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

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(Received September 23rd, 1931.)

IN a recent communication [Hopkins, Kermack and Spragg, 1931] the results were given of a series of experiments on the precipitation of colloidal gold and gum benzoïn by the proteins, gelatin, ovalbumin, serum-albumin, the serum-globulins and oxyhaemoglobin at different concentrations of protein and of hydrogen ions. Observations were also made on the protective actions of these proteins against precipitation of the colloids by 1.25 % sodium chloride. Except in the case of oxyhaemoglobin, the protective power was minimal in the neighbourhood of the isoelectric point of the protein. It seemed desirable to extend the observations to mixtures of two proteins in order to find out, for example, what occurs when conditions are such that one protein alone will precipitate and the other protect. It was decided for this purpose to limit the experiments to the three proteins, gelatin, ovalbumin and oxyhaemoglobin. Of these three, the first two have approximately the same isoelectric point, p_H 4.7, whilst that of the third is considerably higher, p_H 6.8. Further, gelatin has considerable protective power even in the neighbourhood of the isoelectric point, but ovalbumin has not, whilst the behaviour of oxyhaemoglobin is anomalous in that the minimum of protective action is at a p_H somewhat below that of the isoelectric point. These three proteins have been employed in pairs, and in each case two sets of observations have been made, colloidal gum benzoïn being employed as lyophobic colloid in the one case and colloidal gold in the other. As in the previous experiments, it was desired to make readings over a range of p_H values. With each pair of proteins, sets of observations were made in which the p_H was kept constant but the concentration of each protein independently varied. Similar sets of observations were made at other p_H values. With each pair of proteins, either two or three sets of readings were made, each at a constant p_H . The values of p_H employed in the various cases were chosen so as to be likely to yield the maximum amount of information.

In all cases readings were made before and after the addition of sodium chloride.

Although mixtures of proteins are frequently encountered in practice, there appears to be comparatively little recorded in the literature in respect of the precipitating and protective actions of such mixtures. Reference may be made to a paper by Prideaux and Howitt [1931] on the rate of electrophoresis of gold sol in the presence of ovalbumin, gelatin and mixtures of the two. On the acid side of the isoelectric point, the rate of electrophoresis in the presence of the two proteins was considerably less than in the presence of either separately, whilst on the alkaline side, the rate was approximately the same as that observed in the presence of gelatin alone.

EXPERIMENTAL.

The general method of carrying out the experiments was analogous to that employed in the previous work [1931]. To each of a series of tubes were added 0.25 cc. of protein A and 0.25 cc. of protein B, each of suitable concentration, 0.25 cc. of a buffer solution and 1.0 cc. of the lyophobic colloid. The buffer solution contained $N/50$ phosphate and $N/50$ acetate. In any particular experiment the tubes were arranged in the form of a rectangular array. The tubes in any one row contained the same concentration of protein A but various concentrations of protein B, whilst the tubes in any one column contained the same concentration of protein B but various concentrations of protein A. The same buffer mixture was added to all the tubes in any one array, so that the final contents of all the tubes were approximately at the same p_H . This was ensured by previously adjusting the p_H of the protein solutions distributed to the tubes to be approximately that of the buffer mixture employed. To each tube of the array was added 1.0 cc. of either colloidal gum benzoin or of colloidal gold. The tubes were shaken and readings were made after 24 hours. 0.25 cc. of 10 % sodium chloride was then added to each tube, the tubes shaken again and read after a further 24 hours. The p_H of a number of tubes was then determined in order to ascertain the exact p_H , which, although approximately constant throughout the array was not necessarily the same as that of the buffer used, as the latter may be modified by the buffering substances present in the gum benzoin and the gold. As mentioned in the previous paper, the readings in the case of tubes containing appreciable quantities of oxyhaemoglobin could best be made by examining the contents in a beam from an arc lamp, when the presence of particles of gold throughout the solution could readily be detected by means of the Tyndall effect. In recording the results obtained with the gelatin-ovalbumin mixtures and gold sol, two observations were made for each tube, one referring to the colour and the other to the amount of gold which had settled to the bottom of the tube. In the case of experiments with gold in which oxyhaemoglobin was present, analogous observations of colour could only be made when the concentration of oxyhaemoglobin was small. In the previous paper these two methods of reading were not

