denaturation of acid solutions of protein in the same series of anions. Indeed, my experiments showed that the denaturation-velocity of protein in acid solutions is incomparably more increased by potassium thiocyanate than by potassium sulphate; potassium chlorate takes the middle position but it is, in its effect, nearer to potassium sulphate.

The material used was egg-albumin Kahlbaum, the 2 % solution of which had been dialysed during 5 days. The solution obtained was diluted with water and 0.1 *N* HNO₃, so that the content of albumin in all the solutions was 0.41 %, and the content of acid 0.01 mol. per litre. To the solutions such quantity of various salts was added as to render the salt-content of each solution equal to 0.083 mol. per litre. In the presence of KCNS, KCl, K₂SO₄ the average time of denaturation at 50° was found respectively to be 15 secs., 3000 s. and 4650 s.

If albumin solutions contain no free hydrogen-ions but are slightly alkaline, the presence of salts could not evidently accelerate the denaturation (there are no hydrogen-ions in the solution the hydrolytic power of which could be increased), and it is to be expected that the nature of the anion would have no influence upon the denaturation-velocity.

Indeed, my experiments showed that the denaturation-rate in this case is almost equal in the solutions containing salts of various anions. A slightly alkaline solution of egg-albumin Kahlbaum had been dialysed until it no longer coagulated on heating. When 0.1 *N* K₂SO₄, KCl, KI and KOCOCH₃ were added, it coagulated at 75° respectively in 212, 220, 225 and 227 secs.

Concerning the increasing influence of various cations upon the catalytic power of hydrogen-ions in the case of denaturation, it is almost the same for various alkali metals. The acceleration of denaturation by salts of alkaline earths and heavy metals is however so strong, as to make necessary the suggestion that the salts in question first enter into chemical unions with protein which are soluble in water and react with it more easily than the free protein, and therefore are more easily denaturated. The other suggestion that alkaline earths and heavy metals form insoluble compounds with native protein (which are therefore no longer capable of denaturation by heating), the formation of which is accelerated by high temperature as are all other chemical reactions, is less probable, for the temperature coefficients of the reaction were found in my experiments to be too high: 1.28–1.38 per rise of 1°.

A solution of egg-albumin Kahlbaum, after dialysis during 6 days, was diluted with water and salt solutions so that all the solutions to be tested contained 0.3 % albumin. In the presence of 0.1 *N* KCl the average time of denaturation was found, at 75°, to be 2095 secs., while in the presence of 0.1 *N* BaCl₂ denaturation proceeded instantaneously at the same temperature; in the presence of 0.003 *N* BaCl₂ it required 95 secs., 0.003 *N* MgCl₂ 120 secs., 0.0001 *N* HCl 52 secs., while it proceeded instantaneously in the presence of 0.003 *N* HCl.

The study of the influence of salts upon denaturation showed, therefore, that there was no contradiction with the foregoing hypothesis.

(5) THE CAUSE AND THE TEMPERATURE COEFFICIENT OF THE
COAGULATION OF DENATURATED PROTEIN.

As mentioned above, it has long been known, that protein solutions made heat-incoagulable by dialysis acquire the property to coagulate on heating when some electrolyte is added to them. They coagulate also on the addition of electrolytes when preliminarily heated and cooled. The presence of electrolytes is therefore required to produce the second process comprised in heat-coagulation. In order to render this process clear it was first of all necessary to answer the questions, whether the coagulation of denaturated protein is a pure physical process or, at the same time, also a chemical reaction; whether this process is, in its nature, like the other processes of coagulation of suspension-colloids by electrolytes.

In this connection it was interesting to estimate the temperature coefficient of coagulation of denaturated protein and to compare it with that of chemical reactions and of other coagulations.

My experiments showed firstly that the coagulation of denaturated protein proceeds at a certain temperature considerably more rapidly than the denaturation at the same temperature if the protein solution contains a sufficiently great amount of salt.

Thus, in one of my experiments, the average time required to produce the necessary turbidity of 0.57 % solution of dialysed egg-albumin Kahlbaum to which ammonium sulphate was added to the concentration of 0.05 *N* at 65° was found to be 2220 secs., while the average time required to produce the same turbidity of the same albumin solution which had been boiled and to which after cooling the same amount of ammonium sulphate was added (i.e. the time to produce the coagulation) was found to be 10 secs. at 65°.

Further, in accordance with Chick and Martin, my experiments showed that the average temperature coefficient of coagulation of denaturated albumin is usually equal to 1.1 (it varies from 1.08 to 1.2) per temperature-rise of 1°. But sometimes it increases to 1.3. The results of four of my experimental series are the following:

I. Solution of egg-albumin Kahlbaum made non-coagulable on heating (by dialysis) and containing 0.57 % protein was boiled. After cooling $(\text{NH}_4)_2\text{SO}_4$ was added to it to the concentration of 0.025 *N*. The average times of coagulation at 80°, 75°, 70°, 65°, and 60° were found to be equal respectively to 165 secs., 405 s., 943 s., 1922 s., 2800 s. The temperature coefficient = 1.15.

