DISCUSSION

Bacterium 11 differs from *Bacteroides amylogenes* (Doetsch *et al.* 1957) in some of its fermentation reactions, and the fact that it does not form an iodophilic polysaccharide excludes it from classification with this organism. Bryant and his co-workers have described a number of Gram-negative rod-like bacteria isolated from bovine-rumen contents, and bacterium 11 could be classified as a strain of *Butyrivibrio fibrisolvens* (Bryant & Small, 1956*a*). The fact that it rapidly ferments xylan precludes it from inclusion with the species *Succinivibrio dextrinsolvens* (Bryant & Small, 1956*b*) and *Bacteroides succinogenes* (Hungate, 1950), and suggests its inclusion with *Butyrivibrio fibrisolvens*. Although the type species of *B. fibrisolvens* was motile, it was suggested that the 'less actively cellulolytic rod' of Hungate was a *Butyrivibrio* species and this was non-motile (Bryant & Small, 1956*a*). The fermentation reactions of *B. fibrisolvens* strains are variable, but are generally similar to those of bacterium 11, and all are non-iodophilic.

Bacterium 11 differs from *Bacteroides amylogenes* in the products of fermentation of xylose, especially in the formation of lactic acid. However, a lactic acid fermentation of xylose is known, for instance with *Lactobacillus pentosus* (Gest & Lampen, 1952). Although lactic acid is not a final product of the fermentation of xylose by mixed rumen organisms

(Doetsch, Robinson, Brown & Shaw, 1953), the work of Pazur, Shuey & Georgi (1958) suggests that it might be an intermediate product.

SUMMARY

The isolation and properties of a xylanfermenting bacterium from the rumen are described. It was tentatively assigned to the genus *Butyrivibrio*.

REFERENCES

- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Bryant, M. P. & Small, N. (1956*a*). *J. Bact.* **72**, 16.
 Bryant, M. P. & Small, N. (1956*b*). *J. Bact.* **72**, 22.
 Burness, A. T. H. & King, H. K. (1958). *Biochem. J.* **68**, 32*P*.
 Doetsch, R. N., Howard, B. H., Mann, S. O. & Oxford, A. E. (1957). *J. gen. Microbiol.* **16**, 156.
 Doetsch, R. N., Robinson, R. Q., Brown, R. E. & Shaw, J. C. (1953). *J. Dairy Sci.* **36**, 825.
 Doetsch, R. N., Robinson, R. Q. & Shaw, J. C. (1952). *J. Anim. Sci.* **11**, 52.
 Elsdon, S. E. & Lewis, D. (1953). *Biochem. J.* **55**, 183.
 Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
 Gest, H. & Lampen, J. O. (1952). *J. biol. Chem.* **194**, 555.
 Grant, W. M. (1947). *Analyt. Chem.* **19**, 206.
 Hungate, R. E. (1950). *Bact. Rev.* **14**, 1.
 Keeney, M. (1955). *Univ. Maryland Misc. Publ.* **238**, 23.
 Pazur, J. H., Shuey, E. W. & Georgi, C. E. (1958). *Arch. Biochem. Biophys.* **77**, 387.

The Structure of a Disaccharide Liberated by Lysozyme from the Cell Walls of *Micrococcus lysodeikticus*

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Salton (1956) studied in some detail the components produced by the action of egg-white lysozyme on the isolated cell walls of certain Gram-positive micro-organisms. In addition to several large fragments which would not diffuse through cellophan, he found that a considerable proportion of the digest was diffusible. He stated that the main 'small fragment' present in the diffusible fraction was possibly a disaccharide of *N*-acetylglucosamine and the unknown amino sugar present in many cell walls (subsequently characterized, e.g. Strange & Kent, 1959, as muramic acid), and adduced certain evidence to support this idea. The work described here confirms the presence of such an oligosac-

charide in lysozyme digests of cell wall. The supposition that it is a disaccharide is found to be correct and its structure is established.

