SUMMARY

1. In potassium-deficient barley plants 1,4-diaminobutane (putrescine), which had been previously characterized, occurs together with a Sakaguchi- and ninhydrin-positive compound, whose infrared spectrum and chromatographic properties are identical with those of 1-amino-4-guanidinobutane (agmatine).

2. Agmatine and putrescine occur in potassium-deficient red-clover plants.

3. Under conditions of adequate potassium nutrition, agmatine and putrescine were found in barley and clover, and also in cabbage leaves, but the amounts were small relative to those in the potassium-deficient plants.

4. The putrescine concentration in barley seedlings was considerably increased by feeding with agmatine, a much smaller increase being induced by giving ornithine or arginine. Feeding with arginine increased the concentration of a Sakaguchi-positive compound, which was chromatographically indistinguishable from agmatine.

The authors thank Dr C. H. Fawcett and Dr K. Rothwell for producing the infrared spectra.

REFERENCES

- Coleman, R. G. (1953). Ph.D. Thesis: University of London.
- Coleman, R. G. & Hegarty, M. P. (1957). *Nature, Lond.*, **179**, 376.
- Coleman, R. G. & Richards, F. J. (1956). *Ann. Bot., N.S.*, **20**, 393.
- Datta, S. P., Dent, C. E. & Harris, H. (1950). *Biochem. J.* **46**, xlii.
- Engelard, R. & Kutscher, F. (1910). *Zbl. Physiol.* **24**, 479.
- Gale, E. F. (1940). *Biochem. J.* **34**, 853.
- Heyl, F. W. (1919). *J. Amer. chem. Soc.* **41**, 670.
- Irvin, J. L. & Wilson, D. W. (1939). *J. biol. Chem.* **127**, 565.
- Jepson, J. B. & Smith, I. (1953). *Nature, Lond.*, **172**, 1100.
- Kiesel, A. (1922). *Hoppe-Seyl. Z.* **118**, 277.
- Lissitzky, S., Garcia, I. & Roche, J. (1954). *C.R. Soc. Biol., Paris*, **148**, 436.
- Møller, V. (1955). *Acta path. microbiol. scand.* **36**, 158.
- Mourgue, M., Baret, R. & Dokhan, R. (1953). *C.R. Soc. Biol., Paris*, **147**, 1449.
- Nakamura, S. (1944). *J. Biochem., Tokyo*, **36**, 243.
- Richards, F. J. & Berner, E. (1954). *Ann. Bot., N.S.*, **18**, 15.
- Richards, F. J. & Coleman, R. G. (1952). *Nature, Lond.*, **170**, 460.
- Rielländer, A. (1908). *S.B. Ges. ges. Naturw. Marburg*, p. 173.
- Rodwell, A. W. (1953). *J. gen. Microbiol.* **8**, 224.
- Sher, I. H. & Mallette, M. F. (1954). *Arch. Biochem. Biophys.* **53**, 370.
- Shibuya, S. & Makisumi, S. (1953). *J. Jap. biochem. Soc.* **25**, 210.
- Thoai, N., Roche, J. & Robin, Y. (1953). *Biochim. biophys. Acta*, **11**, 403.
- Wolfe, M. (1957). *Biochim. biophys. Acta*, **23**, 186.

Biochem. J. (1962) **84**, 294

Preparation and Properties of the Mucopeptides of Cell Walls of Gram-Negative Bacteria

By J. MANDELSTAM

National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 14 February 1962)

Salton (1953) and Cummins & Harris (1956) showed that the cell walls of Gram-positive bacteria consisted mainly of polymers which were later called mucopeptides by Mandelstam & Rogers (1959). These always contained glucosamine, muramic acid, glutamic acid, alanine, either lysine or α -diaminopimelic acid, and sometimes one or two additional amino acids. The mucopeptides of some bacteria such as *Micrococcus lysodeikticus* were completely digested by lysozyme, but others were attacked only slightly (Salton & Pavlik, 1960).

