Biochem. J. (1968) **106**, 49
Printed in Great Britain

The Cell Wall of *Bacillus licheniformis* N.C.T.C. 6346

ISOLATION OF LOW-MOLECULAR-WEIGHT FRAGMENTS FROM THE SOLUBLE MUCOPEPTIDE

By R. C. HUGHES
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 29 *June* 1967)

1. Soluble mucopeptide was prepared by lysozyme treatment of acid-extracted walls of *Bacillus licheniformis* N.C.T.C. 6346 and separated into fractions differing in molecular size by chromatography on Sephadex G-25 and G-50. 2. About 16% of the weight of soluble mucopeptide has a weight-average molecular weight in excess of 20000. About one half has a weight-average molecular weight of less than 2000 and the balance of soluble mucopeptide is of intermediate size. 3. In the mucopeptide fractions isolated from Sephadex there is a correlation between the weight-average molecular weight, the number of non-reducing muramic acid residues and the proportion of diaminopimelic acid residues recovered after treatment with 1-fluoro-2,4-dinitrobenzene. 4. The extent of cross-linking between peptide side chains is relatively low, even in mucopeptide material of the large molecular size. 5. The small amount of residual phosphorus present in preparations of *B. licheniformis* soluble mucopeptide remains associated mainly with mucopeptide material of large molecular size. 6. The mucopeptide components of lowest molecular weight are not produced as artifacts during the preparation of soluble mucopeptide, but are apparently incorporated in the insoluble mucopeptide present in walls of exponentially growing cells. 7. Soluble mucopeptide isolated in a complex with acidic polymers after lysozyme treatment of walls of *B. licheniformis* N.C.T.C. 6346 and *Bacillus subtilis* W 23 retains a high molecular weight when the covalent bonds between mucopeptide and the acidic polymers are broken. 8. Pure fragments were isolated from *B. licheniformis* soluble mucopeptide. A major component, C1, of the material of smallest size is made up of one residue each of *N*-acetylglucosamine, *N*-acetylmuramic acid, L-alanine, glutamic acid and diaminopimelic acid. The *N*-acetylglucosamine is in β-glycosidic linkage with a reducing *N*-acetylmuramic acid residue. The peptide unit is probably amidated. A quantitatively minor component, C2, has amino acid and amino sugar composition identical with that of component C1, but probably lacks an amide group. Another fragment, B1, is made up of two molecules of component C1 or C2 that are joined together through a molecule of D-alanine.

The isolation of pure wall fractions from disrupted cells of bacteria (Dawson, 1949; Mitchell & Moyle, 1951; Salton & Horne, 1951; McCarty, 1952) led quickly to the identification of the substrate of the bacteriolytic enzyme, lysozyme. The substrate was the mucopeptide component (Perkins & Rogers, 1959) that was found to be present in the walls of all bacteria subsequently examined (Salton, 1964; Rogers, 1966; Martin, 1966). Lysozyme hydrolyses glycosidic linkages in the polysaccharide backbones of chitin (Berger & Weiser, 1957; Hamaguchi & Fumatsu, 1959) and mucopeptide (Salton, 1956, 1957; Salton & Ghuysen, 1960; Ghuysen & Salton, 1960) with the release of soluble fragments. Extensive studies were made on the structures of the soluble fragments originating from mucopeptides of both Gram-positive and Gram-negative bacteria. In the Gram-negative organism *Escherichia coli*, soluble fragments were isolated of relatively low molecular weight (Primosigh, Pelzer, Maass & Weidel, 1961; Weidel & Pelzer, 1964). Under certain conditions the mucopeptide was degraded almost completely into two classes of compounds of different molecular weights (Primosigh *et al.* 1961; Pelzer, Maass & Weidel, 1963; Weidel & Pelzer, 1964). One class had twice the molecular weight of the other. One of the fragments of smaller molecular weight, fragment C6 (Fig. 1), contained

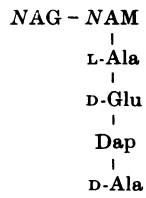

Fig. 1. Structure of a fragment, C6, isolated from *E. coli* mucopeptide after treatment with lysozyme (Weidel & Pelzer, 1964). *NAG*, *N*-Acetylglucosamine; *NAM*, *N*-acetylmuramic acid.

two amino sugars and three amino acids in molar ratios that were identical with those found for the whole mucopeptide (Primosigh *et al.* 1961; Pelzer, 1962). Therefore it was considered to be a basic unit of mucopeptide structure. A fragment, C3, of the higher molecular weight contained two molecules of fragment C6 joined by a cross-link between the peptide side chains (Pelzer, 1963). It has been suggested that similar structures are present in the mucopeptide of *Aerobacter cloacae* (Schocher, Jusic & Watson, 1961; Jusic, Roy & Watson, 1964) and of several other Gram-negative bacteria (Weidel & Pelzer, 1964; Martin, 1966). In contrast, the mucopeptide components of many Gram-positive organisms were degraded by lysozyme and an enzyme of similar specificity isolated from *Streptomyces* into soluble fragments that were mainly of very high molecular weight (Salton, 1956, 1957; Mandelstam & Strominger, 1961; Rogers, 1966). Quantitatively minor fragments of low molecular weight were isolated after treatment with lysozyme of wall preparations containing polymers in addition to mucopeptide (Salton, 1956; Salton & Ghuysen, 1959; Ghuysen & Salton, 1960; Perkins, 1960; Ghuysen, 1960, 1961; Sharon, Osawa, Flowers & Jeanloz, 1966; Mirelman & Sharon, 1966). Some of these had structures similar to the fragments isolated from *E. coli*, showing that the mucopeptides of Gram-negative and Gram-positive organisms were built up from similar structural units. The limited number of *N*- and *C*-terminal groups in mucopeptides isolated from some Gram-positive bacteria (Salton, 1961) shows that the relatively simple structural units may be linked together through their peptide side chains, forming large two-dimensional networks of polysaccharide and polypeptide threads (Rogers, 1962, 1966).

The mucopeptide component of the cell wall of *Bacillus licheniformis* N.C.T.C. 6346 was isolated in pure form by treatment of acid-extracted walls with lysozyme (Hughes, 1968). At least 75% of the muramic acid residues of the soluble mucopeptide carried a free reducing group. Essentially no α-amino groups of the soluble mucopeptide reacted with FDNB,* but a third of the diaminopimelic acid residues were free to do so. This value indicates that the degree of cross-linking between the peptide side chains is not great in *B. licheniformis* mucopeptide, as in some others, unless the side chains are assembled in a way that is completely different from other bacterial mucopeptides. The evidence available from amino acid-sequence analyses of *B. licheniformis* soluble mucopeptide (Hughes, 1968) made this possibility seem unlikely. In the work described here I have attempted to define the extent of heterogeneity in size of the soluble mucopeptide from *B. licheniformis* N.C.T.C. 6346. About half the weight of soluble mucopeptide had a molecular weight less than 2000, about 16% had a very high molecular weight, in excess of 20000, and the balance of soluble mucopeptide was of intermediate size.

