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STUDIES ON HEPARIN DEGRADATION—I

PREPARATION OF [^{35}S] SULPHAMATE DERIVATIVES FOR STUDIES ON HEPARIN DEGRADING ENZYMES OF MAMMALIAN ORIGIN

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Abstract—Methods have been devised for the preparation and characterization of the potassium salts of 2-deoxy-2-[^{35}S] sulphoamino-D-glucose, [^{35}S] sulphoamino-L-serine, ([^{35}S] sulphoamino)-heparin and ([^{35}S] sulphoamino)-chitosan for use in studies on mammalian enzymes involved in the degradation of sulphamate groupings in heparin.

DESPITE the continued use of exogenous heparin as an anticoagulant it is still not clearly understood which metabolic routes are available for the termination of the biological action of this glycosaminoglycan *in vivo* and moreover, which enzymes are responsible for the degradation of the polymer in mammalian tissues. The present work was designed to provide a range of both low and high molecular weight [^{35}S] sulphamate derivatives suitable for the study of factors influencing the degradation of the sulphamate groupings which are associated with the anticoagulant activity of the polysaccharide.

MATERIALS AND METHODS

Reagents

Samples of purified heparin, derived initially from bovine intestinal mucosa (batch Nos. A.50601 and B.45700) were gifts from the Evans Biological Institute, Runcorn, Cheshire. Chitosan[1] was a gift from Dr. D. T. Warner, The Upjohn Co., Kalamazoo, Mich., U.S.A. D-Glucosamine hydrochloride and L-serine were obtained from Koch–Light Ltd. [^{35}S] Labelled chlorosulphonic acid (sp. act. 5·5 mc/mM) was purchased from the Radiochemical Centre, Amersham, Bucks.

General analytical methods

The ester sulphate contents of preparations were measured by a turbidimetric method,[2] after hydrolysis of the materials in 4 N HCl for 6 hr in sealed tubes at 110°. Total N of preparations was determined by the micro-Kjeldahl method. Analyses for C and H were made by the Microanalytical Laboratory, Queen's University of Belfast. Potassium was measured by flame photometry. Analyses for hexosamines and amino acids were made using a Technicon amino acid autoanalyzer after the hydrolysis

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of preparations for 16 hr in 4 N HCl under N_2 in sealed tubes at 110°. Uronic acid was determined by the modified phenol-sulphuric acid procedure[3] and additionally by the carbazole method.[4]

Infra-red spectroscopy

Infra-red spectra were obtained using a Perkin–Elmer Infracord spectrophotometer. Materials were prepared as mulls in Nujol for spectral measurements.

Anticoagulant activity

Measurements of the anticoagulant activity of heparins and their derivatives were made by the Evans Medical Institute, Runcorn, Cheshire.

Paper electrophoresis

The homogeneity of derivatives was assessed by zone electrophoresis on Whatman No. 1 paper. Separations were made in 0·1 M ammonium acetate–acetic acid buffer (pH 4·5) at a potential gradient of 20 V/cm for 2 hr.

Agarose gel electrophoresis

The homogeneity of heparin preparations was also assessed by electrophoresis on agarose gels. Gel slabs were prepared from a 0·5% (w/v) solution of agarose (Seravac Laboratories Ltd.) in 0·05 M barbiturate buffer (pH 8·6) applied to glass plates (16 × 5 cm). The polysaccharides (10 μg) dissolved in 5μl of 0·05 M barbiturate buffer were applied to slots precast along the mid-line of the gel slab. Separations were made in electrophoresis tanks designed to allow cooling of the gel by immersion in petroleum ether (b.p. range 40–60°). Wicks of Whatman No. 1 paper were used to connect the ends of the gel slab to the electrode compartments which contained 0·05 M barbiturate buffer (pH 8·6). A potential difference of 40 V/cm was applied for 30 min. The positions of separated components were detected by staining the gels with a 1% (w/v) solution of toluidine blue in 80% (v/v) aqueous acetone and subsequent destaining in 1% (w/v) aqueous acetic acid.