The walls of Gram-negative bacteria were more complex. They contained lipid (about 20%), polysaccharides and apparently all the amino acids

normally present in protein (Salton, 1953). However, the existence of mucopeptide in these walls was suggested by the fact that both muramic acid and diaminopimelic acid were present. These compounds are not known to occur in any type of polymer except mucopeptide. In addition, Gram-negative cells could be lysed by lysozyme (Repaske, 1958; Zinder & Arndt, 1958), and the materials liberated by lysozyme action contained the hexosamines and amino acids characteristic of mucopeptide (Salton, 1958; Primosigh, Pelzer, Maass & Weidel, 1961).

Later the mucopeptide of *Escherichia coli* was sufficiently separated from protein and polysac-

charide to allow its composition to be determined (Mandelstam, 1961). It contained glucosamine, muramic acid, diaminopimelic acid, lysine, glutamic acid, alanine and serine. In this paper the purification is described in detail, and it is shown that all Gram-negative bacteria examined seem to have the same mucopeptide as *E. coli*.

METHODS

Organisms

The following strains of *E. coli* were used: ML30, K12, M200, B and A.T.C.C. 9637. Mucopeptide was also prepared from other Gram-negative bacteria: *Proteus vulgaris* (N.C.T.C. 4176), *Klebsiella pneumoniae* (N.C.T.C. 5055), *Citrobacter freundii* (N.C.T.C. 9750), *Serratia marcescens* (N.C.T.C. 1377) and *Pseudomonas fluorescens* K.B. 1 (kindly supplied by Dr M. Kogut).

Preparation of mucopeptide

Bacteria were grown with shaking at 35° either in a synthetic medium containing glucose and inorganic salts (Mandelstam, 1958) or in broth (Wright, 1933). The latter medium increased the yield but did not affect the composition of the mucopeptide (see below). The cultures were harvested at 1.5–2.0 mg. dry wt. of bacteria/ml. The procedure followed, and the yield at each step of the purification, is described for a typical strain of *E. coli* (A.T.C.C. 9637). When the method was being developed, the product at each step was dried by washing with acetone and ether, weighed and samples were taken for analysis. In routine preparations the acetone and ether treatments were omitted after step 2. Unless otherwise stated, all procedures were done at room temperature, and the products were washed by centrifuging at 10 000g.

Step 1; breakage of cells and extraction of lipids. Bacteria (16 g. dry wt.) were suspended in water (320 ml.) and 12 ml. batches shaken in the cold room (2°) with Ballotini beads (8 ml., no. 12) in a Mickle disintegrator for 15 min. The beads were removed by filtration through sintered glass and the crude cell wall was sedimented by centrifuging the filtrate for 15 min. The pellet was suspended in water (200 ml.) and heated for 30 min. at 100° to destroy possible lytic activity in the preparation. After centrifuging again, the product was left overnight in 200 ml. of ethanol-ether (3:1, v/v) and then extracted twice more with ethanol-ether (2:1, v/v). The residue was extracted twice with acetone (100 ml.) and ether (100 ml.), and dried at 50°. The yield was 3.0 g.

Step 2; treatment with pepsin. Preliminary experiments had shown that treatment with proteolytic enzymes at this stage, as recommended by Cummins & Harris (1956) for the preparation of mucopeptide from Gram-positive organisms, was ineffective in removing protein. The product from step 1 was therefore first treated for the reduction and alkylation of disulphide bonds in the protein (see Haber & Anfinsen, 1961) by suspending it in 50 ml. of 0.5M-sodium phosphate buffer, pH 8.5, containing urea (30 g.) and 2-mercaptoethanol (1 ml.). After 2 hr. at 35°, 20 ml. of a solution containing iodoacetic acid (7.0 g.), brought to pH 8.5 with NaOH, was added, and the mixture was left for 20 min. The product was washed three times

with water (200 ml.), suspended in 0.05N-HCl (100 ml.) and incubated at 35° with pepsin (20 mg.) for 18 hr. The residue was washed with aq. 2N-NH₃ (100 ml.), which removed more protein, and then with water (100 ml.). The yield (after drying with acetone and ether) was 730 mg. The next three steps were carried out in glass centrifuge tubes.

