XCIV. THE EFFECT OF HYDROGEN ION CONCENTRATION ON THE PRECIPITATING AND PROTECTIVE ACTIONS OF PROTEINS ON COLLOIDAL GOLD AND GUM BENZOIN.

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IN a previous communication [Wright and Kermack, 1923, 1], evidence has been adduced that the power of gelatin to protect colloidal gum benzoïn against precipitation by sodium chloride depends on the p_H and is minimal at the isoelectric point. The variation of protection with p_H was small and so it appeared desirable to confirm the results and to extend the observations to other proteins and to another lyophobic colloid. The present communication deals primarily with the protective action of gelatin, ovalbumin, serum-albumin, the serum-globulins, oxyhaemoglobin and edestin on colloidal gum benzoïn and colloidal gold at various hydrogen ion concentrations. The effect of variation of p_H on the protective power of colloids is of interest not only from the theoretical point of view, but also because such variations may be of importance in biological systems and in technical processes.

The opportunity was taken to make further observations on the action of the proteins on the lyophobic colloids in the absence of salt. It was shown by Wright and Kermack [1923, 1, 2] that colloidal gum benzoïn was precipitated by very small concentrations of gelatin, serum-globulin and oxyhaemoglobin at reactions on the acid side of the isoelectric point, and by somewhat higher concentrations at reactions approaching or corresponding with the isoelectric point [see also Kermack and McCallum, 1924]. Michaelis and Nakashima [1923] almost simultaneously, working with mastic sol, developed a method for the determination of the isoelectric point of proteins in which use was made of the same principle, and in this way obtained values for the isoelectric points of the serum-albumins of various animals. Reinders and Bendien [1925, 1928] have also investigated the reaction between lyophilic and lyophobic colloids at various reactions, and these authors observed that colloidal gold was coagulated by small concentrations of gelatin on the acid side of the isoelectric point. Similar observations were made with other proteins, caseinogen and sodium lysalbuminate. They observed that at p_H 6

or higher all the proteins used exerted no precipitating but only protective action, but the precise effect of variation of p_H on the latter does not appear to have been closely investigated. Especially in view of the proposal of Michaelis and Nakashima to use the phenomenon as a method for the determination of isoelectric points, it seemed desirable to enquire more closely into its general application to various proteins and lyophobic colloids.

EXPERIMENTAL.

The colloidal gum benzoin was prepared as described by Wright and Kermack [1923, 1, p. 637], and the colloidal gold by adding to 100 cc. of distilled water 1 cc. of 1 % solution of photographic gold chloride, 1 cc. of a 1.4 % solution of potassium carbonate (previously dried and ignited), heating to boiling point, and then adding with vigorous shaking, after removal from the flame, 1 cc. of a solution of neutralised 0.40 % formaldehyde.

To each of a series of tubes were added 0.25 cc. of protein of appropriate concentration, 0.5 cc. of acid or alkali or buffer mixture as the case may be, and 1.0 cc. of gold or benzoin sol. After mixing, the tubes were allowed to stand overnight, when, if desired, readings were made in order to record the interaction between the protein and sol in the absence of salt. 0.25 cc. of sodium chloride (10 %) was now added and the tubes shaken and read after a further 24 hours' standing. The readings were recorded in tables similar to those given in previous papers [Wright and Kermack, 1923, 1, 2], and from these readings figures were prepared showing the regions in which complete or almost complete precipitation takes place. To save space, only the figures are given. In the case of the gold, in certain tubes the precipitate was red or purple, and not blue. The regions in which red precipitates were obtained are shown by shading. All the protein concentrations given refer to the final concentrations. As the readings in the absence of salt were made when the volume was $7/8$ of the volume after the addition of the salt, the concentrations of protein should really be $8/7$ of those given, but as the protein concentrations were doubled in consecutive tubes, the correction required is insignificant.