EXPERIMENTAL

Methods

Isolation of walls and preparation of lysozyme digests. *Micrococcus lysodeikticus* (N.C.T.C. 2665) was grown in surface culture and the cell walls were isolated as previously described (Perkins & Rogers, 1959). For preparation of digests the isolated walls (100 mg.) were suspended in 0.1*M*-ammonium acetate buffer, pH 6.1 (25 ml.), and incubated at 37° with lysozyme (crystalline; The Armour

Laboratories) at a final enzyme concentration of 50 µg./ml. for 3 hr. The essentially clear digest was freeze-dried. The residue was dissolved in a small volume of water and dialysed with agitation in cellophan tubes (Visking, 65 mm. diam.) against three changes of distilled water (10 vol.), each time overnight at 2°. The combined diffusible portions and the non-diffusible portion were each freeze-dried. With the diffusible portion the drying process was repeated several times at 0.001 mm. Hg pressure, in order, as far as possible, to remove ammonium acetate.

Paper chromatography and zone electrophoresis. Chromatograms were developed either in butan-1-ol-acetic acid-water (63:10:27, by vol.; organic phase) or in butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951). Carbohydrate material was detected either by the procedure for hexosamines described by Partridge (1948), the final heating after spraying with *p*-dimethylamino-benzaldehyde reagent being omitted, or by aniline phthalate (Partridge, 1949). For preparative purposes Whatman no. 3 chromatography paper was washed before use by the method of Hanes, Hird & Isherwood (1952) to remove peptide contaminants. Zone electrophoresis was carried out on Whatman no. 3 paper either in acetic acid (2N) or collidine-acetate buffer, pH 6.5 (Newton & Abraham, 1954), at 15 v/cm. in an apparatus similar to that described by Kunkel (1954).

Oxidation with alkaline iodine solution. A portion (50 µl.) of a solution containing not more than 1 µmole of material to be examined, an excess (0.4 ml.) of iodine solution (0.01N) and 50 µl. of carbonate-bicarbonate buffer (equal volumes of 0.2M-Na₂CO₃ solution and 0.2M-NaHCO₃ solution, pH 9.9) were mixed together and kept in a glass-stoppered tube at 2° overnight. Then with the tube still kept cool, excess of iodine was removed by passing a stream of CO₂ through the mixture. After acidification with HCl, any further iodine formed by oxidation of liberated HI was again removed by a stream of CO₂, and suitable samples were then hydrolysed in N-HCl in a sealed ampoule at 105° for 2 hr. The HCl was removed in a vacuum desiccator over NaOH flakes, and the material was again evaporated to dryness with two further volumes of water before being used for zone electrophoresis.

Formation of chromogens. *N*-Acetylhexosamines, on heating in alkaline solution, yield chromogens which on addition of Ehrlich's reagent give a purple colour (Morgan & Elson, 1934). In the experiment described below where it was desired to separate the chromogens by electrophoretic or chromatographic procedures, the presence of excess of salt was avoided by using triethylamine or triethylamine-carbonate buffer to provide an alkaline medium which was volatile *in vacuo*. The chromogens produced gave the same absorption curve after the addition of Ehrlich's reagent as those obtained by the use of sodium carbonate or borate. The sample was placed in an ampoule together with either 0.05 ml. of triethylamine (redistilled and stored over NaOH) or 0.1 ml. of triethylamine-carbonate buffer [1.5 ml. of triethylamine and 10 ml. of water, dissolved by addition of CO₂; a 20% (v/v) solution in water gave pH 9.5] and water was added to a final volume of 0.5 ml. The sealed ampoule was heated in a boiling-water bath for the chosen period. After cooling, the water and buffer were removed in a vacuum desiccator over NaOH and H₂SO₄. Chromogens were identified on paper by spraying with Ehrlich's reagent alone (Partridge, 1948) without previous

heating of the paper. Under these conditions *N*-acetylhexosamines themselves give no reaction.

Estimation of hexosamines. After hydrolysis of samples in HCl (4N) for 4 hr. at 105° in sealed ampoules, glucosamine and muramic acid were separated and estimated as described by Perkins & Rogers (1959).

