Biochem. J. (1970) 120, 159–170
Printed in Great Britain

Cell-Wall Thickening in *Bacillus subtilis*

COMPARISON OF THICKENED AND NORMAL WALLS

By R. C. HUGHES, P. J. TANNER and ELAINE STOKES
National Institute for Medical Research, Mill Hill, London N.W.7, U.K.

(*Received* 22 *June* 1970)

1. Incubation of *Bacillus subtilis* 168 *trp* in a glucose–amino acids–salts medium lacking tryptophan leads to an inhibition of cellular growth without affecting cell-wall synthesis. The cell walls increased approximately two- to three-fold in thickness and at the same time the amount of mucopeptide in the cells measured chemically increased to about the same extent. 2. Synthesis of mucopeptide and teichoic acid as measured by the extent of incorporation of radioactivity continued linearly for approximately 1h and then stopped. No reason was found for the strictly limited synthesis of the wall polymers. 3. The initial rates of incorporation of [^{32}P]P_i or [^{3}H]alanine into teichoic acid and of ^{3}H-labelled amino acids into mucopeptide were not appreciably inhibited by the addition of chloramphenicol to the glucose–amino acids–salts medium. 4. There was no selective turnover of the mucopeptide synthesized by the cells in a medium lacking tryptophan on resumption of growth in a complete medium. 5. Wall synthesis taking place during the thickening process was similar to normal wall synthesis proceeding in growing cells. Walls of different thicknesses prepared from cells incubated for various times in incomplete medium did not differ qualitatively in composition. The products of autolysis of thickened walls were isolated and the analyses indicated a close similarity in the details of their mucopeptide structure compared with the mucopeptide of cells growing in the exponential phase.

Incubation of certain Gram-positive bacteria in a suitable medium containing an inhibitor of protein synthesis leads to the phenomenon of 'unbalanced growth' (Shockman, 1965). Early work (Mandelstam & Rogers, 1958; Hancock & Park, 1958) showed that chloramphenicol does not prevent synthesis of cell-wall mucopeptide in *Staphylococcus aureus* although it blocks an increase in protein. Under these conditions cell-division is also inhibited and the increase in mucopeptide mass relative to the cytoplasmic protein content is often observed in the electron microscope as a marked thickening of the cell walls (Bayer & Shockman, 1963; Shockman, 1965; Giesbrecht & Ruska, 1968). An analogous thickening of walls also takes place during incubation of *Staph. aureus* in the presence of tetracycline (Hash & Davies, 1962) and after amino acid starvation of *Streptococcus faecalis* (Shockman, 1965). The chemical structures of the walls that are made in the absence of protein synthesis have not been examined in detail for any organism. However, no significant differences were found in the amino acid and amino sugar compositions of *Staph. aureus* cell walls isolated after growth in the presence or in the absence of chloramphenicol (Mandelstam & Rogers, 1959).

We have examined the walls of *Bacillus subtilis* 168 *trp* after incubation of the organism in a medium lacking tryptophan. The general features of the chemical structure of the cell wall of *B. subtilis* 168 *trp* have been established (Young, Spizizen & Crawford, 1963; Young, Tipper & Strominger, 1964; Hughes, Pavlik, Rogers & Tanner, 1968). The mucopeptide component contains *meso*-diaminopimelic acid, D-glutamic acid, L-alanine, D-alanine, *N*-acetylmuramic acid and *N*-acetylglucosamine and amide groups. The *N*-acetylmuramic acid residues are substituted with tripeptides of amino acid sequence L-Ala-D-Glu-Dap.* A number of these side chains are linked together by a D-alanine residue between a carboxyl group and a free amino group of diaminopimelic acid residues of two tripeptides. The cell wall also contains a teichoic acid consisting of a small proportion of *N*-acetylgalactosamine and approximately equal amounts of phosphorus, glycerol and glucose (Hughes & Tanner, 1968).

