The 'Lorenzan Sulphates'

A NEW GROUP OF VERTEBRATE MUCOPOLYSACCHARIDES

BY J. DOYLE
Marine Station, Millport, Isle of Cumbrae, Scotland

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1. The mucopolysaccharide from the glands of Lorenzini of *Squalus acanthias* has been isolated and purified. 2. The material is a sulphated polysaccharide containing glucosamine, galactosamine, galactose and traces of an uronic acid. 3. This vertebrate polysaccharide is unusual in appearing to have both amino sugars present in the molecule. The amino sugars are *N*-acetylated. 4. This molecule is one of a group of related mucopolysaccharides which vary in composition with the animal species. The generic name 'lorenzan sulphates' is suggested to describe these substances.

The organs of Lorenzini are specialized sense organs which occur in elasmobranch fishes. In the shark-like fishes, they are located entirely in the head regions; in the rays, or skate-like fishes, the ducts can lead over the entire body surface to end in the pores at the trailing edge, an arrangement undoubtedly suited to the geometry and behaviour of the animal. Groups of small ampullae are served by branches of the facial nerve; from the nerve end the ampullae extend into tubes, often many centimetres in length, that end in a pore on the skin surface quite visible to the naked eye and sometimes more than a millimetre in diameter. The ampullae and the tubes are filled with a hyaline jelly which varies in 'stiffness' from species to species. This jelly is in contact with the sea water at the pore, and, at the other end, with the nerve end. The function of this organ is still uncertain, but the arrangement is extremely sensitive to a wide variety of artificially applied stimuli. An understanding of the chemical nature of this jelly might conceivably help in appreciating the physiological role of the system.

Jensen (1956) made a brief study, and found that the jelly contained mucopolysaccharide material, which he concluded, from its behaviour towards hyaluronidases, was a mixture of hyaluronic acid and chondroitin sulphates. Doyle (1963) examined the material from *Squalus acanthias* and performed chemical analyses which contradicted these conclusions, and showed galactose, glucosamine and galactosamine to be present. The small amount of uronic acid found did not agree with the presence of hyaluronic acid, and was not stoicheiometric with the amount of galactosamine. It was decided to extend these investigations by isolating the carbohydrate material and examining its properties. This paper is the first account of this work.

MATERIALS AND METHODS

Collection of jelly. All material was from *Squalus acanthias*. Freshly caught specimens from the Clyde sea area were used, and the jelly was expressed from the pores in the head by pressure with a heavy spatula on the skin surface. About 1g. of wet jelly per adult animal can be collected this way. This represents a small percentage of the material present.

Analysis of the crude jelly. Since all pooled samples of jelly from *Squalus acanthias* showed an amino sugar ratio (glucosamine:galactosamine) 4:1, the possibility that this might be a statistical artifact was examined by determining the ratio for samples from four individual fish.

Preparation of the polysaccharide. A 92g. sample of fresh jelly in 1000 ml. of pH 6·5 buffer was digested with papain at 65° for 24hr. in the presence of activators, as recommended by Scott (1960). The digest was concentrated in a rotating evaporator at 50° to 100ml., filtered through Whatman no. 541 paper and purified on columns of Sephadex G-50. Columns of the Sephadex G-50 were equilibrated with water. Portions (5ml.) of the enzyme digest were added and allowed to drain into the column. This was eluted with water and 4·0ml. fractions were collected. Portions (0·1ml.) were removed for analysis. These were examined for carbohydrates by the anthrone reaction, for metachromasia with Azur A at pH2, and for Cl^- ion (Fig. 1). The anthrone-positive component paralleled that showing metachromasia. The protein components were analysed by the method of Lowry, Rosebrough, Farr & Randall (1951). The anthrone-positive fractions (A) were pooled, concentrated and the crude material was precipitated by adding 5vol. of ethanol and leaving for 48hr. at 5°.

