The Assay and Partial Characterization of Macromolecular Heparin Depolymerase Activity in Rat Small Intestine

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Homogenates of rat small intestine can depolymerize macromolecular rat skin heparin (RS heparin) to products similar in size to commercial heparin [Horner (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 3469–3473]. This activity is attributed to an enzyme provisionally named 'macromolecular heparin depolymerase'. An assay for macromolecular heparin depolymerase activity in rat small intestine has been developed, based on the action of the enzyme on ^{35}S -labelled macromolecular RS heparin. The depolymerized products are separated into two peaks by gel chromatography through columns of Bio-Gel A-15m. The amount of label in the second peak, expressed as a percentage of the total radioactivity, is the index of enzyme activity. The pH optimum was found to be 6.0 and the temperature optimum 45°C. The enzyme was shown to be most stable in 50mM-Tris/maleate buffer containing 1mM-EDTA. Macromolecular heparin depolymerase activity measured as a function of time and substrate concentration produced curves typical of an enzymic reaction. Evidence was obtained demonstrating that the activity did not originate from bacteria in the intestine. Macromolecular heparin depolymerase activity was increased by dilution and storage at 7°C for 24h. This suggests that homogenates of rat small intestine contain an unstable inhibitor of the enzyme.

Rat skin heparin (RS heparin) has been shown to be a macromolecular complex composed of heparin chains linked covalently to a core structure (Horner, 1970b, 1971). This core region appears to be a polypeptide composed of alternating serine and glycine residues with the polysaccharide chains attached to serine units via *O*-glycosidic linkages to xylose residues (Robinson *et al.*, 1978; Horner & Young, 1979). The molecular weight of RS heparin was calculated to be approx. 1.1×10^6 (Horner, 1971), which is more than an order of magnitude greater than that of commercial preparations (Johnson & Mulloy, 1976). High-molecular-weight heparins have also been found in mouse mastocytoma (Ögren & Lindahl, 1971), rat peritoneal mast cells (Horner, 1977; Yurt *et al.*, 1977) and monkey tissues (Horner, 1976).

A preparation from rat small intestine was shown to depolymerize macromolecular RS heparin to products whose molecular size was similar to that of commercial pig mucosal heparin (Horner, 1972).

Abbreviations used: RS heparin, rat skin heparin; PM heparin, pig mucosal heparin.

This depolymerizing activity has been provisionally named 'macromolecular heparin depolymerase'.

In addition to its anticoagulant effect, heparin is known for its antilipaemic action. Heparin stimulates the release of lipoprotein lipase (clearing-factor lipase), an enzyme that hydrolyses the triacylglycerols (triglycerides) of circulating chylomicrons and low-density lipoproteins (Robinson, 1963). When tested for its effect on lipoprotein lipase, RS heparin was found to exhibit poor lipoprotein lipase-releasing activity *in vivo* and actually inhibited this enzyme *in vitro*. However, lower-molecular-weight products formed by the action of macromolecular heparin depolymerase displayed lipoprotein lipase-releasing activity both *in vivo* and *in vitro* (Horner, 1972). Since macromolecular RS heparin may be a precursor form that must be depolymerized *in vivo* before it can exert its physiological effect, a study of macromolecular heparin depolymerase would contribute knowledge to the metabolism and possible physiological role of heparin. In the present paper we describe an assay for measuring macromolecular heparin depolymerase activity in homogenates of rat small intestine. By use of this assay, various properties of the depolymerase activity were studied.

Experimental

Materials

Sodium [^{35}S]sulphate (carrier-free) and PCS scintillation solubilizer were obtained from Amersham-Searle Corp., Arlington Heights, IL, U.S.A.

Agarose gel granules for gel filtration (Bio-Gel A-50m, 100–200 mesh, and Bio-Gel A-15m, 200–400 mesh) were acquired from Bio-Rad Laboratories, Richmond, CA, U.S.A.

Agarose (1%) gel films for electrophoresis (Universal Electrophoresis Films) were supplied by Corning Analytical Chemists, Palo Alto, CA, U.S.A.

Cetylpyridinium chloride was purchased from Matheson, Coleman and Bell, East Rutherford, NJ, U.S.A., and Hyflo Super-Cel (an inert silica filter aid) from Johns-Manville, Denver, CO, U.S.A.

Sodium dextran sulphate 2000 (mean mol.wt. 2×10^6), Blue Dextran 2000 and DEAE-Sephacel were obtained from Pharmacia, Uppsala, Sweden.

