THE SEPARATION OF ACTIVE AND INACTIVE FORMS OF HEPARIN

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SUMMARY

Heparin has been fractionated into two distinct forms. The isolation of these species was accomplished by sucrose density gradient centrifugation of heparin mixed with antithrombin-heparin cofactor. Approximately 1/3 of this mucopolysaccharide was bound to antithrombin-heparin cofactor and had potent anticoagulant activity. This component was clearly separated from the remaining 2/3 of the heparin which could not form a stable complex with antithrombin-heparin cofactor and had minimal anticoagulant activity.

INTRODUCTION

Heparin acts as an anticoagulant by dramatically accelerating the rate at which antithrombin-heparin cofactor neutralizes serine proteases of the hemostatic mechanism (1, 2, 3, 4, 5). This is accomplished via binding of heparin to lysyl residues of antithrombin-heparin cofactor which probably induces a conformational alteration of the inhibitor that renders its reactive site more accessible to the active center of these enzymes (6). Considerable effort has been expended in defining the linear sequence and three dimensional configuration of heparin (7,8,9). However, the precise relationship between its structure and anticoagulant properties remains elusive. Heparin preparations exhibit considerable polydispersity in molecular size (10), variations in the ratio of glucuronic acid to iduronic acid (11), alterations in the amount of ester and N-sulfation (12), and differing extents of N-acetylation (13). Changes in each of these parameters correlate to only a limited degree with anticoagulant potency. Thus, it has been tacitly assumed that a specific, highly defined heparin structure may not be required for anticoagulant activity. In this communication,

we provide the first evidence that only a small fraction of a given heparin preparation can bind to antithrombin-heparin cofactor and is responsible for its distinctive anticoagulant effect. Therefore, we suggest that heparin fractions of this type must be analyzed before meaningful structure-function relationships are likely to emerge.

Materials

Human antithrombin-heparin cofactor and human thrombin were obtained in physically homogeneous form by methods previously reported from this laboratory (6,14). Heparin of porcine intestinal origin (Riker Laboratories Lot # 56913), utilized for the anticoagulant therapy of human subjects, was employed throughout these studies. This mucopolysaccharide was characterized by its susceptibility to degradation by crude heparinase (sonicated flavobacterium heparinum), and resistance to cleavage with chondroitinase ABC (Miles Laboratory). Incubation conditions and assay techniques have been described previously in detail (15). Furthermore, chromatography of this heparin product on DEAE-cellulose demonstrated that the mucopolysaccharide eluted at LiCl concentrations above 1.0 M. This behavior is similar to that obtained with other preparations of this mucopolysaccharide (15). All other chemicals were reagent grade or better.

Methods

Assay of Heparin Concentration: The mucopolysaccharide concentration of a heparin sample was determined colorimetrically by assay of uronic acid via the carbazole method of Bitter and Muir (16) and by a modification of the Azure A method of Jaques (17). When this latter method was utilized, 50 µl of a sample (0-5µg) was added to 1 ml of Azure A (0.01 mg/ml in distilled water). The resultant mixture was agitated and absorbance at 500 nm was measured. Sucrose concentrations from 10% to 50% (w/v) and antithrombin-heparin cofactor levels from 20 µg/ml to 290 µg/ml had, essentially, no effect upon this assay. The heparin concentration of all fractionated samples was determined by comparison with the initial starting material. The standard titration curve of this unfractionated sample exhibited a linear rise in absorbance at 500 nm from 0 to 0.200 as the amount of heparin was increased from 0 to 5 µg.

