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# HEPARIN AND CONCANAVALIN A INTERACTION: ISOLATION OF FRACTION WITH HIGHER ANTICOAGULANT ACTIVITY

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SUMMARY: Heparin and Concanavalin A complexes were studied under different conditions. Interaction was measured by reading the turbidity at 420 nm. The influence of the pH, heparin, and salt concentration was measured. In the presence of salts pH was very critical, and above pH 5.4 the interaction was practically negligible.

At pH 4.6 or 5.2 and the lowest salt concentration compatible with buffering, heparin fractions with different anticoagulant specific activities were detected in the precipitate and in the supernatant, after the interaction. In all cases a significative difference was observed in favor of the heparin isolated from the precipitate.

Possibility of an artifact was eliminated by using adequates blanks and running the coagulation tests in the presence of an excess of Concanavalin A. 

Con A is one of a group of plant proteins, called lectins, that agglutinate a variety of animal cells throughthe binding to carbohydrates of the cell surface (1,2), and to precipitate various neutral polysaccharides such as glycogen(3), and acid polysaccharides like heparin (4). The stereochemical requirements for the interaction between Con A and the polysaccharides in solution have been studied extensively by Goldstein et al.(5,6,7,8). Con A joins to polysaccharides through hydrogen bonds and hydrophobic bonds. However, with charged polysaccharides interaction with Con A is, in contrast highly

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dependent on electrostatic bonds and much less on hydrogen or hydrophobic bonds. The complexes of Con A with neutral polysaccharides are very stable in the presence of a wide salt concentration. Even 4 M NaCl is not sufficient to destroy the complex with Dextran 1335(9). The picture is completely different with charged polysaccharides. Doyle and Kan (10) studied the heparin-Con A complex:optimum turbidity was observed at pH 5.4 and no interaction was detected above pH 5.8. In addition NaCl concentrations approaching 0.1 M completely inhibits the Con A-heparin interaction. This fact is consistent with the idea that the complex is mediated fundamentaly through ionic bonds.

We have made an exhaustive study of the Con A-heparin complex. The influence of pH,and the heparin and salts concentrations were carefully verified.At the lowest sal concentration compatible with buffering heparin fractions with different anticoagulant specific activities were detected in the precipitate and in the supernatant. In all cases a significative difference was observed in favor of the heparin isolated from the precipitate. These results open the way to study the molecular basis of the anticoagulant activity of the heparin with a practical and useful model:the Con A-heparin complex.

## MATERIALS AND METHODS

Con A,type IV,and heparin ammonium salt were purchased from Sigma Chemical Co(St Louis,MO).General laboratory reagents used were analytical grade or better.

Interaction was followed by reading turbidity at 420 nm. Acetate buffer between 3.9 and 5.8 were used for studying the pH influence. For heparin concentration curves a solution in distilled water was used:1 ml of each heparin solution(0.015 to 4.0 mgr/ml) was mixed with 2 ml of acetate buffer 0.1 M,pH 5.0.The reac-

tion was started by adding 1 ml of Con A solution in distilled water (2 mgr/ml).After 30 min at 30°C turbidity was read at 420 nm.In all others experiments the heparin concentration which gives maximum interaction was used.Heparin concentration was determined by an analytical method developed in our laboratory which is based in the reaction of carbohydrates with indol in acid medium(11).In order to obtain maximum color with indol a previous acid hydrolysis was carried out. Anticoagulant activity of heparin was determined by two methods,one described by Freeman et al(12),and the other was that developed by Yin,Wessler and Butler(13).

## RESULTS

The effect of the pH on the interaction was studied and results are shown in Fig.1. A maximum was obtained at pH 5.0 and interaction decreased abruptly at higher pH's. The next step was to find the optimum heparin concentration for the interaction and we found that between 200 µg to 500 µg of heparin,interaction with 2000 µg of Con A gives a plateau,at pH 5.0 and a final buffer concentration of 0.05 M.