An approximately two- to three-fold increase in cell-wall thickness was found after incubation of *B. subtilis* 168 *trp* in a glucose–amino acids–salts medium in the absence of protein synthesis. Walls prepared from cells incubated for different times in

* Abbreviation: Dap (in amino acid sequences), *meso*-diaminopimelic acid.

the defined medium and of varying thickness were found to be very similar in gross chemical composition. The walls of abnormal thickness were readily solubilized during incubation in mildly alkaline solution by the autolytic enzyme present in the wall preparations. The autolytic enzyme, an amidase, hydrolysed the bonds between L-alanine residues in the peptide side chains and *N*-acetylmuramic acid residues in the polysaccharide backbones of mucopeptide, as described for the lytic enzyme present in walls of normal thickness (Young *et al.* 1964; Young, 1966). The products of autolysis of the thickened walls were fractionated and analysed. The results indicate a close similarity in the fine structure of the mucopeptide components of walls of different thickness. While this work was in progress, wall thickening in *B. subtilis* 168 *trp* grown in the presence of chloramphenicol was reported independently by Miller, Zsigray & Landman (1967). Our results differ in certain important aspects from these workers and in particular we can find no inhibition of teichoic acid synthesis by chloramphenicol.

MATERIALS

Pig epididymal β-*N*-acetylglucosaminidase was prepared as described by Findlay & Levvy (1960) to stage 5*b*. In early experiments similar enzyme preparations were kindly made available by Dr H. R. Perkins and Dr D. H. Leaback. Radioisotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Carrier-free [^{32}P]P_i (1mCi) was diluted in 0.1M KH_2PO_4–NaOH, pH7.0 (50ml), before use. [^{3}H]Aspartic acid (specific radioactivity 960μCi/μmol) was diluted with water to 100μCi/ml before use. [^{3}H]Alanine (specific radioactivity 1510μCi/μmol) was diluted with unlabelled alanine to 1μCi/μmol and 100μCi/ml before use.

METHODS

Growth of bacteria and preparation of cell walls. *B. subtilis* 168 *trp* was grown into the middle of the exponential phase (0.5E_{675} units) 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 $MnCl_2$ (0.2%), $CuSO_4$ (0.5%), $ZnSO_4$ (6.5%), $FeSO_4$ (0.5%) and HCl (10%). Walls containing active autolytic enzymes were prepared by differential centrifugation after breakage of the cells by shaking with glass beads in a Braun homogenizer fitted with a cooling device. The walls were washed extensively with water at 4°C and freeze-dried.

Determination of radioactivity. Aqueous samples (0.5ml) were counted for radioactivity in 10ml of dioxan containing naphthalene (18%), 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%) in a Packard Tri-Carb liquid-scintillation spectrometer. Paper strips were counted for radioactivity in 2ml of toluene containing 2,5-diphenyloxazole (0.4%) and 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (0.01%).

Analytical methods. Diaminopimelic acid was determined by the method of Work (1957) after hydrolysis of samples in 4M-HCl at 105°C overnight. Amino acids were also determined in the hydrolysates after paper chromatography in solvents (*B*) and (*C*) (Mandelstam & Rogers, 1959). Amino acids and amino sugars were determined by Dr S. Jacobs with a Beckman–Spinco automatic analyser after overnight hydrolysis of samples in 4M-HCl at 105°C. Acetylamino sugars were determined by the Morgan–Elson reaction (Reissig, Strominger & Leloir, 1955) with a heating time of 3min in potassium borate buffer, pH9.2. Analyses for glucose, glucuronic acid and phosphorus were performed as described by Hughes (1968).

Paper chromatography and electrophoresis. Descending paper chromatography on Whatman 3MM paper was carried out in the following solvent systems: (*A*), isobutyric acid–0.5M-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., upper phase); (*D*), propan-1-ol–aq. 0.2% NH_3 (17:3, v/v); (*E*), butan-1-ol–acetic acid–water (25:6:25, by vol., upper phase). The following buffers were used for electrophoresis on Whatman 3MM paper: (*F*), pyridine–acetic acid–water, pH3.6 or pH6.5, run at 2000V for various times as indicated.

Treatment of oligosaccharides with exo-β-N-acetylglucosaminidase. The procedure of Hughes (1970) was used.

