- Fischer, H. & Zeile, K. (1928). *Liebigs Ann.* **462**, 218.
- Gibson, K. D., Neuberger, A. & Scott, J. J. (1955). *Biochem. J.* **61**, 618.
- Granick, S. & Mauzerall, D. (1958). *J. biol. Chem.* **232**, 1119.
- Grinstein, M. (1947). *J. biol. Chem.* **167**, 515.
- Jackson, A. H. & MacDonald, S. F. (1957). *Canad. J. Chem.* **35**, 715.
- Laver, W. G., Neuberger, A. & Udenfriend, S. (1958). *Biochem. J.* **70**, 4.
- Lockwood, W. H. & Rimington, C. (1957). *Biochem. J.* **67**, 8p.
- MacDonald, D. M. & MacDonald, S. F. (1955). *Canad. J. Chem.* **33**, 573.
- MacDonald, S. F. (1952). *J. chem. Soc.* p. 4176.
- Muir, H. M. & Neuberger, A. (1949). *Biochem. J.* **45**, 163.
- Muir, H. M. & Neuberger, A. (1950). *Biochem. J.* **47**, 97.
- Prasad, K. S. N. & Raper, R. (1955). *Nature, Lond.*, **175**, 629.
- Radin, N. S., Rittenberg, D. & Shemin, D. (1950). *J. biol. Chem.* **184**, 745.
- Robinson, R. (1955). *Structural Relations of Natural Products*, p. 25. Oxford University Press.
- Ropp, G. A. (1950). *J. Amer. chem. Soc.* **72**, 4459.
- Scott, J. J. & Smith, A. T. (1958). *Biochem. J.* **69**, 12p.
- Shemin, D. & Kumin, S. (1952). *J. biol. Chem.* **198**, 827.
- Shemin, D., Russell, C. S. & Abramsky, T. (1955). *J. biol. Chem.* **215**, 613.
- Shuster, L. (1956). *Biochem. J.* **64**, 101.
- Wittenberg, J. & Shemin, D. (1950). *J. biol. Chem.* **185**, 103.

The Isolation, Characterization and Chemical Synthesis of Muramic Acid

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Muramic acid is the trivial name of a hexosamine-like substance which was found in acid hydrolysates of spore peptide (Strange & Powell, 1954) and in the isolated cell walls from a large number of Gram-positive bacteria (Cummins & Harris, 1954, 1956). Recently, Weidel & Primosingh (1957) have found an apparently identical substance in the isolated cell walls of *Escherichia coli*. Park & Strominger (1957) have shown that the acidic hexosamine found by Park (1952) as a constituent of uridine-pyrophosphate-peptide complexes released from penicillin-treated cells of *Staphylococcus aureus* is probably this substance. Muramic acid was isolated in crystalline form from the spore peptide of *Bacillus megaterium* (Strange & Dark, 1956) and provisionally assigned the structure of a 3-O- α -carboxyethylhexosamine (Strange, 1956). Kent (1957) synthesized 3-O- α -carboxyethyl-D-glucosamine from N-acetyl 4:6-O-benzylidene- α -methyl-D-glucosaminide (Neuberger, 1941) and showed it to have very similar properties to natural muramic acid.

This paper presents details of the work previously briefly reported (Strange, 1956; Kent, 1957) on the isolation, structure and synthesis of muramic acid.

METHODS AND MATERIALS

Ion-exchange resins. Amberlite IR-4B (OH) and Amberlite IR-120 (H), both A.R. grade, were obtained from British Drug Houses Ltd. IR-4B (OH) was treated several

times with 2N-HCl until the supernatant solution was no longer coloured. A column of acid-treated resin was washed with water until the effluent liquid was free from acid and then treated with N-NaOH until the effluent liquid was free from Cl⁻ ions. The regenerated resin was washed with de-ionized water until free from alkali and partially dried by passage of CO₂-free air. Resin was purified in this manner just before use; on storage it apparently deteriorated and when an acid solution was mixed with resin which had been prepared for some time, soluble pigmented material was obtained. IR-120 (H) was treated several times with 3.6% (w/v) HCl and freed from acid by washing it in a column with water. M-NaH₂PO₄-Na₂HPO₄ buffer, pH 6.05, was run through the column until the effluent fluid was at the pH of the ingoing buffer. Buffer salts were removed from the resin by washing with de-ionized water until the effluent liquid was free from phosphate. Dowex 50 (cross-linkage, $\times 8$; mesh, 200-400) obtained from Dow Chemical Co., Midland, Mich., U.S.A., was washed with 4N-HCl until the supernatant fluid was colourless and the washed resin was stored in 4N-HCl (Gardell, 1953). 0.3N-HCl was passed through a column of the resin until the acid concentration of the effluent liquid was 0.3N.

