CLXIV. THE PRECIPITATION AND PROTECTION OF VANADIC ACID BY SERUM-PROTEINS.

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ZSIGMONDY [1901] has shown that proteins inhibit the precipitation of red colloidal gold which occurs when salts are added. This effect is however at a minimum at the isoelectric point of the protein. Wright and Kermack [1923, 1, 2] were able to show that proteins on the acid side of the isoelectric point, and in dilute solution, caused precipitation of colloidal gum benzoin. Hopkins, Kermack and Spragg [1931, 1] obtained similar results with gum benzoin and with colloidal gold, which are positive lyophobic colloids. The reactions between acid solutions of protein and negative lyophilic colloids have not previously been studied. While the lyophobic sols depend upon their electric charge alone for their stability, the lyophilic proteins have both an electric charge and an affinity for the dispersion medium. In the neutral and alkaline solutions studied by Zsigmondy, the proteins bear a negative charge like that of the gold particles, but in acid solution the charge on the protein particles becomes positive and being opposite to that of the gold particles, causes neutralisation and precipitation. Although this "electrostatic effect" (as Hopkins, Kermack and Spragg [1931, 2] have called it) changes with p_H , the "proper protective effect" of the protein remains, and so some proteins in higher concentration may still protect lyophobic solutions, even at their isoelectric point. There is too, an intermediate effect of sensitisation, which has been investigated by Pauli and collaborators [see Pauli, 1930]. The reaction between ferric hydroxide sol and proteins described as sensitisation by Reitstötter [1920] is really a case of mutual precipitation of oppositely charged colloids, comparable to some extent with the precipitation of gold sol with acid solutions of proteins observed by Hopkins, Kermack and Spragg, but more nearly comparable with the phenomena described below in which two oppositely charged lyophilic colloids are concerned. The term sensitisation should be limited to those cases where the protein increases the flocculability of a sol. In the present work most of the experiments were carried out in the presence of a low constant concentration of sodium acetate so that sensitisation effects were not sought for and none was observed. This was essential because of the impossibility of preparing buffered solutions of the acids under consideration in the absence of salts and of dissolving serum-euglobulin without salt being

present. The theoretical aspect of the experiments does not suffer from this omission as it will be seen that in acid solution there is always a tendency to precipitation with added protein even without the addition of any salt.

Many hydroxy-acids have a strong tendency to form poly-acids in acid solution. Such acid radicals may be colloidal, and Graham [1864] prepared and investigated colloidal tungstic acid. Silicic acid, metastannic acid and antimonie acid are familiar examples. Britton and German [1930] found that sodium molybdate on acidification to p_H 4.8 begins to form polymolybdic acid in which the acid $H_{10}Mo_{12}O_{46}$ has been identified. Arsenic acid, like phosphoric acid, does not form very complex acids. Antimonie and bismuthic acids form complex colloidal solutions but are unsuitable for study in connection with proteins because they give precipitates on dilution or on change of acidity of their solutions. Vanadium alone of the common elements of the fifth group forms stable poly-acids. Molybdic, tungstic and vanadic acids in addition to combining with themselves to form complex acids all combine with each other.

Düllberg [1903] investigated vanadates and vanadic acid and found that on acidification the simple vanadates formed hexavanadic acid, $H_4V_6O_{17}$, which gave tervalent ions $(HV_6O_{17})'''$. Such an ion would have a molecular weight of almost 600 and it is possible that more complex molecules occur.

The acid solutions of vanadic, molybdic and tungstic acids are negatively charged hydrated semi-colloids. They are quite stable, not being precipitated by salts in any concentration, but precipitated by equivalent amounts of a positive colloid such as $Fe(OH)_3$, but not if excess of the acid is present. In this work the reactions between these positively charged complex acids and acid solutions of proteins with negative charges are studied.

EXPERIMENTAL.

Solutions of the poly-acids were made by mixing decinormal solutions of salts of the acids with decinormal acetic acid in various proportions in order to give the requisite p_H . The solutions were then diluted so that each contained the same amount of the acid radical under consideration. In this way, solutions of various poly-acids in acetate buffer solutions were easily made. A similar technique was used by Bendien [1931] for making solutions of vanadic acids, for the purpose of a "diagnostic test for cancer," but in his case the vanadium present in each solution varied in addition to the hydrogen and sodium ion concentrations. Such solutions would be unsuitable for experimental purposes. The various solutions used were prepared as follows.

