DEMONSTRATION OF ENDOGENOUS HEPARIN IN RAT BLOOD

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1. Introduction

Macromolecular heparin is a multichain form of heparin, the structural integrity of which is dependent upon a polysaccharide core [1]. I have postulated that macromolecular heparin must be depolymerized to become biologically active [1]. Macromolecular heparin is a potent inhibitor of heart lipoprotein lipase (LPL) in vitro [2]. Injected macromolecular heparin has a relatively low potency as an initiator of LPL activity in the blood. This activity appears in the blood rather slowly, compared with low molecular weight heparins (i.e., commercial heparin or enzymically depolymerized macromolecular heparin) [2]. These facts support the concept that depolymerization precedes the release of biologically active heparin from the mast cells. To extend this hypothesis it was necessary to demonstrate endogenous heparin in rat blood.

[^{35}S]heparin has been isolated from dog blood by the carrier technique [3]. The work has been criticised on the grounds that the concentration of heparin which was found was well above the limit of detection by normal isolation procedures, which have failed to show it [4]. In the present work a simular experimental approach was made with rats. Heparin of relatively low molecular weight was recovered, 90% of which was associated with formed elements, principally the platelets.

2. Methods

Each of eight male Wistar rats (body weights 241 ± 8.0 g) received 2 mc of carrier-free [^{35}S] sodium sulfate by intraperitoneal injection and was exsanguinated by cardiac puncture under ether anaesthesia 18 hr later. Blood (total volume 82 ml) was collected into one-ninth volume of anticoagulant (disodium EDTA, 1.5 g per 100 ml 0. 15 M NaCl) using plastic syringes, and transferred to plastic centrifuge tubes. The blood was centrifuged (300 *g* for 20 min) at room temperature and platelet-rich plasma removed with a siliconed pasteur pipet. The remaining blood was respun under the same conditions and more platelet-rich plasma removed. Platelets were obtained by centrifuging at 2000 *g* for 20 min, and platelet-poor plasma pipetted off from the plug of platelets. The red blood cells and buffy layer were gently resuspended in an equal volume of a modified Tyrode's solution containing EDTA but no calcium or magnesium ions [5] and centrifuged at 150 *g* for 20 min. The buffy layer was carefully removed with a siliconed pasteur pipet.

The platelet and buffy layer preparations were resuspended in ethanol. The red blood cells and the plasma were each mixed with 3 vol of ethanol. After standing overnight at 4°C the four products were centrifuged (1000 *g* for 30 min) at 4°C. The supernatants were discarded. The sediments were washed with ethanol and hexane at room temperature and dried under reduced pressure. Each dry product was homogenised with 0. 10 M Tris–HCl, 0.02 M $CaCl_2$, pH 8.0 and incubated with shaking at 40°C. Pronase (10 mg) was added to each. The addition of Pronase was repeated after 24 hr and at this time commercial pig mucosal heparin was added (5 mg to the red blood cells, 2 mg each to the plasma, platelet and buffy layer digests). After 48 hr heparin was recovered by precipitation with cetyl pyridinium chloride in the presence of 1.2 M

