Enzymic Degradation of Heparin. A Glucosaminidase and a Glycuronidase from *Flavobacterium heparinum**

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ABSTRACT: A glucosaminidase and a glycuronidase were isolated and characterized from adapted cells of *Flavobacterium heparinum*. These enzymes were fractionated by Sephadex gel filtration which revealed the presence of a third enzyme characterized as a sulfoesterase. The glucosaminidase degrades heparin to sulfated disaccharides and oligosaccharides. The glycuronidase hydrolyzes the sulfated disaccharides to glucosamine 2,6-disulfate and the sulfoesterase catalyzes the partial desulfation of the trisulfated disaccharide.

Attention is drawn to the importance for the study of the structure of heparin of the consecutive use of these enzyme activities, glucosaminidase, glycuronidase, sulfoesterase, together with the sulfamidase and sulfoesterase previously described. A scheme for the enzymic degradation of heparin by *F. heparinum* enzymes is also presented.

Induced enzymes from *Flavobacterium heparinum* degrade heparin to sulfated monosaccharides and oligosaccharides. These products have been identified as glucosamine, glucosamine *N*-sulfate,[1] glucosamine 2,6-disulfate, two disaccharides with two and three sulfate residues, and sulfated tetra- and hexasaccharides (Dietrich, 1968a,b). The isolation of these compounds suggested a pattern of degradation of heparin in which at least four enzymes would be involved: one degrading heparin to oligo- and disaccharides, another degrading the disaccharides to monosaccharides, and finally sulfatases removing *N*- and *O*-sulfate groups from the molecule. Two of these enzymes have been purified and characterized as a sulfamidase and a sulfoesterase (Dietrich, 1968b). These enzymes are highly specific for glucosamine 2,6-disulfate, which would account for the presence of glucosamine and glucosamine *N*-sulfate as products of heparin degradation by crude enzyme extracts of *F. heparinum*, and suggests that heparin must be previously degraded to monosaccharides in order to be desulfated. The characterization of the sulfamidase as well as its substrate specificity has been recently confirmed by Lloyd *et al.* (1968). Linker and Hovingh (1965) reported the separation of two enzymic activities from *F. heparinum*, one of them capable of degrading heparin to small molecular weight ultraviolet-absorbing products and another converting these products subsequently into monosaccharides. Based on these results these authors postulated that one of the enzymes was an eliminase and the other an hydrolase. Nevertheless, the products of these reactions, with the exception of glucosamine *N*,*O*-disulfate, were not identified, nor the enzymes fully characterized. The products obtained by Linker and Hovingh (1965) differ from the compounds described by Dietrich (1968a) in chromatographic behavior and yield (which was at least one order of magnitude less with the Linker and Hovingh system). Thus the reactions proposed by Linker and Hovingh do not account for the formation of the sulfated oligo-, di-, and monosaccharides reported by Dietrich (1968a).

This paper describes and characterizes a glucosaminidase that degrades heparin to sulfated oligo- and disaccharides and a glycuronidase that hydrolyzes these products to sulfated monosaccharides. Preliminary accounts of this work have been presented (Dietrich, 1968c,d).

Materials and Methods

Preparation of Enzymes. F. heparinum (American Type Culture Collection No. 13,125) was grown with agitation in Trypticase Soy Broth without dextrose, containing 150 mg of heparin (Lederle Laboratories)/l. After growth for 36 hr at room temperature the cells were harvested at 10,000*g* for 15 min, washed with 0.02 M potassium phosphate buffer (pH 7.0), and freeze dried. Dried cells (200 mg) were resuspended in 10 ml of 0.02 M potassium phosphate buffer and treated ultrasonically for 5 min in a Bronwill sonicator at 3°. The suspension was then diluted to 30 ml with the same buffer and centrifuged at 10,000*g* for 30 min. The supernatant fraction was then centrifuged at 100,000*g* for 1 hr. The precipitate formed was discarded and the supernatant was freeze dried. This supernatant fraction (80 mg) was reconstituted with 1 ml of water and applied to a Sephadex G-200

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[1] The abbreviations used are: glucosamine *N*-sulfate, 2-deoxy-2-sulfoamino-D-glucose; glucosamine 2,6-disulfate, 2-deoxy-2-sulfoamino-D-glucose 6-*O*-sulfate; disulfated disaccharide, uronyl-2-deoxy-2-sulfoamino-D-glucose 6-*O*-sulfate; trisulfated disaccharide, 3-sulfouronyl-2-deoxy-2-sulfoamino-D-glucose 6-*O*-sulfate.

