Oral heparin results in the appearance of heparin fragments in the plasma of rats

(oral administration/anticoagulation/angiogenesis)

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ABSTRACT   We have previously shown that angiogenesis inhibition and tumor regression can be accomplished by combinations of heparin or heparin fragments with cortisone [Folkman, J., Langer, R., Linhardt, R. J., Haudenschild, C. & Taylor, S. (1983) Science 221, 719–725]. Oral heparin was also effective in combination with cortisone. We now show that a single oral dose of [³⁵S]heparin or [³H]heparin (15,000 units/kg) results in continuous release of radioactive material into the bloodstream for at least 12 hr. This is associated with the presence of anti-factor Xa activity at a level of ≈0.1 unit/ml. The radioactive material is identified as oligo-, di-, and monosaccharides by its behavior in chromatographic systems, its possession of anti-factor Xa activity, and the effect of treatment with bacterial heparinase. The heparin fragments are extensively metabolized to fragments without anti-factor Xa activity that are readily subject to urinary excretion.

We have previously demonstrated that the combination of heparin, or a heparin fragment, and cortisone inhibited angiogenesis, including the chicken chorioallantoic membrane and the rabbit corneal pocket assay (1). When the combination was used to inhibit tumor growth by parenteral administration to mice, a high rate of hemorrhagic complications was encountered because of the anticoagulant effect of heparin. Other methods of heparin administration were tried, and it was found that the anti-angiogenic effect was maintained when heparin was given orally even though the anticoagulant complications did not occur. This oral regimen inhibited tumor growth and metastasis in some animal tumor models. In an initial experiment to study the absorption of oral heparin, mice were given [³⁵S]heparin in their drinking water. Serum obtained 24 hr later yielded 0.4% of the radioactivity that was present in the drinking water. However, gel filtration chromatography failed to demonstrate radiolabeled heparin fragments with biological activity (1).

The anti-angiogenic activity of oral heparin was somewhat surprising. The literature contains numerous reports of attempts to administer heparin orally (2–4), with little success in achieving clinically useful anticoagulant activity. Attempts have been made to improve enteral absorption in a number of ways: (*i*) use of adjuvants such as EDTA (5), citric acid (6), or dimethyl sulfoxide (7); (*ii*) oral administration in micellar solutions (8) or in liposomes (9); (*iii*) rectal administration with bile salts (10); or (*iv*) administration of low molecular weight heparins (4). No such technique has yet achieved clinical usefulness.

As a result of finding anti-angiogenic activity with oral heparin plus cortisone without anticoagulant activity, we decided to examine the fate of radiolabeled heparin given enterally. Furthermore, by examining two differently labeled heparins (³⁵S and ³H), we sought to gain information about

metabolism of the drug by desulfation and by depolymerization. We now show that a single oral dose of heparin results in continuous release of heparin fragments as large as oligosaccharides (fragments with 10 or more sugars) with anti-factor Xa activity into the bloodstream of rats for at least 12 hr. The heparin fragments are extensively degraded to metabolites without anti-factor Xa activity that are excreted into the urine.

