3-methoxymandelic acid from urine after salicylate ingestion was apparently unchanged (Table 4), and its excretion appeared to remain within physiological limits (two experiments: 330 and 240 $\mu\text{g.}$ in 1 hr. in control urines; 320 and 320 $\mu\text{g.}$ after 100 min.; 440 and 530 $\mu\text{g.}$ after 130 min.). No evidence was obtained that glucose, protein, urinary metabolites of maple syrup urine disease (Dancis & Levitz, 1960) or phenylketonuria exert any effect on the recovery of 4-hydroxy-3-methoxymandelic acid from urine (Table 4).

SUMMARY

1. A method is described for measuring 4-hydroxy-3-methoxymandelic acid, a major metabolite of adrenaline and noradrenaline in human urine. The assay of this acid is a convenient index of catecholamine metabolism.

2. In the procedure, 4-hydroxy-3-methoxymandelic acid is absorbed on a column of anion-exchange resin and, after elution and extraction, is converted into vanillin by autoclaving with dilute acid. The vanillin is measured spectrophotometrically.

3. The accuracy and specificity of this procedure is sufficiently high to make the method suitable for following physiological fluctuations in the low concentration of 4-hydroxy-3-methoxymandelic acid (3.9 ± 0.7 mg./24 hr.) excreted by normal subjects. Mean recoveries from urine $\pm$ s.d. were $73 \pm 6.6\%$. Some factors affecting the recovery are discussed.

4. The value of the procedure as an aid to the clinical diagnosis of the catecholamine-secreting tumour, phaeochromocytoma, is demonstrated.

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Teichuronic Acid: A Mucopolysaccharide Present in Wall Preparations from Vegetative Cells of *Bacillus subtilis*

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Early work (Salton, 1953, 1956; Yoshida *et al.* 1957) showed that preparations of cell-wall material made from *Bacillus subtilis* are likely to be more complicated than those from some other Gram-positive organisms such as *Micrococcus lysodeikticus*, *Staphylococcus aureus* or *Bacillus*

megaterium. Preparations from these last-named organisms revealed, after acid hydrolysis, the presence of only three or four amino acids as major components together with two amino sugars and in some instances one or two hexoses (Salton, 1953; Cummins & Harris, 1956; Salton & Milhaud, 1959). In some strains of the two coccal species up to 80–90% of the wall is made from four amino acids

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and two amino sugars combined as mucopeptides (Perkins & Rogers, 1959; Mandelstam & Rogers, 1959). In contrast, preparations from *B. subtilis* yielded nine amino acids as well as two amino sugars and glucose (Salton, 1956). Moreover, wall preparations from organisms such as *M. lysodeikticus* and *B. megaterium* are rendered almost completely soluble by egg-white lysozyme, whereas preparations from *B. subtilis* are made only partially soluble, leaving material which is said to retain the form of the wall (Salton, 1956). These observations would suggest the presence of at least two components in the wall: one sensitive to and the other resistant to lysozyme. Further work on this problem is presented below.

Also present in wall preparations from *B. subtilis* are the teichoic acids. The discovery and investigation of these substances has been described by Baddiley (1959) and a detailed investigation of teichoic acid from *B. subtilis* published (Armstrong, Baddiley & Buchanan, 1960). In this organism the teichoic acid is a polymer of ribitol phosphate with glucose and alanine attached, the latter in ester linkage.

Teichoic acid can be extracted from the wall preparations by cold trichloroacetic acid solutions, and it was during such treatment we observed that the extracts also contained considerable amounts of a polymer containing a *N*-acetylhexosamine. The extraction of this polymer from the walls led to a reduction in the otherwise high ratio of hexosamine to 3-carboxyethylglucosamine (muramic acid) present in acid hydrolysates of the preparations. Examination of the substance in the extracts which contained hexosamine revealed that it was composed entirely of *N*-acetylgalactosamine and glucuronic acid, thus resembling chondroitin isolated from bovine cornea (Davidson & Meyer, 1955). Examination of the mode of linkage of the hexosamine and hexuronic acid has suggested that the material from *B. subtilis*, which we propose to call teichuronic acid, has in fact α -glycosidic linkages instead of the β -linkages in chondroitin. A brief preliminary account of this work has already appeared (Janczura, Perkins & Rogers, 1960).

MATERIALS AND METHODS

Organism. The strain of *Bacillus subtilis* used was N.C.T.C. 6346.

Growth medium. A casein acid-hydrolysate medium of the following composition was used: Casamino acids (Difco), 10 g.; KH_2PO_4 , 2.72 g.; water to 900 ml. The pH was adjusted to 7.2 with NaOH solution. This medium was sterilized by autoclaving at 120° for 20 min. and then supplemented by 100 ml. of 17 mM- MgSO_4 , 1.0 ml. of a solution of trace metals, containing 0.1 mg. each of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, ammonium molybdate,

$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ /l., and 1 ml. of a solution containing 0.156 g. of $\text{FeSO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 6\text{H}_2\text{O}$ and 0.168 g. of citric acid/100 ml. of water.

Inoculation of medium and growth of cultures. A portion (100 ml.) of the above-described medium was inoculated with 0.25 ml. of a suspension of spores of the organism (containing 3×10^8 viable organisms/ml.) which had previously been incubated at 35° overnight. The small culture was incubated in a 1 l. flask overnight at 35° and shaken to increase aeration. It was then added to the bulk of the above-described medium (4.5 l.) contained in five 5 l. flasks which were again shaken at 35° overnight.

Preparation of suspensions of micro-organisms and cell walls. The cultures, after overnight incubation, were centrifuged at 1000g for 20 min. and the deposited organisms washed with 800 ml. of ice-cold 0.9% NaCl soln. About 10 g. dry wt. of cells was obtained from a 5 l. batch of culture.