Source of muramic acid. Spore-exudate peptide from germinating spores of *B. megaterium* was prepared as described by Strange & Powell (1954). Larger yields of less pure but satisfactory material were obtained if the germinated spores were left suspended in water for several weeks.

Isolation of crystalline natural muramic acid. Spore peptide (500 mg.) was hydrolysed with boiling 6N-HCl (20 ml.) under reflux for 2 hr. The hydrolysate was evaporated to dryness at 30-40° under reduced pressure and the residue was freed from most of the residual HCl by repeated evaporation with water under reduced pressure.

The resulting gum was dissolved in water (15–20 ml.) and the solution shaken with small quantities of IR-4B (OH) until its pH was about 6.5. The mixture was filtered through sintered glass (porosity 5/3) to remove humin and resin. The filtrate was chromatographed on a column containing 80–90 ml. of IR-120 (H) resin, buffered to pH 6.05, water being used for elution, and six fractions each of 10 ml. volume were collected. Samples (0.1 ml.) of the fractions were analysed for hexosamine by the method of Elson & Morgan (1933) and those giving a positive reaction (usually fractions 2, 3 and 4) were combined. The solution was evaporated to dryness under reduced pressure and the residue dissolved in 0.3N-HCl (1 ml.). The acid solution was chromatographed on a column (length 25 cm., diam. 1.25 cm.) containing 80–90 ml. of Dowex 50 (cross-linkage, $\times 8$; mesh, 200–400) with 0.3N-HCl for elution; fractions (approx. 8 ml.) were collected at 15 min. intervals on an automatic fraction collector. Muramic acid left the column after about 120 ml. of 0.3N-HCl had passed through and fractions containing the substance were free from glucosamine and amino acids when examined by paper chromatography. The appropriate fractions were pooled and evaporated to dryness under reduced pressure. The residue was dissolved in water (5 ml.) and the resulting solution shaken with small amounts of IR-4B (OH) until the pH was near 7.0. The mixture was freed from resin by filtration through sintered glass and the filtrate evaporated to dryness under reduced pressure. To the residue dissolved in the minimum volume of water ethanol was added until a faint opalescence appeared. Crystallization commenced on scratching and was allowed to proceed at -8° for at least 24 hr. The crystals were separated by centrifuging at 0° and recrystallized twice from ethanol–water. The product (25–35 mg.) was dried over P_2O_5 *in vacuo* for 1 hr. at 60° .

ANALYTICAL METHODS

C and H determinations were made by Weiler and Strauss, Oxford, and Mr A. C. Thomas of the Chemical Defence Experimental Establishment, Porton, Wilts. Total nitrogen was determined by a micro-Kjeldahl-nesslerization method. Reducing power was determined by the methods of Somogyi (1945) and Hagedorn & Jensen (1923), and addition of HCN by the method of Militzer (1946).

Determination of hexosamine. Total hexosamine was determined by the method of Elson & Morgan (1933) but $m\text{-NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer containing 2% (v/v) of acetylacetone (Immers & Vasseur, 1950) was used (instead of acetylacetone in 0.5N- Na_2CO_3). The colour produced on addition of Ehrlich's reagent was allowed to develop for 10 min. at 70° followed by 10 min. at room temperature (Rondle & Morgan, 1955). These conditions were critical when the 'hexosamine value' of muramic acid was determined with glucosamine as a standard; if the unmodified Elson & Morgan method was used, a much lower value was obtained. Hexosamine was also determined by deamination with HNO_3 followed by reaction with indole (Dische & Borenfreund, 1950), and by steam-distillation with alkaline borate buffer followed by determination of the NH_3 released (Tracey, 1952).