Estimation of apparent N-acetylhexosamine. The method of Reissig, Strominger & Leloir (1955) was employed, but for the oligosaccharide the time of heating was increased until maximum colour yield had been obtained (30 min.) (see Perkins, 1960).

Materials

Muramic acid. This was obtained by isolation on a charcoal-Celite column from a hydrolysate of purified cell walls of *Micrococcus lysodeikticus*.

N-Acetylmuramic acid. This was obtained from a partial acid hydrolysate of the cell walls (Perkins & Rogers, 1959) and was isolated from a preparative chromatogram run on washed paper. The material ran on chromatograms in a variety of solvents in the same position as muramic acid which had been *N*-acetylated, gave colour in the *N*-acetylhexosamine test and on acid hydrolysis yielded muramic acid as the only product identifiable on chromatograms by ninhydrin or aniline phthalate.

Glucosaminic acid. This was a crystalline specimen prepared according to the method of Wolfrom & Cron (1952) and Pringsheim & Ruschmann (1915).

N-Acetylglucosamine and glucosamine hydrochloride. These were commercial specimens.

RESULTS

Isolation of oligosaccharide. A lysozyme digest of cell walls was prepared as described above and separated into diffusible and non-diffusible fractions. The diffusible portion was dissolved in water and placed as a streak on washed paper. The chromatogram was developed with butanol-pyridine-water for 20 hr. at 22°. Thin strips sprayed with aniline phthalate showed material which had not moved far from the origin and a well-defined band which had moved about 14 cm. (*R*_{*N*-acetylglucosamine} 0.85). Both these bands gave a purple colour with the hexosamine spray. The unsprayed region of the paper containing the fast-running component was eluted with water and the eluate dried in a vacuum desiccator. This material was found to be an oligosaccharide corresponding to the 'small fragment' described by Salton (1956).

Composition of the oligosaccharide. The isolated oligosaccharide ran as a single component on chromatograms developed with butanol-acetic acid-water and butanol-pyridine-water (*R*_{*N*-acetylglucosamine} 0.85 in each system) and gave a single anionic band on zone electrophoresis at pH 6.5. On acid hydrolysis (2N-HCl; 2 hr. at 105°) it yielded glucosamine and muramic acid, no amino acids and no glucose. Samples of the isolated material were hydrolysed in HCl and their content of glucosamine and muramic acid was estimated.

On unhydrolysed samples the amount of apparent *N*-acetylglucosamine was determined by comparing the maximal absorption at 585 $m\mu$ obtainable by prolonged heating with the maximal absorption given by known amounts of *N*-acetylglucosamine. As stated by Salton (1956), the absorption curve given by the oligosaccharide under these conditions is essentially the same as that given by *N*-acetylglucosamine. The results given in Table 1 indicate that the oligosaccharide contains equimolecular proportions of glucosamine and muramic acid. From the fact that lysozyme digests of cell walls do not contain appreciable amounts of hexosamine with free amino groups (Ingram & Salton, 1957; Perkins, 1960) it is concluded that both amino sugars in the oligosaccharide are *N*-acetylated.

Free reducing group of the oligosaccharide. Since the compound gave a reaction with aniline phthalate and with *N*-acetylhexosamine reagents, at least one of its component hexosamines must have had a free reducing group. The number of units of the two amino sugars in each molecule was not known, although the fast movement on paper chromatograms suggested that it was not likely to be larger than a disaccharide. An attempt was made to answer these problems by oxidation in the cold with alkaline iodine solution (Kuhn, Gauhe &

Baer, 1954*a*). Under these circumstances the sugar with a terminal reducing group should be oxidized to the corresponding substituted hexonic acid, leaving the rest of the molecule untouched. Examination of the compounds present in an acid hydrolysate of the oxidized product should therefore indicate the number of units present in the original oligosaccharide and which amino sugar possessed a free reducing group.