MATERIALS AND METHODS

Materials. Heparin (sodium salt from porcine intestinal mucosa) was obtained from Abbott (Panheprin lot 40-095 AF, 20,000 units/ml). [*N*-sulfonate-³⁵S]heparin (specific activity, 39 mCi/g; 1 Ci = 37 GBq) was purchased from Amersham. The manufacturer states that sulfate-labeled heparin is prepared by N-resulfation of N-desulfated commercial heparin as described by Levy and Petrcek (11). The resulfated heparin shows no difference in optical rotation, viscosity, sulfur content, infrared data, as well as *in vitro* and *in vivo* anticoagulant potency as compared with the starting material (11). However, ≈1% of the *O*-sulfate is irreversibly removed during the N-desulfation procedure (11). [³H]Heparin (specific activity, 206 mCi/g) was obtained from New England Nuclear. The manufacturer states that tritiated heparin is prepared by reaction with tritiated sodium borohydride as described by Hatton *et al.* (12). This labeling technique relies on the presence of a small proportion of heparin molecules (4–6%), which terminates in a reducing monosaccharide. On reaction with tritiated sodium borohydride, this monosaccharide is reduced to yield an alditol, resulting in tritiation of the C_1 position (12). Most reduced termini are probably of the N-substituted glucosaminitol type. Of the remainder, the majority are uronic acid derivatives (12). It should be noted that heparin molecules of relatively small molecular weight are more likely to react with sodium borohydride than molecules of relatively high molecular weight (12). The radiolabeled heparins were subjected to gel permeation chromatography (Fractogel TSK HW40-S column, 1.5 × 110 cm, with 0.5 M ammonium bicarbonate buffer) and no low molecular weight contaminants were detected. Unlabeled and labeled heparin were mixed to yield the doses indicated for the animal experiments (1 mg of heparin is equivalent to 150 units).

Bacterial heparinase was produced by fermentation of *Flavobacterium heparinum* and purified by using batch hydroxyapatite chromatography (13). Heparinase (heparin lyase, EC 4.2.2.7) is an eliminase that cleaves certain α-glycosidic linkages in heparin, resulting in the formation of

heparin fragments ranging in size from di- to oligosaccharides (14, 15). Heparinase cleavage of radiolabeled heparin results in a product distribution identical to that obtained with unlabeled heparin, as described elsewhere (16).

Animals. For the 5-hr experiments, male Sprague–Dawley rats (300–350 g) were anesthetized by intraperitoneal injection of sodium pentobarbital (Nembutal, 50 mg/kg). The right jugular vein was cannulated according to the method of Weeks and Davis (17) for blood collection. Urine was collected by bladder cannulation with polyethylene tubing (PE-50; o.d., 0.038 inch), as described (16). For the longer experiments, the jugular vein was cannulated and the animals were allowed at least 3 days to recover from the surgery. After administration of heparin, the animals were placed in Nalgene metabolic cages. All animals were fasted overnight prior to the experiments.

Heparin Administration and Sample Collection. Anesthetized rats received 0.5 ml of heparin solution containing unlabeled heparin and either [^{35}S]heparin (75 μCi/ml) or [^{3}H]heparin (210 μCi/ml) at a total dose of 100 mg/kg via a number 5 French pediatric feeding tube (Argyle) introduced into the stomach through the mouth. The solution was allowed to drain by gravity into the stomach. The residual content of the tube was emptied by injection of 0.1 ml of air followed by immediate tube removal. Nine rats were used in the 5-hr studies with [^{35}S]- or [^{3}H]heparin, while two groups of three rats each were used for the long-term studies. An additional group of six rats received the same amount of unlabeled heparin for the anticoagulation studies. As a control against aspiration of the solutions, anesthetized animals received 0.5 ml of 0.4% trypan blue solution (GIBCO) via an orogastric tube in the same manner as the heparin solutions were given. No blue staining of the tracheobronchial tree was noted in dissected specimens up to 5 hr after dye administration. The bowel was stained blue in these specimens, including the colon, showing that bowel peristalsis remained intact under anesthesia. Blood and urine samples were collected as described (16) and stored on ice until conclusion of the experimental period. At the end of the 5-hr experiments, the animals were bled by cardiac puncture. For the long-term experiments, blood and urine samples were collected at various time intervals, approximately every 6–12 hr, for up to 3 days. Blood samples were taken hourly for 12 hr in the group studied for anticoagulant activity.

Radioactive Material in Blood and Urine. Plasma was prepared by centrifugation of citrated whole blood at 3400 × *g*, for 15 min at 4°C. The radioactivity present in plasma and urine was determined by scintillation spectrometry. In all cases, duplicate determinations of two different aliquot sizes were made to ensure counting accuracy.

