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Fractional Precipitation of Serum Proteins by Heavy Metal Ions

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Salts of heavy metals are known to be powerful protein precipitating agents. A large number of investigations on the interaction of metals with proteins have been published, and the fractional precipitation of human serum by addition of zink and barium salts has been reported by Cohn *et al.* [1,2]. The experiments to be described here offer a different approach to a similar problem. A fractional precipitation of equine and bovine serum by means of ions of heavy metals was attempted.

METHODS

Serum was obtained fresh from spontaneously coagulated equine or bovine blood. Solutions of the following salts were used as precipitants: $PbCl_2$ (saturated solution), $CdCl_2$ (0.1 *M*) and $CuCl_2$ (1 *M*). The serum samples were acidified with 1 *N* HCl to a pH where no precipitate appeared when the precipitating solution was added. The solution of the precipitating salt was then added in excess and the pH was increased stepwise by dropwise addition of 1 *N* NaOH with constant stirring. The precipitates obtained were isolated by centrifugation, but they were not washed, because the presence of small amounts of the different serum proteins was favorable for the subsequent identification of the compounds on the electrophoresis diagram. All procedures were performed in the cold (ice bath). The precipitates were dissolved in 0.9 % NaCl by addition of a few drops of HCl. All solutions were dialysed against 0.9 % NaCl to remove heavy metal ions. The solutions were then dialysed against the phosphate buffer to be used during the electrophoresis, which was performed in our Tiselius apparatus[3]. Further details on the procedures appear from the experiments described below and from the legends to the figures.

RESULTS

The precipitates obtained by addition of $PbCl_2$ to bovine serum consisted mainly of albumin and α-globulin. The highest selectivity was obtained at pH 5.0 and the results of such experiments are presented in Fig. 1. When equine serum was precipitated with $PbCl_2$ at pH 6.7, the precipitate also contai-

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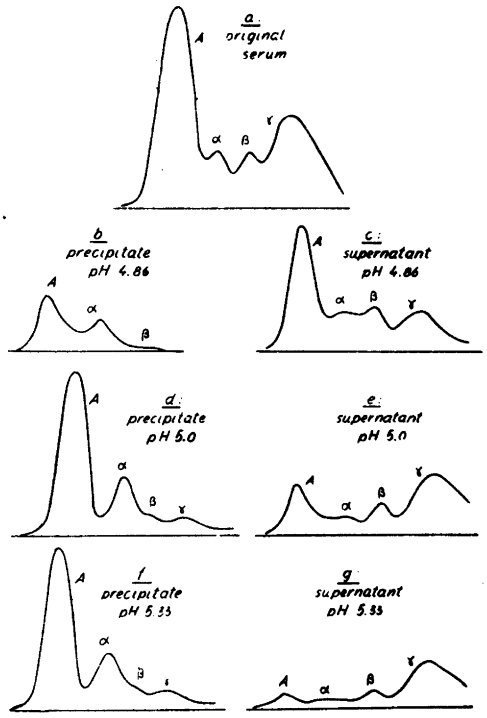

Fig. 1. Precipitation of bovine serum with lead chloride. 15 ml serum + 30 ml saturated $PbCl_2$ + 1 N HCl to pH 4.6. Precipitation by addition of 1 N NaOH. a) Original serum; b) precipitate and c) supernatant at pH 4.86; d) precipitate and e) supernatant at pH 5.00; f) precipitate and g) supernatant at pH 5.33. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Descending patterns. Same relative concentration in all diagrams.

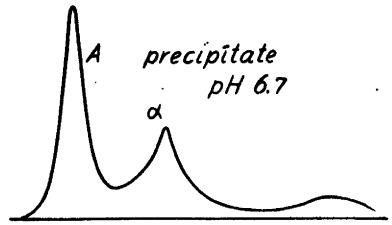

Fig. 2. Precipitate from horse serum with lead chloride. 50 ml serum + 50 ml saturated $PbCl_2$ + AcOH. Addition of 1 N NaOH to pH 6.7. Electrophoresis: NaCl (μ = 0.075) — phosphate (μ = 0.0125) buffer pH 7.6; 150 min., 18.1 mA. Ascending pattern.

ned mainly albumin and α-globulin (Fig. 2). This occurred also when the horse serum had been previously dialysed against 0.9 % NaCl (Fig. 3). When the horse serum was dialysed previously against distilled water less albumin was precipitated, and the content of α-globulin dominated the precipitate (Fig. 4). The precipitate of euglobulin formed during the dialysis against distilled water (*cf.* Svensson [10]) was negligible and no change was detected in the shape of the electrophoretic pattern.