It is necessary in preparing cell walls from many organisms to ensure that lytic enzymes are inactivated, otherwise the yield of cell wall obtained is much reduced and the composition of the residue may not be representative. In the earlier part of this work the enzymes were inactivated by treating the cell suspensions with trichloroacetic acid. Later it was found that very satisfactory yields of wall could be obtained by heating water suspensions of the crude preparations to 100° for 20 min. In the early work treatment with trichloroacetic acid was usually at 0°, but in some instances the cells were first heated with acid for 15 min. at 90°. After the treatment with trichloroacetic acid the cells at a density of 10 mg./ml. were treated with crystalline ribonuclease and trypsin (1 mg./ml.) in 0.05M-buffer, pH 7.6, at 35° in the presence of toluene, followed, after the preparation had been washed, by crystalline pepsin (1 mg./ml.) in 0.05 N-HCl; both treatments were continued until the absorption of ultraviolet light at a wavelength of 280 m μ of the insoluble cell-wall material fell to a minimum. In some other experiments the cells, after treatment with either hot or cold trichloroacetic acid, were shaken with small glass beads of 0.1 mm. diameter on the Mickle (1948) disintegrator for varying times and the insoluble residue was isolated by centrifuging for 10 min. at 10 000g. The residue was resuspended in water and unbroken cells were removed by centrifuging at 1000g for 10 min. The cell-wall material was again treated with the proteolytic enzymes as described above. Finally all the cell-wall preparations were washed in acetone and ether and dried *in vacuo*. The purpose of the above variations in technique was: (1) to stop the action of the active cell-wall-hydrolysing enzymes present in *B. subtilis*; (2) to attempt to remove the material containing a wide range of amino acids from the mucopeptide and thus to obtain wall preparations resembling those from other organisms, such as the cocci, which consist of mucopeptides containing only four or five amino acids. However, the composition of the material obtained by the types of treatment outlined above was qualitatively always the same in its amino acid and amino sugar composition. Experiments in which the quantitative composition of the wall material was measured showed that the relative amounts of the various amino acids were also approximately constant. As will be shown later, the quantitative amino sugar composition was dependent upon previous treatment.

In later experiments it was found that cell-wall preparations of the same composition were obtained without the use of trichloroacetic acid by the Cummins & Harris (1956) technique, provided that the following modifications were introduced: (1) all procedures between the end of incubating the cultures and the differential centrifuging of the cell walls were performed at 0–4°; (2) the suspension of cell walls once obtained was raised to 100° for 20 min. before treatment with enzymes, as has previously been described for the preparation of mucopeptide from *S. aureus* (Mandelstam & Rogers, 1959); (3) treatment with trypsin and ribonuclease was continued overnight (i.e. 16 hr.), but when teichuronic acid was to be isolated the treatment with pepsin used by Cummins & Harris (1956) was omitted and the preparations were not dried. When the hot-water method was used the isolated wall material accounted for 20–25% of the dry weight of the organism.

Hyaluronidase and mucopolysaccharides. The bovine testicular hyaluronidase was Rondase (Evans Medical Supplies, London) and the staphylococcal hyaluronidase was that present in supernatants from cultures of *Staphylococcus aureus* strain 524 SC incubated for 18 hr. at 35° under the conditions previously described (Rogers, 1953). The hyaluronic acid was kindly given to us by Dr J. E. Scott, and the chondroitin sulphate A used for preparation of chondroitin was from Dr Helen Muir, to whom we are grateful. Chondroitin was prepared from chondroitin sulphate A by the method of Kantor & Schubert (1957). The product obtained contained less than 0.1% of sulphur.

Lysozyme digestion of walls. Unless otherwise stated in the text, the walls were suspended at 1 mg./ml. in 0.1M-ammonium acetate solution adjusted to pH 6.5. This suspension was incubated at 35° and crystalline egg-white lysozyme (Armour Laboratories) equivalent to 50 µg./ml. was added.

Infrared spectra. Infrared absorption was measured in compressed KCl disks on a Perkin-Elmer double-beam instrument with rock-salt prism.

Analytical methods

Hexosamine. This was estimated after hydrolysis for 4 hr. at 100° with 4N-HCl by the method of Rondle & Morgan (1955), after separation when necessary from 3-carboxyethylglucosamine as described by Perkins & Rogers (1959).

Hexuronic acid. The colorimetric estimation of Dische (1947) was used.

Phosphorus. This was estimated by the technique of Allen (1940).

Amino acids. These were estimated after paper chromatography by a modification of the method of Kay, Harris & Entenman (1956).

Paper electrophoresis. The apparatus of Kunkel (1954) was used with a potential difference of 15 v/cm. applied for 2–3 hr. The following buffers were used: collidine-acetic acid, pH 6.5 (Newton & Abraham, 1954); 2 N-acetic acid.

Paper chromatography. Descending chromatography on Whatman no. 3 paper was used throughout the work. Two solvent systems were used: (a) butan-1-ol-acetic acid-water (6.3:1.0:2.7, by vol.); (b) butan-1-ol-pyridine-water (6.4:3, by vol.).

RESULTS

Mucopeptide and protein content of walls

Preliminary experiments showed that when the wall preparations were hydrolysed for 16 hr. with 6N-HCl at 100° and the hydrolysate was examined by two-dimensional chromatography, 15 or 16 ninhydrin-positive areas could be distinguished. The relative positions of those areas which could be clearly identified corresponded to those for alanine, aspartic acid, glutamic acid, glycine, α -diaminopimelic acid, valine, leucine or isoleucine or both, threonine, serine, lysine, phenylalanine, proline, glucosamine and muramic acid. Of the amino acids, alanine, glutamic acid, glycine, aspartic acid and the leucine-isoleucine pair were in greatest molar concentration. Next came α -diaminopimelic acid, valine and phenylalanine at about one-third to one-quarter of the concentration, and there were smaller amounts of serine, lysine, tyrosine and proline.