In the second part of the work the structures of the fragments of low molecular weight were examined. A preliminary account of part of this work has been published (Hughes, 1967).

MATERIALS AND METHODS

Analytical methods. Total hexosamine and phosphorus were estimated as described by Hughes (1965). Muramic acid was separated from glucosamine by adsorption on charcoal by a modification (Hughes, 1968) of the method described by Perkins & Rogers (1959). Amino acids were determined with a ninhydrin reagent (Mandelstam & Rogers, 1959) after paper chromatography in solvent *B* or *C* (below). In certain cases, after acid hydrolysis, amino acids and amino sugars were estimated with the autoanalyser. Optical configurations of the amino acids were identified with specific enzymes (Hughes, 1968).

Paper chromatography. Descending chromatography on washed Whatman no. 1 and no. 3 paper was carried out in the following solvent systems: *A*, isobutyric acid–0·05 N-NH_3 (5:3, v/v); *B*, butan-1-ol–pyridine–water (6:4:3, by vol.); *C*, butan-1-ol–acetic acid–water (4:1:5, by vol.); *D*, propan-1-ol–0·2% NH_3 (17:3, v/v). Thin-layer chromatography on silica gel employed chloroform–methanol–acetic acid (85:14:1, by vol.) (Ghuysen, Tipper, Birge & Strominger, 1965).

Paper electrophoresis. This was carried out in the following buffer systems: N-acetic acid; 0·1 M-sodium acetate, pH 3·6; 0·1 M-sodium phosphate, pH 6·5. The applied voltage was 15 v/cm. and electrophoresis was for 4 hr.

Reaction of fragments with FDNB. Mucopeptide material (1 mg.) in dilute $NaHCO_3$ solution (0·75 ml.) was treated with 2·5% (w/v) FDNB in ethanol (1·50 ml.). After 16 hr. at 35° excess of reagent was extracted with ether and the aqueous layer was freeze-dried. Dinitrophenylated mucopeptide fragments were hydrolysed in 6 N-HCl at 105° for 16 hr. DNP-amino acids were separated by paper chromatography in solvent *B*, *C* or *D* and by thin-layer chromatography. The spots were eluted with aq. 1% $NaHCO_3$ and

* Abbreviations: FDNB, 1-fluoro-2,4-dinitrobenzene; Dap (in amino acid sequences), αε-diaminopimelic acid residue.

the extinction was read at 360mμ. Mono-DNP-diaminopimelic acid moved to a position just ahead of alanine in solvents *B* and *C* and was well separated from the amino acids, dinitrophenol and ether-soluble DNP-amino acids; ϵ_{360} for mono-DNP-diaminopimelic acid was taken as 16400 (Takebe, 1965).

Reduction of fragments with sodium borohydride. A mixture of soluble mucopeptide material (0·2ml., 5mg./ml.) and aq. 1% $NaBH_4$ (0·3ml.) was kept overnight at 35°. The excess of reagent was destroyed by the addition of 12N-HCl (0·25ml.). Hexosamines were separated and analysed in the acidified mixtures after they had been heated at 100° for 4hr.

Treatment of fragments with crude glycosidases. A crude enzyme mixture from snail juice (Industrie Biologique Française, S.A., Gennevilliers, Seine, France) was dialysed against water overnight in the cold. Mucopeptide material (0·2ml., 5mg./ml.) was treated with the dialysed enzyme (0·2ml.) at 35° overnight. A small portion of the mixture was examined by paper chromatography in solvents *B* and *C*. The remaining material was treated with 3% (w/v) $NaBH_4$ (0·1ml.) at 35° for 5hr. Glucosamine and muramic acid were determined after acid hydrolysis. Controls containing only mucopeptide or enzyme were subjected to the whole procedure.

Determinations of molecular weight. These were done by Dr P. Charlwood, with a Spinco model E ultracentrifuge near 20° and the short-column equilibrium method of Yphantis (1960). Most experiments were done in 12mm. double-sector or multi-channel cells with solution columns 0·8 or 1·0mm. high, but samples with molecular weights below 1000 were studied in 30mm. cells and 1·5mm. columns. The speeds used are given in Table 4.

Since the smaller mucopeptide fragments could not be dialysed to equilibrium with solvent, all the samples were prepared by mixing 1vol. of an aqueous solution of mucopeptide (free of salts and other small molecules) with 0·25vol. of stock buffer, which consisted of 0·5M-NaCl in sodium phosphate buffer, pH7·0 and *I* 0·1. The comparison solvent was prepared by mixing 1vol. of water with 0·25vol. of stock buffer. Mucopeptide concentrations were determined either in a differential refractometer or by layering solvent on solution in a double-synthetic-boundary cell and measuring the Rayleigh fringes. The concentration figures given in Table 3 assume a specific refractive increment of 0·0016, but the molecular weights are independent of the accuracy of this assumption.

In all calculations a partial specific volume of 0·69 was used (Primosigh *et al.* 1961).

Cell walls and preparation of soluble mucopeptide. B. licheniformis N.C.T.C. 6346 was grown in the usual medium (Hughes, 1965). *B. subtilis* W23 was grown in a medium containing casein hydrolysate (0·5%), yeast extract (0·5%), $MgSO_4$ (1mM), β-glycerophosphate (60mM) and glucose (1%). This medium was supplemented with a salts solution (0·02ml./l.) containing $CuSO_4$ (0·5%), $ZnSO_4$ (6·5%), $FeSO_4$ (0·5%), $MnCl_2$ (0·2%) and HCl (10%). Cells were harvested at the end of the exponential phase and walls were isolated by a method that included a heating step to inactivate autolytic enzymes, as described by Hughes (1968). The soluble fraction from lysozyme treatment of acid-extracted walls contained all of the mucopeptide component of the starting walls (Hughes, 1968). Soluble mucopeptide was also prepared in pure form without prior extraction of the walls with trichloroacetic acid. Walls (846mg.) of *B. licheniformis* were treated directly with lysozyme and the soluble fraction was separated by chromatography on a column (2cm.×120cm.) of DEAE-cellulose (Hughes, 1965). A part of the mucopeptide fraction, L1 (170mg.), was eluted at low salt concentration and was clearly separated from a mucopeptide fraction that was eluted together with teichuronic acid and teichoic acid (Hughes, 1965).