Step 3; extraction with formic acid. The residue was repeatedly extracted with 20 ml. portions of formic acid (90%, v/v) and centrifuged for 30 min. at 3000g. The supernatant from each extraction was treated with ether (3 vol.). The white flocculent material (mainly protein) was usually negligible after the third or fourth extraction. The residue was washed twice with ether (30 ml.) to remove formic acid. The yield was 307 mg.

Step 4; extraction with phenol. The product was extracted twice with phenol (90%, w/w) at 20° for 30 min. After centrifuging at 3000g, the supernatant could be poured cleanly from the mucopeptide residue, which remained as a clear gel at the bottom of the tube. The phenol-soluble material could be precipitated with excess of ether. It contained protein and polysaccharide. When the mucopeptide gel was treated with ether, a white powder was again obtained. The yield (after drying with acetone and ether) was 255 mg.

Step 5; extraction with 'copper ethylenediamine'. The residue still contained appreciable amounts of protein and polysaccharide which could be partly removed by extracting three times with 10 ml. of 'copper ethylenediamine' [6% (w/v) Cu(OH)₂ and 8% (w/v) ethylenediamine]. The extractions were done at room temperature and not left for longer than 30 min. Higher temperatures or more prolonged extraction led to loss of some mucopeptide. This was probably due to the high pH of the solution. The mucopeptide formed a blue copper complex. This was dissociated by repeated washing with 20 ml. amounts of HCl (0.05N) until extracts were colourless. The yield (after drying) was 100 mg.

To determine whether the extraction procedure was likely to damage the mucopeptide, steps 2–5 were carried out on the mucopeptides of *M. lysodeikticus* and *Staphylococcus aureus* 524. Quantitative analysis of the hexosamines and amino acids (see below) showed that these mucopeptides were unaltered by the treatment.

Analytical methods

Amino acid composition. The amino acid composition of mucopeptide was determined with hydrolysed samples by quantitative paper chromatography, as described by Mandelstam & Rogers (1959). Chromatograms were developed for 2 days in butanol-acetic acid-water (63:10:27, by vol.). This gave good separation of diaminopimelic acid, lysine, serine, glutamic acid and alanine. Serine was distinguished from glycine by eluting the glycine-serine region of the chromatogram and running the extracted material on paper with water-saturated *m*-cresol.

To obtain an indication of the amount of protein contamination, a separate chromatogram was run for 18 hr. in the butanol-acetic acid-water solvent, and the leucine-isoleucine spot was measured.

Hexosamines. Samples were hydrolysed with HCl (4N) for 4 hr. at 100°, and glucosamine and muramic acid were determined by the method of Perkins & Rogers (1959).

RESULTS

The degree of purification was indicated by the progressive increase in the content of muramic acid and the concurrent fall in the amount of leucine-isoleucine. Other amino acids characteristic of protein (phenylalanine, valine, etc.) decreased in the same way as the leucine-isoleucine fraction, but were only occasionally measured.

The course of one preparation is shown in Table 1, and the analysis of the final product is

Table 1. *Purification of mucopeptide from Escherichia coli (A.T.C.C. 9637)*

Mucopeptide was prepared from bacteria (16 g. dry wt.), as described in the Methods section. The degree of purification is indicated by the increase in muramic acid content. The leucine-isoleucine content was used as an indicator of protein contamination.

Product	Wt. (mg.)	Muramic acid content (μ g./mg.)	Leucine- isoleucine content (μ g./mg.)
From step 1	3000	<10	90
From step 2	730	23	66
From step 3	307	48	18
From step 4	255	52	12
From step 5	100	128	6

Table 2. *Composition of mucopeptide from Escherichia coli (A.T.C.C. 9637)*

The mucopeptide was prepared and analysed as described in the Methods section. Molar ratios were calculated relative to glutamic acid. The values in parentheses refer to the *N*-acetylated hexosamine.

