Invertebrate Connective Tissue

ISOLATION OF D-ARABINOSE FROM SPONGE ACIDIC POLYSACCHARIDE

By RICHARD L. KATZMAN, ELWIRA LISOWSKA* AND ROGER W. JEANLOZ

Laboratory for Carbohydrate Research, Departments of Biological Chemistry and Medicine, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. 02114, U.S.A.

(Received 31 March 1970)

1. D-Arabinose, an extremely rare sugar in the animal kingdom, was isolated from a complex acidic polysaccharide obtained from the connective tissue of the sponge *Hippospongia gossypina*. A crystalline derivative of arabinose, the 1-benzyl-1-phenylhydrazone, was prepared and characterized by its melting point, optical rotation, and mixed melting point with authentic D- and L-enantiomers. 2. The distribution of arabinose within the animal kingdom is discussed, and a biological role is proposed for the spongonucleotides.

We have recently begun a study of the chemistry of the collagen, acidic polysaccharide and glycoprotein present in the connective tissue of three invertebrates, the sea cucumber, the sea anemone and the sponge (Katzman & Jeanloz, 1969; Katzman, Bhattacharyya & Jeanloz, 1969; Katzman & Jeanloz, 1970a,b). In the present paper we describe the isolation and characterization of D-arabinose from an acidic polysaccharide from a sponge.

EXPERIMENTAL

Chemicals. D-Arabinose and cetyltrimethylammonium bromide were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). L-Arabinose was supplied by Pfanstiehl Chemical Co. (Waukegan, Ill., U.S.A.). 1-Benzyl-1-phenylhydrazine hydrochloride was a product of Eastman Kodak Co. (Rochester, N.Y., U.S.A.). Authentic D- and L-arabinose 1-benzyl-1-phenylhydrazones were prepared as described below.

Methods and equipment. All optical rotations were measured with a Perkin-Elmer no. 141 polarimeter. Melting points were determined with a Mettler FP2 apparatus and correspond to the 'corrected melting point'. Sugars were determined by g.l.c. (Perkin-Elmer model 900) of the per(trimethylsilyl)ated methyl glycosides on columns of 3% OV-1 and 3% OV-17 coated on 60/80 mesh Gas-Chrom Q (Applied Science Laboratories, College Station, Pa., U.S.A.). The methyl glycosides were prepared by heating samples with an internal standard of inositol in 5M-HCl in methanol at 65°C for 12h. Samples were injected at 120°C and the temperatures of the columns were raised by 5°C/min. The peak areas and retention times were recorded with a digital integrator (Kent Instruments Ltd., Luton, Beds.,

U.K.). Colorimetric procedures were used for the determination of hexuronic acid (Dische, 1947) and sulphate (Spencer, 1960).

Isolation of acidic polysaccharide. Six live sheepswoll sponges were obtained from Mr L. S. Smitzes, Tarpon Springs, Fla., U.S.A., with the assistance of Mr E. Riley of the American Sponge and Chamois Co., New York, N.Y., U.S.A. and identified as *Hippospongia gossypina* by Dr W. D. Hartman of the Yale University Peabody Museum. They were cut into small pieces and washed well with water. Considerable care was taken to remove foreign matter (shrimps, bits of shellfish etc.) from each piece of sponge with the aid of a large magnifying glass and forceps. The cleaned and dried sponge pieces weighed 87.3g. The pieces were digested for 48 h at 37°C in 5 litres of 0.1M-NaHCO₃ containing 2g of Difco (Detroit, Mich., U.S.A.) trypsin, essentially as described by Gross, Sokal & Rougie (1956). The digest was saturated with chloroform and toluene. Spongins A and B, two morphologically distinct collagen fibres of the sponge, were separated from the trypsin digest by centrifugation as described by Gross *et al.* (1956).

