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The Isolation of D-Fucosamine (2-Amino-2,6-dideoxy-D-galactose) from Polysaccharides of *Bacillus*

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Until 1954, D-glucosamine was the only amino sugar known to occur in bacteria. More than ten amino sugars have been isolated from bacteria since then (Sharon, 1964). Most bacterial strains appear to contain only two compounds of this class, namely glucosamine and muramic acid. A limited number of bacterial species is, however, capable of producing a larger variety of amino sugars. These include species of the genus *Bacillus*, which, in addition to glucosamine and muramic acid, form galactosamine (Sharon, 1957), a diamino sugar (Sharon & Jeanloz, 1960) and D-fucosamine.

The present paper describes the isolation and identification of D-fucosamine (2-amino-2,6-dideoxy-D-galactose) from polysaccharides produced by two organisms, *Bacillus licheniformis* and *Bacillus subtilis*. This amino sugar was found for the first time in a specific lipopolysaccharide of the Gram-negative *Chromobacterium violaceum* (NCTC 7917) (Crumpton & Davies, 1958). It was subsequently isolated from *B. licheniformis* by Sharon, Shif & Zehavi (1962). [*B. licheniformis* was previously known as *B. subtilis* (ATCC 9945). In the last edition of the *American Type Culture Collection, Catalogue of Cultures* (1958), it has been designated as *Bacillus licheniformis* (ATCC 9945). Indeed, tests in our Laboratory have shown that this organism exhibits scant growth in glucose broth under anaerobic conditions, a property characteristic of *B. licheniformis* (Breed, Murray & Smith, 1957). The organism obtained from Dr Baddiley (Armstrong, Baddiley & Buchanan, 1960) did, however, grow well under the same conditions, and is properly called *B. subtilis*.] A brief report on the occurrence of fucosamine in an unidentified *Bacillus* species and in *Bacillus cereus* has appeared (Leatherwood, Rollins, Kulkarni & Wheat, 1963), and L-fucosamine has been found in the *Pneumococcus* type V polysaccharide (Williams, 1960; Barker, Brimacombe, How, Stacey & Williams, 1961).

A preliminary account of this work has been given (Sharon *et al.* 1962). The final proof of the structure of D-fucosamine, by its synthesis from D-galactosamine, has been described in a preliminary note (Zehavi & Sharon, 1963).

EXPERIMENTAL

Materials. D-Fucosamine hydrochloride from *C. violaceum* was given by Dr D. A. L. Davies and by Dr R. W. Wheat. An amino sugar from *Bacillus* sp., later identified as fucosamine (Leatherwood *et al.* 1963), was obtained from Dr R. W. Wheat. A polysaccharide preparation from *B. subtilis* (Armstrong *et al.* 1960, p. 615) was kindly given to us by Dr J. Baddiley and Dr J. J. Armstrong. The nature of the sugar components of this polysaccharide was not known at the time. Diamino sugar (4-acetamido-2-amino-2,4,6-trideoxyhexose) from *B. licheniformis* (ATCC 9945) was prepared essentially as described by Sharon & Jeanloz (1960).

Preparation of polysaccharide. B. licheniformis (ATCC 9945) was grown in bottles (4 l.) with aeration in a peptone (2%)–sodium chloride (0·5%) liquid medium, as described by Armstrong *et al.* (1960). The bottles contained 1 l. of medium each, and were aerated at a rate of 3 l./min. Cells were collected by centrifugation and washed with cold peptone–sodium chloride medium. The succeeding steps were carried out at a temperature of 0–5°. Then 2 vol. of 10% (w/v) trichloroacetic acid was added to the packed cells, mixed well and the thick suspension was left overnight. On the next day it was centrifuged for 30 min. at 12 000*g*, the supernatant solution was poured off, and the cells were extracted twice more with equal volumes of 5% (w/v) trichloroacetic acid. The combined extracts were not viscous, in contrast with the extracts obtained from the same organism grown on Sauton's medium at 37° (Sharon & Jeanloz, 1960).

The solution was then extracted with ether to remove the trichloroacetic acid. On the addition of 3 vol. of 95% (v/v) ethanol to the aqueous solution, a white precipitate separated. The precipitate was collected by centrifugation, washed successively with aqueous ethanol, ethanol and ether, and dried *in vacuo*. The yield was 25 mg./l. of growth medium. No attempt was made to purify this crude polysaccharide.

