An Unusual Polysaccharide in Chondroid Tissue of the Snail *Busycon*: Polyglucose Sulphate

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It is commonly accepted that if a tissue looks like cartilage it is to be considered cartilage. The characterization of tissues must, however, also take into consideration their chemical composition. Cartilage tissue is defined as tissue which, besides fulfilling the histological requirements of possessing a hyaline metachromatic matrix, can also be extracted to yield one or more of the chondroitin sulphates (A and C) and keratosulphate. These polysaccharides can be characterized by acid hydrolysis to yield respectively D-galactosamine, D-glucuronic acid and sulphuric acid or D-glucosamine, galactose and sulphuric acid.

The rasping organ (radula) in snails is supported by a chondroid apparatus, the odontophore. It was noted that the chondroid odontophore of the marine snail, *Busycon caniculatum*, does not contain chondroitin sulphate (Lash, 1959). Evidence has now been obtained which indicates that the polysaccharide present in this chondroid matrix is actually a polyglucose sulphate.

MATERIALS AND METHODS

The odontophores were dissected from the probosces and all attached muscles were removed. The tissues were stored in 100 vol. of acetone at 4°. Before analysis the tissues were removed from the acetone and allowed to dry at room temperature.

Extraction of the polysaccharide. Since the alkaline-extraction method of Einbinder & Schubert (1950) degraded the polysaccharide, enzymic digestion of the whole tissue with trypsin was employed (cf. Schiller, Mathews, Jefferson, Ludowieg & Dorfman, 1954). Odontophores were placed in dialysis sacs containing phosphate buffer, trypsin (approx. 5 mg./g. of odontophores) and a little thymol. The sac was suspended in 2 l. of potassium phosphate buffer (0.1M, pH 7.8) containing some thymol and stirred magnetically. Tryptic digestion was continued for a total period of 5 days at 38°, the dialysis medium being renewed after 2 days. Protein was removed from the contents of the dialysis sac by addition of 1/3 vol. of 40% (w/v) trichloroacetic acid. Precipitated protein was removed after 30 min. by centrifuging. The supernatant was dialysed against distilled water for 3 days at 4° and

concentrated at 28° under reduced pressure in a vacuum desiccator.

Ionophoresis. A small volume of the concentrated extract was spotted on Whatman no. 3MM paper and submitted to ionophoresis in 0.2M-sodium borate buffer, pH 8.6, with a potential gradient of 12V/cm. The paper strip was stained for metachromatic areas by immersion in 0.15% (w/v) thionin in 65% (v/v) ethanol, acidified with approx. 0.1% (v/v) acetic acid. Excess of stain was washed out with either 95% (v/v) ethanol or running tap water. Heparin (Abbott Laboratories, North Chicago, Ill., U.S.A.) and chondroitin sulphate from various animal tissues (bovine trachea, costal cartilage and nasal septa; chick embryonic cartilage; marine cartilaginous fishes) were run simultaneously as controls.

Analysis for monosaccharides. The dried extract was hydrolysed with N-sulphuric acid for 4 hr. in sealed tubes at 105°. The hydrolysate was neutralized with solid barium carbonate, centrifuged and concentrated under reduced pressure. Hydrolysates were examined for reducing sugars by the following procedures. (a) Paper chromatography on Whatman no. 1 paper, irrigated with butanol-acetic acid-water (4:1:5, v/v) for 18 hr., or with pyridine-ethyl acetate-water (1:2:2, v/v) for 18 hr. The chromatograms were stained with either aniline oxalate or with ammoniacal silver nitrate. (b) Paper ionophoresis on Whatman no. 3MM paper in either potassium phthalate (0.2M, pH 6.0) or sodium borate (0.2M, pH 8.6) buffer (Consden & Stanier, 1952). (c) Glucose was detected with the specific glucose oxidase, notatin, by the detection of generated hydrogen peroxide with horse-radish peroxidase, tartrazine (FD & C yellow no. 5) and o-tolidine (Comer, 1956). Test papers (TesTape, Eli Lilly and Co., Indianapolis, Ind., U.S.A.) impregnated with these combined enzymes and reagents were immersed in neutralized solutions of the acid hydrolysate. Analysis for 2-aminohehexoses by the Elson-Morgan colour reaction (Blix, 1948; Gardell, 1958) was carried out after destruction of the bulk of the neutral sugars by hydrolysing the tissue with 4N-hydrochloric acid for 18 hr. at 105° (cf. Werner, 1953). Uronic acid content was tested by the carbazole reaction (Dische, 1947, 1950, 1955).