Constituent	Content (μ g./mg. of product)	Molar ratio
Glutamic acid	148	1.0
Diaminopimelic acid	134	0.71
Lysine	28	0.19
Serine	34	0.31
Alanine	132	1.50
Glucosamine	142 (175)	0.79
Muramic acid	128 (150)	0.51

shown in Table 2. If it is assumed that glucosamine and muramic acid were originally present in the mucopeptide in the *N*-acetyl form, the constituents listed in the Table account for 80 % of the preparation (less if account is taken of gain in weight during hydrolysis). The preparation also contained about 5 % of protein. This assumes that leucine-isoleucine represents about 10–12 % of the protein. There was also 1.2 % of phosphorus. In addition, chromatography of an acid hydrolysate, followed by spraying with ammoniacal silver nitrate, revealed the presence of a number of reducing sugars. Since the amount of these sugars varied considerably from one preparation to another, they were not considered to be an integral part of the mucopeptide, and neither their identity nor their amount has been investigated further.

Amounts of mucopeptide. The yield of mucopeptide varied from 0.2 to 1 %. This appeared to be due to differences in the original mucopeptide content of the cells, since muramic acid and diaminopimelic acid measurements showed that very little mucopeptide was lost during the preparation. Similar variability could occur in the same bacterial strain grown under different conditions. Thus *E. coli* ML30 grown in minimal medium contained only 0.2 % of mucopeptide. Growth of the cells in broth increased the amount of mucopeptide to about 1 %, but did not alter its composition.

Composition of mucopeptide. The molar ratios for mucopeptides of a number of Gram-negative organisms are shown in Table 3. Relative to glutamic acid, the ratios of diaminopimelic acid, alanine and muramic acid were fairly constant, and did not differ from the mean by more than about 10 %. However, lysine and glucosamine varied over a twofold and serine over a fourfold range (Table 3), and this variation could occur in different preparations from the same bacterial strain.

Action of lysozyme. The effect of lysozyme on the Gram-negative cell wall at various stages of treatment was followed by turbidity measurements in the Spekker absorptiometer (Filter H508). The

Table 3. *Molar ratios of constituents of mucopeptides from different Gram-negative organisms*

The mucopeptides were prepared and analysed as described in the Methods section. DAP, diaminopimelic acid; GM, glucosamine; MA, muramic acid.

	Glu	DAP	Lys	Ser	Ala	GM	MA
<i>E. coli</i> A.T.C.C. 9637	1.0	0.71	0.19	0.31	1.50	0.79	0.51
<i>E. coli</i> M200	1.0	0.67	0.21	0.16	1.47	0.65	0.47
<i>E. coli</i> B	1.0	0.74	0.23	0.13	1.47	0.70	0.45
<i>E. coli</i> K12	1.0	0.68	0.20	0.15	1.50	0.95	0.50
<i>E. coli</i> ML30	1.0	0.70	0.23	0.14	1.50	1.0	0.55
<i>P. vulgaris</i> N.C.T.C. 4175	1.0	0.73	0.20	0.20	1.60	0.75	0.51
<i>K. pneumoniae</i> N.C.T.C. 5055	1.0	0.76	0.15	0.40	1.46	0.70	0.52
<i>C. freundii</i> N.C.T.C. 9750	1.0	0.70	0.21	0.10	1.50	0.52	0.47
<i>Ser. marcescens</i> N.C.T.C. 1377	1.0	0.78	0.12	0.14	1.60	0.90	0.56
<i>Ps. fluorescens</i> K.B. 1	1.0	0.76	0.18	0.18	1.50	0.60	0.55

sample was suspended at about 1 $\mu\text{g.}/\text{ml.}$ in 0.05M-sodium phosphate buffer, pH 6.0, and then incubated with crystalline egg-white lysozyme (100 $\mu\text{g.}/\text{ml.}$) at room temperature. The reason for the high concentration of lysozyme was that during the mucopeptide purification some material was introduced which, under the above conditions, was sufficient to inactivate 20–50 $\mu\text{g.}$ of lysozyme/ml. The lysozyme thus affected was then without action on the mucopeptide of *M. lysodeikticus*, which is normally attacked rapidly at a concentration of 10 $\mu\text{g.}$ of lysozyme/ml.

The turbidity of the crude wall obtained after step 2 was not measurably affected by lysozyme. After step 3 it showed about a 30 % fall in turbidity. This was increased to about 60 % after step 4. The

mucopeptide after the final step was reduced in turbidity about as much as the mucopeptide of *M. lysodeikticus* (Fig. 1).