Preparation of soluble mucopeptide from growing cultures of B. licheniformis. Cultures (3l.) were grown to the end of the exponential phase in the usual medium (Hughes, 1965). Trichloroacetic acid (50%, w/v) (100ml./l. of culture) was added and the mixture was shaken at 35° overnight. The insoluble residue obtained after centrifugation was again extracted at 35° overnight with 5% (w/v) trichloroacetic acid (100ml.), washed with water by centrifugation and suspended in 10mM-ammonium acetate (25ml.). Lysozyme (5mg.) and a few drops of toluene, to prevent bacterial growth, were added. After 16hr. at 35° the insoluble fraction was removed by centrifugation. The soluble fraction containing mucopeptide (253mg.) was dried from the frozen state. The large insoluble residue (2·06g.) contained no detectable muramic acid after acid hydrolysis and hence was devoid of mucopeptide.

Sephadex G-25 chromatography of soluble mucopeptide. Sephadex G-25 (medium grade) (Pharmacia Fine Chemicals) was suspended in 0·1M-pyridine–acetic acid buffer, pH5·1, and fines were decanted. The gel was poured into a column (2·1cm.×139cm.) and washed extensively with buffer. Soluble mucopeptide dissolved in water (4·0ml.) was applied to the top of the column and was eluted with buffer at a flow rate of 40ml./hr. The column was operated at room temperature and fractions (2·5ml.) were collected. Suitable portions (0·1ml. or 0·2ml.) were analysed for total hexosamine after acid hydrolysis. Up to 150mg. of soluble mucopeptide was successfully fractionated on the column. Appropriate fractions were pooled and volatile buffer salts were removed by drying from the frozen state.

Sephadex G-50 chromatography of soluble mucopeptide. The column (2·0×130cm.), containing Sephadex G-50 (medium grade) (Pharmacia Fine Chemicals), was prepared and run as described for chromatography on Sephadex G-25.

RESULTS

Fractionation of soluble mucopeptide isolated from acid-extracted cell walls of B. licheniformis

A typical fractionation of soluble mucopeptide on Sephadex G-25 is shown in Fig. 2. Three fractions, A, B and C, containing hexosamines, were consistently obtained in the same relative proportions, if soluble mucopeptide isolated from acid-extracted walls was used as starting material (Table 1). The three fractions from Expt. 1 (Table 1) were passed separately through the column of Sephadex G-25 (Fig. 3). The greatest part of each fraction was eluted from the column in a position that was closely similar to that found previously. The overall yield of mucopeptide material after rechromatography was at least 80%. Analyses of typical preparations of fractions A, B and C are shown in Table 2. Table 2 includes for comparison

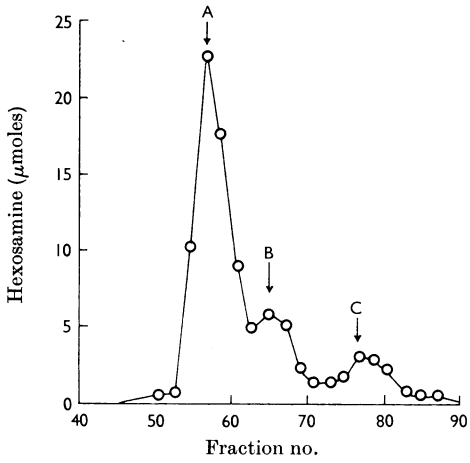

Fig. 2. Fractionation of *B. licheniformis* soluble mucopeptide on Sephadex G-25. Soluble mucopeptide (115 mg.), dissolved in water (4·0 ml.), was applied to the column (2·1 cm. × 139 cm.) and eluted with 0·1 M-pyridine–acetic acid buffer, pH 5·1. Fractions (2·5 ml.) were collected and portions (0·1 ml.) were analysed after acid hydrolysis for total hexosamine.

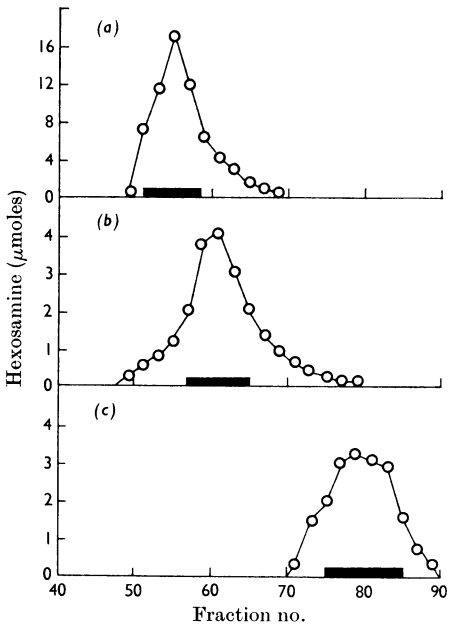

Fig. 3. Rechromatography on Sephadex G-25 of fractions A, B and C obtained from soluble mucopeptide (Fig. 2). (*a*) Fraction A (66 mg.), (*b*) fraction B (31 mg.) and (*c*) fraction C (21 mg.), dissolved in water (2·5 ml.), were passed in turn through the column and portions of the fractions tested for total hexosamine, as described in Fig. 2. The fractions that were made by pooling tubes are indicated by blocks along the abscissa.

an analysis of unfractionated soluble mucopeptide of *B. licheniformis* (Hughes, 1968). The soluble mucopeptide fragments present in fractions A and B were of a closely similar composition to that of unfractionated soluble mucopeptide. However, fraction C contained almost exactly one molar proportion of alanine relative to the other amino acids instead of the more than 1·5 molar proportions present in fractions A and B and in soluble mucopeptide. Reaction with D-amino acid oxidase showed that fractions A, B and C (Table 2) contained respectively 70, 39 and 18 μmoles of D-alanine/100 mg. of material. An approximately equimolar amount of L-alanine relative to glutamic acid and diaminopimelic acid was found to be present in all three fractions. Unfractionated soluble mucopeptide also contained equimolar amounts of L-alanine, glutamic acid and diaminopimelic acid (Hughes, 1968). A sample (81·5 mg.) of fraction A (Table 1, Expt. 2) was applied to a column of Sephadex G-50. Material containing hexosamines was eluted in a broad asymmetrical peak (Fig. 4). The peak was divided into four fractions, A1–4. Separate experiments showed that fractions B and C (Fig. 2) were eluted from the column of Sephadex G-50 as single peaks at tubes 81–91 and tubes 96–105 respectively. Fractions A1, A2, A3 and A4 (Fig. 4) contained 28%, 33%, 16% and 23% respectively of the material applied to the column. Since fraction A represented 57% of the weight of unfractionated soluble mucopeptide (Table 1), it is calculated that respectively 16%, 19%, 9% and 13% of the weight of unfractionated soluble mucopeptide was recovered in fractions A1, A2, A3 and

Table 1. *Fractionation of soluble mucopeptide from* B. licheniformis *cell walls*

Soluble mucopeptide was prepared from acid-extracted walls by lysozyme treatment and separated into fractions A, B and C by chromatography on Sephadex G-25 (Fig. 2). The results obtained in two separate experiments starting from different preparations of walls are shown. In Expt. 2 the material eluted first from Sephadex G-25, fraction A, was separated further on Sephadex G-50 to give fractions A1–4 (Fig. 4).

