Native Heparin from Rat Peritoneal Mast Cells*

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[*S]Heparin was produced in vitro by incubation of rat peritoneal mast cells with [*S]sulfate and in vivo by injection of [*S]sulfate into rats. The [*S]heparin together with nonlabeled heparin in the mast cells was isolated in native form by mild methods that avoided the use of proteolytic enzymes or high alkali concentrations. The heparin had low anticoagulant activity. Incubations of mast cells with [*S]sulfate for less than several hours in vitro resulted in [*S]heparin of approximately Mr = 200,000 to 400,000 based on gel filtration, while longer incubations yielded [*S]heparin of approximately Mr = 750,000 that was similar to the nonlabeled heparin in the mast cells. When [*H]serine was included in the in vitro incubations, [*H]-labeled material was found to co-chromatograph with the [*S]heparin. None of the heparin could be degraded by any of several proteolytic enzymes, but incubation for 14 h at 25° with 0.5 M NaOH degraded all samples to a size of approximately Mr = 40,000. One-third of the [*H]-serine label continued to co-chromatograph with the [*S]heparin after alkali treatment, while the remaining two-thirds appeared as smaller molecules completely separated from the [*S]heparin. Thus, native heparin of the mast cell may be an unusual proteoglycan that is resistant to proteolytic enzymes.

MATERIALS AND METHODS

Chondroitin 6-sulfate, superspecial grade, average molecular weight 60,000 (Miles Laboratories, Elkhart, Ind.); hyaluronic acid (Grade I, from human umbilical cord), pancreatein, papain, o-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.); porcine intestinal heparin, 130 units/mg (Schwarz/Mann, Orangeburg, N. J.); trypsin (Worthington Biochemical Co., Freehold, N. J.); pronase and deoxyribonuclease (Calbiochem, San Diego, Calif.); benzoyl-phenylalanine-p-nitroanilide (S-2200, AB Bofors, Malmö, Sweden); carrier-free H,[*S]SO₄ and L-[*H]serine, 3.2 Ci/mmol (New England Nuclear, Boston, Mass.); Metrizamide, analytical grade (Gallard-Schlesinger Chemical Manufacturing Corp., New York, N. Y.); hexadimethrine bromide (Polybrene, Abbott Laboratories, North Chicago, Ill.); bovine topical thrombin (Parke-Davis, Detroit, Mich.); Sepharose 4B, Sephadex G-25 and G-50 (Pharmacia Fine Chemicals, Piscataway, N. J.); Dowex 1-X2 (100 to 200 mesh, chloride form) and DEAE-cellulose (Bio-Rad Laboratories, Richmond, Calif.) were obtained as noted. Subtilisin and elastase were gifts from Dr. C. Stouffer (Proctor and Gamble & Co., Cincinnati, Ohio) and Dr. C. Franzblau (Boston University, Boston, Mass., respectively). Mucopolysaccharide reference standards of chondroitin sulfate and heparin were obtained from Dr. M. B. Matthews (University of Chicago, Chicago, Ill.). Purified heparinase prepared from Flavobacterium heparinum was supplied by Dr. A. Linker (University of Utah, Salt Lake City, Utah) and chondroitinase ABC was obtained from Miles Laboratories, Elkhart, Ind.

The standard method of dialysis and concentration of rat mast cell heparin before enzyme incubations or between chromatography steps consisted of dialysis of the sample against 2 liters of distilled water for 90 min with two water changes followed by lyophilization. Under these dialysis conditions there was 95 to 100% recovery of the sample; more extensive dialysis caused a decrease in recovery, particularly of higher molecular weight fractions. Radioactivity was measured with a liquid scintillation spectrometer.

Measurement of Heparin—Heparin was measured by metachromasia, uronic acid content, anticoagulant activity in whole plasma, and capacity to serve as an antithrombin cofactor. Metachromasia was determined by the method of Jaques et al. (6). Uronic acid content of commercial and mast cell heparin was estimated by the modified carbazole reaction of Bitter and Muir (7). The anticoagulant activity of heparin was determined in a partial thromboplastin time assay (8) in which heparin was added to normal human plasma 5 min before the kaolin activation step. Antithrombin heparin cofactor activity was determined by a modification of the method of Blombäck et al. (9) for determining human plasma antithrombin activity by keeping the anti-thrombin content constant and varying the amount of heparin.