Examination of the effect of egg-white lysozyme upon these preparations confirmed other work (Salton, 1956), showing that the turbidity of the suspensions could be reduced only to about 30–40% of the initial value, and that further incubation or addition of enzyme caused no further digestion of the cell walls. The immediate deduction, however, that this decrease in turbidity indicates that 30–40% of the wall preparation was left unhydrolysed is not supported by isolation of the residue. When the suspension remaining after 24 hr. incubation with lysozyme in the presence of toluene was centrifuged at 10 000g for 10 min. a small deposit was obtained and a clear supernatant fluid. The deposit was washed once with water and then transferred to a tared tube with a minimum quantity of water, mixed with ethanol and centrifuged at 1000g for 20 min. Finally it was washed with acetone and ether and dried *in vacuo*. In two different experiments the weight of this deposit represented 7.2% and 11.1% of the weight of the cell walls. This figure agrees well with that of 7% reported by Salton & Marshall (1959) as 'insoluble wall residue'.

Examination was then made of the composition of the material dissolved by the action of lysozyme and of the residue. The supernatant obtained after digestion of the wall material with lysozyme was dried and the ammonium acetate removed *in vacuo*. A weighed amount of both the residue and the soluble material was then hydrolysed with 6N-HCl at 105° for 16 hr. The dried hydrolysates after solution in water were examined for the distribution of ninhydrin-staining areas by chromatography in two directions. Table 1 shows the results obtained compared with those for the whole wall. It will be seen that the only amino acids occurring in

large amounts in the part of the wall material rendered soluble by lysozyme were $\alpha\epsilon$ -diaminopimelic acid, glutamic acid and alanine. A very small amount of glycine equivalent to about 0.04 times the amount of alanine was constantly present in a large number of experiments. Its significance is unknown but a similar small amount of glycine in whole wall material from a different organism has already been reported (Rogers & Perkins, 1959). It is notable that all the diaminopimelic acid and all of both the amino sugars are found in the soluble part. Thus it seems reasonable to suppose that the mucopeptide in this organism contains as major constituents alanine, $\alpha\epsilon$ -diaminopimelic acid, glutamic acid, muramic acid and glucosamine. The

residue to which this is attached contains at least 12 amino acids and probably has a normal protein composition. This result is to be contrasted with that obtained by Salton & Marshall (1959), who reported the presence of only traces of four amino acids other than alanine, glutamic acid and diaminopimelic acid in cell-wall preparations from *B. subtilis*. The protein is resistant to both trypsin and pepsin whilst still associated with the mucopeptide, but no work has been done to see whether it is resistant after removal of the mucopeptide by lysozyme.

Recognition of the presence of a hexosamine-containing polymer apart from mucopeptide

Table 1. *Composition of the residue and material dissolved from the cell-wall preparations from Bacillus subtilis by lysozyme*

The + signs give a semi-quantitative indication of the amounts of the various substances. For comparison the results from an examination of the untreated wall material are also included. tr., Trace; —, Not known.

Substance	Whole wall material	Wall material after lysozyme treatment	
		Soluble	Insoluble
Alanine	+++	+++	++
Aspartic acid	+	0	+++
Diaminopimelic acid	+++	+++	0
Glutamic acid	+++	+++	+++
Glycine	+	+	+++
Leucine	}	0	+
Isoleucine			
Lysine	tr.	0	tr.
Methionine	+	—	—
Phenylalanine	+	0	++
Proline	+	—	tr.
Serine	+	0	tr.
Threonine	+	0	++
Tyrosine	+	0	tr.
Valine	+	0	+
Glucosamine	++	++	0
Muramic acid	+	+	0

During the course of analysis of a number of cell-wall preparations a very considerable variation in the molar ratio of unsubstituted hexosamine to 3-carboxyethylglucosamine was noted. This ratio was sometimes as great as 3 or 4 to 1 and sometimes small enough to indicate nearly equimolar amounts of the two substances. Further examination of this situation showed that the lowest ratios were correlated with the use of hot 5% trichloroacetic acid (see Methods section) during preparation of the walls, whereas material with the highest ratio resulted from the method in which there was no treatment with trichloroacetic acid. It therefore seemed possible that trichloroacetic acid was removing a substance containing unsubstituted hexosamine rather than 3-carboxyethylglucosamine. To test this possibility walls prepared after treatment of the crude product with hot water alone (see Methods section) were subjected to various treatments with 5% (w/v) trichloroacetic acid. As will be seen from Table 2, the molar ratio of the hexosamine to 3-carboxyethylglucosamine was 3.6:1 in an untreated wall preparation and by treatment with hot trichloroacetic acid the ratio could be reduced to approximately 1:1.

Table 2. *Treatment of cell-wall preparation with trichloroacetic acid*

The cell walls were first prepared by the method in which the lytic enzymes were inactivated by raising the temperature of a suspension in water to 100° for 20 min. In Expt. 2, walls already extracted three times with 5% trichloroacetic acid at 37° for 24 hr. were further extracted three times with fresh portions of 5% trichloroacetic acid, each time for 15 min. at 90°.

Expt.	Treatment	Temperature	Time	μ mole/mg.		Ratio (A/B)
				Hexosamine (A)	3-Carboxyethyl-hexosamine (B)	
1	None	—	—	0.93	0.26	3.6
	5% Trichloroacetic acid	20°	18 hr.	0.84	0.31	2.7
		90	15 min.	0.63	0.31	2.0
		90	60 min.	0.65	0.39	1.7
2	5% Trichloroacetic acid	90	15 min. (three times repeated)	0.81	0.70	1.2

Examination of trichloroacetic acid extracts

In order to examine the nature of the substances extracted by trichloroacetic acid solution, 200 mg. of cell-wall preparation (made with hot-water treatment alone to inactivate the lytic enzymes) was mixed with 10 ml. of 5% (w/v) trichloroacetic acid. This suspension was shaken for 16 hr. at room temperature, the cell-wall material was then deposited by centrifuging and re-extracted with trichloroacetic acid twice more under the same conditions. The extracts were combined and four to five times their volume of acetone was added. A gluey precipitate (about 8 mg.) was obtained which was dried with ether. This material was dissolved in 2.0 ml. of water and 0.7 ml. applied as a band to Whatman no. 3 paper and subjected to electrophoresis with 2M-acetic acid as buffer. The applied potential was 15 v/cm. and the time 3 hr. After drying, a longitudinal 1 cm. strip of paper was cut off and stained according to the method of Rydon & Smith (1952). Two anionic zones appeared, the slower (A) having moved 6 cm. and the faster (B) 14 cm.; the region of the origin also showed some staining. The areas corresponding to the two zones A and B in the remainder of the paper which had not been stained were separately eluted with water and the eluates dried. The resulting materials will be temporarily referred to as A and B.