Further purification. Portions were treated by Sevag's method to remove residual protein, and then by treatment

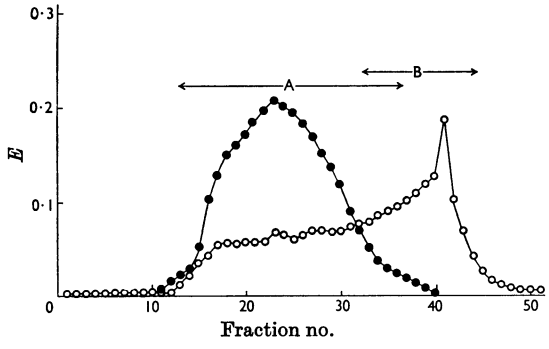

Fig. 1. Separation of major components of papain digest of Lorenzini jelly. Column, Sephadex G-50, 30cm. × 2·5cm.; eluent, water. Fraction volume, 4ml. A, Metachromatic zone; B, Cl^- ion. ●, Anthrone analysis; ○, protein.

on a column of DEAE-cellulose. DEAE-cellulose columns (2cm. × 20cm.) in the chloride form were used. Columns were packed in 0·1M-NaCl. Portions estimated to contain 15mg. of crude polysaccharide were added to the column and washed in with 5ml. of water. The column was then washed with 100ml. of 0·1M-NaCl. The metachromatic material appearing with a further 100ml. volume of 1·0M-NaCl was concentrated at 50° *in vacuo* to 10ml. and the chloride removed by treatment on Sephadex G-50 as before.

Chemical analyses. Analysis of individual amino sugars was by Gardell's (1953) method, and total hexosamine analysis was performed by the technique of Boas (1953). Sulphate analysis (as total oxidizable sulphur) was by the method of Jones & Letham (1954). Nitrogen analysis was by the Kjeldahl method, with a Markham-type still. Neutral hexose was assayed by the anthrone method (Trevelyan & Harrison, 1952). Acetyl-group analysis was by the method of Ludowieg & Dorfman (1960). Uronic acid analysis was by the method of Bitter & Muir (1962).

Paper electrophoresis. This was carried out on 2·5cm.-wide strips of filter paper (Whatman no. 3) in 0·1M-veronal buffer, pH8·6, for 90min. at 15v/cm. Strips were dried and then stained in 1% Azur A in 0·01N-HCl. Excess of dye was washed off with dilute acetic acid. Ox-nasal-septum chondroitin sulphate (a mixture of the isomers) was run on parallel strips simultaneously as controls. Mixed solutions of the chondroitin sulphate and the Lorenzini-jelly polysaccharide were run in addition. For preparative purposes 30 runs were performed with the purified polysaccharide, five strips being used per run, and the position of the metachromatic bands was located by staining the outer millimetre of the two outer strips. The areas corresponding to the stained zone were cut out, pooled with all the other pieces and eluted with water, and the extracts were then dialysed against distilled water. The solution was then made 4·0N with respect to HCl, and hydrolysed for 14hr. at 105°. The evaporated solution was then subjected to fractionation and analysis for amino sugars according to the method of Gardell (1953).

Fractional precipitation of the polysaccharide. The technique of fractional precipitation of the calcium salts of chondroitin sulphate and keratosulphate as developed by Meyer, Linker, Davidson & Weissmann (1953) was used. The free acid (30mg.) was prepared by passing the polysaccharide through a column (2cm. × 20cm.) of Zeo-Karb 225 cation-exchange resin in the H^+ form, washing with 50ml. of water. This was then concentrated to a volume of 4·0ml., and adjusted to an acetic acid concentration of 0·5N, and calcium acetate added to make a 5% concentration. Ethanol was added with stirring to various known concentrations, and the material left in this condition for 24hr. at 4° in between each addition. Any precipitated material was spun off and retained for analysis before further adjustments to the ethanol concentration were made.

Paper chromatography. The polysaccharide material was hydrolysed for 5hr. in N-H_2SO_4 at 105° in a sealed tube. Barium carbonate was added to neutrality, and the filtered solution concentrated and added to a small (2cm. × 20cm.) column of Zeo-Karb 225 cation-exchanger. The acid and neutral sugar components were eluted with water. Descending chromatography was used with ethyl acetate–propan-1-ol–water (7:1:2, by vol.) and butanol–pyridine–water (5:3:2, by vol.) as the solvent systems. Portions of neutralized hydrolysate untreated by the resin technique were also used to identify the amino sugars by the method of Stoffyn & Jeanloz (1954), with the butanol–pyridine–water system.

Identification of neutral sugars. To confirm the chromatographic findings, attempts were made to form a derivative. The hydrolysate was freed from amino sugars by passing the neutral solution through a 2cm. × 20cm. column of Zeo-Karb 225 (H^+ form) and washing the column with 50ml. of water; the total eluate was then concentrated at 50° *in vacuo* to near-dryness and taken up in 1·0ml. of water. A portion equivalent to 25mg. of purified polysaccharide was used. A portion (0·5ml.) of aqueous solution of the neutral sugar was mixed with 0·5ml. of ethanol, 0·05ml. of α-methylphenylhydrazine and 0·05ml. of 50% acetic acid. The solution was kept at 37° for 6hr. and then at 0° overnight. The crystals formed were washed with absolute ethanol and ether and dried in air. Standard and mixed melting points were made on the preparation and on authentic galactose α-methylphenylhydrazone prepared in the same way.