Column chromatography. Ion-exchange chromatography was performed on columns (2cm×40cm) of DEAE-cellulose in pyridine–acetic acid buffers, pH5.1. The resin (Whatman grade DE11) was washed as recommended by the manufacturers before equilibration with 0.01M-pyridine–acetate buffer, pH5.1. After application of the sample (containing up to 150mg of autolysed cell walls) the column was eluted with 0.01M-pyridine–acetate buffer, pH5.1 (100–150ml) and fractions (5.0ml) were collected. Then an approximately linear gradient was established by running 5M-pyridine–acetate buffer, pH 5.1, into a mixing vessel containing 0.01M-pyridine–acetate buffer, pH5.1 (1 litre). Sephadex G-25 (Pharmacia, Uppsala, Sweden) was suspended in 0.1M-pyridine–acetate buffer, pH5.1, and fines were decanted. Samples (up to 4ml) for chromatography were applied to the top of a column (2cm×140cm) of the washed Sephadex G-25 and eluted with the same buffer at room temperature and a flow rate of 30ml/h. Fractions (2.5ml) were collected and suitable portions were analysed. Buffer salts were removed from peak fractions by freeze-drying.

Incubation of bacteria in 'wall medium'. Cells were harvested from bacterial cultures by centrifugation at 3000*g* for 10min at room temperature. The cells were washed twice by centrifugation at room temperature with water (approx. 100ml each) and finally suspended in 0.1M-KH_2PO_4–NaOH buffer, pH7.0 (one-tenth of the volume of the original culture), previously warmed to 35°C. The final cell density was 1.5–2mg dry wt. of bacteria/ml. In the standard incubation mixture the cell suspension (200ml) was added to a 2 litre flask warmed to 35°C and containing a 'wall medium' comprising DL-alanine (4ml, 10mg/ml), DL-glutamic acid (8ml, 5mg/ml), DL-aspartic acid (20ml, 2mg/ml), M-$MgSO_4$ (0.4ml) and glucose (5ml, 40%, w/v). When indicated, chloramphenicol (50μg/ml, final concentration) was also present. The flasks were shaken at 35°C.

Measurement of mucopeptide synthesis. Mucopeptide

synthesis was measured by the extent of incorporation of [^{3}H]aspartic acid into diaminopimelic acid residues in mucopeptide. [^{3}H]Aspartic acid (20μCi) was added to an incubation mixture made up as described above. Duplicate or triplicate samples (5ml each) of the incubation mixture were removed at various times and pipetted into 15ml glass centrifuge tubes containing trichloroacetic acid (0.5ml, 50%, w/v). The tubes were vigorously shaken at 35°C overnight. After centrifugation at 12500*g* for 15min the precipitates were treated as described by Rogers (1967) for preparation of the mucopeptide. The pure mucopeptide fractions were suspended finally in water (5.0ml) and portions (0.5ml) were removed for radioactive counting. Samples (2.0ml) were also hydrolysed overnight in 4M-HCl at 105°C. The hydrolysates were dried and dissolved in water (1.0ml). Portions were taken for radioactive counting and for determination (Work, 1957) of the diaminopimelic acid content to obtain the specific radioactivity of diaminopimelic acid. In early experiments the hydrolysates were streaked on to sheets of Whatman 3MM paper and subjected to chromatography in solvent (*B*). The band on the chromatograms corresponding to diaminopimelic acid was eluted with water, freeze-dried and dissolved in water (1.0ml). Suitable samples were taken for determination of the specific radioactivity of diaminopimelic acid. It was found that at least 97% of the radioactivity incorporated from [^{3}H]-aspartic acid into mucopeptide during incubation in the wall medium was present as diaminopimelic acid. In later experiments, therefore, the chromatographic isolation of diaminopimelic acid was omitted.