Both materials were examined: (1) hexosamine was estimated after hydrolysis for 4 hr. at 100° with 4N-HCl; (2) ninhydrin-positive areas were noted after chromatography of the hydrolysates in two dimensions; (3) a hydrolysate made with 2N-HCl acting for 2 hr. at 100° was examined for hexose after chromatography in one direction with butanol-acetic acid-water as solvent; (4) total phosphorus was estimated; (5) hexuronic acid was estimated on the unhydrolysed material.

No significant amounts of amino acids were present in either of the materials, except a trace of alanine in substance B. Hexosamine and hexuronic acid tests were positive in material A. In material B there was phosphorus and glucose but no hexosamine or hexuronic acid. Material B was likely to be teichoic acid, which had already been isolated from wall preparations of *B. subtilis* (Armstrong *et al.* 1960) by extraction with trichloroacetic acid. Further examination of several preparations showed that the molar ratio of phosphorus to glucose varied from 1.2 to 0.8 and that solutions of the material showed no specific absorption of ultraviolet in the region of 260 m μ . The almost complete absence of alanine from substance B seemed at first anomalous since the teichoic acid contains about 1 mole of this amino acid/mole of phosphorus. Further consideration of the great

alkali-lability of the bond by which alanine is linked to teichoic acids (Armstrong *et al.* 1960), however, suggested the probability that it had been removed during the treatment of the wall preparations with trypsin and ribonuclease at pH 7.6. It was considered that substance B was teichoic acid and it was not examined further.

Substance A yielded only hexosamine and hexuronic acid on hydrolysis. It is this substance we propose to call teichuronic acid.

Isolation and properties of teichuronic acid

The following method was adopted as routine for the preparation of teichuronic acid. It is not claimed that this is the best method and indeed it is likely to reduce the size of the molecule considerably from that of the substance in the cell-wall material itself. It does, however, yield a reasonable amount of mucopolysaccharide in a satisfactory state of purity.

Cell-wall material was prepared from the cells obtained from 5 l. of culture by the method which involves heating a water suspension to inactivate the lytic enzymes (cf. Methods section). About 1.2 g. dry wt. of material was used. It was suspended without first drying in 150 ml. of 5% (w/v) trichloroacetic acid and left at room temperature overnight (i.e. about 15–18° for 16 hr.). This removes relatively little teichuronic acid but a lot of the teichoic acid and the first extract was discarded. The walls were then resuspended in a further 150 ml. of 5% trichloroacetic acid and left at 35° for 16 hr. This extraction at 35° was repeated three more times. The combined extracts (two to four) were then extracted three times with an equal volume of ether to remove the trichloroacetic acid, and the pH was adjusted to 7.0 by the addition of a few drops of N-NaOH. The volume of the extracts was then reduced to 100 ml. by evaporation at a pressure of 15 mm. Hg, the temperature of the outside bath being not greater than 45°. The pH of the solution was then adjusted to 3.2 with 2N-H₂SO₄ (about 0.15 ml. was required) and a 1% (w/v) solution of cetylpyridinium bromide added until no further precipitation occurred. The suspension was then warmed to 30° to agglutinate it and the precipitate was removed by centrifuging, washed with 0.1% cetylpyridinium bromide and dissolved in 20 ml. of 0.3M-Na₂SO₄. A very slight amount of material which failed to dissolve was deposited by centrifuging and discarded. The supernatant was placed in a tube of cellophane and dialysed against 0.05M-Na₂SO₄ for 16 hr. with stirring. The suspension was then centrifuged and a very oily precipitate formed. This was dissolved in 20 ml. of 0.2M-Na₂SO₄ and 40 ml. of ethanol added. The thick precipitate of Na₂SO₄ crystals

was removed. A further 40 ml. of ethanol was then added and on the addition of a few crystals of potassium acetate a further precipitate formed. This was deposited by centrifuging, washed twice with ethanol, once with ether and dried *in vacuo* (yield 81 mg.). Various batches of material had the analyses indicated in Table 3. Neither phosphorus nor sulphur could be detected.

The material as isolated consisted of a white powder readily soluble in water to form somewhat viscous solutions. When dialysed against water in Visking cellophan tubing none of the material passed through the membrane. When subjected to electrophoresis on paper at pH 2.4 it moved further towards the anode than hyaluronic acid. At pH 6.5 it moved anionically more rapidly than hyaluronic acid and less than chondroitin sulphate. It remained at the origin at pH 2.0. These results probably indicate that the effective negative charge on the teichuronic acid molecule is greater than that on hyaluronic acid but less than that on chondroitin sulphate. In assessing the value of the evidence for this, however, it should be borne in mind that from the method of isolation the molecular size of teichuronic acid might be expected to be considerably smaller than that of the other two mucopolysaccharides, and this may lead to alterations in the rate of movement on paper.

Behaviour of teichuronic acid on a cellulose ion-exchange material

The homogeneity of the preparations of teichuronic acid was further demonstrated by chromatography on a column of diethylaminoethyl-cellulose. The column was prepared as described by Rogers & Perkins (1960) and equilibrated with 0.01M-ammonium acetate buffer, pH 5.0. It was eluted with a gradient of the same buffer, starting with 1 l. of 0.01M-ammonium acetate in the mixing flask and using concentrations of firstly 0.5M and secondly 5M in the reservoir. Fractions (2.5 ml.) were collected and samples were taken for estimation of uronic acid. Fig. 1 shows that a single sharp peak was obtained. This peak also contained the hexosamine.