Hydrolysis. The polysaccharide was hydrolysed in a boiling-water bath for 6 hr. with 1 N-H_2SO_4, at a concentration of 20 mg./ml. After hydrolysis, the hydrolysate was neutralized with solid $BaCO_3$. The $BaSO_4$ formed was removed by filtration and washed with hot water, and the combined filtrates were evaporated to dryness in a desiccator under vacuum.

Ion-exchange chromatography. Hydrolysates were fractionated on columns (0·9 cm. × 30 cm.) of Dowex 50 (X8; H^+ form), according to the method of Gardell (1953). Samples of the fractions were analysed by the Elson–Morgan reaction, as described by Gardell (1953), with D-glucosamine hydrochloride (Nutritional Biochemicals Corp.) as standard.

Paper chromatography. Single-dimensional descending paper chromatography was used with Whatman no. 1 paper and the following solvent systems: A, butan-1-ol–acetic acid–water (25:6:25, by vol.); B, butan-1-ol–ethanol–water (4:1:1, by vol.).

Sugars were applied in 10–20 μg. amounts in volumes of 2 μl. The sugar spots were revealed with silver nitrate by the method of Trevelyan, Procter & Harrison (1950), as modified by Sharon & Jeanloz (1960); amino sugars were also detected by ninhydrin [0·25% (w/v) solution in acetone].

RESULTS

The elution diagram of an acid hydrolysate of the polysaccharide from *B. licheniformis* (ATCC 9945) is given in Fig. 1(*a*). For comparison, the elution diagram of a mixture of D-glucosamine, D-galactosamine and the diamino sugar of *B. licheniformis* (ATCC 9945) is also given (Fig. 1*b*). It is evident that the polysaccharide contains, in addition to glucosamine and galactosamine, another amino sugar, whose effluent volume is different from that of the diamino sugar present in a polysaccharide isolated from *B. licheniformis* (ATCC 9945) grown on a synthetic medium in stationary cultures at 37° (Sharon, 1957; Sharon & Jeanloz, 1960).

Ion-exchange chromatography of an acid hydrolysate of the polysaccharide of *B. subtilis* (Armstrong *et al.* 1960) also showed the presence of an amino sugar (Fig. 1*c*) with an effluent volume similar to that of the new compound found in the polysaccharide of *B. licheniformis* (ATCC 9945). In the polysaccharide of *B. subtilis*, however, no

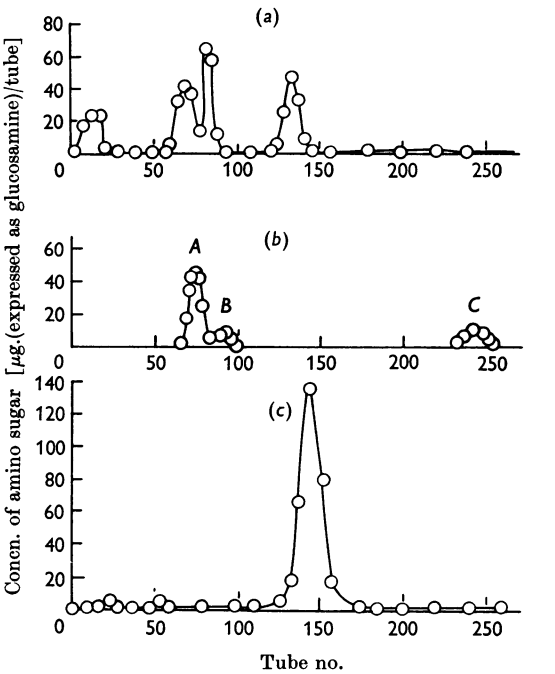

Fig. 1. Separation of amino sugars on columns (0·9 cm. × 30 cm.) of Dowex 50 (X8; H^+ form) (Gardell, 1953). The eluent was 0·3 N-HCl; 1 ml. fractions were collected at a rate of 3 ml./hr. (*a*) Acid hydrolysate of 100 mg. of polysaccharide from *B. licheniformis* (ATCC 9945). (*b*) Synthetic mixture of 1 mg. of D-glucosamine hydrochloride (*A*), 0·3 mg. of D-galactosamine hydrochloride (*B*) and 1 mg. of diamino sugar (*C*). (*c*) Acid hydrolysate of 62 mg. of polysaccharide from *B. subtilis* (Armstrong *et al.* 1960).

significant amounts of other amino sugars were present. Paper-chromatographic analysis of the hydrolysate revealed only one major component, which was reducing and gave a ninhydrin-positive reaction. Only minor amounts of other reducing components were present in this polysaccharide.