Measurement of teichoic acid synthesis and mucopeptide synthesis. The incorporation of [^{3}H]alanine into the ester-linked D-alanine residues of teichoic acid and into the D- and L-alanine residues of mucopeptide was followed. Washed cells were suspended at approx. 1.5–2mg dry wt./ml in 0.1M-KH_2PO_4–NaOH buffer, pH7.0. Portions (200ml) were added to 2 litre flasks containing the wall medium made up as described above and supplemented with [^{3}H]alanine (4μCi). Certain flasks also contained chloramphenicol (50μg/ml final concentration). The flasks were removed at various times from the shaker and cooled by pouring on crushed ice. The cells were obtained by centrifugation and washed twice with cold alanine (0.1mg/ml, 20ml each), followed by water (20ml). The washed cells were suspended finally in water at 2°C at a concentration of approx. 20mg dry wt. of bacteria/ml for the preparation of cell walls. During the preparation the walls were treated for 20min at 100°C to inactivate autolytic enzymes. The yield of walls was approx. 50–80mg of walls from one incubation mixture. A sample (approx. 20mg) of each wall preparation was suspended in 0.5M-HCl (5ml) and shaken overnight at 35°C to extract teichoic acid. The suspension was centrifuged at 12500*g* for 30min and the residue was re-extracted with 0.5M-HCl (5ml). The supernatants were combined and contained at least 90% of the total phosphorus of the walls. The residue containing mucopeptide was washed with three portions (each 5ml) of water by centrifugation. The residue was finally drained well and suspended in water (5.0ml). Portions (2ml) of the acid-soluble and insoluble fractions were hydrolysed in 4M-HCl at 105°C overnight. After drying, the hydrolysates were dissolved in water (0.1ml) and streaked on Whatman 3MM paper for chromatography in solvent (*B*). The region of the chromatograms containing alanine was eluted with water, and the eluates were freeze-dried and dissolved in water (1.0ml). Portions were removed for radioactive counting. The content of alanine was determined with ninhydrin and the specific radioactivity was derived. It was found that alanine accounted for at least 95% of the radioactivity incorporated from [^{3}H]alanine into the cell walls during incubation in the wall medium. In later experiments the specific radioactivity of alanine in the teichoic acid and mucopeptide fractions was obtained as follows. The acid hydrolysates were dissolved in water (0.2ml). Portions (0.1ml) were taken for radioactive counting and other portions (0.02ml) were used for quantitative paper chromatography in solvent (*B*). In some experiments teichoic acids were removed from the isolated cell walls (approx. 20mg of each sample) by extraction with 0.1M-NaOH (5.0ml) at 35°C for 16h as described by Hughes & Tanner (1968). After centrifugation the soluble fraction containing teichoic acid and the insoluble mucopeptide were analysed as before.

Teichoic acid biosynthesis by [^{32}P]P_i incorporation. Teichoic acid biosynthesis was also followed by the incorporation of [^{32}P]P_i into the glycerophosphate units of the polymer backbone. In these experiments the incorporation of [^{3}H]alanine into both teichoic acid and the mucopeptide was measured as described above, simultaneously with the incorporation of [^{32}P]P_i into teichoic acid. The standard incubation mixtures in addition contained [^{32}P]P_i (100μCi) and [^{3}H]alanine (4μCi). In some flasks chloramphenicol was also present at a final concentration of 50μg/ml. After the chosen incubation times the flasks were removed from the shaker, the solutions were chilled and centrifuged. The cells were washed with ice-cold non-radioactive 0.1M-KH_2PO_4–NaOH buffer, pH7.0 (20ml) containing unlabelled alanine (0.1mg/ml) and suspended finally in water at about 20mg/ml for preparation of the cell walls. Samples (5mg) of the purified walls were hydrolysed in 6M-HCl at 105°C overnight, dried and dissolved in water (2.5ml) for determination of the specific radioactivity of phosphorus. Other samples (25mg) of each cell-wall preparation were extracted with dilute 0.1M-NaOH (5.0ml) as described previously and the specific radioactivities of alanine in the teichoic acid and mucopeptide fractions were determined. The specific radioactivity of phosphorus was also determined in the isolated teichoic acid fractions and the values were within 5% of those obtained by direct analysis of unfractionated cell walls.

Autolysis of isolated cell walls. In experiments to follow the release of amino groups during autolysis, cell walls (approx. 10mg) were suspended in 0.05M-sodium borate buffer, pH8.9, at 4mg/ml and incubated with shaking at 35°C. At frequent intervals the turbidity of the suspensions was measured and, less frequently, samples (0.2ml) were removed and boiled for 30min to inactivate the autolytic enzyme. The boiled samples were then mixed with fluorodinitrobenzene (0.05ml, 1.3%, v/v, in ethanol) and potassium tetraborate (0.05ml, 10%, w/v) and diluted to 0.5ml with water. After incubation at 35°C overnight, the reaction mixtures were acidified with conc. HCl (0.25ml) and extracted with ether. The ether extracts contained free dinitrophenylalanine derived from the ester-linked alanine residues of teichoic acid that are

hydrolysed at 100°C under the alkaline pH conditions used for autolysis. The acidified mixtures were hydrolysed at 105°C overnight and re-extracted with ether. The ether extracts and aqueous solutions were dried, dissolved in water (0.10ml) and examined by paper chromatography.