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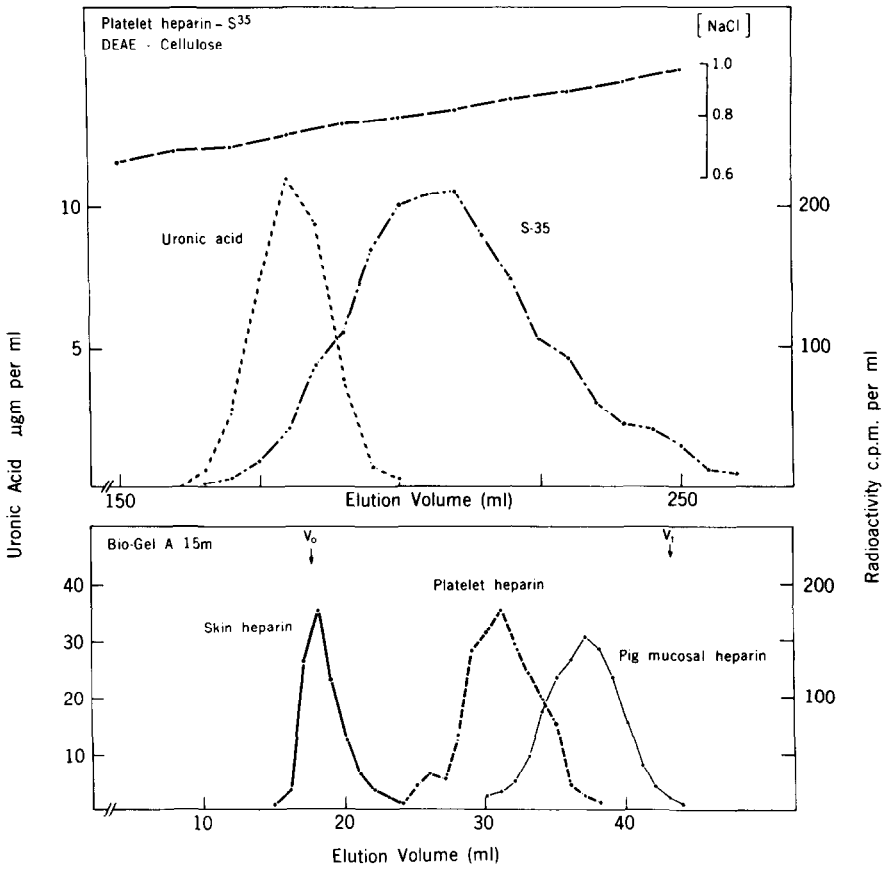

Fig. 1. Upper panel: elution of carrier pig mucosal heparin and endogenous [^{35}S]heparin, recovered from rat platelets after digestion with Pronase and precipitation with cetyl pyridinium chloride, from a column of microgranular DEAE-cellulose (Whatman DE 32, dimensions 1.5 cm × 20 cm, volume 35 ml) with a linear NaCl gradient at pH 2.5 [1]. NaCl concentration was determined with a conductivity meter. Lower panel: gel-filtration of samples of macromolecular [^{35}S]heparin from rat skin, [^{35}S]heparin from rat platelets and pig mucosal heparin on a column of Bio-Gel A-15 m agarose gel granules (100–200 mesh, dimensions 1.5 cm × 24 cm, volume 43 ml) equilibrated with 1.0 M NaCl. V_0 = void volume, determined with tobacco mosaic virus. The pig mucosal heparin was quantitated by uronic acid determinations [6] and the rat heparins by measuring ^{35}S radioactivity.

NaCl, using general procedures described previously [1]. Each partially purified heparin preparation was applied to a column of microgranular DEAE-cellulose (Whatman DE 32) and eluted in a NaCl gradient at pH 2.5 [1]. The carrier heparin was measured by a carbozole method for uronic acid [6] and endogenous heparin by determining ^{35}S radioactivity. The endogenous and carrier heparins were combined and reprecipitated with ethanol. Their relative molecular sizes were estimated by gel filtration on a column of Bio-Gel A-15 m agarose gel granules.

To confirm that the endogenous polyanionic products were heparins, samples were hydrolysed with 0.04 N HCl [7] or degraded with nitrous acid [8]. The products of these reactions were separated on a column of Sephadex G-25.