Oxidation with sodium periodate. Jeanloz & Forchielli (1951) studied the periodate oxidation of hyaluronic acid and showed that at pH 4.5 and 5° over-oxidation was prevented. Under these conditions hyaluronic acid consumed about 0.3 mole of oxidant/repeating unit in 25 hr. To see whether teichuronic acid resembled hyaluronic acid in general structure, and in particular whether it had an excessive proportion of end groups characteristic of a branched-chain polymer, we oxidized each of these mucopolysaccharides with periodic acid under the conditions described above, and measured the disappearance of periodate. The results (Table 4) show that teichuronic acid and hyaluronic acid behave similarly toward periodate oxidation. The slightly greater over-oxidation of teichuronic acid may indicate that the degree of polymerization of the sample as isolated was less than that of the hyaluronic acid used. It is known (see discussion by Rogers, 1961) that the small periodate consumption observed with hyaluronic acid does not preclude the presence of vicinal OH

Fig. 1. Gradient chromatogram on *NN*-diethylaminoethyl-cellulose column. Gradient was made by running (a) 0.5M- and (b) 5M-ammonium acetate into 1 l. of 0.01M-ammonium acetate in a closed vessel. Both buffers were pH 5.0. Teichuronic acid was estimated by measuring the amount of glucuronic acid by the method of Dische (1947).

Table 3. *Analytical data for different batches of teichuronic acid*

Calculated values for a polymer of *N*-acetylgalactosamine and glucuronic acid are: for the free acid, 47.2% of galactosamine, 51.2% of glucuronic acid; for the potassium salt, 42.9% of galactosamine, 46.5% of glucuronic acid.

Sample	Batch	Galactosamine (%)	Glucuronic acid (%)	Molar ratio (glucuronic acid/galactosamine)
Potassium salt	1	33.2	38.4	1.07
	2	32.2	38.1	1.09
Free acid	—	43.9	44.8	0.93
Material from electrophoresis strip	(A ₂)	—	—	1.04
	(A ₄)	—	—	1.01

groups and therefore of substitution on positions other than C-3 on the hexose molecules in the polysaccharide, since a C-4 linkage has now been shown to occur on the glucuronic acid moiety of hyaluronic acid (Meyer, 1957, 1958). The same reasoning may apply to teichuronic acid.

Action of hyaluronidases. Since teichuronic acid resembled hyaluronic acid and chondroitin in many respects it was interesting to see whether it would be attacked by hyaluronidases. As there was also the possibility, mentioned below, that teichuronic acid might contain α - rather than β -glycosidic linkages, it might have been expected that enzymes which normally attack β -linked mucopolysaccharides would be without effect upon it.

Solutions of the various substrates were prepared at such a concentration that they yielded a conveniently dense precipitate with serum reagent at pH 4.2 (Tolksdorf, McCready, McCullagh & Schwenk, 1949). The substrates were incubated at pH 6.0 and 37° with suitable dilutions of either testicular hyaluronidase (stock solution 1500 i.u./ml.) or the supernatant from an overnight culture of *Staphylococcus aureus* 524 SC, which is known to contain much hyaluronidase (Rogers, 1953). Incubation was continued either for 10 min. or, if

little action had occurred, overnight. The final turbidity obtained upon addition of acetate buffer, pH 4.2, and serum reagent was read on a Spekker colorimeter with a neutral Ilford filter H 508. In addition to hyaluronic acid and teichuronic acid, chondroitin which has the same gross chemical composition as teichuronic acid but is known to be β -linked was also used as a substrate. The results are given in Table 5. Testicular hyaluronidase attacked hyaluronic acid with extreme readiness and chondroitin at about one-twentieth of the rate, but was virtually without effect on teichuronic acid. Staphylococcal filtrates behaved similarly with respect to the first two substrates, but also showed some action on teichuronic acid. The inability of testicular hyaluronidase to attack teichuronic acid offers some support for the idea that the configuration of its glycosidic linkages is different. It is not clear whether the attack by unpurified staphylococcal filtrates can be attributed to the hyaluronidase which they are known to contain, or to some other enzyme.

Components of teichuronic acid

Nature of the amino sugar. A hydrolysate of teichuronic acid was prepared by heating with 4N-HCl for 4 hr. at 100° and, after the acid had been removed *in vacuo*, the hexosamine was separated by paper chromatography with the solvent system butanol-pyridine-water. It produced a single ninhydrin-positive spot with R_f slightly less than that of glucosamine. The eluted hexosamine was then oxidized with ninhydrin according to the technique of Stoffyn & Jeanloz (1954). The pentoses formed were separated by zone electrophoresis in 0.025M-borate buffer, pH 9.0. The only pentose which could be recognized by this technique was lyxose; no arabinose was formed. This clearly indicated that the original hexosamine was not glucosamine, but might be galactosamine or talosamine. The hexosamine was

Table 4. *Periodate oxidation of hyaluronic acid and teichuronic acid*

Oxidation was performed in 0.1M-acetate buffer, pH 4.5, at 5°.

Substance	Time (hr.)	Periodate consumed (mole/repeating unit)
Teichuronic acid	3.5	0.20
	5.5	0.28
	22	0.56
Hyaluronic acid	3.5	0.24
	5.5	0.26
	22	0.42

Table 5. *Action of hyaluronidase on teichuronic acid and chondroitin*

Enzyme action was measured by the decrease in turbidity obtained after addition of serum reagent at pH 4.2. Stock enzyme solutions were (a) testicular hyaluronidase (1500 i.u./ml.) and (b) supernatant from an overnight culture of *Staphylococcus aureus* 524 SC. The dilutions of the enzyme were made in 0.4% gelatin.

