Studies on the Heparin Sulphamidase Activity from Rat Spleen

INTRACELLULAR DISTRIBUTION AND CHARACTERIZATION OF THE ENZYME

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A sulphamidase activity present in rat spleen capable of hydrolysing *N*-[³⁵S]sulphated heparin was studied. This activity was associated with the lysosomal fraction. Studies *in vivo* showed that the rat is capable of significantly desulphating heparin. Lysosomes in all the major tissues can effectively accumulate heparin. The heparin sulphamidase and arylsulphatase activities from rat spleen were separated by isoelectric focusing. Heparin sulphamidase was a distinct entity from all the arylsulphatase activities.

N-[³⁵S]sulphated heparin is metabolized by mammals, as measured by the release of inorganic SO₄²⁻ (Lemaire *et al.*, 1967; Lloyd *et al.*, 1968). Several mammalian tissues can liberate SO₄²⁻ from *N*-[³⁵S]sulphated heparin and this activity is different from all the various arylsulphatases (EC 3.1.6.1) found in the same tissues (Friedman & Arsenis, 1972). The present results establish the intracellular distribution of the heparin sulphamidase (EC 3.—.—) in rat spleen. The enzyme was purified and compared with the arylsulphatases (EC 3.1.6.1), and its role in the degradation of heparin was studied both *in vivo* and *in vitro*. Heparin sulphamidase (EC 3.—.—) is reported to be deficient in some patients with the Sanfillipo syndrome (Matalon & Dorfman, 1973).

Materials and Methods

Materials

The following materials were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.: cytochrome *c* (horse heart; type III), phenolphthalein β -glucuronide, *p*-nitrophenyl β -D-galactopyranoside, *p*-nitrocatechol sulphate, *p*-nitrophenyl *N*-acetyl- β -D-glucosaminide, *p*-nitrophenyl α -D-mannoside, bovine haemoglobin (type II), heparin (Na⁺ salt; grade I), NAD⁺, NADPH, catalase (from ox liver), bovine serum albumin and alcohol dehydrogenase from yeast.

The following materials were purchased from Calbiochem, Los Angeles, Calif., U.S.A.: *p*-nitrophenyl phosphate, *p*-nitrophenyl sulphate, and *N*-[³⁵S]sulphated heparin.

Amersham-Searle, Arlington Heights, Ill., U.S.A., provided Na₂³⁵SO₄ and *N*-[³⁵S]sulphated heparin in the final stages of the work.

Sprague-Dawley or Holtzman rats (300±100g) were used for all the experiments.

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Methods

Assays. Acid phosphatase (EC 3.1.3.2) with *p*-nitrophenyl phosphate as substrate was measured from the *p*-nitrophenolate released (Arsenis & Tousler, 1968). Biuret-positive material released in the trichloroacetic acid supernatant from denatured haemoglobin was used to assay cathepsin D activity (EC 3.4.23.5) (Bowers *et al.*, 1967). *N*-Acetyl- β -D-glucosaminidase (EC 3.2.1.30), β -D-galactosidase (EC 3.2.1.23) and α -D-mannosidase (EC 3.2.1.24) activities were measured by the *p*-nitrophenolate ion released (Sellinger *et al.*, 1960; Frohwein & Gatt, 1966). Phenolphthalein β -glucuronide was used as the substrate for β -glucuronidase (EC 3.2.1.31) (Gianetto & de Duve, 1955). Cytochrome oxidase (EC 1.9.3.1) was assayed by the spectrophotometric method of Cooperstein & Lazarow (1951), by the rate of oxidation of reduced cytochrome *c*. NADPH-cytochrome *c* reductase (EC 1.6.2.4) was assayed by following the reduction of cytochrome *c* by NADPH (Sottocassa *et al.*, 1967). Catalase (EC 1.11.1.6) was assayed by the procedure of Martin & Ames (1961) or by a colorimetric assay (Baudhuin *et al.*, 1964). Alcohol dehydrogenase (EC 1.1.1.1) and glutamate dehydrogenase (EC 1.4.1.2) activities were measured by the increase in the absorption at 340nm caused by the reduction of NAD⁺ (Martin & Ames, 1961; Beaufay *et al.*, 1959). Protein in cell extracts was determined by the method of Lowry *et al.* (1951), and protein in the fractions after electrofocusing was determined from measurement of the absorption at 260 and 280nm (Warburg & Christian, 1942).

