ACTA CHEMICA SCANDINAVICA 8 (1954) 901—908

Fractional Precipitation of Serum Proteins by Means of Specific Anions

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This paper presents an investigation of the fractional precipitation of serum proteins with protein precipitating anions as described in a previous report [1]. The method constitutes a general application of procedures, which have been used occasionally by different authors in the purification of various proteins [2-8]. A selective precipitation could be produced by acidification of a protein solution containing such ions. By subsequent neutralisation and dialysis the protein compounds formed could be split again and the proteins regenerated. Serum from different species, as well as the effect of different anions and varying conditions of precipitation have been studied.

MATERIALS AND PROCEDURES

The anions tested here were sulfosalicylate, tungstate, molybdate, phosphomolybdate, ferrocyanide, silicofluoride and metaphosphate. Most of the investigations were performed with bovine serum obtained from recalcified oxalated ox plasma. Experiments were also made with human and horse serum and with samples of antidiphtheria or antitetanus horse serum (kindly supplied by *The State Serum Institute*, Copenhagen). Before precipitation the serum samples were diluted with approximately equal volumes of phosphate buffer $M/15$ pH 7.6 containing NaCl to $\mu = 0.2$.

Three different procedures were used in performing the precipitations. The first two of these methods were generally used in preliminary experiments. The third method became the method of choice when more detailed information was necessary.

Method I: Various amounts of precipitating anion were added in the form of solutions of the free acid, and the resulting pH values were recorded. *Method II:* A certain pH value was maintained by means of HCl during addition of the precipitating anion, which was used in the form of a solution of the sodium salt. *Method III:* An excess of the precipitating anion (in solution as sodium salt) was added between pH 7 and pH 9, and the precipitation was achieved by adding HCl. All precipitations were performed in the cold (ice bath). A cooled centrifuge was used for the separation of the precipitates, which were redissolved in an appropriate amount of 0.9 % sodium chloride by addition of small amounts of solid $NaHCO_3$. After thorough dialysis against phosphate — sodium chloride buffer all precipitates and supernatants were analysed by electrophoresis in our Tiselius apparatus [9]. None of the precipitates were washed because the presence of a

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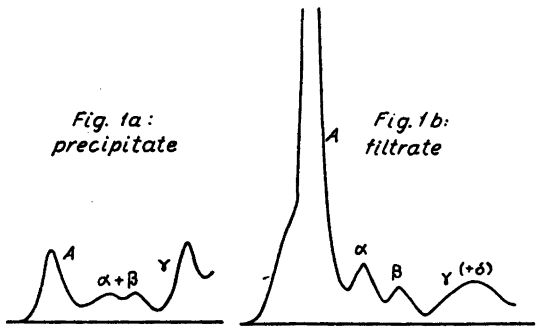


Fig. 1. Precipitate (a) and supernatant (b) from precipitation of bovine serum with tungstate. 20 ml serum + 20 ml NaCl-phosphate buffer pH 7.6 + 0.6 ml 1 M sodium tungstate brought to pH 6.4 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer, pH 7.6; a. 150 min., 18.8 mA; b. 154 min. 19.0 mA. Ascending pattern.

small amount of albumin in the samples facilitated the comparison of the various electrophoresis diagrams. With a few exceptions the mobilities of the protein components remained completely unchanged by the fractionation procedures.

RESULTS

The experiments presented in the following are illustrative examples. They represent only a small number of the experiments performed.

Precipitation of bovine serum with tungstate. A solution of sodium tungstate (0.6 ml 1 *M* tungstate) was added to four 40 ml samples of diluted bovine serum and precipitated by addition of 1.20, 1.55, 1.70 and 2.05 ml 1 *N* HCl respectively (Method III). The resulting pH values obtained were 6.4, 6.0, 5.6 and 5.2 respectively. The composition of precipitate and supernatant in the first sample (pH 6.4) is shown in Fig. 1. The precipitate contained the bulk of the γ-globulin and only small amounts of albumin and other globulins. The electrophoresis patterns obtained from the precipitates and supernatants resulting from the other samples showed that the composition was only slightly changed by decreasing the pH-values to 5.2. At this point appreciable quantities of albumin and of α- and β-globulin appeared in the precipitate (Fig. 2).