II. Solution of egg-albumin Kahlbaum. Content of albumin = 0.41 %. Content of ammonium sulphate added after boiling and cooling the solution = 0.05 *N*. The average times of coagulation: at 65° 10 secs., at 60° 40 secs., at 55° 138 secs., at 53° 251 secs., at 50° 687 secs., at 47° 1630 secs. The temperature coefficient = 1.33.

III. 0.14 % solution of recrystallised egg-albumin, made non-coagulable on heating by dialysis. Content of ammonium sulphate added after boiling and cooling to the solution = 0.1 *N*. The average times of coagulation were: at 45° 72 secs., at 40° 152 secs., at 35° 425 secs., at 30° 1200 secs. The average temperature coefficient = 1.2.

IV. Egg-white had been dialysed till the absence of heat-coagulation, diluted with water fivefold, boiled and cooled. $(\text{NH}_4)_2\text{SO}_4$ was then added to the concentration of 0.1 *N*. The average times of coagulation were: at 60° 35 secs., at 50° 297 secs., at 45° 687 secs., at 40° 1325 secs. The average temperature coefficient = 1.18.

As known, the denaturated protein is regarded as a suspension-colloid (see above). It was therefore interesting to compare the temperature coefficients found with those of coagulation of other suspension-colloids. But there are yet no investigations dealing with the dependence of coagulation upon temperature. Scattered up and down a number of observations only pointed out that colloidal solutions are generally less stable at higher temperatures [see, for example, Zsigmondy, 1912].

I therefore made some experiments with colloidal solutions of arsenic trisulphide and lecithin by means of the method above described, which, in the case of arsenic, was a little modified; the vessel for the colloidal solution was observed with the intact horizontal microscope (small magnification) and the time-intervals were noted which were required to produce a complete coagulation. This was marked by the appearance of orange particles of arsenic trisulphide, all through the liquid, on the pure white ground.

A 0.5 % colloidal solution of arsenic trisulphide (obtained by passing sulphuretted hydrogen into water containing powdered As_2O_3), from which H_2S had been removed by a current of air, was centrifuged until particles visible under the microscope were absent. Then ammonium sulphate was added to the resulting colloidal solution to the concentration of 0.01 mol. per litre.

The average times of coagulation were found to be at 85° 228 secs., at 75° 401 secs., at 65° 601 secs., at 55° 1110 secs. The temperature coefficient = 1.05 (per rise of 1°).

In another experimental series where 0.4 % solution of arsenic trisulphide was used and the concentration of added salt (NaCl) was 0.04 mol. per litre, the average times of coagulation were: at 60° 19 mins., at 50° 29 mins., at 40° 39 mins., at 30° 56 mins. and at 17.3° 86 mins. The temperature coefficient was therefore 1.04 (per rise of 1°).

1 % solution of lecithin in water was made by mixing an ethereal solution of lecithin with water and removing ether by blowing air through the solution. Twenty-four hours after the preparation ammonium sulphate was added to the solution (concentration 0.08 *N*). The average times of coagulation were at 90° 106 secs., at 80° 500 secs., at 70° 910 secs., at 60° 1960 secs. The average temperature coefficient was therefore 1.10. When the concentration of

lecithin was 0.5 %, the times of coagulation were: at 90° 376 secs., at 80° 606 secs., at 70° 1356 secs., at 60° 3290 secs. The average temperature coefficient was therefore 1.07 (per rise of 1°).

From the experiments cited it may be seen that the temperature coefficient of coagulation of denaturated albumin is greater than that of arsenic trisulphide or lecithin but it is nearer to that of lecithin, which is known to occupy an intermediate position between hydrophilic and hydrophobic colloids (resp. emulsoids and suspensoids). In the case of arsenic trisulphide the temperature coefficient of coagulation is like that of the diffusion of salts. The temperature-rise only increases the quantity of salt acting upon the colloidal particles. The temperature effect, in the case of lecithin coagulation, is however more complex. The temperature influences in this case not only the salt quantity acting upon the colloidal particles, but also the degree of dispersion and therefore the reacting surface of the colloidal particles, and the coefficient is greater.

The most complex effect of temperature is that upon the coagulation of denaturated protein. The temperature influences probably, in this case, not only the dispersion of the colloid and the acting salt quantity, but also the rate of reaction between salt and protein. For, a formation of loose chemical compounds ("adsorption compounds") of salts with denaturated protein during the coagulation is very probable (see below).

(6) INFLUENCE OF ACIDS AND ALKALIES UPON THE COAGULATION OF DENATURATED PROTEIN.