Heparin sulphamidase activity was assayed as described by Friedman & Arsenis (1972). For the arylsulphatase assay, nitrocatechol sulphate (Roy, 1953) or *p*-nitrophenyl sulphate (Sellinger *et al.*, 1960) were used as substrates.

Preparation of enzyme suspensions. In all cases, the rats were killed by decapitation. The spleen was

quickly cleaned of excess of fat and immersed in ice-cold 0.15M-KCl and weighed. After the spleen was rinsed in 0.15M-KCl, it was homogenized with one up-and-down stroke of a Potter-Elvehjem-type homogenizer driven at 1000rev./min followed by three up-and-down strokes of a Dounce manual homogenizer. Fractionation of rat spleen suspensions by differential centrifugation was performed essentially by the procedure developed for rat liver (de Duve *et al.*, 1955). For each fraction thus prepared, total activity of various enzymes was measured after the enzymes had been completely released by treating the fractions with a final concentration of 0.1% Triton X-100 or by freezing and thawing three times in solid CO₂-acetone.

The mitochondria and lysosomal fraction (M+L) was prepared by homogenizing the spleen, sedimenting the nuclear fraction and centrifuging the cytoplasmic suspension at 17500rev./min for 10min in the Spinco type 40 rotor.

In the density-gradient experiments, a mitochondrial and lysosomal fraction was layered over a linear sucrose gradient of density 1.10–1.24 (23.15–50.4%, w/w). Both the heavy and the light sucrose solutions contained 0.15M-KCl, which was effective in preventing non-specific aggregation (Bowers & de Duve, 1967). The sample suspended in 0.2M-KCl was layered on the top of the gradient and centrifuged in the SW. 25.2 swinging-bucket rotor in the Beckman model L-2 centrifuge for 6h at 25000rev./min. Enzymes from the various fractions were released by freezing and thawing three times in solid CO₂-acetone. Protein and subcellular marker enzymes in the different fractions were then assayed.

The molecular weight of heparin sulphamidase was determined, with alcohol dehydrogenase and catalase as the marker enzymes, as described by Martin & Ames (1961).

Stability studies on the heparin sulphamidase and arylsulphatase activities were done on cytoplasmic suspensions after the spleen had been homogenized as described above and the nuclear debris removed. The resulting cytoplasmic suspension was then frozen and thawed three times and divided into four sub-fractions. Each subfraction was adjusted to pH 5.0 or 7.4. These subfractions were then preincubated at 0° or 37°C for various time-intervals up to 72h. Samples from all four preincubated subfractions were assayed for heparin sulphamidase under the standard assay conditions and the results were expressed as percentages of initial activity.

Study of N-[³⁵S]sulphated heparin in vivo. Two male Sprague-Dawley rats (370±10g) were each injected with 700µg (3×10⁶c.p.m.) of N-[³⁵S]-sulphated heparin, dissolved in 1ml of 0.9% NaCl, via the penis vein while the rats were under mild ether anaesthesia. One rat was killed by decapitation after 30min, and the other by decapitation after 4 days.

A differential centrifugation was performed on the kidney, liver and spleen of each rat as described (de Duve *et al.*, 1955), and arylsulphatase was used as the lysosomal marker enzyme for these studies. In addition, the amount of ³⁵S radioactivity (primarily associated with heparin) was measured in each fraction in a Packard liquid-scintillation counter (Brostrom & Jeffay, 1970), and the distribution compared with the distribution of arylsulphatase in these tissues. Also the faeces, urine, blood plasma and blood cells were collected and assayed for ³⁵S radioactivity.

On some of the fractions, primarily the urine of the animal killed after 4 days, the relative percentage of free inorganic SO₄²⁻ (based on the injected radioactivity) was determined by paper electrophoresis as described by Friedman & Arsenis (1972).

Release of sulphamidase activity in vitro. A rat spleen was carefully homogenized in 0.20M-KCl and centrifuged in the Spinco type 40 rotor as described above and a mitochondrial and lysosomal (M+L) pellet was obtained by centrifuging the 10000g-min supernatant at 17500rev./min for 10min (200000g-min). This pellet was then resuspended and the suspension was preincubated for various time-intervals up to 2½h at 37°C. After the preincubation the suspension was centrifuged at 17500rev./min for 10min and the supernatant solution was assayed for heparin sulphamidase activity. The centrifugation was thought necessary because of the subsequent prolonged incubation. Results are expressed as percentages of the total activity, which is the activity released after Triton X-100 treatment.