Chondroitinase ABC was acquired from Miles Laboratories, Elkhart, IN, U.S.A.

Pig mucosal heparin (PM heparin) was supplied by Canada Packers, Toronto, Ont., Canada, and bovine serum albumin by Calbiochem, San Diego, CA, U.S.A.

All other reagents were of analytical grade.

Analytical methods

Uronic acid was determined by the carbazole method of Bitter & Muir (1962) with D-glucuronolactone as standard. Protein was determined by the procedure of Lowry *et al.* (1951), with bovine serum albumin as standard. Heparin concentration was measured by a modification of the metachromatic Azure A assay method of Jaques *et al.* (1949) as described by Lam *et al.* (1976), with pig mucosal heparin as standard.

Radioactivity was counted with a Nuclear-Chicago mark I liquid-scintillation system. PCS solubilizer (10ml/glass vial) was employed as the scintillation medium.

Gel electrophoresis was conducted in 1% agarose films as described by Horner (1975).

Gel chromatography was performed at room temperature on columns of Bio-Gel A-15m (200–400 mesh) (0.9cm $\times$ 27cm). Samples to be chromatographed were dissolved in 1.0M-NaCl (0.5ml) and applied to the columns, which were equilibrated and eluted with the same solvent. A flow rate of 4ml/h was maintained. Fractions were collected by drop-counting with an LKB Ultrarac type 7000 fraction collector.

DEAE-cellulose anion-exchange chromatography was carried out at 7°C on columns (1.5cm $\times$ 20cm) of DEAE-Sephacel (Cl^- form) equilibrated with 0.3M-NaCl adjusted to pH2.5 with HCl. The samples to be fractionated were dissolved in this solution, applied

to the columns, and then eluted with a linear gradient formed by adding 190ml of 2.0M-NaCl, pH2.5, to 190ml of 0.3M-NaCl, pH2.5. Fractions (5ml) were collected by drop-counting.

Preparation of substrate

Batches of ^{35}S -labelled RS heparin were prepared from groups of six to eight male Wistar rats (body weight 200–300g). Each rat was injected intraperitoneally with 2.0mCi of sodium [^{35}S]sulphate in 0.15M-NaCl (1.0ml). The animals were killed with ether 18h later and their skins were quickly removed and frozen. The labelled macromolecular RS heparin was prepared as previously described for unlabelled RS heparin (Horner, 1971), with minor modifications. Bio-Gel A-50m was used for gel filtration instead of Sagavac 4F. A portion (50–100 μl) of each fraction eluted from Bio-Gel A-50m was taken for scintillation counting of radioactivity. The elution pattern showed two peaks, the first and larger of which was macromolecular RS heparin (Horner, 1971). Fractions comprising this peak were combined and the ^{35}S -labelled RS heparin was precipitated with 2vol. of ethanol. The uronic acid content of the final product was determined by the carbazole reaction. The ^{35}S -labelled RS heparin was dissolved in 0.15M-NaCl at a concentration of 1.0mg/ml and stored at -20°C . The specific radioactivity of different preparations ranged from 25×10^4 to 74×10^4 c.p.m./mg of RS heparin. The homogeneity of each preparation was checked by electrophoresis in 1% agarose gel at pH3.0.

Preparation of homogenate

Groups of six to eight rats (body weight 200–350g) were killed with ether. Their small intestines, from the stomach to the caecum, were quickly removed and placed in ice-cold 50mM-Tris/maleate, pH7.4, containing 0.15M-NaCl and 1mM-EDTA. The intestines were slit lengthwise with the blunt end of dissection scissors, rinsed extensively with the above buffer to remove contents and cut into small pieces. The wet weight of the tissue was determined after straining on a fine steel wire mesh. The wet weight averaged 8g per rat. Fresh buffer (50mM-Tris/maleate/1mM-EDTA, pH7.4) was added in a wet weight/buffer ratio of 3g to 4ml.

The tissue was homogenized in a Polytron PT 10–35 instrument with a PT 20-ST generator (Brinkmann Instruments). Ice/water was used as coolant at all times. Batches (20ml) of intestine resuspended in the buffer were homogenized in a polycarbonate centrifuge tube (13cm $\times$ 4.2cm) for 30s at half speed, re-cooled for 2.5min and homogenized for 30s at full speed. To ensure complete homogenization, combined with the full sonication effect provided by the Polytron instrument, homogenization was repeated in a smaller polycarbonate centrifuge tube

(15.3cm×2.0cm). Samples (15ml) of the initial homogenate were given two 30s treatments at full speed, separated by a 2.5min re-cooling period.

