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Enzymic Degradation of Heparin

A SULPHAMIDASE AND A SULPHOESTERASE FROM *FLAVOBACTERIUM HEPARINUM*

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A sulphamidase and a sulphoesterase were isolated from adapted cells of *Flavobacterium heparinum*. These enzymes were partially purified from the 'heparinases' present in the bacterial extracts and characterized. The sulphamidase has a high specificity for glucosamine *N*-sulphate and glucosamine 2,6-disulphate. The activity decreases sharply with increasing molecular weight of the substrates tested. The sulphamidase and the sulphoesterase activities were distinguished from each other by their different sensitivities to concentration of phosphate ion and to temperature. The importance of these enzymes in the study of the structure of heparin is discussed.

Degradation of heparin by enzymes from adapted cells of *Flavobacterium heparinum* has led to the identification of several structurally related degradation products by Linker & Sampson (1960), Linker & Hovingh (1965) and Dietrich (1968*a*). Among these products, glucosamine *N*-sulphate, glucosamine 2,6-disulphate, di- and tri-sulphated disaccharides and oligosaccharides were isolated by Dietrich (1968*a*). Besides these compounds it was also observed by J. C. Karapally & C. P. Dietrich (unpublished work) that free glucosamine is liberated during the enzymic degradation. The presence of glucosamine and glucosamine *N*-sulphate among the degradation products of heparin could lead to the interpretation that these sugars are part of the intact molecule of heparin and would be released from heparin by the action of the 'heparinases' present in the crude enzymic extract. A second possibility is that they could have originated from one of the major isolated degradation products such as glucosamine 2,6-disulphate by a secondary enzymic degradation. If the latter is true, one would expect the presence of a sulphamidase and a sulphoesterase in the enzymic system. The existence of such enzymes in *F. heparinum* was suggested by Korn & Payza (1956) and by Korn (1957), who observed the appearance of free amino groups as well as an increase in periodate-oxidizable products after degradation of heparin by *F. heparinum*. Sulphoesterase activity has been described in *F. heparinum* as well as in other micro-organisms that split sulphate groups from mucopolysaccharides or their degradation products: this was reviewed by Dodgson (1966). The present paper describes the isolation and characterization of a sulphamidase and a sulphoesterase from *F. heparinum*. A preliminary account of this work has appeared (Dietrich, 1968*b*).

MATERIALS AND METHODS

Micro-organism. Slants of *F. heparinum* were generously given by Dr A. Linker, and also purchased from the American Type Culture Collection (collection no. 13125). The cells were initially transferred to 50ml. of trypticase-soy-broth-without-dextrose medium containing 150mg. of heparin/l. After growth for 36hr. at room temperature, 10ml. of the cell suspension was transferred to 3l. of the same medium and further agitated for 24hr. The cells were then harvested and washed twice with 0·02M-sodium acetate buffer, pH7·0, and freeze-dried.

Preparation of enzyme fractions. A sample (100mg.) of freeze-dried cells was resuspended in 10ml. of 0·02M-sodium acetate buffer, pH7·0, and treated ultrasonically for 5min. in a Bronwill Sonicator with cooling. The suspension was diluted to 30ml. with buffer and centrifuged at 10000*g* for 15min. The supernatant was collected and centrifuged at 100000*g* for 1hr. The pellet formed was washed twice with 30ml. portions of acetate buffer with subsequent centrifugation and finally resuspended in 2ml. of the same buffer. The supernatant of the 100000*g* centrifugation was freeze-dried. All the operations were carried out at 3°. Both fractions were kept at −20° without loss of activity.