The influence of salt concentration in the interaction is visualized in Fig.2:above 100 mM interaction is

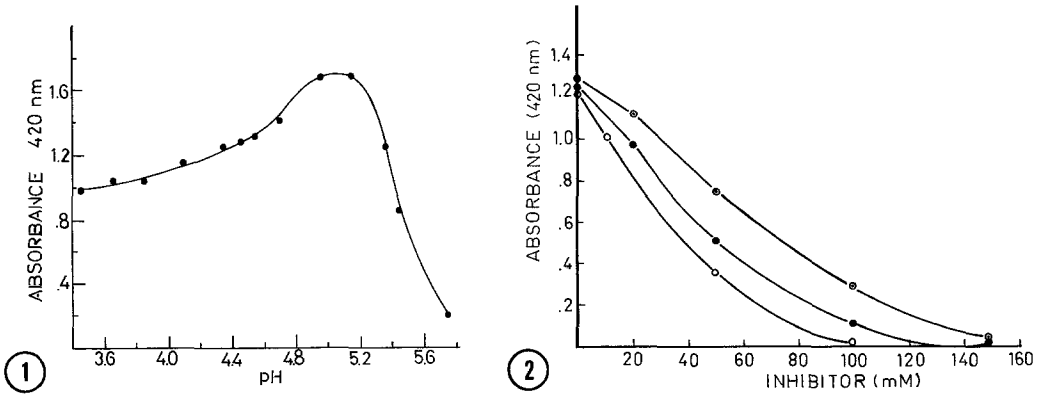


Figure 1: The influence of the pH on the Con A-heparin interaction.
50 mM final buffer concentration.

Figure 2: The influence of salt concentration on the interaction Con A-heparin.
⊙-⊙-⊙: LiCl
o-o-o: KCl
●-●-●: NaCl

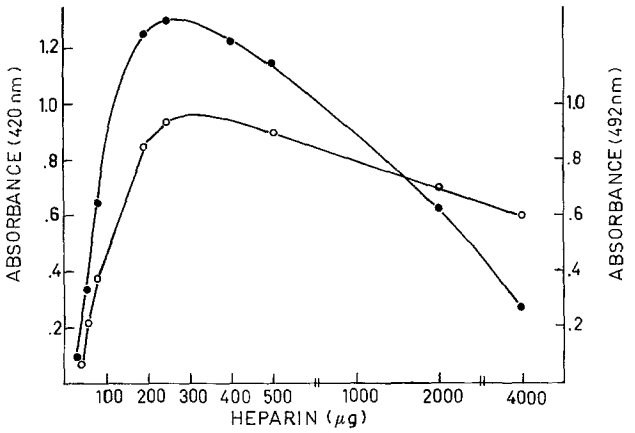


Figure 3: The influence of the heparin concentration on the interaction with Con A.
Conditions: see Materials and Methods.
After interactions the suspensions were centrifuged at 3000 rpm for 20 min and the heparin concentrations were determined in the precipitates.

negligible. With the optima conditions for the interaction fixed,a study of the heparin which binds to Con A was undertaken. The suspension was centrifuged at 3000 rpm for 20 min and heparin was analyzed in the precipitate and in the supernatant by the indol-HCl method(11). As Fig.3 shows,a close relationship was found between the interaction and the amount of heparin present. In parallel experiments the biological activities of both fractions were determined(12,13)and then the corresponding specific activities calculated and expressed as units of biological activity per mgr of heparin. The results are shown in Table I and,in all cases a significative difference in the specific activities of both fractions were found.

