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Notes on the Fractionation and Colorimetric Assay of Commercial Heparin

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The barium salt of heparin was fractionated by means of precipitation with acetone into two different sodium salts as reported by Kuizenga and Spaulding [1]. By means of electrophoresis the present authors separated commercial heparin into two distinctly different fractions, both of which exerted anticoagulant and metachromatic activities [2]. Continued fractionation studies have been performed with a view to gain more insight into the anticoagulant and metachromatic capacities of different fractions obtained, and this forms the subject of the present paper. When comparing the anticoagulant and metachromatic activities of heparin preparations or fractions of purified heparin it should be recalled that these activities are segragated and cannot be ascribed to the same group of the heparin molecule; the anticoagulant effect may be due to a central nitrogen bond [3], the electric charge of the molecule [4], or some other characteristic. The metachromatic reaction is on the other hand referable to the ester sulphate groups of heparin [5]. The segragation mentioned above was studied in previous recrystallization experiments [2]. Additional data on the inhomogeneity of purified heparin with reference to the varying degree of esterification may be obtained from recent report by Jorpes and Gardell [6].

EXPERIMENTAL

Serial precipitation experiments were performed on commercial heparin (Vitrum, Sweden) using the organic precipitants acetone, alcohol, and dioxane. Increasing amounts of the precipitants were added to a row of glass tubes, each containing 1 ml of a 1 per cent heparin solution in 0.9 per cent sodium chloride. The concentration increment of the precipitants between two sub-

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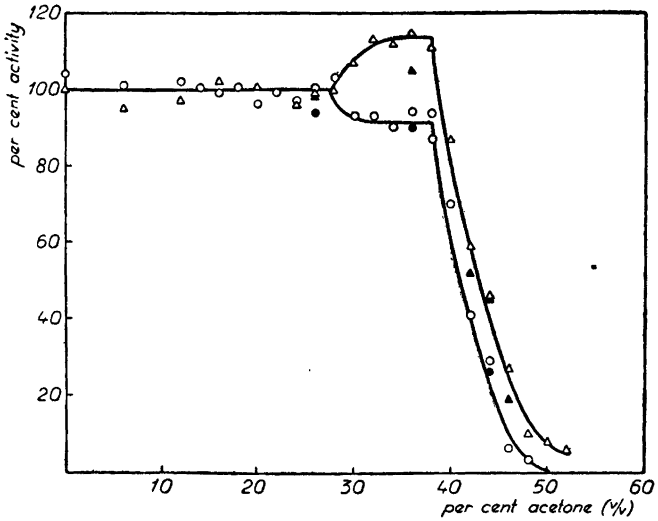

Figure 1. Precipitation diagram of heparin Vitrum (lot F 1946) using acetone as precipitant.

○ *anticoagulatiang activity of the supernatant.*

△ *metachromatic activity of the supernatant.*

solid points: anticoagulant- and metachromatic activity of the samples used for electrophoresis experiments (see Table 1).

sequent tubes was 2 per cent. The precipitants were carefully pipetted into the tubes, which were then gently shaken and immediately corked to avoid evaporation. To prevent contamination with the cork a small piece of soft tin foil was wrapped around the latter, and this thightening proved to be effective. The tubes were allowed to stand in a water thermostat at 20° C until the precipitate had collected. From every tube samples of 0.100 ml. of the supernatant liquid were transferred to a row of 10 ml. volumetric flasks; the organic precipitate was removed by suction in vacuum, and the flasks were then filled with 0.9 per cent sodium chloride solution. This series of solutions was used for the determination of anticoagulant activity by means of the thrombin method of Jaques and Charles [7], and for the assay of metachromatic reaction by the method of MacIntosh [8] using toluidine blue (Grübler, equivalent to Asure A). All experiments were performed at pH 7.

