Native Heparin from Rat Peritoneal Mast Cells*

ROGER W. YURT,* R. WESLEY LEID, JR.,$ AND K. FRANK AUSTEN
From the Departments of Medicine, Harvard Medical School and Robert B. Brigham Hospital, Boston, Massachusetts 02120

JEREMIAH E. SILBERT**
From the Connective Tissue-Aging Research Laboratory, Veterans Administration Outpatient Clinic, and Tufts University School of Medicine, Boston, Massachusetts 02108

*This investigation was supported in part by Grants AI-07722, AI-10356, HL-17382, and AM-07031 from the National Institutes of Health.
$Postdoctoral Trainee supported by Training Grant AM-07031 from the National Institutes of Health.
$National Research Service Awardee (5 P22 A101697). National Institutes of Health.
†To whom reprint requests should be addressed.

[14S]Heparin was produced in vitro by incubation of rat peritoneal mast cells with [14S]sulfate and in vivo by injection of [14S]sulfate into rats. The [14S]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 [14S]sulfate for less than several hours in vitro resulted in [14S]heparin of approximately $M_r = 200,000$ to 400,000 based on gel filtration, while longer incubations yielded [14S]heparin of approximately $M_r = 750,000$ that was similar to the nonlabeled heparin in the mast cells. When [3H]serine was included in the in vitro incubations, [3H]-labeled material was found to co-chromatograph with the [14S]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 $M_r = 40,000$. One-third of the [3H]serine label continued to co-chromatograph with the [14S]heparin after alkali treatment, while the remaining two-thirds appeared as smaller molecules completely separated from the [14S]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), pancreatic, 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-phenylalanyl-valyl-arginine p-nitroanilide (S-2160, AB Bofors, Molindal, Sweden); carrier-free H$_3$SO$_4$ and L-$^3$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, III.); 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 Chicago, Chicago, Ill.). Uranic acid pentasodium was obtained from Fisher Scientific Company, Fair Lawn, N. J.; N-lauryl sarcosine (Aldrich Chemical Co., Milwaukee, Wis.); benzoyl-phenylalanine, 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.).

The rat mast cell granule, which contains heparin, releases histamine (1) and biologically active peptides (2) upon immunologic challenge of the mast cell. Although the exact fate of the heparin during this release phenomenon is not certain, ultrastructural data do indicate the extrusion of granules (3, 4) which are presumed to contain heparin. The native structure of heparin within these mast cells has not been defined, although a preliminary communication has reported that it might be a proteoglycan (5).
Native Heparin from Rat Mast Cells

commercial heparin. The cells were sedimented at 400 \times 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 \times 10^6 to 8 \times 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 \times 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 \times 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 \times 10^6 to 5 \times 10^6 mast cells in 1.0 ml of the Tyrode's buffer were mixed with 10 mcCi of [35S]sulfate in 1.0 ml of Hank's 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 mcCi of [35S]sulfate and 1.0 mcCi of 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 mcCi 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 with 0.5 M NaCl and eluted in stepwise fashion with 0.1 to 1.0 M LiCl 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 had minimal 35S and 3H content, and most of the material was larger than at 1.0 M NaCl eluate had minimal 35S and 3H content, and most of the material was larger than at 1.0 M NaCl eluate of Sepharose 4B (Fig. 3). The [35S]heparin isolated after a 1-h incubation with [3S]sulfate 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.

RESULTS

Isolation of Labeled Mast Cell Heparin - The extract from 3 \times 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 assessed 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 35Sulfate incorporation into the heparin-like material present. Based on the uronic acid determinations, the total recover of heparin-like material was approximately 930 

Another sample of the [35S]heparin was filtered on a column of Sephrose 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. Another sample of the [35S]heparin was filtered on a column of Sephrose 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 \times 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 Sephrose 4B (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 at 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 vivo formed [35S]heparin 14 days after injection of [35S]sulfate.

Mast 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 

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).
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 $M_r = 40,000$.

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 $M_r = 750,000$) 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 poly saccharide core rather than to a polypeptide core.

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 $^3$H in the 3.0 M NaCl eluate coincident with $^{35}$S and metachromasia. Recchromatography of the 3.0 M NaCl eluate again showed a distinct peak of $^3$H, $^{35}$S, and metachromasia which was now completely separated from tritiated contaminants. An aliquot was filtered on Sepharose 4B (Fig. 4A), and $^3$H 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 $^3$H-labeled material continued to co-chromatograph with this $^{35}$S peak, while the other two-thirds of the $^3$H presented in a completely separate further retarded peak with essentially no $^{35}$S (Fig. 4B). Fractions 37 to 40 containing this separated $^3$H-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 $^3$H were found at 40%, 65%, and 89% column bed volume where polypeptides of less than $M_r = 4,000$ would filter.

Fig. 2. Gel filtration of rat peritoneal mast cell heparin on Sepharose 4B. The mast cell heparin and a standard of phenol red were filtered on a column (1 x 60 cm) of Sepharose 4B with 2.0 M NaCl eluant at a flow rate of 4 to 5 ml/h. Fractions (1 ml) were collected and assayed for $^{38}$S (O- - -O), metachromasia (W- - -W), uronic acid (A- - -A), and protein (O- - -O). The positions of blue dextran, chondroitin 6-sulfate (approximately $M_r = 60,000$), and commercial heparin (approximately $M_r = 12,000$), which were filtered separately on the same column, are shown.

Fig. 3. Gel filtration on Sepharose 4B of [35S]heparin formed during timed incubations. [35S]heparin was isolated and filtered as in Fig. 2 with internal standards of blue dextran and phenol red. In vitro incubations were for 1 h (A), 2 h (B), and 4 h (C), while in vivo labeling (D) was carried out for 14 days.

Fig. 4. Filtration on Sepharose 4B of [3H, 35S]heparin. Samples were chromatographed on Sepharose 4B as described in Fig. 2 and fractions were assayed for $^{35}$S (O- - -O) and [3H (O- - -O). Internal standards of blue dextran, chondroitin 6-sulfate, and phenol red are indicated. A, chromatogram of $^3$H and $^{35}$S doubly labeled heparin; B, chromatogram of $^3$H and $^{35}$S doubly labeled heparin after incubation with 0.5 M NaOH.
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. 4), 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 $^{3}H$ 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


Access the most updated version of this article at http://www.jbc.org/content/252/2/518

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/252/2/518.full.html#ref-list-1