In larger-scale experiments cell walls (100mg approx.) were suspended (4mg/ml) in 20mM-$(NH_4)_2CO_3$, pH8.9, and shaken at 35°C. The turbidity was measured at 675nm and after overnight incubation the extinction had decreased to 5% or less of the initial value. The small residue was removed by centrifugation and washed several times with water. Supernatants were combined, freeze-dried and dissolved in water (5.0ml).

Electron microscopy. Electron microscopic examination of cells incubated in wall medium was kindly carried out for us by Mr I. D. J. Burdett of this Institute.

All samples were fixed for 1h by adding an equal volume of cold 5% (w/v) glutaraldehyde to the cells in medium. The fixative was buffered with 0.05M-sodium cacodylate buffer, pH6.9, containing 10mM-$CaCl_2$. After an overnight wash in buffer (two changes), the samples were post-fixed for 2h with 1% (w/v) OsO_4 (Ryter & Kellenberger, 1958). Centrifuged pellets were enrobed in 2% agar (in Ryter–Kellenberger buffer) and soaked for 2h in 0.5% (w/v) uranyl acetate dissolved in the same buffer. The samples were dehydrated in ethanol, washed in propylene oxide and embedded in Epikote 812 resin. Sections were cut with glass knives on an LKB Ultratome and picked up on bare copper grids (400 mesh). The sections were stained with a saturated solution of uranyl acetate in 50% (v/v) ethanol, and covered with a thin layer of carbon before examination in the electron microscope.

Microscopy was performed with a Philips EM300 electron microscope, at an accelerating potential of 60kV, with double-condenser illumination and an anti-contamination trap. A 50μm objective aperture was used as a routine.

RESULTS

Stability of B. subtilis 168 trp *cells in wall medium.* It was first necessary to show that the washed cells isolated from actively growing cultures of *B. subtilis* did not lyse when incubated at pH7.0 in the absence of protein synthesis. Exponential-phase cells were washed twice with water at room temperature and suspended in 0.1M-potassium phosphate–sodium hydroxide buffer, pH7.0, at approx. 2mg dry wt. of bacteria/ml. Portions (50ml) of the cell suspension were added to flasks containing respectively no additions, glucose alone, a mixture of glucose and ammonium chloride, and a mixture containing glucose, alanine, glutamic acid and aspartic acid. The flasks were shaken at 35°C. The turbidity of the incubation mixtures was measured at intervals and the results are shown in Fig. 1. The cells lysed rapidly upon incubation at pH7 when no additions were made. The lysis was almost entirely blocked when glucose or glucose plus ammonium chloride was present. In the complete wall medium containing three amino acids and glucose, no cell lysis was found for at least 4h and a small but definite increase in the turbidity was observed. No spores were visible over 4h of incubation in wall medium.

Synthesis of mucopeptide in the complete wall medium. The incorporation of [^{3}H]aspartic acid into the mucopeptide fraction of *B. subtilis* 168 *trp* cells during incubation in the complete wall medium is shown in Fig. 2. The labelling of the isolated mucopeptide increased approximately linearly for the first 45min and then stopped after about 60min. The extent of mucopeptide synthesis during the incubation period was also measured by chemical determination of diaminopimelic acid and of total hexosamines in the mucopeptide fractions. The original yield of diaminopimelic acid was 24μmol from approx. 400mg dry wt. of bacteria and this increased during incubation to a maximum value of about 70μmol (Fig. 2). A similar increase was measured in the amount of hexosamine material present.

A very similar result to that shown in Fig. 2 was obtained when chloramphenicol (50μg/ml, final concentration) was included in the incubation mixture.