The homogenate was centrifuged at 12100g at 4°C for 30min. The pH of the supernatant was adjusted from 6.8 to 6.0 by the dropwise addition of 5M-maleic acid with vigorous magnetic stirring. The protein concentration of this final supernatant was determined by the method of Lowry *et al.* (1951).

Assay for macromolecular heparin depolymerase activity: standardized conditions

Samples (5.0ml) of the enzyme preparation were pipetted into 25ml Erlenmeyer flasks. After a preincubation period of 10min at 37°C, the macromolecular ³⁵S-labelled RS heparin (100μg) was added and incubation was continued in a metabolic shaker bath at 37°C for the required time. The pH after 8h of incubation was approx. 5.9. Incubations were stopped by transferring the mixture quickly to 40ml centrifuge tubes and adding 5.0ml of 2.0M-NaCl and 3 vol. of ethanol. The tubes were left at 4°C overnight. The precipitated material was spun down at 12100g for 30min at room temperature and air-dried overnight.

To recover labelled RS heparin, each precipitate was stirred magnetically at room temperature for 12h with 6ml of 2.4M-NaCl containing dextran sulphate 2000 (0.33mg/ml). After centrifuging at 12000g for 30min at room temperature the supernatant was removed and a second extraction of the insoluble material was carried out as above. Finally a third extraction was made with 5ml of 2.4M-NaCl. The three supernatants were combined and the NaCl concentration was adjusted to 1.2M by adding distilled water.

The next stage involved the use of cetylpyridinium chloride by using the principles described by Scott (1960). A 14ml volume of cetylpyridinium chloride solution (1%, w/v, in 1.2M-NaCl) was added slowly to the combined supernatants, with constant magnetic stirring, in tubes that were warmed to 35°C in a water bath to facilitate complex-formation.

This mixture was transferred to a 25ml plastic centrifugal filter assembly (Filterfuge; Millipore Corporation) fitted with a 1.2μm (type RA) Millipore filter and containing Hyflo Super-Cel (0.25g). Most of the clear filtrate passed through the filter by gravity. Final traces were removed by centrifuging at 200g for 10min at room temperature. The filter pad was washed with two 2ml portions of warm 0.1% cetylpyridinium chloride in 1.2M-NaCl. The heparin-cetylpyridinium chloride complex was then dissociated with four 2ml portions of warm (40°C) 2.4M-NaCl. The washings and extractions were carried out in the same way as the initial filtration. The combined extracts were adjusted to 1.0M-NaCl with distilled water, and heparin was precipitated by

adding 3 vol. of ethanol. The carrier dextran sulphate was not displaced from its cetylpyridinium chloride complex by 2.4M-NaCl and was therefore retained on the filter pad. After standing at 4°C overnight, the heparin precipitate was spun down at 900g for 90min and the supernatant discarded. The precipitation of heparin from 1.0M-NaCl by ethanol was repeated and the product was dried under reduced pressure at room temperature. The percentage recovery of radioactivity was expressed relative to a 100μg sample of undegraded substrate.

Each recovered depolymerization product was dissolved in 1.0M-NaCl (0.5ml), and the ³⁵S-labelled heparins were fractionated by gel filtration on Bio-Gel A-15m columns. A sample (100–500μl) was taken from each 0.59ml fraction, mixed with PCS solubilizer in a glass vial and its radioactivity counted for 20min. Elution curves drawn from the radioactivity data showed two peaks, the second containing heparin chains released from the partially degraded macromolecular heparin present in the first peak. The percentage of total radioactivity in the second peak was taken as the index of macromolecular heparin depolymerase activity. The methods described above were used in most experiments and are referred to as standard conditions.

