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STUDIES ON HEPARIN DEGRADATION—II

THE METABOLIC FATE OF THE POTASSIUM SALTS OF ([^{35}S] SULPHOAMINO)-HEPARIN, ([^{35}S] SULPHOAMINO)-CHITOSAN, 2-DEOXY-2-[^{35}S] SULPHOAMINO-D-GLUCOSE AND [^{35}S] SULPHOAMINO-L-SERINE IN THE RAT

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Abstract—The metabolic fate of the potassium salts of ([^{35}S] sulphoamino)-heparin, ([^{35}S] sulphoamino)-chitosan, 2-deoxy-2-[^{35}S] sulphoamino-D-glucose and [^{35}S] sulphoamino-L-serine has been investigated following administration to free-ranging rats. The intravenous and intraperitoneal injection of ([^{35}S] sulphoamino)-heparin resulted in the appearance in the urine of unchanged ester together with substantial amounts of inorganic $^{35}SO_4^{2-}$ ions. In contrast only minimal quantities of inorganic $^{35}SO_4^{2-}$ ions were present in the urine following the injection of 2-deoxy-2-[^{35}S] sulphoamino-D-glucose which was rapidly excreted. No degradation of the sulphamate groupings of ([^{35}S] sulphoamino)-chitosan and [^{35}S] sulphaomino-L-serine occured when examined in the same way. The metabolism of ([^{35}S] sulphoamino)-heparin was also investigated in rats with bile and ureter cannulae and analysis of urine, bile and blood samples revealed the ability of theses animals to degrade this compound with the appearance of inorganic $^{35}SO_4^{2-}$ ions.

STUDIES on the *in vivo* degradation of [^{35}S] heparin, prepared by wholly-biosynthetic means, have formed the subject of several earlier reports.[1–4] Unfortunately, the dual labelling of the *O*-sulphate and sulphamate (*N*-sulphate) moieties in biosynthetic [^{35}S] heparin automatically precludes the use of this material in experiments designed to assess the relative stabilities of these groupings in biological system.[5–7] Furthermore, in view of the difficulties encountered during the purification of [^{35}S] heparin from natural sources on a small scale, allowance must also be made for the possibility that such biosynthetically-labelled preparations may be contaminated with alternative [^{35}S] sulphated acidic glycosaminoglycans. Finally, [^{35}S] heparin labelled biologically suffers the disadvantage of having only a relatively low specific radioactivity.

Preliminary work[6,8] has already established that these difficulties can be resolved by the use of semi-chemically synthesized [^{35}S] heparin, labelled specifically by the chemical introduction of [^{35}S] sulphamate groupings[9] and designated for practical purposes as ([^{35}S] sulphoamino)-heparin. The present report describes observations on the metabolism of ([^{35}S] sulphoamino)-heparin in the rat together with parallel *in vivo* studies on the structurally related carbohydrate derivatives ([^{35}S] sulphoamino)-chitosan and 2-deoxy-2-[^{35}S] sulphoamino-D-glucose in the same species. Investigations into the metabolism of [^{35}S] sulphoamino-L-serine were included for reasons

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discussed previously[10] and because the corresponding analogue L-serine *O*-[^{35}S] sulphate is known to undergo extensive degradation in the rat.[11]

MATERIALS AND METHODS

Sulphamate derivatives

[^{35}S] labelled sulphamate derivatives were prepared and characterized as described by Lloyd *et al.*[10] The specific radioactivities of these preparations were in the range 1·9–4·8 mc/mM of S.

Experimental animals

Rats of the M.R.C. hooded strain were used throughout the investigation. Animals were in the age range 3–4 months and were maintained on a standard diet. The animals were injected either intraperitoneally or intravenously via the tail vein while the animals were under light ether anaesthesia. After recovery, animals were housed in metabolism cages designed for the separate collection of urine and faeces. The animals were allowed water without restriction during the course of the experiments.