clearly distinguished. When gold is precipitated by an inorganic salt the colour is changed from red to blue and precipitation follows. This precipitate is always quite blue. In the previous paper it was noted that in the presence of protein the precipitate was frequently red or purple, although all the gold had been deposited. The diagrams given showed the regions in which complete or almost complete colour change, or complete or almost complete precipitation, had been observed. It has become clear during the present work that it is desirable to distinguish between these two phenomena. As a consequence readings have been made in the case of gold, firstly on the basis of colour change alone, and secondly on the basis of degree of precipitation alone. The diagrams which are given have been prepared from the latter readings. It has been found that the two sets of readings did in fact follow each other fairly closely with regard to the general shape of the zones, but not with regard to the exact position of the zones. It also appears from the present results that the precipitates marked red or purple (by shading) in the previous paper, although distinctly reddish as compared with the normal blue precipitate of gold, were nevertheless considerably bluer than the unprecipitated gold. It follows from these considerations that the exact limits of precipitation of gold recorded in the new diagrams are not always completely in agreement with those given in the previous paper. This refers more particularly to the case of gelatin and gold. Fig. 8 shows the results when the new method of recording is employed. (Contrast with Fig. 1 A in the previous paper.) It will be noted that on the acid side of the isoelectric point, protection now occurs in the presence of lower concentrations of gelatin. This is because a tube showing an almost complete colour change is now in the non-precipitated region, as under these conditions with very low protein concentration, precipitation does not occur in general until the colour change is complete.

The experimental results are shown in Figs. 1 A to 8. The thick lines, whether continuous or dotted, represent approximately the concentrations at which partial precipitation occurs, so that they separate zones of precipitation from those of non-precipitation. The shading indicates that side of the line on which is the zone of precipitation. Each line is distinguished by a number, *e.g.* 5-6, which denotes the p_H value to which the line refers. The significance of dotted and continuous lines, thick and thin lines, will be explained in the discussion.

DISCUSSION.

According to the theory of colloids which has been elaborated in recent years by Krulyt and others, the stability depends on two factors, (1) the existence of an electric charge on the particles, and (2) the existence of groups which have an affinity for the medium employed for the continuous phase. In the case of a lyophobic colloid protected by a lyophilic colloid such as protein, the effects of the protein may be exerted in two different ways, (1) by modifying the electric charge on the particles, and (2) by forming a film round the

particles, so modifying the surface forces that the particles will not adhere together even though electrically neutral. In this paper, we propose to refer to the former as the "electrostatic effect" and to the latter as the "proper protective effect." For example, the "proper protective effect" of ovalbumin is relatively small, and that of gelatin relatively great. It has been shown in the previous paper [Hopkins, Kermack and Spragg, 1931] that when a single protein is present these two factors explain completely the behaviour of certain lyophobic colloids in the presence of proteins, although, with other lyophobic colloids such as gold, the behaviour is less simple. The results of the present work indicate that this conclusion, so far as concerns colloidal gum benzoin, may be extended to the case where two proteins are present simultaneously. It is entirely in harmony with the above principles that the proper protective effect should be additive and should always exert itself whatever the electrostatic condition of the particles may be. The electrostatic effect is also additive in itself, but is subordinate to the combined proper protective effect.