When $CdCl_2$ was added the precipitation took an entirely different course. A precipitate obtained with bovine serum at pH 6.9 contained mainly γ-globulin. Appreciable amounts of α- and β-globulin were also precipitated and appeared as a single peak on the electrophoresis diagram. Albumin was almost totally retained in the supernatant (Fig. 5).

When the precipitation was performed with cupric ions a heavy green precipitate appeared during dialysis of the solutions against 0.9 % NaCl. The filtrates from these mixtures were blue-green and contained appreciable

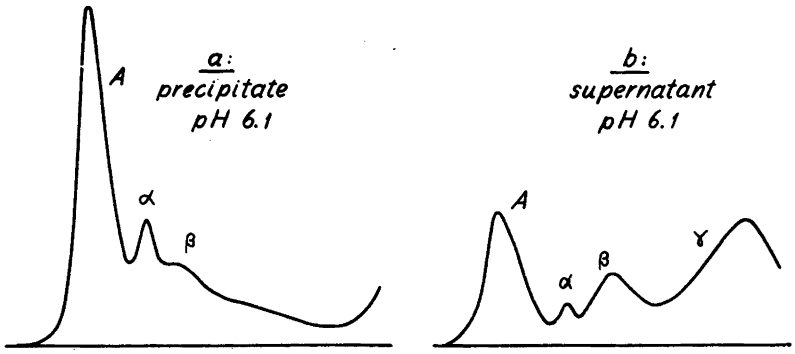

Fig. 3. Precipitate (a) and supernatant (b) from precipitation of horse serum (dialyzed against 0.9 % NaCl) with lead chloride. 20 ml dialyzed serum + 40 ml saturated $PbCl_2$ + AcOH. Addition of 0.5 N NaOH to pH 6.1. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Concentration of precipitate relative to supernatant: 0.96. Ascending patterns.

amounts of protein. Evidently copper forms very slightly dissociable compounds with the proteins. In order to completely remove the copper the solutions were dialysed against sodium cyanide containing solutions. In this manner clear solutions were obtained, but the procedure was discontinued because we found that dialysis of normal serum against solutions containing cyanide produced a change of the electrophoretic pattern of the serum proteins. This was not reversed by a subsequent dialysis against cyanide free solutions. It follows from these experiments that the copper ion is less suitable for the fractional precipitation of serum proteins. A comparison of the results obtained at pH 4.5 with and without the addition of cyanide indicates that the copper protein compounds which precipitate during dialysis contain the bulk of the γ-globulins (Fig. 6).

It appears from these results that it is possible to achieve a fractional precipitation of serum proteins by means of ions of heavy metals. However, the specificity of the precipitations could be improved by combining the method

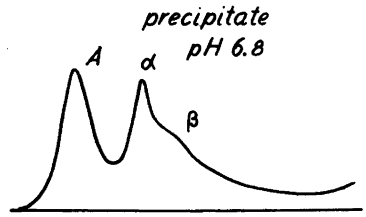

Fig. 4. Precipitate from dialyzed horse serum with lead chloride. 20 ml serum (dialyzed against distilled water) + 40 ml saturated $PbCl_2$. Addition of 0.5 N NaOH to pH 6.8. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Ascending pattern.

here described with other methods of fractional precipitation of proteins. The following description of the use of a lead precipitation in combination with other methods may serve as an illustration of the possibilities of the method.

To 1 volume of bovine serum (electrophoresis curve in Fig. 7 a) was added 2 vol. of saturated $PbCl_2$ and enough 1 *N* HCl to prevent precipitation. After adjusting the pH to 5.0 with 1 *N* NaOH the precipitate was removed by centrifugation and dissolved in 0.9 % NaCl by addition of a small amount of 1 *N* HCl. The precipitate showed the electrophoresis diagram in Fig. 7b. A small amount of saturated ammonium sulphate was now added in order to precipitate the lead as sulphate. After centrifugation the solution was neutralized, and further amounts of $(NH_4)_2SO_4$ were added to 55 % saturation. The precipitate obtained contained the rest of the globulins, mainly α-globulin (Fig. 7c). The filtrate consisted almost entirely of albumin (Fig. 7d) and was precipitated by dilution to 40 % saturation with $(NH_4)_2SO_4$ and addition of an equal volume of 2 % tricresol in water. The final concentration of 1 % tricresol and 20 % saturation with $(NH_4)_2SO_4$ had in a previous paper [4] been found favorable for the selective precipitation of albumin. The precipitate was dissolved in a small amount of phosphate buffer and dialysed until free from cresol. The electrophoresis diagram showed an albumin preparation of high purity containing only traces of other components (Fig. 7e).