DEAE-Sephadex ion-exchange chromatography. DEAE-Sephadex A-50 (capacity 3.5±0.5mequiv./g; particle size 40–120µm) was swollen in 5mM-Tris-HCl buffer, pH 8.0, and repeatedly washed until it was equilibrated at that pH. The resin was poured into a water-jacketed column (25cm×4cm) at 4°C. A spleen cytoplasmic extract, treated with 0.1% Triton X-100, was applied in a minimum volume to the column and washed with 75ml of 5mM-Tris-HCl–50mM-NaCl, pH 8.0. This was followed by a linear salt gradient generated by mixing 100ml of 5mM-Tris-HCl–50mM-NaCl, pH 8.0, and 100ml of 50mM-Tris-HCl–260mM-NaCl, pH 8.0.

The flow rate of the column was 10ml/h and 5ml fractions were collected by an automated fraction collector. These fractions were then assayed for protein and various enzymic activities. NaCl concentrations were estimated by assuming linearity of the gradient.

Isoelectric focusing. Isoelectric focusing was carried out by using 1% Ampholine pH gradients of pH 3–10 or 4–6 (LKB 8100 Ampholine Electro-focusing Instruction Manual). When the Ampholine gradient of pH 3–10 was used, the anode was at the top of the column and the cathode at the bottom, but with the Ampholine gradient of pH 4–6 the polarity

was reversed. The density gradient was arranged manually and the sample replaced the light solution in some of the middle fractions. The electrofocusing was carried out for approx. 24h at 0°C. For the first 2h, the voltage was set at 250V; then it was increased to 500V until the end of the run. Fractions (5ml) were collected with a peristaltic pump, the pH was measured, and assays on these fractions were performed. Protein in these fractions was measured by reading their absorption at 260 and 280nm.

Heparin sulphamidase was purified as a routine as follows. A rat spleen was homogenized in ice-cold water and the debris spun down after treatment with Triton X-100 (0.1 % final concentration) in the Sorvall centrifuge (ss-34 rotor) at 12000rev./min for 10min. The crude cytoplasm was adjusted to pH5 with a few drops of pH4-6 Ampholine, and the suspension was centrifuged in the Sorvall apparatus as above. This pH5.0 supernatant solution was used in the electrofocusing experiment. These steps are critical since all

the precipitate must be removed before focusing. In the assay for heparin sulphamidase, all the activity is recovered in the pH5.0 supernatant.

Results

Intracellular localization of heparin sulphamidase

Differential-centrifugation studies. Fig. 1 shows the distribution of several marker enzymes in the various fractions obtained after differential centrifugation of a suspension prepared by homogenization as described above. The ordinate, relative specific activity, is the percentage of total activity in each fraction divided by the percentage of total protein in the same fraction, and the abscissa is the percentage of protein in each fraction.

The pattern of distribution in the various subcellular fractions of heparin sulphamidase is the same as that of arylsulphatase and α -mannosidase, two

Fig. 1. Distribution patterns of various rat spleen enzymes in the different subcellular fractions obtained by differential centrifugation of a whole spleen homogenate suspension

Abbreviations are as follows: N, nuclear fraction; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, high-speed supernatant fractions. Enzymes tested are labelled as follows: (a) arylsulphatase; (b) α -mannosidase; (c) cathepsin D; (d) acid phosphatase; (e) catalase; (f) NADPH-cytochrome c reductase; (g) cytochrome oxidase; (h) heparin sulphamidase. % protein is the percentage of protein in a fraction compared with the sum of the protein in all the fractions.

Fig. 2. Distribution patterns of different rat spleen enzymes in the various subcellular fractions obtained by differential centrifugation of a whole spleen homogenate suspension from a rat injected with Triton WR-1339

Enzymes tested are labelled as follows: (a) arylsulphatase; (b) heparin sulphamidase; (c) β -galactosidase; (d) glutamate dehydrogenase; (e) cathepsin D; (f) acid phosphatase. All other abbreviations are as in Fig. 1.

known lysosomal enzymes in spleen. There are dual peaks of activity in the mitochondrial and lysosomal fractions, with a slightly higher relative specific activity in the mitochondrial fraction. The lysosomal enzymes β -glucuronidase, β -galactosidase and *N*-acetyl- β -D-glucosaminidase are not shown in Fig. 1; however, the same distribution pattern for these enzymes was obtained as for arylsulphatase and α -mannosidase.