Alternatively, animals were restrained and kept under light nembutal anaesthesia permitting the insertion of bladder and bile duct cannulae (see ref. 12 for details). In one series of experiments the administration of ([^{35}S] sulphoamino)-heparin was followed by withdrawal of duplicate blood samples (100 μl) from the penal vein at 30 min intervals. In a parallel series of experiments no blood samples were taken. Instead, bile and urine samples voided at 30 min intervals were collected. The samples of blood, bile and urine were retained for subsequent analysis.

Measurement of radioactivity in urine, bile and blood

Collected urines were filtered through glass wool "plugs", the appropriate filtrates and washings diluted to a known volume and either used immediately or stored at −12°. For the determination of the total [^{35}S] sulphate content of the urines, a portion of the diluted urine was hydrolysed in N HCl and precipitated as $Ba^{35}SO_4$ according to the method of Lloyd.[13] Precipitated $Ba^{35}SO_4$ was placed at "infinite thickness" in plastic planchets having a surface area of 1 cm^2 for the measurement of radioactivity.

It was noted early in the investigation that ([^{35}S] sulphoamino)-heparin, 2-deoxy-2-[^{35}S] sulphoamino-D-glucose and ([^{35}S] sulphoamino)-chitosan all coprecipitated to varying degrees in the standard method for the determination of inorganic $^{35}SO_4^{2-}$ ions based on precipitation of $Ba^{35}SO_4$ without prior hydrolysis. For this reason the inorganic $^{35}SO_4^{2-}$ ion content of urine samples after injection of these [^{35}S] sulphamate derivatives was measured by concentrating a portion of the diluted urines which were reduced to 500 μl by evaporation under reduced pressure at 37°. Duplicate samples (20 μl) of the concentrated solution were then applied to a sheet of Whatman No. 100 paper and separated by electrophoresis for 2 hr at a potential of 20V/cm in 0·1 M ammonium acetate–acetic acid buffer at pH 4·5. Separation of standards consisting of mixtures of the appropriate [^{35}S] sulphamate derivatives and known amounts of inorganic $^{35}SO_4^{2-}$ ions, were made at the same time under identical conditions of electrophoresis. The strips were dried and the positions of the radioactive zones determined using a Radiochromatogram Scanner (Packard Model 7201) in 4π operation and under gas-flow. The relative amounts of radioactivity corresponding to

zones of the undegraded [^{35}S] sulphamate preparation under test and inorganic $^{35}SO_4^{2-}$ ions were obtained by measurement of the areas under the appropriate portions of the trace on the chart recordings. These values were then used to calculate that proportion of the injected dose excreted as inorganic $^{35}SO_4^{2-}$ ions based on measurements of the "total [^{35}S] sulphate" content of a sample of the same urine.

Determinations of the total [^{35}S] sulphate and inorganic $^{35}SO_4^{2-}$ ions content of bile and blood samples were carried out in a similar way with due allowance being made for the smaller sample size and the generally lower contents of radioactivity.

Measurement of radioactivity of faeces and carcass

The total [^{35}S] contents of faeces and carcass were determined as precipitated $Ba^{35}SO_4$ following oxidation in fuming HNO_3 according to the method of Young *et al.*[14]

RESULTS

Free-ranging animals

Paper electrophoresis of rat urine concentrates, obtained 24 hr after both the intravenous and intraperitoneal injection of ([^{35}S] sulphoamino)-heparin into free-ranging rats, revealed the presence in each instance of two zones of radioactivity