The ratio of the peak volume at which the new amino sugars were eluted from the Dowex 50 columns to that of glucosamine ($R_{Glucosamine}$) was 1·9. This value was very close to that found for D-fucosamine by Crumpton (1959) and for L-fucosamine by Williams (1960), suggesting that the new amino sugar is fucosamine.

From the elution curves it was calculated that the polysaccharide of *B. licheniformis* (ATCC 9945) contained about 1 % of the unknown amino sugar, with similar amounts of glucosamine and galactosamine, whereas the polysaccharide of *B. subtilis* contained about 7 % of the new component.

The contents of the tubes corresponding to the peaks with $R_{Glucosamine}$ 1·9 of several ion-exchange chromatograms of each of the polysaccharides were pooled and evaporated to dryness *in vacuo*. Each of the resulting materials was dissolved in a small amount of water, and the sugar crystallized from a water–methanol–acetone mixture. The colourless crystals were washed with acetone and dried *in vacuo*. From 500 mg. of *B. licheniformis* (ATCC 9945) polysaccharide we obtained 5 mg. of crystalline material; 100 mg. of the *B. subtilis* polysaccharide also gave 5 mg. of the crystalline amino sugar.

On paper-chromatographic analysis in two solvent systems, butan-1-ol–acetic acid–water (A) and butan-1-ol–ethanol–water (B), both compounds gave single spots, revealed with silver nitrate or with ninhydrin. The two compounds had the same mobility. This mobility was different from that of glucosamine, galactosamine or the diamino sugar, but was identical with that of D-fucosamine from *C. violaceum* (Table 1), as well as that of the amino sugar isolated from *Bacillus* sp. by Wheat and his co-workers and later also identified as fucosamine (Leatherwood *et al.* 1963).

Further proof for the identity of the isolated amino sugars with fucosamine was obtained by ninhydrin degradation experiments, according to the method of Stoffyn & Jeanloz (1954). The ninhydrin degradation products of the two compounds isolated by us were reducing, could not be revealed by ninhydrin, and showed mobilities on paper identical with those of the ninhydrin degradation product of D-fucosamine from *C. violaceum* and of the fucosamine from *Bacillus* sp. (Table 1).

All the fucosamine preparations investigated gave a positive Elson–Morgan reaction (Gardell, 1953) with a maximum absorption at 535 mμ, indicating the absence of substitution at C-3. Deamination with nitrous acid of the two fucosamine preparations isolated by us, under the conditions described by Crumpton & Davies (1958), yielded a product that gave a positive sulphuric acid–cysteine reaction (Dische & Shettles, 1948). A strong absorption with a maximum at 405 mμ was found, which is characteristic of methylpentoses. When D-fucosamine from *C. violaceum* was tested in the same manner, it also gave an absorption maximum at 405 mμ [Crumpton & Davies (1958) report a maximum at 400 mμ], whereas D-glucosamine gave the maximum absorption at 415 mμ characteristic of hexoses.

The physical constants of the newly isolated amino sugar preparations were very close to those of D-fucosamine. For the amino sugar from *B. licheniformis* (ATCC 9945), m.p. 177–183° (decomp.) and $[\alpha]_D^{25} + 74°$ (*c* 0·18 in water) were found. The values for the amino sugar from the polysaccharide obtained by Armstrong *et al.* (1960) were m.p. 176–183° (decomp.) and $[\alpha]_D^{25} + 81°$ (*c* 0·1 in water). Crumpton & Davies (1958) reported m.p. 170–175° (decomp.) and $[\alpha]_D^{20} + 91 \pm 2°$. Synthetic D-fucosamine (Zehavi & Sharon, 1963), had m.p. 192° (decomp.) and $[\alpha]_D^{25} + 92°$ (*c* 0·2 in water). The infrared-absorption spectra of the four preparations were almost completely superimposable.