Treatment with hyaluronidase. Purified polysaccharide (15mg.) was incubated for 5 days at 37° in the presence of toluene, with 2·5mg. of testicular hyaluronidase (British Drug Houses Ltd., Poole, Dorset) in 4ml. of acetate buffer, pH6·5, made 0·15M with respect to NaCl. The experiment was performed twice, at different times and with different polysaccharide preparations. The effects of the enzyme on the amino sugar ratio were examined by separating the small-molecular material from the large on a column (22cm. × 1·7cm.) of Sephadex G-75. The sample was applied and the column eluted with water. Fractions (1ml.) were collected, and the eluate was examined by the Molisch test, and by checking for metachromasia by spotting with Azur A in 0·01N-HCl on a white glazed porcelain tile. The metachromatic fractions were pooled in two lots arbitrarily divided for analysis. Fractions 6–16, and fractions 17–28, were taken for quantitative amino sugar analysis by Gardell's (1953) method. The small-molecular material, judged by chloride tests, started at fraction 30.

Table 1. *Hexosamine analysis (Gardell, 1953) on native Lorenzini jelly and on preparations*

Figures are expressed as a percentage of the total hexosamine.

		Glucosamine	Galactosamine
(A) Crude jelly from individual fish	(1)	80·5	19·5
	(2)	78·8	21·2
	(3)	79·0	21·0
	(4)	79·7	20·3
(B) Electrophoretically purified material	(1)	78·8	21·2
	(2)	79·7	20·3

Table 2. *Chemical analyses of purified Lorenzini-jelly polysaccharide preparation (LS/2)*

Figures are for the sodium salt.

	Percentage	Molar ratio
Galactose	30·0	1·0
Uronic acid	2·85	0·08
Total hexosamine	28·6	0·96
Acetyl	7·4	1·03
Sulphate	14·5	0·91

Mild alkali treatment. Purified material (10mg.) was dissolved in 0·1N-NaOH, then heated at 40° for 1hr. The solution was neutralized and dialysed against several changes of veronal buffer, and subjected to paper electrophoresis as already described, ox-nasal-septum chondroitin sulphate and untreated polysaccharide being used as control strips during the run.

Infrared spectrum. Infrared-spectral analysis was performed by the courtesy of the Department of Chemistry, University of Birmingham. Polysaccharide (1·5mg.) was used with 300mg. of KBr in a KBr disk.

RESULTS

The hexosamine analyses on crude jelly of the individual fish and for the various purifications and fractionations are shown in Table 1.

Preparation of polysaccharide. Crude jelly (92g.) gave 2·82g. of crude powder as the sodium salt. This crude material had a total hexosamine content of 23·9%. The original jelly had 10·5μg. of hexosamine/mg. wet wt. of jelly, the recovery thus being 70% at this stage. A further 0·51g. of material from the ethanolic solution was collected on standing for some weeks. This gives a yield of crude material of 83% in terms of the starting material. The nitrogen content of this material (designated LS/1) was 4·34%, and the further purification steps (Sevag's deproteinizing method), followed by purification on ion-exchange columns, reduced this nitrogen figure to 2·95%. This purified material was designated LS/2.

Chemical analysis. The analytical results for LS/2 are given in Table 2 together with the molar ratios relative to galactose. The figures are uncorrected for hydrolytic degradation.

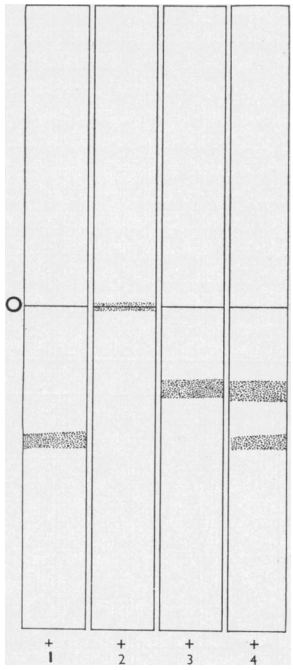

Fig. 2. Paper electrophoresis diagram of mucopolysaccharides from Lorenzini jelly. See the text for conditions. O, Origin. 1, Chondroitin sulphate. 2, Native Lorenzini jelly. 3, Purified lorenzan sulphate. 4, 1 + 3.