Assay of enzymes. A typical incubation mixture contained: 1μl. of 0·4M-phosphate buffer, pH7·0; 1μl. of 0·1M-$MgCl_2$; 5μl. of enzyme (10mg./ml.); 5μl. of [*N-sulphate*-^{35}S]heparin (5mg./ml., 1·9mc/g.) or other substrates and additions as indicated, in a final volume of

20 μl. For assay of the sulphoesterase the phosphate buffer was replaced by sodium acetate buffer of the same concentration and pH. After inactivation by heating (100° for 1 min.) the reaction mixtures were spotted on a thin-layer chromatogram (Distillation Products, Rochester, N.Y., U.S.A.) and were chromatographed in isobutyric acid–1 M-NH_3 soln. (5:3, v/v) for 4 hr. In some instances electrophoresis was also used to separate inorganic sulphate, glucosamine 2,6-disulphate and glucosamine *N*-sulphate: the reaction mixture was spotted on Whatman 3MM filter paper and the electrophoresis was run in 0·3 M-pyridine–acetate buffer, pH 4·5, for 1 hr. at 300 v. To detect these substances and other products radioautographs were prepared by exposure to Kodak Royal Blue X-ray film for 3 days. The areas of the chromatogram containing the radioactive material were then cut and counted in 10 ml. of 0·5% (w/v) 2,5-diphenyloxazole in toluene in a Beckman LS-100 liquid-scintillation spectrometer. For thin-layer plates the solution was 0·5% (w/v) 2,5-diphenyloxazole–10% (w/v) naphthalene in dioxan. The plastic chromatogram sheet containing the radioactivity was cut and counted with 0·5 ml. of water and 10 ml. of dioxan scintillation solution. No quenching was observed with either scintillation liquid.

Chemicals. [*N-sulphate*-^{35}S]Heparin was obtained from Calbiochem (Los Angeles, Calif., U.S.A.), with a specific radioactivity of 1·9 mc/g. Hydrolysis of this compound with 0·04 N-HCl for 1 hr. at 100° releases 95–98% of its radioactivity as inorganic sulphate. The compound has the same electrophoretic mobility as other commercial heparin samples, and has an anticoagulant activity of 160 i.u./mg. by the U.S.P. assay. *N-sulphate*-^{35}S-labelled glucosamine *N*-sulphate, glucosamine 2,6-disulphate and *N*-sulphated disaccharides, tetrasaccharide and hexasaccharide were prepared from [*N-sulphate*-^{35}S]heparin by degradation with crude enzymes of *F. heparinum*, as previously described for non-radioactive degradation products (Dietrich, 1968*a*). The radioactive glucosamine *N*-sulphate was further purified from contaminants by electrophoresis. Chemically synthesized glucosamine *N*-sulphate was a gift from Professor A. S. Perlin, McGill University, Montreal, Canada.

Other methods. Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Reducing sugars were detected on thin-layer and paper chromatography by the $AgNO_3$ reagent. Oligosaccharides and heparin were detected by the toluidine blue reagent as described by Dietrich (1968*a*). Amino sugars were measured after acid hydrolysis (4 N-HCl at 100° for 4 hr.) by the method of Glick (1958). The specific radioactivity of the substrates was calculated by relating the content of glucosamine in a compound to its amount of radioactivity. The measured counts were corrected for the natural decay of ^{35}S (half-life 88 days).

RESULTS

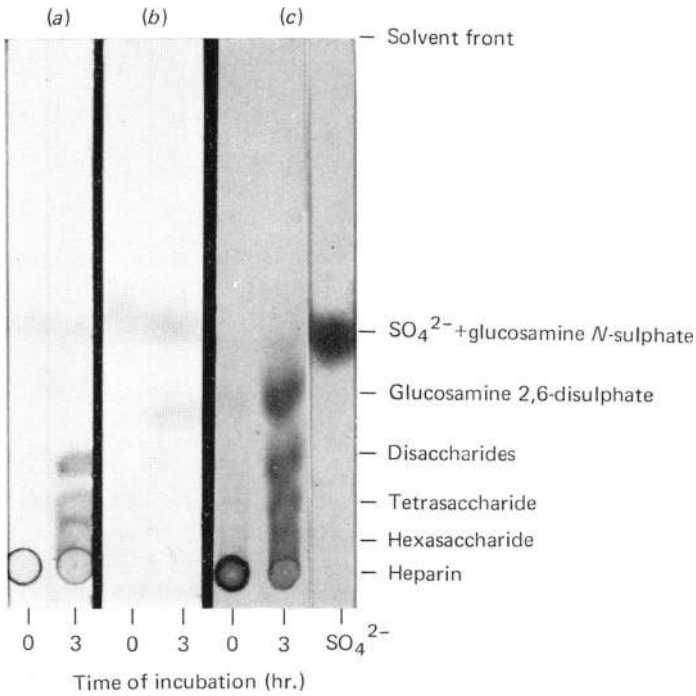
N-sulphate-^{35}S-labelled degradation products from [N-sulphate-^{35}S]heparin. Plate 1 shows a thin-layer chromatogram of the degradation products of heparin obtained by incubation of radioactive heparin with the 10000*g* supernatant. The labelled products had the same chromatographic mobilities as the ones obtained from non-labelled heparin. Further, all the compounds that stained with either toluidine blue dye or silver nitrate reagent showed the presence of *N*[^{35}S]-sulphate groups. Glucosamine *N*-sulphate and inorganic sulphate were also products of this degradation, although they were not separated by this chromatographic system. The resolution of these two products was easily accomplished by electrophoresis. The appearance of inorganic [^{35}S]sulphate in this incubation indicated the presence of a sulphamidase in the crude enzyme extract. To find whether the sulphamidase acted directly on heparin or on its degradation products, a separation of the activities characterized as 'heparinases' from the sulphatase activities was necessary. This was achieved by centrifugation of the extract at 100000*g*, as shown in Table 1.