Spongin B (yield 60.6g) contained 3.0% galactose, 2.3% glucose, 0.3% mannose and 0.5% hexosamine. Arabinose was not detected. Spongin A (yield 2.1g) contained 1.3% arabinose, 1.7% galactose, 1.0% glucose, 1.3% mannose, 2.4% fucose, 1.6% galactosamine, 2.3% glucosamine, 1.0% hexuronic acid, 1.7% sulphate and 7.8% hydroxyproline. The arabinose could not be released from the insoluble Spongin A by further treatment with trypsin or other proteolytic enzymes, including collagenase. The supernatant fluid from the trypsin digest was concentrated to 150ml by rotary evaporation at 45°C. A precipitate was removed by centrifugation. The supernatant fluid was then dialysed against water and the dialysis residue was again clarified by centrifugation. To the dialysis residue was added an equal volume of 2% (w/v) cetyltrimethylammonium bromide. The coacervate was collected by centrifugation, washed with water, converted into the potassium salt by solubilization in 0.5M-KSCN and precipitated with 3 vol. of ethanol. The

* Permanent address: Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wrocław, Poland.

precipitated material was washed with ethanol until free of SCN^- (tested with FeCl_3) and dried with diethyl ether. The yield was 1.28 g and the arabinose content 4.1%. This material was further purified by mild alkaline hydrolysis (0.1 M-KOH, 80°C, 1 h) followed by dialysis. The dialysis residue was then precipitated from 1% potassium acetate solution by the addition of ethanol. The fraction precipitating between 18% and 23% ethanol was collected and dried with ether. This fraction (yield 455 mg) contained 8.2% arabinose, 12% fucose, 8% galactose, 1.1% mannose, 0.6% xylose, 6.3% hexosamine, 12% hexuronic acid, 22% sulphate and 3% amino acid.

Isolation of arabinose. The purified acidic polysaccharide (442 mg) was hydrolysed at a concentration of 0.5% in M-HCl at 100°C for 65 min. The acid was removed by rotary evaporation at 40°C. The resulting syrup was deionized by passage down a mixed-bed ion-exchange resin consisting of equal portions of Dowex 1 (X2; 50–100 mesh; acetate form) and Dowex 50 W (X2; 50–100 mesh; H^+ form). On elution with 3–4 column volumes of water, the neutral sugars were quantitatively recovered (yield 123 mg, arabinose content 24%). The neutral sugar fraction (110 mg in 1 ml of 50% ethanol) was applied as a strip (40 cm $\times$ 0.4 cm) to a sheet of Whatman 3 MM paper, together with appropriate standards, and developed in the descending direction for 24 h with ethyl acetate–pyridine–water (10:3:2, by vol.). Guide strips were cut from the sheet and stained with aniline hydrogen phthalate (Partridge, 1949). The pink areas of the chromatogram corresponding to the arabinose standard were eluted with water. Removal of the water by rotary evaporation gave a syrup (yield 21 mg) containing 86% arabinose, 0.2% fucose and 0.3% mannose.

Preparation of the 1-benzyl-1-phenylhydrazone of arabinose. The crude arabinose syrup (5 mg) was dissolved in 50 μl of water. To this solution was added 5.5 mg of 1-benzyl-1-phenylhydrazine hydrochloride in 0.15 ml of ethanol–acetic acid (19:1, v/v). Crystallization occurred spontaneously at room temperature within 2–3 days. After 1 week the crystals were washed with water, 50% propan-2-ol, and ether, and dried *in vacuo* at 80°C overnight, yielding 7 mg of the 1-benzyl-1-phenylhydrazone.