Denaturated proteins are known to be soluble in alkalies and in strong acids [Cohnheim, 1904], so that the formation of compounds of denaturated proteins with alkalies and acids is undoubted. From the solution of denaturated albumin in weak potassium hydroxide the protein can be precipitated by ammonium sulphate. The precipitate is however easily soluble in a stronger solution of this alkali, but a greater quantity of salt precipitates protein anew. This precipitate is also soluble in more alkali, etc. The increase of alkali concentration produces therefore a continuous change of colloidal properties of denaturated albumin owing evidently to the formation of alkali compounds of protein, and the greater the alkali concentration of the solution the more alkali these compounds contain.

We should therefore expect that alkalies and acids would have a considerable influence upon the coagulation-velocity of denaturated albumin. My experiments showed, indeed, that, generally, acid strongly increases and alkali strongly diminishes the coagulation-rate of denaturated albumin. Moreover 0.00005 *N* HNO₃ increases this rate more strongly than 0.0001 and even 0.0002 *N* acid so that the increasing effect cannot be explained by the influence of hydrogen-ions; it is evidently due to the formation of acid compounds of denaturated protein the colloidal properties of which change with

the amount of acid contained in them. The same result was also found for the alkali effect. The decrease of coagulation-velocity is in this case not at all proportional to the hydroxyl-ion concentration: 0.001 *N* hydroxyl-ion concentration, in the case of ammonia, produces a similar diminution of velocity as 0.011 *N* hydroxyl-ion concentration in the case of potassium hydroxide. Conversely the like molecular concentrations of ammonia and potassium hydroxide bring about a like decrease of coagulation-velocity so that the decreasing effect of alkali cannot be explained except by the formation of alkali compounds of denaturated albumin having other colloidal properties than the free albumin.

The experiments were made with egg-albumin Kahlbaum, the solutions of which had been dialysed during many days and no longer coagulated on heating.

I. A solution of albumin (containing 2.07 % protein) was boiled and, after cooling, diluted fivefold partly with water, partly with nitric acid. To the solutions obtained potassium sulphate was added to the concentration of 0.083 mol. per litre. The average time of coagulation was found at 75°, in the absence of acid, to be 2700 secs., whilst it was found with 0.00005 *N* acid to be 102 secs., with 0.00010 *N* acid 236 secs., 0.00020 *N* acid 178 secs. But in the presence of 0.01 *N* acid the coagulation proceeded instantaneously.

II. A solution of albumin (containing 3.2 % protein) was boiled and, after cooling, diluted fivefold partly with water, partly with solutions of potassium hydroxide or of ammonia of certain concentrations. Ammonium sulphate was added to the concentration of 0.06 *N*. The coagulation proceeded at 70°, in the absence of alkali, instantaneously, while the average time of coagulation, with 0.0018 *N* ammonia (H-ion concentration = 0.00031 *N*), was 370 secs., with 0.018 *N* ammonia (H-ion concentration = 0.00100 *N*), 760 secs., of 0.2 *N* ammonia (H-ion concentration = 0.00400 *N*), 2200 secs. In the presence of 0.018 *N* KOH (H-ion concentration = 0.01100 *N*) it was 770 secs.

My experiments showed further, that the temperature coefficient of coagulation is in the presence of acid still less than that of coagulation of denaturated albumin in the presence of alkali and even than that of coagulation of arsenic trisulphide. So in a series of experiments, in which dialysed egg-albumin Kahlbaum in the presence of 0.0001 mol. nitric acid per litre was tested, the coagulation time was found to be equal to 237 secs. at 75°, to 400 secs. at 65°, to 720 secs. at 55°. So that the average temperature coefficient was 1.05. In another series, in which the acid concentration was 0.0002 mol. per litre, the coagulation time was: 760 secs. at 75°, 1597 secs. at 55° and 1720 secs. at 50°. The average temperature coefficient of coagulation was, therefore, 1.02. In the presence of 0.018 mol. ammonia per litre this coefficient was found to be 1.12 and in the presence of 0.18 mol. ammonia per litre 1.19.

We might thus suggest, that the colloidal state of acid compounds of

denaturated albumin is nearer to that of typical suspension colloids, while that of alkali compounds is nearer to the state of emulsion colloids. The free denaturated albumin seems to occupy an intermediate position between the alkali compounds and the acid compounds.

Finally, my experiments showed that the acid compounds of denaturated albumin are not identical with the denaturated acid compounds of native albumin, formed in the presence of the same concentration of acid. Its colloidal state is different.

One portion of solution of dialysed egg-albumin containing 2.07 % protein and not coagulating on heating was diluted two and a half-fold with water and boiled up. After cooling, to this protein solution a solution of potassium sulphate and diluted nitric acid were added, altogether in such quantities that the original albumin solution was diluted fivefold. The concentration of acid was 0.0002 mol. per litre, that of potassium sulphate 0.083 mol. The average time of coagulation at 65° was found to be 240 secs., at 55° 365 secs.