Substrate	Enzyme	Dilution	Incubation time	Decrease in turbidity (%)
Hyaluronic acid	Testicular	40	10 min.	42
	Bacterial	100	10 min.	84
Teichuronic acid	Testicular	2	10 min.	0
	Testicular	2	24 hr.	13
	Bacterial	4	10 min.	—
	Bacterial	4	24 hr.	37
	Bacterial	4	24 hr.	37
Chondroitin	Testicular	2	10 min.	36
	Testicular	2	24 hr.	100
	Bacterial	4	10 min.	16
	Bacterial	4	24 hr.	53

then *N*-acetylated under conditions similar to those described by Cardini & Leloir (1957), and run on a paper chromatogram with butanol-pyridine-water as solvent. The single spot giving the purple colour characteristic of *N*-acetylated hexosamines (Partidge, 1948) corresponded in position with authentic *N*-acetylglactosamine, and was clearly distinguished from the glucosamine and talosamine derivatives (Crumpton, 1959). Since the *N*-acetylated material gave the Morgan-Elson reaction for *N*-acetylhexosamines, it could not have been the 2:4-diacetamidodideoxyhexose isolated from *B. subtilis* by Sharon & Jeanloz (1958, 1960), because their material, in common with other 2-acetamidohexoses substituted in the C-4 position, did not give this reaction (Kuhn, Gauhe & Baer, 1954).

Hexuronic acid. As mentioned above, teichuronic acid gave a positive carbazole reaction (Dische, 1947) for hexuronic acid. It is known that the various hexuronic acids give very different ratios of colour intensity in the carbazole and orcinol reactions (Khym & Doherty, 1952), only glucuronic acid and galacturonic acid giving ratios of approximately 1.0 (Hoffman, Linker & Meyer, 1956). Iduronic acid, guluronic acid and mannuronic acid give carbazole/orcinol ratios of 0.22, 0.30 and 0.13 respectively. It was found that a sample of teichuronic acid gave a carbazole/orcinol ratio of 1.1, suggesting that either glucuronic acid or galacturonic acid was present. To distinguish between these possibilities, samples of teichuronic acid were hydrolysed in 0.5N-HCl at 100° for 3 hr. and the hydrolysates were run on paper chromatograms in butanol-acetic acid-water and ethyl acetate-acetic acid-water (3:1:3, by vol.) (Rao, Beri & Rao 1951). The hydrolysates produced spots corresponding only to glucuronic acid and its lactone and not to galacturonic acid, which in any case does not form a lactone. Hence we concluded that the hexuronic acid component of teichuronic acid was glucuronic acid.

Nature of the glycosidic bonds

The material isolated from the trichloroacetic acid extracts, either by paper electrophoresis as described earlier in this paper or by precipitation by cetylpyridinium bromide, did not diffuse through cellophan membranes and behaved generally like a substance of relatively high molecular weight (e.g. concentrated solutions showed detectable viscosity). It therefore seemed likely from the analyses that teichuronic acid was a polymer made up of equimolar amounts of *N*-acetylglactosamine and glucuronic acid. The position and configuration of the supposedly glycosidic bonds were, of course, unknown.

Optical rotation. As can be seen from Table 3,

the amounts of *N*-acetylglactosamine and glucuronic acid present in the various preparations amounted to about 80 % of the dry weight of the material. In order to calculate the specific optical rotation it was assumed that the remaining 20 % of the material represented ash and water. On this basis $[\alpha]_D^{25}$ had a value of +38° to +40°. The positive direction of rotation is opposite from that of chondroitin (Davidson & Meyer, 1955; Kantor & Schubert, 1957) or that of hyaluronic acid, both of which have a high negative rotation. This result suggested to us that in contrast to these two polysaccharides the configuration of one or both types of glycosidic bond in teichuronic acid might be α instead of β . Further work, however, was clearly necessary to establish this.

Acid hydrolysis. When hyaluronic acid or chondroitin sulphate is hydrolysed with dilute acids disaccharides with the structure 3-*O*- β -glucuronosyl-2-deoxyaminohexose can be isolated. Weissman, Rapport, Linker & Meyer (1953) hydrolysed hyaluronic acid with 0.5N-H₂SO₄ at 100° for 3 hr. and obtained 20–30 % yield of crystalline hyalobiuronic acid. We treated a sample of teichuronic acid in the same way, hydrolysing for 20, 40, 60 and 90 min. Chromatographic examination of the products showed only unchanged material and progressively increasing amounts of free galactosamine, together with free *N*-acetylhexosamine in the early samples. The *N*-acetylhexosamine ran in the same position as an authentic sample of *N*-acetylated galactosamine. There was no sign of any appreciable yield of disaccharide, in contrast with the results of Weissman *et al.* (1953) with hyaluronic acid. This result suggests the presence in teichuronic acid of the more acid-labile α -linkage.

Further partial hydrolyses were performed in an attempt to identify the point of attachment of the glucuronic acid residues to those of *N*-acetylglactosamine. As mentioned above, the yields of disaccharide were very small, but nevertheless it still proved possible to apply the technique of Cifonelli & Dorfman (1958). In this method samples of mucopolysaccharide are partially hydrolysed in HCl and the colour produced by the hydrolysates in the Elson-Morgan reaction is examined. A low ratio for the relative light-absorption at 540 m μ and at 510 m μ indicates the presence of substituents on the C-3 position of the hexosamine. Glucosamine hydrochloride gives a value of 1.38.