Total reducing sugar was measured on hydrolysates of the extracted odontophore polysaccharide. The dried polysaccharide was redissolved in water with shaking at 4° and adjusted to about pH 7.0 by the addition of sodium bicarbonate. Samples were hydrolysed with N-hydrochloric acid in sealed tubes at 105° for 4 and 6 hr., neutralized with 2N-sodium hydroxide and analysed colorimetrically by Nelson's (1944) modification of the micro-Somogyi method. Glucose standards were heated with N-hydrochloric acid and neutralized under the same conditions.

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α -Amino groups were measured colorimetrically with ninhydrin (Moore & Stein, 1948), glucosamine hydrochloride and glycine being used as standards.

Sulphate determination. The micro method of Bertolacini & Barney (1957), employing barium chloranilate (Fisher Scientific Co., Philadelphia, Pa., U.S.A.), was used to determine sulphate content in hydrochloric acid hydrolysates of the odontophore polysaccharide. Since uronic acids interfere with the barium chloranilate colour reaction (M. W. Whitehouse and J. W. Lash, unpublished observations), the sulphate content in hydrolysates of chondroitin sulphate and heparin was estimated spectrophotometrically after precipitations with benzidine hydrochloride (Anderson, 1953).

Anticoagulation tests. Blood (0.5 ml.) was drawn from the right ventricle of a male albino rat (Wistar strain) and transferred immediately to small test tubes containing 0.1 ml. of the solutions being tested for anticoagulant properties. Approx. 1% solutions of the sodium salts of heparin, chondroitin sulphate and the odontophore extract were used. Blood was considered to be clotted when it failed to run down the sides of the inverted tube.

RESULTS

Properties of the odontophore extract

The fresh odontophore contains a large amount of myoglobin, which is readily extracted with water or dilute salt solutions (Lash, 1959). The chondroid matrix remaining after extraction of the myoglobin exhibits strong metachromasia in histological sections. Attempts to extract the metachromatic material directly by using various salt solutions (potassium chloride, potassium carbonate) led to degradation of the molecule and loss of metachromasia. The metachromatic material was readily released into solution after tryptic digestion. This extract gave a pronounced metachromatic spot when it was dried on filter paper and stained with the ethanolic thionin. Table 1 shows that this extract contains an acidic component with an ionophoretic mobility slightly greater than that of chondroitin sulphate. The mobilities of chondroitin sulphate and heparin are comparable with the values reported by Pasternak & Kent (1952).

This metachromatic material behaved as a typical acidic polysaccharide, being precipitated from solutions of low ionic strength by hexamminocobaltichloride (Mathews & Dorfman,

1953), cetyltrimethylammonium bromide (Cetavlon) (Bera, Foster & Stacey, 1955) and certain other organic bases (neomycin, spermine).

Additional evidence for the similarity between the odontophore polysaccharide and other acidic polysaccharides was obtained by testing for anti-coagulant properties. Table 1 shows that the odontophore polysaccharide exhibits marked anti-coagulant activity. Untreated blood coagulated in 1–2 min., whereas chondroitin sulphate, heparin and the odontophore polysaccharide considerably delayed the clotting time. Chondroitin sulphate and the odontophore polysaccharide delayed the clotting time for 2 and 3 hr. respectively. Heparin-treated blood failed to clot after 3 hr.

The presence of ester-bound sulphate was shown by the colour reaction with barium chloranilate after acid hydrolysis. Phosphate ions did not give a positive reaction and had no effect upon the sulphate determinations. Both acetone-extracted whole tissue and the dialysed products of trypsin digestion were hydrolysed separately in 4N-hydrochloric acid. These hydrolysates gave a positive colour reaction for SO_4^{2-} ions. These findings indicate that the acidity and metachromatism of the dialysed extract is due to ester sulphate groupings, and not to macromolecular phosphates.