Vanadic acid. Na_3VO_4 solution (12.2 g. sodium vanadate (Kahlbaum) per litre) was mixed with varying amounts of $N/10$ acetic acid to give solutions varying in p_H from 3.2 to 5.4. Twenty solutions each with different proportions of acid and salt and different p_H were made. Each was then diluted so as to have the same concentration of vanadium (1 gram-atom V per 300 litres).

Molybdic acid and tungstic acid. *M*/70 ammonium molybdate (17.6 g. $(\text{NH}_4)\text{Mo}_7\text{O}_{24} \cdot 4\text{H}_2\text{O}$) and *M*/10 sodium tungstate (33.0 g. Na_2WO_4 per litre) were mixed with acetic acid. Ten solutions varying in p_{H} from 3.25 to 5.5 were made, each containing 1 gram-atom molybdenum or tungsten respectively per 300 litres.

The proteins were separated from normal human sera by dialysis and precipitation. The serum was dialysed in closed collodion sacs which were continuously raised and lowered gently in a vessel through which distilled water at a temperature below 8° was flowing at a rate of 700–800 cc. per hour.

The movement of the collodion sacs was so arranged that a bubble of air passed through the protein solution from end to end of the collodion sac each time it was raised or lowered. By this means the inside surface of the collodion was continually swept and dialysis was much accelerated. After the salts were removed from the serum in this way (2 to 3 days), the serum was centrifuged to remove the precipitated euglobulin. The clear liquid was then saturated with carbon dioxide in the cold when most of the pseudoglobulin was precipitated, and was removed by means of the centrifuge. The remaining solution contained the albumin possibly contaminated with a little pseudoglobulin, but the contamination is probably less significant than the change brought about by precipitation with strong ammonium sulphate solution [see Svedberg, 1931]. The albumin solution was used as obtained. The euglobulin precipitate was washed with distilled water and then dissolved in *M*/10 sodium acetate, in which it dissolved with difficulty, and only weak solutions could be prepared. The protein concentrations of the solutions used were determined by accurate estimation of the refractive index in a Zeiss interferometer and calculated by means of the data obtained by Adair and Robinson [1930].

The method of observing precipitation was similar to that of Hopkins, Kermack and Spragg [1931, 1], except that the ratio of volumes was that adopted by Bendien [1931]. Protein solutions made up to 0.4 cc. with water in each case were placed in a small tube and 2 cc. of the poly-acid solution were added with shaking. The tubes were allowed to stand for 24 hours and the formation or otherwise of a precipitate was then observed. Slight turbidity unaccompanied by any settling was not considered to be precipitation. The results are expressed graphically after the manner used by Hopkins, Kermack and Spragg [1931, 1]. The lines in the figures represent the boundary between precipitation and protection; points are marked at the minimum concentration of protein where it appeared that protection had occurred.

Albumin.

The results obtained with vanadic acid and serum-albumin are shown in Fig. 1. It will be seen that albumin is able to inhibit protection if it is present in sufficient concentration in solutions more acid than p_{H} 4.0. This effect is similar to that obtained by Hopkins, Kermack and Spragg with colloidal gold, in which the protective action was minimum about the isoelectric point. In

the experiments with colloidal gold much lower concentrations of albumin give protection than in the present experiments. This difference is largely due to difference in concentration of the sols used. (The gold sol used contained 1 gram-atom Au in 3000 litres.) The point of minimum protection with vanadic acid appears to occur at a p_H slightly more acid than with gold sol. The stable sol which is produced with excess albumin and vanadic acid was shown to be positively charged by means of cataphoretic experiments. Two platinum electrodes connected to a 6-volt battery were placed in a U-tube containing such a sol. After the current had been passing for an hour the yellow colour of the vanadic acid-protein complex had disappeared from the liquid surrounding the positive electrode and was deposited round the negative electrode. A similar experiment with vanadic acid in the absence of protein showed the vanadic acid to be negatively charged as would be expected from the ionisation of $H_3(HV_6O_{17})$.