The anticoagulant activity found in the supernatants was expressed in per cent of the calculated activity which would have been found provided no precipitation had occurred, and these values were plotted as ordinates. The corresponding concentrations of the precipitants used in the tubes were

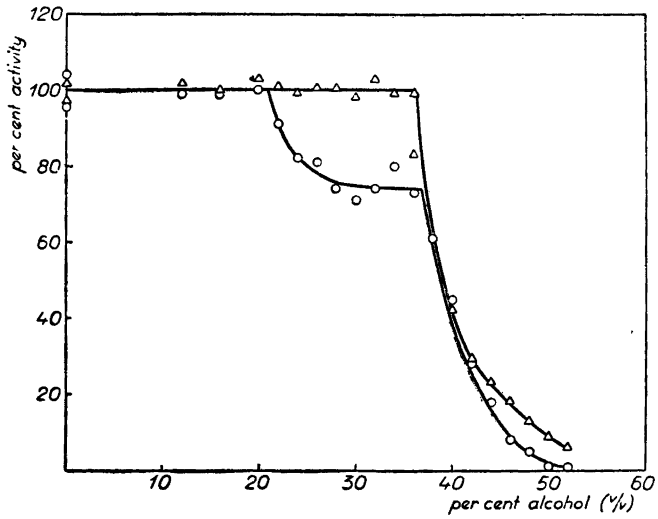

Figure 2. Precipitation diagram of heparin Vitrum (lot F 1946) using alcohol as precipitant.

○ *anticoagulant activity of the supernatant.*

△ *metachromatic activity of the supernatant.*

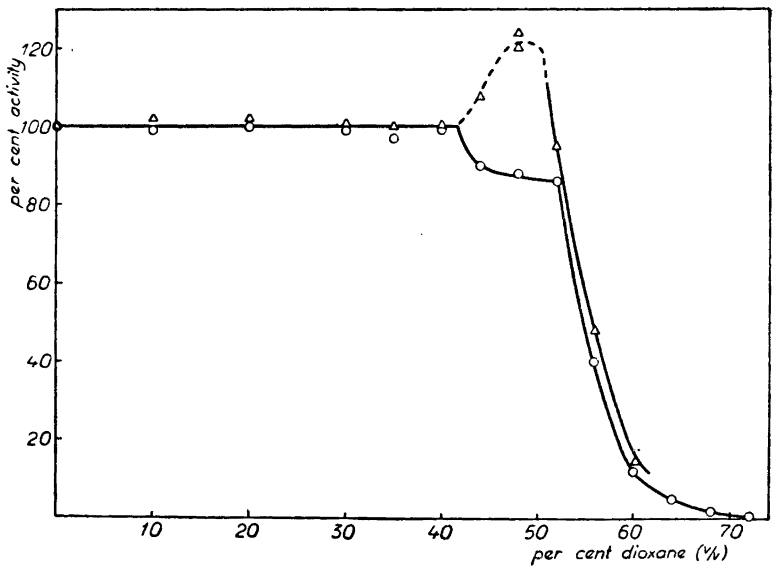

Figure 3. Precipitation diagram of heparin Vitrum (lot VII : 44) using dioxane as precipitant.

○ *anticoagulant activity of the supernatant.*

△ *metachromatic activity of the supernatant.*

plotted along the abscissa, and in this way precipitation diagrams were obtained for acetone, alcohol, and dioxane as precipitants (Figs. 1—3).

All diagrams were similar in type and evidence the inhomogeneity of commercial heparin. Partial precipitation of the active material was found to appear after the addition of 20—40 per cent of alcohol and acetone, and after 40—50 per cent of dioxane, and this was followed by a decrease in anticoagulant activity of about 10—20 per cent. Following the addition of larger volumes of the precipitants, the actual precipitation of active material rapidly took place. The values obtained by means of the metachromatic assay for heparin showed a consistent divergence from those just mentioned, but only during the first phase of partial precipitation (Figs. 1—3).