In the case of colloidal gum benzoin, which itself has a p_H of about 3.8, the various organic acids present in low concentration have a sufficient buffering effect to stabilise the p_H over the range employed in these experiments. In the case of colloidal gold, however, it was usually necessary to employ small concentrations of buffer mixtures in order to obtain satisfactory results. The concentration of buffers was small ($N/200$) in comparison with that of the sodium chloride employed to effect the precipitation, so that any effect of the ions of the buffer in sensitising or protecting the gold would be small in comparison with the effect of the sodium and chlorine ions. The exact buffers used are stated in the figures. The p_H measurements were usually made colorimetrically by means of the B.D.H. capillator, and in some cases confirmed by means of the quinhydrone electrode.

It is to be emphasised that the transition from complete protection to complete precipitation is usually a gradual one, and that, further, in this transition region the exact amount of precipitation depends on the precise conditions of the experiment. The aim of these experiments was, therefore, to determine broadly the approximate conditions under which precipitation and protection take place, and the figures should be interpreted accordingly.

Gelatin. Isoelectric gelatin was prepared according to the method of Loeb [1922, p. 35], and the protein content determined by drying to constant weight at 100° . Dilutions in distilled water were prepared and the experiment carried out as described above. The results are shown in Figs. 1 A and 1 B.

Fig. 1 A. Action of gelatin on gold sol. Final concentration of buffer used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of gelatin p_H 4.7.

Fig. 1 B. Action of gelatin on gum benzoin. No buffers used.

Ovalbumin. Crystalline ovalbumin (Merck), 1 g. in 100 cc. of distilled water, was filtered to remove a small amount of flocculent insoluble material, and the requisite dilutions in distilled water prepared. The results are shown in Figs. 2 A and 2 B.

Fig. 2 A. Action of ovalbumin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of ovalbumin p_H 4.6–4.8.

Fig. 2 B. Action of ovalbumin on gum benzoin. No buffers used.

Serum-albumin, pseudoglobulin and euglobulin. A quantity of human plasma, separated from oxalated blood, was fractionated by ammonium sulphate precipitations. The fibrinogen was removed by quarter saturation and

the serum-globulins in the filtrate precipitated by half saturation, filtered and washed with half-saturated ammonium sulphate. The albumin was precipitated by full saturation. Each fraction was dissolved in distilled water, reprecipitated, washed, redissolved in water and dialysed through a collodion membrane until practically free from sulphate. The euglobulin, which had precipitated during dialysis, was separated from the pseudoglobulin and was dissolved in 0.75 % sodium chloride solution. The protein present in each solution was determined by means of the Folin-Wu method as in the determination of serum-proteins.

Fig. 3 A. Action of serum-albumin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-albumin p_H 4.7-4.9.

Fig. 3 B. Action of serum-albumin on gum benzoin. No buffers used.

The results appear in Figs. 3 A and 3 B (serum-albumin), 4 (serum-pseudoglobulin) and 5 (serum-euglobulin). In the case of euglobulin the original solution necessarily contained sodium chloride required to effect solution,

but the dilutions were made with distilled water so that in the lower concentrations of protein the concentrations of salt were correspondingly reduced.

Fig. 4. Action of serum-pseudoglobulin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-pseudoglobulin p_H 4.7–4.8.

In any case the final concentration of salt added was considerably greater than that present in the most concentrated protein solution used, so that,

Fig. 5. Action of serum-euglobulin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of serum-euglobulin p_H 5.5.

although the concentration of salt in the final mixture was not constant, the variations were relatively small.

Oxyhaemoglobin. Human red blood corpuscles, centrifuged from oxalated plasma were washed several times with 0.9 % salt solution, mixed with distilled water to cause haemolysis and repeatedly centrifuged to remove stromata. The concentration of oxyhaemoglobin in the dialysed solution was

determined by using the Zeiss strophophotometer to ascertain the extinction coefficient, the filter S 51 ($\mu\mu$ 490–530) being employed. The concentration of haemoglobin in a particular solution was found by the Kjeldahl method (total nitrogen), and from the extinction coefficient of this solution it was calculated that a 3 cm. layer of a 0.01 % solution had an absorption coefficient of 0.1358. The percentage concentration of any solution is therefore given by the formula $x = \frac{0.01\sigma}{0.1358}$, where σ is the observed extinction coefficient for a 3 cm. layer.