Fig. 1. Precipitation and protection of vanadic acid.

• Albumin
+ Euglobulin

Euglobulin.

The effect on euglobulin is however different. In all concentrations used euglobulin was precipitated in solutions more acid than p_H 5.2. Trials were made with $N/1000$ HCl present, giving a final p_H of 3.0, but precipitation still occurred. Experiments with half and quarter concentrations of vanadium with the maximum amount of globulin (0.05 %) also gave precipitates. Solutions more alkaline than p_H 5.4 were not precipitated at any concentration tried.

Pseudoglobulin.

The effect obtained with pseudoglobulin was different from that with albumin. Precipitation occurred at all hydrogen ion concentrations above p_H 5.0. Increase of concentration did not result in protection in any case,

Fig. 2. Globulin in mg. held in solution by 100 mg. albumin in vanadic acid solution.

+ Pseudoglobulin (mg.)
• Euglobulin (mg.)

so that pseudoglobulin is like euglobulin in its reaction to vanadic, tungstic and molybdic acids. It was also found that pseudoglobulin added to albumin which was itself protected caused precipitation. Albumin could however protect pseudoglobulin and the amount protected by a definite amount of albumin was practically the same as the amount of euglobulin protected, as can be seen from Fig. 2. The experiments described with albumin, euglobulin and pseudoglobulin and vanadic acid were repeated with tungstic and molybdic acids, and exactly the same results obtained. The globulin always gave a precipitate if the p_H were between 3.0 and 5.0. Increase in concentration of globulin did not give protection. On the other hand excess of albumin caused protection as it did with vanadic acid. The experiments were also repeated with fresh horse serum-proteins in place of human serum-proteins and the same results obtained. A preparation of commercial horse serum sterilised with chloroform gave albumin with little protective action. On the other hand a preparation of crystalline horse serum-albumin prepared in 1914, for which I am indebted to Dr H. Chick, had protective action identical with that of normal human serum-albumin. The addition of salts which might increase the charge on globulin and thus increase its protective action was tried. Globulin solutions with small additions of sodium chloride, barium acetate, aluminium chloride and sodium citrate were treated with vanadic acid in the usual way. In all cases precipitation occurred.

Mixtures of proteins.

When mixtures of albumin and euglobulin or pseudoglobulin were used precipitation occurred unless albumin were present in considerable excess. The amount of globulin which albumin could hold in solution varied with the p_H as can be seen in Fig. 2. The precipitation that occurs when there is sufficient albumin present to protect itself but not the globulin, involves all the vanadic acid, but not all the protein. A yellow precipitate settles, leaving a clear liquid above, which contains protein. The protein remaining in solution is not precipitated by half-saturated ammonium sulphate and must therefore be albumin.

Some experiments were also carried out with vanadate solution made in the concentrations described by Bendien. Similar results were obtained. Albumin in sufficient concentration inhibited precipitation and globulin was always precipitated. If a little globulin was added to a normal human serum previously giving a precipitate in Bendien's solution No. 8, precipitation could be produced with his solution 7, 6, 5 or 4, according to the amount added, e.g. 0.2 cc. serum containing 5.2 % albumin and 2.7 % globulin just gave a precipitate with 2 cc. Bendien solution No. 8; with the addition of 0.04 cc. of 0.38 % serum-globulin, precipitation occurred in tube 7, with 0.10 cc. in tube 6, with 0.14 cc. in tube 5, and with 0.20 cc. in tube 4.

Bendien [1931, p. 27] says that increased globulin in serum and the precipitation are not parallel. There would however appear to be no doubt that

changes in concentration are the main cause of differences in precipitation. Another cause of such differences is the total protein content—if the albumin content were sufficiently low precipitation would occur with all vanadic acid solutions even without globulin being present. Still another cause of differences in precipitation lies in differences in the alkali reserve of sera. It is possible for the alkali reserve of serum to vary from 25 to 75 volumes CO_2 per 100 volumes serum, *i.e.* from 0.02 *N* to 0.06 *N* alkali, a possible deviation of 0.04 *N*. Now the difference in acid content of the Bendien solutions (1 to 12) is 0.05 cc. *N*/10 acid per 10 cc., *i.e.* 0.0005 *N*. In the test, serum is diluted 10 times with the vanadic acid, so that the maximum differences in alkali reserve of serum can produce considerable differences in the final acidity and so in the precipitation. The Bendien test gives no real insight into the three factors which are involved, namely, albumin-globulin ratio, total protein content and alkali reserve, but merely indicates whether serum is normal or not.