Repaske (1958) found it necessary to add ethylenediaminetetra-acetic acid to enable lysozyme to act on the walls of intact *E. coli*. With the purified mucopeptide this reagent was unnecessary, and its inclusion in the incubation mixture did not enhance the activity of the enzyme.

Action of papain. In an attempt to remove residual protein from the mucopeptide obtained after extraction with 'copper ethylenediamine', the preparations were treated as follows. Mucopeptide (30–50 mg.) was suspended in 10 ml. of 0.05M-sodium phosphate buffer, pH 7.0, containing 10 mg. of cysteine hydrochloride, 0.05 ml. of 0.2M-ethylenediaminetetra-acetic acid and 1.0 mg. of papain [twice recrystallized by the method of Kimmel & Smith (1954)]. Suspensions were incubated at 35° for 18 hr., during which there was no change in the turbidity of the suspensions. The mucopeptide, after being washed well with water and dried, usually weighed about 90 % of the starting material. It is possible that some of the loss of product occurred in decanting the supernatants during the washing.

With K12 mucopeptide, the leucine-isoleucine content of the residue showed that about half of the contaminating protein had been removed (see Table 4). Papain treatment removed a substantial part of the glucosamine, some of the lysine and virtually all of the serine, the concentration of which was decreased to that of the amino acids of the protein contamination. The relative amounts of glutamic acid, muramic acid, diaminopimelic acid and alanine remained the same.

The amounts and molar ratios for K12 mucopeptide are shown in Table 4. The product after papain treatment contained equal amounts of muramic acid and glucosamine (0.5 mole/mole of glutamic acid). The mucopeptides from *Klebsiella*, *Pseudomonas* and *Proteus* were all affected in the same way, i.e. the glucosamine was decreased to 0.5 mole/mole of glutamic acid, the lysine to about

Fig. 1. Action of lysozyme on mucopeptides of *Escherichia coli*. Mucopeptides from *E. coli* M200 (●) and *E. coli* B (○) at about 1 mg./ml. were incubated at room temperature (18°) with lysozyme (100 $\mu\text{g.}/\text{ml.}$). For comparison the digestion of mucopeptide of *M. lysodeikticus* with lysozyme (10 $\mu\text{g.}/\text{ml.}$) is also shown (Δ).

Table 4. *Effect of papain on the composition of mucopeptide*

Mucopeptide prepared from *E. coli* K12 was treated with papain in the presence of cysteine and ethylenediaminetetra-acetic acid for 18 hr. at 35°. The molar ratios of the constituents have been calculated relative to glutamic acid. Treatment with papain decreased the leucine/isoleucine content from 7 to 3 $\mu\text{g.}/\text{mg.}$

	Before papain treatment		After papain treatment	
	($\mu\text{g.}/\text{mg.}$)	(moles)	($\mu\text{g.}/\text{mg.}$)	(moles)
Glutamic acid	148	1.0	148	1.0
Diaminopimelic acid	134	0.70	142	0.75
Lysine	28	0.19	19	0.13
Serine	14	0.13	4	0.04
Alanine	132	1.50	140	1.55
Glucosamine	156	0.87	98	0.48
Muramic acid	128	0.51	150	0.53

0.1–0.15 mole, and the serine to a negligible amount. The molar ratios of the residues from all these strains of bacteria after papain treatment did not differ significantly from the values shown for K12 in Table 4 (last column).

Since the papain might have been contaminated with lysozyme from the papaya latex, the reaction was carried out in the presence of iodoacetamide (10 mM) or mercuric chloride (mM). These inactivate papain but not lysozyme (Kimmel & Smith, 1954). In the presence of these inhibitors papain had no effect. It therefore appears that the changes produced are not the result of lysozyme action but are due to the papain itself.