DISCUSSION

It was reported previously that protein precipitating anions, such as sulphosalicylate and tungstate, can be used in the fractional precipitation of serum proteins [5]. In a recent paper the applicability of this method in the fractionation of serum of different animal species was demonstrated [6]. The anions form dissociable compounds with the proteins, which may be selectively precipitated at appropriate pH values.

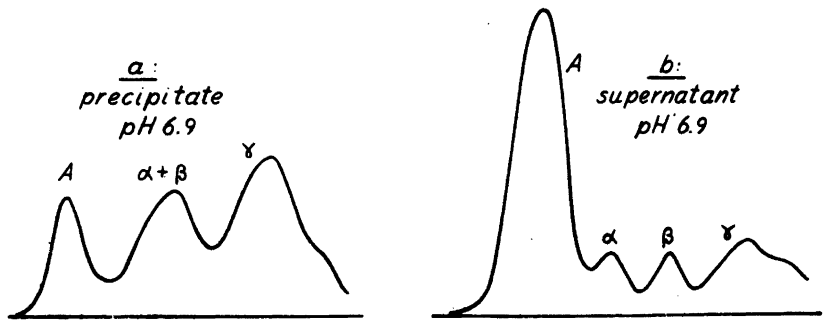

Fig. 5. Precipitate (a) and supernatant (b) from precipitation of bovine serum with cadmium chloride. 15 ml serum (same as Fig. 1a) + 15 ml 0.9 % NaCl + 7.5 ml 0.1 M $CdCl_2$ + 2.4 ml 0.25 N HCl. Addition of 1 N NaOH to pH 6.9. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Concentration of precipitate relative to supernatant: 2.7. Descending patterns.

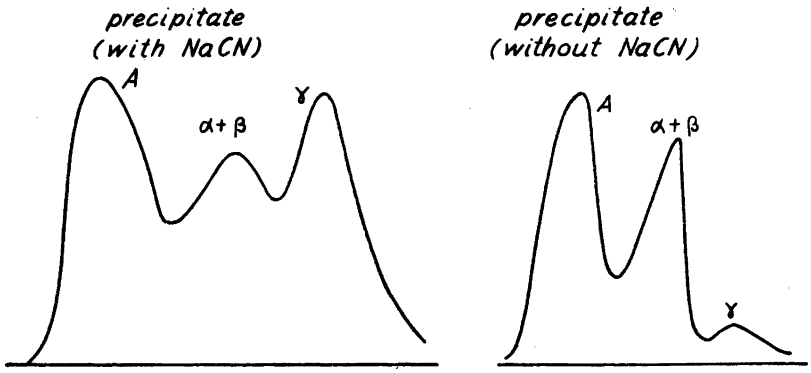

Fig. 6. Precipitate from bovine serum with copper chloride dialyzed subsequently against 0.9 % NaCl with (a) and without (b) added NaCN (0.1 %). 40 ml serum + 40 ml 0.9 % NaCl + 2 ml 1 N HCl + 2 ml 1 M $CuCl_2$. Addition of 1 N NaOH to pH 4.5. Electrophoresis: NaCl (μ = 0.075) — phosphate (μ = 0.025) buffer pH 7.5; 150 min., 18.2 mA. Descending patterns.