The 100000*g* 'washed pellet' had the higher sulphamidase activity, whereas the 'heparinases'

Table 1. *Activities of sulphatases and 'heparinases' in protein fractions from* F. heparinum

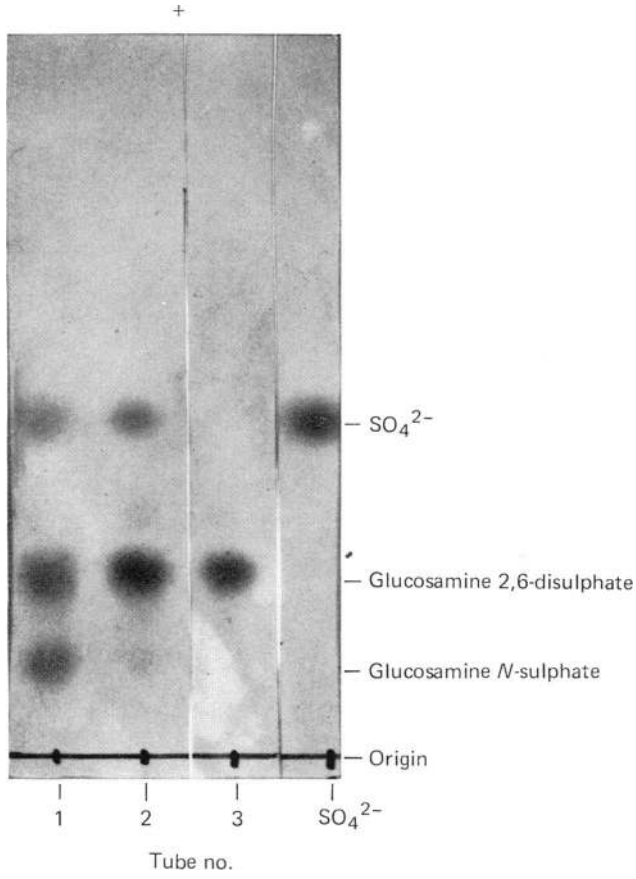
The sulphamidase and sulphoesterase activities were assayed as follows. The assay mixture contained glucosamine 2[^{35}S],6-disulphate (75000 counts/min.) in 5 mM-$MgCl_2$–0·03 M-sodium acetate buffer, pH 7·0, with about 50 μg. of protein fraction as indicated, in a final volume of 20 μl. The reactions were stopped by heating at 100° after 3 hr. incubation at 25°, and the incubation mixtures were spotted on Whatman 3MM filter paper and subjected to electrophoresis. Released inorganic [^{35}S]sulphate (sulphamidase activity) and glucosamine *N*[^{35}S]-sulphate (sulphoesterase activity) were located by radioautography and counted. Activity is expressed in mμmoles of product/mg. of protein/hr. Assays of the 'heparinases' were carried out as described above, except that [*N-sulphate*-^{35}S]heparin was used as substrate and the glucosamine 2[^{35}S],6-disulphate formed was isolated by thin-layer chromatography instead of electrophoresis. This compound was considered to be the end product of the degradation by the 'heparinases'.