The experiment in Fig. 2 was carried out with a more dilute suspension (0.5mg dry wt./ml) of cells to decide whether insufficient aeration caused synthesis of mucopeptide to stop after 60min. This,

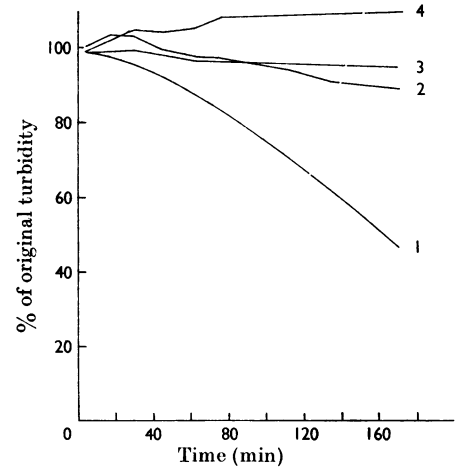


Fig. 1. Stability of *B. subtilis* 168 cells at pH7. The cells were suspended at approx. 2mg dry wt./ml in 0.1M-KH_2PO_4–NaOH buffer, pH7. Portions (50ml) were added to flasks containing (1) no additions; (2) glucose (1.25ml, 40%, w/v); (3) glucose (1.25ml, 40%, w/v) and NH_4Cl (1.0ml, 1%, w/v); (4) glucose (1.25ml, 40%, w/v), aspartic acid, glutamic acid and alanine (each 1ml, 1%, w/v). The flasks were shaken at 35°C and turbidity was measured at intervals.

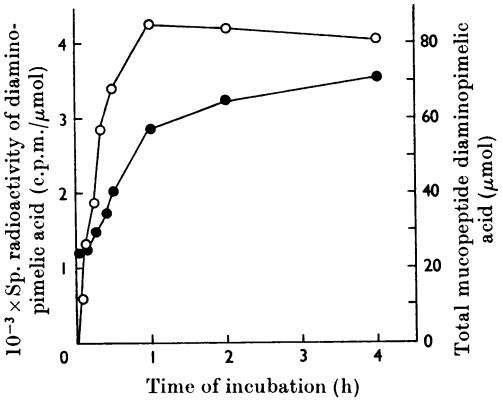


Fig. 2. Synthesis of mucopeptide in the wall medium. *B. subtilis* 168 *trp* cells (2 mg dry wt./ml) in 0.1 M-KH_2PO_4–NaOH buffer, pH 7 (200 ml), were incubated at 35°C in a glucose–amino acids–salts mixture containing [^{3}H]aspartic acid. The flask was shaken at 35°C, and at intervals portions (5 ml) were removed for preparation of mucopeptide. The content (●) and specific radioactivity (○) of diaminopimelic acid in the fractions were obtained as described in the Methods section.

however, was found not to be so. There was a relatively rapid increase in the total amount of mucopeptide as determined chemically, and synthesis then stopped. In another experiment cells were incubated at 2 mg dry wt. of bacteria/ml in complete wall medium for 120 min when the incorporation of [^{3}H]aspartic acid into mucopeptide was found to have stopped. The cells were harvested by centrifugation, washed twice with water and finally resuspended in 0.1 M-phosphate buffer, pH 7.0, at 2 mg dry wt./ml. The cells were added to fresh wall medium at 35°C and the incorporation of [^{3}H]-aspartic acid into mucopeptide was followed. The total radioactivity in the isolated mucopeptide fractions increased by only 9% after further incubation for 120 min.

To follow the turnover of mucopeptide synthesized in wall medium, cells were incubated in the usual way for 120 min in the presence of [^{3}H]aspartic acid. The cells were recovered, washed and resuspended in casein hydrolysate medium at 35°C. At zero time and after one doubling in cell number, samples of cells were removed for preparation of mucopeptide. The specific radioactivity of diaminopimelic acid had decreased during outgrowth to approx. 58% of the value found at the beginning of the incubation in growth medium. This result showed that there was no very rapid selective turnover of the mucopeptide synthesized in the absence of protein synthesis.

Electron microscopy of cells incubated in wall medium for various times. Samples (4 ml) of cells were removed from the incubation mixture described in Fig. 2 and after fixation and sectioning were examined in the electron microscope by Mr I. D. J. Burdett. Typical examples of the sections examined are reproduced in Plates 1 and 2. A striking increase in the thickness of the cell walls was found after incubation in wall medium in the absence of protein synthesis. The general appearance of the walls after staining for electron microscopy also changed, as shown in Plates 1 and 2 and Table 1. Measurements of the overall thickness of the walls and the various sub-layers are given in Table 1. The overall thickness of the wall approximately doubled during incubation.