Total amino plus hydroxyl groups. The sample (5 mg.) was heated with 0.1 ml. of acetic anhydride–pyridine (1:4, v/v) for 1 hr. at 100° in a sealed tube in parallel with a similar tube containing acetylating mixture alone. After heating,

the contents of the tubes were diluted to 10 ml. with water. Samples (2.5 ml.) of each were titrated with 0.01N-NaOH, with phenolphthalein as indicator and a stream of CO_2 -free air. The amount of acetic anhydride consumed by the substance was determined by difference.

Periodate oxidation. Consumption of periodate was determined as described by Jeanloz & Forchielli (1951); near-theoretical results were obtained for glucosamine when the conditions proposed by these authors were used. Formic acid and formaldehyde production were measured by the methods of Potter & Hassid (1948) and O'Dea & Gibbons (1953) respectively.

α -Amino groups. The manometric method of Van Slyke, Dillon, MacFadyen & Hamilton (1941) was used.

Alkoxy groups. The Zeisel method was used.

Degradation with ninhydrin. The paper chromatographic method of Stoffyn & Jeanloz (1954) was used. Products were detected with anisidine hydrochloride in butanol (Hough, Jones & Wadman, 1950), aniline phthalate (Partridge, 1949) or benzidine–acetic acid (Horrocks, 1949).

Paper chromatography. Whatman chromatographic paper no. 1 or 4 was used. Solvent systems employed (all v/v) were phenol–water (3:1), collidine–lutidine–water (4:1:5), pyridine–ethyl acetate–water (1:2:2), *tert*-butanol–6N-HCl–water (70:1:29) and butanol–acetic acid–water (4:1:5).

Electrometric titration. Carbon dioxide-free air was blown through a solution of the sample in a small cell connected by a bridge of saturated KCl solution to a standard electrode. A micro-glass electrode dipped in the solution and pH changes occurring on titration with 0.01N-NaOH or 0.01N-HCl were measured in a model no. 23 pH meter (Electronic Instruments Ltd.).

Infrared spectrophotometry. Infrared spectra were obtained by Mr L. C. Thomas of the Chemical Defence Experimental Establishment, Porton, Wilts., who used a Perkin–Elmer double-beam instrument, model no. 21.

RESULTS

Behaviour of muramic acid on paper chromatograms. On two-dimensional paper chromatograms with phenol–water in an atmosphere of NH_3 , followed by collidine–lutidine–water, the position occupied by the spot corresponding to muramic acid varied but the substance was always distinguishable from glucosamine or galactosamine (Strange & Powell, 1954; Cummins & Harris, 1956). On single-dimensional chromatography muramic acid was easily separated from these hexosamines with either phenol–water in the absence of NH_3 or *tert*-butanol–HCl–water (Strange & Powell, 1954). It reacted with ninhydrin, ammoniacal AgNO_3 , Elson & Morgan reagents and weakly with sugar-spray reagents such as aniline phthalate, anisidine hydrochloride or benzidine–acetic acid. It gave no reaction with reagents for keto sugars.

Behaviour of muramic acid in the Elson & Morgan reaction. The colour produced by muramic acid after condensation with alkaline acetylacetone and addition of Ehrlich's reagent was salmon-pink

and quite distinct from that produced by glucosamine or galactosamine (Strange & Powell, 1954). This characteristic colour reaction was utilized during the isolation of the substance; the ratio $E_{530\text{ m}\mu}/E_{520\text{ m}\mu}$ for the muramic acid derivative was 0.89–0.93, whereas for glucosamine or galactosamine chromophores the ratio was 1.06–1.08 (Strange & Dark, 1956).

Isolation and composition of muramic acid. Acid hydrolysates of spore peptide from *B. megaterium* contained glucosamine, muramic acid, alanine, glutamic acid, α -diaminopimelic acid and acetic acid. The method of Gardell (1953) for the separation of hexosamines on columns of Dowex 50 was not applied to the separation of muramic acid and glucosamine because the peaks at which the sugars are eluted by 0.3N-HCl on a preparative scale overlap somewhat. Therefore glucosamine was removed by absorption on to IR-120 (Na/H), buffered to pH 6.05, before chromatography of the hydrolysate on Dowex 50. Attempts to crystallize muramic acid hydrochloride by concentration of the 0.3N-HCl eluates from the Dowex 50 column were unsuccessful. However, after treatment with IR-4B (OH) to form the free base, crystallization occurred fairly readily.