Dilutions in distilled water were prepared and the experiments carried out in the usual manner. Owing to the colour of oxyhaemoglobin it was difficult or impossible to make readings of the precipitation of gold in the ordinary way. It was found, however, that by observing the tubes in a beam from an arc lamp, the presence of particles of colloidal gold throughout the solution could readily be detected by the Tyndall effect. Tubes in which sedimentation of gold had not occurred gave a marked scattering of incident light. In the experiments with gum benzoin this difficulty did not arise, and readings could be easily made in the ordinary way. The results are given in Figs. 6 A and 6 B. It was found that in the tubes in which the p_H was 5 or

Fig. 6 A. Action of oxyhaemoglobin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of oxyhaemoglobin p_H 6.7–6.8.

less, a brown colour developed, clearly due to the splitting of the oxyhaemoglobin into haematin and globin. The results in this region should therefore be interpreted with caution.

Edestin. Several attempts were made to carry out experiments with edestin from hemp seed (Merck), but no satisfactory results could be obtained, owing to the very small solubility of this protein in distilled water in the neighbourhood of the isoelectric point. In the most concentrated solutions prepared no protection of gold or benzoin was observed between p_H 3.5 and 7.5. Above and below this range protection was observed with 0.05 % edestin. In another experiment in which, in order to obtain higher concentrations of edestin, the

protein was dissolved in 5 % sodium chloride and the solution adjusted to various reactions from p_H 3.0 to 8.5 by the addition of a suitable acetate-phosphate buffer mixture ($M/50$), no protection was observed when an equal volume of gold or benzoin sol was added to these mixtures. The final concentration of edestin in these experiments ranged from 0.1 to 0.0002 %, that of

Fig. 6 B. Action of oxyhaemoglobin on gum benzoin. No buffers used.

salt being constant throughout. The gold in all the tubes was completely precipitated, but, in the more concentrated solutions and the more alkaline of these, the precipitates were distinctly red and graded steadily to blue, being quite blue in the dilute solutions (all reactions) and in acid solutions (all concentrations).

DISCUSSION.

As the results obtained with oxyhaemoglobin are in some respects anomalous, these will be discussed separately after dealing with the other proteins.

Precipitation in the absence of added electrolytes (excluding the experiments with oxyhaemoglobin). The observations are in agreement with those previously made by Wright and Kermack [1923, 1] and Michaelis and Nakashima [1923]. In the presence of small quantities of proteins on the acid side of the isoelectric point, and therefore positively charged, the discharge of the negative colloid takes place and precipitation occurs. When the concentration of protein is very low, precipitation is observed only at reactions well on the acid side of the isoelectric point, as otherwise the protein adsorbed on the negative colloid bears an insufficient number of positive charges to discharge the colloid. As the concentration of protein is increased the p_H at which precipitation occurs approaches more nearly to the isoelectric point. It is not to be expected that the limit p_H at which precipitation occurs will always coincide exactly with the isoelectric point, as it depends for example on such

factors as the degree of adsorption of the protein on the particles, the rate at which ionisation increases as the isoelectric point is left, and the protective power of the protein. If this latter were sufficiently great, it might prevent precipitation at a concentration of protein insufficient to bring the zone of precipitation up to the isoelectric point. There is also the possibility that the isoelectric point of the protein might be altered by adsorption, for example as the result of denaturation. Further, if we are to compare the isoelectric point of the protein with the limiting p_H at which precipitation occurs when the concentration of protein is increased, it is necessary to define the latter unambiguously. In the case of gelatin and mastic, or gelatin and gum benzoin, there is no ambiguity, as when the concentration of protein is increased the zone of precipitation narrows down and converges on a particular point which is not, however, necessarily the maximum value of p_H at which precipitation occurs. With lower concentrations of protein precipitation may occur at a higher p_H , although the mean p_H of the zone obtained at any particular protein concentration does not exceed, but tends to approach, the limiting p_H (see Fig. 1 B). In the case of a protein which, in the neighbourhood of the isoelectric point, brings about precipitation, even at high concentrations, it is clear that the mean p_H of the zone must be taken. The actual width of the zone itself at a given protein concentration depends on various factors, *e.g.* the concentration of buffer used, or the stability of the lyophobic colloid, but the mean p_H is relatively constant. We shall therefore consider the "limit mean" p_H of the zone in relation to the isoelectric point. It appears from the diagrams that when colloidal gum benzoin is used, this "limit mean" p_H does in fact approximately agree with the isoelectric point, but when gold is employed the agreement is not so good, and in the case of oxyhaemoglobin there is a discrepancy of more than one unit of p_H .