Expt. no.	Fraction	Proportion of total soluble mucopeptide (%, w/w)
1	A	56
	B	26
	C	18
2	A	57
	A1	16
	A2	19
	A3	9
	A4	13
	B	25
	C	18

Table 2. *Analysis of fractions obtained by chromatography of* B. licheniformis *soluble mucopeptide on Sephadex G-25*

Fractions A, B and C were prepared as described in Table 1 and purified further by rechromatography on Sephadex G-25, as shown in Fig. 3. After acid hydrolysis the amino acid compositions of the fractions were determined by quantitative paper chromatography. Amino sugars were separated on charcoal and estimated as described in the Materials and Methods section. The data relating to soluble mucopeptide were obtained by the same techniques and are taken from Hughes (1967*b*).

	Composition (μmoles/100mg. of dry material)				Molar ratios			
		Fractions				Fractions		
Substance	Soluble mucopeptide	A	B	C	Soluble mucopeptide	A	B	C
Diaminopimelic acid	114	117	100	93	0·91	1·00	1·03	0·98
Glutamic acid	125	116	97	95	1·00	1·00	1·00	1·00
Alanine	206	181	163	104	1·65	1·56	1·68	1·09
Glucosamine	94	84	74	84	0·76	0·73	0·77	0·88
Muramic acid	83	93	94	101	0·67	0·80	0·97	1·06

A4 (Table 1). The chemical compositions of fractions A1–4 did not differ significantly from that of fraction A (Table 2).

Molecular weights of the fractions isolated from Sephadex. The molecular weights of fractions A, B and C are presented in Table 3. The results shown were obtained in two separate experiments starting from different batches of cell walls. The values obtained for the weight-average molecular weights of fraction C were closely reproducible. Fraction B had approximately twice the weight-average molecular weight of fraction C. The weight-average molecular weight of fraction A varied in the two experiments from 6650 to 10360 (Table 1). This difference is understandable, since fraction A includes the material that is totally excluded from the column. Any difference in the pattern of hydrolysis of glycosidic linkages in the mucopeptide during the treatment with lysozyme would be reflected in the weight-average molecular weight of fraction A. It is of interest, however, that the yields of fractions B and C in the two experiments shown in Table 1 were identical. This strongly suggested that certain regions of insoluble mucopeptide were attacked with great ease by lysozyme, producing reproducible amounts of fragments of low molecular weight. Fractions A1, A2, A3 and A4 (Table 1, Expt. 2) were estimated to have weight-average molecular weights, measured at 25980rev./min., of 20510, 8860, 5320 and 3780 respectively. The weighted-mean value was calculated from these figures and the relative contributions of A1–4 to the weight of fraction A. The value of 10500 obtained was in very good agreement with the experimental value of 10360 measured at 20410 rev./min. (Table 3).

Free N*-terminal and reducing groups present in the Sephadex fractions.* It was thus known that the

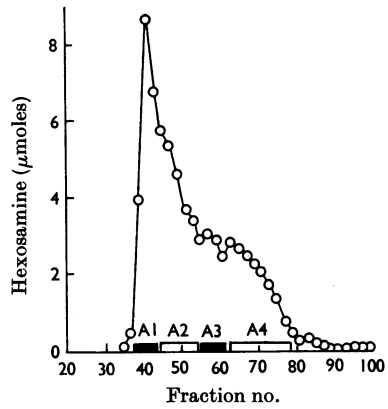

Fig. 4. Chromatography of fraction A (Fig. 2) on Sephadex G-50. Fraction A (81·5mg.) in water (2·0ml.) was passed through the column and portions of the fractions were tested for total hexosamine, as described in Fig. 2. The fractions that were made by pooling tubes are indicated by blocks along the abscissa.

soluble mucopeptide could be separated into fractions of different molecular size. In *B. licheniformis* mucopeptide (Hughes, 1968), as in other bacterial mucopeptides (Pelzer, 1962; Weidel & Pelzer, 1964; Martin, 1966), diaminopimelic acid is involved in forming cross-linkages between peptide side chains. Further, lysozyme treatment of acid-extracted walls of *B. licheniformis* did not liberate all of the reducing groups of muramic acid residues present in the polysaccharide chains of mucopeptide. Even after prolonged enzymic treatment, about 20% of the total muramic acid residues remained in glycosidic linkage (Hughes, 1968). It was decided to determine the number of free

Table 3. *Molecular weights of fractions of soluble mucopeptide isolated from Sephadex*

Preparation of the fractions is described in Table 1. In Expt. 1 the fractions were rechromatographed on Sephadex G-25. Weight-average molecular weights were determined as described in the Materials and Methods section.

Expt. no.	Fraction	Concn. (mg./ml.)	Speed (rev./min.)	Mol.wt.
1	A	3·7	26000	7170
		3·7	39500	6650
	B	3·1	42000	1720
	C	3·0	42000	936
2	A	3·2	20410	10360
	A1	3·1	25980	20510
	A2	3·2	25980	8860
		3·2	39460	8300
	A3	2·6	25980	5320
		2·6	39460	5120
	A4	2·6	25980	3780
		2·6	39460	3660
	B	3·7	42040	2153
	C	8·1	42040	895

Table 4. *Number of free amino groups of diaminopimelic acid residues and free reducing groups of muramic acid residues in Sephadex fractions of soluble mucopeptide*

Fractions were prepared as described in Table 1. After treatment with FDNB the samples were hydrolysed and analysed for amino acids and DNP-amino acids as described in the Materials and Methods section. Reduction with $NaBH_4$ and analysis of the reduced derivatives were carried out as described in the Materials and Methods section. N.D., Not determined.