DISCUSSION

From the colour reactions and the chromatographic and physical properties of the amino sugar preparations isolated from two *Bacillus* polysaccharides, it is evident that these compounds are identical with D-fucosamine. The optical rotation of the isolated compounds is somewhat low, probably owing to the presence of impurities. Because of

Table 1. *Paper-chromatographic data for* D-*fucosamine and its ninhydrin degradation product*

Experimental details are given in the text. The solvents used were: A, butan-1-ol–acetic acid–water (25:6:25, by vol.); B, butan-1-ol–ethanol–water (4:1:1, by vol.).

Compound	$R_{Glucose}$ Solvent A	$R_{Glucose}$ Solvent B
D-Fucosamine:		
(I) from *B. licheniformis* (ATCC 9945)	1·32	0·98
(II) from *C. violaceum*	1·34	0·95
(III) synthetic (Zehavi & Sharon, 1963)	1·36	0·95
Ninhydrin degradation product:		
of (I)	2·36	2·52
of (II)	2·36	2·53
Diamino sugar from *B. licheniformis*	1·8	1·4
D-Glucosamine hydrochloride	0·75	0·61
D-Galactosamine hydrochloride	0·72	0·60

shortage of material, no further purification, by repeated crystallization or other means, could be undertaken. This shortage of material was due to the following: (*a*) the fucosamine content of the polysaccharides was low, and (*b*) the amount of polysaccharide produced by the bacteria and their fucosamine content varied considerably in different batches of the same organism.

Similar difficulties have been encountered by other workers. Thus Leatherwood *et al.* (1963) reported that spores grown from dried organisms of their *Bacillus* sp. did not yield fucosamine-producing cultures. Armstrong *et al.* (1960) found that the amount of teichoic acid that can be extracted from whole bacteria varied in different batches of the organisms; when the strain of *B. subtilis* used by them was grown in the presence of a relatively high concentration of glucose (2 %) direct extraction of the whole cells yielded no teichoic acid. Instead, a considerable amount of polysaccharide was obtained from the trichloroacetic acid extract of this culture, and this polysaccharide was the one used in the present studies. Attempts made by us to increase the yield of polysaccharide and of fucosamine by growing *B. licheniformis* (ATCC 9945) on the peptone–sodium chloride medium to which glucose (2 %) was added were not successful.

In contrast with difficulties in getting uniform yields of D-fucosamine from *B. licheniformis* (ATCC 9945), no difficulties of this type were encountered in the production of the diamino sugar (Sharon & Jeanloz, 1960). Both the amount of polysaccharide extracted from cells grown at 37° on Sauton's medium, and the content of the diamino sugar, varied only to a very slight extent. In this polysaccharide, however, no fucosamine could be found. On the other hand, we never found the diamino sugar in polysaccharide prepared from cells grown under aeration at 22°, according to Armstrong *et al.* (1960).

B. licheniformis is thus capable of synthesizing several amino sugars. Two of these, D-fucosamine and the diamino sugar, have common structural features; both are 2-amino-6-deoxyhexoses. Preliminary experiments (N. Sharon & U. Zehavi, unpublished work) indicate that the configuration at C-2 of the diamino sugar is the same as that of D-fucosamine and of D-glucosamine. These three compounds differ therefore only in C-3 to C-6, and it is very likely that they are metabolically interrelated. In view of the pathways shown to operate in the biosynthesis of 6-deoxyhexoses (Blumsom & Baddiley, 1961; Glaser, 1963; Ginsburg, 1964), it is tempting to speculate that a compound of the type of 'nucleoside diphosphate 2-acetamido-6-deoxy-4-ketohexose' is the key intermediate in the metabolism of these amino sugars. Such a compound may be formed in a manner analogous, for example, to the formation of thymidine 5′-diphosphate 6-deoxy-4-ketoglucose from thymidine 5′-diphosphate glucose (Okazaki, Okazaki, Strominger & Michelson, 1962) or of GDP-6-deoxy-4-ketomannose (Ginsburg, 1961). By stereospecific reduction it will yield the corresponding derivative of D-fucosamine, whereas reductive amination will lead to the formation of nucleoside diphosphate diamino sugar. A reaction of the latter type has been shown to take place in the formation of thymidine 5′-diphosphate 4-acetamido-6-deoxyhexose from the corresponding 4-keto derivative, and glutamine served as the donor of the amino group (Matsuhashi, 1963; Strominger, Matsuhashi & Dietzler, 1963). It may be noted that the diamino sugar is formed on a medium rich in glutamic acid and ammonia.