Triton WR-1339 was injected into a rat, the spleen subfractionated as described above and the subcellular fractions were assayed for marker enzymes (Fig. 2). Arylsulphatase, β -galactosidase and heparin sulphamidase still show dual peaks of activity in the mitochondrial and lysosomal fractions. However, the lysosomal fraction has a slightly higher relative specific activity than the mitochondrial fraction. The 'light' lysosomal enzymes, cathepsin D and acid

phosphatase, still show dual peaks of activity, with the lysosomal fraction having higher relative specific activities.

Glutamate dehydrogenase, a mitochondrial enzyme, is unaffected by the Triton WR-1339 treatment.

Sucrose-density-gradient studies. When a mitochondrial and lysosomal fraction was spun to equilibrium in a sucrose density gradient, two major bands were obtained which were tan in colour: a sharp, clearly defined band at *d*1.20, and a more diffuse band at *d*1.16. Various enzymic assays were performed on the four fractions obtained from the gradient and the results are shown in Fig. 3. Owing mainly to the generally accepted stability of most lysosomal enzymes the recovery for all hydrolases was more than 95%. The recoveries for catalase and cytochrome oxidase were 85% and 90% respectively.

Arylsulphatase, β -galactosidase and heparin sulphamidase have approximately equal relative specific activities in fractions II and IV. Cathepsin D and acid phosphatase show a much higher relative specific activity in fractions II and III compared with that in fraction IV. Cytochrome oxidase and catalase also show a much higher relative specific activity in fractions II and III. Besides the two major bands at *d*1.16 and *d*1.20, a faint white band was found at *d*1.13 which was high in a α -mannosidase activity.

Subcellular distribution of injected *N*-[35 S]sulphated heparin. Studies on *N*-[35 S]sulphated heparin were performed *in vivo*. Fig. 4 shows the intracellular distribution of injected *N*-[35 S]sulphated heparin in the various fractions obtained by differential centrifugation of a whole liver or spleen homogenate solution. At 30min after injection of the radioactive heparin, the radioactivity was associated with the mitochondrial and lysosomal fraction in liver and spleen, and the distribution pattern was the same as that for arylsulphatase. Kidney (not shown in the figure) also showed the same pattern for both radioactivity and arylsulphatase. Table 1 shows that blood plasma after 30min had a very high radioactivity and contained over 30% of the injected radioactivity, whereas liver at this point contained 7% of the injected label.

After 4 days, the radioactivity (expressed as 35 S c.p.m./g of tissue) was significantly decreased in kidney, liver and spleen (Table 1). However, the radioactivity was still associated with the mitochondrial and lysosomal fraction. Table 1 shows the decrease of 35 S radioactivity in the mitochondrial lysosomal fraction for kidney, liver and spleen.

The percentage of the radioactivity that was in the form of free inorganic SO_4^{2-} was also determined in the mitochondrial and lysosomal fraction after 4 days. It was found that this fraction from all the tissues examined contained radioactive inorganic SO_4^{2-} .

The plasma had no appreciable radioactivity associated with it 4 days after injection. Of the injected radioactivity 50% was recovered in the urine collected

Fig. 3. Enzyme distribution in the various fractions obtained after an isopycnic-density-gradient centrifugation in a linear gradient of the mitochondrial and lysosomal fraction from rat spleen

The particles were layered over a sucrose gradient of density 1.10–1.24. After centrifugation the gradient was separated into four fractions which were assayed for arylsulphatase (a), heparin sulphamidase (b), β -galactosidase (c), acid phosphatase (d), cytochrome oxidase (e), catalase (f) and cathepsin D (g). Data are plotted as described by de Duve *et al.* (1955).

up to 4 days afterward, of which 30% was in the form of inorganic SO_4^{2-} .

Purification and separation of arylsulphatase and heparin sulphamidase activities

DEAE-Sephadex chromatography. This was performed as described in the Materials and Methods section. It was observed (Fig. 5) that arylsulphatase had two peaks of activity, a major peak that was eluted at approx. 0.20M-NaCl and a minor peak that was eluted at approx. 0.25M-NaCl. The heparin sulphamidase activity gave a distinct peak which was eluted at 0.23M-NaCl.