Precipitation of colloidal gum benzoin. We shall first consider the pair of proteins, ovalbumin and gelatin. In the previous paper, dealing with single proteins, it was found convenient to represent the results diagrammatically in the form of graphs, one ordinate representing concentration of protein, the other p_H . The regions over which complete or almost complete precipitation occurred were then marked out. As, in the present instance, there are two proteins, a three-dimensional model is required to give a similar representation. It was found possible from the observations to construct a model of this kind showing the regions precipitated, the three variables being concentration of gelatin, concentration of ovalbumin and p_H . It should be added that, in constructing this model, considerable help was gained by taking into account the degree of precipitation in tubes which were not exactly at the boundary line between precipitation and non-precipitation. It is difficult to give an adequate

Fig 1 A. Action of ovalbumin on gum benzoin. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of ovalbumin p_H 4.6-4.8.

representation of a model of this kind by two-dimensional diagrams, but some indication of its general nature may possibly be afforded by the "contour" diagrams given. Considering for the moment Figs. 1 A and 1 B, these refer to

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

ovalbumin and gelatin respectively alone, and so give a picture of the two adjacent faces of the model, which meet in an edge representing the p_H axis corresponding to zero concentration of both proteins. The region of precipitation in the three-dimensional model was in the form of a zone separating a region in which the particles possessed a positive charge from one in which they possessed a negative charge. This zone naturally had an under surface bounding the region of positive charge and an upper surface bounding the region of negative charge. The lines shown in Fig. 2 A may be regarded as contours drawn on these surfaces, each at a constant p_H . The parts of the contours which refer to the upper surface are represented by continuous lines and those referring to the lower surface by dotted lines. The thick continuous and dotted lines were obtained directly from the observations as mentioned in the experimental section. The thin continuous and dotted lines have been interpolated with the help of the model, and serve to indicate more fully the nature of the two surfaces, upper and lower. As mentioned in the experimental section, shading indicates on which side of the contours precipitation occurred.

The zone of precipitation comprises the region in which the particles would be expected to have a very low or zero charge, but it does not extend to any concentration of gelatin above a certain critical concentration. At this concentration the proper protective effect begins to function, so that, even at the isoelectric point, precipitation does not occur. Although the proper protective effect of the ovalbumin is not sufficient to prevent agglutination of the particles when their charge is zero, nevertheless it is not altogether non-existent. This is shown by the fact that, in presence of large concentrations of ovalbumin the

concentration of gelatin required for complete protection is not quite so great as when ovalbumin is absent (see Fig. 2 A).

Fig. 2 A. Action of mixtures of gelatin and ovalbumin on gum benzoin at various $[H']$.

Fig. 2 B. Protective action of mixtures of gelatin and ovalbumin on gum benzoin at various $[H']$ in presence of 1.25 % NaCl.

In the case of mixtures of gelatin and oxyhaemoglobin, the same general result is obtained, and in the three-dimensional model the chief difference as compared with the gelatin-ovalbumin system is that the zone of precipitation is moved to a region of higher p_H values in that part of the system which refers to low gelatin and high oxyhaemoglobin concentrations (see Fig. 3 A).

In the case of ovalbumin-oxyhaemoglobin mixtures, the zone of precipitation is extended in the direction of higher ovalbumin concentration in consequence of the low proper protective effect of this protein. Since the proper

Fig. 3A. Action of mixtures of gelatin and oxyhaemoglobin on gum benzoin at various $[H^+]$.

Fig. 3B. Protective action of mixtures of gelatin and oxyhaemoglobin on gum benzoin at various $[H^+]$ in presence of 1.25% NaCl.

protective effects of these two proteins are small, the stability is almost entirely controlled by the electrostatic effects (see Fig. 4A).

Protection of colloidal gum benzoin. The results observed in the presence of sodium chloride may be discussed in the light of the same principles. The chief

function of salt is to reduce the charge on the particles which have no film of protein round them, or which have adsorbed only small quantities of protein.

Fig. 4A. Action of mixtures of ovalbumin and oxyhaemoglobin on gum benzoin at various $[H']$.