The acetyl group is evidently as *N*-acetyl, presumably on the hexosamine, for the purified material gave a negative ninhydrin reaction, but after treatment with 0·1N-sulphuric acid for 1hr. at 100° the ninhydrin reaction was strongly positive.

Electrophoresis. Fig. 2 shows a metachromatic band travelling at about half the rate of mammalian chondroitin sulphate. Untreated (protein-bound) jelly material does not move from the origin. Mixtures of chondroitin sulphate and the purified preparation are readily separated.

Preparative electrophoresis. The hexosamine ratio of the material eluted from the metachromatic bands after electrophoresis on duplicate sets of experiments was the same as that in the crude jelly and the purified preparation (Table 1).

Fig. 3 shows the results of analysis of the amino sugar analysis by the Gardell (1953) technique. The separation shown is typical of all such analyses reported.

Ethanol precipitation. The yields of calcium salts at various concentrations of ethanol, and hexosamine analysis of the fractions, are given in Table 3.

Paper chromatography. Galactose was the only neutral sugar found. The R_{Glc} values were 0·87 in the ethyl acetate solvent system and 0·89 in the other. Trace amounts of uronic acids were seen, but no positive identification of the nature of the uronic acid was made. The amino sugar analysis gave R_{Glc} 1·75 for lyxose (galactosamine) and 1·31 for arabinose (glucosamine).

α-Methylphenylhydrazone. The chromatographic evidence was confirmed by the formation of the α-methylphenylhydrazone derivative. This had m.p. 179–180° (uncorrected) and gave mixed m.p. 179–181° with the authentic galactose derivative.

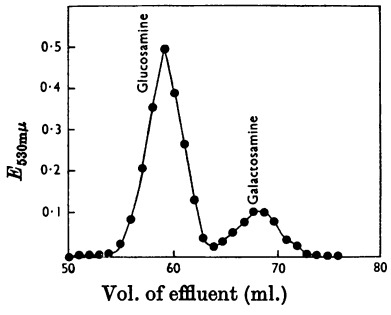

Fig. 3. Typical chromatographic analysis of the amino sugars of purified mucopolysaccharide from Lorenzini jelly of *Squalus acanthias*. The analysis is of the ethanolic (50%) precipitate of the calcium salt; 2·35mg. of the calcium salt was applied to the column. The method is according to Gardell (1953).

Hyaluronidase treatment. The effect of this enzyme on the polymer is not great, inasmuch as about 90% of the macromolecule was recovered as such after treatment. Its behaviour on gel-filtration columns, and its metachromasia, were consistent with a sulphated polysaccharide. The amino sugar ratios after enzyme treatment and fractionation on the Sephadex column were slightly changed from that of the purified material (Table 4).

Alkali treatment. Electrophoresis results were as for untreated material. No change in electrophoretic mobility was observed after this treatment,

Table 4. *Effects of hyaluronidase on the amino sugar ratio of purified Lorenzini-jelly mucopolysaccharide*

The material after enzyme treatment was separated on Sephadex G-75 columns from low-molecular-weight material, and the fractions of macromolecular material were arbitrarily separated into two groups on the basis of their elution from the Sephadex column. Results of duplicate experiments are reported.

	Total hexosamine (%)	
	Glucosamine	Galactosamine
Expt. 1		
Fractions 6–16	74·4	25·6
Fractions 17–28	82·5	17·5
Expt. 2		
Fractions 6–16	74·6	25·4
Fractions 17–28	83·3	16·7

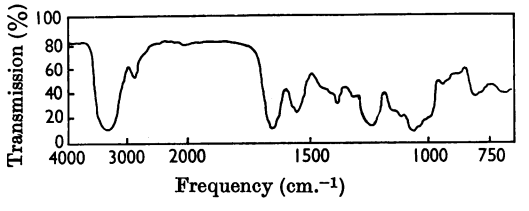

Fig. 4. Infrared spectrum of mucopolysaccharide from Lorenzini jelly of *Squalus acanthias*.

Table 3. *Yields of calcium salts mucopolysaccharide, at various concentrations of ethanol, and hexosamine analysis of the fractions*

Final concn. of ethanol (%)	Yield of calcium salt (mg.)	Total hexosamine (%)	
		Glucosamine	Galactosamine
25	—	—	—
35	—	—	—
50	17·5	77·9	22·1
66	10·9	82·4	17·6
80	—	—	—

and only one metachromatically staining band was found.