The purity of recrystallized muramic acid used for analysis was confirmed by paper chromatography with the five solvent systems given in Methods. Dried crystalline material contained: C, 40.5; H, 7.1; N, 5.3%; no detectable P or S. The analyses corresponded to $\text{C}_6\text{H}_{19}\text{O}_5\text{N}$, mol.wt. 269. A determination of molecular weight by Barger's (1904) method showed that the value was not a multiple of 269. The substance was dextrorotatory ($[\alpha]_D^{20} 109^\circ$; c, 2 in water).

With glucosamine as a standard, estimation of the pure substance by the methods of Elson & Morgan (1933), Dische & Borenfreund (1950) and Tracey (1952) gave 'hexosamine values' of 56, 54 and 64% respectively (Strange & Dark, 1956). The finding that muramic acid gave a positive reaction for hexosamine by the three methods strongly suggested that it was a 2-deoxyamino sugar with a carbonyl group vicinal to an amino group. Confirmation of the presence of a reducing group was obtained by determinations of reducing power and addition of HCN, which gave results of 29 and 23%, as glucose, respectively.

The behaviour of muramic acid on a column of IR-120 resin, buffered to pH 6.05, suggested that it possessed an acidic group. Electrometric titration confirmed the presence of a carboxyl group and also of an amino group, the pK values of which were approximately 2.6 and 8.8 respectively. No α -amino nitrogen was detected by the ninhydrin- CO_2 method of Van Slyke *et al.* (1941). Degradation with ninhydrin (Stoffyn & Jeanloz, 1954) gave a

product which, on paper chromatography with pyridine-ethyl acetate-water or collidine-lutidine-water as solvents, reacted as a pentose with spray reagents for sugars but which had an R_f less than that of arabinose, ribose, lyxose or xylose (Strange & Dark, 1956). On paper electrophoresis at pH 8.6, the derivative moved 4 cm. to the anode, whereas ribose moved 1 cm. to the cathode (220 v, 2.5 hr.). It appeared that the derivative was a pentose with an acidic group.

Determination of hydroxyl plus amino groups by quantitative acetylation gave a value of 3.8. The isolated acetylated product gave a Morgan & Elson (1934) reaction for *N*-acetylhexosamine and the absorption spectrum of the coloured derivative was identical with that of the derivative of *N*-acetylglucosamine.

The following results were obtained from periodate-oxidation experiments, the molecular weight of muramic acid being assumed to be 269; consumption of periodate, 2.6 moles; production of formic acid, 1.7 moles; production of formaldehyde, 0.93 mole; NH_3 was also released but was not determined. The formation of formaldehyde on oxidation of muramic acid with periodate showed that a primary alcohol group was present vicinal to an actual or potential secondary alcohol group.

The above results suggested that muramic acid was a substituted 2-deoxyamino hexose with a glycosidic hydroxyl at position 1, a primary hydroxyl at position 6 and an actual or potential secondary hydroxyl at position 5. The periodate-oxidation studies showed that the other hydroxyl group was on either C-3 or C-4 of the hexosamine. Thus a substituent carrying a free carboxyl group was attached to either C-3 or C-4 and, because of the stability of muramic acid in hot, moderately strong mineral acids, the attachment was probably by means of a C-C or ether linkage. No volatile alkyl halide was obtained on heating the substance with constant-boiling HI in the Zeisel method, but on heating with HI+red P, a fatty acid was released. In a qualitative experiment, muramic acid (2–3 mg.) was heated with constant-boiling HI (0.5 ml.) and a small amount of red P in a sealed tube for 0.5 hr. at 130° . After cooling, the solution was extracted several times with small volumes of ether. The extracts were pooled and extracted with aqueous Na_2CO_3 solution containing $\text{Na}_2\text{S}_2\text{O}_3$. The aqueous extract was acidified with dilute HCl and extracted with ether. The final ether extract was evaporated to dryness under reduced pressure and a small residue was obtained which smelled strongly of a fatty acid, probably propionic acid. A trace of phenolphthalein was added and the residue was neutralized by the addition of aq. N-NH_3 solution from a micropipette. Examination of the neutralized residue by paper

Fig. 1. Synthesis of muramic acid.