Fig. 4B. Protective action of mixtures of ovalbumin and oxyhaemoglobin on gum benzoin at various $[H']$ in presence of 1.25 % NaCl.

The salt may, however, also affect both the electrostatic and the proper protective effects of the protein. As shown in the previous paper, the minimum protective actions of ovalbumin and gelatin are in the neighbourhood of the isoelectric point, whilst that of oxyhaemoglobin is at about p_H 6, somewhat below the isoelectric point. Mixtures of ovalbumin and gelatin behave in

additive fashion, the protective effect of each accentuating that of the other, with one exception. When very small quantities of gelatin are present, very slightly more ovalbumin is required to protect than when no gelatin is present. This may be due to the effect of a very small quantity of gelatin in the surface layer of the particles, quite inadequate in itself to produce any appreciable protective action, so altering the surface forces that the quantity of ovalbumin adsorbed becomes decreased (see Fig. 2 B).

Systems of oxyhaemoglobin and gelatin, or of oxyhaemoglobin and ovalbumin also in general behave additively. The results obtained with these two systems are very similar to each other (see Figs. 3 B and 4 B). This similarity is particularly marked, as, in the presence of salt, high concentrations of ovalbumin exercise a protective action, even at the isoelectric point, although the minimum at this point is rather more marked than in the case of gelatin. Since, in the absence of salt, ovalbumin precipitates at the isoelectric point even in high concentrations, this result implies a certain amount of peptisation by the sodium chloride, similar to that previously noted in respect to oxyhaemoglobin at p_H 6.5. In the previous paper, the observations on ovalbumin in the absence of salt were not recorded, so that peptisation was not noted. The most satisfactory explanation of the anomalous behaviour of oxyhaemoglobin in the presence of salt, as recorded in the previous paper and confirmed in this one, is that the proper protective effect is minimal not at the true isoelectric point, but at about one unit of p_H below. This shift in the minimum of proper protective effect may or may not be accompanied by a corresponding change in the apparent isoelectric point in the presence of sodium chloride, similar to that noted by Michaelis and Szent-Györgyi [1920] in the case of caseinogen.

The general results in the two systems, oxyhaemoglobin-gelatin and oxyhaemoglobin-ovalbumin, are in harmony with the principles enunciated above, namely, that the proteins independently affect the electric charge, and also independently and additively exercise their proper protective effects. In accordance with the curious effect of salt on the protective action of ovalbumin, it is found that the similar peptising action of salt on oxyhaemoglobin is not inhibited by the presence of ovalbumin, although it seems to be inhibited by the presence of moderate quantities of gelatin.

Precipitation and protection of colloidal gold. Anomalous effects of the type referred to above in connection with oxyhaemoglobin and gum benzoin were more marked when this protein was used with gold. This was referred to in the previous paper. The further complication arises here, that, as mentioned in the experimental section of this paper, the degree of colour change and the extent of precipitation do not necessarily run parallel in the presence of protein. When the precipitation results for two proteins *plus* gold are examined, they are found broadly to conform with the principles enunciated above in the case of gum benzoin, with the modification necessitated by the existence of the anomalies exhibited in the case of oxyhaemoglobin. When the colour change results are examined, they are also found to be in accordance with the above

principles. The lack of parallelism between colour change and precipitation is most marked in the presence of gelatin on the acid side of the isoelectric point, where considerable colour change without flocculation takes place, whilst in

Fig. 5A. Action of mixtures of gelatin and ovalbumin on gold sol at various $[H^+]$.

Fig. 5B. Protective action of mixtures of gelatin and ovalbumin on gold sol at various $[H^+]$ in presence of 1.25 % NaCl.

the neighbourhood of the isoelectric point of the protein, complete flocculation is observed without equally complete colour change. These remarks are true of mixtures both in the presence and absence of salt.