Gel filtration on Sephadex G-100

Gel filtration on Sephadex G-100 was used to assess the physical characteristics of each of the polysaccharide preparations. Sephadex G-100 previously swollen in 0·2 M tris–HCl buffer (pH 7·8) was used to prepare a column (28 × 1 cm). The polysaccharides (4 mg) dissolved in 0·5 ml of 0·2 M tris–HCl buffer (pH 7·8) were applied to the Sephadex bed, which was then eluted with the same tris–HCl buffer at a flow rate of 10 ml/hr. The eluate was collected in 1-ml fractions. In the case of heparin preparations elution of carbohydrate was followed using the carbazole reaction[4] and in appropriate instances the radioactivities of eluates were also measured (see below). The gel filtration of chitosan and its derivative was followed by analyzing fractions of the eluate by the method of Langunoff and Warren.[5]

Methods of radioisotope measurement

For the measurement of radioactivities [^{35}S] labelled derivatives were hydrolysed in 4 N HCl for 4 hr at 100° and liberated inorganic $^{35}SO_4^{2-}$ ions then precipitated as $Ba^{35}SO_4$.[6] The radioactivities of the precipitates were measured as "infinitely thick

layers" using a gas-flow counting system (D.47) in windowless operation, in conjunction with automatic scaling equipment (Nuclear–Chicago Corp., Ill., U.S.A.). Corrections were made for background, coincidence and decay and sufficient counts were recorded to ensure a standard error of less than 2 per cent. Radioactive areas on paper strips after electrophoresis were detected using a Packard Model 7201 gasflow chromatogram scanner in 4π operation. Radioactive areas on agarose gels were detected with the same instrument operated in the 2π mode.

EXPERIMENTAL AND RESULTS

Trimethylamine-[^{35}S] sulphur trioxide reagent

Chloro-[^{35}S] sulphonic acid (0·6 ml) was mixed cautiously with dry chloroform (1·0 ml) at −5° and the mixture then added in a drop-wise manner to anhydrous trimethylamine (3·5 ml) in dry chloroform (1·0 ml) previously cooled to −5°. Vigorous stirring was used throughout the addition, which was accomplished in a period of 30 min. The white trimethylamine-[^{35}S] sulphur trioxide complex which precipitated during this period was collected by centrifuging and then washed several times by suspension in dry chloroform at −5°, with intermediate centrifuging, before drying *in vacuo* over $CaCl_2$. Yields of the radioactive product in the range 1·2–1·3 g were usually obtained by this method.

Preparation and characterization of low molecular weight [^{35}S] sulphamates

Potassium 2-deoxy-2-[^{35}S] sulphoamino-D-glucose. This derivative was prepared in the manner described by Lloyd and co-workers[7] except that trimethylamine-[^{35}S] sulphur trioxide was used as sulphating agent in place of the less stable pyridine-[^{35}S] sulphur trioxide employed in the original procedure. The yield of product was increased to 49 per cent of theoretical, compared to 28 per cent for the original method. In addition use of the trimethylamine-based reagent eliminated the production of the contaminating "dye" materials (see Baumgarten[8]), which are by-products of the reaction of pyridine-sulphur trioxide in alkaline aqueous media, greatly simplifying purification of the reaction mixture. Chromatography of the [^{35}S] sulphamate derivative on Dowex-1 ion-exchange resin, a necessary step in the original method,[8] was found to be unnecessary. Potassium 2-deoxy-2-[^{35}S] sulphoamino-D-glucose obtained by the modified procedure showed only one radioactive component on paper electrophoresis and had a sp. act. of 4·8 mc/mM. On analysis the product gave C, 22·8; H, 4·8; total N, 4·3; hexosamine N, 4·3; SO_4^{2-}, 30·1; K, 12·6. $C_6H_{12}O_8NSK{\cdot}1H_2O$ requires C, 22·8; H, 4·4; N, 4·4; SO_4^{2-}, 30·5; K, 12·4. The infrared spectrum was identical with that previously recorded.[7]