Results

Comments on the assay method for macromolecular heparin depolymerase activity

Incubations were carried out under standard conditions for periods ranging from 1 to 10h and the products were fractionated on Bio-Gel A-15m columns. As a control, a sample of substrate was incubated with buffer only for 24h. Several typical elution profiles are shown in Fig. 1. In most cases two peaks were obtained, but these always overlapped. This is an inevitable consequence of the polydisperse nature of the products. Therefore, in cases where enzymic depolymerization was either low or high (see 2 and 6h incubations; Fig. 1), there existed the difficulty of determining where the division between peaks should be made. The dividing point for assignment of fractions to the two peaks was standardized by reference to an example where the separation into two peaks was distinct (see 4h incubation; Fig. 1). The assignment of fractions to the second peak was based on this criterion for every depolymerized product. The percentage of total radioactivity present in the second peak was used as the index of depolymerase activity. This is defined as percentage depolymerization (Table 1). When multiple samples of a single product were passed through the same column, or through different columns of the same volume, the calculated values for percentage depolymerization varied by less than

Fig. 1. Bio-Gel A-15m column chromatography of ^{35}S -labelled products obtained from timed incubations. Macromolecular ^{35}S -labelled RS heparin was incubated with homogenates of rat small intestine as described in the Experimental section ($\blacktriangle$, 2 h; $\square$, 3 h; $\bullet$, 4 h; Δ , 6 h; $\circ$, 100 μg of ^{35}S -labelled RS heparin incubated with buffer only for 24 h; essentially the same profile was obtained for a boiled control incubated under the same conditions). The recovered products were fractionated on a column (0.9 cm $\times$ 2.7 cm) of Bio-Gel A-15m eluted with 1.0 M-NaCl. The void volume (V_0) was determined with Blue Dextran 2000. The ^{35}S content of each fraction (volume 0.59 ml) is expressed as a percentage of the total radioactivity in the sample. Division into two peaks is indicated by the vertical broken line. The percentage of total radioactivity present in the second peak is taken as the index of macromolecular heparin depolymerase activity. The arrow indicates the peak position of a sample of PM heparin chromatographed on the same column and determined quantitatively by the carbazole reaction (Bitter & Muir, 1962).

Table 1. Macromolecular heparin depolymerase activity during timed incubations

The percentage depolymerizations and percentage recoveries were calculated as outlined in the Experimental section. The gel filtration elution profiles of four of the products are shown in Fig. 1.

Time of incubation (h)	Recovery (%)	Depolymerization (%)
1	99.1	8.5
2	100	14.3
3	100	35.3
4	87.1	58.5
5	98.7	76.8
6	97.2	87.4
8	89.2	95.1
10	78.7	97.3

2%. Consequently it was considered that the assay method was sufficiently reproducible for the purposes of studying this enzyme.

In the control sample incubated with buffer only, 3.6% of the total radioactivity was found in the area of the second peak. It is probably indicative of slight contamination of the substrate with lower-molecular-weight heparin from rat skin; 3.6% was the highest value ever obtained. Other batches of labelled RS heparin gave control values as low as 1.4%.

The use of dextran sulphate in the recovery procedure was found to be very important. Typical high recovery values are given in Table 1. If the use of dextran sulphate was omitted the recoveries were less than 40%.

The mean protein concentration of ten enzyme preparations made under standard conditions was $27.4 \pm 2.5 \text{ mg/ml}$ (mean $\pm$ S.D.). This indicates that the method of preparation was reproducible.

Effect of various buffers on macromolecular heparin depolymerase activity

Depolymerase activity was determined in homogenates of small intestine prepared with the standardized homogenization technique except that nine different buffers were used. These are listed in Table 2. All buffers (except the three Tris buffers) were prepared as described by Dawson *et al.* (1969). Each buffer also contained 1 mM-EDTA. The pH values chosen for comparison were close to 7.0 and/or 6.0, as the effective range of the buffer permitted. Before homogenization, intestines were washed in the appropriate buffer system, containing 0.15 M-NaCl, at pH 7.4. As this was not feasible with the sodium acetate buffer system, the intestines were washed with 50 mM-Tris/HCl, pH 7.4, containing 0.15 M-NaCl. Homogenates were then prepared in the NaCl-free buffers listed in Table 2. All incubations were for 8 h at 37°C. The results listed in Table 2 clearly show better activity in Tris buffers. Tris/maleate was the best of the three Tris buffers tested and was therefore used in our standardized assay. Other buffers used inhibited activity in the order citrate > acetate > phosphate > imidazole.