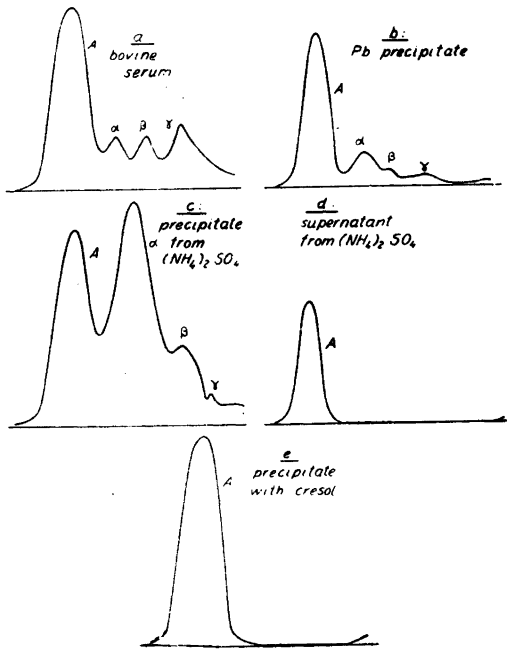

Fig. 7. Preparation of bovine serum albumin. a) Original serum; b) Lead precipitate at pH 5.0; c) Ammonium sulfate precipitate from solution of b; d) Filtrate from ammonium sulfate precipitation; e) Precipitate from d) with tricresol. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.6; 120 min., 18.0 mA. Descending patterns. Relative concentrations: a) 1.0; b) 1.2; c) 8.1; d) 1.2; e) 3.2

These results suggested a study of the applicability of protein precipitating cations for the same purpose. The work was initiated in 1947 but had to be abandoned again until recently. In the meantime the results of Cohn's group on the fractional precipitation of proteins by means of various metal ions appeared [1,2]. Our results together with those of Cohn's group show that it is possible to perform a fractional precipitation of serum proteins by heavy metal ions.

While Cohn produced fractional precipitation in human serum with zink and barium salts, we have used bovine and equine serum and have studied lead, cadmium and copper as precipitants, because these salts are known as potent precipitants of proteins.

Our results show a considerable difference in the action of the various salts on the serum proteins. Addition of lead chloride to bovine serum caused a precipitation of albumin and of α-globulin (at pH 5.0). The precipitate obtained with cadmium (at pH 6.9) contained mainly globulins, especially γ-globulin. Copper produced a rather unspecific precipitation, but a very insoluble compound was formed with the γ-globulin, from which copper could be removed only by dialysis against solutions containing sodium cyanide. However, dialysis against sodium cyanide changed the electrophoretic pattern of normal serum, so that this method could not be recommended though it has been used by Kunkel [7].

Heavy metals have been proposed as reagents in the turbidimetric estimation of elevated concentrations of globulin (especially γ-globulin) in human sera. Thus Kunkel [7] used highly diluted serum and small concentrations of copper or zink sulphate to produce a turbid precipitate of γ-globulin. He found that Hg, Pb, Cd and U salts produced a similar effect. Under the conditions used in our experiments (bovine or equine serum in high concentrations) Pb precipitated selectively albumin and α-globulin.

Lower dilutions were used by Wunderly and Wuhrmann [8] in their cadmium test for increased globulin content in human serum. This reagent gives a positive reaction not only with γ-globulin, but also with increased α-globulin concentration. This is in accordance with the results of cadmium fractionation of bovine serum recorded in the present paper.

Doladilhe [9] reported that lead chloride precipitated mainly the euglobulins when added to dialysed horse serum. He drew this conclusion from ammonium sulphate precipitations. We have tried above to reproduce his experiments, following the precipitation by electrophoretic analysis. It will be seen that our results are not in accordance with those of Doladilhe. Typical for our results with lead precipitation was the separation of albumin and α-globulin in the precipitate. Though the composition varied to some extent with the experimental conditions (pH and salt concentration), γ-globulin did not appear in the precipitate. Doladilhe has not specified his conditions as to pH, salt concentration and concentration of lead, but it appears difficult to explain all discrepancies between our results and his solely on this basis.

A great deal of work has appeared on the combination of metals with proteins. An important result of these investigations has been the realization that apart from the free carboxylic groups also the thiol group and the various basic groups play a role in the formation of metal compounds. Probably the

marked differences in the behaviour of the cations studied here (Cd^{++}, Cu^{++}, Pb^{++}) are caused by variations in the affinities to the various metal combining groups in the proteins as well as by variations in the solubilities of the compounds formed. It appears possible to modify the solubilities during the precipitations by varying the salt concentrations, and thereby to enhance the selectivity of the fractionation procedure. The method therefore appears adaptable and has probably a wide range of applicability. The example recorded on the preparation of bovine albumin shows its usefulness in combination with other methods of protein fractionation.

SUMMARY

1. Cadmium, copper and lead chloride were used as precipitants in the fractionation of bovine and horse sera.

2. Lead showed the highest selectivity, precipitating albumin and α-globulin from bovine as well as from horse serum.

3. Cadmium precipitated mainly globulins. Copper lacked selectivity under the conditions used. With the γ-globulin fraction it formed a compound containing firmly bound copper.

4. The preparation of pure bovine serum albumin is described, using lead precipitation in combination with other methods.

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