HEPARINASE ACTIVITY IN RAT LIVER

Bradley Arbogast[1], John J. Hopwood[2,3] and Albert Dorfman

Departments of Pediatrics and Biochemistry
Joseph P. Kennedy, Jr. Mental Retardation Research Center
Pritzker School of Medicine
University of Chicago
Chicago, Illinois 60637

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SUMMARY

Heparin was degraded to oligosaccharides by an endoglycosidase present in rat liver lysosomes. Inorganic sulfate equivalent to approximately one sulfamide bond cleaved per heparin chain was also released in incubations of N-[^{35}S]heparin with crude lysosomal preparations. There was no evidence of exoglycosidase or further sulfamidase activity although oligosaccharides approaching the size of tetrasaccharide were produced. The endoglycosidase has a broad pH-dependence with optimum activity observed at pH 4.4 and intermediate activity at pH 5.5 and 3.8.

Considerable progress has been achieved in the isolation and purification of bacterial heparinases, but mammalian heparinase has not been reported since the early work of Jaques and co-workers (1,2). They detected heparinase activity in liver preparations but the assay method utilized was difficult and perhaps inconclusive. The release of $^{35}SO_4$ from N[^{35}S]heparin has been reported (3-6). The only enzyme reported with *in vitro* activity toward heparin is sulfamidase (7-9). Heparan sulfate, a glycosaminoglycan similar to heparin, has been shown to be resistant to the action of lysosomal glycosidases and hyaluronidase in liver and kidney at acid pH's suitable for hyaluronic acid and chondroitin sulfate degradation (10). At pH 5.6, heparan sulfate is degraded by an endoglycosidase present in rat liver

[1] Present Address: University of Pennsylvania, Department of Medicine, Philadelphia, Pennsylvania 19104

[2] Kennedy Fellow in the Neurosciences

[3] Present Address: The Adelaide Children's Hospital, Department of Chemical Pathology, North Adelaide, S. A. 5006, Australia

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lysosomal preparations (11). Heparin was not degraded under these conditions (11). Most recently Klein et al. (12) have presented evidence suggesting the participation of an endoglucosaminidase and endohexuronidase in the degradation of heparan sulfate by cultured human skin fibroblasts. The enzyme responsible for the cleavage of macromolecular heparin is inactive on heparin of the size range used in this study (13). This paper reports the demonstration of in vitro endoglycosidase degradation of heparin by rat liver lysosomal preparations.

MATERIALS AND METHODS

Incubations with N-[^{35}S]Heparin. Mitochondrial-lysosomal (M and L) fractions were prepared from adult Sprague-Dawley rat livers using differential centrifugation. The rat liver was minced, homogenized in 0.25M sucrose with two strokes of a Potter-Elvehjem homogenizer. The suspension was centrifuged at 900 x g for 10 min to remove the nuclear fraction. The supernatant solution was centrifuged at 9000 x g for 10 min to sediment the lysosomes and mitochondria. A 200 µl portion of the M and L pellet from one rat liver suspended in 1 ml of distilled water was mixed with 5 µl N-[^{35}S]heparin (120,000 cpm, 8.7 mg/ml (Amersham/Searle Corp.)), 20 µl of toluene and 50 µl of 0.15M NaCl/0.1M Na acetate buffer, pH 4.5, and kept at 37° for 18 hrs in a shaking water bath. Twenty µl aliquots of the incubation mixture were electrophoresed on paper strips (Whatman No. 3) (14) to determine the amount of inorganic sulfate released. Each incubation mixture was then diluted with an equal volume of 3M NaCl and centrifuged at 9,000 x g for 10 min, and the supernatant solution was applied to a column of Sephadex G-200 or G-50 as described in the legends to figures. Eighty-90% of the counts were recovered in the supernatant solution.