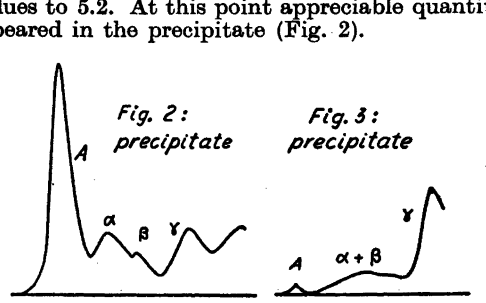


Fig. 2. Precipitate from bovine serum with tungstate. 20 ml serum + 20 ml NaCl-phosphate buffer pH 7.6 + 0.6 ml 1 M sodium tungstate brought to pH 5.2 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15)-phosphate (μ = 0.05) buffer pH 7.6; 150 min., 190. mA. Ascending pattern.

Fig. 3. Precipitate from bovine serum with tungstate at pH 6.2, redissolved in NaCl — phosphate buffer and reprecipitated at pH 7.0. Procedure similar to that in Figs. 1 and 2. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.6; 160 min., 19.0 mA. Ascending pattern.

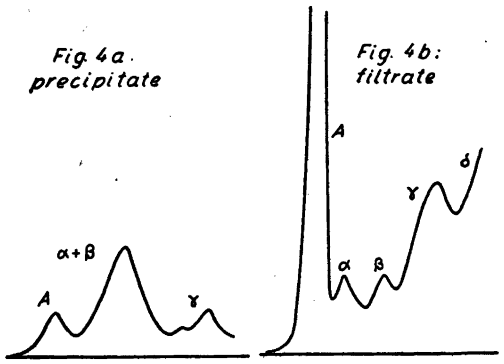


Fig. 4. Precipitate (a) and supernatant (b) from precipitation of bovine serum with sulfosalicylate. 20 ml serum + 20 ml NaCl — phosphate buffer pH 7.6 brought to pH 4.0 with 1 N HCl and precipitated with 3.8 ml 0.2 M sodium sulfosalicylate. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.6; a. 150 min., 18.8 mA; b. 129 min., 18.7 mA. Ascending pattern. Concentration of precipitate relative to supernatant: 2.8.

A sample of bovine serum was precipitated with tungstate at pH 6.2 (Method III), redissolved in phosphate buffer and reprecipitated with tungstate at pH 7.0. The electrophoretic patterns showed that the precipitate after the second precipitation consisted of almost pure γ-globulin (Fig. 3).

In another experiment acid was added to the supernatant from the first tungstate precipitation. Bovine serum was first precipitated with tungstate at pH 6.8 and the precipitate removed. To the supernatant more acid was added in order to complete the precipitation of γ-globulin. However, the resulting precipitate contained only traces of γ-globulin thus indicating a maximal precipitation of γ-globulin at pH 6.8. Even after precipitation at pH 4.7 the supernatant still contains a certain amount of γ-globulin.

This observation prompted a further investigation of the action of tungstate on a solution of γ-globulin. A sample containing mainly γ-globulin and only small amounts of the other protein components was therefore prepared from 120 ml bovine serum by repeated precipitation with 40 % saturated ammonium sulfate. The sample (35 ml) was dialyzed against 0.9 % sodium chloride, mixed with 30 ml phosphate-sodium chloride buffer and 20 ml 0.1 *M* sodium tungstate, and precipitated by lowering the pH to 5.4 with 0.5 *N* HCl (Method III). Refractometric measurements showed that about 2/3 of the γ-globulin had been precipitated. The filtrate, containing the rest, was now concentrated *in vacuo* to 10 ml and precipitated with 8.5 ml buffer, 6 ml 0.1 *M* tungstate, and lowering the pH to 5.4 with HCl. Refractometric measurements showed that the precipitated γ-globulin represented again 2/3 of that originally present. This incomplete precipitation of γ-globulin by means of tungstate is possibly caused by the solubility of the protein compound formed and is probably not caused by a qualitative difference in the precipitation of the components of the γ-globulin.

Precipitation of bovine serum with sulfosalicylic acid. When sulfosalicylate was used as precipitating anion results different from those obtained with tungstate were found. The electrophoretic patterns of the precipitate and supernatant obtained after addition of 3.8 ml 0.2 *M* sodium sulfosalicylate to 40 ml diluted serum at pH 4.0 are presented in Fig. 4 (Method II). In contrast to the results obtained with tungstate the precipitate consisted chiefly of α- and β-globulin. At lower pH values increasing amounts of albumin were precipitated, while most of the γ-globulin remained in solution. It is remarkable in this experiment, that in the diagram of the precipitate no resolution was obtained between α- and β-globulin. They appear as a single peak with an intermediate mobility. This indicates that the described fractionation procedure involves changes in the protein molecule, which may not be completely reversed by subsequent dialysis.