Assay of Anticoagulant Activity: The anticoagulant activity of the unfractionated heparin preparation was assumed to be equivalent to the U.S.P. unitage cited on its label. The potency of all fractionated heparin samples was determined by comparison with this starting material. To construct a standard titration curve, 50 µl of antithrombin-heparin cofactor (50 µg/ml) were added to 20 µl aliquots of various dilution of the reference sample. The resultant mixtures were incubated for 1-2 min at 37°. Then 280 µl of 0.135 M NaCl in 0.05 M Tris-Imidazole (pH 8.3) was admixed with the latter solutions prior to the addition of 50 µl of human thrombin (~60 N.I.H. units/ml or ~25µg/ml). After 30 sec. of incubation at 37°, 300 µl of substrate were introduced and the reaction mixtures were incubated for an additional 60 sec. at 37°. The substrate consisted of Benzoyl-Phen-Val-Arg-p-Nitroanilide. HCl (AB Bofors, Nobel Division, Molndal, Sweden), and polybrene dissolved in H_2O with each component at a final concentration of 0.33 mg/ml. Finally, 300 µl of glacial acetic acid was added to quench the action of thrombin upon the tripeptide and the extent of amidolysis was quantitated by measuring the absorbance of samples at 405 nm. All determinations were performed in triplicate. The titration curve of the heparin reference standard exhibited a linear decline

in absorbance from ~0.8 to ~0.2 when heparin concentrations ranged from 0 to 1 unit/ml. To measure the anticoagulant activity of a fractionated heparin sample, it was diluted to approximately 0.5 units/ml, and assayed with the above technique by comparison with the reference standard.

Fluorometric Assay of Protein Concentration (18): Fifty µl of a sample (0 to 100 µg of antithrombin-heparin cofactor) was added to 250 µl of 0.2 M sodium borate buffer (pH 9.0). Then 100 µl of a solution containing fluorescamine dye (0.1 mg/ml in acetone) was injected while vortexing the solution and the final volume of the resultant mixture was brought to 2 ml with the buffer described above. The fluorescence of this solution was read at 475 nm after excitation at 390 nm. A standard titration curve exhibited a linear increase in relative fluorescence from 0 to 1000 as the level of antithrombin-heparin cofactor was varied from 0 to 100 µg. Concentrations of heparin below 140µg/ml have essentially no effect on this assay of protein concentration.

RESULTS AND DISCUSSION

Samples containing 100 µg of antithrombin-heparin cofactor or 10 µg of heparin were examined separately by sucrose density gradient centrifugation. As shown in Fig. 1 (upper and middle panel), single, narrow, non-overlapping peaks of either protein, or heparin were apparent. When both components were mixed together at the final concentrations and solvent conditions previously employed, two major alterations in the pattern of component distribution were noted (Fig. 1, lowest panel). First, the profile of the antithrombin-heparin cofactor concentration revealed a significant degree of polydispersity with large amounts of protein present at high sedimentation velocities (Fractions 2-11). In addition, a shift in the sedimentation velocity of the antithrombin-heparin cofactor peak to a slightly higher value was apparent and this subtle finding was noted during many centrifugation analyses. Second, heparin is no longer present as a discrete peak but is distributed throughout the density gradient. Approximately 1/3 of this component is located under the antithrombin heparin cofactor peak suggesting that it has been drawn into this region due to strong interactions with the inhibitor. Approximately 2/3 of the heparin is present at its original position in the density gradient and therefore does not appear to be capable of forming a stable complex with antithrombin-heparin cofactor. This is surprising since a two to three fold molar excess of the inhibitor was present in the original reaction mixture. The data suggested that heparin bound to the inhibitor might represent an active species while heparin unable to complex with antithrombin-heparin cofactor