Potassium [^{35}S] sulphoamino-L-serine. L-Serine (1·0 g) was dissolved in 20 ml of water and the pH of the solution adjusted to 9·5 by the addition of 2 N NaOH. Trimethylamine-[^{35}S] sulphur trioxide reagent (1·0 g) was added to the well-stirred solution over a period of 2 hr at 55°. During the period of the addition the pH of the mixture was kept between 9 and 10 by the addition of solid anhydrous sodium bicarbonate (total 0·93 g). The reaction mixture was kept at 55° for 20 hr. At the end of this period the colourless solution was passed through a column of Amberlite CG-120 (H^+ form; 100–120 mesh; 10 × 1·5 cm) to remove Na^+ ions, residual L-serine and traces of dissolved trimethylamine. The acid eluate and washings from the column were

pooled and adjusted to pH 8·0 with saturated aqueous $Ba(OH)_2$ to precipitate $^{35}SO_4^{2-}$ ions as $Ba^{35}SO_4$. Precipitated material was removed by centrifuging and the clear supernatant then passed through a column of Amberlite CG-120 (H^+ form; 100–200 mesh; 10 × 1·5 cm) to remove excess Ba^{2+} ions. The acid eluate and washings were pooled and adjusted to pH 7·5 with 2 N KOH. The volume of the solution was then reduced to 4·0 ml and 10 vol. of ethanol added to precipitate the product. The white, flocculent precipitate was collected by centrifuging and then redissolved in a 60% (v/v) ethanol–water mixture (10 ml). Crystallization was induced by the drop-wise addition of acetone to incipient turbidity followed by cooling to 4°. The product was recrystallized from 60% (v/v) ethanol–water as above, filtered and dried *in vacuo* over $CaCl_2$. Potassium [^{35}S] sulphoamino-L-serine prepared in this way (yield 51 per cent of theoretical) was homogeneous on paper electrophoresis and had a sp. act. of 4·6 mc/mM. Analysis of the product gave C, 13·2; H, 218; total N, 5·4; amino acid N, 5·35; SO_4^{2-}, 36·4; K, 29·8. $C_3H_6O_6NSK_2$ requires C, 13·7; H, 2·3; N, 5·4, SO_4^{2-}, 36·6; K, 29·7. The infra-red spectrum of potassium [^{35}S] sulphoamino-L-serine (see Fig. 1)

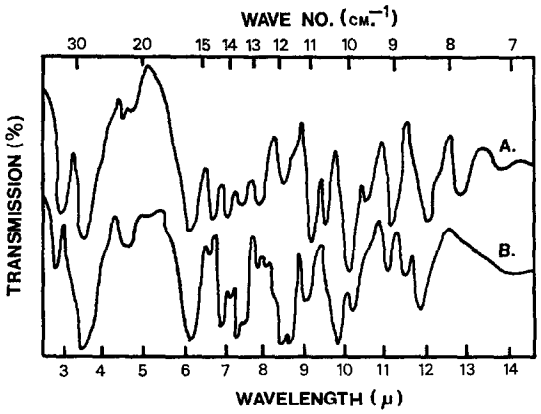

FIG. 1. Infra-red spectra of L-serine (curve A) and [^{35}S] sulphoamino-L-serine (curve B). Spectra are displaced vertically to facilitate comparison.

exhibited a strong absorption at 3320 cm^{-1}, attributable to vibrations involving the unsubstituted hydroxyl grouping, as well as a band of strong absorption in the range 1150–1250 cm^{-1} (maximum 1200 cm^{-1}), previously attributed to vibrations involving S—O linkages in the N-SO_3^- system.[7] These findings were in agreement with the existence of a sulphamate (N-sulphate) grouping rather than an O-sulphate grouping in the labelled derivative.

Preparation and characterization of high molecular weight [^{35}S] *sulphamates*

Potassium ([^{35}S] *sulphoamino*)*-heparin.* (a) *Preparation of the* [^{35}S] *labelled derivative.* Both batches of commercial heparin used in the study were found to be contaminated with trace amounts of Cl^- and SO_4^{-2} ions. Removal of these contaminants was achieved by passing 100 ml of a 5% (w/v) aqueous solution of the polysaccharide through a column of Amberlite CG 400 (OH^- form; 100–200 mesh; 20 × 3 cm). The eluate and washings were combined, adjusted to pH 7·5 with 2 N acetic acid and the volume of the solution then reduced to 20 ml by freeze-drying. Precipitation of the

polysaccharide was achieved by the addition of 10 vol. of absolute ethanol and the flocculent precipitate collected by centrifuging. The precipitates were washed twice by suspension in ethanol, with intermediate centrifuging and finally by suspension in ether, followed by centrifuging before drying *in vacuo* over $CaCl_2$. Portions of both purified polysaccharides were retained for analysis (see Table 1).