Infrared analysis. The 1240cm.$^{-1}$ (S=O stretching) characteristic of sulphate is present (Fig. 4). At 1550 and 1640cm.$^{-1}$ there are strong absorptions attributable to the presence of *N*-acetyl residues. There is not much evidence for either ionized or non-ionized carboxylic acid functions which give absorptions at 1736 and 1230cm.$^{-1}$ (CO_2H) and 1410cm.$^{-1}$ (CO_2^-).

DISCUSSION

The constancy of composition, particularly as it is expressed in the amino sugar ratios, both of pooled samples and from some individual animals, provides grounds for suspecting the existence of a true chemical compound, and not a mixture of, say, a chondroitin sulphate and keratan sulphate. In view of the very low uronic acid figures, it is difficult to account for the results in this way. The presence of uronic acid was in doubt for some time during this study. However, its presence has been repeatedly indicated by the Bitter & Muir (1962) technique, and sample blanks were used to compensate for non-specific colour formed by charring of the neutral sugars. The paper-chromatographic evidence for the presence of uronic acids would not be sufficient alone. The infrared-spectral analysis shows little or no sign of uronic acid. The galactose, total hexosamine, acetyl and sulphate appear in a molar ratio close to unity. The behaviour of the substance on gel-filtration columns, and to dialysis, and its metachromasia indicate that the molecule is large; no measurements of size have yet been made, although they would clearly be desirable. The nitrogen figures for the purified material (LS/2) indicate that the material is not entirely free from residues of protein material. It is doubtful, in view of recent structural studies on the linkage of protein to polysaccharide, whether any mild proteolytic preparative technique can free the polysaccharide entirely from amino acid residues. This is a consequence of mild preparative techniques. The evidence from this study can be accounted for by the hypothesis of a single molecular compound involving both amino sugars. Electrophoretic evidence is clear-cut, and even treatment with sodium hydroxide failed to demonstrate a separation of a chondroitin sulphate and a keratan sulphate. The finding of both amino sugars in the original ratio in this electrophoresis band is difficult to account for on the basis of any known mixture. The results of the calcium salt fractionation confirm this; no precipitation occurred below 50% ethanol concentration, a range in which one would expect chondroitin sulphate to be precipitated. Other ways of accounting for the presence of galactosamine and for the constancy of composition seem less probable.

The nature of the neutral sugar as galactose is in no doubt, and this establishes this molecule as a closer relative of keratan sulphate than of the chondroitin sulphates. It is noteworthy that the first recorded description of what was clearly keratan sulphate was also in tissue from an elasmobranch fish (Hisamura, 1938).

The behaviour of the purified material towards testicular hyaluronidase is interesting. Jensen (1956) observed changes in viscosity with testicular hyaluronidase, and also with bacterial hyaluronidase. From this, he concluded that the jelly was a mixture of chondroitin sulphate and hyaluronic acid. This, from the analytical findings reported earlier (Doyle, 1963) and in this paper, is wrong. Indeed, the enzymic evidence reported in this study shows the molecule to be to a large extent resistant to the testicular enzyme. A small variation in the amino sugar ratio was observed, the relatively larger molecules (those fractions eluted first from the gel-filtration column) being slightly enriched in galactosamine with respect to the low-molecular-weight material. This was not observed with material untreated by the enzyme. The change is not great, however, but may be of interest in subsequent structural studies. The division into two groups was quite arbitrary, as the anthrone analysis of the column effluent gave no indication of more than one peak. It is possible that although no great chemical degradative changes have taken place, yet some depolymerization may have occurred. This would account for Jensen's (1956) findings. Keratan sulphate itself, which would appear to be the closest known relative of this molecular species, is unaffected by testicular hyaluronidase.

The infrared analysis supports generally the analytical findings and agrees in assigning the position of acetyl groups to the amino group of the amino sugar. It also underlines the differences between this material and known mucopolysaccharides.

The function of this jelly in the sense organ is uncertain; so indeed to some extent is the function of the organ itself. This lack of understanding, however, is no reflection on the importance of the organ to the animal. The nature, size and arrangement of this sensory apparatus point to its being important, and it is receiving the attention of physiologists. It would be possible to suppose that the mucopolysaccharide might bind pharmacologically active bases and release them to the nerve ending on changes of temperature or salinity, and thus account for some of the physiological findings mechanistically. The proposed name 'lorenzan sulphates' (followed by the species of the

animal) is formed from the name of the Italian biologist who first described these organs, and the name ending is made consistent with modern terminological practice. Thus the compound described in this preliminary study would be 'lorenzan sulphate (*Squalus acanthias*)'.

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