Table 1. *Optical rotation and chromatographic behaviour of natural and synthetic muramic acid and the synthetic isomer*

	Average values derived from several experiments		
	Optical rotation $[\alpha]_D^{20}$	R_F^*	$R_{\text{glucosamine value}}^\dagger$ on Zeo-Karb 225 column eluted with 0.33N-HCl
Natural muramic acid	+109	0.53	1.10
Synthetic muramic acid	+109	0.53	1.10
Stereoisomer of muramic acid	+ 52	0.44	0.87

* Values obtained with Whatman no. 1 paper and phenol-water as solvent.

† Values in this column have been reported by Crumpton (1958). The $R_{\text{glucosamine value}}$ relates the elution characteristics of the substance to those of glucosamine run at the same time.

by muramic acid when run under the same conditions. Full details are given in the Experimental section.

The product was shown to be identical with natural muramic acid in the following respects: (1) It gave the same ratio (0.93) for $-\log$ transmissions at 530 and 520 $m\mu$ in the Immers & Vasseur (1950) modification of the Elson & Morgan (1933) test. (2) When eluted from an analytical column of Zeo-Karb 225 it produced a peak identical in shape and position with that given by natural muramic acid when run under precisely similar conditions. (Crumpton, 1958). (3) It behaved identically with muramic acid on paper chromatograms in phenol-water and *tert.*-butanol-6N-HCl-water. (4) On oxidation with ninhydrin it gave a pentose type of material which behaved identically with the oxidation product of natural muramic acid on a paper chromatogram in ethyl acetate-pyridine-water and in paper electrophoresis. (5) The infrared-absorption spectra (2-15 μ) of the synthetic and the natural material were identical.

In all these tests, the synthetic material behaved in a markedly different manner from glucosamine and slightly but significantly different from the isomer mentioned above. Optical rotations and chromatographic data for the two synthetic isomers and natural muramic acid are given in Table 1.

DISCUSSION

The analytical and synthetic work leave no doubt that muramic acid is 3-*O*- α -carboxyethyl-D-glucosamine or its enantiomorph. On biological grounds it would seem unlikely that this acid would be a derivative of L-glucosamine. It has not been found possible so far to degrade muramic acid to D-glucosamine but the enantiomorph configuration is effectively ruled out by the fact that muramic acid is strongly dextrorotatory: $[\alpha]_D^{20} + 109 \pm 3^\circ$ (2%, w/w, in water). This synthesis leaves ambiguous the configuration of the asymmetric carbon atom of the lactic acid moiety, nor is the

use of an optically active lactic acid derivative likely to resolve this point without extensive kinetic studies of the condensation to determine its mechanism. However, muramic acid is more strongly dextrorotatory than α -glucosamine, the more strongly dextrorotatory of the anomers of glucosamine ($[\alpha]_D^{20}$ 100; Westphal & Holzmann, 1942), and than α -glucosamine hydrochloride. If the laws of optical superposition hold even approximately in this case, the lactic acid moiety must be dextrorotatory. The infrared spectrum shows that muramic acid as isolated is in the 'zwitterion' form. Purdie & Walker (1895) have shown that D(-)-lactic acid gives dextrorotatory salts. It follows therefore that the configuration of the ionized lactic moiety in muramic acid is very probably D.

Structurally, muramic acid is closely related to neuraminic acid (Gottschalk, 1955). Oxidation at the asymmetric carbon atom of the lactic acid moiety of muramic acid in its open-chain form, followed by an internal aldol condensation, converts the structure into that of neuraminic acid.

So far muramic acid has always been found associated with amino acids, glucosamine, or galactosamine or both. In the cell wall it could be attached to an amino acid by a peptide linkage and to hexosamine by a glycosidic linkage. Strange & Powell (1954) found that hydrolysis of spore peptide with 0.5N-hydrochloric acid released only 66% of the hexosamine found after hydrolysis with 6N-hydrochloric acid, suggesting that a proportion of the amino sugars present were not attached by glycosidic linkages.

EXPERIMENTAL

Starting materials

N-Acetyl-4:6-O-benzylidene- α -methyl-D-glucosaminide. Synthesized as by Neuberger (1941).