Expt. no.	Fraction	Diaminopimelic acid recovered as the free amino acid after FDNB treatment (% of total)	Muramic acid recovered after $NaBH_4$ treatment (% of total)
1	A	52	28
	B	38	20
	C	5	4
2	A	N.D.	N.D.
	A1	60	48
	A2	40	48
	A3	31	36
	A4	30	37
	B	33	17
	C	6	0

amino groups of diaminopimelic acid residues and free reducing groups of muramic acid residues in the various fractions isolated from Sephadex. A clear correlation was found between the number of these residues in any particular fraction and the molecular weight of the fraction (Table 4). Fraction C was the fraction of smallest molecular size (Table 3). Essentially all of the diaminopimelic acid residues were reactive with FDNB, and an equivalent amount of mono-DNP-diaminopimelic acid was obtained. A proportion of the diaminopimelic acid residues present in fractions A and B did not react with the reagent; fraction A contained more of these residues than B. Similarly the number of these residues was greatest (60%) in the fraction, A1, of highest molecular weight. The total number of diaminopimelic acid residues unreactive with FDNB detected in fractions A, B and C was rather lower than expected from analysis of unfractionated soluble mucopeptide. About 60% of the diaminopimelic acid of unfractionated soluble mucopeptide was engaged in cross-linkage, but only about 43% of the total number of diaminopimelic acid residues present in fractions A, B and C were linked. A small amount of highly cross-linked mucopeptide may have been lost during chromatography. Since the yield of material from the column of Sephadex G-25 was high, this substance could not have contributed greatly to the weight of mucopeptide.

Reduction of fraction C eliminated essentially all of the muramic acid residues (Table 4). Fractions A and B and fractions A1–4 contained muramic acid residues that were unaffected by treatment with sodium borohydride. Fraction A was treated for several days with lysozyme, as described in the Materials and Methods section, in an attempt to expose more of the reducing groups of the muramic acid residues. However, the treated material was eluted from Sephadex G-25 in the position found previously and contained an unchanged proportion of muramic acid residues that were resistant to reduction. The glucosamine contents of fractions A, B and C were unchanged after reduction.

Phosphorus content of the Sephadex fractions. Soluble mucopeptide isolated from acid-extracted cell walls of *B. licheniformis* contained a small amount of phosphorus (Hughes, 1968). After fractionation of soluble mucopeptide containing 12 μmoles of phosphate/100mg. on Sephadex G-25, 85% of the phosphate was recovered in fraction A (Fig. 2). The remainder was present in fraction B, and fraction C contained no detectable phosphate.

Fractionation of soluble mucopeptide isolated from the lysozyme-soluble fraction of B. licheniformis *cell walls*

It was thought that the extraction of walls with 5% (w/v) trichloroacetic acid employed during the preparation of soluble mucopeptide might lead to a breakdown of glycosidic or peptide linkages with the production of fragments of low molecular weight. Therefore a sample of soluble mucopeptide was prepared by a procedure that did not involve drastic methods. Previous work (Hughes, 1965) has shown that treatment of *B. licheniformis* cell walls with lysozyme released into a soluble fraction all the mucopeptide, teichoic acid and teichuronic acid of the walls. The soluble material was resolved by chromatography on DEAE-cellulose into two main fractions. In the first of these, fraction L1, 55% of the mucopeptide component of walls was eluted in pure form. The fraction contained no phosphorus (Hughes, 1965). A second fraction was eluted later from the column and contained the rest of the mucopeptide together with teichoic acid and teichuronic acid. A sample of fraction L1 (154mg.) was separated on Sephadex G-25 (Fig. 5). Three fractions, A, B and C, were obtained in a way exactly analogous to that found previously with soluble mucopeptide prepared after acid treatment of walls. However, the amount of fraction A relative to fraction C was much less than found earlier (Fig. 2). The amounts of material in fractions A, B and C (Fig. 5) were 38%, 32% and 30% of the total recovered from the column. Therefore it is calculated that the yields of fractions A, B and C (Fig. 5) were respectively 21%, 18% and 17% of the total weight of the wall mucopeptide.

The molecular weights of fractions A, B and C obtained from fraction L1 (Fig. 5) were determined at 20000, 42000 and 42000rev./min. respectively. Values of 6620, 1978 and 981 respectively were obtained. The compositions of the fractions (Table 5) were similar to those found previously (Table 2). Further analysis showed that 63% and 42% of the diaminopimelic acid residues of fractions A and B respectively were recovered after treatment with FDNB. Respectively 24% and 16% of the muramic acid residues of A and B were stable to reduction with sodium borohydride. All the diaminopimelic acid residues and muramic acid residues of fraction C (Table 5) carried either free amino groups or free reducing groups that were reactive with FDNB or sodium borohydride respectively. By these criteria the two samples of fraction C, isolated by both of the methods so far described, appeared to be identical. The yield of fraction C isolated from fraction L1 was very similar to that obtained from walls after acid treatment, and the yield of fraction B was in fact less than before. It was therefore concluded that no

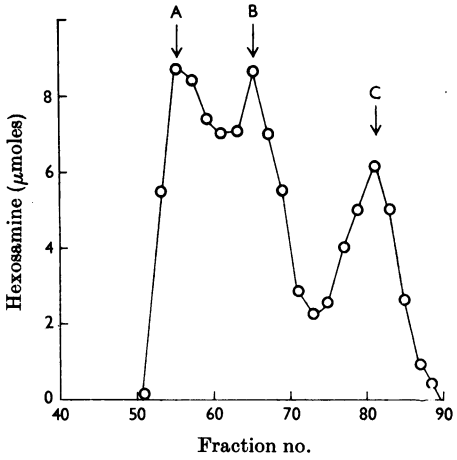

Fig. 5. Chromatography of fraction L1 on Sephadex G-25. Walls of *B. licheniformis* were treated with lysozyme and the soluble fraction was applied to a column of DEAE-cellulose. The pure mucopeptide fraction, eluted at low salt concentration and representing 55% of the total wall mucopeptide, was separated further by chromatography on Sephadex G-25. This Figure shows the elution pattern from Sephadex G-25; portions of the fractions were tested for total hexosamine as described in Fig. 2.

Table 5. *Analysis of fractions obtained by Sephadex G-25 chromatography of the soluble mucopeptide, L1*

B. licheniformis walls were treated with lysozyme and the soluble fraction was separated by chromatography on DEAE-cellulose. The pure mucopeptide fraction, L1, was eluted from the column at low salt concentration and separated on a column of Sephadex G-25 to give fractions A, B and C (Fig. 5). Analytical methods are given in the Materials and Methods section.

Substance	Molar ratios of fractions		
	A	B	C
Diaminopimelic acid	0·85	1·00	1·00
Glutamic acid	1·00	1·00	1·00
Alanine	1·40	1·39	1·01
Glucosamine	0·90	0·91	0·93
Muramic acid	0·81	0·80	0·70

drastic change had occurred in the mucopeptide structure during extraction of the walls with trichloroacetic acid.