Effect of pH on macromolecular heparin depolymerase activity

Macromolecular heparin depolymerase activity was measured at six pH values ranging from 5.5 to 8.0 as follows. A batch of intestinal homogenate was prepared by standardized conditions. The pH of the 12100g supernatant was 6.7. Samples were adjusted to pH values of 5.5, 6.0 and 6.5 with saturated maleic acid (5M) and to pH values of 7.0, 7.5 and 8.0 with 5M-NaOH. Duplicate determinations of depolymerase activity were then made at each pH value, by using standard conditions with an incubation time of 6 h. pH values were then quickly meas-

Table 2. *Effect of various buffers on macromolecular heparin depolymerase activity*

Homogenates were prepared in the various buffers by using the standardized homogenization conditions (see the Experimental section). All buffers (except those containing Tris) were prepared as described by Dawson *et al.* (1969). Each buffer also contained 1 mM-EDTA.

Buffer used for homogenization	Initial pH of homogenate	pH after 8 h incubation	Depolymerization (%)
Citric acid/ Na_2HPO_4			
(a) pH 7.0	6.7	6.5	8.9
(b) pH 6.0	6.1	6.0	9.6
$\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$			
(a) pH 7.0	7.0	6.3	22.9
(b) pH 6.0	6.2	6.2	17.3
Sodium acetate/acetic acid			
pH 6.0	6.2	6.3	15.2
Imidazole/HCl			
pH 7.4	7.4	7.1	40.5
50 mM-Tris/HCl			
pH 7.4	7.0	6.8	57.1
50 mM-Tris/acetate			
pH 7.4	6.8	6.7	52.0
50 mM-Tris/maleate			
pH 7.4	6.8	6.5	77.3
	(also) 6.0*	5.9	79.8

* Adjusted from pH 6.8 after homogenization by addition of 5 M-maleic acid.

Fig. 2. *Effect of pH on macromolecular heparin depolymerase activity*

Homogenates of rat small intestine were prepared under standard conditions (see the Experimental section) and assayed with different buffers as described in the text. The incubation time was 6 h. Each point represents the mean of duplicate determinations.

5.9, in homogenates with an initial pH of 6.0. Thus 50 mM-Tris/maleate, pH 6.0, was shown to be an effective buffer and its use was incorporated into the standard assay conditions, described in the Experimental section.

Effect of substrate concentration on macromolecular heparin depolymerase activity

Samples (5 ml) of a standard intestinal preparation were incubated with various amounts of substrate, ranging from 5 to 300 μg . Incubation times of 2 and 4 h were used. The results, illustrated in Fig. 3, show that a plateau in activity (indicative of zero-order kinetics) was reached essentially at 100 μg . For this reason, as well as economy of substrate, this amount of substrate was incorporated into the standard conditions.

Effect of temperature on macromolecular heparin depolymerase activity

Standardized assay mixtures were incubated at five different temperatures ranging from 25 to 50°C for 4 h. Optimum activity was obtained at 45°C, as shown in Fig. 4. At the optimum temperature there is a fine balance between optimal thermal activation and increased denaturation of the enzyme. It was decided that incubation at this temperature would be inappropriate. Subsequently all incubations were performed at 37°C, as described for standard conditions.

ured at room temperature before the addition of NaCl and ethanol to stop the reaction.

The effect of pH on depolymerase activity is summarized in Fig. 2, in which post-incubation pH values are plotted against percentage depolymerization. Optimal activity was obtained at a final pH of

Fig. 3. Effect of substrate concentration on macromolecular heparin depolymerase activity

Increasing amounts of ^{35}S -labelled RS heparin were incubated for 2h (○) and 4h (●) with homogenates of small intestine as described in the Experimental section. Each point is the mean of duplicate determinations.

Fig. 4. Effect of temperature on macromolecular heparin depolymerase activity

Homogenates of small intestine were prepared under standard conditions (see the Experimental section) and duplicate samples were incubated at the temperatures indicated for 4h.

Incubation with intestinal contents

Since the intestine contains an abundant bacterial flora (Dubos *et al.*, 1965), the possibility existed that the depolymerase activity was of bacterial origin. To check this possibility, the intestinal contents from a group of rats were removed and strained on cheese-

cloth. The contents and washed intestines were then homogenized separately and identically, as described for standard conditions. The protein concentration of the 12100g supernatant from intestinal contents was 16mg/ml. The results of incubations to measure macromolecular heparin depolymerase activity are presented in Table 3. The activity from intestinal contents was low compared with activity from the intestines. The activity of the intestinal contents after 24h of incubation was comparable with that of the intestinal homogenate incubated for only 2h. Depolymerase activity has been detected previously both in the mucosa and in the wall of the intestines (E. Young, unpublished work). The sloughing off of mucosa into the intestinal lumen during normal rapid mucosal turnover or by mechanical means during the cleaning of the intestines probably accounts for the low activity observed in the intestinal contents, rather than activity from a bacterial source.