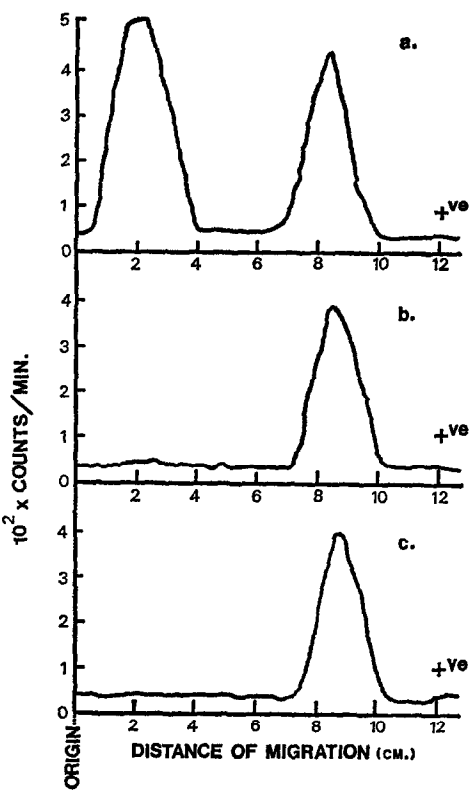

FIG. 1. Paper electrophoretograms of a 12 hr rat urine concentrate sample following the intravenous injection of ([^{35}S] sulphoamino)-heparin. (a) Untreated rat urine sample, (b) rat urine sample treated with 5-aminoacridine, (c) sodium $^{35}SO_4^{2-}$ ions. For practical details see text.

corresponding in mobility to unchanged [^{35}S]-heparin and inorganic $^{35}SO_4^{2-}$ ions respectively (see Fig. 1). Conclusive evidence was sought establishing the identity of the faster moving zone with inorganic $^{35}SO_4^{2-}$ ions, especially since *in vivo* depolymerization of ([^{35}S] sulphoamino)-heparin might also contribute *N*-[^{35}S] sulphated oligosaccharide fractions of increased mobility on paper electrophoresis. Dodgson *et al.*[15] have already recorded the efficiency of 5-aminoacridine as a precipitant for a wide range of sulphated materials in urine (see also Muir[16]). For this reason, the urine sample obtained 24 hr after the intravenous injection of 2 mg of ([^{35}S] sulphoamino)-heparin into a rat was diluted to 100 ml. To a portion of the diluted urine (50 ml) was added 2 ml of a saturated aqueous solution of 5-aminoacridine hydrochloride at 20°. The whole was then cooled to 2° and kept at this temperature for 2 hr before removing precipitated material by centrifuging at 6000 *g* (av.) for 30 min at 2°. A sample (15 ml) of the clear supernatant was then reduced to 500 μl by rotary evaporation under reduced pressure and duplicate portions (20 μl) of the concentrated material then examined by paper electrophoresis (see Fig. 1). In such preparations only the zone corresponding in electrophoretic mobility to inorganic $^{35}SO_4^{2-}$ ions remained. Duplicate samples (15 ml) of the 5-aminoacridine-treated urine preparations were transferred to modified 50 ml centrifuge tubes[17] and 3 ml of 0·15 M K_2SO_4 added to each. To the mixtures were added 5 ml of 4 N HCl followed by 4 ml of aq. 10% (w/v) $BaCl_2$. After careful mixing the samples were kept at 2° for 6 hr. Precipitated material was separated by centrifuging at 3000 *g* (av.) for 30 min at 2° and the supernatant decanted carefully in each case, before concentrating to 500 μl by rotary evaporation under reduced pressure. Samples (20 μl) of the concentrated supernatants were then separated by electrophoresis. No radioactive zones were detected with the radiochromatogram scanner in these instances. The corresponding precipitates were then washed three times by suspension in water (40 ml) and once with acetone (40 ml), with intermediate centrifuging, before drying at 110°. The dried precipitates were then plated at "infinite thickness" in plastic planchettes of 1 cm^2 area and the radioactivities measured with the gas-flow counting system. The radioactivities of the precipitates averaged 6245 $\pm$ 30 counts/min after correction for coincidence and background. The precipitates were removed from the planchettes, resuspended in water and washed as above before centrifuging, drying, plating and recounting as described. There was no significant change in the average counting rate when precipitates were treated three times in this way, indicating that they contained $Ba^{35}SO_4$ and establishing the nature of the faster zone.

The results of experiments after the intravenous and intraperitoneal injection of ([^{35}S] sulphoamino)-heparin are recorded in Table 1. The figures represent the distribution of radioactivity corresponding to several different doses of the [^{35}S]-labelled polymer in the "total [^{35}S] sulphate" and "inorganic $^{35}SO_4^{2-}$ ion" fractions of rat urine over a period of 48 hr. At the end of this time the animals were killed and the proportion of radioactivity retained in the carcass and excreted with the faeces was also determined.