N-Desulphation of heparin preparations was achieved under conditions known to minimize hydrolysis of *O*-sulphate linkages in the polysaccharide.[9] A 4% (w/v) aqueous solution of heparin (100 ml) was passed through a column of Amberlite CG-120 (H^+ form; 100–200 mesh; 30 × 3 cm). The acid eluate and washings from the column were combined and then kept at 55° for 24 hr. After this period the solution was passed through a column of Amberlite CG-400 (OH^- form; 100–200 mesh; 30 × 3 cm) to remove SO_4^{2-} ions liberated as a result of the acid hydrolysis of the sulphamate groupings. The eluate and washings from the column were combined and adjusted to pH 7·5 by adding 2 N KOH. The solution was then lyophilized to dryness and finally dried *in vacuo* over $CaCl_2$. Yields of *N*-desulphated heparin in the range 3·2–3·5 g were usually obtained. Portions of the products were retained for analysis (see Table 1).

[^{35}S] Sulphation of the free amino residues of *N*-desulphated heparin was achieved in the following way.[9] *N*-Desulphated heparin (1·3 g) was dissolved in water (25 ml) and the solution adjusted to pH 9·5 by the addition of 2 N NaOH. The solution was then warmed to 55° before the addition of trimethylamine-[^{35}S] sulphur trioxide (1·3 g) and sodium bicarbonate (1·1 g) with vigorous stirring. The alkaline solution was then kept at 55° for 24 hr. After cooling to 15° the solution was passed through a column of Amberlite CG-400 (OH^- form; 100–200 mesh; 10 × 2 cm) to remove inorganic CO_3^{2-} and SO_4^{2-} ions. The pooled eluate and washings were then passed through a column of Amberlite CG-120 (H^+ form; 100–200 mesh; 10 × 2 cm) to remove Na^+ ions and residual trimethylamine. The final eluate was adjusted to pH 7·5 with 2 N KOH and then dialyzed exhaustively against changes (equivalent to 100 vol.) of distilled water in a shaking dialyzer at 4° for 16 hr to remove low molecular weight components. The solution from the dialysis bag was then lyophilized to dryness and finally dried *in vacuo* over $CaCl_2$. Yields of the labelled polymer, designated for practical purposes as ([^{35}S] sulphoamino)-heparin, in the range 1·1–1·3 g were obtained by this method. The radioactivities of the preparations were in the range 1·7–1·9 mc/mM of S. Analytical figures are recorded in Table 1.

(b) *Characterization of* ([^{35}S] *sulphoamino)-heparin.* The methods used for the characterization of the ([^{35}S] sulphoamino)-heparin preparations were chosen deliberately to provide conclusive evidence for the close similarity of the products of chemical modification and the initial commercial materials (see Table 1). First and foremost, the values obtained for the ester sulphate contents were consistent with the complete restoration of sulphamate groupings during the preparation of the [^{35}S] labelled derivatives. This feature was confirmed independently by the return of anticoagulant activity for the ([^{35}S] sulphoamino)-heparins, contrasting sharply with the properties of the *N*-desulphated heparins. Furthermore, both [^{35}S] labelled heparin preparations showed only single zones of radioactivity on paper electrophoresis indicating the absence of inorganic $^{35}SO_4^{2-}$ ions. The intermediary stages in the method of preparation also excluded the possibility that any inorganic $^{35}SO_4^{2-}$ ions were "bound" to the labelled polymers as an exchangeable anion increment as suggested by Helbert and Marini.[10] Lastly, electrophoresis on agarose gels indicated that

TABLE 1. ANALYTICAL FIGURES FOR HIGH MOLECULAR WEIGHT PREPARATIONS

	Ester sulphate (%)	Total N (%)	Glucosamine N (%)	Uronic acid		Electrophoretic mobility*	Anticoagulant activity (IU/mg)
				Phenol-H_2SO_4 (%)	Carbazole (%)		
Beef mucous heparins:							
Batch no. A-50601	38·1	2·45	21·7	28·1	42·9	1·0	149
Batch no. B.45700	38·7	2·37	22·1	27·8	42·3	1·0	153
N-Desulphated heparins:							
Batch no. A.50601	32·1	2·61	27·5	30·2	49·4	0·82	8
Batch no. B.45700	30·1	2·52	26·2	31·4	48·6	0·84	6
([^{35}S] Sulphoamino)-heparins:							
Batch no. A.50601	38·5	2·42	21·5	27·9	42·1	1·0	137
Batch no. B.45700	38·4	2·33	22·8	28·0	41·8	1·0	145
([^{35}S] Sulphoamino)-chitosan	30·4	5·21	66·7	—	—	0·68	0

* Mobility on agarose gel electrophoresis, using British Standard mucosal heparin as reference.

the ([^{35}S] sulphoamino)-heparins had mobilities identical with those of the initial polysaccharides, the mobilities of the *N*-desulphated materials being significantly lower. In such separations the radioactive zone corresponded identically with the positions of the polysaccharides revealed by staining with toluidine blue.