RESULTS

The optical rotation, $[\alpha]_D^{20} -92^\circ\text{C}$ (c 0.7 in water), of the crude arabinose syrup suggested that the arabinose was the D-enantiomer. The crystalline 1-benzyl-1-phenylhydrazone derivative had m.p. 170–172°C and $[\alpha]_D^{20} +14^\circ\text{C}$ (c 0.5 in methanol). Literature melting points of 174°C and 168.8–169.8°C are recorded for D-arabinose 1-benzyl-1-phenylhydrazone; values for the specific rotation of this compound are $+14.6^\circ\text{C}$ and $+14.4^\circ\text{C}$ (Vogel & Georg, 1931). When mixed with authentic crystals of D-arabinose 1-benzyl-1-phenylhydrazone, m.p. 170.5–172°C was recorded; when mixed with authentic crystals of the L-enantiomer, m.p. 171–173°C, the melting point was depressed to 149–152°C. These results conclusively demonstrate that the acidic polysaccharide of *H. gossypina* contains D-arabinose. [Although D-arabinose may be pro-

duced by the acid-catalysed decarboxylation of D-altruronic or L-galacturonic acid, these hexuronic acids do not exist in nature (Percival, 1963). Further, the hexuronic acid value of the purified polysaccharide did not significantly decrease after the acid hydrolysis.]

DISCUSSION

Arabinose is a very common sugar in the plant kingdom, existing in homo- as well as hetero-polysaccharides (Horton & Wolfrom, 1963). It is also found in plant cell-wall glycoproteins, where it participates in a glycoprotein linkage with hydroxyproline (Lampert, 1969). Arabinose is also found in bacteria, and is a highly characteristic component of the surface-layer polysaccharide of certain groups (Davies, 1955; Romano & Sohler, 1956; Cummins & Harris, 1956; Cummins, 1958). Arabinose has been isolated from *Corynebacterium diphtheriae* and was shown to be the D-enantiomer (Holdsworth, 1952).

Arabinose is very rarely encountered in the animal kingdom. Gross, Dumsha & Glazer (1958) reported arabinose to be a component of the collagen from a coelenterate and an echinoderm, as well as a poriferan. We have examined connective tissue and collagen from animals in these phyla and found arabinose only in the sponge. Rosenberg & Zamenhof (1962) reported arabinose to be present in a polysaccharide isolated from the digestive gland of the squid. The identification, however, was based solely on its paper chromatographic mobility and colorimetric reactions.

Stary, Wardi, Turner & Allen (1965) reported the isolation of L-arabinose from an acidic polysaccharide from human brain. Although the acidic polysaccharide was termed 'hyaluronate peptide', its relationship to hyaluronic acid is unclear. Margolis (1967) reported that bovine brain hyaluronic acid contains 'less than 0.2%' arabinose. Partially purified rooster comb hyaluronic acid contains a protease-susceptible fraction that definitely contains arabinose (D. Swann, unpublished work). Arabinose has also been detected in a brain glycolipid fraction obtained by chloroform–methanol (2:1, v/v) extraction (G. Hauser, unpublished work). We have noted the sporadic phylogenetic distribution of acidic polysaccharides (Katzman & Jeanloz, 1969); thus, although arabinose is present in the connective tissue of vertebrata and porifera, it is not necessarily present in all or any of the intervening phyla.

Two decades ago Bergmann & Feeney (1950, 1951) and Bergmann & Burke (1955) isolated D-arabinofuranosyl nucleosides of uracil and thymine (named spongonucleosides) from extracts of a sponge. Since only D-ribose and 2-deoxy-D-erythro-

pentose (deoxyribose) were known to be the sugar components of nucleic acids, the possible biological role of the spongonucleosides elicited considerable interest, and their chemistry and metabolism were studied extensively (Cohen, 1966). Cohen (1963) has developed an elegant theory in which he proposes that cancer and ageing are caused by the irreversible inactivation of critical nucleic acid polymers by the incorporation of arabinosyl nucleotides, but experimental proof for this theory is still lacking. [Bergmann, Watkins & Stempien (1957) have examined the RNA and DNA of sponge, and could not detect any arabinose. Despite nearly twenty years of intensive investigation, arabinose has never been conclusively demonstrated to be incorporated into any biological nucleic acid polymer (Cohen, 1966, pp. 80–82).] We propose that a possible biological function of the spongonucleosides is to serve as precursors to activated sugars in the biosynthesis of mesogleal arabinose-containing acidic polysaccharide in the sponge. Sponge cells possess pyrimidine nucleoside phosphorylase activity (Cohen, 1966, p. 35). The D-arabinose 1-phosphate liberated by this enzyme may be converted into an activated nucleotide glycosyl donor, which is then incorporated into acidic polysaccharide. We realize that this proposal is not in accord with the current concepts of carbohydrate metabolism, but this hypothesis could be tested by studying the biosynthesis of sponge acidic polysaccharide.