Anticoagulant Activity. The anticoagulant activity was determined by a factor Xa chromogenic assay (Kabi Diagnostica, Stockholm, Sweden). The procedure followed was that recommended by the manufacturer. In this assay, the decrease in absorption at 405 nm is linear in the range of 0–5 μg of heparin per ml of plasma. The detection limit is ≈0.05 unit of heparin (0.3 μg) per ml. The plasma was also assayed for anticoagulant activity by the activated partial thromboplastin time (aPTT) assay (Ortho Diagnostics). The procedure was essentially as recommended by the manufacturer except that the test plasma was assayed directly without dilution, and the mixture of plasma and activated Thrombofax reagent was incubated at 37°C for 15 min rather than for 5 min. This results in increased coagulation times and better reproducibility of the aPTT values. Since base-level aPTT values are quite variable in rats (18), a standard curve was prepared for each rat using plasma collected prior to the experiment. In the aPTT assay, the time for clot formation is linear with the heparin concentration in the range of 0–4 μg of heparin per ml of plasma. The detection limit is ≈0.1 unit

of heparin (0.6 μg) per ml. All samples were measured in duplicate.

Identification of Heparin Fragments. The urine samples were filtered and subjected to gel permeation chromatography. The plasma samples were adjusted to pH 3.0 with 5 M HCl before application to a Bio-Rad AG 50W-X2 (200–400 mesh) column (1.5 × 10 cm) equilibrated with dilute HCl (pH 3.0). At this pH, only the strongly negatively charged heparin fragments or other glycosaminoglycans will pass through the cation exchange column and elute in the void volume. The recovery of radioactivity after ion exchange chromatography was ≈70%. No attempt was made to elute remaining material. However, preliminary experiments in which radiolabeled heparin fragments of known size distribution were added to plasma and passed through the ion exchange column showed no detectable differences between the added and the eluted material. After cation exchange chromatography, radioactive fractions were pooled and subjected to gel permeation chromatography. Each sample was examined at least twice. The heparin fragments were identified by comparing their elution profiles with those of heparin di-, tetra-, hexa- and octasaccharides (1, 19); glucosamine; and blue dextran.

Recovery of Biological Activity. Portions of oligo-, di-, or monosaccharide fractions were pooled, freeze-dried, and redissolved in plasma at an estimated concentration of 0.35 unit/ml, as calculated from the dpm in the substance. The factor Xa and aPTT assays were then performed as described above.

RESULTS

Radioactive Material Is Continuously Released into the Blood of Rats After a Single Dose of Oral [^{35}S]- or [^{3}H]heparin. When [^{35}S]heparin (total, 5000 units) was given orally to rats, radioactive material was continuously released into the bloodstream for at least 12 hr (Fig. 1A). The radioactivity in the blood corresponded to 0.1–0.2% of the dose during the first 12 hr. By 24 hr, ≈0.4% of the dose was present, while the radioactivity decreased to ≈0.2% of the dose by 36 hr. The first part of the curve corresponded closely to what was observed in rats (*n* = 5) during the 5-hr experiments (results not shown). The constant release of radioactive material into the bloodstream and its subsequent clearance into the urine
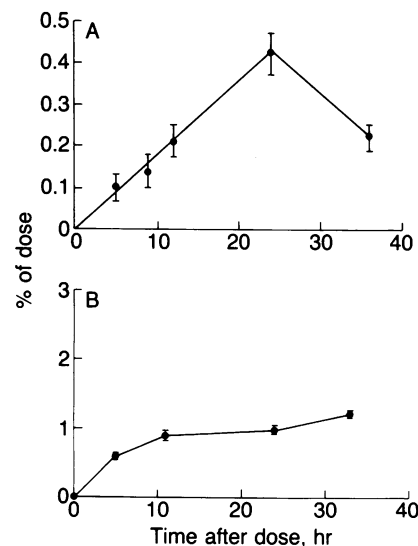

FIG. 1. Appearance of radioactivity in the plasma of rats after an oral dose (100 mg/kg) of either [^{35}S]heparin (*A*), or [^{3}H]heparin (*B*). The values are averages from three rats (±SD).

was indicated by the accumulation of ^{35}S-labeled material in the urine for the first 36 hr, after which time it leveled off (Fig. 2A). A total of 17% of the initial dose had accumulated in the urine at this time.