Fractionation of soluble mucopeptide isolated from B. subtilis *cell walls*

Since fraction L1 accounts for only 55% of the total mucopeptide of *B. licheniformis* walls, it seemed that the other mucopeptide fragments, isolated in covalent linkage with the acidic cell-wall polymers after lysozyme treatment of intact walls (Hughes, 1965), would be mainly of high molecular weight and similar to fraction A (Fig. 2). It was technically difficult to demonstrate this point with walls of *B. licheniformis*. This is because these walls contain teichuronic acid, a polymer of high molecular weight containing galactosamine (Janczura, Perkins & Rogers, 1961; Hughes, 1966). The cell wall of *B. subtilis* W 23 contains very little galactosamine (Young, 1965). Soluble mucopeptide was isolated from acid-extracted walls of this organism and was found to be very similar to *B. licheniformis* soluble mucopeptide in amino acid and amino sugar composition. *B. subtilis* W 23 cell walls were previously shown to contain also a glucosylated ribitol teichoic acid (Chin, Burger & Glaser, 1966). The mucopeptide and teichoic acid components account for greater than 80% of the weight of the walls.

When isolated cell walls (32mg.) of *B. subtilis* W 23 were treated with lysozyme, the mucopeptide and teichoic acid components were made completely soluble. The soluble fraction was separated by chromatography on DEAE-cellulose as described previously for the lysozyme-soluble fraction of intact walls of *B. licheniformis* (Hughes, 1965). A pure mucopeptide component, fraction L1, that contained 46% of the total mucopeptide of the walls, and a mucopeptide–teichoic acid component were obtained. The mucopeptide–teichoic acid complex was heated in 0·5N-hydrochloric acid at 35° for 16hr. to break the labile covalent linkages between the two components (Hughes, 1965). The hydrolysed mixture was then separated on Sephadex G-25 with the result shown in Fig. 6. All the mucopeptide material was eluted as a sharp peak in the position of fraction A (Fig. 2) and was totally excluded from the gel. Teichoic acid was eluted later from the column as a broad peak, and the mucopeptide peak contained only 6% of the total phosphorus recovered from the column. The elution pattern from Sephadex G-25 of the pure mucopeptide component, L1, of *B. subtilis* W 23 (Fig. 6) was very similar to that found for the fraction L1 of *B. licheniformis* mucopeptide (Fig. 5).

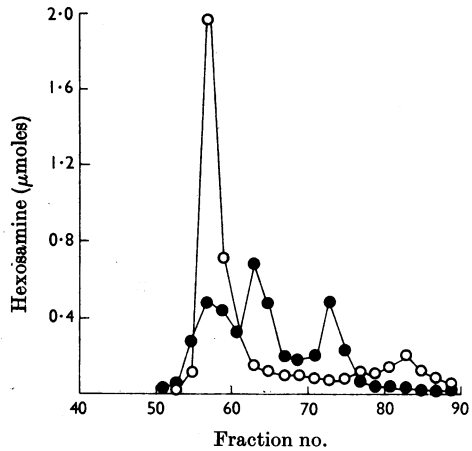

Fig. 6. Chromatography on Sephadex G-25 of mucopeptide fractions from *B. subtilis* W 23. The portion of walls made soluble with lysozyme was separated by chromatography on DEAE-cellulose into a pure mucopeptide fraction and a mucopeptide–teichoic acid complex, as described in the text. The mucopeptide–teichoic acid fraction was heated in dilute acid to break the complex. The pure mucopeptide fraction (7·1 μmoles of equivalent glucosamine) and the acid-treated complex (9·1 μmoles of glucosamine equivalent) were applied separately to the column and eluted as described in Fig. 2. Fractions (2·5ml.) were collected and analysed after acid hydrolysis for total hexosamine. Pure mucopeptide (●); mucopeptide–teichoic acid complex (○). Teichoic acid was eluted from the column between tubes 60 and 80.

Fractionation of soluble mucopeptide isolated from growing cultures of B. licheniformis

Bacterial cells and cell walls contain enzymes that are active on mucopeptide (Martin, 1966). Mucopeptide prepared from isolated walls of *B. licheniformis* might have been modified during preparation of the walls, although every effort was made to limit this effect. To eliminate the possibility, trichloroacetic acid was added directly to growing cultures of the organism. Presumably growth and enzymic activity were immediately stopped. Soluble mucopeptide was prepared from the insoluble residue by treatment with lysozyme. The chromatography on Sephadex G-25 of soluble mucopeptide prepared by this means was identical with that shown in Fig. 2. The compositions of the three fractions, A, B and C, obtained are presented in Table 6. Within experimental error, fraction C contained, after hydrolysis, equimolar amounts of diaminopimelic acid, glutamic acid, L-alanine, glucosamine and muramic acid, and was identical in composition with the preparations obtained from isolated walls (Tables 2 and 5). Fractions A, B and C accounted for 48%, 27% and 25% respectively of the total weight of material recovered from the column.

Table 6. *Analysis of three fractions obtained by chromatography on Sephadex G-25 of soluble mucopeptide isolated from growing cells of* B. licheniformis

Soluble mucopeptide was isolated from growing cultures as described in the text. Fractionation was carried out on Sephadex G-25 and the fractions A, B and C obtained were analysed as described in the Materials and Methods section.

	Molar ratios of fractions		
Substance	A	B	C
Diaminopimelic acid	1·04	1·09	0·84
Glutamic acid	1·00	1·00	1·00
Alanine	1·46	1·42	0·97
Glucosamine	0·92	1·04	1·07
Muramic acid	0·92	1·00	0·97

Isolation and chemical composition of homogeneous fragments from B. licheniformis *soluble mucopeptide*

Fractions obtained from Sephadex were examined by paper chromatography in solvent *A*. Closely similar results were obtained with fractions isolated from soluble mucopeptide prepared either from whole cells (Table 6) or from isolated cell walls (Tables 2 and 5). The material present in fraction A and in fractions A1–4 remained immobile or moved very slowly during chromatography. Fraction B was resolved into a major component, B1 (R_{Dap} 0·32) and a minor component (R_{Dap} 0·18) where R_{Dap} represents the mobility relative to $\alpha\epsilon$-diaminopimelic acid. Similarly fraction C gave one major component, C1 (R_{Dap} 0·71), and a minor component, C2 (R_{Dap} 0·46). A small amount of material with mobility similar to C2 was also present in fraction B. Fractions B and C isolated from the soluble mucopeptide of *B. subtilis* W 23 contained components with very similar mobilities to the compounds obtained from *B. licheniformis*. Relatively large amounts of fractions B1, C1 and C2 were prepared from *B. licheniformis* mucopeptide by large-scale paper chromatography.