DISCUSSION

These experiments show that the mucopeptides of Gram-negative bacteria are in general similar to those of the Gram-positive bacteria. They contain the same two hexosamines and a limited range of amino acids, and they are attacked by lysozyme. There are, however, some noteworthy differences between the two types of mucopeptide. In none of the Gram-negative strains examined did the mucopeptide amount to more than about 1% of the bacterial mass, whereas in Gram-positive organisms the mucopeptide is usually 10–20% [see, for example, Mandelstam & Rogers (1959)]. Apart from being a minor constituent of the cell, the Gram-negative mucopeptide is also a small part of the wall. In *E. coli* ATCC 9637 the mucopeptide is only about 3% of the cell-wall fraction (Table 1), and in many of the strains it is even less. Nevertheless, the integrity of the mucopeptide seems to be necessary for maintaining the rigidity and strength of the wall, because removal of the mucopeptide by lysozyme produces osmotically fragile spheroplasts (Repaske, 1958), and penicillin, which inhibits synthesis of mucopeptide, also produces spheroplasts (Lederberg, 1956).

The values in Table 3 show that the relative amounts of glutamic acid, diaminopimelic acid, alanine and muramic acid are about the same in all the mucopeptide preparations. This is another difference between mucopeptides from Gram-negative and Gram-positive organisms. In the latter there is considerable variability from one strain to another. Even in closely related organisms such as different strains of lactobacilli (Ikawa & Snell, 1960) or of micrococci (see Rogers & Perkins, 1959), the amino acid ratios are so inconstant that it is difficult to find any two strains that do not differ appreciably from one another.

The variability in serine, lysine and glucosamine (see Table 3) is found occasionally even in different preparations from the same strain. This suggests that these components may, in part at least, belong

to another polymer. This interpretation is suggested by the finding that papain eliminated the differences and reduced all the ratios to those shown in Table 4 (last column), so that the residues after papain treatment appeared to be identical, although the bacteria from which they had been derived differed considerably. These observations should be considered in conjunction with those of Primosigh *et al.* (1961), who treated *E. coli* walls with lysozyme and found that the fragments liberated contained glucosamine, muramic acid, diaminopimelic acid, glutamic acid and alanine.

One can conclude that the serine and part of the lysine are probably not constituents of the Gram-negative mucopeptide. The rest of the lysine could be part of the mucopeptide; the alternative explanation, that it is part of another molecule which cannot be removed by the present fractionation procedures, is not ruled out.

The experiments with lysozyme show that the apparent resistance of the walls of Gram-negative bacteria to lysozyme is due to the other polymers which protect the mucopeptide. Once these have been removed, the mucopeptide is readily digested by the enzyme and the addition of ethylenediaminetetra-acetic acid, as recommended by Repaske (1958), is no longer necessary. Indeed, the mucopeptides from Gram-negative organisms are much more susceptible to the action of lysozyme than are many of the mucopeptides from Gram-positive organisms tested by Salton & Pavlik (1960).

SUMMARY

1. A method is described for the preparation of the mucopeptides of Gram-negative bacteria in an almost pure form.

2. The mucopeptides contain muramic acid, glutamic acid, α -diaminopimelic acid and alanine. The relative amounts of these constituents are the same in all the mucopeptides isolated. This is in contrast with the variability found in mucopeptides of Gram-positive organisms.

3. Glucosamine, lysine and serine, which are also present, are more variable. Papain acts on the mucopeptide, removing some of the glucosamine and lysine and almost all of the serine. The residues after papain treatment seem to be the same in all the mucopeptides from Gram-negative organisms treated.

4. The mucopeptides are readily digested by lysozyme; the addition of ethylenediamine-tetra-acetic acid is unnecessary.

'Copper ethylenediamine' was kindly provided by Dr S. G. Smith of the British Cotton Industry Research Association. I am much indebted to Miss R. Coyle for technical assistance.