Samples of teichuronic acid were hydrolysed in 2N-HCl at 100° for various periods and the hydrolysates were examined in the Elson-Morgan reaction (Rondle & Morgan, 1955). The comparative values for light-absorption at 540 m μ and 510 m μ are given in Table 6, together with the results of Cifonelli & Dorfman (1958) on hyaluronic acid and chondroitin sulphate A. The result for

15 min. hydrolysis makes it probable that the glucuronic acid is linked to the galactosamine at the C-3 position. After standing overnight, the coloured solution from the sample hydrolysed for 15 min. showed an increase in light-absorption (also characteristic of hexosamines substituted at C-3) (Crompton, 1959) and the absorption curve shown in Fig. 2 was obtained. Substituents at C-3, C-4 and C-6 give absorption maxima in the Elson-Morgan reaction at 510, 530 and 540 $m\mu$ respectively. This evidence points to the presence of a 3-glucuronosyl-*N*-acetylgalactosamine unit in teichuronic acid.

Table 6. *Relative absorption at 540 and 510 $m\mu$ in Elson-Morgan reaction given by partial hydrolysates of mucopolysaccharides*

2 *N*-HCl at 100° was used for the hydrolysis. Extinctions were read immediately after development of the colour in the Randle & Morgan (1955) reaction.

Time of hydrolysis	$E_{540\text{ m}\mu}/E_{510\text{ m}\mu}$		
	Hyaluronic* acid	Chondroitin* sulphate A	Teichuronic acid
15 min.	—	—	0.77
30 min.	0.37	0.45	0.95
60 min.	0.44	0.48	0.91
120 min.	0.72	0.71	0.98
16 hr.	1.15	1.20	—

* Results obtained by Cifonelli & Dorfman (1958).

Fig. 2. Absorption curves in Elson-Morgan reaction. The 15 min. partial hydrolysate of teichuronic acid (see Table 6) was treated by the Randle & Morgan (1955) procedure. The absorption curve was prepared after the final solution had stood overnight. O, Teichuronic acid hydrolysate; Δ, glucosamine hydrochloride.

Alkaline hydrolysis. It was found that samples of teichuronic acid, although diffusible and clearly macromolecular, gave exceptionally high values for free *N*-acetylhexosamine under the conditions of the Morgan-Elson reaction, as modified by Reissig, Strominger & Leloir (1955). For instance, after heating for 15 min. at 100° in borate buffer, pH 9.0, a sample of teichuronic acid gave a reaction with Ehrlich's reagent corresponding to the presence of 22% of its weight as *N*-acetylgalactosamine, compared with the theoretical total content of 58%. A sample of hyaluronic acid in a parallel experiment gave no colour at all. Once again it appeared that the glycosidic links in teichuronic acid were of exceptional lability. Some confirmation that breakdown was taking place under the alkaline conditions was obtained as follows. A sample of teichuronic acid was heated for 4 min. in a sealed ampoule at 100° with triethylamine (Perkins, 1960). Chromatography of the product compared with untreated material showed that the alkaline treatment had liberated much of the same Morgan-Elson chromogen given by free *N*-acetylhexosamines, observable as a spot with R_f 0.60 in butanol-pyridine-water. The untreated teichuronic acid had not moved from the origin.

Infrared spectroscopy. Examination of infrared spectra provided further evidence for the configuration of the glycosidic linkages in teichuronic acid. Barker, Bourne, Stacey & Whiffen (1954) showed that among many glycosides and polysaccharides, including some amino sugar glycosides, α -linked compounds were characterized by an absorption maximum at about 844 cm^{-1} , whereas a β -link produced a peak at about 890 cm^{-1} , though this latter peak might easily be obscured by others. We compared the infrared spectrum of a sample of chondroitin with that of teichuronic acid. Both substances were used as the free acid.

The results are shown in Fig. 3. A peak at 845–865 cm^{-1} , present in the spectrum of teichuronic acid and absent (or shifted to the region 875–900 cm^{-1}) in that of chondroitin, suggests that α -glycosidic links occur in teichuronic acid.

DISCUSSION

In confirmation of the results obtained by Salton (1953, 1956) and Yoshida *et al.* (1957) we find that the cell walls of the strain of *B. subtilis* which we have examined contain materials composed of more amino acids than do similar preparations from other Gram-positive organisms. Irrespective of the method of preparation of the wall material it contained about 7–10% of protein material which was not made soluble either by lysozyme or by prolonged incubation with trypsin or pepsin. The amino acid content of this material appeared in no

Fig. 3. Infrared spectra. Upper curve, teichuronic acid; lower curve, chondroitin. Each compound was examined in the form of the free acid.

way to distinguish it from other proteins. Also in the wall is a much larger amount of mucopeptide similar to that in wall preparations from other micro-organisms. This mucopeptide contains glutamic acid, alanine, α -diaminopimelic acid, hexosamine and 3-*O*-carboxyethylglucosamine; it is rendered soluble by the action of egg-white lysozyme. In addition to these components are the polyribitol phosphate-glucose compound, called by Baddiley and his co-workers (Baddiley, 1959; Armstrong *et al.* 1960) teichoic acid, and the mucopolysaccharide described in the present paper as teichuronic acid. Thus in the wall preparations from *B. subtilis* strain 6346 made by a technique similar to that of Cummins & Harris (1956) there are at least four polymeric substances. Nothing, of course, is yet known about the arrangement or importance of these substances for the functioning of the wall surrounding the living organisms. It is known (Salton, 1953; Barkulis & Jones, 1957; Freimer, Krause & McCarty, 1959) that in streptococci, for example, M protein adheres very firmly to the cell-wall material, and it is possible that any substance of the right properties which is destined to be exported from the cell into the external medium may be partially retained by the cell wall.

The insoluble protein of the cell wall is presumably present as a structural feature in the organism but the exact status of the apparently water-soluble teichoic acid and teichuronic acid is thus less clear. These may be held in the meshes of the insoluble wall structure by forces other than covalencies and may not play any essential role in maintaining the structure of the wall. On the other hand, even if this is true, the presence of these large electro-negatively charged molecules might be expected to have considerable influence on the 'permeability' of the cell wall to other macromolecules such as proteins during the course of their exportation from the cell.