Samples of other ^{35}S-labelled glycosaminoglycans were recovered from the same rats for comparative purposes. Macromolecular heparins were isolated from skin [1] and peritoneal mast cells [9]. Dermatan sulfate, which is the major component of the pre-heparin peak eluted from DE 32 in the preparation of heparin from rat skin [1] was precipitated as the calcium salt from 25% ethanol [10]. Chondroitin

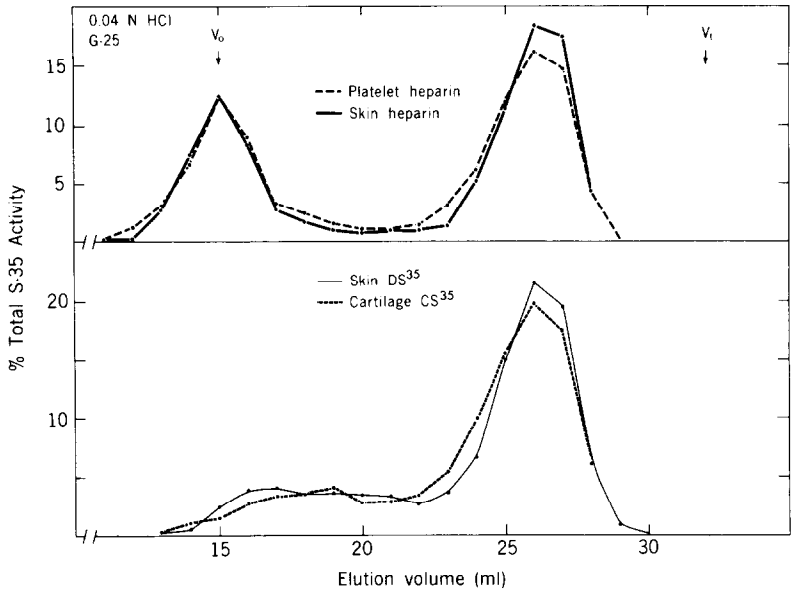

Fig. 2. Gel-filtration of samples of platelet [^{35}S]heparin, skin [^{35}S]heparin, skin [^{35}S]dermatan sulfate and cartilage [^{35}S]chondroitin sulfate after hydrolysis with 0.04 N HCl at 98°C for 2 hr [7]. The column of Sephadex G-25 (dimensions 0.9 cm × 50 cm, vol 32 ml) was equilibrated with 1.0 M NaCl. The ^{35}S content of each fraction is expressed as a percentage of the total counts in the sample. V_0 = void volume, determined with Blue Dextran 2000.

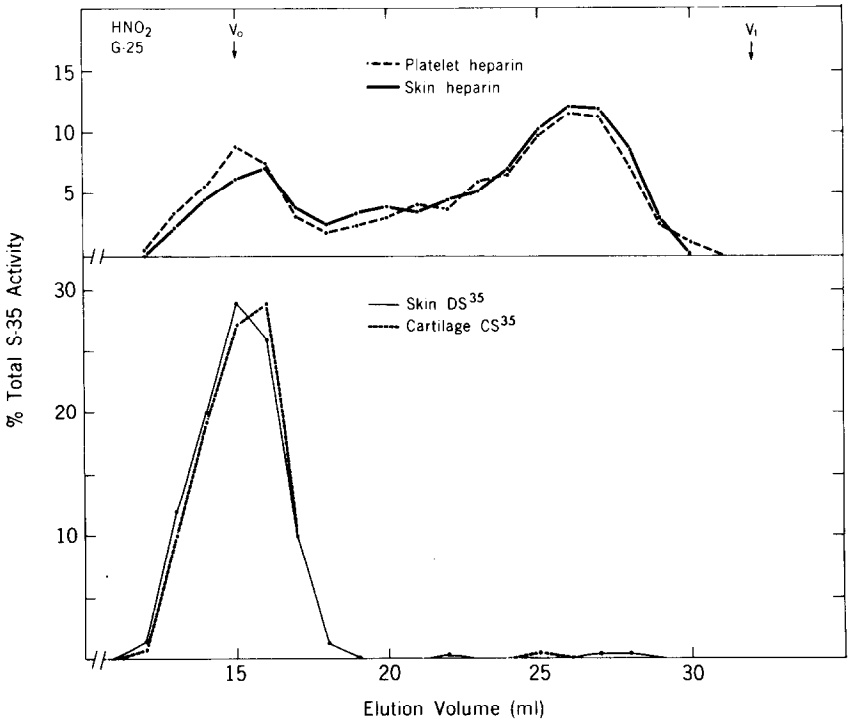

Fig. 3. Gel-filtration of samples of the same four ^{35}S-labeled glycosaminoglycan preparations (see legend to Fig. 2) on the same Sephadex G-25 column, after treatment with nitrous acid at room temperature for 90 min [8].

sulfate was recovered from Pronase-digested sternums by precipitation from 0.5 M NaCl with cetyl pyridinium chloride.