Another point which should be noted in connection with the precipitation of gold is that the precipitates were not infrequently red or purple in colour instead of blue. As the blue colour of gold precipitated by inorganic salts may be taken as an indication of the adhesion of the gold particles together, it would seem that the purple or red colour of these precipitates indicates that the gold particles were still virtually separate. The red precipitate showed itself chiefly in the immediate neighbourhood of the isoelectric point and in the presence of moderate concentrations of protein. It would appear that the protein micelles become aggregated round the gold particles and, as a result of this, they sediment relatively more easily. When the isoelectric point is reached and the charge on the protein virtually disappears, these aggregates, each containing a gold particle, may sediment, even although the pure protein is quite stable under these conditions. The gold particles will, however, be carried down in a dispersed condition, and a red precipitate will be formed. The zone of red precipitation was especially wide in the case of serum-euglobulin, and this is in agreement with the fact that this protein is relatively easily precipitated from solution.

Protection in the presence of electrolytes (excluding the experiments with oxyhaemoglobin). On the addition of salt (sodium chloride) any particles of the lyophobic colloid which have adsorbed no protein or only very small amounts will tend to lose their charges and will then be free to aggregate, and in the case of gold the colour will change to blue. Salt, in the concentration employed in these experiments, will have comparatively little direct effect on the proteins. It is not to be expected that the red region of precipitation will be markedly extended, and, further, the entanglement of the particles in the precipitated protein where this has occurred will prevent the change of colour to blue. The charge of the particles which are surrounded with protein will depend primarily on the relation of the p_H of the medium to the isoelectric point of the protein. Disregarding for the moment the tubes in which red precipitation occurred before the addition of salt, it appears in general that the region of blue precipitation occurs in more and more concentrated protein as the isoelectric point is approached. To this extent there is evidence that the protective action of the protein decreases in the neighbourhood of the isoelectric point. We may as a working hypothesis assume that the amount of adsorption of the protein is, at a first approximation, independent of the hydrogen ion concentration in the neighbourhood of the isoelectric point, and that an approximately similar film is formed whatever the reaction. At some distance from the isoelectric point, the protective effect of this film is reinforced by the charge which it carries. As the isoelectric point is approached this charge decreases, and so the apparent protective power is less. At the isoelectric point itself the charge is zero just as it was before the addition of salt, so that no change in the stability is effected by the addition of salt at this point. This accords with experiment except in the case of haemoglobin.

In the neighbourhood of the isoelectric point, approximately the same results are obtained with benzoin as with gold, except that in this case the precipitates are white in every instance, and there is no apparent difference corresponding to that of red and blue precipitates with gold. Protection is minimal at the isoelectric point and increases on each side. At some distance on each side gum benzoin differs rather remarkably from gold in one respect. With gold the maximum amount of protection on the alkaline side is much greater than on the acid side; with gum benzoin, on the other hand, approximate equality on each side was observed. Thus, for example, with serum-albumin at p_H 6.0 to 6.1 protection was observed at dilutions of 1/512 % and 1/64 % with gold and gum benzoin respectively, whereas at p_H 2.4 the corresponding dilutions were 1/16 % and 1/128 % respectively. It appears from these results that at p_H 2.4 eight times as much serum-albumin is required to protect gold as is required to protect gum benzoin, whereas at p_H 6.1 only one-eighth as much is required. It is rather difficult for any satisfactory explanation to be advanced, but it would appear that in acid solution there is relatively little adsorption of the positively charged protein ions by the gold.