Nature of the monosaccharide component

Acid hydrolysis of the extract gave a single component, identified as glucose by paper ionophoresis in borate buffer, pH 8.6, and paper chromatography in the pyridine-ethyl acetate solvent system. Both of these procedures separate glucose from galactose, mannose and the 2-aminoheptoses. Confirmation of the identity of the monosaccharide as D-glucose was obtained with the specific glucose oxidase, notatin, and detection of the enzymically generated hydrogen peroxide. Hydrolysis of the precipitated complexes formed with Cetavlon or hexamminocobaltic ions yielded glucose as the only monosaccharide component. No trace of a uronic acid or aminosugar was found in any of the hydrolysates. The Dische colour reaction for uronic acid and the Elson-Morgan colour reaction for 2-aminoheptoses were both negative.

Table 1. *Properties of the odontophore polysaccharide and some other acidic polysaccharides*

	Anticoagulation	Sulphate content (%)	Ionophoretic mobility*
Odontophore polysaccharide	+++	35	-12.2
Chondroitin sulphate (sodium salt)	++	15	-11.8
Heparin (sodium salt)	+++	44	-14.5

* Mobility = $\mu \times 10^{-5} \text{ cm.}^2/\text{sec.} \cdot 1/\sqrt{c}$ at 22°.

The odontophore extracts and the tissue itself gave no colour reactions with iodine, excluding the possibility of the polysaccharide being glycogen or any other 1:4- α -linked polyglucose.

This evidence indicates that the acidic polysaccharide extracted from the chondroid matrix of the odontophore is either a polyglucose sulphate or a mixture of polyglucose sulphates with very similar ionophoretic mobilities.

Analytical results

In a typical extraction, 640 mg. of dried odontophores yielded 174 mg. of dry protein-free polysaccharide. This represents a polysaccharide sulphate content of 27% for the odontophore. The acidic polysaccharide content of hyaline mammalian cartilage is reported to be approx. 20% of the dry weight (Meyer, Hoffman & Linker, 1959).

After hydrolysis with hydrochloric acid for 4 hr. SO_4^{2-} ions equivalent to 35% (average of 33, 34 and 37%) of the unhydrolysed polysaccharide were liberated. Unhydrolysed solutions of the polysaccharide failed to react with the barium chloranilate reagent, indicating no measurable content of free SO_4^{2-} ions.

The reducing equivalent, calculated as glucose, of 4 hr. hydrolysates was found to be 57% (average of 58.8, 54.2 and 57.2%), not increased by extension of the duration of hydrolysis to 6 hr. The reducing equivalent of unhydrolysed solutions of the polysaccharide sulphate, after neutralization with sodium bicarbonate, corresponded to 0.51%. The minimum size of the macromolecule must therefore correspond to a degree of polymerization of at least 100. The content of reducing sugar and of SO_4^{2-} ions measured after hydrolysis accounts for approx. 92% of the dry weight of the extracted polysaccharide. That very little protein or polypeptide was associated with this material was indicated by the feeble ninhydrin reaction (corresponding to only 0.08% α -amino content) given by 4- and 6-hr. hydrochloric acid hydrolysates.

DISCUSSION

Comparison of the odontophore polysaccharide with chondroitin sulphate and heparin

The polyglucose sulphate extracted from the odontophore has many molecular properties resembling those of a chondroitin sulphate from mammalian cartilage. The two polysaccharides exhibit strong metachromasia, similar ionophoretic mobilities and similar precipitation reactions.

The odontophore polyglucose sulphate has likewise appreciable antihaemocoagulant properties. Other sulphated polysaccharides have been shown to act as anticoagulants: dextran sulphates

(Ricketts, 1952), cellulose sulphates (Chargaff, Bancroft & Stanley-Brown, 1936; Karrer, Koenig & Usteri, 1943), glycogen and starch sulphates (Bergstrom, 1936) and other polyglucose sulphates (Kent, 1953; Wood & Mora, 1958). These anticoagulant properties are dependent upon molecular size and degree of sulphation, rather than upon any specific molecular structure.