Tetrahydrofuran. Tetrahydrofuran was treated with SnCl_4 and anhydrous CaCl_2 , and allowed to stand in the dark for 24 hr. and fractionally distilled. The distillate

(64–66°) was treated with sodium wire, and allowed to stand overnight and again treated with fresh sodium wire with occasional shaking for several hours. The supernatant was carefully blown over by dry air into a flat-bottomed flask protected by a CaCl_2 tube, treated with 0.5 g. of NaH and allowed to stand overnight. It was then treated with successive small (0.05 g.) amounts of NaH until there was no appreciable evolution of H_2 .

Ethyl α -DL-iodopropionate. This was prepared by refluxing ethyl 2-bromopropionate (British Drug Houses Ltd.) with a small excess of NaI in dry acetone overnight. After cooling, the product was filtered from NaBr, and the solvent removed and the residue distilled, b.p. 39°/2 mm. Hg.

Ethyl α -DL-iodo-orthopropionate. Attempts to prepare this substance by a method analogous to that used by Beyerstedt & McElvain (1937) for the preparation of ethyl α -iodo-orthoacetate from the bromo-orthoacetate were unsuccessful. With anhydrous methanol as solvent considerable amounts of NaBr were formed, showing that halogen exchange had taken place but appreciable quantities of free iodine were also formed. After removal of solvent, the product was extracted from inorganic material with ether and in one experiment the free iodine was removed from the ethereal layer by successive extractions with saturated aqueous KI solution until the aqueous layer was colourless. After drying and removal of solvent, the product was distilled but yielded only a small quantity of distillate (0.8 ml. from 6 ml. of bromo-ortho ester); b.p. 45–57°/4 mm. Hg. Despite the fact that this distillate had about the expected refractive index (n_D^{20} 1.458) (as also had the residue from the still, n_D^{20} 1.461) and contained iodine, its boiling point is too low for ethyl α -iodo-orthopropionate. In a second experiment, the free iodine was removed from the ethereal solution, prepared as above by shaking with Hg metal. On distillation, the yield was much improved but the distillate had the same characteristics.

For the condensation described below, ethyl α -iodo-orthopropionate was prepared by refluxing 0.001 mole of α -bromopropionate (Walters & McElvain, 1940) with 0.001 mole of NaI in 40 ml. of purified tetrahydrofuran for 40 hr. The product was cooled, filtered from NaBr and used directly.

Synthesis of muramic acid

The synthesis of muramic acid from *N*-acetyl-4:6-*O*-benzylidene- α -methyl-D-glucosaminide and ethyl α -DL-iodo-orthopropionate has been briefly described (Kent, 1957). The glucosaminide in dry, deperoxidized tetrahydrofuran was treated with excess of NaH at reflux temperature with stirring and after 7 hr. the sodio derivative was separated from the heavier excess of NaH by decantation under anhydrous conditions. It was then stirred under reflux with a preparation of ethyl α -DL-iodo-orthopropionate (prepared from 1.5 equiv. of the bromo-orthopropionate as described above). The two expected isomers, having the alternative configurations about the C atom of the lactic acid moiety, were obtained in approximately equal amounts. The derivative of the isomer of muramic acid, being sparingly soluble in cold tetrahydrofuran, largely separated as a solid at the end of the condensation and could be centrifuged off. After hydrolysis of the supernatant and removal of glucosamine on an

IR-120 column buffered to pH 6.05, the muramic acid was separated from the remaining isomer on a Dowex 50 column. Later it was found that the simple ester, ethyl α -DL-iodopropionate, could successfully replace the ortho ester but, in this case, the two isomers formed are both soluble in tetrahydrofuran and they had to be separated by careful fractionation on the Dowex 50 column, from which they issue in two well-defined peaks with a small overlap. By both these methods, muramic acid could be prepared as a freeze-dried powder in 20% theoretical yield based on the glucosaminide (25 mg. from 0.001 mole). The methods depending on the separation of two solids were obviously cumbersome and prone to loss, so improvements were sought. It was found that although ethyl α -iodopropionate reacted rapidly in boiling tetrahydrofuran with NaH, as shown by a brisk evolution of H_2 , its rate of reaction with the sodio derivative of the glucosaminide was at least comparable. The method was therefore modified as follows.