Incubation with [^{35}S][^{3}H]Heparin. The labeled heparin (a generous gift from Dr. M. Höök, Uppsala, Sweden) was prepared by incubation of mouse mastocytoma tissue with $^{35}SO_4$ and [^{3}H]glucosamine (15). A 300 µl portion of the M and L suspension described above was mixed with 20 µl of toluene, 300 µl of 0.1M Na acetate buffer, and 50 µl of [^{35}S][^{3}H]heparin (11,000 cpm of ^{3}H, 2000 cpm of $^{35}SO_4$ containing less than 5 µg of uronic acid.) The reaction mixture was incubated with shaking at 37° for the times and under the conditions described in the legends to figures. The supernatant solution obtained from centrifugation of the incubation mixture at 9000 x g for 10 min was applied to a column of Sephadex G-200 or Sephadex G-25 and eluted as described in the legends to figures. The void volume (V_o) and the total volume (V_t) of columns were determined using Blue Dextran (Pharmacia) and inorganic $^{35}SO_4$ respectively. Aliquots of each fraction were counted in a Packard Tricarb Liquid Scintillation Spectrometer as previously described (16).

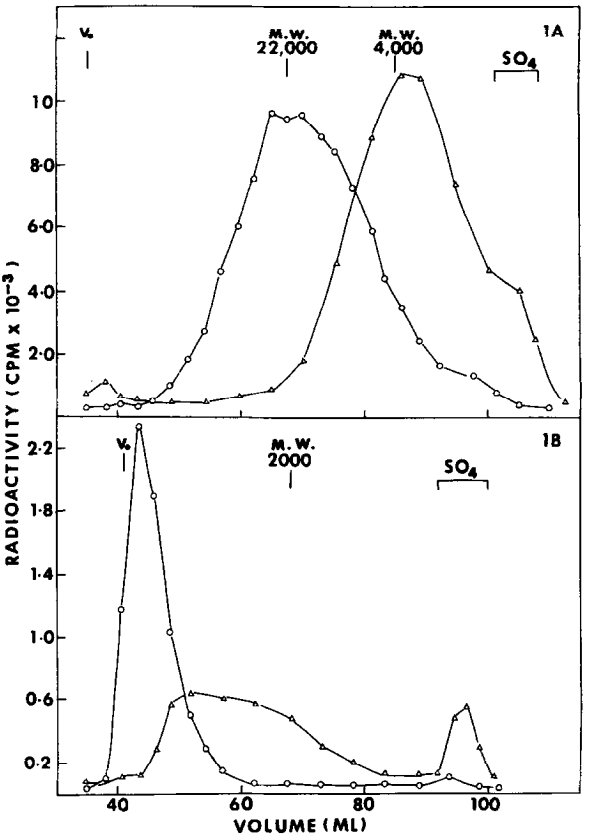

Fig. 1. Elution profiles from a (1 x 200 cm) column of (A) Sephadex G-200 of the products obtained after incubation for 18 hr of N-[^{35}S]heparin with the M and L fraction from rat liver or (B), Sephadex G-50 of the pooled and lyophilized radioactive material recovered from Sephadex G-200 of the reaction products obtained after incubation of N[^{35}S]sulfated heparin with the M and L fraction from rat liver (Δ—Δ) or with boiled enzyme blank (o—o). The elution position of fragments obtained by partial nitrous acid deamination of commercial heparin are indicated. The molecular weights of the two large fragments (Fig. 1A) were determined by sedimentation equilibrium ultracentrifugation; the smaller fragment (Fig. 1B) was a hexasaccharide, molecular weight about 2000. SO_4 indicates the elution volume of inorganic sulfate measured with $^{35}SO_4$. Columns were eluted with 0.2M pyridine acetate buffer, pH 5.0.