Isoelectric focusing. When a rat spleen cytoplasmic extract was subjected to isoelectric focusing on a pH3–10 Ampholine gradient (Friedman & Arsenis, 1972) the results were as follows. Arylsulphatase, with nitrocatechol sulphate as substrate and assayed at pH5.0, gave two peaks of activity. Their apparent isoelectric points were pH6.30 and 4.30 respectively.

It was also observed that the heparin sulphamidase activity gave a peak at pI4.70, and therefore appears to be different from the other arylsulphatase activities.

Fig. 6 shows the results of isoelectric focusing of spleen cytoplasmic extract in a pH4–6 Ampholine gradient.

Heparin sulphamidase gave a clearly distinctive peak at pI4.7, thus making it possible to obtain heparin sulphamidase activity free of arylsulphatase.

Purification of heparin sulphamidase. Table 2 shows the purification of heparin sulphamidase. Some 82% of the original activity was recovered with a 13-fold purification. Less than 2% of the original arylsulphatase activity was recovered in the final fraction.

Kinetic properties of heparin sulphamidase

Stability. The stability of heparin sulphamidase was tested at 0° and 37°C, and at two different pH values. The enzyme is stable at 0°C at either pH7.4 or 5.0, but loses activity at 37°C (Fig. 7). In addition, no

differences were observed when the assays were performed under a layer of toluene.

Kinetic data. Various kinetic properties of heparin sulphamidase are presented in Fig. 8.

(a) Activity (expressed as percentage of SO_4^{2-} released) plotted against enzyme (protein) concentration, was linear up to a certain point, after which linearity ceased. It appears that the maximum percentage of sulphate breakdown is 5% of the total *N*-sulphate in heparin regardless of how much enzyme is present.

Fig. 4. Studies on *N*-[^{35}S]sulphated heparin *in vivo*

The intracellular distribution of intravenously injected *N*-[^{35}S]sulphated heparin (a and c) and arylsulphatase (b and d) in the various fractions obtained by differential centrifugation of a whole rat spleen (a and b) or rat liver (c and d) homogenate suspension 30 min after injection is shown. The relative specific activity in each fraction is plotted against the percentage of protein in the same fraction (de Duve *et al.*, 1955). Other abbreviations are as in Fig. 1.

(b) The progress curve of the heparin sulphamidase reaction follows the general form seen with most enzymes in which the velocity decreases with time. In this case, this is the result of a decrease in the degree of saturation of the enzyme with the substrate rather than enzyme denaturation.

(c) Activity (expressed as percentage of SO_4^{2-} released) plotted against pH, shows the pH optimum for the reaction to be 5.1.

(d) Normal Michaelis kinetics were observed when activity at 37°C (expressed as μg of SO_4^{2-} released/h per ml of enzyme) was plotted against substrate concentration. A K_m value of $9 \times 10^{-6}\text{M}$ is found for heparin sulphamidase by assuming a molecular weight for heparin of 14000 (Fig. 8). Since only 5% of the substrate is available to the enzyme, this should be taken into consideration for the determination of the actual K_m value. V_{max} is $0.26 \mu\text{g}$ of SO_4^{2-} released/h per ml of enzyme, whereas in the presence of 10mM-SO_4^{2-} , which is a non-competitive inhibitor, V_{max} is $0.12 \mu\text{g/h}$ per ml of enzyme. The heparin concentrations were 67, 133, 200 and $667 \mu\text{g/ml}$ and the corresponding activities were 0.04, 0.07, 0.10 and $0.2 \mu\text{g}$ of SO_4^{2-} released/h per ml of enzyme. When 10mM inorganic SO_4^{2-} was present the corresponding activities were 0.02, 0.03, 0.04 and $0.07 \mu\text{g}$ of SO_4^{2-} released/h per ml of enzyme.

Effects of incubation temperature. Fig. 9 shows the effect of different incubation temperatures on heparin sulphamidase activity. The optimum temperature for the usual incubation time of 24 h is 37°C.

Effects of fresh enzyme or new substrate. Fig. 10 shows the effect of adding fresh enzyme or new substrate to heparin sulphamidase incubation mixtures that have reached their maximal 5% breakdown. The addition of fresh enzyme to the incubation mixture had very little, if any, effect in increasing enzymic activity over 5%. However, if new substrate was

Table 1. Distribution of ^{35}S radioactivity in the various rat tissues 30 min or 4 days after injection of *N*-[^{35}S]sulphated heparin (3×10^6 c.p.m.) via the penis vein

The amount of ^{35}S radioactivity was measured in the whole tissue suspension and in the mitochondrial plus lysosomal fraction prepared by differential centrifugation. At 4 days after injection 50% of the injected radioactivity was recovered in the urine; of this approx. 30% was in the form of inorganic SO_4^{2-} .