Fractions C1 and C2. Fraction C had a molecular weight of about 900. After hydrolysis, one molar proportion of it yielded one mole each of L-alanine, glutamic acid, diaminopimelic acid, glucosamine and muramic acid (Table 2). Identical compositions were found for fractions C1 and C2. Fractions C1 and C2 represented 85% and 15% of the total diaminopimelic acid recovered after paper chromatography. The diaminopimelic acid residue present in fractions C1 and C2 reacted quantitatively with FDNB, and an equimolar amount of a mono-DNP derivative of diaminopimelic acid was isolated after acid hydrolysis. No other DNP-amino acid derivative was found, even after milder hydrolysis in 2N-hydrochloric acid at 100° for 2hr. Under these conditions, DNP derivatives of glucosamine and muramic acid would be expected to survive the acid hydrolysis. The muramic acid residue of fractions C1 and C2 were completely reduced by treatment with sodium borohydride. Treatment of fraction C1 with a crude preparation containing β-*N*-acetylglucosaminidase released *N*-acetylglucosamine, which was identified by paper chromatography. A portion of fraction C1, after treatment with the glycosidase, was reduced with sodium borohydride. After acid hydrolysis less than 10% of the glucosamine content of C1 was recovered. These results suggested that the *N*-acetylglucosamine residue in C1 was joined by a β-glycosidic linkage to an *N*-acetylmuramic acid residue that carried a free reducing group. The disaccharide, *N*-acetylglucosaminyl-β-(1→4)-*N*-acetylmuramic acid, has been isolated from the cell-wall mucopeptides of *Micrococcus lysodeikticus* (Sharon *et al.* 1966) and *Staphylococcus aureus* (Tipper, Ghuysen & Strominger, 1965). The sequence of amino acids in fractions C1 and C2 was not studied directly. However, the tripeptide L-Ala-Glu-Dap was isolated from unfractionated soluble mucopeptide after mild acid hydrolysis and evidence was obtained that in the mucopeptide L-alanine was linked to muramic acid (Hughes, 1968). Fractions C1 and C2 were homogeneous during paper electrophoresis at pH 1·9, pH 3·6 and pH 6·5, and at each pH value C1 was less acidic than C2. The difference in charge between C1 and C2 could be explained by loss of an amide group from the tripeptide side chain of fraction C1. Soluble mucopeptide was found to contain 0·93 molar proportions of amide groups (Hughes, 1968). Since fraction C accounts for at least 18% of the weight of soluble mucopeptide (Table 1), it was therefore likely that the bulk of fraction C also contained 1 mole of amide/mole. Recently fragments identical in composition and chromatographic properties with C1 and C2 have been isolated from *B. licheniformis* A.T.C.C. 9945 (N. Sharon, personal communication).

Fraction B1. Fraction B appeared to be a dimer of fraction C. Thus the weight-average molecular weight was approximately double that of fraction C (Table 3). The chemical compositions of the two fractions were very similar except that fraction B contained approx. 1 mole of D-alanine/mole in addition to approx. 2 moles of L-alanine, glutamic acid, diaminopimelic acid, glucosamine and muramic acid/mole. D-Alanine is probably involved in the cross-linkage between peptide side chains (Hughes, 1968), and it was therefore not surprising to find this amino acid in fraction B. Fraction B1 accounted for 78% of the diaminopimelic acid content of fraction B and had a very similar chemical composition. After treatment with FDNB, 53% of the diaminopimelic acid content was converted into mono-DNP-diaminopimelic acid.

This value strongly suggests that one of the diaminopimelic acid residues of fraction B1 is involved in a cross-linkage between the monomeric units. The units are probably joined together by a molecule of D-alanine from the *C*-terminus of one tripeptide to an amino group of the diaminopimelic acid residue of a second tripeptide.

DISCUSSION

The clearest evidence for the pattern of breakdown of bacterial mucopeptide by lysozyme was obtained by Weidel and his colleagues (Pelzer *et al.* 1963; Weidel & Pelzer, 1964; Takebe, 1965). After degradation with lysozyme, 27% of the weight of the mucopeptide from *E. coli* was recovered as fragments C6 (Fig. 1) and C5. Fragment C5 is closely similar to fragment C6 except for the absence of a molecule of D-alanine (Primosigh *et al.* 1961). This residue is removed enzymically during preparation of the cell walls (Leutgeb & Weidel, 1963). About 53% of the weight was recovered as dimers of C6, namely fragments C3 and C4. About 18% was of higher molecular weight (Pelzer *et al.* 1963).

Fragments identical in composition with fragment C5 of *E. coli* were present in fraction C isolated from *B. licheniformis* mucopeptide after lysozyme treatment. No trace of a fragment similar to fragment C6 (Fig. 1) was found. The possibility that fraction C was produced by enzymic action during preparation of the walls of *B. licheniformis* was ruled out, and this structure appears to be a normal structural unit of the mucopeptide. The yield of fraction C isolated from *B. licheniformis* soluble mucopeptide was similar to the combined amount of C5 and C6 obtained from *E. coli* mucopeptide. Fraction B1 had a closely similar molecular weight to that of the *E. coli* fragment C3, and was built up in a similar way. It differed, however, in containing 1, not 2, moles of D-alanine/mole. In *E. coli* non-cross-linked mucopeptide is built up by polymerization of a nucleotide precursor containing a pentapeptide side chain that is terminated by the sequence D-Ala-D-Ala. Formation of the peptide cross-linkages present in fully formed mucopeptide and in fragment C3 accompanies the enzymic splitting of the bond between these D-alanine residues. In a later step the terminal D-alanine residue of the second pentapeptide unit is presumably removed enzymically (Izaki, Matsuhashi & Strominger, 1966). *B. licheniformis* fraction B1 could arise by the same mechanism leading to a molecule similar to *E. coli* fragment C3, followed by enzymic removal of the D-alanine residue that is not involved in the cross-linkage between peptide side chains. It is noteworthy that the D-alanine carboxypeptidase present in *E. coli* does not remove such a D-alanine residue from the fragment C3 molecule (Pelzer, 1963; Martin, 1966). No structure similar in composition to fraction B1 has been isolated from *E. coli* mucopeptide (Primosigh *et al.* 1961; Weidel & Pelzer, 1964).

A greater proportion of fragments of high molecular weight was produced by lysozyme from *B. licheniformis* mucopeptide than was obtained from *E. coli* mucopeptide (Pelzer *et al.* 1963). However, a relatively low degree of cross-linking between peptide chains was present, even in material of the highest molecular weight, fraction A1 (Table 4). These fragments then are made up predominantly of units similar to the fragments in fractions B and C and joined together by glycosidic linkages between the disaccharide moieties. To a certain extent they may contain structures in which more than two peptide strands are joined together. It is impossible to determine from the present results the exact contribution that these last structures make to the weight of *B. licheniformis* mucopeptide, but it is not likely to be large. Fragments of molecular weight similar to fractions A3 and A4 (Table 3) were isolated from *E. coli* mucopeptide by a limited treatment with lysozyme (Leutgeb & Weidel, 1963). Glycosidic linkages present in the fragments were broken down by further treatment with lysozyme, and monomer and dimer molecules were obtained. In a similar way fractions A1–4 contain muramic acid residues in glycosidic linkage (Table 4), although these were apparently not cleaved after retreatment with lysozyme under the conditions used in the present work.