(Pharmacia, Upsala, Sweden) column (1.8 × 50 cm) previously equilibrated with 0.02 M phosphate buffer (pH 7.0). The proteins were then eluted with the same buffer. After the void volume of the column (about 30 ml), 3-ml fractions were collected. In some instances the phosphate buffer was substituted by 0.02 M ammonium carbonate buffer. Essentially the same fractionation was obtained. The fractions obtained were then assayed with the various substrates as described below. All the operations were carried out at 3°.

Assay of Enzymes. A typical incubation mixture contained 20 mM phosphate buffer (pH 7.0), 5 mM $MgCl_2$, 50 μg of enzyme, 25 μg of heparin (0.25 mCi/g) or other substrates, and additions as indicated, in a final volume of 30 μl. After inactivation by heating the reaction mixtures were spotted on Whatman No. 1 filter paper and chromatographed in isobutyric acid–1 M NH_3 (5:3, v/v) for 24 hr (solvent A) or butanol–acetic acid–water (10:3:6 v/v) (solvent B). In some instances electrophoresis and thin-layer chromatography were also used as already described (Dietrich, 1968a). In order to detect the radioactive products radioautograms were prepared by exposure to Kodak Royal Blue X-ray film (Eastman Kodak Co.) for 3 days. The areas of the chromatogram containing the radioactive material were then cut out and counted in 10 ml of a solution of 5 g of diphenyloxazole in 1 l. of toluene in a Beckman liquid scintillation spectrometer (LS-100). The measured counts were corrected for the natural decay of ^{35}S (half-life 88 days).

Chemicals. Heparin *N*-[^{35}S]SO_4 was obtained from Calbiochem (Los Angeles, Calif.) with a specific activity of 0.25 mCi/g. (Hydrolysis of this compound with 0.04 N HCl for 1 hr at 100° releases 95–98% of its radioactivity as inorganic sulfate.) The compound had the same electrophoretic mobility as the other commercial heparins and an anticoagulant activity of 160 IU/mg by the USP assay. *N*-[^{35}S]SO_4-labeled glucosamine 2,6-disulfate, *N*-sulfated disaccharides, tetra- and hexasaccharides were prepared from *N*-[^{35}S]SO_4-labeled heparin by degradation with crude enzymes from *F. heparinum* as previously described for nonradioactive degradation products (Dietrich, 1968a). These radioactive products have the same electrophoretic and chromatographic mobilities in three solvent systems as the degradation products obtained from five different commercial heparin preparations. These commercial heparin preparations were obtained from Nutritional Biochemicals Co., Eli Lilly Co., Abbott Laboratories, Lederle Laboratories, and Upjohn Co.

Other Methods. Protein was measured by the method of Lowry *et al.* (1951). Reducing sugars were detected on thin-layer and paper chromatography by the $AgNO_3$ reagent. Nonradioactive oligosaccharides and heparin were detected by the toluidine blue reagent as described previously (Dietrich, 1968a).

Results

N-[^{35}S]SO_4-Labeled Degradation Products from Heparin N-[^{35}S]SO_4. Figure 1 shows a paper chromatogram of the degradation products of heparin obtained with crude supernatant fraction of *F. heparinium*. Each of the products after purification migrates as a single component in solvent A (shown in Figure 1), in solvent B, on thin-layer chromatography, and on electrophoresis. Furthermore they are indistinguishable from the products obtained by enzymic degradation from five different commercial heparin preparations in those systems. They have been identified as glucosamine 2,6-disulfate, disulfated disaccharide, and trisulfated dissaccharide (Dietrich, 1968a). A radioactive tetra- and a hexasaccharide were also isolated using the same purification procedure. All these products as well as heparin were used subsequently as substrates to test the activity of the several enzymes involved in the degradation.