The second portion of the same solution of egg-albumin was directly diluted fivefold with diluted nitric acid so that the acid concentration was also 0.0002 mol. per litre. Subsequently the solution was boiled up and cooled; afterwards potassium sulphate was added to the concentration of 0.083 mol. per litre. The average time of coagulation at 65° was found to be 320 secs., at 55° 660 secs.

(7) INFLUENCE OF THE DEGREE OF DISPERSION OF DENATURATED ALBUMIN
UPON ITS COAGULATION-VELOCITY.

My experiments relating to the comparison of the time required to produce a certain degree of denaturation of an albumin solution with the time required to bring about the coagulation of the same albumin denaturated by boiling led to an interesting observation which is in accordance with the fact cited above (found by Lillie) that salts lower the degree of dispersion of albumin solutions.

My experiments showed, namely, that the appearance of the definite turbidity of a 0.57 % solution of dialysed egg-albumin Kahlbaum, containing 0.1 mol. potassium chlorate per litre, required on an average 220 secs. at 75°, and 1630 secs. at 71°. However, the same turbidity appeared in 1380 secs. at 75° and in 2020 secs. at 71°, when before adding potassium chlorate the same albumin solution had been boiled and cooled. In both cases the turbidity marked the coagulation of denaturated albumin, but in the first case this coagulation, following the denaturation, proceeded in a lapse of 220 and 1630 secs. (or still more rapidly), while in the second case this coagulation required 1380 and 2020 secs.

It is evident that the colloidal properties of denaturated albumin formed in the solution containing 0.1 mol. potassium chlorate were unlike those of denaturated albumin formed in the solution containing no potassium chlorate, for the former albumin required less time to be coagulated than the latter.

This phenomenon can only be explained by a greater degree of dispersion of the denaturated albumin formed in absence of potassium chlorate. Lillie showed that the degree of dispersion of albumin in the presence of salts is smaller than in the absence of it, even before the denaturation takes place. The denaturation of an albumin of a smaller degree of dispersion leads therefore to the formation of a denaturated albumin which also has a smaller degree of dispersion.

In agreement with the results obtained by Lillie, my experiments showed that the presence of potassium chlorate even in concentration of 0.01 mol. per litre affects the colloidal properties of albumin. So in one of my experiments a 1 % solution of dialysed egg-albumin Kahlbaum (not coagulating on heating) was boiled and, after cooling, diluted with an equal volume of 0.2 *N* KCl. This albumin solution coagulated at 75° on an average in 1380 secs. On the other hand, to the same solution of albumin Kahlbaum KCl was first added to the concentration of 0.01 mol. per litre and the solution was then boiled and after cooling diluted with an equal volume of 0.19 *N* KCl. In this case the protein solution coagulated at 75° on an average in 225 secs., though the concentration of KCl was in both cases the same, namely 0.1 mol. per litre.

The coagulation-velocity of denaturated albumin formed in the presence of potassium chloride was found therefore to be almost sixfold as great as the coagulation-velocity of denaturated albumin formed in the absence of salt. The result obtained corresponds precisely with the value of diminution of osmotic pressure of albumin solution by potassium chloride found by Lillie.

(8) INFLUENCE OF SALTS UPON THE COAGULATION OF DENATURATED ALBUMIN.

In the preceding part of this paper it has several times been pointed out that protein solutions from which salts have been removed by dialysis do not coagulate on heating, but they become coagulable when salts are added to them. Conversely Pauli and his pupil Handovsky affirm that after a very long dialysis the serum (*viz.* serum-albumin) acquires the property to coagulate on heating without adding salts. Salts added to such serum bring about a rise of its "coagulation temperature," and thiocyanates and iodides make it even heat-uncoagulable [Pauli, 1908; Pauli and Handovsky, 1908, 1909].

These authors call the protein contained in their serum "amphoteric protein," pointing out that it conducts the electrical current not more than distilled water and shows no cataphoresis. It is however a pity that the authors did not define the salt content of their protein, as for instance, by determining the quantity of ash, for salts might not have been dissolved in the water of the serum but adsorbed on the protein particles. Then after the denaturation of albumin they might be liberated anew and produce the coagulation.

At all events, it is impossible to regard the small salt content of "amphoteric protein" of Pauli, as the cause of the possibility of its heat-coagulation, and to suppose that the absence of heat-coagulation of dialysed protein solutions which has been observed by all other authors is due to the incompleteness of the dialysis. Indeed, the addition of salts to the "amphoteric protein" could cause only a rise of coagulation temperature, and only thiocyanates and iodides (normally absent in natural liquids) made, in the experiments of Pauli, the heat-coagulation of serum impossible.

In order to render the influence of salts upon the coagulation of denaturated protein clear, I made experiments with egg-albumin and serum-albumin Kahlbaum. We consider first the influence of salts upon the coagulation of egg-albumin.