Radioactivity also appeared in the plasma of rats that received [^{3}H]heparin. By 24 hr, ≈1% of the initial dose was present. The rate of appearance increased for the first 12 hr, after which it remained approximately steady for up to 33 hr (Fig. 1B). The radioactive material that accumulated in the urine of rats that received [^{3}H]heparin appeared to level off by 33 hr, at which time ≈10% was present (Fig. 2B).

Identification of Radioactive Material as Heparin Oligo-, Di-, and Monosaccharides. The elution profiles of radioactive material present in the plasma of rats 5 hr after oral doses of [^{35}S]- or [^{3}H]heparin are shown in Fig. 3 A and B, respectively. The majority of the material eluted in the same positions as oligo-, di-, and monosaccharide markers. Minor peaks represent material the size of octa- and tetrasaccharides.

Radioactive material excreted into the urine during the first 5 hr after heparin administration was also subjected to gel permeation chromatography as shown in Fig. 4. It can be seen that a general shift toward lower molecular weight material has taken place when compared with the plasma profiles. Practically all the radioactivity in the urine of rats that received [^{35}S]heparin eluted in the same position as di- or monosaccharides, with only a minor part eluting as higher molecular weight fragments (Fig. 4A). In contrast, higher molecular weight material could be detected in the urine of rats that received [^{3}H]heparin in addition to disaccharides and monosaccharides (Fig. 4B). In both cases, material of less than monosaccharide size was present, possibly due to opening of saccharide rings and partial degradation of the terminal monosaccharide unit. The material that eluted as oligosaccharides was collected and half of it was reapplied to the same column, where it eluted in the same position. The other half was degraded with bacterial heparinase and reapplied to the column. The radioactive material then eluted as fragments of predominantly di- and tetrasaccharide size, as was the case for the parent heparin (16).

The Heparin Fragments in the Bloodstream Have Anti-Factor Xa Activity. The anti-factor Xa activity appearing in the plasma of rats after an oral dose of heparin is shown in Fig. 5. Weak anti-factor Xa activity was detectable after 30 min and remained constant at a level of ≈0.1 unit/ml for at
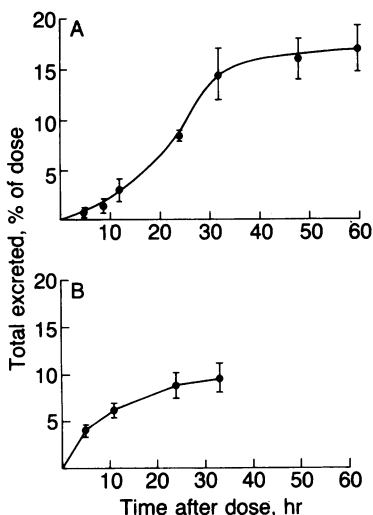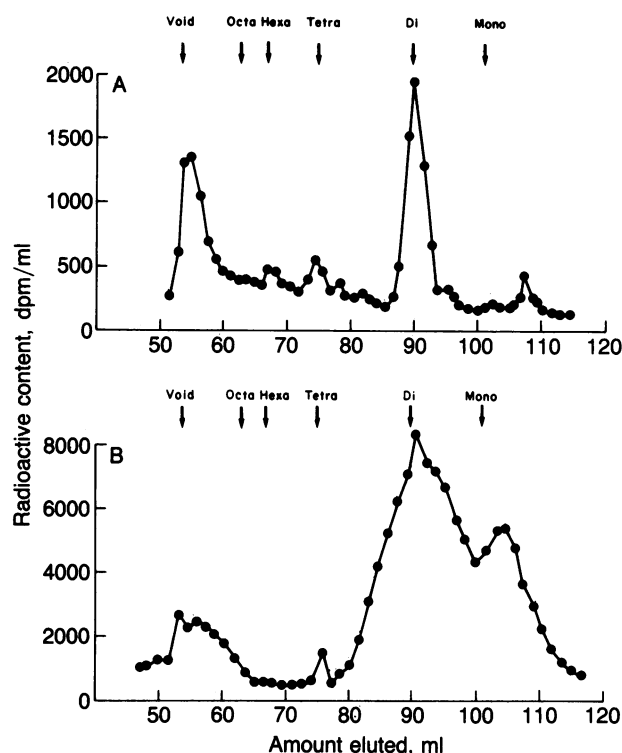