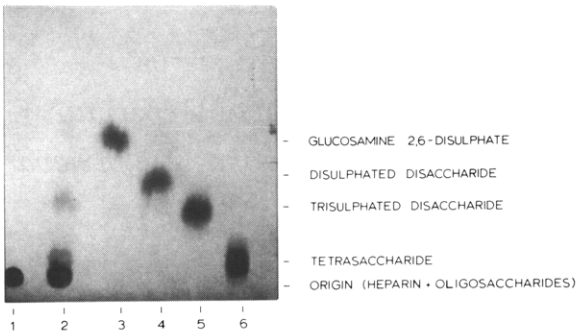

FIGURE 1: Chromatography of the purified radioactive products obtained from heparin *N*-[^{35}S]SO_4. Heparin *N*-[^{35}S]SO_4 (50 μg) was incubated with 50 μg of the supernatant of 100,000*g* in 5 mM $MgCl_2$ and 0.03 M potassium phosphate buffer (pH 7.0). After 3-hr incubation (tube 2) or at zero time (tube 1) the reaction was stopped by heating (100° for 1 min), spotted on Whatman No. 1 filter paper, and chromatographed in solvent A for 48 hr together with the purified fractions obtained from a large-scale incubation. Glucosamine 2,6-disulfate (3), disulfated disaccharide (4), trisulfated disaccharide (5), and sulfated tetrasaccharide (6) were localized in the chromatogram by radioautography.

Activity of the 100,000g Supernatant Fraction on Heparin and Oligosaccharides. When heparin is the substrate, glucosamine 2,6-disulfate is the major product accumulated after a long incubation using excess of supernatant enzymes (Figure 2). At early stages of incubation the tetrasaccharide is also produced in amounts comparable with glucosamine 2,6-disulfate but subsequently decreases. The two disaccharides are not accumulated to a great extent under these conditions. When the experiment is performed with excess of substrate the accumulation of higher amounts of disaccharides occurs (Figure 1). This result seems to indicate the existence of two enzymes in the degradation process, a glucosaminidase degrading heparin to disaccharides and a glycuronidase that rapidly converts these compounds into glucosamine 2,6-disulfate. The relative accumulation with time of disaccharides would probably be due to different rates of formation and degradation of these compounds by the two enzymes. The degradation of the hexasaccharide proceeds at a much slower rate as compared with heparin and results