We thank Miss E. Burr and Mr K. Linsley for technical assistance and Dr J. F. Codington and Dr J. Gross for discussions and advice. This study was supported by the National Institutes of Health Grants AM-03564 and CA-18166. This is paper no. 3 in the series 'Invertebrate Connective Tissue' (paper no. 2: Katzman & Jeanloz, 1970a) and is publication no. 507 of the Robert W. Lovett Memorial Group for the Study of Crippling Diseases, Harvard Medical School at the Massachusetts General Hospital, Boston, Mass. 02114, U.S.A.

REFERENCES

- Bergmann, W. & Burke, D. C. (1955). *J. org. Chem.* **20**, 1501.
- Bergmann, W. & Feeney, R. J. (1950). *J. Am. chem. Soc.* **72**, 2809.
- Bergmann, W. & Feeney, R. J. (1951). *J. org. Chem.* **16**, 981.
- Bergmann, W., Watkins, J. C. & Stempien, M. F. (1957). *J. org. Chem.* **22**, 1308.
- Cohen, S. S. (1963). *Perspect. Biol. Med.* **6**, 215.
- Cohen, S. S. (1966). In *Progress in Nucleic Acid Research and Molecular Biology*, p. 1. Ed. by Davidson, J. N. & Cohen, W. E. New York: Academic Press Inc.
- Cummins, C. S. (1958). *J. gen. Microbiol.* **18**, 173.
- Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
- Davies, D. A. L. (1955). *Biochem. J.* **59**, 696.
- Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
- Gross, J., Dumsha, B. & Glazer, N. (1958). *Biochim. biophys. Acta*, **30**, 293.
- Gross, J., Sokal, Z. & Rougvie, M. (1956). *J. Histochem. Cytochem.* **4**, 227.
- Holdsworth, E. S. (1952). *Biochim. biophys. Acta*, **9**, 19.
- Horton, D. & Wolfrom, M. L. (1963). In *Comprehensive Biochemistry*, vol. 5, p. 185. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
- Katzman, R. L., Bhattacharyya, A. K. & Jeanloz, R. W. (1969). *Biochim. biophys. Acta*, **184**, 523.
- Katzman, R. L. & Jeanloz, R. W. (1969). *Science, N.Y.* **166**, 758.
- Katzman, R. L. & Jeanloz, R. W. (1970a). In *The Chemistry and Molecular Biology of the Intercellular Matrix*, p. 217. Ed. by Tristram, R. G. & Balazs, E. A. New York: Academic Press Inc.
- Katzman, R. L. & Jeanloz, R. W. (1970b). *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 599.
- Lamport, D. T. A. (1969). *Biochemistry, Easton*, **8**, 1155.
- Margolis, R. U. (1967). *Biochim. biophys. Acta*, **141**, 91.
- Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
- Percival, E. (1963). In *Comprehensive Biochemistry*, vol. 5, p. 74. Ed. by Florkin, M. & Stotz, E. H. Amsterdam: Elsevier Publishing Co.
- Romano, A. H. & Sohler, A. (1956). *J. Bact.* **75**, 478.
- Rosenberg, E. & Zamenhof, S. (1962). *J. Biochem., Tokyo*, **51**, 274.
- Spencer, B. (1960). *Biochem. J.* **72**, 435.
- Stary, Z., Wardi, A. H., Turner, D. L. & Allen, W. S. (1965). *Archs Biochem. Biophys.* **110**, 388.
- Vogel, H. & Georg, A. (1931). *Tabellen der Zucker und ihrer Derivate*, pp. 126–127. Berlin: J. Springer.