Analyses for uronic acid by the phenol-sulphuric acid procedure and for hexosamine with the autoanalyzer were also in accord with close constitutional identity of both batches of heparin and the [^{35}S] labelled preparations derived from them. It was also considered noteworthy that both unlabelled and labelled preparations gave similar results for uronic acid in the carbazole reaction in which heparin is known to behave aberrantly.[4] There was no evidence for the presence of dermatan sulphate in any of the preparations as only D-glucosamine could be detected in analyses of acid hydrolysates of each of the polymers with the autoanalyzer. Moreover, as expected from the prolonged exposure of the polymers to alkaline conditions during the [^{35}S] sulphation procedure, the same analyses failed to reveal the presence of residual peptide material, particularly L-serine[11] in the [^{35}S] labelled preparations.

Infra-red spectroscopic analysis was also used in the comparison of the materials. The spectra of the original polysaccharides and of the corresponding ([^{35}S] sulphoamino)-derivatives were indistinguishable from one another (see Fig. 2). On the other

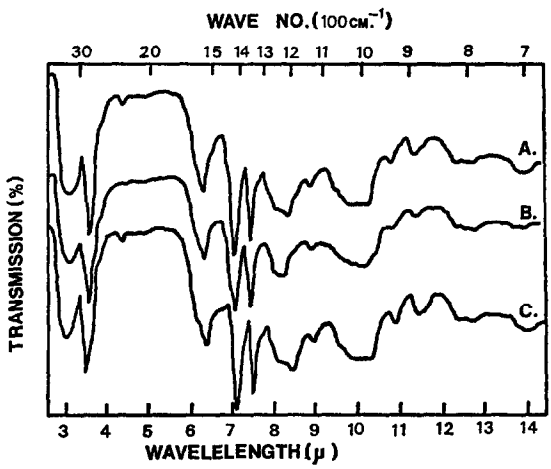

FIG. 2. Infra-red spectra of heparin and derivatives. Commercial beef mucous heparin, batch no. A.50601—curve A. *N*-Desulphated heparin derived from batch no. A.50601—curve B. ([^{35}S] Sulphoamino)-heparin derived from batch no. A.50601—curve C. Spectra are displaced vertically to facilitate comparison. Essentially identical spectra were given by heparin batch no. B.45700 and its derivatives.

hand, minor differences in the region 1200–1250 cm^{-1} consistent with the removal of the natural sulphamate groupings and accompanied by a shift to higher frequencies of the position of maximum absorption of the band attributable to vibrations involving the —SO_3^- system, were always observed in spectra of samples of *N*-desulphated heparins.[7,12]

Both of the original commercial heparins were retarded on filtration through Sephadex G-100 (Fig. 3a). In each case the elution profiles were indicative of a substantial molecular weight polydispersity and indeed, of an unusually high apparent