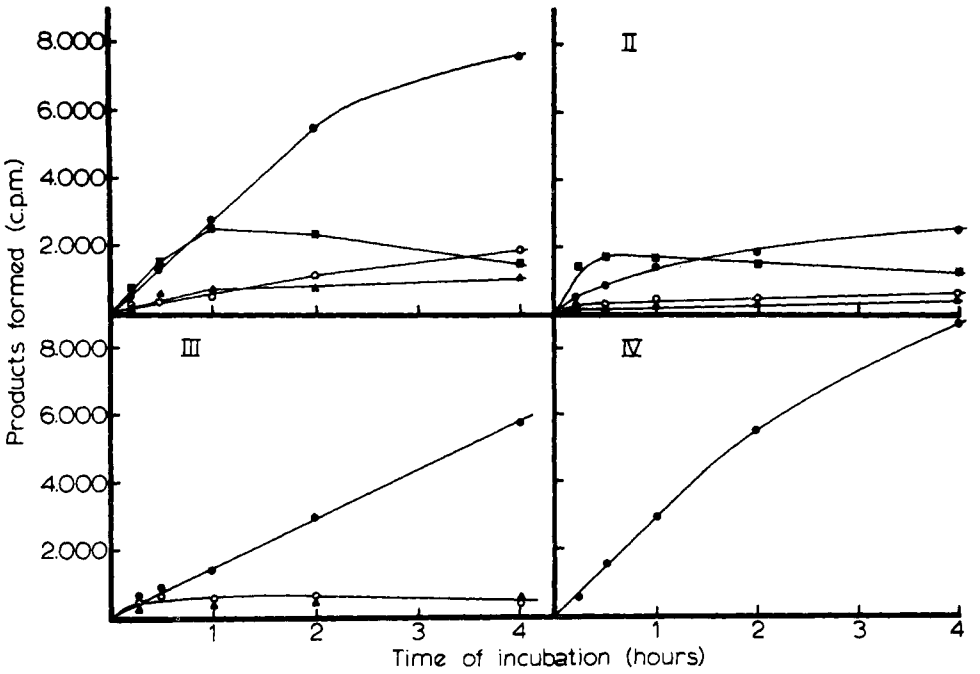

FIGURE 2: Time course of degradation of heparin and degradation products by supernatant of 100,000*g*. The incubation mixture contained: 500 μg of supernatant enzymes and 200 μg (100,000 cpm) of *N*-[^{35}S]SO_4-labeled compounds in 5 mM $MgCl_2$ and 0.04 M phosphate buffer (pH 7.0) to a final volume of 110 μl. Aliquots of 20 μl were heated at the times indicated, spotted on paper, and chromatographed in solvent A. The following substrates were incubated: I, heparin, II, hexasaccharide; III, tetrasaccharide; and IV, trisulfated disaccharide. The products: glucosamine 2,6-disulfate (●—●), disulfated disaccharide (▲—▲), trisulfated disaccharide (○—○), and tetrasaccharide (■—■) were localized in the chromatogram by radioautography and counted.

also in an early accumulation of the tetrasaccharide. When the tetrasaccharide is used as substrate, the degradation rate is intermediate between heparin and hexasaccharide and glucosamine 2,6-disulfate is the only product accumulated. The trisulfated disaccharide is degraded to glucosamine 2,6-disulfate at the same rate as heparin. These results indicate that heparin is the best substrate for the glucosaminidase, which in turn has low affinity for the oligosaccharides. Another observation is that the tetrasaccharide seems to be degraded faster than the hexasaccharide to glucosamine 2,6-disulfate. If one postulates the existence of a second enzyme, the glycuronidase, specific for the disaccharides, this faster formation of product can be explained by the fact that the tetrasaccharide is split into two disaccharide units while the hexasaccharide produces only one disaccharide at a time resulting in an apparent lower affinity for the enzyme. Thus the concentration of the disaccharide may be the limiting factor for the formation of glucosamine 2,6-disulfate. In order to prove these assumptions a fractionation of the enzymes was necessary.

Fractionation of 100,000g Supernatant by Sephadex G-200. Five different enzymic activities could be demonstrated in the supernatant of 100,000*g* centrifugation. Four of these are illustrated in Figure 3. Each fraction of the Sephadex column was tested with heparin, disaccharides, and glucosamine 2,6-disulfate as substrate. Heparin is converted into oligosaccharides and disaccharides by the action of a glucosaminidase. The end product of this enzyme is mainly trisulfated disaccharide (see Figure 4). This enzyme precedes in the elution pattern a glycuronidase which hydrolyzes the two disaccharides to glucosamine 2,6-disulfate. The glycuronidase appears split into two peaks of activity for which there is no explanation at this moment. Both disaccharides were tested with all the Sephadex fractions and identical elution patterns were observed. A sulfamidase which desulfates glucosamine 2,6-disulfate to glucosamine 6-sulfate and a sulfoesterase which desulfates the 6-sulfate of glucosamine 2,6-disulfate are the first enzymes to be eluted. These enzymes are partially precipitated by 100,000*g* centrifugation and have been already described (Dietrich, 1968b,c). Another sulfoesterase could be identified by the present fractionation method (see Figure 3). It converts the trisulfated disaccharide to disulfated disaccharide. This enzyme activity could be detected between the glucosaminidase and glycuronidase fractions and it does not act upon glucosamine 2,6-disulfate. The activity obtained for this enzyme should be considered only approximate since the presence of the glycuronidase hydrolyzing both disaccharides does not permit its quantitative measurement. The partially separated activities of these enzymes upon heparin and trisulfated disaccharide are illustrated in Figure 4. Fraction 3 of the Sephadex column containing mostly the glucosaminidase degrades heparin to oligosaccharides and trisulfated disaccharide. Almost no degradation of trisulfated disaccharide was observed when incubated