FIG. 3. Gel permeation chromatography of radiolabeled material present in the plasma of rats 5 hr after an oral dose of either [^{35}S]heparin (A) or [^{3}H]heparin (B). A portion of each sample was applied to a Fractogel-TSK HW-40(s) column (1.5 × 110 cm) equilibrated with 0.5 M ammonium bicarbonate. The elution was performed at a flow rate of 0.1 ml/min. Fractions (1 ml) were collected, and the radioactive content of each fraction was determined by liquid scintillation spectrometry. Void, Octa, Hexa, Tetra, Di, and Mono denote the respective elution volumes of blue dextran; heparin octa-, hexa-, tetra-, and disaccharides; and glucosamine.

least 12 hr after dosing. By 24 hr, no anti-factor Xa activity was detectable. No aPTT activity could be demonstrated at any time point. When the various peaks shown in Fig. 3 were examined for anticoagulant activity, the oligosaccharide peak was shown to possess ≈70% of heparin's anticoagulant activity as measured by anti-factor Xa activity, whereas no aPTT activity could be detected. No anti-factor Xa activity was detectable in the mono- and disaccharide fractions.

Metabolism of the Heparin Fragments Decreases Their Anticoagulant Activity. In addition to urinary clearance, the heparin fragments were subject to metabolic degradation, which decreased their anti-factor Xa activity. By 24 hr, ≈0.4% of the initial dose was present in the blood of rats that received [^{35}S]heparin and 1% in rats that received [^{3}H]heparin (Fig. 1). However, no anti-factor Xa activity was detectable. The oligosaccharides in the plasma (Fig. 3) that possessed anti-factor Xa activity were degraded to di- and monosaccharides with no anti-factor Xa activity, as shown by the urine profiles (Fig. 4). In addition to depolymerization of higher molecular weight material, N-desulfation occurred as indicated by the presence of ^{3}H-labeled oligosaccharides (the ^{3}H label is at the reducing end of the molecule), while ^{35}S-labeled material of the same size was not demonstrable (the ^{35}S label is on the glucosamine residues) as shown in Fig. 4. No anti-factor Xa activity was demonstrated in oligosaccharide fractions present in the urine.

FIG. 2. Appearance of radioactivity in the urine of rats after an oral dose (100 mg/kg) of either [^{35}S]heparin (A) or [^{3}H]heparin (B). The values are averages from three rats (±SD).

DISCUSSION

These experiments demonstrate the following: (i) Heparin fragments are continuously released into the bloodstream of