In order to elucidate whether the first drop in anticoagulant activity could be ascribed to the precipitation of a singel one of the heparin components found during electrophoresis (2, α and β), further precipitation experiments were performed on larger samples of heparin *(Vitrum)* together with additional electrophoresis investigations. As previously described, serial precipitation was performed with acetone but at larger concentration invervals. From each tube 20 ml. samples were taken of the supernatant liquid, freed from acetone by suction in vacuum, and then dialyzed against a phosphate buffer at pH 6.8, and finally subjected to electrophoresis. Smaller samples were used for control determinations of the anticoagulant and metachromatic activities of the supernatants. The latter values were plotted in Fig. 1 (solid points) and corroborate the previous ones.

The results obtained from electrophoresis experiments (Table 1) indicate that the reciprocal concentration of the two main components α and β (2) contained in the supernates is not significantly changed in the course of the precipitation, nor are the concentration values of the components differing from those of the original material.

Table 1. Relative concentrations of heparin components contained in the supernates during precipitation with acetone.

Amount of acetone in per cent		0	38	42	44	46
Relative concentration of heparin components .... in per cent	α	66	72	60	72	73
	β	34	28	40	28	27

These experiments were repeated using alcohol as precipitant. Following the precipitation at an alcohol concentration of 36 per cent, the supernatant

liquid was found to contain 66 per cent of α and 34 per cent of β, and the precipitate obtained held 70 per cent of α and 30 per cent of β. The electrophoresis experiments thus indicate that the first drop in anticoagulant activity reported in the precipitation curves (Figs. 1—3), could not be explained by the precipitation of a single one of the heparin components α or β.

DISCUSSION

Present attempts to separate commercial heparin by means of gentle precipitation into two components corresponding to those previously found [2] in electrophoresis experiments have not been successful. On the contrary, both components were found to be precipitated concurrently, and further in a characteristic two-step fashion. No reasonable explanation of this fact can so far be advanced. Another precipitant acting more smoothly might achieve such a segragation of the actual components *Cf.* [6].

However, the results are of definite interest in other respects. Following the gradual addition of acetone (Fig. 1) and dioxane (Fig. 3) an increase in metachromatic capacity was found in the supernates parallel to the first drop in anticoagulant activity. Later on, during rapid precipitation of the remaining solute both activities are decreasing at the same rates. Available data do not justify an interpretation of this interesting segragation between the anticoagulant and metachromatic capacities of the material contained in the supernates. Several possibilities have to be considered. Commerical heparin is known to be inhomogeneous and impurities may occur, which might be able to obscure the metachromatic reaction. With reference to the last mentioned possibility the following experiments were made.

In two points of the dioxane precipitation diagram (Fig. 3) corresponding to 48 and 52 per cent dioxane content, the amount of the precipitate obtained was roughly estimated by drying an aliquote amount of the supernatant, and found to agree with the observed drop in anticoagulant activity calculated as heparin. This means that a possible admixture could only constitute a smallish fraction of the original substance, which in that case would explain why such an admixture was not observed in previous electrophoresis experiments [2].

In addition, when heparin dialysed for a week was tested according to the method of MacIntosh [8] the anomalous increase in metachromatic activity had disappeared. This might indicate that some impurity was removed by dialysis.

For lack of sufficient information of the mechanism and kinetics of the metachromatic staining reaction further discussion will be postponed. Difficulties evidently exist for the assay of heparin by means of the metachromatic reaction [5] as well as by using more indirect methods of determination [9].

SUMMARY

Serial precipitation experiments on commercial heparin (Vitrum) are reported using the organic precipitants acetone, alcohol, and dioxane. A similar segragation of heparin into two components as demonstrated in previous electrophoresis experiments was not obtained. Both components were found to be precipitated concurrently in a two-step fashion. During the first phase of precipitation a remarkable discrepancy was observed between the anticoagulant and metachromatic activity of the remaining solute material. This may be due to a variety of factors, and some attention was paid to the possible precipitation of some impurity, which might hamper the metachromatic reaction. This unexplained phenomenon constitutes a source of error in colorimetric assays of heparin.

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