It was suggested that the probable explanation lay in the small adsorption

of the protein on the gold, and the poor protective action of these proteins on the acid side as compared with that on the alkaline side of the isoelectric point suggests that this small adsorption may be characteristic of the protein cations.

Fig. 6A. Action of mixtures of gelatin and oxyhaemoglobin on gold sol at various $[H^+]$

Fig. 6B. Protective action of mixtures of gelatin and oxyhaemoglobin on gold sol at various $[H^+]$ in presence of 1.25 % NaCl.

The proper protective effect of the proteins will be measured by the quantity required to prevent precipitation when the gold particles are discharged and the protein molecules are in the neutral condition. The much greater protective action when the p_H is somewhat greater or less than corresponds to the isoelectric point must be regarded as the combined effect of the proper protective

effect of the protein along with stabilisation due to the existence of like charges. On this basis we may consider for example the results obtained with oxyhaemoglobin and gelatin at p_H 5.6 (see Figs. 6 A and 6 B). In the absence of salt,

Fig. 7 A. Action of mixtures of ovalbumin and oxyhaemoglobin on gold sol at various $[H^+]$.

Fig. 7 B. Protective action of mixtures of ovalbumin and oxyhaemoglobin on gold sol at various $[H^+]$ in presence of 1.25 % NaCl.

there is no precipitation with gelatin alone. In the presence of a sufficient concentration of oxyhaemoglobin, however, precipitation occurs with zero or very small concentrations of gelatin. But with larger concentrations of gelatin the negatively charged gelatin ions reinforce the negative charge on the particles and in this way prevent discharge by the positively charged oxyhaemoglobin.

As the concentration of oxyhaemoglobin increases up to a point, rather more gelatin is required to prevent precipitation. In the presence of salt and absence of oxyhaemoglobin gelatin protects the gold at a concentration of 1/128,000 % and over. At a somewhat lower p_H gelatin does not protect at 1/8000 % concentration. It seems probable that the protective action of gelatin at p_H 5.6 is accentuated by the negative charge borne by the protein at this p_H . When, however, small quantities of oxyhaemoglobin are present, the positive charge on this protein will tend to neutralise the negative charge on the gelatin. The protection of the colloid will then have to be effected largely by the proper

Fig. 8. Action of gelatin on gold sol. Final concentration of buffers used $N/400$ acetate + $N/400$ phosphate. Isoelectric point of gelatin p_H 4.7.

protective effect of the gelatin assisted by the comparatively small proper protective effect of the oxyhaemoglobin, the latter on the acid side of the isoelectric point. Thus, protection does not take place in the presence of 1/10,000 % oxyhaemoglobin until the concentration of gelatin exceeds 1/8000 %, and this quantity of gelatin increases as the concentration of oxyhaemoglobin increases. The concentration of gelatin never exceeds 1/500 % even when the concentration of oxyhaemoglobin is as high as 1/10 %. The limiting factor here may be the proper protective effect of the gelatin. The results obtained with the other mixtures of proteins conform to the same principles. In the case of gelatin and ovalbumin the results obtained are similar to those obtained with this mixture of proteins and gum benzoin (Figs. 5 A and 5 B) whilst those obtained with ovalbumin and oxyhaemoglobin are similar to those obtained with gelatin and oxyhaemoglobin with gold (Figs. 7 A and 7 B).

SUMMARY.

1. Experiments have been performed on the precipitation of colloidal gold and gum benzoin by mixtures of ovalbumin and gelatin, ovalbumin and oxyhaemoglobin, and of oxyhaemoglobin and gelatin at various hydrogen ion concentrations.

2. Observations have also been made on the protective action of these mixtures at various hydrogen ion concentrations against precipitation of the lyophobic colloids by 1.25 % sodium chloride.

3. It appears that the results previously obtained with the single proteins can be extended to apply to the above mixtures. If the effect of a protein on the charge of the lyophobic particles is distinguished from its proper protective effect, the two proteins would appear to exercise each of these two influences additively.

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