Isolation, Labeling, and Extraction of Mast Cells—Cells from 40 to 50 male or female Sprague-Dawley rats, each weighing 200 to 300 g, were collected by lavage of the peritoneal cavity of each rat with 20 ml of Tyrode's buffer containing 0.1% gelatin and 20 mg/liter of
commercial heparin. The cells were sedimented at 400 × g for 15 min at room temperature, pooled, and washed twice with Tyrode's buffer containing 0.1% gelatin and 30 mg/liter of deoxyribonuclease. This Tyrode's buffer was used routinely. Approximately 3 × 10^6 to 8 × 10^6 cells in 1 ml of the Tyrode's buffer were layered on each of 40 2.0-ml bands of 22.5% w/v Metrizamide (density 1.125 g/ml) and centrifuged at room temperature for 15 min at 400 × g at the buffer-Metrizamide interface (10). The cells remaining at this interface were aspirated and discarded; the cells in the pellet were resuspended, pooled, washed with and resuspended in the Tyrode's buffer at 5 × 10^6 cells/ml. Of the nucleated cells in this preparation, 90 to 95% were mast cells and the mast cell yield was approximately 10^7 per rat.

For in vitro radiolabeling, 4 × 10^6 to 5 × 10^6 mast cells in 1.0 ml of the Tyrode's buffer were mixed with 10 μCi of [35S]sulfate in 1.0 ml of Hanks' balanced salt solution with Eagle's basal medium. After incubation for 2 h at 37°C the cells were washed and resuspended in the Tyrode's buffer. For double labeling experiments, 0.2 μCi of [35S]sulfate and 1.0 μCi of 1-L-[3H]serine were used in vitro in an otherwise standard procedure. In vitro labeling was performed by injecting each of 30 rats subcutaneously with 2.0 ml of [35S]sulfate in 0.5 ml of sterile saline (11); after 14 days the cells were harvested, and the mast cells were purified as described for unlabeled cells.

Heparin was extracted by freezing and thawing the cells six times followed by the addition of NaCl to a final molarity of 1.0, and of NaOH to a final molarity of 0.05. Samples were neutralized after 30 min at 25°C by the dropwise addition of 1 M HCl.

RESULTS

Isolation of Labeled Mast Cell Heparin — The extract from 3 × 10^6 mast cells was applied directly to a column of Dowex 1 equilibrated in 1 M NaCl and eluted in stepwise fashion with 1.0, 3.0, and 4.0 M NaCl (12). Portions of each fraction were assayed for 35S, protein (by absorbance at 280 nm), uronic acid, and metachromasia. The 1.0 M NaCl effluent contained a major portion of the total protein and approximately two-thirds of the 35S but was essentially without uronic acid or metachromasia. The material eluting with 3.0 M NaCl contained a small amount of protein and one-third of the 35S which co-chromatographed exactly with the material showing metachromasia and the presence of uronic acid, indicating [35S]sulfate incorporation into the heparin-like material present. Based on the uronic acid determinations, the total recovery of heparin-like material was approximately 930 μg. Calculated from the specific activity of the [35S]sulfate in the incubation mixture and assuming 2.5 sulfates/disaccharide repeating unit the amount of labeled material synthesized in the 2-h incubation was estimated as 0.08 pg or 0.009% of the mast cell store of heparin-like material. On the basis of uranic acid, and metachromasia, the material eluting with 3.0 M NaCl contained a small amount of protein and one-third of the 35S which co-chromatographed exactly with the material showing metachromasia and the presence of uronic acid, indicating [35S]sulfate incorporation into the heparin-like material present. Based on the uronic acid determinations, the total recovery of heparin-like material was approximately 930 μg. Calculated from the specific activity of the [35S]sulfate in the incubation mixture and assuming 2.5 sulfates/disaccharide repeating unit the amount of labeled material synthesized in the 2-h incubation was estimated as 0.08 pg or 0.009% of the mast cell store of heparin-like material. On the basis of uronic acid content, mast cell heparin-like material had approximately 85% of the metachromasia of commercial heparin, but only about 20% of the antithrombin cofactor activity and 10% of the anticoagulant activity. The 4.0 M NaCl eluate had minimal 35S and no uronic acid or metachromasia. All further experiments were performed only with material which had previously been isolated from Dowex 1 by 3 M NaCl elution, as described above.