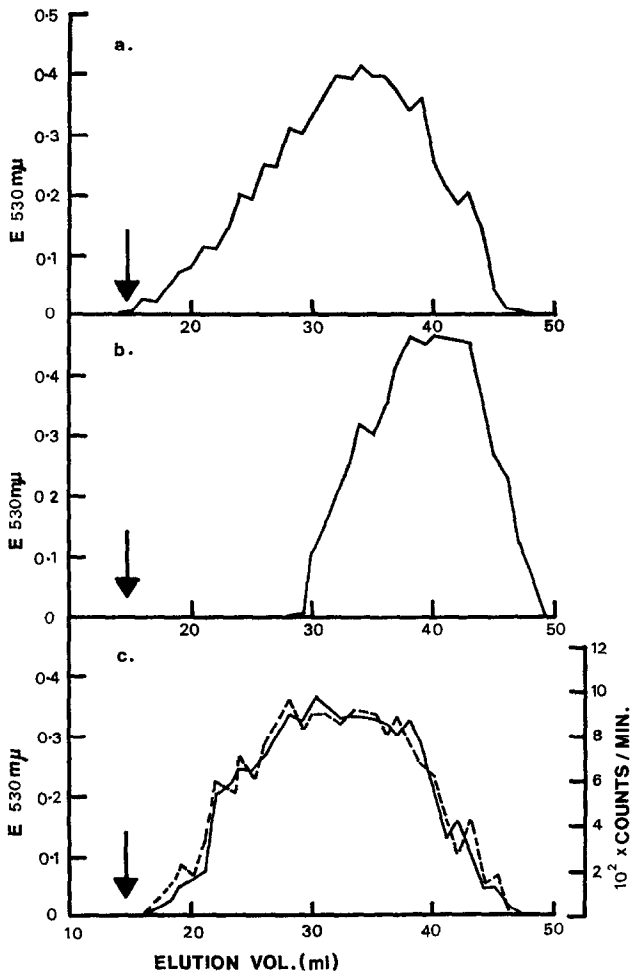

FIG. 3. Elution profiles obtained following the gel-filtration of heparin (batch no. A.50601) and its derivatives on Sephadex G-100. Experimental points have been omitted for the sake of clarity. (a) Original heparin. (b) *N*-Desulphated heparin. (c) ([^{35}S] Sulphoamino)-heparin. ——, elution of carbohydrate as measured by the carbazole reaction[4] at 530 mμ; ——, elution of radioactivity. The arrow in each case indicates the elution volume of a sample of blue Dextran. Essentially similar results were obtained with heparin batch no. B.45700 and its derivatives.

molecular weight. However, in this latter context it is now recognized that the behaviour of heparin on filtration through cross-linked dextran gels is governed not only by the molecular weight of the polysaccharides, but also by the large radius of gyration conferred on the anionic polysaccharide chains by the stiffly-extended conformation which they are likely to assume in solution.[13, 14] The elution profiles obtained following the gel filtration of the *N*-desulphated heparin derivatives differed appreciably from those recorded with the original polysaccharides (Fig. 3b). At first sight these seemed to indicate a reduction both in apparent molecular weight and the degree of polydispersity of the polysaccharides. Subsequent consideration focussed attention on the fact that the *N*-desulphation of heparin was accompanied by the creation of cationic

centres in the otherwise intensely anionic polysaccharide chains. Such a state of affairs would be expected to contribute to a collapse of the original extended macromolecular configuration favoured by the formation of intramolecular ionic bonds leading to a more compact polymeric structure and a substantially decreased axis of gyration. Equally, *N*-re-[^{35}S] sulphation would be expected to reverse this physical effect. This view was confirmed by the elution patterns recorded following the gel filtration of ([^{35}S] sulphoamino)-haparin preparation (Fig. 3c). In both instances the profiles of carbohydrate elution closely resembled those obtained with the original materials, indicating the restoration of the large axis of gyration with no detectable change in apparent molecular weight for the [^{35}S] labelled polysaccharides. In these separations the elution of carbohydrate and radioactivity corresponded closely. Radioactive contaminants of low molecular weight were not detected.

Potassium ([^{35}S] sulphoamino)-chitosan. (a) *Preparation of the [^{35}S] labelled polymer.* Chitosan (87 mg) was suspended in 20 ml of water and the suspended material then dissolved by the addition of 3 ml of conc. HCl. The chitosan hydrochloride was then converted to a flocculent precipitate of free chitosan by the gradual addition of 20 ml of 4 N NaOH. The pH of the suspension was then adjusted to pH 9·5 and the whole stirred at 55° vigorously during the addition of 1·5 g of trimethylamine-[^{35}S] sulphur trioxide. The pH of the reaction mixture was kept at 9·5 during the addition by periodic adjustments with 2 N NaOH. The whole was then kept at 55° for 20 hr during which the original flocculent precipitate dissolved completely. At the end of this period the volume of the solution was reduced to 10 ml by rotary evaporation under reduced pressure at 37° and the concentrated material then dialyzed exhaustively against several changes of distilled water (equivalent to 100 vol.) in a shaking dialyzer at 4° for 16 hr to remove low molecular weight components. The contents of the dialysis bag was then passed through a column of Amberlite CG-120 (H^+ form; 100–200 mesh; 10 × 2 cm) to remove residual trimethylamine and Na^{2+} ions. The acid eluate and washings were pooled, adjusted to pH 7·5 by the addition of 2 N KOH and concentrated to 5 ml by rotary evaporation under reduced pressure at 37°. Potassium acetate (80 mg) was added to the concentrate followed by 5 vol. of absolute ethanol. Precipitated material was collected by centrifuging, redissolved in 5 ml of water and reprecipitated by the addition of 5 vol. of absolute ethanol. The precipitate was collected by centrifuging, washed twice by suspension in ethanol and once by suspension in ether, with intermediate centrifuging and finally dried *in vacuo* over $CaCl_2$ under reduced pressure. Radioactivities of successive preparations were in the range 4·1–4·4 mc/mM of S.