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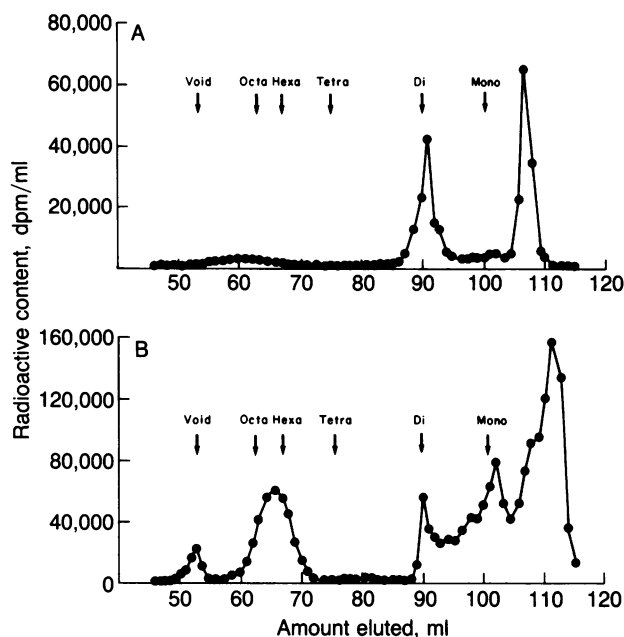
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FIG. 4. Gel permeation chromatography of radiolabeled material excreted into the urine of rats during the first 5 hr after an oral dose of either [³⁵S]heparin (*A*) or [³H]heparin (*B*). Each sample was analyzed as described (legend of Fig. 3). Void, Octa, Hexa, Tetra, Di, and Mono denote the respective elution volumes of blue dextran; heparin octa-, hexa-, tetra-, and disaccharides; and glucosamine.

rats for at least 12 hr after a single oral dose of heparin. (*ii*) The anti-factor Xa activity appearing in the plasma is associated with the presence of fragments up to oligosaccharide size. (*iii*) Metabolism of these heparin fragments results in loss of their anticoagulant activity.

The gastrointestinal tract appears to degrade heparin to fragments that are absorbed into the circulation. This is suggested by two observations: (*i*) While heparin [average molecular weight, 15,000 (20)] exhibits anticoagulant activity as measured by aPTT as well as by factor Xa assays, the fragments found in plasma after oral heparin only possess anti-factor Xa activity. While the presence of some fragments with aPTT activity cannot be totally excluded, their concentration would have to be very low, since no aPTT activity was detected even in the oligosaccharide fraction isolated from plasma. Anti-factor Xa activity has been associated with fragments as small as tetrasaccharides (16, 21), while thrombin activity, as measured by aPTT, is associated with fragments of at least tetradodecasaccharide size, corresponding to a molecular weight of ≈4000 (22). (*ii*) The recovery of radioactivity in the urine is ≈10% of the dose in rats treated with [³H]heparin, but it is 17% in rats treated with [³⁵S]heparin. This is in contrast to what has been reported for
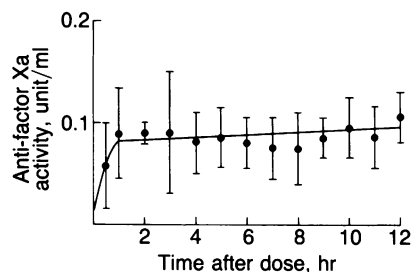

FIG. 5. Appearance of anti-factor Xa activity in the plasma of rats (*n* = 6) after an oral dose of heparin (100 mg/kg). The points represent average values (±SD).

rats receiving heparin intravenously, where the urinary excretion of the two isotopes is almost the same (16, 23). Since [³H]heparin is labeled only at the reducing end, while the ³⁵S label is evenly distributed throughout the molecule, this would be the expected result if heparin were cleaved to lower molecular weight fragments in the gastrointestinal tract. If a certain fraction of heparin fragments were released into the bloodstream, the heparin fragments generated by cleavage of [³⁵S]heparin would always be radiolabeled, while only fragments containing the reducing end of [³H]heparin would be radiolabeled. Finally, Lund *et al.* (24) have described heparin-degrading activity partially purified from canine intestinal mucosa, while Young and Horner (25) have described macromolecular heparin depolymerase activity in the rat intestine.