Identification of 35S-labeled material as heparin was confirmed by degradation by purified heparinase. This degradation was established by filtration on Sephadex G-50 (13). Chondroitinase ABC (14) had no effect on the labeled material.

A sample of the [35S]heparin together with glycosaminoglycan standards was chromatographed on a column of DEAE-cellulose, with a logarithmic gradient of 1.0 M LiCl followed by a similar gradient of 2.0 M LiCl (15). The [35S]heparin began to elute with the ascending limb of the commercial heparin but was also found in later fractions (Fig. 1). There was little [35S] material in the area of the chondroitin 4-sulfate standard where heparan sulfate would also be found (13).

Another sample of the [35S]heparin was filtered on a column of Sepharose 4B and fractions assayed for [35S]heparin, protein, uronic acid, and metachromasia. As shown in Fig. 2, [35S]heparin presented as a broad peak, with an estimated molecular weight, based on glycosaminoglycan standards, of 200,000. The size of the unlabeled heparin assayed by metachromasia and uronic acid content was approximately M_r = 750,000 and obviously considerably larger than the small fraction of the total heparin formed during the 2-h in vitro incubation.

Size Changes of [35S]Heparin during Synthesis — Longer incubations with [35S]sulfate were conducted to see if larger heparin would be formed. For these experiments 6 × 10^6 mast cells were incubated in the usual incubation mixture from which MgSO_4 had been deleted to increase the specific activity of the [35S]sulfate. Portions of 0.3 ml were taken at 1, 2, 4, and 14 h. [35S]Heparin was extracted, isolated by Dowex 1 chromatography, and filtered with internal standards on Sepharose 4B (Fig. 3). The [35S]heparin isolated after a 1-h incubation (Fig. 3A) exhibited a wide range of sizes; after 2 h (Fig. 3B) most of the material was larger than 1 h, and after 4 h (Fig. 3C) the [35S]heparin was found primarily in the location previously shown to contain the bulk of the mast cell heparin (see Fig. 2). Even at the end of 4 h, however, there still was some low molecular weight material. At the end of the 14-h incubation (not shown) the pattern was identical with that of Fig. 3D, which is the chromatogram of in vitro formed [35S]heparin (14 days after injection of [35S]sulfate).

Mask Cell Heparin as a Possible Proteoglycan — [35S]Heparin was incubated with proteolytic enzymes to see if it would be reduced in size, as occurs when chondroitin sulfate proteoglycan is treated with proteolytic enzymes (16). Incubations with 100 μg of trypsin, chymotrypsin, pancreatin, or subtilisin were carried out for 14 h at 37°C in 200-ml volumes of 0.05 M Tris, pH 8.2, made isonotic with NaCl. Other samples were incubated with papain at 65°C after the method of Heinigard and Hascall (17) for chondroitin sulfate proteoglycan. Incubation with 50 μg of pronase at 50°C was carried out for 48 h with 20 μg more of pronase being added at the halfway point. Treatment with 1.0 mg of elastase was carried out at 37°C for 1 h.

FIG. 1. DEAE-cellulose chromatography of [35S]heparin. [35S]-heparin and standards of 3 mg of chondroitin 4-sulfate and 5 mg of commercial heparin were eluted from a column (1 × 5 cm) of DEAE-cellulose by a 0.1 to 1.0 M LiCl logarithmic gradient, followed by a similar gradient of 2.0 to 2.0 M LiCl. For this gradient the mixing flask contained 150 ml of 0.1 M LiCl and the reservoir contained 1 M LiCl, which was changed to 2.0 M LiCl at Fraction 81. Fractions (2 ml) were collected at 10 to 15 ml/h and assayed for radioactivity (O---O) and uronic acid content (O- - - O).
there a change in the filtration characteristics of the [35S]heparin on Sepharose 4B after treatment with any of the above enzymes as compared to untreated material, indicating that none had a measurable effect on the size of the native molecule. Proteolytic activities of the various enzymes under the above conditions were confirmed by parallel incubations with bovine serum albumin or by elastin in the case of elastase.