SUMMARY

1. The isolation of D-fucosamine from polysaccharides extracted from *Bacillus licheniformis* and *Bacillus subtilis* is described.

2. The amino sugar preparations were identified by their behaviour on paper and ion-exchange chromatograms, by their colour reactions and by their physical properties.

3. The possible metabolic interrelations between D-fucosamine and the other amino sugars produced by *B. licheniformis* are discussed.

We thank Professor J. Baddiley, F.R.S., and Dr J. J. Armstrong for samples of the *B. subtilis* polysaccharide, and Dr A. L. Davies and Dr R. W. Wheat for the various samples of D-fucosamine. Additional thanks are also due to Professor J. Baddiley, F.R.S., for the strain of *B. subtilis* used in his Laboratory. This paper is part of a Thesis to be submitted by Mr Uri Zehavi to the Hebrew University, Jerusalem, in partial fulfilment of the requirements for the Ph.D. degree. This investigation was supported by Grant E-3528 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, U.S. Public Health Service.

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Biochem. J. (1964), **93**, 214

The Chemistry of Xanthine Oxidase

10. THE INHIBITION OF THE BOVINE ENZYME BY PURINE 6-ALDEHYDE*

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A number of workers have reported on the powerful inhibition of xanthine oxidase by 2-amino-4-hydroxypteridine 6-aldehyde (Hofstee, 1949; Lowry, Bessey & Crawford, 1949; Kalckar, Kjeldgaard & Klenow, 1950; Byers, 1952). Since this compound contains two potential substrate groups, namely the pteridine nucleus and the aldehyde group, it was of interest to study an analogous compound containing the purine ring system and an aldehyde group. The preparation of purine 6-aldehyde (Giner-Sorolla, Zimmerman & Bendich, 1959) prompted us to examine the effects of this compound on the aerobic oxidation of xanthine by highly purified bovine milk xanthine oxidase. The results presented here show that the purine aldehyde is a powerful inhibitor of this enzyme, but the kinetics of the reactions are unusual.

MATERIALS

The purine 6-aldehyde hydrochloride hydrate was a gift from Dr A. Giner-Sorolla of the Sloan–Kettering Institute, New York. It was stated to be 95% pure and the concentrations given in this paper have been calculated on that basis. A stock solution was prepared by dissolving 10·3 mg. in water to 5 ml. This was stored at 4°, the resulting precipitate being redissolved (by warming to room temperature) before diluting a portion to a suitable concentration for use in the following experiments. The purine aldehyde was found to be stable for about 1 month under these conditions.

* Part 9: Gilbert & Bergel (1964).

Pyrophosphate buffers have been used throughout and were prepared by mixing 50 mM solutions of tetrasodium pyrophosphate (British Drug Houses Ltd., A.R. grade) and disodium dihydrogen pyrophosphate (British Drug Houses Ltd., laboratory grade) to give the required pH.

All water was glass-distilled and then deionized by a mixed-bed ion-exchange column.

Xanthine (Roche Products Ltd.) was stored at room temperature as a 10 mM solution in 20 mM-NaOH.

Xanthine oxidase, prepared from buttermilk by the method of Gilbert & Bergel (1964), was used as the eluate (stage AM5) having a ratio E_{280}/E_{450} 5·3 and, originally, a ratio activity/$E^{1\,cm.}_{450}$ 95 (these ratios correspond to the 'PFR' and 'AFR' values respectively of Avis, Bergel & Bray, 1955). During several months' storage at 4° some denaturation occurred and the enzyme was therefore occasionally centrifuged and passed through a Sephadex G-25 column (Pharmacia, Uppsala, Sweden; cf. Porath & Flodin, 1959) to remove free flavin. This process also removed an inhibitor, presumably that reported by Bray (1959). The ratio activity/$E^{1\,cm.}_{450}$ was 35 at the time these experiments were carried out.

METHODS

Except where stated otherwise, the rate of oxidation of xanthine was measured by determining the rate of increase of extinction at 295 mμ over the initial 1–4 min. of the reaction. A Unicam SP. 500 spectrophotometer with thermostatically-controlled cell housing (23·5±0·2°) was used for these measurements, all buffer solutions being pre-incubated in the apparatus before the addition of enzyme, inhibitor and substrate. The standard (optimum) xanthine concentration was 0·1 mM.