The solutions of egg-albumin (5–10 %) were usually centrifuged and filtered. They had a slight alkaline reaction (litmus) and were almost water-clear. After drying a part of the solutions in vacuum and afterwards in the drying-stove the dry albumin was found to contain about 3 % ash, of which the most part (2 %) was water-soluble (KCl, NaCl, K_2SO_4 , K_2CO_3). The solutions coagulated on heating.

The dialysis was executed by means of the thinnest parchment paper which could be purchased. If the dialysis was prolonged an antiseptic was added to the solutions tested and the dialyser was placed in a covered vessel. The solution layer in the dialyser never exceeded $\frac{1}{2}$ cm. in depth.

After 24 hours of dialysis (distilled water was changed each two hours) the protein solutions usually became neutral (litmus) in reaction and then lost the property of coagulating on heating (if the protein-content was relatively great, *e.g.* 10 %) or they lost this property only after two or three days of dialysis (if the protein-content was small).

The dialysed solutions were usually centrifuged and filtered anew till they became water-clear. The content of protein and of mineral substances in the solutions (which did not coagulate on heating) is set out below.

Number of tested solutions	Original concentration of albumin	Duration of dialysis	Concentration of albumin after dialysis	Content of mineral substances in dry albumin after dialysis	Content of soluble mineral substances in g. per 100 cc. of solution after dialysis
				%	
1	10	1	7.5	1.8	0.05
2	4.9	2	3.29	1.1	0.02
3	4.1	5	2.07	1.2	0.01

In order to answer the question whether solutions of egg-albumin which no longer coagulate on heating would acquire the property to coagulate anew by a still more prolonged dialysis, solutions 1 and 3 were employed for a further dialysis.

To solution No. 1 an excess of chloroform was added as an antiseptic. After 10 days of dialysis at 20° this solution showed heat-coagulation anew.

But it acquired an unpleasant scent and, in spite of the chloroform, contained many bacteria. The heat-coagulation was evidently due in this case to organic acids or salts which had been formed by the bacteria. Indeed, after a dilution of the solution with water the heat-coagulation disappeared anew, but it reappeared if some ammonium sulphate was added to it.

To solution No. 3 an emulsion of melted thymol in water was added as an antiseptic. The dialysis lasted three weeks. The solution contained after such dialysis 0.99 % albumin. The dry albumin (drying in vacuum) was found to contain 0.8 % ash of which about a half was soluble in water. The solution contained 0.008 % dissolved mineral substances and coagulated on heating. An unpleasant scent and bacteria were however absent.

The albumin solution thus obtained showed the required degree of coagulation (turbidity like that of the ground glass, see above) at 75° in 27 secs. After dilution of the solution with water to double the volume, the same degree of coagulation was observed at 75° in 700 secs., at 80° in 370 secs., at 70° in 1260 secs. The striking increase of coagulation-time after a relatively considerable dilution with water indicates that the very small amount of salts contained in the albumin, which was sufficient to bring about the coagulation of protein in 27 secs. at 75°, after the dilution with water, could no longer produce the coagulation in this time. At the same time, the small temperature coefficient—1.12 and 1.14 per temperature-rise of 1°—showed that the coagulation-velocity, after the dilution with water, was smaller than the denaturation-velocity.

When this albumin solution was diluted fivefold with water, it ceased to coagulate even on boiling, whereas it coagulated easily if some ammonium sulphate was first added to it. Similarly it coagulated if after boiling and cooling this salt was added to it.

It seemed, therefore, that the heat-coagulation of denaturated egg-albumin has two optimal concentrations of salts present in the solution. Namely the coagulation on heating takes place when this concentration is more than 0.1 % (viz. about 0.01 *N*), it ceases to be observed when this concentration is between 0.01 and 0.05 % (viz. about 0.001 and 0.006 *N*), it appears anew when this concentration diminishes to 0.008 % (viz. about 0.0008 *N*), and, finally, it ceases again to appear when the salt-concentration becomes about 0.001 % (viz. about 0.0001 *N*).

Similarly, two most favourable salt-concentrations, viz. two coagulation-zones, were observed also on the other colloids, as for example, on lecithin and cholesterol [Porges and Neubauer, 1908]. Nevertheless, my experiments could not confirm the existence of any coagulation-zones in the case of heat-coagulation of protein.

They showed that a gradual increase of salt-content produces, instead of a retardation or a cessation of heat-coagulation, an acceleration of this coagulation of protein. In my experiments, the above solution of albumin containing 0.008 % dissolved mineral substances was diluted with an equal

volume of solution of potassium chlorate of various concentrations and the heat-coagulation time was determined by the previous method.