The dry glucosaminide (0.32 g., 0.001 mole) was introduced into a well-dried 250 ml. three-necked flask, fitted with mercury-seal stirrer and reflux condenser protected by a CaCl_2 and a soda-lime tube. Approximately 40 ml. of dry, deperoxidized tetrahydrofuran was blown in by a stream of dry air and the mixture warmed with stirring until solution was complete. NaH (approx. 0.05 g.) was added and the mixture stirred at gentle reflux for 7 hr., NaH (0.05 g.) being added at the end of 2, 3, 4 and 6 hr. Ethyl α -iodopropionate (0.9 g., 0.004 mole) was then added and the stirring and refluxing were continued for 20 min., when most of the sodio derivative went into solution. The product was cooled to room temperature, which moderates the reaction between the ester and NaH, and was then decanted under anhydrous conditions from excess of NaH, the latter being washed by decantation twice with 10 ml. of tetrahydrofuran. The combined fluid product was treated with ethyl α -iodopropionate (0.22 g., 0.001 mole), then refluxed for 1 hr. and, after cooling, centrifuged to remove a small amount of solid. The solvent was removed by distillation, eventually at the water pump, and the residue (partly solid, partly liquid) treated with light petroleum (b.p. 40–60°) and scratched until wholly solid. The light petroleum was poured off and the residue triturated twice with light petroleum to remove as much ester as possible. It was then dried, taken up in 10 ml. of ice-cold methanol and filtered. The methanol was removed by distillation, eventually at the water pump, and the residue dried *in vacuo* over H_2SO_4 (1 hr.).

The dried product was hydrolysed with 6*N*-HCl (5 ml.) for 2 hr. and the hydrolysate was extracted with ether until the ethereal layer was colourless, thereby removing benzaldehyde and much free iodine. The aqueous layer was evaporated to dryness *in vacuo*, then taken up in a small volume of water and again evaporated to dryness *in vacuo*. The product was dissolved in 2–3 ml. of water and shaken with small quantities of IR-4B (OH) until the pH rose to 7. It was then filtered through a no. 4 sintered-glass filter, the resin was washed and the combined filtrates were reduced to 5 ml. if necessary. This filtrate was applied to a column (20 cm. $\times$ 1.5 cm. diam.) of IR-120, buffered to pH 6.05, as described under Methods, and was eluted with water (50 ml./hr.), 10 ml. fractions being collected. A portion (0.05 ml.) of each fraction was tested for amino sugar by the Elson & Morgan test. The positives were bulked and evaporated to dryness *in vacuo*. The residue was taken up

in the minimum volume of 0.3N-HCl, placed on a column (20 cm. $\times$ 2.0 cm. diam.) of Dowex 50 ($\times$ 8; mesh, 200-400) previously equilibrated with 0.3N-HCl and eluted with 0.3N-HCl (15 ml./hr.), 5 ml. fractions being collected.

Elson & Morgan tests on samples (0.1 ml.) of the eluates showed that the isomer left the column after approx. 200 ml. of 0.3N-HCl had passed through (peak 230-250 ml., according to exact column size and flow rate). Muramic acid followed (peak 270-310 ml.), there being a small overlap of the peaks. Those fractions containing muramic acid which from the shape of the peaks were shown to be free from isomer were bulked, evaporated to dryness *in vacuo*, taken up in about 2 ml. of water and neutralized (pH 7) with IR-4B (OH). The resulting solution was filtered from resin and freeze-dried. Yield 45 mg.

For crystallization, the product was dissolved in the absolute minimum volume of 50% (v/v) ethanol-water, absolute ethanol added until a faint turbidity formed and the mixture allowed to stand at -8° for 24 hr. Losses were considerable.

The crystalline product contains some NaCl, which is only slowly removed by repeated crystallization. After two recrystallizations it still contains about 1% of inorganic material. As reported for the natural acid (see Results), drying for a short period at 60° *in vacuo* leaves some water and prolonged drying at this temperature does not quantitatively remove it. Drying for any prolonged period at 100° leads to sintering. A sample dried *in vacuo* over P_2O_5 at 80° for 24 hr. gave the following figures, adjusted for ash content: C, 43.1; H, 7.0; N, 5.5 (Dumas, variation 5.2-5.6). $C_8H_{17}O_7N$ requires C, 43.0; H, 6.8; N, 5.6%; $[\alpha]_D^{20} + 109 \pm 3^\circ$ (2%, w/w, in water).