DISCUSSION.

It can be seen from these experiments that solutions of vanadic acid of varying acidity could be used as a rough guide in determining the ratio of albumin and globulin in a solution such as serum. With increase of acidity more globulin can be held in solution and the p_H at which precipitation occurred would indicate the ratio in which the two proteins were present. It is to this fact that the “diagnostic test for cancer” devised by Bendien [1931] is due, although this author did not appear to be aware of the true nature of his test.

The results of the experiments on the separate proteins can be explained mainly by means of their electric charge and their stability as lyophilic colloids. The proper protective effect, as distinct from the electrostatic effect, in the present experiments, however, appears small, being probably of the same order as the protective action of polysaccharides, because the negative colloid is itself lyophilic and hydrated. Hardy's [1899] original work showed that the main cause of stability of protein solutions is their electric charge; when this charge is removed, as at the isoelectric point, the solution becomes unstable. Kruyt and De Jong [1922] have been able to show that with increased charge there is an increase in the hydration, with which is bound up the proper protective effect. It is however in the electrostatic effect of the protein molecule that an explanation of the results described above is to be found.

If the charge on the protein molecules added (*i.e.* the total charge present, which is the product of the charge per molecule and the protein concentration) is small compared with that on the vanadic acid present then the protein charge is neutralised and precipitation occurs. If the charge on the protein is great enough then it can itself neutralise the vanadic acid charge and if any surplus electrostatic charge remains, precipitation need not occur. Although a stable sol is produced with excess of protein over vanadic acid, the

same does not occur with large excesses of vanadic acid. Both colloids are lyophilic and the difference in effect must be due to difference in size of the particles. The serum-albumin particles are probably 50 times as heavy as those of vanadic acid and for that reason cannot be held in solution by vanadic acid.

The difference in action of globulin and albumin could be attributed to difference in the respective charges. It has been shown [Cohn, 1925] that globulin has relatively few groups capable of ionisation on the acid side of the isoelectric point. The difference between the two proteins in this respect is really very considerable. About the isoelectric point serum-albumin requires 28×10^{-5} mols acid per g. protein to change the acidity by 1 unit of p_H . Serum-globulin under the same conditions requires but 5×10^{-5} mols acid to produce the same change in acidity. The charge on globulin in acid solution will probably therefore be about one-sixth of that present on serum-albumin under similar conditions. It will therefore have but a small charge in solution and this is neutralised by the positive vanadic acid. The charge on the globulin is not great enough to reverse the charge of the poly-acid. This effect is also helped because, owing to the low solubility, it is not possible to use concentrated solutions of globulins.

This however leaves the behaviour of the mixed proteins unexplained. The globulin molecules must have some negative charge and this charge should help the albumin to protect the solution against precipitation. In all experiments in acid solution however antagonism occurs. The effect can only be explained by some specific precipitation of euglobulin by these poly-acids. This precipitation may be related to the fact that globulin is soluble in salt solutions, but not soluble in the absence of salts. There is possibly some definite compound formed between euglobulin and salt and possibly complex acids, *e.g.* vanadic acid, combine in the same way giving an insoluble derivative.

Vanadic acid precipitates serum-globulin and this precipitation may be inhibited by serum-albumin as all precipitations are inhibited by the protective action of such a colloid.

SUMMARY.

1. Vanadic, molybdic and tungstic acids form negative lyophilic semi-colloidal solutions.
2. Different serum-proteins react with such solutions in different ways. Serum-albumin is precipitated in low concentrations, but in higher concentrations there is no precipitation, protection occurs and a stable positive colloid is formed. Serum-globulin however causes precipitation in all available concentrations between p_H 3.0 and 5.3.
3. If euglobulin is added to an amount of albumin great enough to cause protection when a poly-acid is added alone, precipitation will occur. If sufficient excess of albumin is added the globulin will be held in solution. The

amount of globulin which a definite amount of albumin can protect increases with increase in acidity of the solution.

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