When potassium chlorate was substituted by potassium thiocyanate, the phenomenon remained the same, and only very strong concentrations of this salt (2.5 mol. per litre) brought about a diminution of coagulation-velocity. A suppression of heat-coagulation by adding KCNS could never be observed. At a concentration of KCl of 0.003 *N* the temperature coefficient was found to be equal to 1.17; and only at the concentration of 0.01 *N* did this coefficient attain the value of the normal denaturation coefficients 1.35–1.40, and the denaturation began to proceed more slowly than the coagulation of the denaturated albumin. The same was also observed, when a sufficient amount of KCNS (0.1 mol. per litre) was added to the solution. On the other hand an excess of this salt (2.5 mol. per litre) retards the coagulation anew and the denaturation begins to proceed more rapidly than the coagulation (the temperature coefficient = 1.04).

The albumin contained in the solution which had been dialysed during four weeks is therefore not identical with that contained in the solution which had been dialysed during 5–7 days. After denaturation the former albumin coagulates more easily under the influence of salts than the latter. The degree of dispersion of the former is probably smaller than that of the latter. At all events the very prolonged dialysis evidently alters the original albumin. Both albumins are however "amphoteric" (see Pauli), for neither the one nor the other showed in my experiments cataphoresis by an electrical current of 110 volts; but both migrated to the anode after denaturation (according to the increase of alkalinity of the solution).

We pass now to the experiments with serum-albumin.

A 5 % solution of serum-albumin Kahlbaum was centrifuged and filtered. The slightly alkaline solution obtained was very faintly opalescent, coagulated on heating and contained 3.07 % dry residue of which 0.25 % was mineral matter.

The dry protein therefore contained 8.4 % mineral substances. The soluble part of the latter consisted of sodium chloride, potassium chloride, sodium carbonate, etc., the insoluble part was also insoluble in acids (SiO_2 ?).

The albumin solution obtained was dialysed during 20 hours. After the dialysis it was centrifuged and filtered anew. It reacted neutral (litmus) and no longer coagulated on heating, but only became opalescent. It now contained 2.4 % dry residue, of which 0.07 % was mineral and 0.05 % water-soluble. The dry albumin contained now about 3 % mineral substances.

After adding salts to this albumin solution heat-coagulation could again be produced on heating.

The high temperature coefficient of heat-coagulation (= 1.55) after adding potassium chloride showed that 0.1 mol. per litre of this salt was already

sufficient to make the coagulation of the denaturated albumin proceed more swiftly than the denaturation.

In fact, heating the solution of dialysed serum-albumin after a preliminary boiling and cooling and the subsequent addition of potassium chloride to a concentration of 0.1 mol. per litre, showed in my experiments that the coagulation of the denaturated albumin at 70° required on the average only 35 secs., at 68° 50 secs., at 65° 90 secs., etc. On the other hand the same concentration of potassium thiocyanate only brought about a coagulation which proceeded slower than the denaturation, and the temperature coefficient was found in this case to be low. Only between 65° and 70° did the denaturation-velocity begin to be greater than the coagulation-velocity and the temperature coefficient increased, while the coagulation-time became equal to that observed in the presence of potassium chloride.

Further dialysis renders the solution of serum-albumin heat-coagulable again, and even after 6 weeks of dialysis the solution coagulated on heating. At the same time even after 5 days of dialysis the filtered solution contained 1.28 % dissolved solids, of which 0.02 % was mineral; the dry albumin therefore now contained 1.4 % mineral substances (half of which was soluble in water). Further dialysis however altered this salt-content of the albumin solution very little.

Similarly to the solutions of egg-albumin those of serum-albumin, when dialysed during a long time, had a low temperature coefficient of heat-coagulation, the denaturation proceeding evidently more rapidly than the coagulation of the denaturated albumin. The heat-coagulation at 70° required in my experiments on the average 840 secs. and at 80° 510 secs.; the temperature coefficient per rise of 1° was thus found to be equal to 1.05.

Further, a very small concentration of salts (KCl or KCNS) was found in my experiments to accelerate the coagulation of denaturated serum-albumin, and the increase of salt concentration accelerated it so strongly that its velocity became greater than the denaturation-velocity, and the temperature coefficient was found high again and like that observed for solutions of serum-albumin which had not been dialysed (1.69).

The results obtained in my experiments on serum-albumin are therefore like those obtained on egg-albumin. The difference is only quantitative. The solution of serum-albumin loses its mineral substances considerably more readily by dialysis than the solution of egg-albumin. After two days of dialysis the solution of serum-albumin has lost the greater part of its mineral matter; and after 5 days only one-twelfth of this remains in the solution.

Further, the disappearance of heat-coagulation of serum-albumin is observed when the salt-content of the solution is between 0.03 and 0.07 %, while the absence of heat-coagulation of egg-albumin takes place when this content is between 0.01 and 0.05 %. On the other hand the addition of salts

(even of potassium thiocyanate) to the solutions of both albumins, made coagulable on heating by a very prolonged dialysis, cannot render them incoagulable on heating anew.