The reason for incomplete splitting of glycosidic linkages during the preparation of *B. licheniformis* soluble mucopeptide is unknown, but inhibition of lysozyme action has been attributed to several factors including the presence of teichoic acids (Brumfitt, Wardlaw & Park, 1958; Mandelstam & Strominger, 1961; Perkins, 1965). The presence of residual phosphorus in the acid-insoluble residue of walls used to prepare soluble mucopeptide may prevent a complete cleavage of glycosidic linkages that are potentially susceptible to lysozyme. Indeed, after lysozyme treatment of whole walls of both *B. licheniformis* N.C.T.C. 6346 and *B. subtilis* W 23, a large part of the mucopeptide material present in fraction A (Fig. 2) remained as a covalent complex with the teichoic acids. The yield of fractions B and C from intact walls after treatment with lysozyme was similar to that obtained from the residue of walls remaining after removal of the acidic polymers. No fragments of low molecular weight were found in the mucopeptide that was associated with teichoic acid in intact walls of *B. subtilis* W 23, even after breakage of the linkages between the mucopeptide and teichoic acid. It is

still probable that fragments similar to those present in fractions B and C were incorporated in that part of mucopeptide linked to teichoic acid. If this is so, the glycosidic linkages joining these mucopeptide units together were not cleaved by lysozyme.

I am grateful to Dr H. J. Rogers for his encouragement throughout this work, to Mr P. Thurman for excellent technical assistance and to Dr P. Charlwood for the ultracentrifugal analyses and for helpful discussion.

REFERENCES

Berger, L. R. & Weiser, R. S. (1957). *Biochim. biophys. Acta*, **26**, 517.
Brumfitt, W. A., Wardlaw, A. C. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1783.
Chin, T., Burger, M. M. & Glaser, L. (1966). *Arch. Biochem. Biophys.* **116**, 358.
Dawson, I. M. (1949). *Symp. Soc. gen. Microbiol.* **1**, 119.
Ghuysen, J. M. (1960). *Biochim. biophys. Acta*, **40**, 473.
Ghuysen, J. M. (1961). *Biochim. biophys. Acta*, **47**, 561.
Ghuysen, J. M. & Salton, M. R. J. (1960). *Biochim. biophys. Acta*, **40**, 462.
Ghuysen, J. M., Tipper, D. J., Birge, C. H. & Strominger, J. L. (1965). *Biochemistry*, **4**, 2245.
Hamaguchi, K. & Fumatsu, M. (1959). *J. Biochem., Tokyo*, **46**, 1659.
Hughes, R. C. (1965). *Biochem. J.* **96**, 700.
Hughes, R. C. (1966). *Biochem. J.* **101**, 692.
Hughes, R. C. (1967). *Biochem. J.* **102**, 26P.
Hughes, R. C. (1968). *Biochem. J.* **106**, 41.
Izaki, K., Matsuhashi, M. & Strominger, J. L. (1966). *Proc. nat. Acad. Sci., Wash.*, **55**, 656.
Janczura, E., Perkins, H. R. & Rogers, H. J. (1961). *Biochem. J.* **80**, 82.
Jusic, D., Roy, C. & Watson, R. W. (1964). *Canad. J. Biochem.* **42**, 1553.
Leutgeb, W. & Weidel, W. (1963). *Z. Naturf.* **18**b, 1065.
McCarty, M. (1952). *J. exp. Med.* **96**, 569.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Mandelstam, M. H. & Strominger, J. L. (1961). *Biochem. biophys. Res. Commun.* **5**, 466.
Martin, H. H. (1966). *Annu. Rev. Biochem.* **35**, 457.
Mirelman, D. & Sharon, N. (1966). *Biochem. biophys. Res. Commun.* **24**, 237.
Mitchell, P. & Moyle, J. (1951). *J. gen. Microbiol.* **5**, 981.
Pelzer, H. (1962). *Biochim. biophys. Acta*, **63**, 229.
Pelzer, H. (1963). *Z. Naturf.* **18**b, 950.
Pelzer, H., Maass, D. & Weidel, W. (1963). *Naturwissenschaften*, **50**, 722.
Perkins, H. R. (1960). *Biochem. J.* **74**, 182.
Perkins, H. R. (1965). *Biochem. J.* **95**, 876.
Perkins, H. R. & Rogers, H. J. (1959). *Biochem. J.* **72**, 647.
Primosigh, J., Pelzer, H., Maass, D. & Weidel, W. (1961). *Biochim. biophys. Acta*, **46**, 68.
Rogers, H. J. (1962). *Symp. biochem. Soc.* **22**, 55.
Rogers, H. J. (1966). *Brit. med. Bull.* **22**, 185.
Salton, M. R. J. (1956). *Biochim. biophys. Acta*, **22**, 495.
Salton, M. R. J. (1957). *Bact. Rev.* **21**, 82.
Salton, M. R. J. (1961). *Biochim. biophys. Acta*, **52**, 329.
Salton, M. R. J. (1964). *The Bacterial Cell Wall*, pp. 133–155. Amsterdam, London and New York: Elsevier Publishing Co.
Salton, M. R. J. & Ghuysen, J. M. (1959). *Biochim. biophys. Acta*, **36**, 552.
Salton, M. R. J. & Ghuysen, J. M. (1960). *Biochim. biophys. Acta*, **45**, 355.
Salton, M. R. J. & Horne, R. W. (1951). *Biochim. biophys. Acta*, **7**, 177.
Schocher, A. J., Jusic, D. & Watson, R. W. (1961). *Biochem. biophys. Res. Commun.* **6**, 16.
Sharon, N., Osawa, T., Flowers, H. M. & Jeanloz, R. W. (1966). *J. biol. Chem.* **241**, 223.
Takebe, I. (1965). *Biochim. biophys. Acta*, **101**, 124.
Tipper, D. J., Ghuysen, J. M. & Strominger, J. L. (1965). *Biochemistry*, **4**, 468.
Weidel, W. & Pelzer, H. (1964). *Advanc. Enzymol.* **26**, 193.
Young, F. E. (1965). *Nature, Lond.*, **207**, 104.
Yphantis, D. A. (1960). *Ann. N.Y. Acad. Sci.* **88**, 586.