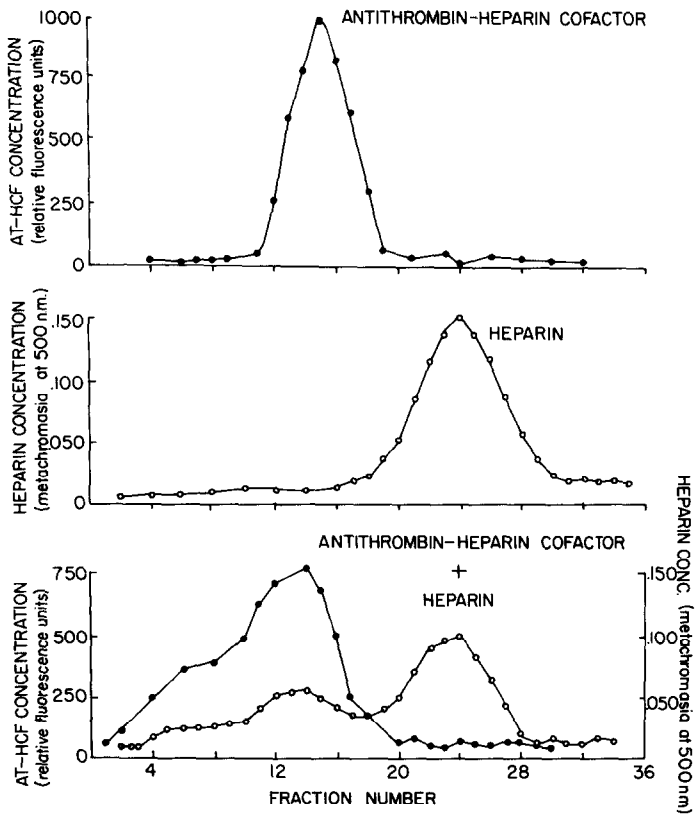

Fig. 1

Sucrose density gradient centrifugation of antithrombin-heparin cofactor and heparin. Heparin and/or antithrombin-heparin cofactor were utilized at final concentrations of 33.3 µg/ml and 333 µg/ml, respectively. Each component had previously been extensively dialyzed against 0.15 M NaCl in 0.01 M sodium phosphate (pH 7.5). Approximately 300 µl of these solutions were overlayed on 4.7 ml of 10% to 15% (w/v) sucrose density gradients in which the solvent was 0.15 M NaCl in 0.01 M sodium phosphate (pH 7.5). After centrifugation at 224,000 x g for 20 hours, 4°, the bottom of each tube was punctured and fractions of ~120µl were collected for analyses. See materials and methods for additional details.

could signify a chemically similar but inactive form of this mucopolysaccharide

To validate this hypothesis, it was necessary to quantitate the anti-coagulant activity of each of these heparin species. Therefore, fractions

TABLE I

SPECIFIC ANTICOAGULANT ACTIVITY OF HEPARIN SAMPLES
(Anticoagulant Activity per mg of heparin)[a]

Experiment	Unfractionated Heparin	Heparin Bound to Antithrombin-heparin cofactor	Heparin unable to bind to antithrombin-heparin cofactor
1	155	388	19
2	155	365	52
3	155	348	43

[a]The heparin content was determined by measurement of uronic acid and by quantitation of the metachromic reaction with Azure A. Since similar results were obtained with each procedure, the average value of both techniques was utilized to calculate specific anticoagulant activities.

were collected either from areas of the density gradient free of protein (Fractions 21-28) or from regions in which heparin was bound to antithrombin-heparin cofactor (Fractions 3-15). In order to accumulate sufficient material for analyses, it was necessary to obtain fractions from six to twelve individual density gradients. Heparin fractions unable to bind to antithrombin-heparin cofactor were pooled, extensively dialyzed and assayed for anticoagulant activity as well as heparin content. As shown in Table I, the average specific activity (anticoagulant activity per mg of heparin) obtained during three separate experiments was 38 units/mg. Heparin fractions which had bound to antithrombin-heparin cofactor were also pooled and extensively dialyzed. Prior to quantitating the anticoagulant activity of these samples, it was necessary to free them of protein. This processing was necessary so that significant amounts of antithrombin-heparin cofactor were not introduced into the assay system since this would invalidate the measurements obtained. To this end, samples containing ~0.6 mg of antithrombin-heparin cofactor and ~25µg of heparin were adsorbed to a DEAE-cellulose column (0.85 x 1.8cm); the chromatographic matrix was washed with 5 ml of 0.25 M of LiCl to differentially elute protein and the mucopolysaccharide was harvested with 2 M LiCl.

approximately 70% of the heparin could be recovered by this procedure with only ~1% of the initial antithrombin-heparin cofactor present in these fractions. Additional experiments demonstrated that the specific activity of heparin was not altered by the above technique.