Heparin-derived material is released into the plasma for at least 12 hr after a dose of either [³H]- or [³⁵S]heparin. However, the estimated plasma concentration differs depending on which isotope is used. It is interesting that the same difference is found when the two isotopes are given intravenously. For example, the amount of radioactivity in the blood of rats dosed with [³H]heparin (150 units/kg) has been reported to be 18%, 10%, and 7% of the dose 1, 2, and 5 hr, respectively, after dosing (23), while the similar values in rats dosed with [³⁵S]heparin are 8%, 4%, and 2% (16). This suggests the presence of desulfated heparin-derived material associated with formed blood components or with plasma proteins. Once in the bloodstream, the heparin fragments are extensively metabolized, which results in eventual loss of anticoagulant activity. Degradation by endogenous heparinases is suggested by the presence of di- and monosaccharides in the plasma (Fig. 3). These small fragments will preferentially be excreted into the urine because of their molecular size (16). This is supported by the present finding of almost exclusively low molecular weight fragments in the urine.

The presence of ³H-labeled oligosaccharides in the urine but not the corresponding ³⁵S-labeled oligosaccharide fractions (Fig. 4) suggests extensive desulfation of *N*-sulfate groups. This is further supported by constant urinary excretion of radioactive material in rats receiving [³⁵S]heparin, while practically no further excretion takes place in rats that received [³H]heparin after 24 hr. Constant slow release of ³⁵S-labeled inorganic sulfate into the urine has been demonstrated in rats receiving [³⁵S]heparin fragments (16) or [³⁵S]heparin (26) intravenously, while the urinary excretion of ³H-labeled material is found to be almost completed within 5 hr after the dose (23). The desulfation is most likely a result of the action of endogenous heparin *N*-desulfatases, previously demonstrated in rat spleen and liver (26, 27), from which the released sulfate is either excreted into the urine or transferred to endogenous mucopolysaccharides via the sulfate pool (28).

The anticoagulant activity of heparin has been shown to be dependent both on size and on the sulfate groups, particularly the N-sulfated ones (29). This corresponds to the present finding of absence of anti-factor Xa activity in the urine. It is also consistent with the absence of anticoagulant activity in the plasma 24 hr after dosing, since previous studies have shown that the radioactivity at this time is associated with low molecular weight material of no more than disaccharide size (1).

We have previously shown that intravenous heparin administration to rats (150 units/kg) results in an initial plasma level of ≈2 units/ml, which is rapidly reduced with a biologic half-life of 50 min (16). The present study shows that a single oral dose of heparin (15,000 units/kg) results in a continuous low level of anti-factor Xa (≈0.1 unit/ml) for at least 12 hr. For new functions of heparin being discovered (for review, see ref. 30), it is not known whether a continuous dose of heparin differs from intermittent bolus doses in biologic

efficacy. There may be biological systems—for example, angiogenesis modulation—in which continuous exposure to the drug may be necessary. In this case, the oral route of administration might be more effective.

It has also been shown that low blood levels of heparin may be useful in preventing thrombosis. Sagar *et al.* (31) demonstrated that women taking oral contraceptives had increased risk of thromboembolic complications with surgery due to diminished levels of anti-thrombin III. This risk was normalized with a single subcutaneous injection of 2500 units of heparin, which achieved a peak plasma heparin concentration of 0.05 unit/ml. Since the animals in the present study achieved plasma heparin levels of 0.1 unit/ml for up to 12 hr, a single oral dose of heparin preoperatively may be of use as prophylaxis against perioperative thrombotic complications in high-risk patients. These potential uses for oral heparin deserve more study.

In summary, this work demonstrates that fragments of heparin appear in the bloodstream when the drug is given orally. Although this work does not support the idea that oral heparin may be of use as a clinical anticoagulant, it raises the strong possibility that the oral route of heparin administration may be effective in the prophylaxis against thrombosis. Finally, the demonstration of heparin fragments in the bloodstream after oral administration raises the possibility that these fragments can be delivered with an appropriate steroid to inhibit angiogenesis.

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