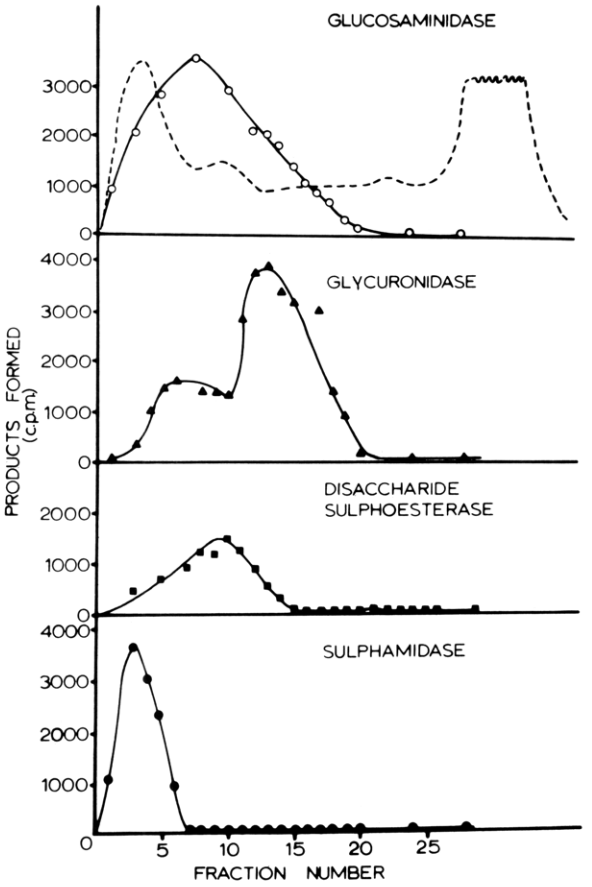

FIGURE 3: Fractionation of enzymes from supernatant of 100,000*g* by Sephadex G-200. Sephadex G-200 fractions (20 μl) containing 0.02 M phosphate buffer were incubated with 40 μg of *N*-[^{35}S]SO_4 radioactive substrates as specified below containing 7 mM $MgCl_2$. The reaction mixtures were spotted on paper, chromatographed in solvent A, and radioautographed. The products formed were counted and plotted. Heparin *N*-[^{35}S]SO_4 was used as substrate for the assay of the glucosaminidase (○—○). The products that moved from the origin (from mono- to tetrasaccharide) were considered to be the result of activity of this enzyme. *N*-[^{35}S]SO_4-labeled trisulfated disaccharide was used as substrate to measure the activity of the glycuronidase (▲—▲). Radioactive glucosamine 2,6-disulfate formed from this reaction was counted. The amount of disulfated disaccharide formed from the trisulfated disaccharide represented the activity of the disaccharide sulfoesterase (■—■). The sulfamidase (●—●) activity was measured by the release of inorganic sulfate (identified by electrophoresis) from glucosamine 2,6-disulfate. The reaction mixtures were incubated for 18 hr at room temperature except for the one containing heparin as substrate (glucosaminidase) which was incubated for 6 hr. The dotted line represents the content of protein in these fractions (2000 cpm = 2 mg/ml of protein).