RESULTS AND DISCUSSION

Incubation of N-[^{35}S]heparin with the M and L fraction from rat liver resulted in a decrease in average MW of the heparin from approximately 22,000 to less than 4,000 (Fig. 1A). Chromatog

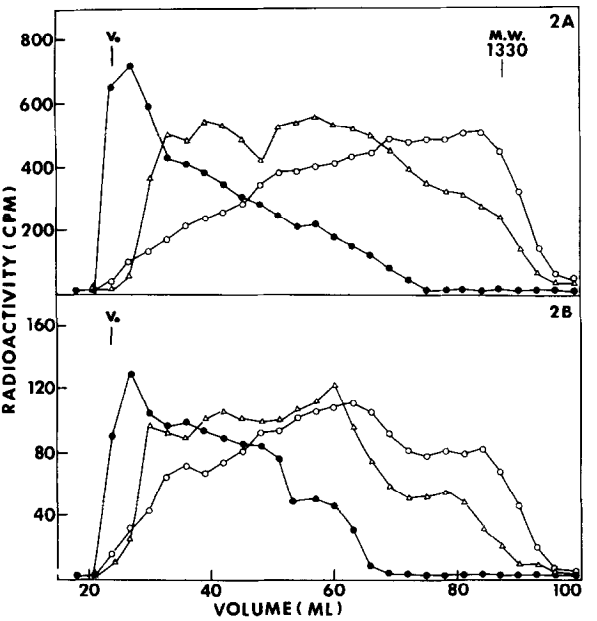

Fig. 2. Elution profiles from a column (1 x 140 cm) of Sephadex G-200 of reaction products from incubation of [^{3}H][^{35}S]heparin with M and L fraction at different buffer pH's and for 18 hr. Column was eluted with 1.0M NaCl in 0.1M Tris-HCl, pH 8.0. Fraction volumes were approximately 1.5 ml, ●—● boiled blank, Δ—Δ pH 5.5, o—o pH 4.4. Profile from pH 3.8 incubation (not shown) had an intermediate pattern between pH 4.4 and pH 5.5 profiles. The peak elution position of a heparin fragment obtained by deamination of commercial heparin with nitrous acid is indicated. The fragment was a tetrasaccharide, molecular weight approximately 1330. (A) Plot of [^{3}H]GlcN cpm. (B) Plot of [^{35}S]cpm.

raphy of the radioactive material, recovered from the G-200 column, on Sephadex G-50 demonstrated that the products of degradation of N-[^{35}S]heparin are largely oligosaccharides (Fig. 1B). Inorganic $^{35}SO_4$ released as measured by paper electrophoresis, was never more than 6% of the total sulfate counts in agreement with the findings of Dietrich (7) and Friedman and Arsenis (9).

Sulfate was liberated early in the degradation of [^{35}S]heparin, but there was a progressive decrease in MW of heparin without further liberation of inorganic sulfate (data not shown).

Incubation of [^{3}H][^{35}S]heparin with the M and L fraction

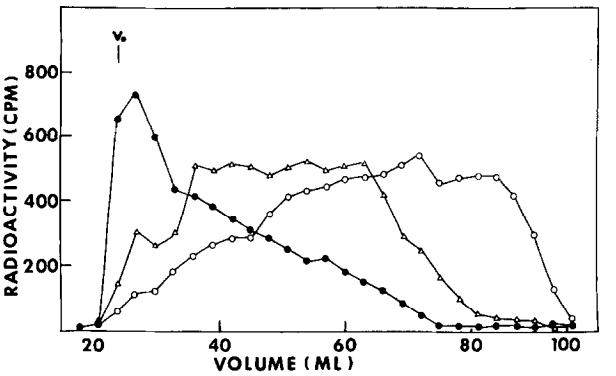

Fig. 3. Elution profiles from a column (1 x 140 cm) of Sephadex G-200 of the reaction products from incubation of [^{3}H][^{35}S]heparin with the M and L fraction at pH 4.4 for different times at 37°. Approximately 1.5 ml fractions were eluted with 1.0M NaCl in 0.10M Tris-HCl, pH 8.0, ●—● boiled blank, Δ—Δ 30 min, o—o 4 hr. Twenty-four hour profile (not shown) was similar to the 4 hr profile shown in this figure and the 18 hr profile (pH 4.4) shown in Fig. 2A.