The results obtained after the injection of 2-deoxy-2-[^{35}S] sulphoamino-D-glucose, ([^{35}S] sulphoamino)-chitosan and [^{35}S] sulphoamino-L-serine were notably different from those recorded with the [^{35}S]-labelled natural polymer (see Table 2). In all experiments it was found that the greater part of the injected material was excreted in an apparently unchanged form within a short time interval following administration.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN URINE, FAECES AND CARCASS FOLLOWING THE INJECTION OF ([^{35}S] SULPHOAMINO)-HEPARIN INTO FREE-RANGING MALE RATS

			Injected radioactivity recovered in:												
			Urine (%)										Faeces carcass (%)		
			12 hr		24 hr		36 hr		48 hr		Total				Total
	Dose (mg)	Body wt. (g)	T	I	T	I	T	I	T	I	T	I	T	T	T
a. Intravenous															
	0·2	282	38·8	18·1	13·1	12·9	6·8	1·6	5·5	1·2	64·2	33·8	1·4	33·3	98·9
	1·0	225	54·4	18·9	7·2	7·2	3·5	3·5	2·5	2·5	67·6	32·1	2·6	14·9	95·1
	2·0	230	43·4	11·5	9·8	9·8	3·7	3·7	1·9	1·9	58·8	26·9	1·6	30·5	90·9
b. Intraperitoneal															
	0·2	291	47·6	15·3	21·4	16·1	7·2	7·2	3·1	3·1	79·3	41·7	2·7	14·4	96·4
	1·0	239	44·9	14·9	12·6	12·6	6·4	6·4	3·7	3·7	67·6	37·6	1·2	27·7	96·5
	2·0	258	42·5	9·2	13·6	10·1	6·9	5·5	5·1	4·9	68·1	29·7	1·3	28·2	98·2

(Mean values are given for experiments involving groups of three animals.)
T = Total [^{35}S] sulphate. I = Inorganic $^{35}SO_4^{2-}$ ions.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN URINE, FAECES AND CARCASS FOLLOWING THE INTRAPERITONEAL INJECTION OF 2-DEOXY-2-[^{35}S] SULPHOAMINO-D-GLUCOSE, ([^{35}S] SULPHOAMINO)-CHITOSAN AND [^{35}S] SULPHOAMINO-L-SERINE INTO FREE-RANGING MALE RATS

			Injected radioactivity recovered in:												
			Urine (%)										Faeces carcass (%)		Total
			12 hr		24 hr		36 hr		48 hr		Total				
	Dose (mg)	Body wt. (g)	T	I	T	I	T	I	T	I	T	I	T	T	T
a. 2-deoxy-2-[^{35}S] sulphoamino-D-glucose															
	1·6	258	82·3	0·8	1·03	—	0·04	—	0·02	—	83·4	0·8	1·4	19·6	104·3
	3·2	210	82·5	1·2	3·1	—	0·92	—	0·48	—	87·1	1·2	0·5	15·7	103·2
b. ([^{35}S] sulphoamino)-chitosan															
	1·2	213	61·2	0	4·3	0	2·8	0	1·2	0	69·5	0	2·1	27·3	98·9
c. [^{35}S] sulphoamino-L-serine															
	2	200	—	—	76·0	0	—	—	2·4	0	78·4	0	1·3	5·8	80·5

(Mean values are given for experiments involving groups of three animals.)
T = Total [^{35}S] sulphate. I = Inorganic $^{35}SO_4^{2-}$ ions.

Inorganic $^{35}SO_4^{2-}$ ions were not detected in rat urine concentrates during these experiments.