After elution from DEAE-cellulose, the samples were extensively dialyzed and assayed for anticoagulant activity as well as heparin content. As shown in Table I, the average specific activity obtained during three separate experiments was 367 units/mg. This is considerably higher than the specific activity of the unfractionated heparin (155 units/mg). Fractionation of these two heparin forms was dependent upon the presence of antithrombin-heparin cofactor. Attempts to duplicate this isolation procedure with bovine serum albumin, a plasma protein similar in size to antithrombin-heparin cofactor, were unsuccessful. Indeed, as judged by sucrose density gradient centrifugation, heparin does not interact with this plasma component.

In summary, our data would indicate that the heparin preparation examined consisted of two distinct forms which differ greatly in their ability to bind to and activate antithrombin-heparin cofactor. The first form constituted approximately 2/3 of the chemical mass of the unfractionated heparin and did not bind to antithrombin-heparin cofactor under the condition of these experiments. This fraction was responsible for only ~15% of the total anticoagulant activity of the starting material. The relatively low anticoagulant potency may be an intrinsic property of this molecular species or could be due to residual contamination with the more active form of heparin. The second form, comprising 1/3 of the remaining chemical mass of unfractionated heparin, bound tightly to antithrombin-heparin cofactor and contained approximately 85% of the total anticoagulant activity of the starting material. The heparin utilized in these studies was indistinguishable from preparations employed by other investigators, as judged by susceptibility to enzyme degradations and elution from DEAE-cellulose. Therefore, we suspect that other heparin products will exhibit similar

cytes. In the 5 systems, the mitogens exhibit decreasing activity in the following sequence: human ≈ bovine » rabbit ≈ rat ▸ mouse lymphocytes. Comparing thymidine incorporation at the dose optima, lima bean lectin is a potent mitogen towards human peripheral blood and bovine lymph node lymphocytes comparable to the lectins Con A and PHA. Similar results were obtained for lymphocytes from human tonsils (data not shown). The shift of the stimulation dose optimum in serum free cultures (Fig. 2b) can be explained by the competitive binding of serum factors. Rabbit and rat cells show only a weak, mouse lymphocytes a very weak effect. Thus, lima bean lectins stimulate lymphocytes in a species specific manner. Similarly, Wang et al. report different response behaviour of mouse and human lymphocytes towards Con A [7], and Novogrodsky et al. find species specific differences for the pea nut agglutinin [11]. One reason for these differences generally found is the presence and accessibility of the lectin specific sugar residues on the cells. With the lima bean lectins, binding studies to the cells shall be performed to correlate binding and mitogenicity.

In all species tested, LIM 247, the isolectin with 4 sugar binding sites, is a by far better mitogen than the divalent lectin. For human and bovine lymphocytes, LIM 247 exhibits stimulation indices up to 200, whereas LIM 124 shows only indices up to 10. For the other species tested, only the tetravalent compound has a significant effect. A number of at least 4 sugar binding sites seems to be necessary in our systems to achieve good activation. Similarly, succinylated dimeric Con A shows only about half the stimulating effect of the native tetrameric tetravalent molecule [7], and the aggregated soy bean lectin stimulates better than the native divalent compound [12].

13. Cifonelli, J.A. and King, J. (1973) *Biochim. Biophys. Acta.* 320:331-341.
14. Rosenberg, R.D. and Damus, P.S. (1976) *Meth. in Enzy.* Acad.Press Vol. 45
15. Lewis, R.G., Spencer, A.F., and Silbert, J.E. (1973) *Biochem. J.* 134:455-463.
16. Bitter, T. and Muir, H.M. (1962) *Anal. Biochem.* 4:330-334.
17. Jaques, L.B., Monkhouse, F.C. and Stuart, M.J. (1949) *J. Physiol.* (Lond) 109:41-48.
18. Udenfriend, S., Stein, S., Bohlen, P. and Dairman, W. (1972) *Science.* 178:871-872.