Effect of enzyme concentration (dilution) on macromolecular heparin depolymerase activity

An intestinal preparation was made as described under standard conditions. The resultant supernatant was regarded arbitrarily as 100%. Dilutions containing 75, 50, 25 and 12.5% supernatant were made with 50mM-Tris/maleate, pH 6.0, containing 1mM-EDTA. Each 5.0ml sample was assayed for macromolecular heparin depolymerase activity, with an incubation time of 5h. The results are shown in Fig. 5. Unexpectedly, greater activity was obtained with the 1:1 dilution of supernatant.

Stability of macromolecular heparin depolymerase in the cold and frozen

A depolymerase preparation was made by the standard method. A control sample was immediately assayed for macromolecular heparin depolymerase activity by incubation at 37°C for 5h. Other samples were either kept in the cold-room at 7°C or frozen immediately in their incubation flasks, with acetone/solid CO_2 , and subsequently stored at -20°C. After

Table 3. Incubation with intestinal contents

Homogenates of the intestinal contents and washed small intestine were prepared identically and assayed for macromolecular heparin depolymerase activity as described in the Experimental section.

Homogenate	Time of incubation (h)	Depolymerization (%)
Intestinal contents	4	12.6
Intestinal contents	24	16.5
Intestine	2	14.3
Intestine	4	58.5

Fig. 5. Effect of dilution on macromolecular heparin depolymerase activity

A standard homogenate of rat small intestine (see the Experimental section) was designated as 100% concentration and dilutions of this preparation, made with 50mM-Tris/maleate / 1mM-EDTA, pH6.0, were assayed for depolymerase activity. The incubation time was 5h.

Fig. 6. Stability of macromolecular heparin depolymerase activity in the cold (7°C) or frozen (-20°C)

Samples of a standard homogenate of rat small intestine (prepared as described in the Experimental section) were assayed immediately (control) or after storage at 7°C or -20°C for the periods indicated. Assay mixtures were incubated for 5h.

various storage times, these samples were assayed for depolymerase activity in the same way as the control. Before incubation, the frozen samples were permitted to thaw for 1h at 7°C. The results of these experiments are summarized in Fig. 6. Unexpectedly, the samples stored cold or frozen displayed increases in activity. The increase for the samples kept at 7°C was almost 30%, and the enzyme was stable at this temperature for up to 1 week. The frozen samples did not display such a prominent increase (approx. 20%) during the

first week, but at the end of 30 days the increase was similar to that observed for the 7°C samples. These experiments also showed that the enzyme can withstand freezing and thawing.

Influence of dilution and cold storage on macromolecular heparin depolymerase activity

Portions of a standard enzyme preparation were (i) assayed immediately, (ii) kept at 7°C for 24h and then assayed, or (iii) diluted with an equal volume of 50mM-Tris/maleate/1mM-EDTA, pH6.0, kept at 7°C for 24h and then assayed. In all three cases 5ml samples were incubated for various times from 1 to 10h. The results of these incubations are shown in Fig. 7. Clearly, the samples that were diluted 1:1 with buffer and kept at 7°C for 24h demonstrated the best activity. The effects of storage at 7°C and dilution on depolymerase activity appear to be additive.

Specific radioactivity of ³⁵S-labelled RS heparin and its depolymerized products

The specific radioactivities of macromolecular ³⁵S-labelled RS heparin and its depolymerized products were determined to discount the possibility of the action of a sulphate-transferring enzyme rather than a depolymerase.

Assay mixtures were incubated under standard conditions for 1, 2 and 3h. For each time interval five 5ml portions of homogenate were used. Each depolymerized product was recovered by standardized procedures with minor modifications. The

Fig. 7. Influence of dilution and storage at 7°C on macromolecular heparin depolymerase activity

A standard enzyme preparation was assayed as described in the Experimental section for depolymerase activity. Samples were incubated immediately (▲), stored at 7°C for 24h and then assayed (○), or diluted 1:1 with 50mM-Tris/maleate/1mM-EDTA, pH6.0, stored at 7°C for 24h and then assayed (●).