Protein fraction	Activity (mμmoles/mg./hr.) Sulphamidase	Sulphoesterase	'Heparinases'
Supernatant (10000*g*)	29·3	65·9	562·0
Supernatant (100000*g*)	21·2	28·4	660·6
100000*g* 'pellet'	62·1	46·1	28·4
100000*g* 'pellet', twice washed	62·6	42·9	18·4



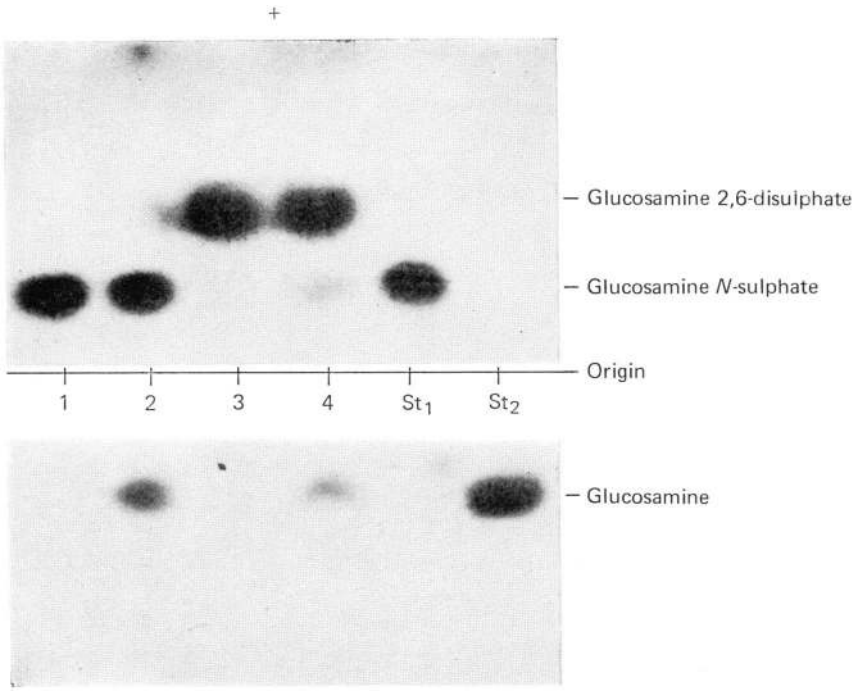
EXPLANATION OF PLATE 1

N-sulphate-^{35}S-labelled products obtained by enzymic degradation of [*N-sulphate*-^{35}S]heparin. The incubation mixture contained 50 μg. of ultrasonically treated cells and 100 μg. of heparin or 25 μg. of [*N-sulphate*-^{35}S]heparin in 5 mM-$MgCl_2$–0·02 M-phosphate buffer, pH 7·0, in a total volume of 20 μl. The reaction was stopped by heating at 100° for 1 min. after incubation for 3 hr. at 25°, or at zero time. The samples were centrifuged and the supernatant of the non-radioactive sample was spotted in duplicate on two thin-layer sheets; 10 μl. of the sample containing the radioactive heparin was spotted on another thin-layer sheet. After being developed for 4½ hr. in isobutyric acid–aq. NH_3 soln., the chromatograms were dried and stained: (*a*) with toluidine blue; (*b*) with $AgNO_3$ reagent. (*c*) Radioautography of the thin-layer containing the radioactive degradation products. SO_4^{2-} denotes inorganic sulphate.



EXPLANATION OF PLATE 2

Release of inorganic [^{35}S]SO_4 and glucosamine *N*[^{35}S]-sulphate from glucosamine 2[^{35}S],6-disulphate by the action of *F. heparinum* sulphatases. Glucosamine 2[^{35}S],6-disulphate (50000 counts/min.) was incubated with 40 μg. of 100000*g* 'pellet' in 5 mM-$MgCl_2$, and 0·03 M-sodium acetate buffer, pH 7·0 (tube 1), or 0·03 M-phosphate buffer, pH 7·0 (tube 2), in a total volume of 20 μl. The reaction was stopped by heating at 100° for 1 min. after incubation for 1 hr. at 25°, or at zero time (tube 3). The samples were spotted in Whatman 3MM filter paper and subjected to electrophoresis and radioautographed. SO_4^{2-} denotes inorganic sulphate.