It should also be noted that we have examined only one strain of *B. subtilis* and it seems improbable that one could generalize to other strains of the organism. Nevertheless, sufficient has been done to show that attempts to suggest structures for mucopeptides on the basis of overall analyses of the unfractionated cell-wall preparations are unlikely to be of value because components of different unrelated polymers may all contribute to the final analytical figures. Thus when cell-wall preparations show molar ratios of apparent glucosamine to muramic acid considerably in excess of

1.0, the strong possibility should be considered that polymers such as mucopolysaccharides or teichoic acids as well as mucopeptide might be present; teichoic acids from organisms such as *Staphylococcus aureus* contain *N*-acetylglucosamine in place of glucose (Baddiley, 1959). Likewise molar ratios of alanine to glutamic acid present in cell-wall material should be scrutinized to see that none of the alanine is contributed by teichoic acid rather than by mucopeptide. Cell-wall material may contain up to 60% or more of teichoic acid, which may contribute considerable amounts of D-alanine according to the method of preparation.

The mucopolysaccharide that is described here yields galactosamine and glucuronic acid when it is hydrolysed under the appropriate conditions. The amino sugar is likely to have been present in the original material as the *N*-acetyl compound since *N*-acetylgalactosamine could be recognized as a constituent of partial hydrolysates made by treatment with dilute acid. Together *N*-acetylgalactosamine and glucuronic acid make up about 80–90% of the weight of dried samples. The remaining small amount of the material is presumably ash.

The relationship of this substance to other known mucopolysaccharides is one of great interest because the components, *N*-acetylgalactosamine and glucuronic acid, are the same as those in chondroitin (Davidson & Meyer, 1955). This latter substance was isolated from the cornea of bovine eyes, and can also be produced by removing the sulphate from chondroitin 4- or 6-sulphate (Kantor & Schubert, 1957). The latter substances are prominent components in mammalian connective tissue. Teichuronic acid differs from chondroitin in the following ways: (a) direction of optical rotation; (b) ease of acid hydrolysis; (c) possession of an absorption band at about 850 cm.⁻¹; (d) resistance to testicular hyaluronidase. On the basis of these differences we would suggest the possibility that whereas the glycosidic bonds in chondroitin are β , those in teichuronic acid are α in configuration; the evidence for this is, of course, not complete.

The position of at least some of the glucuronosyl-*N*-acetylgalactosamine bonds is likely to be 1–3, as judged by the reaction of partial acid hydrolysates in the Cifonelli & Dorfman (1958) test. Unfortunately the amounts present under all the hydrolytic conditions examined were so small that no isolation could even be attempted. No evidence has been obtained about the position of the *N*-acetylgalactosaminosyl-glucuronic acid bond.

The properties and composition of teichuronic acid clearly distinguish it from the polysaccharide isolated also from *B. subtilis* cells by Sharon (1957). The material isolated by this author gave no

reaction for hexuronic acids and contained galactose and the diamino hexose later identified by Sharon & Jeanloz (1958, 1960). It was isolated from whole cells by extracting them with trichloroacetic acid in a manner similar to that used here for extracting teichuronic acid from cell walls.

SUMMARY

1. Cell-wall preparations made from vegetative cells of *Bacillus subtilis* strain 6346 contain at least four polymers: (a) a mucopeptide consisting of glucosamine, 3-carboxyethylglucosamine (muramic acid), glutamic acid, α -diaminopimelic acid, alanine and probably a trace of glycine; (b) an insoluble protein; (c) teichoic acid; (d) a mucopolysaccharide called here teichuronic acid.

2. Teichuronic acid consists of equimolar amounts of *N*-acetylgalactosamine and glucuronic acid.

3. The glycosidic linkage between at least part of the glucuronic acid and the *N*-acetylgalactosamine in teichuronic acid is likely to be on C-3 of the amino sugar.

4. The properties of teichuronic acid strongly suggest that the glycosidic bonds are α in configuration.

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The Specificity of Malic Dehydrogenase from Higher Plants

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Evidence has been presented (Davies & Kun, 1957) supporting the view that malic dehydrogenase present in ox-heart mitochondria is an α -hydroxydicarboxylic acid dehydrogenase, catalysing the following reactions between substrate and diphosphopyridine nucleotide (DPN⁺):

L-Malate + DPN⁺

$\rightleftharpoons$ oxaloacetate + reduced DPN (DPNH) + H⁺ (1)

D(-)- or meso-Tartrate + DPN⁺

$\rightleftharpoons$ oxaloglycollate + DPNH + H⁺ (2)

Oxaloglycollate + DPN⁺

$\rightleftharpoons$ dioxosuccinate + DPNH + H⁺ (3)

Tartronate (hydroxymalonate) + DPN⁺

$\rightleftharpoons$ mesoxalate (oxomalonate) + DPNH + H⁺ (4)

α -Hydroxyglutarate + DPN⁺

$\rightleftharpoons$ α -oxoglutarate + DPNH + H⁺ (5)

Reactions (1)–(4) have been demonstrated in higher plants and tentative evidence that separate

enzymes catalyse reactions (1), (2) and (4) has been presented (Stafford, 1956, 1957). Whilst admitting that proof of specificity requires the separation of enzyme activities Stafford produced two types of evidence to support the view that malic dehydrogenase and hydroxymalonate dehydrogenase are separate enzymes: (a) During the purification of hydroxymalonate dehydrogenase, the relative activities with oxomalonate, dioxosuccinate and oxaloacetate were not constant; (b) hydroxymalonate is a competitive inhibitor of malic dehydrogenase.

It is probable that the concentration of oxomalonate and dioxosuccinate used by Stafford was well below saturation and in consequence her assays would be sensitive to errors in concentration of the unstable keto acids. The observation of competitive inhibition, contrary to Stafford's argument, supports the view that a single enzyme is involved and quantitative evidence for this view will be presented.

Delbruck, Schimassek, Bartsch & Bücher (1959) have suggested that animal cells contain two distinct malic dehydrogenases: one located in the

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