The isomer of muramic acid

If the ortho ester was used, the material insoluble in tetrahydrofuran was hydrolysed with 6N-HCl and worked up as for muramic acid. The product contained only traces of muramic acid and started to issue from the Dowex 50 column after about 200 ml. After evaporation to dryness and neutralization with IR-4B (OH) it was isolated as a freeze-dried powder. If ethyl α -iodopropionate was used in the preparation, the first peak from the Dowex column, suitably cut to avoid contamination with muramic acid, was treated similarly. Yields by both methods were about equal to the yields of muramic acid; $[\alpha]_D^{20}$ (freeze-dried product) $+52^\circ$ (2%, w/w, in water).

The R_F of the isomer in phenol-water was slightly less than that of muramic acid, and the R_F for its ninhydrin-oxidation product in ethyl acetate-pyridine-water was slightly greater than that of the similar product from muramic acid. A comparison of the two isomers is shown in Table I.

SUMMARY

1. The isolation of crystalline muramic acid from spore peptide is described.

2. Analyses of the natural product suggests that it is a 3-O- α -carboxyethylhexosamine.

3. Synthesis of a 3-O- α -carboxyethyl-D-glucosamine is described. The product is identical with natural muramic acid. The α -carboxyethyl group probably has a D-configuration.

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REFERENCES

- Barger, G. (1904). *J. chem. Soc.* **85**, 286.
 Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
 Beyerstedt, F. & McElvain, S. M. (1937). *J. Amer. chem. Soc.* **59**, 1273.
 Crumpton, M. J. (1958). *Biochem. J.* **69**, 25P.
 Cummins, C. S. & Harris, H. (1954). *Biochem. J.* **57**, xxxii.
 Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
 Dische, Z. & Borenfreund, E. (1950). *J. biol. Chem.* **184**, 517.
 Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
 Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
 Gottschalk, A. (1955). *Nature, Lond.*, **176**, 881.
 Hagedorn, H. C. & Jensen, B. N. (1923). *Biochem. Z.* **135**, 46.
 Horrocks, R. H. (1949). *Nature, Lond.*, **164**, 444.
 Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
 Immers, J. & Vasseur, E. (1950). *Nature, Lond.*, **165**, 898.
 Jeanloz, R. W. & Forchielli, E. (1951). *J. biol. Chem.* **188**, 361.
 Kent, L. H. (1957). *Biochem. J.* **67**, 5P.
 Kuhn, R., Gauhe, A. & Baer, H. H. (1954). *Ber. dtsh. chem. Ges.* **87**, 289, 1138, 1553.
 Militzer, W. E. (1946). *Arch. Biochem.* **9**, 91.
 Morgan, W. T. J. & Elson, L. A. (1934). *Biochem. J.* **28**, 988.
 Neuberger, A. (1941). *J. chem. Soc.* p. 50.
 O'Dea, J. F. & Gibbons, R. A. (1953). *Biochem. J.* **55**, 580.
 Park, J. T. (1952). *J. biol. Chem.* **194**, 885.
 Park, J. T. & Strominger, J. L. (1957). *Science*, **25**, 99.
 Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
 Potter, A. L. & Hassid, W. Z. (1948). *J. Amer. chem. Soc.* **70**, 3488.
 Purdie, T. & Walker, J. W. (1895). *J. chem. Soc.* **67**, 616.
 Reid, R. L. & Lederer, M. (1952). *Biochem. J.* **50**, 60.
 Rondle, C. J. M. & Morgan, W. T. J. (1955). *Biochem. J.* **61**, 586.
 Somogyi, M. (1945). *J. biol. Chem.* **86**, 655.
 Stoffyn, P. J. & Jeanloz, R. W. (1954). *Arch. Biochem. Biophys.* **52**, 373.
 Strange, R. E. (1956). *Biochem. J.* **64**, 23P.
 Strange, R. E. & Dark, F. A. (1956). *Nature, Lond.*, **177**, 186.
 Strange, R. E. & Powell, J. F. (1954). *Biochem. J.* **58**, 80.
 Tracey, M. V. (1952). *Biochem. J.* **52**, 265.
 Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
 Walters, P. M. & McElvain, S. M. (1940). *J. Amer. chem. Soc.* **62**, 1482.
 Weidel, von W. & Primosingh, J. (1957). *Z. Naturf.* **12**, 441.
 Westphal, O. & Holzmann, H. (1942). *Ber. dtsh. chem. Ges.* **75**, 1274.