Both heparin and the chondroitin sulphates have β -glycosidic linkages. Both the native odontophore polysaccharide and partially desulphated preparations resulting from mild alkaline treatment gave no colorations with iodine and had low optical rotation. This suggests that the odontophore polyglucose sulphate is not 1:4- α -glycosidically linked. Further studies are in progress in an attempt to establish whether or not the odontophore polysaccharide is related to cellulose.

Comparative biochemistry of polysaccharide sulphates

In higher animals, heteropolysaccharide sulphates are found principally in the connective tissues. These animal polysaccharides include the chondroitin sulphates (A, B and C), keratosulphate, heparin and the heparitin sulphates (Meyer, Davidson, Linker & Hoffman, 1956; Kent & Whitehouse, 1955; Linker, Hoffman, Sampson & Meyer, 1958), all of which contain either D-glucosamine or D-galactosamine. These compounds have been designated 'mucopolysaccharides' (Stacey, 1946; Meyer, 1955). Some related mucopolysaccharides have been isolated from marine invertebrates. Thus a series of heparin-like compounds, the mactins, have been extracted from clam tissues (Burson *et al.* 1956) and a polysaccharide somewhat resembling keratosulphate has been detected in the mucin secreted by the hypobranchial gland of *Busycon caniculatum* (Bacila & Ronkin, 1952; Kwart & Shashoua, 1957; Shashoua & Kwart, 1959). Limacoin sulphate, from the mucin of a Japanese snail, contains ester sulphate, L-fucose, galactose, mannose, galacturonic acid and glucosamine (Masamune & Yosizawa, 1956).

The mucopolysaccharides synthesized by marine invertebrates resemble in many respects some of the polysaccharide sulphates found in marine algae (reviews by Mori, 1953; Hirst, 1958; Richardson & Dewar, 1958). These plant polysaccharides are typified by carrageenin and agar-agar (polygalactose sulphates from the red seaweeds), the fucoidins (polyfucose sulphates from the brown seaweeds) and the mucilage from *Dilsea edulis* (a heteropolysaccharide containing D-galactose, glucuronic acid, xylose, 3:6-anhydrogalactose and ester sulphate).

Vasseur (1948, 1950) has found that the coatings of sea-urchin eggs contain either polyfucose or

polygalactose sulphate, depending upon the species of echinoderm. Polyglucose sulphates are found in the mucin of the marine snail *Charonia lampas* (Soda & Tereyama, 1948; Egami *et al.* 1955), and, as this report indicates, in the odontophore of the marine snail *Busycon*. Whereas these polyhexose sulphates were found in either mucin or egg coats, i.e. associated with tissues of epithelial origin, the polyglucose sulphate of *Busycon* is found in typical connective tissue, the odontophore. In this respect it serves a supportive function, comparable to the role of chondroitin sulphate in the cartilage of higher animals and the cellulose in the cell wall of plants. This comparison may not be adventitious, since 'cellulose sulphate' has been found to be present in the mucin of another marine snail, *Charonia lampas* (Soda & Tereyama, 1948; Egami *et al.* 1955). The probable ubiquity of cellulose is becoming more and more apparent, with its detection in certain animals including the higher mammals (Hall, Lloyd, Saxl & Happey, 1958).

Similarities between the odontophore and cartilage

The chondroid odontophore is in many respects similar to mammalian cartilage; histologically it exhibits a typical hyaline metachromatic matrix, and it contains a polysaccharide sulphate. These similarities, however, are less important than the biochemical differences between typical mammalian cartilage and the chondroid odontophore. On the basis of its chemical composition alone, the odontophore cannot be considered normal cartilage tissue.