EXPLANATION OF PLATE 3

Release of glucosamine from glucosamine *N*-sulphate or glucosamine 2,6-disulphate by the action of the sulphatases. Glucosamine *N*-sulphate (100 μg.) (tubes 1 and 2) or glucosamine 2,6-disulphate (100 μg.) (tubes 3 and 4) was incubated with 40 μg. of 100000*g* 'pellet' in 5 mM-$MgCl_2$, and 0·03 M-potassium phosphate buffer, pH 7·0, or 0·03 M-sodium acetate buffer, pH 7·0, respectively, in a total volume of 20 μl. The reaction was stopped by heating at 100° for 1 min. after incubation for 6 hr. at 25° (tubes 2 and 4), or at zero time (tubes 1 and 3). The samples were spotted in Whatman 3MM filter paper and subjected to electrophoresis. The paper was then dried and stained with $AgNO_3$ reagent. St_1, Glucosamine *N*-sulphate; St_2, glucosamine.

Table 2. *Substrate specificity of the sulphamidase*

Assays were performed as described for Plate 2, with 0·03 M-potassium phosphate buffer, pH 7·0, and about 50000 counts/min. of each substrate. The inorganic [^{35}S]sulphate formed was located by radioautography and counted.

N-sulphate-^{35}S-labelled substrate	Inorganic [^{35}S]sulphate released (counts/min.)
Glucosamine *N*-sulphate	6320
Glucosamine 2,6-disulphate	4734
Glucuronylglucosamine 2,6-disulphate	972
Tetrasaccharide from heparin	308
Hexasaccharide from heparin	286
Heparin	828

remained in the supernatant of the 100000*g* centrifugation. Radioactive glucosamine *N*-sulphate was also found in the incubation with 100000*g* 'washed pellet', indicating the presence of a sulphoesterase that hydrolysed the 6-sulphate group from glucosamine 2,6-disulphate. The sulphoesterase activity was also particle-bound. The two enzymes were distinguished from each other by their different properties, described below.

Substrate specificity of the sulphamidase. From the experiments described above there was no indication whether heparin or the degradation products were suitable substrates for the sulphamidase. Only after the separation of 'heparinases' from the sulphatase activities was it possible to perform a substrate-specificity test: this is reported in Table 2. Glucosamine *N*-sulphate and glucosamine 2,6-disulphate were indeed the best substrates for the sulphamidase. There was also some release of inorganic sulphate from the *N*-sulphated oligosaccharides and heparin. This could be explained by the contamination of my sulphamidase preparation with some 'heparinases' activities (Table 1). These enzymes degrade the oligosaccharides and heparin to glucosamine 2,6-disulphate, from which inorganic sulphate would ultimately be released. Nevertheless the possibility that the oligosaccharides and heparin could be poor substrates for the sulphamidase was not completely ruled out.

Sulphamidase and sulphoesterase activities in the 100000g pellet from adapted cells of F. heparinum. When glucosamine 2[^{35}S],6-disulphate was incubated with the 100000*g* 'pellet' in the absence of phosphate two radioactive products were formed that were identified as glucosamine *N*[^{35}S]-sulphate and inorganic [^{35}S]sulphate (Plate 2 and Table 3). When this incubation was carried out in the presence of phosphate, inorganic [^{35}S]sulphate but no glucosamine *N*[^{35}S]-sulphate was detected. The formation of glucosamine *N*[^{35}S]-sulphate was due to the activity of the sulphoesterase. Since the

Table 3. *Sulphamidase and sulphoesterase activities in the 100000g 'pellet'*

These are the quantitative results obtained from the experiment illustrated in Plate 2.