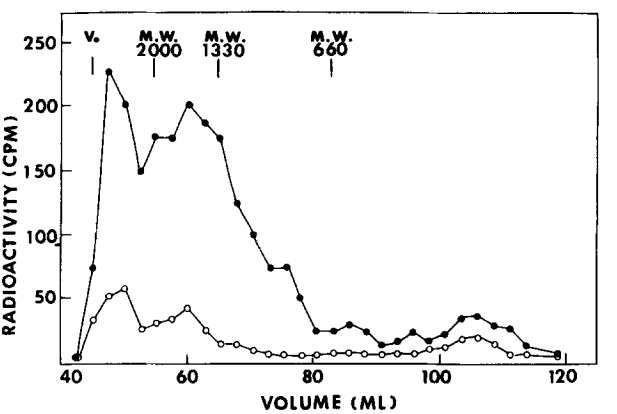

Fig. 4. Elution profile from a column (1 x 200 cm) of Sephadex G-25 of the reaction products from incubation of [^{3}H][^{35}S]heparin with the M and L fraction and buffer at pH 4.4 for 4 hrs at 37° followed by a further 4 hrs at 37° after the addition of another 300 l of the M and L fraction. Indicated are the peak elution positions of heparin fragments obtained by partial deamination of commercial heparin with nitrous acid. The fragments were hexasaccharide, tetrasaccharide and disaccharide, molecular weights of about 2,000, 1,300 and 660 respectively. Approximately 3.0 ml fractions were eluted with 0.2M NaCl in 0.10M Tris-HCl, pH 8.0, ●—● [^{3}H]GlcN cpm, o—o $^{35}SO_4$ cpm.

also demonstrated a marked reduction in the chain length as shown by gel chromatography of the digestion products on Sephadex G-200 (Figs. 2 and 3) and Sephadex G-25 (Fig. 4).

The effect of pH on the degradation of [^{3}H][^{35}S]heparin by lysosomal preparations is shown in Fig. 2. Degradation is most marked at pH 4.4. However, breakdown of the heparin substrate at lower and higher pH also produced oligosaccharides. Heparin degradation at pH 4.4 and lower suggests that this heparinase activity may be different from the activity observed by Höök et al. (11) for the degradation of heparan sulfate using a solubilized enzyme preparation isolated from rat liver. Their preparations did not degrade heparin and were inactive toward heparan sulfate at pH below 5.0 (11). In agreement with their findings, lysosomal enzyme solutions prepared by their freeze-thaw method did not significantly degrade [^{3}H][^{35}S]heparin. However, the pellet obtained after the freeze-thaw procedure did degrade [^{3}H][^{35}S]heparin although at a considerably reduced level compared to that shown in Figs. 2A and 2B. These results suggest that the heparin endoglycosidase activity in rat liver is not solubilized or is unstable under these conditions of isolation. However, it has been found that although a single freeze-thaw cycle releases lysosomal enzymes, repeated freezing and thawing results in incorporation of lysosomal enzymes into vesicles (Lazo, P. and Horecker, B. L., personal communication).

The extent of degradation of [^{3}H][^{35}S]heparin increased with time of incubation up to 4 hrs. Incubation for longer periods of time did not significantly increase the extent of degradation of this substrate (Fig. 3). Addition of extra M and L fraction after 4 hrs of incubation and incubation for a further 4 hrs yielded degradation products of which approximately 66% were tetra-

to octasaccharide in size as estimated by gel chromatography (Fig. 4). The absence of extensive exoglycosidase and sulfatase activity in these preparations suggests that these enzymes, if present, are not stable or are inactive under the assay conditions used.

The present study confirms the work of Jaques and co-workers on the presence of a mammalian heparinase in liver (1,2). Further, the nature of the endoglycosidase activity and the extent of degradation have been clarified.

ACKNOWLEDGEMENTS

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