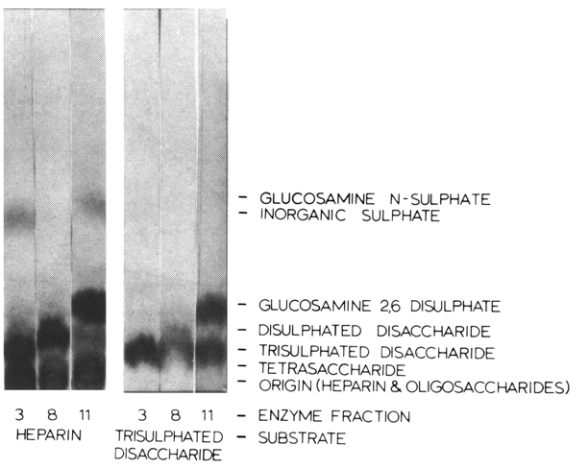

FIGURE 4: Enzymic activities of some Sephadex fractions upon heparin and trisulfated disaccharide. Assays were carried out as described in Figure 3 except that the time of incubation for the glucosaminidase activity was also 18 hr. Also the reaction mixtures were chromatographed in solvent A for 24 hr only.

with this fraction. Fraction 8, which contains the glucosaminidase and disaccharide sulfoesterase, when incubated with heparin produces accumulation of disulfated disaccharide. It is worth mentioning that in this experiment 80% of the incubated radioactive heparin was transformed into disaccharides. When the trisulfated disaccharide is incubated with this fraction, formation of disulfated disaccharide is observed. Fraction 11 contains mostly glycuronidase and some glucosaminidase. This fraction converts heparin and the trisulfated disaccharide into glucosamine 2,6-disulfate without accumulation of any intermediate product.

Properties of the Glucosaminidase and Glycuronidase. In order to study some properties of the two enzymes another Sephadex G-200 column fractionation was performed using ammonium carbonate as eluent. The individual fractions obtained were assayed with the substrate in the same way as described in Figure 3. The fractions containing mostly the activities of glucosaminidase, of disaccharide sulfoesterase, and of glycuronidase were pooled separately, lyophilized, and resuspended in sodium acetate buffer 0.01 M. The products formed by these fractions are described in Table I. Pooled fraction I (tubes 5–7) contained most

TABLE I: Products Formed from Heparin *N*-[^{35}S]SO_4 by Degradation with Pooled Enzyme Fractions of Sephadex G-200.[a]

Enzyme Fraction	Products Formed (cpm)		
	Trisulfated Disaccharide	Disulfated Disaccharide	Glucosamine 2,6-Disulfate
I	5457	1026	634
II	1236	3940	1233
III	1118	1196	4663

[a] Protein fraction (50 μg) was incubated with 75 μg of heparin *N*-[^{35}S]SO_4 in 5 mM $MgCl_2$ and 0.03 M phosphate buffer (pH 7.0) to a final volume of 40 μl. After 18-hr incubation at room temperature the reaction mixtures were heated at 100° for 1 min and 20 μl was spotted on Whatman No. 1 paper and chromatographed. The products formed in this reaction were analyzed as described in Figure 3.

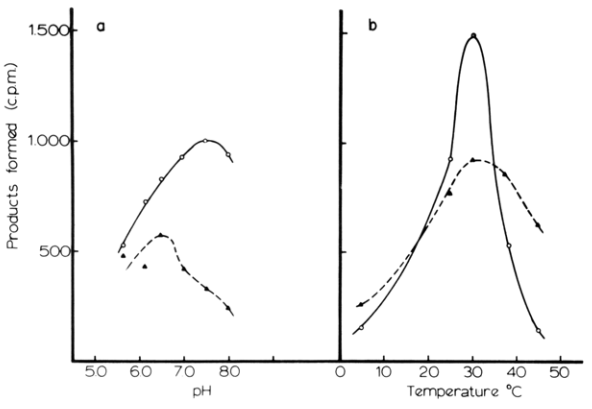

FIGURE 5: Effects of pH and temperature on the activities of the glucosaminidase and the glycuronidase. Glucosaminidase (○—○) or glycuronidase (▲—▲) fractions (20 μg) were incubated with 50 μg of heparin *N*-[^{35}S]SO_4 and *N*-[^{35}S]SO_4-labeled trisulfated disaccharide, respectively. The incubation mixtures containing 5 mM $MgCl_2$ and 0.02 M potassium phosphate buffer (pH 7.0) were incubated for 3 hr at room temperature. The products formed from each of the enzymes were then analyzed and measured as described in Figure 3.