System	Product formed (counts/min.): Glucosamine *N*[^{35}S]-sulphate	Product formed (counts/min.): Inorganic [^{35}S]sulphate
100000*g* 'pellet' in sodium acetate buffer	18125	11632
100000*g* 'pellet' in sodium phosphate buffer	1699	11482

[^{35}S]sulphate label was in the 2-position of glucosamine 2,6-disulphate the release of inorganic [^{35}S]sulphate was due to the activity of the sulphamidase. The sugars released by the action of the sulphamidase and the sulphoesterase were also studied with non-labelled substrates. As shown in Plate 3, glucosamine *N*-sulphate was converted into glucosamine by the sulphamidase in the presence of phosphate, which inhibits the sulphoesterase activity. When this incubation was carried out in the absence of phosphate and with glucosamine 2,6-disulphate as substrate two products were formed: glucosamine *N*-sulphate and glucosamine. The formation of glucosamine *N*-sulphate is accounted for by the sulphoesterase activity and the formation of glucosamine was due to the action of both the sulphamidase and the sulphoesterase.

No accumulation of glucosamine 6-sulphate (which remains at the origin in the electrophoresis) was noticed in this experiment. This result suggests that this compound (formed by the action of the sulphamidase) might be a better substrate for the sulphoesterase than glucosamine 2,6-disulphate.

Several properties distinguish these two enzymes. Fig. 1 shows that the optimum temperature for the sulphoesterase was about 45° whereas that for the sulphamidase was 25°. The sulphoesterase was readily inhibited by phosphate in low concentration (0·03 M), whereas the sulphamidase was inhibited only at higher concentration (Fig. 2). The differences in properties between the two enzymes enabled me to determine their apparent K_m values without any further purification. The apparent K_m values were $1{\cdot}3 \times 10^{-4}$ M for the sulphamidase and 8×10^{-5} M for the sulphoesterase, when glucosamine 2,6-disulphate was used as substrate (Fig. 3).

Other properties of the enzymes. The activity of both enzymes showed a broad pH range between 6 and 8 with an optimum at about pH 7. Both enzymes were quite stable during the incubation period and were activated by Mg^{2+} ions with optimum concentration about 1 mM.

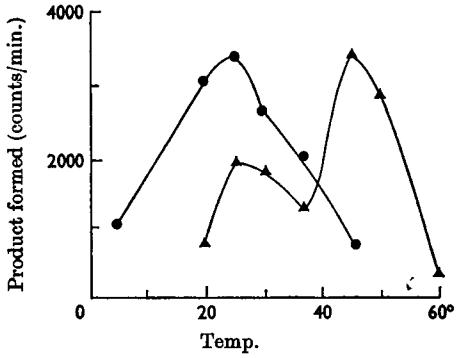


Fig. 1. Sulphamidase (●) and sulphoesterase (▲) activities at different temperatures. Glucosamine 2[^{35}S],6-disulphate (50000 counts/min.) was incubated with 40 μg. of 100000g 'pellet' in 5 mM-$MgCl_2$–0·03 M-sodium acetate buffer, pH 7·0, in a total volume of 20 μl. After incubation for 30 min. at the temperatures indicated, the reaction was stopped by heating (100° for 1 min.) and the mixture was subjected to paper electrophoresis and radioautographed. The inorganic [^{35}S]sulphate released (sulphamidase activity) and glucosamine N[^{35}S]-sulphate released (sulphoesterase activity), located by radioautography, were cut from the paper and counted.

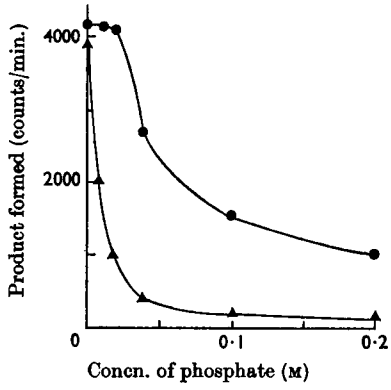


Fig. 2. Effect of phosphate concentration in the sulphamidase (●) and sulphoesterase (▲) activities. Assays were carried out as described in Fig. 1 except that the temperature of incubation was 25° for the sulphamidase activity and 45° for the sulphoesterase activity, and phosphate was added at the concentrations indicated.