3. Results and Discussion

The elution of platelet [^{35}S]heparin and carrier pig mucosal heparin from a DE 32 column is illustrated in fig. 1 (upper panel). The reason for the elution of the heparin at a higher salt concentration than the carrier pig mucosal heparin is not known, but rat skin heparin shows exactly the same behaviour [11].

The elution patterns for gel filtration on Bio-Gel A-15 m (fig. 1, lower panel) show that the endogenous platelet heparin is larger than pig mucosal heparin, but much smaller than macromolecular heparin. It is thus of similar size to the lower molecular weight fraction obtained by gel filtration of enzymically degraded macromolecular heparin [2].

Hydrolysis of heparin with 0.04 N HCl yields *N*-desulfated heparin and inorganic sulfate [7]. The gel filtration patterns of hydrolysed platelet and skin heparins shown in fig. 2 are virtually identical, the material eluting in the void volume being *N*-desulfated heparin. Under the same conditions, dermatan sulfate and chondroitin sulfate were completely degraded to lower molecular weight products.

N-desulfation of a glucosamine residue in heparin with nitrous acid [8] is accompanied by cleavage of the adjacent glycosidic bond [12]. Therefore, more low molecular weight products are obtained by degradation with nitrous acid than by hydrolysis with 0.04 N HCl. Glycosaminoglycans which have no *N*-sulfate groups are resistant to nitrous acid under these conditions. Fig. 3 shows gel filtration patterns on Sephadex G-25 after nitrous acid treatment. The patterns for platelet heparin and skin heparin are the same, both having been extensively degraded. In contrast, dermatan sulfate and chondroitin sulfate were not broken down.

Thus the presence of *N*-sulfate in platelet heparin in the same proportion found in authentic heparin from the skin was established by two different methods of degradation. The platelet product therefore meets chemical criteria for heparin.

I have used the platelet product to illustrate the methods used to isolate and characterize the endogenous heparin in blood. The reason for this is that 50% (in terms of ^{35}S radioactivity) of the heparin recovered was in the platelet product, 30% was recovered from the erythrocytes, 10% from the buffy layer and 10% from the plasma. Thus 90% of the blood heparin was associated with formed elements. The high proportion associated with platelets is particularly interesting because they contain a protein with very high binding affinity for heparin, platelet factor 4 [13].

The [^{35}S]heparin radioactivity in blood was 143 cpm per ml. The specific activities of carrier-free heparins recovered from the skin and peritoneal mast cells of the same rats were 1100 and 8400 cpm per μg uronic acid respectively. Making the possibly unwarranted assumption that the specific activity of the heparin in blood is within this range, the concentration of heparin in blood can be estimated. By these criteria, it is in the range 1.7 to 13 μg uronic acid per 100 ml, 5.5 to 42 μg heparin per 100 ml, or, assuming an anticoagulant activity of 160 units per mg, 0.88 to 6.7 units per 100 ml.

With the present experimental design, the use of carrier heparin is obligatory. This precludes direct chemical analyses and anticoagulant assays of the endogenous heparin. Obviously a more direct method of determining the concentration of heparin in blood is needed. However, this study has demonstrated the presence of heparin in blood and its association with the formed elements, particularly the platelets. Furthermore, the heparin in blood is of relatively low molecular weight, supporting the concept that depolymerization of the macromolecular form probably precedes its release into the blood.

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