(b) *Characterization of ([^{35}S] sulphoamino)-chitosan.* Analyses for the preparations are recorded in Table 1. The figures are averages for three preparations, with ranges in parentheses. In agreement with previous findings for unlabelled (sulphoamino)-chitosan[1] the analyses were in accord with the complete substitution of all of the free amino residues present in the original chitosan. The materials moved as single radioactive zones on paper electrophoresis and no evidence was obtained for contamination of the preparations with inorganic $^{35}SO_4^{2-}$ ions. The results of infrared spectroscopy (Fig. 4) again favour *N*-[^{35}S] sulphation, as opposed to the formation of *O*-[^{35}S] sulphate derivatives, as the frequency of maximum absorption attributable to the $-SO_3^-$ system occurs at 1200 cm^{-1} rather than 1240 cm^{-1} (see Lloyd and coworkers[7,15,16]).

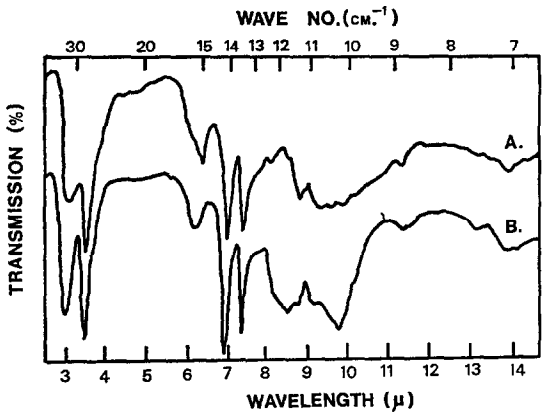

FIG. 4. Infra-red spectra of chitosan (curve A) and ([^{35}S] sulphoamino) chitosan (curve B). Spectra are displaced vertically to facilitate comparison.

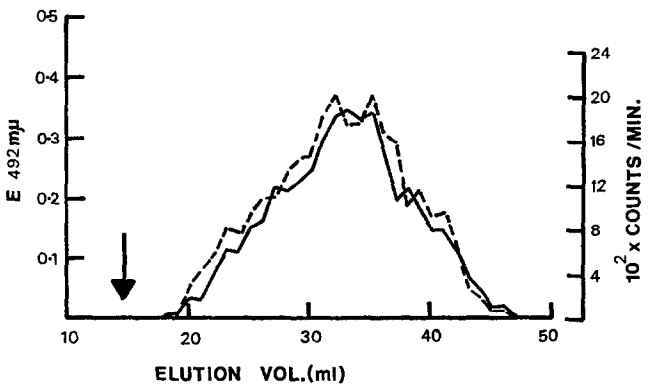

FIG. 5. Elution profiles obtained following the gel filtration of ([^{35}S] sulphoamino)-chitosan on Sephadex G-100. Experimental points have been omitted for the sake of clarity. ——, elution of hexosamine measured by the method of Lagunoff and Warren at 492 mμ; ——, elution of radioactivity. The arrow indicates the elution volume of a sample of blue Dextran.

The insolubility of chitosan in the buffer used for gel-filtration studies precluded an examination of the molecular weight characteristics of this material. On the other hand, the behaviour of the soluble ([^{35}S] sulphoamino)-chitosan (Fig. 5) on Sephadex G-100 indicated a combination of polydisperse macromolecular properties and a large radius of gyration for the polysaccharide [^{35}S] sulphamate in solution. Elution of carbohydrate and radioactivity again corresponded closely.