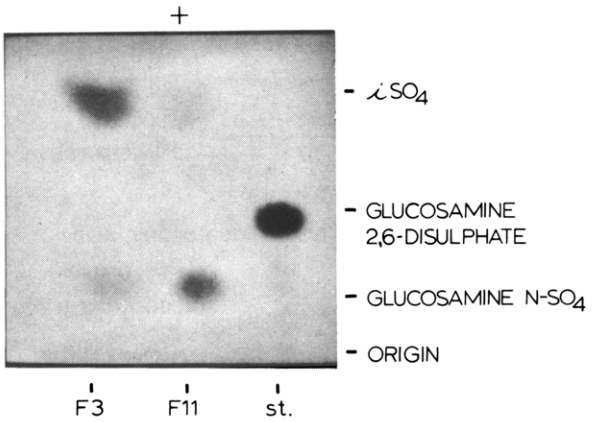

FIGURE 6: Electrophoretic mobility of fast-moving components produced from heparin by purified enzyme fractions of *F. heparinum* (for details, see text).

glucosaminidase activity. Fraction II (tubes 8–10) contained the glucosaminidase with disaccharide sulfoesterase activities and fraction III (tubes 14–19) contained most glycuronidase activity with some glucosaminidase. The glucosaminidase is activated by $MgCl_2$ and phosphate. The extent of activation for both ions at 5×10^{-3} M concentration is about 30%. The lcyguronidase is insensitive to these ions at this onccentration.

The pH optimum of activity for the glucosaminidase is 7.5 and for the glycuronidase around 6.5 (Figure 5a). The temperature optimum of activity for both enzymes is 30°. It is interesting to note that the glucosaminidase is very sensitive to temperature changes compared with the glycuronidase which is again relatively insensitive (Figure 5b).

Product Identification. The di- and trisulfated disaccharides as well as glucosamine 2,6-disulfate formed by the action of the purified enzymes have the same electrophoretic and chromatographic mobilities in solvents A and B as those formed by the action of the crude enzyme extract. A fast-moving component is formed by the action of fractions 11–15 (see Figure 4) with the same chromatographic mobility of glucosamine *N*-sulfate in solvent A. This material was eluted and subjected to electrophoresis exhibiting again the mobility of glucosamine *N*-sulfate (Figure 6). This monosaccharide could not be formed by desulfation of glucosamine 2,6-disulfate since there are no monosaccharide sulfatases present in these fractions. Another alternative would be that glucosamine *N*-sulfate is one of the components of the native molecule of heparin. Table II shows the amount of glucosamine *N*-sulfate obtained from heparin as compared with glucosamine 2,6-disulfate also derived from heparin in the same experiment. The results indicate that a proportion of 1 glucosamine *N*-sulfate to about 10 glucosamine 2,6-disulfate would be present in the heparin molecule. Fractions 1–5 from the Sephadex G-200 column also form another fast-moving component which was identified as inorganic sulfate (Figure 6). The formation of this compound is due to the action of the sulfamidase also present in these fractions.

In order to find $AgNO_3$-reacting sugars as products of heparin degradation other than those labeled with radioactive sulfate, a semilarge-scale incubation was prepared with 200 μg of heparin *N*-[$^{35}SO_4$] as substrate and the three enzyme fractions separately. The reaction mixture was incubated for 24 hr containing the usual salts. The reaction mixture was then chromatographed in solvent A, radioautographed, and developed with silver nitrate reagent. Only very faint spots could be detected with the silver nitrate reagent which were devoid of radioactivity. It was roughly calculated that at least 90% of the products reacting with silver nitrate also contained radioactive sulfate.

TABLE II: Monosaccharides Obtained as Products of Incubation of Heparin with Monosaccharide Sulfatase-Free Glucosaminidase and Glycuronidase.[a]

Monosaccharide Product	cpm	Ratio
Glucosamine 2,6-disulfate	3495	9.6
Glucosamine *N*-sulfate	361	1.0

[a] Products obtained by incubating for 18 hr the fraction 12 from the Sephadex G-200 column with heparin as described in Figure 3.