Since the linkage region (18) of many proteoglycans is known to be sensitive to alkali, the [35S]heparin was treated with 0.5 m NaOH at room temperature for 14 h. After this alkali treatment the [35S]heparin eluted from DEAE-cellulose at a lower ionic strength, closer to the profile of commercial heparin than to that of the native heparin. When assessed on Sepharose 4B, the alkali-treated [35S]heparin filtered in fractions indicating a size of approximately $M_r = 40,000$.

The marked reduction in size caused by alkali treatment suggested that the native heparin might be a proteoglycan with alkali labile linkages of glycosaminoglycan to protein. In order to further investigate this possibility, mast cells were doubly labeled by incubation with L-[3H]serine and [35S]sulfate. When chromatographed on Dowex 1, there was a peak of $^3H$ in the 3.0 m NaCl eluate coincident with $^{35}S$ and metachromasia. Rechromatography of the 3.0 m NaCl eluate again showed a distinct peak of $^3H$, $^{35}S$, and metachromasia which was now completely separated from tritiated contaminants. An aliquot was filtered on Sepharose 4B (Fig. 4A), and $^3H$ and $^{35}S$ were largely coincident. After alkali treatment, the [35S]heparin was reduced in size to an approximate molecular weight of 40,000, based on the chondroitin 6-sulfate standard of average $M_r = 60,000$. Approximately one-third of the $^3H$-labeled material continued to co-chromatograph with this $^{35}S$ peak, while the other two-thirds of the $^3H$ presented in a completely separate further retarded peak with essentially no $^{35}S$ (Fig. 4B). Fractions 37 to 40 containing this separated $^3H$-labeled product were pooled, lyophilized, resuspended in 0.1 m ammonium acetate buffer, pH 8.0, and applied to a column of Sephadex G-25. Broad overlapping peaks of $^3H$ were found at 40%, 65%, and 89% column bed volume where polypeptides of less than $M_r = 4,000$ would filter.

**DISCUSSION**

A simple technique avoiding proteolytic enzymes or prolonged alkali treatment has been used to obtain heparin in a native form from rat peritoneal mast cells. The molecule was much larger (approximately $M_r = 700,000$) (Fig. 2) than the heparin found in commercial preparations or that found in preparations from mouse mast cell tumors (19) and bovine liver capsule (20-23). In this respect, rat peritoneal mast cell heparin was similar to that previously found in rat skin and other tissues (24) and termed "macromolecular" heparin; the latter was considered to consist of glycosaminoglycan chains connected to a polysaccharide core rather than to a polypeptide core.
The chromatographic characteristics of the $^{35}S$-labeled material on DEAE-cellulose (Fig. 1) indicated that heparin was the major if not the only $^{35}S$-labeled macromolecule. This is supported by the capacity of purified heparinase to degrade the labeled material.

The time-related growth in size of this $^{35}S$-heparin (Fig. 3) is quite different from the changes described with heparin from mouse mast cell tumors where an endoglycuronidase appears to cause a progressive time-related reduction in size (19, 25). Moreover, the growth of the heparin is related to its availability for release by immunologic challenge from the cells, since native heparin is released unchanged in size with a preference for older molecules (26).

Alkali is known to cleave the xylosyl-serine linkages that occur in proteoglycans such as chondroitin sulfate. Furthermore, a xylosyl-serine component has been found in heparin preparations as portions of single glycosaminoglycan chains attached to peptides (27). Since the rat mast cell heparin is reduced in size by alkali treatment (Fig. 4I, it might be suggested that there are similar linkages present, and that rat mast cell heparin may be a proteoglycan. This is supported by the finding with gel filtration that after alkali treatment approximately two-thirds of the $^3H$ label in the native heparin can be separated from that still associated in the $^{35}S$-labeled glycosaminoglycan (Fig. 4B). Nonetheless, the resistance to degradation by proteolytic enzymes is unusual.

The rat peritoneal mast cell material fulfills criteria for heparin such as metachromasia, high charge (Fig. 1), and susceptibility (or resistance) to degradation by specific polysaccharidases. As previously reported by other investigators (28), it has a low anticoagulant activity. In this respect it may be comparable to the heparin of low anticoagulant activity recently shown to be present as a large proportion of commercial heparin preparations (29).

REFERENCES


Native heparin from rat peritoneal mast cells.
R W Yurt, R W Leid, Jr and K F Austen


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