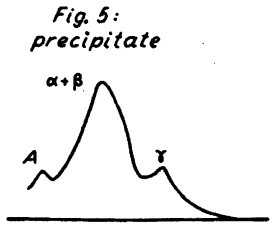


Fig. 5. Precipitate from bovine serum with sulfosalicylate. 19 ml serum + 19 ml NaCl — phosphate buffer pH 7.6 brought to pH 4.0 with 2.28 ml 10 per cent sulfosalicylic acid. Electrophoresis: NaCl (μ = 0.10) — phosphate (μ = 0.10) buffer pH 7.6; 75 min., 22 mA. Descending pattern.

Similar results were obtained when sulfosalicylic acid was added to the buffered serum according to Method I. The amounts used in such an experiment were 1.50, 1.70, 2.28, 3.10 and 3.95 ml 10 % sulfosalicylic acid to 38 ml diluted serum, and the corresponding pH values were 4.7, 4.3, 4.0, 3.5 and 3.0. The composition of the precipitate obtained at pH 4.0 is shown in Fig. 5.

Precipitation of human serum with tungstate. When sodium tungstate was added to diluted human serum (Method III) results as presented in Fig. 6 were obtained. It appears, that the β-globulin, and to a lesser degree the α-globulin, is precipitated first, followed at lower pH values by albumin and some γ-globulin. The range investigated was from pH 7.2 to pH 6.0. This result differs from that obtained by precipitation of bovine serum with tungstate but is similar to the results found when sulfosalicylic acid was used on bovine serum. This observation indicates a fundamental species difference in the properties of the serum proteins. It is interesting to note that the selective precipitation of α- and β-globulins also in this case is characterized by the appearance of one single peak with a mobility slightly greater than that of the β-globulin in the original sample. Neither the precipitate nor the supernatant showed the usual β-anomaly after the fractionation procedure.

Precipitation of bovine and human serum by other anions. Among the other precipitants, molybdate, phosphomolybdate, and ferrocyanide showed properties similar to those of tungstate though the selectivity of the precipitations was slightly less. Metaphosphate precipitated γ-globulin rather selectively. Fig. 7 illustrates the composition of the precipitate after adding 2.3 ml 10 % HPO_3 to 40 ml buffered bovine serum (pH 4.8). The precipitate consists almost exclusively of γ-globulin. Experiments with silicofluoride showed this substance to be a completely unspecific precipitant. One experiment on human serum is presented in Fig. 8. Precipitate and supernatant contain all the pro-

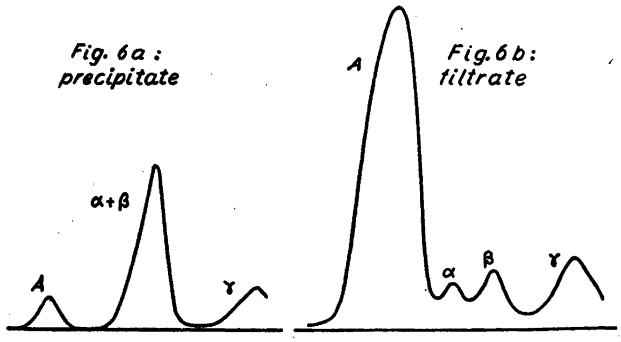
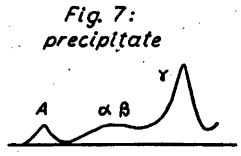


Fig. 6. Precipitate (a) and supernatant (b) from precipitation of human serum with tungstate. 10 ml serum + 14 ml NaCl — phosphate buffer + 6.2 ml 0.1 M Sodium tungstate brought to pH 6.35 with 0.2 N HCl. Electrophoresis: NaCl (μ = 0.025) — phosphate (μ = 0.075) buffer pH 7.55; 120 min., 18.0 mA. Descending pattern. Concentration of precipitate relative to supernatant: 3.1.

Fig. 7. Precipitate from bovine serum with metaphosphate. 20 ml serum + 20 ml NaCl — phosphate buffer brought to pH 4.8 with 2.3 ml 10 % metaphosphoric acid. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer; pH 7.5; 150 min., 18.5 mA. Ascending pattern.



tein components in about equal proportions. Similar results were obtained with bovine and horse serum. Silicofluoride may thus be considered unsuited for serum protein fractionation.