Animals with urine and bile cannulae

In these experiments both urine and bile were collected over a 1 hr period following the intravenous injection of ([^{35}S] sulphoamino)-heparin. The total volume of urine collected during the period ranged from 1·6 to 2·4 ml The patterns of excretion of "total [^{35}S] sulphate" and "inorganic $^{35}SO_4^{2-}$ ions" in the urines at various time intervals during the experiments, following the injection of the [^{35}S]-labelled polymer are recorded in Table 3. It was observed consistently that radioactivity excreted in the first 2 hr of the experiment was confined exclusively to the zone corresponding in electrophoretic mobility with ([^{35}S] sulphoamino)-heparin in electrophoretograms of the concentrated urines. Thereafter, the proportion of urinary radioactivity corresponding to inorganic $^{35}SO_4^{2-}$ ions increased substantially. Finally, despite the fact that volumes of bile in the range 3·5–4·3 ml were secreted during the 7 hr period, the quantity of radioactivity excreted by this route did not exceed 2·2 per cent of the injected dose.

Parallel experiments on immobilized animals involving the removal of blood samples showed that the total radioactivity of blood declined rapidly in the first 2 hr following injection (see Fig. 2). At subsequent time intervals only negligible quantities of radioactivity could be detected. The combined results of the experiments indicated that urinary excretion was not wholly responsible for the rapid decline of blood

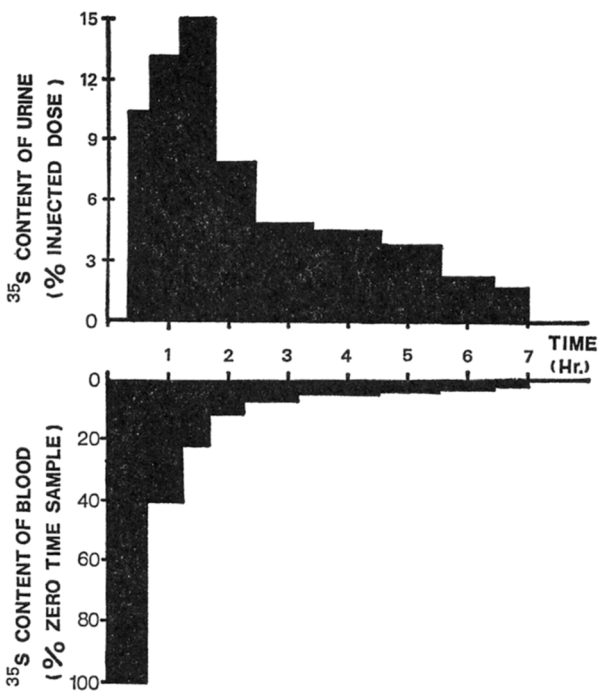

FIG. 2. Analysis of blood and urine samples following the intravenous injection of ([^{35}S] sulphoamino)-heparin into rats with bile duct and ureter cannulae. See text for practical details.

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN THE URINE FOLLOWING THE INJECTION OF ([^{35}S] SULPHOAMINO)-HEPARIN (2·0 mg per 250 g body wt.) INTO ANAESTHETISED RATS WITH BILE-DUCT AND BLADDER CANNULAE

		Injected radioactivity (%) recovered in urine after:									Total ^{35}S excreted in 7 hr
		0·5 hr	1 hr	1·5 hr	2 hr	3 hr	4 hr	5 hr	6 hr	7 hr	
Animal 1	T	8·4	11·7	15·2	7·9	4·8	3·9	3·6	2·4	2·2	58·8
	I	—	—	—	4·6	4·5	3·9	3·6	2·4	2·2	21·4
Animal 2	T	10·8	13·1	15·6	6·7	5·6	4·3	4·3	3·8	2·7	66·9
	I	—	—	—	5·8	5·4	3·8	4·3	3·8	2·7	25·8

T = Total [^{35}S] sulphate. I = Inorganic $^{35}SO_4^{2-}$ ions.

radioactivity and are consistent with the existence of a variety of "storage" sites for the injected [^{35}S]-labelled polymer and its degradation products in rat tissues.[18,19]

DISCUSSION

The findings detailed above establish the presence in rat tissues of an entirely novel mammalian enzyme system which has the ability to degrade the sulphamate groupings of exogenous ([^{35}S] sulphoamino)-heparin and thus exhibits the characteristics of a "heparin sulphamidase". Enzyme systems tentatively designated as heparin sulphamidases have only been demonstrated previously in extracts of the microorganism *Flavobacterium heparinum* adapted by prior growth in media containing heparin.[20,21] Moreover, in view of (a) the known reabsorption of inorganic $^{35}SO_4^{2-}$ ions by the renal tubules in rat[22] and (b) the retention and utilization of inorganic $^{35}SO_4^{2-}$ ions for the biosynthesis of a variety of materials in the intact animal, the figures obtained for the various inorganic $^{35}SO_4^{2-}$ ion fractions must be considered as not wholly representing the total extent to which *N*-de-[^{35}S] sulphation occurs *in vivo*. It should also be noted that the process of degradation is not affected materially by the route of injection of the polymer.