Samples of the isolated oligosaccharide were oxidized with cold alkaline iodine solution and the product was hydrolysed in HCl. The hydrolysate was examined by zone electrophoresis in acetic acid. This system gave a clear separation of glucosamine, glucosaminic acid, muramic acid and its oxidized product. It was found that *N*-acetylmuramic acid treated as described above did not yield a product which could be identified with ninhydrin, although iodine was used up and free muramic acid (after acid hydrolysis) disappeared. The procedure of Rydon & Smith (1952), however, which detects amide linkages by treatment with Cl_2 followed by a starch-iodide spray, showed a clear characteristic band after electrophoresis of the product obtained from *N*-acetylmuramic acid by oxidation and acid hydrolysis. It seemed possible that this product might have been due to spontaneous lactam formation, but this point has not been investigated further. The results of these procedures are set out in Table 2. Since no glucosaminic acid at all was formed from the oligosaccharide it can be concluded that C-1 of each molecule of *N*-acetylglucosamine was bound in a glycosidic linkage. Since the oxidized product of muramic acid was clearly present, the free reducing group of the oligosaccharide must have been that of *N*-acetylmuramic acid. Furthermore, as at the same time all the free muramic acid had disappeared, the material must have been a disaccharide rather than a tetrasaccharide or higher polymer. This is in accord with the relatively rapid movement of the material on paper chromatograms.

Table 1. *Composition of the disaccharide material and amount of 'apparent N-acetylglucosamine'*

Sample no.	Before acid hydrolysis	After acid hydrolysis	
	Apparent <i>N</i> -acetylglucosamine* (mole)	Glucosamine (mole)	Muramic acid (mole)
1	0.37	1.0	0.85
2	0.39	1.0	0.90

* 'Apparent *N*-acetylglucosamine' is the maximal absorption at 585 $m\mu$ obtainable by prolonged heating in the Reissig, Strominger & Leloir (1955) conditions compared with the maximal absorption given by *N*-acetylglucosamine.

Table 2. *Zone electrophoresis of products of alkaline iodine oxidation followed by acid hydrolysis*

Electrophoresis was performed on paper at 15 v/cm. for 250 min. in 2*N*-acetic acid.

Material	Treatment	Method of detection	Distance moved towards cathode (cm.)
Glucosamine hydrochloride	None	Ninhydrin	24.2
Glucosaminic acid	None	Ninhydrin	10.0
Muramic acid	None	Ninhydrin	14.8
<i>N</i> -Acetylglucosamine	Alkaline I_2 + acid hydrolysis	Ninhydrin	10.0
		Cl_2 + starch-KI	10.0 (none at 2.8)
<i>N</i> -Acetylmuramic acid	Alkaline I_2 + acid hydrolysis	Cl_2 + starch-KI	2.8
Oligosaccharide	Alkaline I_2 + acid hydrolysis	Cl_2 + starch-KI	2.8
		Ninhydrin	24.2 (none at 10.0)

Linkage between C-1 of N-acetylglucosamine and N-acetylmuramic acid. As has been shown above, the free reducing group of the disaccharide is that of *N*-acetylmuramic acid. Since C-2 is occupied by the acetamido grouping, and C-3 by the lactic acid side chain (Strange & Kent, 1959), and assuming a pyranose ring to be present, only C-4 or C-6 remains available for attachment to the *N*-acetylglucosamine. It has been established for other *N*-acetylhexosamines that a substituent on C-4 precludes the formation of chromogens under the alkaline conditions of the Morgan-Elson reaction (Kuhn, Gauhe & Baer, 1954*b*). Since the disaccharide under discussion gave a Morgan-Elson reaction (Table 1) it might therefore be assumed that the linkage was (1:6). However, it was known (Perkins, 1960) that under the conditions of the reaction alkaline hydrolysis of glycosidic bonds could precede chromogen formation in lysozyme digests of cell walls, and that alkali would set free *N*-acetylglucosamine from the disaccharide (see below). It is not known whether *N*-acetylmuramic acid substituted on C-4 will undergo alkaline hydrolysis in this way. Nevertheless, it has been shown that when *N*-acetylhexosamine substituted on C-6 is treated with alkali, a chromogen can be formed to which the substituent is still attached (Kuhn *et al.* 1954*b*), a reaction which could not occur with a substituent on C-4. An attempt was therefore made to isolate, after alkaline treatment of the disaccharide, chromogenic material to which *N*-acetylglucosamine was still linked.