Precipitation of horse serum. When normal horse serum was precipitated with tungstate, results similar to those obtained with bovine serum were obtained. Thus in an experiment where normal horse serum was precipitated at pH 5.8 with tungstate according to Method III, the first precipitate consisted mainly of γ-globulin accompanied by small amounts of α- and β-globulins (Fig. 9). However, varying results were obtained when immune sera were used. Thus when antidiphteria or antitetanus serum were precipitated by tungstate the α- and β-globulins were precipitated first (at pH 5.6) together with some γ-globulin (Fig. 10). At lower pH values increasing amounts of γ-globulin and albumin were found in the precipitates.

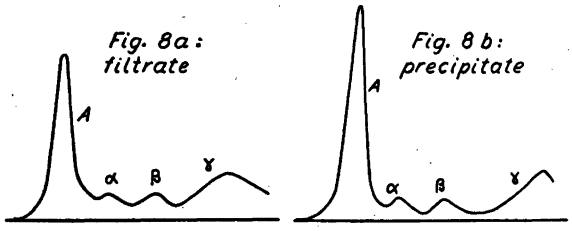
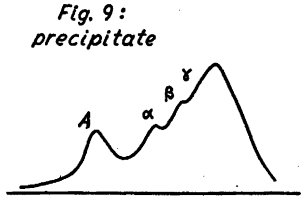


Fig. 8. Precipitate (a) and supernatant (b) from precipitation of human serum with silicofluoride. 20 ml serum + 40 ml saturated neutral Na_2SiF_6 brought to pH 6.5 with 0.5 N HCl. Electrophoresis: NaCl-phosphate buffer pH 7.6; a. 150 min., 18.2 mA; b. 150 min., 18.0 mA. Ascending pattern.

Fig. 9. Precipitate from normal horse serum with tungstate. 20 ml serum + 20 ml NaCl — phosphate buffer + 0.7 ml 1 M sodium tungstate brought to pH 5.8 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.5; 151 min., 18.0 mA. Descending pattern.



DISCUSSION

The fractional precipitation of serum proteins has always been a mattes of much importance, and in the course of time several different procedurer have been tried. In the methods mostly used the fractionation is achieved through the addition of various amounts of certain salts, such as ammonium sulfate, sodium sulfate and others. Large scale preparation of several serum protein components has also been accomplished by precipitation with alcohol in a procedure mainly developed by Cohn *et al.*[10]

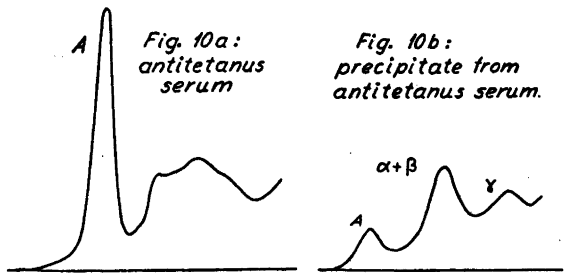


Fig. 10. Heat sterilized antitetanus serum from horse (a) and precipitate obtained with tungstate (b). 20 ml serum + 20 ml NaCl — phosphate buffer + 0.7 ml 1 M sodium tungstate brought to pH 5.6 with 1 N HCl. Electrophoresis: NaCl (μ = 0.15) — phosphate (μ = 0.05) buffer pH 7.5; a. 150 min., 18.2 mA; b. 154 min., 18.8 mA. Ascending pattern. Concentration of precipitate relative to original serum: 3.8.

The method of serum protein fractionation with specific anions differs from most other procedures of fractionation in that it is not based on the different solubilities of the native proteins. We may suppose that the added anions combine reversibly with the basic groups of the protein molecules. By addition of acid the carboxylic groups are deprived of their negative charge and the protein compounds will precipitate according to their solubilities, which will depend to a high degree on the character and number of bound anions.

In spite of the promising possibilities of this method it has so far only been used in a few cases, such as the purification of mare serum gonadotropin [2,3], precipitation of egg albumin [4], and the purification of renin [5,6], of cathepsin [7], and of pepsin inhibitor [8].

In the present paper an attempt has been made to achieve a fractionation of the serum proteins from different species. The various anions tested showed a considerable variability in selectivity. The best separations were obtained with tungstate, sulfosalicylate, and metaphosphate. From bovine serum γ-globulin was precipitated, when tungstate was added at pH of about 6.4, or metaphosphate at pH 4.8. In contrast to this the addition of sulfosalicylate at pH 4.0 caused a precipitation of α- and β-globulin. It is thus evident that the composition of the precipitated proteins as well as the appropriate pH depend on the anion used. Furthermore a distinct species difference could be demonstrated. The fractionation of human serum with tungstate caused a precipitation of α- and β-globulin very similar to that obtained from bovine serum with sulfosalicylate, and in contrast to the γ-globulin precipitated from bovine serum by tungstate. Similar variations were encountered with normal horse serum and heat treated horse immune sera, the main constituents of the tungstate precipitates being γ-globulin in the first case, α- and β-globulin in the second.