The results of studies with immobilized animals have several features of interest. Firstly, the experiments both confirmed and extended the observations made with free-ranging animals, allowing especially observations on the excretion of radioactivity at relatively short time intervals after the administration of the [^{35}S]-labelled heparin. Secondly, they revealed that urinary excretion was a primary means for the disposal of [^{35}S]-polysaccharide fractions in the initial period following injection. On the other hand, catabolism of the polymer was favoured in later stages leading to the production of inorganic $^{35}SO_4^{2-}$ ions. Lastly, they showed that biliary excretion was apparently only a minor route for the disposal of [^{35}S] heparin or its degradation products in the rat. Consequently, a possible pathway for the production of urinary inorganic $^{35}SO_4^{2-}$ ions involving the secretion of the [^{35}S] polymer into the intestine via the bile, its catabolism by organisms of the intestinal flora and subsequent reabsorption of degradation products preceding urinary excretion can now be discounted.

The rapid elimination of radioactivity comprised exclusively of unchanged material following the injection of 2-deoxy-2-[^{35}S] sulphoamino-D-glucose or ([^{35}S] sulphoamino)-chitosan (essentially a β, 1–4 linked *poly*, 2-deoxy-2-[^{35}S] sulphoamino-D-glucose) and [^{35}S] sulphoamino-L-serine raises several important issues. Thus, in common with the *O*-[^{35}S] sulphate esters of hexoses and *N*-acetylhexosamines[13,23,24] the *N*-[^{35}S] sulphate derivative of D-glucosamine appears to have a considerable resistance to degradation by mammalian enzyme systems *in vivo*, regardless of whether it is administered in a monomeric or a model-polymeric form. Considered in the simplest way, these findings might be used to attribute some degree of specificity to the system responsible for the liberation of inorganic $^{35}SO_4^{2-}$ ions from 2-deoxy-2-[^{35}S] sulphoamino-D-glucose units when they are incorporated into the polymer chain of ([^{35}S] sulphoamino)-heparin. However, a considerable body of evidence has now emerged which establishes that the metabolism of low molecular weight [^{35}S] sulphate esters *in vivo* in mammals to yield inorganic $^{35}SO_4^{2-}$ ions is governed not only by the presence or absence of an appropriate catabolic enzyme system, but also by the

relative efficiency of their transport across biological membranes. Thus, the paradoxical situation exists that 2-hydroxy-5-nitrophenyl sulphate (nitrochatechol sulphate) which is extensively degraded *in vitro* by the arylsulphatases A and B of lysosomal origin, is eliminated rapidly and in an unchanged form in the urine when the labelled analogue is administered to free-ranging rats. These findings contrast sharply with those gathered following studies on several other low molecular weight esters such as *p*-nitrophenyl *O*-[^{35}S] sulphate,[25] L-serine *O*-[^{35}S] sulphate and L-threonine *O*-[^{35}S] sulphate[11,26] glycyl-L-serine *O*-[^{35}S] sulphate and L-serylglycine *O*-[^{35}S] sulphate,[27] and finally oestrone [^{35}S] sulphate,[28] all of which are extensively degraded to yield inorganic $^{35}SO_4^{2-}$ ions by mammalian tissues both *in vivo* and *in vitro*. The patterns of cortisone 21-[^{35}S] sulphate metabolism is representative of the opposite extreme where observations of *in vivo* desulphation cannot be duplicated *in vitro*.[29] It will be clear therefore that resolution of the problem of the substrate specificity of rat heparin sulphamidase must await the isolation and assessment of the properties of the system *in vitro*.

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