The odontophore of *Busycon* is remarkable in other respects. It contains myoglobin, whereas the respiratory pigment for the rest of the organism is haemocyanin (Lash, 1959). Even though *Busycon* synthesizes elsewhere a mucopolysaccharide characteristic of mammalian connective tissue (containing D-glucosamine, D-galactosamine and ester sulphate) in the hypobranchial gland (Shashoua & Kwart, 1959), this completely atypical structural polysaccharide is synthesized in the chondroid matrix of the odontophore. It would appear that this tissue is deficient in the metabolic apparatus for transforming glucose into 2-aminohexoses and other monosaccharide components of normal connective-tissue polysaccharides.

SUMMARY

1. A polyglucose sulphate has been extracted by tryptic digestion from the odontophore of the marine snail *Busycon caniculatum*. D-Glucose was the sole monosaccharide component detected in acid hydrolysates.

2. Although the odontophore is a chondroid tissue, no evidence could be found for the occur-

rence therein of either a chondroitin sulphate or keratosulphate.

3. The odontophore polysaccharide was compared with heparin and chondroitin sulphates with respect to precipitation reactions, anticoagulation properties and ionophoretic mobility.

4. The odontophore polyglucose sulphate may be a cellulose sulphate.

5. The histological and chemical basis for defining a tissue as cartilaginous are discussed. On the basis of its biochemical properties, the odontophore cannot be considered as normal cartilage.

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Metabolism of Nicotinamide and Nicotinic Acid in *Corcyra cephalonica* St.

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Sundaram, Radhakrishnamurty & Sarma (1954) and Sundaram & Sarma (1954) reported that in the larva of the rice moth, *Corcyra cephalonica* St., *N*'-methylnicotinamide is not an excretory product of nicotinic acid and nicotinamide as it is in many higher animals. The end products of the vitamin metabolism in this insect are principally nicotinic acid and to a lesser but significant extent nicotinuric acid. It was inferred that the deamidation of nicotinamide is an important pathway in the metabolism of the vitamin in *Corcyra*. Somewhat similar observations have been recently made for the mould *Neurospora crassa*, which neither methylates the vitamin nor conjugates it with glycine but disposes of it predominantly as free nicotinic acid, deamidating nicotinamide in the process (Sundaram, Rajagopalan & Sarma, 1958).

The results of a study of the metabolism of ¹⁴C-labelled nicotinic acid and nicotinamide in *Corcyra* and the enzymes mediating the deamidation and the conjugation reactions are presented in this paper.

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EXPERIMENTAL

The stock colony of *Corcyra cephalonica* St. was maintained on whole wheat-flour diet as described earlier (Sarma, 1943).

Determination of nicotinuric acid excreted by Corcyra larvae. The experimental diet consisted of salt-extracted wheat flour, salts mixture and vitamins (Sarma, 1943) and was supplemented with nicotinic acid or nicotinamide (30 mg./100 g.). Larvae (10–12 days old), hatched on a whole wheat-flour diet, were maintained on the experimental diet until the major portion of the diet had been consumed. The excreta were then separated from diet particles. A portion (100 mg.) of the powdered excreta was autoclaved with 5 ml. of water at 15 lb./in.² for 1 hr. The mixture was then adjusted to pH 4.5, made to volume and filtered. The filtrate was brought to pH 7.0 and assayed microbiologically for nicotinic acid and its tertiary derivatives with *Lactobacillus arabinosus* 17–5 (Snell, 1951) and *Leuconostoc mesenteroides* 9135 (Krehl, De La Huerge, Elvehjem & Hart, 1946). *L. arabinosus* responds to both nicotinic acid and nicotinuric acid, whereas *L. mesenteroides* 9135 specifically measures only free nicotinic acid (Johnson, 1945). When the supplement to the larval diet was nicotinamide, a second 100 mg. lot of the larval excreta was autoclaved with 0.6N-H₂SO₄. Nicotinamide, but not nicotinuric acid, is hydrolysed to nicotinic acid under these conditions (Johnson, 1945). An assay of the hydrolysed sample with *L. mesenteroides* would therefore give the combined nicotinic acid and nicotinamide content of the excreta.

Metabolism of [¹⁴C]nicotinic acid and [¹⁴C]nicotinamide in Corcyra. Batches (1 g.) of the experimental diet were supplemented with nicotinic acid or nicotinamide at a