It was occasionally found that the separations were accompanied by changes in the β-globulin fraction. The β-anomaly of the human serum disappeared, and in several cases α- and β-globulin moved as a rather broad single peak, indicating some degree of complex formation. These effects are not necessarily due to real changes in the proteins such as denaturation, but

might be explained by the removal of certain components by the precipitation procedure.

When the precipitations were interrupted after only a small amount of protein had been precipitated, it was possible to obtain these first fractions with a high degree of purity. In this case, however, the filtrate contained an appreciable amount of the fraction in question. Attempts to achieve a more complete precipitation by lowering the pH were not successful, as this led to the precipitation of considerable amounts of other fractions as well. Some experiments indicate that the solubility of the anion compounds does not entail a quantitative precipitation, even at the optimal pH-value. These difficulties might be overcome by the addition of neutral salts or of a small amount of alcohol as used in the fractionation procedures of Cohn *et al.* [10,11] It is thus probable that the optimal conditions and the most suitable agents have as yet not been found and that suitable variations may lead to much more complete separations.

Three precipitation procedures were used. The first of these was considered to be of least value, as pH and concentration of the precipitating anion could not be varied independently. The second method, in which the precipitant was added at a constant pH value, should be preferred, if the differences between the protein compounds formed depended mainly on the affinity to the added anion. The third procedure, in which the precipitation was performed at different pH values in the presence of an excess of anion, should be preferred if the compounds differed in their solubility and in the position of their isoelectric points. In those instances where different precipitation procedures were used with the same anion, no significant differences in the composition of the precipitates could be noted. It must be admitted that only few such experiments were performed as it seemed most promising to work with an excess of anion as in procedure III, which was therefore generally preferred.

Cohn *et al.*[11] have described a fractionation procedure, which takes advantage of the specific combination of the proteins from human serum with various metals and of the characteristic solubilities of the compounds. In the present paper the possibility and value of using specific anions for similar fractionations has been demonstrated. The method may prove a valuable supplement to existing methods for protein fractionation.

SUMMARY

1. The low solubility of compounds between proteins and inorganic and organic anions has been used in the fractionation and isolation of serum proteins.

2. The course of the fractionation depends on the character of the anion as well as on the species from which the serum sample was obtained.

3. The investigation shows that the precipitation by means of specific anions deserves consideration as a method supplementing the generally applied methods for serum protein fractionation.

This investigation was supported by grants from "*Teknisk kemisk Fond*", Copenhagen.

REFERENCES

1. Astrup, T. and Birch-Andersen, A. *Nature* **160** (1947) 637.
2. Rinderknecht, H., Noble, R. L. and Williams, P. C. *Biochem. J. (London)* **33** (1939) 381.
3. Rimington, C. and Rowlands, J. W. *Biochem. J. (London)* **35** (1949) 736; **38** (1944) 54.
4. Perlmann, G. and Hermann, H. *Biochem. J. (London)* **32** (1938) 926.
5. Dexter, L., Haynes, F. W. and Bridges, W. C. *J. Clin. Invest.* **24** (1945) 62.
6. Astrup, T. and Birch-Andersen, A. *Nature* **160** (1947) 570.
7. Anson, M. L. *J. Gen. Physiol.* **23** (1940) 695.
8. Herriot, R. M. *J. Gen. Physiol.* **24** (1941) 325.
9. Astrup, T. and Brodersen, R. *Acta Chem. Scand.* **1** (1947) 1.
10. Cohn, E. J., Strong, L. E., Hughes, Jr. W. L., Mulford, D. L., Ashworth, J. N., Melin, M. and Taylor, H. L. *J. Am. Chem. Soc.* **68** (1946) 459.
11. Cohn, E. J., Gurd, F. R. N., Surgenor, D. M., Barnes, B. A., Brown, R. K., Derouaux, G., Gillespie, J. M., Kahnt, F. W., Lever, W. F., Liu, C. H., Mittelman, D., Mouton, R. F., Schmid, K. and Uroma, E. *J. Am. Chem. Soc.* **72** (1950) 465.

Received February 13, 1954.