Samples of disaccharide were heated at 100° with triethylamine-carbonate buffer for various periods. After removal of the buffer the products were subjected to paper chromatography in butanol-acetic acid-water, and the unheated paper was sprayed with Ehrlich's reagent. With increasing time of heating in the alkali a slow-running chromogen first appeared, and then disappeared to be replaced by a fast-running chromogen ($R_{N\text{-acetylglucosamine}}$ 0.75 and 1.75 respectively). The latter chromogen is identical with that obtained from *N*-acetylglucosamine or *N*-acetylmuramic acid alone. The slow-running chromogen was separated from unchanged disaccharide and from liberated *N*-acetylglucosamine as follows.

Chromogenic material was prepared by heating a sample of disaccharide with triethylamine-carbonate buffer at 100° for 60 min. in a sealed ampoule. The material was dried in a vacuum desiccator and subjected to zone electrophoresis on washed paper at pH 6.5. In this way chromogenic material, which moved slightly towards the cathode, was easily separated from unchanged disaccharide, which moved several centimetres towards the

anode. The compact chromogen band was eluted, dried, transferred to washed paper and run on a chromatogram in butanol-acetic acid-water overnight. Three bands were detectable on marker strips: free *N*-acetylglucosamine and, clearly separated from it, slow and fast-running chromogens as before.

The chromogen bands were eluted from the part of the paper which had not been sprayed, hydrolysed in 2*N*-HCl for 2 hr. at 105°, freed of HCl and again applied to a chromatogram and run in butanol-acetic acid-water with markers of muramic acid and glucosamine. The fast chromogen gave some weak ninhydrin-positive spots which corresponded to neither marker; the slow chromogen gave a clear spot of glucosamine, and in addition similar weak spots which seemed to derive from acid hydrolysis of free chromogen. Evidently the slow-running material consisted of chromogen derived from the *N*-acetylmuramic acid portion of the molecule which was still linked to unchanged *N*-acetylglucosamine. It is concluded therefore that the disaccharide isolated from lysozyme digests of cell wall has the composition 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid.

DISCUSSION

The work of Salton (1956), demonstrating the liberation by lysozyme from sensitive cell walls of an oligosaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid, has been confirmed and his suggestion that it might be a disaccharide has been substantiated. He also showed that free reducing groups not present in the isolated cell wall are progressively set free by the action of lysozyme. The present work shows that a considerable proportion (see also Perkins, 1960) of this reducing material is a disaccharide, the reducing group of which is that of *N*-acetylmuramic acid. Assuming that only one type of bond is attacked by lysozyme, then that bond must be a glycosidic link from *N*-acetylmuramic acid, possibly to *N*-acetylglucosamine, although there is no direct evidence for this at the moment. However, Berger & Weiser (1957) have shown that chitin, which is known to be a β -1:4-linked poly-*N*-acetylglucosamine, is attacked by egg-white lysozyme. It seems possible therefore that by analogy the link attacked in bacterial cell walls may be 4-*O*- β -(*N*-acetylmuramosyl)-*N*-acetylglucosamine.

At present it is not known whether an α - or a β -linkage is present in the 6-*O*-(*N*-acetylglucosaminyl)-*N*-acetylmuramic acid which has been isolated from the lysozyme digests of cell wall. It seems likely that this point might be elucidated by the use of α - or β -*N*-acetylglucosaminidases in conjunction with their specific inhibitors.

SUMMARY

1. An oligosaccharide has been isolated from lysozyme digests of the cell walls of *Micrococcus lysodeikticus*, and has been shown to contain only *N*-acetylglucosamine and *N*-acetylmuramic acid, thus confirming the work of Salton (1956).

2. Oxidation of this oligosaccharide with alkaline iodine solution, followed by acid hydrolysis, has shown that it is a disaccharide, the reducing terminus of which is *N*-acetylmuramic acid.