Discussion

The results obtained so far indicate that heparin is first degraded to sulfated disaccharides and oligosaccharides by the action of a glucosaminidase. The two disaccharides found are then degraded to glucosamine

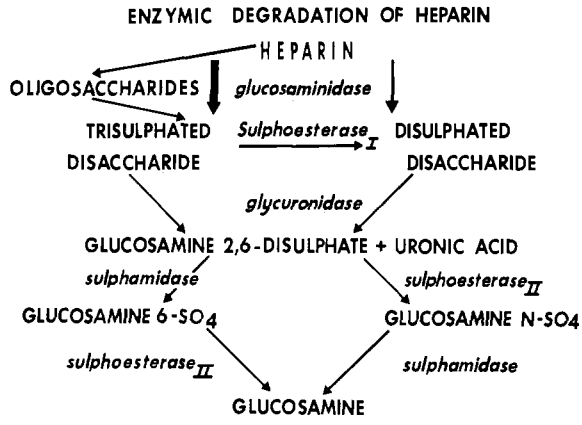

FIGURE 7: Scheme of the enzymic degradation of heparin by *F. heparinum* (for details, see text).

2,6-disulfate and uronic acid by the action of the glycuronidase. Glucosamine 2,6-disulfate is then desulfated to glucosamine by the action of a sulfamidase and a sulfoesterase (Dietrich, 1968b,c). The proposed steps in the degradation of heparin by *F. heparinum* enzymes are shown in Figure 7. The desulfation of the uronic acid residue seems to occur, at least partially, at the disaccharide level. It is difficult to understand the results obtained by Linker and Hovingh (1965) when compared with the results presented in this paper. These authors used the same method of fractionation of the enzymes but nevertheless the products obtained varied widely from those described in here. They observed the formation of compounds which had much faster chromatographic mobilities than glucosamine 2,6-disulfate in solvent B. The di- and oligosaccharides described in this paper had a much slower migration than glucosamine 2,6-disulfate, in the same chromatographic system. A possible interpretation for this discrepancy could be the following: the glucosaminidase in our system forms mainly trisulfated disaccharide. This enzyme is contaminated with two sulfatases which could slowly remove some of the sulfate groups from the disaccharide forming small amounts of compounds with faster chromatographic mobilities. This interpretation would also explain the small yield of degradation products obtained by those authors.

The question whether the glucosaminidase described above acts as an eliminase still remains unsolved. We are attempting to characterize, by another approach, the second product of the glycuronidase activity upon the disaccharide, the uronic acid residue, since the radioactive methods used in this paper do not permit such characterization. Only traces of compounds devoid of radioactivity were formed from heparin by the partially purified enzymes. These results could indicate that the uronic acid released undergoes other transformations such as the ones described by Ashwell *et al.* (1960), in *Escherichia coli*. Cifonelli and Dorfman (1962), Radhakrishnamurthy and Berenson (1963), and Wolfrom *et al.* (1968) have identified iduronic acid in heparin. Further, Perlin *et al.* (1968) have demonstrated by different chemical methods that the amounts of iduronic acid present in the heparin molecule were substantial. The significance of these findings will eventually be determined when the uronic acid residue or its products obtained from heparin with *F. heparinum* enzymes can be properly analyzed.

Other interesting observations derived from the fractionation of the glucosaminidase and glycuronidase concern the structure of heparin. The isolation of an enzyme fraction free from sulfatases (containing only the glucosaminidase and glycuronidase) able to degrade heparin to monosaccharides has made it possible to verify that glucosamine *N*-sulfate, although in small proportion when compared with glucosamine 2,6-disulfate, seems to be an integral part of the molecule of heparin. Danishefsky *et al.* (1968) have recently demonstrated by chemical degradation the presence of glucosamine 2,6-disulfate and glucosamine *N*-sulfate in the heparin molecule. Also, the isolation of the glucosaminidase which is able to degrade heparin almost completely to disaccharides indicates that these units are a fundamental part of the heparin molecule.

Acknowledgments

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