Possibility of an artifact produced by the presence of Con A,heparin or an unknown umpurity was eliminated in two ways:a) Adequates blanks were run with the same amounts of Con A and heparin used in the interaction but without any ions. In these conditions interaction

TABLE I

Isolation of an heparin fraction with higher specific activity

| EXP | SPECIFIC ACTIVITY U/mgr | | RATIO |
|---|---|---|---|
| | PRECIPITATE | SUPERNATANT | |
| 1 | 220 | 95 | 2.3 |
| 2 | 157 | 105 | 1.5 |
| 3 | 174 | 57.5 | 3.0 |
| 4 | 247 | 73.5 | 3.4 |
| 5 | 230 | 82.5 | 2.8 |
| 6 | 254 | 108 | 2.3 |
| 7 | 226 | 53 | 4.0 |
| 8 | 291 | 44 | 6.6 |
| 9 | 344.8 | 142.8 | 2.4 |

Conditions: see Materials and Methods.
After interactions the suspensions were centrifuged at 3000 rpm for 20 minutes and biological activities and heparin concentrations were determined in precipitates and supernatants.(11,12).
All results by duplicate.

was practically null($L_{420\ nm}$=0.015 against distilled water),and an average specific activity between that found in the precipitate and the supernatant was detected in all cases. b) An excess of Con A was added before doing the coagulation tests. As Table II shows,in all cases,no significative differences in the coagulation time were found.The same results were obtained when Con A was added to the reaction mixtures containing precipitate or supernatant aliquots.(not shown).

On the other hand,in regard the analytical method for measuring heparin,addition of as much as 1 mgr of Con A does not affects the calibration curve(11).

TABLE II

Influence of Concanavalin A on the coagulation time

| METHOD | ADDITION | | TIME | |
|---|---|---|---|---|
| | Protein | ug | seconds | % |
| I.Yin et al. | none | - | 23 | 100 |
| | | 0.312* | 22.5 | 98 |
| | Con A(D) | 0.624 | 23.5 | 102 |
| | | 2.08 | 21 | 91 |
| | Con A(D)$Ca^{2+}$ | 0.312* | 23 | 100 |
| | Con A(D)$Ca^{2+}Mn^{2+}$ | 0.312* | 22.5 | 98 |
| II.Freeman et al. | | | minutes | |
| | none | - | 14,25 | 100 |
| | | 7.5 | 13.5 | 94.7 |
| | Con A(D) | 15* | 15.2 | 106.6 |
| | | 22.5 | 14.5 | 101.7 |

Method I: Experiments by duplicate
Method II: Results with 15 ug of Con A are the average of four experiments.
In all cases appropriate dilutions of standard heparin were added to the normal human plasma in the presence of the amount of Con A showed.
Con A(D): Con A demetallized by treatment with 1 N HCl.
Con A(D)Ca: Demetallized Con A plus 3 mM $Ca^{2+}$.
Con A(D)Ca.$Mn^{2+}$: Demetallized Con A plus 3 mM $Ca^{2+}$ and 7.5 mM $Mn^{2+}$.

*Maximum amount present in the aliquot used if all Con A in the original incubation mixture would remain in the precipitate or in the supernatant.

## DISCUSSION

In our laboratory we are studying the relationship between the chemical structure of heparin and its anticoagulant properties. At molecular level,heparin interacts with the plasma protein,antithrombin III,in a very specific way,and induces a conformational change in the protein molecule which is responsible of the dramatic acceleration observed in thrombin inactivation.A clue to the research on heparin came about twelve years ago when Lam,Silvert and Rosemberg(14) found that only 1/3 of

the heparin binds antithrombin III,and that this fraction is responsible of the anticoagulant activity. An octasaccharide was isolated which has high affinity for antithrombin III(15),and its structure was partially characterized(16).

Our interest in Con A-heparin complexes came from the work of Randoff and Danishefsky(17) on the location on heparin of the oligosaccharide section responsible for accelerating the neutralization of thrombin and factor Xa by antithrombin III. They found that these groups are located at,or near,the non reducing end of the heparin chain.As Con A recognizes also the non reducing end of the polysaccharide molecule,we supposed that the lectin would bind to the active part of the heparin molecule. We were able to demonstrate that this was the case:we fractionated heparin by the precipitation technique and obtained a fraction with high activity in the precipitate, and a fraction with low activity in the supernatant.These results open the way to the study of the molecular basis of the anticoagulant activity of heparin with a practical model: the Con A-heparin complex.

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