3. A study of the Morgan-Elson chromogens formed by heating the disaccharide with alkaline solutions has shown that its structure is 6-*O*-(*N*-acetylglucosaminy)-*N*-acetylmuramic acid.

REFERENCES

- Berger, L. R. & Weiser, R. S. (1957). *Biochim. biophys. Acta*, **26**, 517.
 Hanes, C. S., Hird, F. J. R. & Isherwood, F. A. (1952). *Biochem. J.* **51**, 25.
 Ingram, V. M. & Salton, M. R. J. (1957). *Biochim. biophys. Acta*, **24**, 9.
 Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
 Kuhn, R., Gauhe, A. & Baer, H. H. (1954*a*). *Chem. Ber.* **87**, 289.
 Kuhn, R., Gauhe, A. & Baer, H. H. (1954*b*). *Chem. Ber.* **87**, 1138.
 Kunkel, H. G. (1954). *Meth. biochem. Anal.* **1**, 141.
 Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
 Newton, G. G. F. & Abraham, E. P. (1954). *Biochem. J.* **58**, 103.
 Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Perkins, H. R. (1960). *Biochem. J.* **74**, 186.
 Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647.
 Pringsheim, H. & Ruschmann, G. (1915). *Ber. dtsch. chem. Ges.* **48**, 680.
 Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
 Rydon, H. N. & Smith, P. W. G. (1952). *Nature, Lond.*, **169**, 922.
 Salton, M. R. J. (1956). *Biochim. biophys. Acta*, **22**, 495.
 Strange, R. E. & Kent, L. H. (1959). *Biochem. J.* **71**, 333.
 Wolfrom, M. L. & Cron, M. J. (1952). *J. Amer. chem. Soc.* **74**, 1715.

Substances Reacting as Hexosamine and as *N*-Acetylhexosamine Liberated from Bacterial Cell Walls by Lysozyme

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It has long been known that egg-white lysozyme acts upon certain Gram-positive micro-organisms and their isolated cell walls to yield substances giving the Morgan & Elson (1934) reaction for *N*-acetylhexosamine (Epstein & Chain, 1940; Salton, 1956). Salton (1956) also demonstrated that at least some of the colour given in this reaction was due to the presence of an oligosaccharide of *N*-acetylglucosamine and *N*-acetylmuramic acid (see Perkins, 1960), but he did not give any quantitative figures for the amount of '*N*-acetylhexosamine' that he found. It has also been reported by Brumfitt, Wardlaw & Park (1958; cf. Table 1 in their paper) that material reacting in the Elson & Morgan (1933) test as 'glucosamine' is liberated by lysozyme from the cell walls of *Micrococcus lysodeikticus*. This result seemed to contradict the work of Ingram & Salton (1957), who treated lysozyme digests of bacterial cell walls with fluorodinitrobenzene and found no dinitrophenylglucosamine and only traces of dinitrophenylmuramic acid after acid hydrolysis of the product. In the work re-

ported here these conflicting reports have been further examined. It has been found that direct measurements of '*N*-acetylhexosamine' and 'hexosamine' are an unreliable guide to the composition of lysozyme digests. In order to obtain quantitative information about the various fragments liberated by lysozyme, the mixture of compounds produced by the action of lysozyme on the cell walls of *M. lysodeikticus* has been fractionated and, after acid hydrolysis, the distribution of hexosamine between the fractions has been studied.

METHODS AND MATERIALS

Micro-organisms. The organisms used were *Micrococcus lysodeikticus* (N.C.T.C. 2665), *Bacillus subtilis* (N.C.T.C. 6346) and *Staphylococcus* strain 11 which was partially sensitive to lysozyme (Rogers & Perkins, 1959).

Preparation of cell walls. Cell walls were prepared essentially according to the method of Cummins & Harris (1956) as described by Perkins & Rogers (1959).

Action of lysozyme. Isolated cell walls were incubated at 37° with lysozyme (crystalline; The Armour Laboratories)