Tissue	Radioactivity (c.p.m.)			
	30 min after injection		4 days after injection	
	Whole	Mitochondrial+lysosomal	Whole	Mitochondrial+lysosomal
Kidney (per g)	45 000	13 700	14 300	2200
Liver (per g)	26 800	14 200	6500	3000
Spleen (per g)	17 460	11 000	12 500	5700
Blood plasma (per ml)	1 000 000	—	20	—
Urine (per ml)	—	—	1 000 000*	—

* Urine collected up to 4 days.

Fig. 5. Resolution of heparin sulphamidase and arylsulphatase activities by DEAE-Sephadex A-50 chromatography

The spleen cytoplasmic extract was applied to the column and washed with 5 mM-Tris-HCl-50 mM-NaCl, pH 8.0, and then with a linear gradient generated by mixing 100 ml of 5 mM-Tris-HCl-50 mM-NaCl, pH 8.0, and 100 ml of 50 mM-Tris-HCl-260 mM-NaCl, pH 8.0. Fractions (approx. 5 ml) were collected with an automatic fraction collector and assayed for heparin sulphamidase (■), arylsulphamidase (●) and proteins (▲). NaCl concentrations (----) were estimated by assuming linearity.

Fig. 6. Resolution of heparin sulphamidase (○) and arylsulphatase (●) activities from rat spleen by isoelectric focusing in a pH4-6 Ampholine gradient

The anode was at the bottom of the column and on the left side of Fig. 6. Fractions (5 ml) were collected with a peristaltic pump and assayed.

added, a new burst of enzymic activity was observed, until another 5% of the substrate was hydrolysed.

Molecular weight of heparin sulphamidase

Fig. 11 shows the results of sucrose-density-gradient centrifugation of heparin sulphamidase, alcohol dehydrogenase (mol.wt. 150000), catalase (mol.wt. 250000) and haemoglobin (mol.wt. 66000). From the known molecular weights the molecular weight of heparin sulphamidase is 120000 ± 5000 .

Effects of various compounds on heparin sulphamidase

F^- , SO_4^{2-} , SO_3^{2-} , CN^- and P_i , all at 5 mM final concentration, were individually included in the heparin sulphamidase assay mixture (Table 3).

SO_3^{2-} completely inhibited the reaction, whereas CN^- had no effect. Both SO_4^{2-} and P_i inhibited the reaction by 20%, and F^- inhibited by 30%.

Glucose 6-sulphate and chondroitin sulphate, both O-sulphate compounds, had no effect on the sulphamidase. Sulphaminouracil, a low-molecular-weight N-sulphate compound, also had no effect on the heparin sulphamidase. Nitrocatechol sulphate completely inhibited the sulphamidase, and p-nitrophenyl sulphate inhibited the reaction by 20%.

Discussion

Rats can degrade N-[^{35}S]sulphated heparin (Lloyd *et al.*, 1968); inorganic $^{35}SO_4^{2-}$ appeared in the urine of rats after an injection of N-[^{35}S]sulphated heparin (Levy & Petracek, 1962). In the present studies, no diminution of enzymic activity was observed when other mucopolysaccharides with a high content of O-sulphate, such as the chondroitin sulphates, were added to the incubation mixture containing the radio-active heparin. Glucose 6-sulphate was also without effect. Thus it seems likely that the release of SO_4^{2-} observed was due to a heparin sulphamidase.

The results presented confirm that rat spleen exhibits heparin sulphamidase activity, which is associated with the lysosomes.

The dual peaks of activity shown by the lysosomal enzymes are of interest. All of the lysosomal enzymes that were studied, except for acid phosphatase and cathepsin D, have their activities in the mitochondrial and lysosomal fractions, with a slightly higher specific activity in the mitochondrial fraction.

Spleen also contains at least two arylsulphatases confined to the lysosomes and a slightly basic arylsulphatase associated with the microsomal fraction.

Table 2. *Purification of heparin sulphamidase*

A cytoplasmic extract after treatment with Triton X-100 was adjusted to pH 5 with pH4-6 Ampholine, subjected to electrofocusing and fractionated. Each fraction was assayed for heparin sulphamidase, arylsulphatase and protein.