Precipitation and protection by oxyhaemoglobin. The reaction between oxyhaemoglobin and gum benzoin in the absence of salt has already been described by Wright and Kermack [1923, 1], who used sheep's oxyhaemoglobin, and these results have been confirmed using human oxyhaemoglobin, the "limit mean" p_H found in this case being about 6.6. After the addition of sodium chloride the absence of protection was observed between p_H 5.4 and 6.0. The zone of minimum protection is thus distinctly to the acid side of the isoelectric point, which is about 6.8 [Michaelis and Airila, 1921; Geiger, 1931]. A cataphoretic experiment in a U-tube with the oxyhaemoglobin employed in the precipitation experiments showed that it was in fact positively charged at p_H 6.3. It was very curious to find that, on the addition of salt, partial or complete re-dispersion occurred in certain tubes at p_H 6.0 to 6.6. The results in the case of gold were even more unexpected. In the absence of salt the "limit mean" p_H at which precipitation occurs is about p_H 5.8 or even lower. When very little buffer was present, precipitation extended from p_H 5.0 to 5.5 in the higher concentrations of oxyhaemoglobin, and from p_H 5.0 to 6.0 in the lower concentrations. In an experiment carried out with phosphate buffers added to control p_H a widening of this zone occurred, but in the higher concentrations of protein, no precipitation took place above p_H 6.2, although at lower concentrations precipitation was observed from p_H 4.6 to 7.2. It is clear that, in the case of gold, the centre of the zone of precipitation is well below the isoelectric point, and without added buffers the whole zone may be well below it. When salt is added, very little change occurs on the alkaline side of the zone, except a widening in very low concentrations, but on the acid side extensive precipitation occurs and there is practically no protection. It has, however, to be remembered that the zone in the absence of salt extends down to p_H 5.0, and that below this p_H dissociation of oxyhaemoglobin into its components takes place, so that too much significance should not be attached to these observations below p_H 5.0.

It is clear that with this protein distinct abnormalities occur; in particular, it sometimes behaves as though its isoelectric point were well below the generally accepted value. There is no doubt that the gold particles in presence of haemoglobin or other proteins are positively charged in moderately acid and negatively in moderately alkaline solution. In the case of proteins other than oxyhaemoglobin, the zone of precipitation coincides with a zero charge on the protein, so that, on the acid side of this zone the particles are positively charged and on the alkaline side negatively charged. When the zone of precipitation coincides with the isoelectric point, this is entirely reasonable. But when the zone of precipitation is on the acid side, it follows that between this zone and the isoelectric point the particles must be negatively charged whilst the protein itself is positively charged. An experiment in which an attempt was made, by means of the ultramicroscope, to observe the charge on the gold particles in the presence of oxyhaemoglobin at p_H 6.3, indicated that it was in fact negative, but as it was relatively small, it was

difficult to ascertain its sign with absolute certainty. As mentioned above, a cataphoretic experiment by the U-tube method on the oxyhaemoglobin alone, showed that this protein had a positive charge at this p_H . Reference to previous work [Wright and Kermack, 1923, 1, 2; Kermack and McCallum, 1924; Michaelis and Nakashima, 1923] makes it clear that a region in which the particles are negatively charged while the protein is positively charged normally exists when the concentration of protein is sufficiently low, for in this case the zone of precipitation lies well below the isoelectric point. The explanation in this instance is clearly that the protein, being present in low concentration, must be at a p_H well below the isoelectric point in order that it should be sufficiently positively charged to discharge the negatively charged lyophobic particles completely. It is possible, then, that the abnormalities observed with oxyhaemoglobin may be due to the small adsorption of this protein on the colloidal particles in the neighbourhood of the isoelectric point. If only small adsorption takes place, it is possible for the positively charged protein to exist in stable equilibrium with negatively charged particles.