We have thus to conclude that in both cases albumin is altered by a very prolonged dialysis in such a manner that, after denaturation it shows a greater susceptibility to salts than before. In what however this alteration consists, is still unknown. It is probable that the degree of dispersion of both albumins became smaller by lapse of time, or that a small part of the salts, contained in albumin solutions, are chemically united with the native albumin and the prolonged dialysis decomposes such salt compounds. Free albumin, after denaturation, is in this latter case more susceptible to salts than its unions with salts.

It was formerly pointed out that Pauli and Handovsky affirm that they have found the addition of salts to "amphoteric albumin" to bring about an increase of its "coagulation-point." This observation contradicts the results of my experiments which showed, as mentioned, an increasing effect of salts upon the coagulation-velocity of albumins. But nevertheless, according to Pauli and Handovsky, my experiments showed that potassium thiocyanate and iodide in strong concentration (more than 1 mol. per litre) diminish the coagulation-velocity of denaturated albumin and in the case of serum-albumin can even make it incoagulable on heating when they are present in almost saturated solution. This phenomenon can be explained by the formation of chemical compounds of these salts with denaturated serum-albumin. This albumin is still soluble in a strong and boiling solution of potassium thiocyanate [Pauli and Handovsky, 1908]¹.

Moreover, the results of my experiments concerning the influence of dilution with water upon the coagulation-velocity of denaturated albumin and particularly upon that of denaturated serum-albumin also contradict the result obtained by Pauli and Handovsky. These authors point out that an increasing concentration of protein produces a considerable rise of the coagulation-point. My experiments show however that dilution of heat-coagulable albumin solutions which had been very long dialysed diminishes the coagulation-velocity and a sufficiently strong dilution makes it so small as to render it inaccessible to observation.

What is the cause of all these contradictions? Could it not lie in that conjuncture which had taken place in the experiments of Pauli and Handovsky, who, before determining the coagulation-point of serum dialysed 6 weeks, left it to settle during 3-5 months [1908, pp. 416-8]? It is possible that during such a long time the serum-albumin was altered under the influence of water and the oxygen of the air. In one of my experiments, at least, a solution of serum-albumin, having been dialysed during 6 weeks and coagu-

¹ The degree of dispersion of compounds of albumin with thiocyanates and iodides is probably only greater than that of free denaturated albumin, a sufficient amount of ammonium sulphate making them coagulable anew.

lating on heating lost this property and became slightly alkaline after standing during one year (thymol as antiseptic).

We now pass to the experiments on the influence of salts upon the coagulation-velocity of denaturated albumin dialysed during some days. In these experiments albumin solutions were dialysed until they no longer coagulated on heating without the addition of salts.

We have seen above that increase of salt concentration accelerated the coagulation of denaturated albumin which had been very thoroughly dialysed and coagulated on heating. By virtue of low temperature coefficients we have, indirectly, concluded that in this case of heat-coagulation we have been dealing with the coagulation of denaturated albumin. To investigate the influence of salt concentration upon the coagulation of denaturated albumin not coagulating on heating, without the addition of salts, is evidently much simpler. The solutions of dialysed albumin were in my experiment first boiled up and, after cooling, mixed with salt solutions of a certain concentration; the albumin solutions obtained were then tested at certain temperatures in the thermostat. I will cite here an example of my experiments.

A solution of egg-albumin Kahlbaum containing 2.85 % protein and not coagulating on heating (owing to dialysis during 5 days) was boiled up, and, after cooling, diluted fivefold with solutions of ammonium sulphate of various concentrations so that the albumin solutions obtained contained 0.025, 0.05, 0.1 and 0.15 mol. ammonium sulphate per litre. At 47° the times required to produce the standard turbidity were found to be respectively equal (on an average) to 10,600 secs., 1620 s., 40 s. and 17 s.

The experiment cited shows that the dependence of the coagulation-rate upon the salt concentration of the solution is not like the dependence of the adsorption of salt upon the salt concentration. The coagulation is therefore not a simple adsorption of electrolytes whose ions electrically discharge the colloidal protein particles.

According to the well-known adsorption-isotherm $\frac{x}{m} = ac^n$, the adsorbed quantity of dissolved substance increases more slowly than the concentration of this substance in the solution. In my experiment however the increase of the salt concentration of twofold was accompanied by an increase of coagulation-velocity of ten- or fourteen-fold.

As mentioned above Hardy showed that the denaturated protein can be precipitated by adjusting the reaction so as to render the particles iso-electric with the solution; but when the protein is made negative the valency of the cation is of importance for its precipitation. It has already been pointed out that the colloidal particles in the solution of egg-albumin Kahlbaum, which has been dialysed for some days (4-6 days), are iso-electric with the solution (no cataphoresis was observed) and that they become negative after denaturation. According to Hardy we should therefore expect that the cation only would be important in the coagulation of denaturated protein of the albumin solution in question. My experiment showed however that not only cations

but also anions are of importance in the process. The results of experiments are set out below.