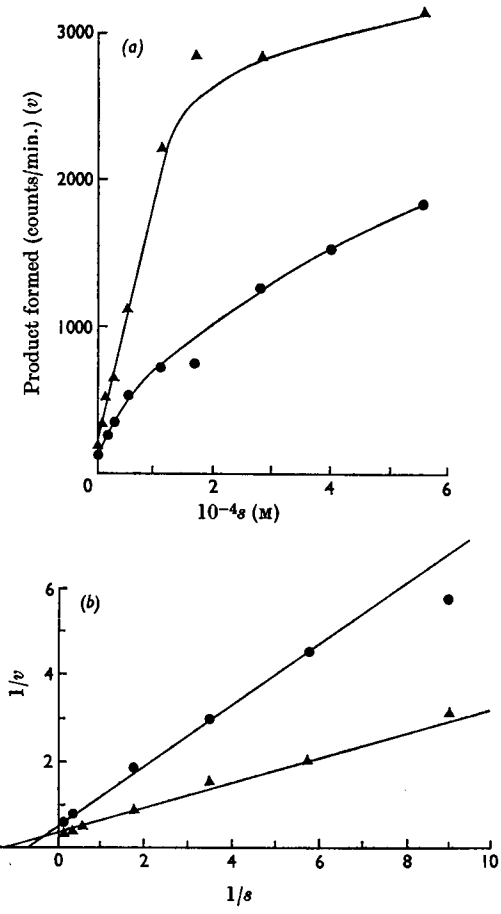


Fig. 3. (*a*) Dependence of reaction velocity on the concentration of glucosamine 2[^{35}S],6-disulphate, with the 100000g 'pellet'. Assays were carried out as described in Fig. 1 except that the temperature of incubation was 25° for the sulphamidase activity (●) and 45° for the sulphoesterase (▲) activity. Phosphate buffer instead of acetate buffer was used for the sulphamidase assay at a final concentration of 0·03 M. (*b*) Lineweaver–Burk plot of the results shown in (*a*). The K_m values are $1{\cdot}3\times10^{-4}$ M for sulphamidase and 8×10^{-5} M for sulphoesterase.

DISCUSSION

The major difficulty in the study of the sulphatases from *F. heparinum* has been the lack of adequate substrates for these enzymes (Dodgson, 1966). By the use of the products of enzymic degradation of heparin as substrates, combined with the use of [*N-sulphate*-^{35}S]heparin and its *N-sulphate*-^{35}S-labelled degradation products, it was possible to overcome this difficulty and disclose some of the characteristics of the two enzymes.

The isolated sulphamidase has a high specificity for glucosamine 2,6-disulphate and glucosamine *N*-sulphate, and is not inhibited by phosphate. It is temperature-sensitive with almost no activity at 45°. Korn & Payza (1956), using crude extracts of *F. heparinum*, observed that at 45° there was no degradation of heparin. They suggested that this was due to the inactivation of a sulphamidase

present in the crude extract. These authors suggested that the sulphamidase would be the first enzyme to act on heparin, removing *N*-sulphate groups from the molecule and rendering it susceptible to the action of hydrolases that are not temperature-sensitive. The hydrolases referred to are those that hydrolyse chondroitin sulphate and hyaluronic acid, and are also present in the crude extract.

My observations on the specificity of the sulphamidase towards glucosamine 2,6-disulphate rather than heparin suggests a different sequence in the degradation process in which the molecule would be first hydrolysed to glucosamine 2,6-disulphate. The fact that Korn & Payza (1956) did not obtain any degradation of heparin at 45° could be explained by the presence of specific temperature-sensitive 'heparinases' in the *F. heparinum* grown on heparin.

The sulphoesterase activity was inhibited by low concentration of phosphate and had optimum activity at 45°. These properties are similar to those described for the *Flavobacterium* and *Proteus* sulphoesterases.

The results obtained with these two enzymes permit some important considerations of the fine structure of heparin. The specificity of the sulphamidase and sulphoesterase towards glucosamine 2,6-disulphate suggests that the desulphation occurs at the monosaccharide level. Thus glucosamine and glucosamine *N*-sulphate seem to be formed from glucosamine 2,6-disulphate and not to be present as such in the heparin molecule.

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