Table 4. *Specific radioactivities of macromolecular ^{35}S -labelled RS heparin and its depolymerized products*
 Macromolecular ^{35}S -labelled RS heparin was incubated with homogenates of rat small intestine, prepared by standardized conditions, for 1, 2 and 3 h. The products were recovered, purified and analysed by procedures described in detail in the text (see the Experimental section). Each determination was done in triplicate. The values given are the means ($\pm$ s.d.) for these determinations.

Sample	Radioactivity (c.p.m./50 μl)	Heparin ($\mu\text{g}/50 \mu\text{l}$)	Specific radioactivity (c.p.m./ μg of heparin)
^{35}S -labelled rat skin heparin	3064 $\pm$ 13	5.00 $\pm$ 0.09	613 $\pm$ 11*
1 h incubation product	2571 $\pm$ 23	4.21 $\pm$ 0.04	611 $\pm$ 8
2 h incubation product	2716 $\pm$ 14	4.43 $\pm$ 0.01	613 $\pm$ 3
3 h incubation product	2785 $\pm$ 18	4.44 $\pm$ 0.06	627 $\pm$ 9

* The standard deviation of each specific-radioactivity value was derived from the standard deviations of the corresponding radioactivity and heparin concentrations by the treatment of fractional (percentage) errors described by Wilson (1952). When compared with macromolecular ^{35}S -labelled RS heparin and with each other, there are no significant differences between any of the specific radioactivities by Student's *t* test ($P > 0.05$).

2.4M-NaCl washes containing dextran sulphate were pooled for each incubation period before the addition of 70 ml of 1% cetylpyridinium chloride in 1.2M-NaCl. After passage through the filter assembly, the heparin-cetylpyridinium chloride complex was dissociated with seven 2 ml portions of warm 2.4M-NaCl, and the heparin was precipitated from 1.0M-NaCl with 3 vol. of ethanol.

The depolymerized products were digested with chondroitinase ABC to remove traces of chondroitin sulphates and/or dermatan sulphate detected by gel electrophoresis in 1% agarose films. Each product was dissolved in 10 ml of chondroitinase ABC buffer (Saito *et al.*, 1968) containing 1.7 units of enzyme and incubated at 37°C. After 24 h, each incubation mixture was adjusted to 1.0M-NaCl with solid salt and 2 mg of dextran sulphate was added. Then 1% cetylpyridinium chloride (6 ml) was added, and heparin was recovered as described above.

After precipitation with ethanol each depolymerized product was further purified by DEAE-cellulose anion-exchange chromatography (see the Experimental section). A 50 μl sample was taken from each 5 ml fraction for counting of radioactivity. Those fractions comprising a peak of radioactive material were combined and precipitated from 1.0M-NaCl with 3 vol. of ethanol. A 100 μg sample of ^{35}S -labelled RS heparin was likewise precipitated. The heparin precipitates were spun down, washed with 75% (v/v) ethanol followed by ethanol and then dried. After these purification procedures, approx. 70–80% of the original radioactivity was recovered for each depolymerized product. The purity of each product was confirmed by gel electrophoresis in 1% agarose films. The products of incubation for 1, 2 and 3 h were depolymerized 29, 47 and 68% respectively.

Each depolymerized product and the macromolecular ^{35}S -labelled RS heparin was dissolved in water to a concentration appropriate for the deter-

mination of heparin concentration by the metachromatic Azure A method. Heparin concentrations and radioactivity were determined in 50 μl samples. The results are shown in Table 4. The close agreement between the specific radioactivities of macromolecular ^{35}S -labelled RS heparin and of the depolymerized products from 1, 2 and 3 h of incubation indicates that homogenates of rat small intestine contain macromolecular heparin depolymerase activity rather than sulphate-transferring activity.

Discussion

The present work has demonstrated some of the properties of a macromolecular heparin depolymerase from rat small intestine and described an assay method for this enzyme. The substrate was a macromolecular heparin from rat skin (Horner, 1971). Heparins of a similar macromolecular nature have also been identified in human and monkey tissue (Horner, 1970a, 1976), in rat peritoneal mast cells (Horner, 1977; Yurt *et al.*, 1977) and in mouse mastocytoma tissue (Ögren & Lindahl, 1971, 1975, 1976). The molecular weight of this mastocytoma macromolecular heparin is 69000: smaller than RS heparin but substantially larger than PM heparin (Ögren & Lindahl, 1971). An enzyme has been isolated from the particulate fraction of the same tissue with the ability to degrade this substrate into chains similar in size to commercial heparin (Ögren & Lindahl, 1975). This enzyme has been named 'heparin-liberating enzyme' (Ögren & Lindahl, 1976). It has also been shown to degrade RS heparin (Lindahl *et al.*, 1977), but the products have not been characterized. This enzyme differs from macromolecular heparin depolymerase in several respects. Its pH optimum is 5.0 (versus 6.0 for the intestinal depolymerase), and it is most active in an acetate