I. The material used was egg-albumin Kahlbaum, a solution of which had been dialysed for 6 days (albumin-content 2.8 %). It was boiled and after cooling diluted with solutions of salts (fivefold). The average times of coagulation at 75° were found to be 2100 secs. in the presence of 0.1 *N* KCl, 1450 secs. at 0.1 *N* NH₄Cl, 1180 secs. at 0.1 *N* NaCl, 1057 secs. at 0.1 *N* LiCl; 1287 secs. at 0.003 *N* BaCl₂, 1320 secs. at 0.003 *N* MgCl₂, 1080 secs. at 0.1 *N* FeCl₃, 2540 secs. at 0.1 *N* AlCl₃. In the presence of 0.02–0.1 *N* BaCl₂ or MgCl₂ the coagulation proceeded instantaneously. In the presence of 0.003 *N* FeCl₃ very slowly.

II. The same solution of egg-albumin. The average times of coagulation at 75° were found to be 215 secs. in the presence of 0.1 *N* K₂SO₄, 826 secs. at 0.1 *N* potassium tartrate, 1070 secs. at 0.1 *N* KNO₃, 1363 at 0.1 *N* KCl, 1406 secs. at 0.1 *N* KBr, 1826 secs. at 0.1 *N* KI and 3826 secs. at 0.1 *N* KCNS.

Therefore, the cations act upon the coagulation-velocity of denaturated albumin not at all proportionally to their valency; the trivalent cations (Al, Fe) produce an acceleration of this velocity almost equal to that produced by the univalent ions. The bivalent cations (Ba, Mg) are however strikingly active, producing in the concentration of 1/33 of the univalent ions an almost equal acceleration of coagulation-velocity. In the single series of cations of equal valency the accelerating effects are as follows: Li > Na > NH₄Cl > K; Ba > Mg and Fe > Al. In all cases the temperature coefficients are relatively great (1.1), and then the suggestion emerges that the different effects of cations are due to some chemical influence produced by these cations. The latter could, for example, form adsorption compounds with denaturated protein. The yellow colour of the precipitate brought about by ferric chloride directly confirms this suggestion. At all events, the observed effect of cations shows that the coagulation of denaturated albumin cannot simply be regarded as a process of electrical discharge of protein particles by the ions. The same results also from the observed effect of various anions.

In spite of the slight negative charge carried by the particles of denaturated albumin the anions employed can be placed in the following series:

Both the series of ions (that of univalent cations and that of anions) are the well-known lyotropic series of ions which were found for all precipitations of emulsion-colloids.

The observed difference in effect of single anions becomes still sharper when the solution of denaturated albumin is made more alkaline by adding potassium hydroxide. In this case, as already mentioned, protein certainly forms compounds with alkali, which are definitely nearer to the emulsion-colloids than the free denaturated albumin (see above). In my experiments

the coagulation of denaturated albumin proceeded at 75° instantaneously when the solution contained 0.001 mol. per litre potassium hydroxide and 0.5–1 mol. K_2SO_4 ; whilst the average time of coagulation was 180 secs. in the presence of the same quantity of alkali and 1 mol. KCl, it was 1220 secs. with 1 mol. KNO_3 . In the presence of 1 mol. of KI or KCNS the coagulation did not appear at all. KCNS produces a coagulation only when it saturates the solution.

As already mentioned, dialysed albumin shows, after denaturation, a cataphoresis to the anode, so that it is probable that the reaction of the solution becomes in this case slightly alkaline (see above, Hardy). But it was pointed out that even very slight concentrations of alkali can cause the formation of compounds with denaturated protein. It is therefore possible that the lyotropic series of ions found is observed only when the coagulation of alkali compounds of protein is investigated. In order to study the influence of ions upon the coagulation of alkali-free albumin, I added to the albumin solution, after denaturation, nitric acid to the concentration of 0.0002 mol. per litre. This concentration is not sufficient for the formation of acid-compounds of albumin (see above). Nevertheless the lyotropic series of anions was perceptible even in this case.

If in my experiments the acid concentration increased to 0.004 mol. per litre and above, the denaturated albumin formed compounds with acid (see above) and the lyotropic series of anions became indefinite, but the valency of the anions came to the foreground. Potassium sulphate, for instance, was found to accelerate the coagulation considerably more strongly than potassium chloride; on the other hand, according to its lyotropic property (the series is in this case inverse) potassium thiocyanate had a still greater effect upon the coagulation than potassium sulphate. The results obtained by Hardy were confirmed only in part.

The study of the influence of salts upon the coagulation-velocity showed generally that the process of coagulation of denaturated albumin is not simply a pure physical phenomenon of discharge of colloidal protein particles owing to an adsorption of electrolytes (ions), but that it is, at least partly, a chemical phenomenon, in which not only electrical properties but also chemical properties of salts are significant.

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