DISCUSSION

Considered as a member of the widespread group of sulphated polysaccharides in Nature, the polysaccharide heparin exhibits three distinct features of uniqueness. Firstly, it is the only major representative of the group in mammals which exists normally in an intracellular environment, apparently contributing to the framework of the granular cytoplasmic inclusions of mast cells, the other members have a largely extracellular distribution in the amorphous ground substance of the connective tissues.

Secondly, amongst the polysaccharides whose structural organization has been established with some certainty, heparin has the distinction of being the most anionic polymer found in mammals. Each tetrasaccharide period within the polymer is conceived as bearing seven potentially acidic groupings (see Dodgson and Lloyd[17] for recent review). Two of these arise from the carboxyl groups of the constituent hexuronic acids, three from the esterification of hydroxyl groups with *O*-sulphate moieties and a further two contributed by the substitution of amino groups with covalently-bound sulphate giving rise to sulphamate residues. ω-Heparin, mactins A and B and the heparan sulphates[17] are each possible competitors for the structural distinctions associated with heparin, particularly since they also contain sulphamate residues, However, these polymers remain poorly defined in contemporary chemical terms. Finally, heparin holds the distinction of being the sole representative of the group of acidic glycosaminoglycans of mammalian origin to be produced on a commercial scale for use as a therapeutic agent in man. Thus, in addition to the knowledge which must be gathered regarding the biological "turnover" of endogenous heparin, the acquisition of information regarding natural methods available for the disposal of exogenous heparin is obviously of prime importance.

The physical, analytical and biological criteria applied in the present work to the characterization of ([^{35}S] sulphoamino)-heparin clearly established the close structural similarity of the semi-chemically synthesized derivative and the original natural polymer. In the light of previous studies[17] it must be presumed that the labelled derivative has a polymeric structure based on the tetrasaccharide period shown in Fig. 6.

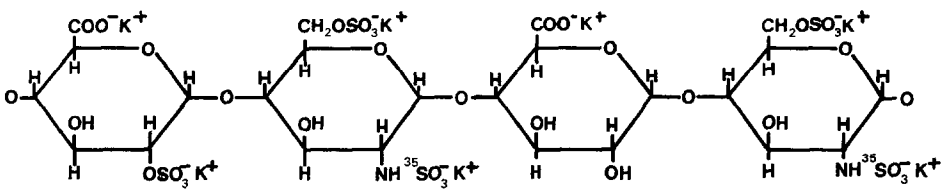

FIG. 6. Structure of the tetrasaccharide repeating period of ([^{35}S] sulphoamino)-heparin.

([^{35}S] Sulphoamino)-chitosan was selected as a polymeric "model" of the 2-deoxy-2-[^{35}S] sulphoamino-D-glucose residues which occur in labelled heparin. Derived structurally from the naturally occurring polysaccharide chitin the structure of ([^{35}S] sulphoamino)-chitosan is presumed to be that of a *poly*,β1:4-2-deoxy-2-[^{35}S] sulphoamino-D-glucose as shown in Fig. 7.

Although having a molecular weight approximating to that of ([^{35}S] sulphoamino)-heparin, the chitosan derivative is notably different from heparin in the structural

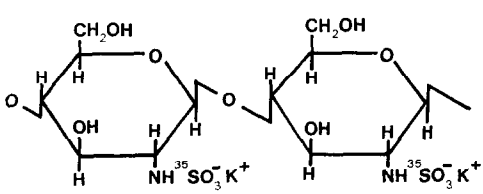

FIG. 7. Structure of the disaccharide period of ([^{35}S] sulphoamino)-chitosan.

FIG. 8. Structures of 2-deoxy-2-[^{35}S] sulphoamino-D-glucose (I) and [^{35}S] sulphoamino-L-serine (II).

sense since it is a wholly β-linked polymer and is devoid of hexuronic acid and *O*-sulphate moieties. The monosaccharide derivative, 2-deoxy-2-[^{35}S] sulphoamino-D-glucose (Fig. 8) provided a low molecular weight alternative for studies on factors influencing the biological stability of carbohydrate sulphamate linkages.

Finally, [^{35}S] sulphoamino-L-serine (see Fig. 8) was acquired with the intention of providing a [^{35}S] sulphamate based on an amino acid structure designed, in the long term, as a derivative suitable for probing the catalytic events occurring at the active centre of sulphamate-degrading enzymes.

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