REFERENCES

- Cummins, C. S. & Harris, H. (1956). *J. gen. Microbiol.* **14**, 583.
- Haber, E. & Anfinsen, C. B. (1961). *J. biol. Chem.* **236**, 422.
- Ikawa, M. & Snell, E. E. (1960). *J. biol. Chem.* **235**, 1376.
- Kimmel, J. R. & Smith, E. L. (1954). *J. biol. Chem.* **207**, 515.
- Lederberg, J. (1956). *Proc. nat. Acad. Sci., Wash.*, **42**, 574.
- Mandelstam, J. (1958). *Biochem. J.* **69**, 103.
- Mandelstam, J. (1961). *Nature, Lond.*, **189**, 855.
- Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
- Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647.
- Primovich, J., Pelzer, H., Maass, D. & Weidel, W. (1961). *Biochim. biophys. Acta*, **48**, 68.
- Repaske, R. (1958). *Biochim. biophys. Acta*, **30**, 225.
- Rogers, H. J. & Perkins, H. R. (1959). *Nature, Lond.*, **184**, 520.
- Salton, M. R. J. (1953). *Biochim. biophys. Acta*, **10**, 512.
- Salton, M. R. J. (1958). *J. gen. Microbiol.* **18**, 481.
- Salton, M. R. J. & Pavlik, J. G. (1960). *Biochim. biophys. Acta*, **39**, 398.
- Wright, H. D. (1933). *J. Path. Bact.* **37**, 257.
- Zinder, N. D. & Arndt, W. F. (1958). *Proc. nat. Acad. Sci., Wash.*, **42**, 586.

Biochem. J. (1962) **84**, 299

Inhibition of Cell-Wall-Mucopeptide Formation in *Escherichia coli* by Benzylpenicillin and 6-[p(-)- α -Aminophenylacetamido]penicillanic Acid (Ampicillin)

BY H. J. ROGERS AND J. MANDELSTAM
National Institute for Medical Research, Mill Hill, London, N.W. 7

(Received 14 February 1962)

Duguid (1946) suggested, on the basis of cytological observations, that penicillin might interfere with cell-wall synthesis. Lederberg (1956) noted that, when *Escherichia coli* was treated with penicillin, the bacteria were converted into osmotically fragile spheroplasts, and suggested that the drug acted by inhibiting the formation of cell wall. However, most of the evidence for the mode of action of penicillin depends on work done with staphylococci. Park (1952) showed that penicillin caused accumulation of uridine-linked peptides which are believed to be precursors of cell-wall mucopeptide (Park & Strominger, 1957). Inhibition of mucopeptide synthesis by penicillin in washed staphylococci has been shown by Park (1958), Mandelstam & Rogers (1959) and Rogers & Jeljaszewicz (1961).

However, work with Gram-negative bacteria has produced divergent views on penicillin action. Trucco & Pardee (1958) found no inhibition of [14 C]glucose incorporation into the wall of *E. coli*; Meadow (1960), also working with *E. coli*, reported that penicillin did not inhibit incorporation of α -diamino[14 C]pimelic acid into the cell wall; Hugo & Russell (1960*a, b*, 1961) showed that loss of viability in Gram-negative organisms treated with penicillin differed from the incidence of spheroplast formation, and concluded that the lethal effect of the drug was not a consequence of fragility. The original suggestion by Lederberg (1956) was, however, supported by Nathenson & Strominger

(1961), who showed that penicillin inhibited the incorporation of tritiated diaminopimelic acid by about 50–75%.

The present work, reported in a preliminary form by Rogers & Mandelstam (1961), was undertaken to obtain further evidence on the mode of action of penicillin in *E. coli*. Penicillin was found to inhibit the incorporation of carbon from [14 C]-glucose into bound diaminopimelic acid. So far as is known, bound diaminopimelic acid occurs only in the cell-wall mucopeptides, and these experiments therefore support the contention that penicillin has the same action in *E. coli* as in staphylococci.

METHODS

Organism. *E. coli* K12 was grown at 35°, with shaking, in a mineral-salts medium (Mandelstam, 1962*a*) with glucose (10 g./l.) as carbon source, and harvested when the culture had reached a density of about 0.5 mg. dry wt. of bacteria/ml.

Incorporation of carbon from [14 C]glucose. The bacteria were washed twice with mineral-salts medium (no glucose) and suspended at 1.0 mg. dry wt./ml. in 100 ml. of the same mineral-salts medium containing [14 C]glucose [10–20 μ C; final concn., 0.2% (w/v)]. Sucrose (20%, w/v) was present to prevent lysis of any spheroplasts that might be formed. The flasks (500 ml.) were shaken at 35° for a suitable period, usually 60 min., and incorporation was stopped by adding trichloroacetic acid to give a final concentration of 5% (w/v). A zero-time sample was obtained by adding trichloroacetic acid immediately after the cells