An alternative explanation might be based on the observation that in certain cases proteins are denatured when adsorbed on a surface [Loeb, 1923] and that the isoelectric point of the denatured protein might differ from that of the native protein. It might be that the adsorbed oxyhaemoglobin is denatured and now possesses an isoelectric point about one unit of p_H below that of the native protein. This hypothesis, however, appears to be untenable, in the first place, because it does not explain the difference in the "limit mean" p_H values in the cases of gold and of benzoin, and in the second place, as the result of experiments made to determine the isoelectric point of oxyhaemoglobin denatured by heating to 90° for 5 minutes. By means of ultra-microscopic observations it was found that the particles had a positive charge at p_H 6.0, and a barely detectable positive charge at p_H 6.4. At p_H 6.5 and at p_H 7.0 no charge was discernible, whilst at p_H 7.5 the particles bore a small but negative charge. Heat-denaturation is of course not necessarily the same as surface denaturation, but the above two considerations appear to render the explanation unlikely.

It may be noted that, when a low concentration of calcium chloride (0.05*N*) was used in place of the usual concentration of sodium chloride employed in the experiments with colloidal gold and oxyhaemoglobin, a somewhat different result was obtained. The effect of the addition of calcium chloride was to extend the zone of precipitation on the alkaline as well as on the acid side. This fits in with the view that the particles of gold covered with oxyhaemoglobin are negatively charged at p_H 6.0 or more, as in that case the bivalent calcium ions would in this region effect precipitation more readily than the univalent sodium ions.

General. These results confirm the previous findings reported by Wright and Kermack [1923, 1] and almost simultaneously by Michaelis and Nakashima [1923] to the effect that, when low concentrations of protein are employed,

a zone of precipitation occurs on the acid side of the isoelectric point, and that, as the concentration of protein is increased, this zone approaches the isoelectric point, but does not pass it. As mentioned in the introduction, Michaelis and Nakashima have gone so far as to suggest that these observations form the basis of a method for the determination of the isoelectric point of soluble proteins, and have applied it in the case of a number of serum-albumins. It is to be observed, however, that they had apparently tested out the principle only over a limited range, namely with gum mastic and gelatin, and it is doubtful, in view of the variability of the isoelectric point of gelatin, how far their result, namely p_H 4.65, can be considered as a decisive proof of the validity of the method. The results now obtained with oxyhaemoglobin and gold show that, at least when certain lyophobic colloids are used, quite erroneous results may be obtained. Thus, although there is little doubt as to the general nature of the phenomena observed, there does not seem to be sufficient basis for applying the principle without qualification, as suggested by Michaelis and Nakashima, to the quantitative determination of the isoelectric points of proteins.

SUMMARY.

1. Observations have been made on the precipitation of colloidal gold and gum benzoin by gelatin, ovalbumin, serum-albumin, pseudoglobulin, euglobulin and oxyhaemoglobin at different concentrations of protein and of hydrogen ions. The observations confirm and extend the results previously obtained. In very low concentrations of protein, precipitation occurs on the acid side of the isoelectric point, but as the concentration of protein increases the zone of precipitation approaches the isoelectric point, and when sufficient protein is present, the centre of the zone, except in the case of oxyhaemoglobin, is at a point not significantly different from the isoelectric point.

2. In the presence of 1.25 % sodium chloride, minimum protection by these proteins, except oxyhaemoglobin, is observed at a reaction not significantly different from the isoelectric point. In the case of gold the precipitates observed in the neighbourhood of the isoelectric point are usually red in colour.

3. With oxyhaemoglobin abnormal results were obtained in respect both of precipitation and protection. This protein behaved as if its isoelectric point lay below its real value of about p_H 6.8. The anomaly is more pronounced in the case of gold than of gum benzoin. The explanation does not appear to depend on a change in the isoelectric point of the protein following on denaturation as a result of adsorption on the lyophobic particles, but may be due to small adsorption of the protein in the neighbourhood of the isoelectric point.

4. The results with oxyhaemoglobin and gold suggest that the method proposed by Michaelis and Nakashima for the determination of the isoelectric point of proteins is not of general application, and, in particular, may give erroneous results when gold is employed.

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