Step	Total activity (μg of SO_4^{2-} - released/h)	Yield (%)	Specific activity (μg of SO_4^{2-} released/h per mg of protein)	Purification (fold)	Yield of arylsulphatase
(1) Crude cytoplasm	5.7	100	0.015	1.00	100
(2) pH 5.0 supernatant	6.3	111	0.035	2.35	72
(3) Peak at pH 4.30-5.05 Ampholine	4.7	82	0.195	13.0	1.8

Fig. 7. *Thermal stability of heparin sulphamidase*

The activity of purified heparin sulphamidase was measured after preincubation for various time-intervals at pH 7.4 (a) or 5.0 (b) and at 0° (■) or 37°C (●). Percentage of remaining activity is plotted against time of preincubation.

Fig. 8. *Kinetic properties of purified heparin sulphamidase*

The conditions are described in the Materials and Methods section. (a) Percentage of SO_4^{2-} released plotted against amount of protein, (b) percentage of SO_4^{2-} released plotted against time of incubation, (c) percentage of SO_4^{2-} released plotted against pH of incubation and (d) reciprocal plot of μg of SO_4^{2-} released/h per ml of enzyme against the reciprocal of substrate concentration. Control (●) and plus 10 mM SO_4^{2-} (○). The intercept of the line gives $1/K_m$.

Fig. 9. Heparin sulphamidase activity as a function of incubation temperature

Heparin sulphamidase was incubated at 25° (○), 37° (●), 50° (■) and 70°C (Δ) for various time-intervals and the amount of SO₄²⁻ released was plotted against time of incubation.

Fig. 10. Effect of addition of fresh substrate or fresh enzyme on the heparin sulphamidase activity

A standard incubation mixture for assaying heparin sulphamidase (●) was incubated for 70h and then divided into three equal parts. To the first buffer was added (●), to the second fresh enzyme (■) and to the third fresh substrate (○) at the time shown by the arrow. Samples were removed and the percentage of SO₄²⁻ released was determined. Data were corrected for dilutions and then plotted as shown.

This situation also occurs in ox liver (Roy, 1960). Rat spleen has two major lysosomal populations which differ not only in density but in certain enzymes as

Fig. 11. Molecular-weight determination of heparin sulphamidase on the basis of sucrose-density-gradient centrifugation of rat spleen heparin sulphamidase and known marker enzymes

Catalase (0.05mg), alcohol dehydrogenase (0.1mg) and heparin sulphamidase (300µg of SO₄²⁻ released/h), mixed in 0.3ml of 0.01M-Tris-HCl buffer, pH7.4, were layered on a sucrose gradient. After 15h of centrifugation at 38000rev./min at 5°C, the gradient was fractionated and analysed for heparin sulphamidase (●), alcohol dehydrogenase (○) and catalase (Δ).

Table 3. Effect of various compounds on the heparin sulphamidase peak of activity obtained after isoelectric focusing with pH4-6 Ampholine

Compound	Activity (% of control value)
NaF (5mm)	70
Na ₂ SO ₄ (5mm)	80
Na ₂ SO ₃ (5mm)	0
NaCN (5mm)	100
NaH ₂ PO ₄ (5mm)	80
Chondroitin sulphate (10mg/ml)	100
Glucose 6-sulphate (5mm)	100
Nitrocatechol sulphate (20mm)	0
Nitrophenyl sulphate (20mm)	80
Sulphaminouracil (20mm)	100
Cystine (20mm)	100
Control	100

well (Bowers & de Duve, 1967). It was suggested that the light lysosomal population originates from the spleen lymphocytes, whereas macrophages contain heavy lysosomes. Our preliminary results based on spleen cell separation strengthen this hypothesis.

Lysosomes appear to be able to accumulate heparin in all the tissues examined. The decrease in the total radioactivity observed after a few days in the various organs examined supports the view that heparin was cleared by these tissues and that these tissues contain an enzyme capable of releasing inorganic SO_4^{2-} from heparin (Friedman & Arsenis, 1972).

SO_4^{2-} release from heparin *in vitro* appears to be limited to only 5% of the total sulphate (Dietrich, 1970), although the intact animal is capable of releasing approx. 40% of the injected N -[^{35}S]sulphated heparin radioactivity. The reason for this difference is unknown.

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