buffer (Ögren & Lindahl, 1975). Acetate inhibits macromolecular heparin depolymerase (see Table 2). The mastocytomal heparinase can be purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ (Ögren & Lindahl, 1975), which inactivated the intestinal depolymerase (E. Young, unpublished work). The mastocytomal enzyme has been tentatively identified as an endoglycuronidase on the basis of the reduction of the polysaccharide degradation products with NaB^3H_4 (Ögren & Lindahl, 1975). The nature of the bond cleaved by the depolymerase is not known. Both macromolecular heparin depolymerase and the heparin-liberating enzyme release heparin chains from a multichain macromolecular substrate. Additional enzymes are involved in the further degradation of the heparin chains to oligosaccharides. Such heparin endoglycosidases have been identified in the lysosomes of rat liver (Arbogast *et al.*, 1977) and of rat spleen (Höök *et al.*, 1977) and in homogenates of human platelets (Hedlin *et al.*, 1977).

Instead of a depolymerase, it could be argued that the activity observed involved a sulphate-transferring enzyme. This type of enzyme might transfer the radioactive sulphate from the macromolecular RS heparin to endogenous low-molecular-weight heparins in rat small intestine. However, experiments to demonstrate heparin in rat intestine have revealed only trace amounts of a low-molecular-weight heparin-like molecule (E. Young, unpublished work). Also, the results from the present study have shown that the specific radioactivities of ^{35}S -labelled RS heparin and its enzymically depolymerized products are almost identical. These observations support the concept of a depolymerase rather than of a sulphate-transferring enzyme.

In the assay procedure the use of dextran sulphate is obligatory to obtain good heparin recoveries. This indicates that in the intestinal homogenate supernatant heparin is strongly bound in some type of complex. It requires a more highly sulphated compound to displace heparin from this complex. A polypeptide similar to that isolated from peritoneal mast-cell granules (Bergqvist *et al.*, 1971) and platelets (Da Prada *et al.*, 1976) may be responsible for the heparin binding, but this has not been studied.

In earlier work with rat small intestine (Horner, 1972) depolymerase activity was solubilized by high-speed homogenization, followed by ultrasonication. In the present work we have used the Polytron homogenizer, which was designed to combine the effects of mechanical shearing and ultrasonic disruption of tissues. Our experience is that the efficiency of release of depolymerase activity is critically dependent on the condition of the Polytron generator. Comparison of the data in Tables 1 and 4 demonstrates this dramatically. The data in Table 1 were obtained with a PT 20-ST generator that had been in use for about 2 years: the

values for depolymerization after 1, 2 and 3 h of incubation were 8, 14 and 35 % respectively. The data in Table 4 were obtained with a new PT 20-ST generator: the values for depolymerization after 1, 2 and 3 h of incubation were 29, 47 and 68 % respectively. We suspect that the ultrasonication effect is decreased in older Polytron generators.

The activity of the depolymerase as assayed in our experiments was increased when diluted to 50 % or when kept at 7°C for 24 h. These observations suggest that an inhibitor that became less effective on dilution or denatured on standing in the cold was involved.

The isolation of ^{35}S -labelled macromolecular RS heparin has always been accompanied by a minor fraction of lower-molecular-weight heparin with the same specific radioactivity as that of the major macromolecular fraction (A. A. Horner, unpublished work). In monkey tissues, heparins ranging from high to low molecular weights have been found (Horner, 1976). Macromolecular heparin depolymerizing activity has also been reported in human intestine and spleen (Ögren & Lindahl, 1975) and in rat serum (Young *et al.*, 1978). These observations support the hypothesis that lower-molecular-weight chains are released from a macromolecular precursor by heparin depolymerases. Since macromolecular RS heparin is an inhibitor of lipoprotein lipase activity whereas depolymerized chains are activators (Horner, 1972), these depolymerases may act physiologically by controlling the availability of low-molecular-weight heparin chains to activate lipoprotein lipase.

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