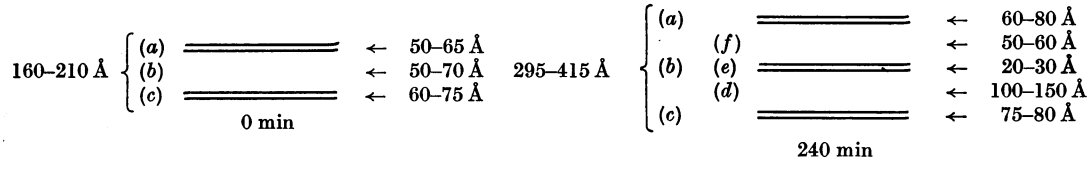
Synthesis of teichoic acid in the wall medium. Teichoic acid synthesis was followed by the incorporation of [^{3}H]alanine into the ester-linked alanine residues of the polymer and by the incorporation of [^{32}P]P_i into the polyglycerol phosphate chains (Fig. 3). The formation of mucopeptide could be followed at the same time by the extent of incorporation of the labelled amino acid, since it is possible to extract quantitatively the teichoic acid from *B. subtilis* 168 cell walls with dilute acid or with dilute sodium hydroxide. It was not possible to measure chemically the synthesis of teichoic acid since the yield of cell walls was certainly not quantitative because of losses during preparation. However, the synthesis of teichoic acid proceeded linearly during incubation for at least 60 min in the complete wall medium, as judged by the incorporation either of [^{3}H]alanine or of [^{32}P]P_i. A linear incorporation of the amino acid into mucopeptide was also observed during the 60 min of incubation, as expected. The plateau of incorporation of amino acid label into the wall polymers or of phosphorus into teichoic acid was not reached in the particular experiment shown in Fig. 3. Analytical results (described below) of walls prepared from cells incubated in wall medium for 180 min showed little difference in the relative amounts of the mucopeptide and teichoic acid polymers as compared with walls incubated for only 2.5 min. These results showed clearly that the balance between mucopeptide and teichoic acid synthesis was maintained during prolonged incubation in wall medium, and that both teichoic acid and mucopeptide synthesis eventually stopped.

Effect of chloramphenicol on wall synthesis. The experiment described above was carried out by using cells incubated in the wall medium and starved of tryptophan. We set up at the same time a similar experiment in which the cells were incubated in wall medium containing chloramphenicol. The cells were incubated for 15, 30 and 60 min before preparation of the walls. The incorporation of [^{3}H]alanine into mucopeptide was virtually unaffected by the presence of chloramphenicol (Fig. 3).

Table 1. *Measurement of wall thickness as estimated from electron micrographs (Plate 2)*

Cells incubated for various times in the wall medium (Fig. 2) were prepared for electron microscopy as described in the Methods section. The values given represent the range observed in a number of different sections of cells incubated for the particular time in wall medium. The meaning of each value is made clear by the diagram below.

Time of incubation in wall medium (min)	Thickness (Å) Inner layer (*c*)	Middle layer (*b*)			Outer layer (*a*)	Total
		(*d*)	(*e*)	(*f*)		
0	60–75		50–70		50–65	160–210
15	75		75–90		50–75	200–250
30	50–75		75–110		60–80	185–265
		30	15–20	50–60		
60	50–75		130–155		60–80	240–310
		50–75	20–30	50–60		
120	50–75		120–180		60–90	230–345
		50–90	20–30	50–60		
240	75–80		170–255		60–80	295–415
		100–150	20–30	50–60		



Similarly, under these conditions there was no inhibition of teichoic acid synthesis as measured by incorporation of the amino acid or of [^{32}P]P_i.

Stability of cells incubated in wall medium. Cells that had been kept in the wall medium for several hours were noticeably more stable during later manipulation than the cells taken from an exponentially growing culture. These cells, presumably with thickened cell walls, were found to lyse significantly more slowly than control cells (Fig. 4). However, after incubation in phosphate buffer at pH 7 and 35°C for 24h cell lysis was complete, regardless of the length of previous incubation in the wall medium.

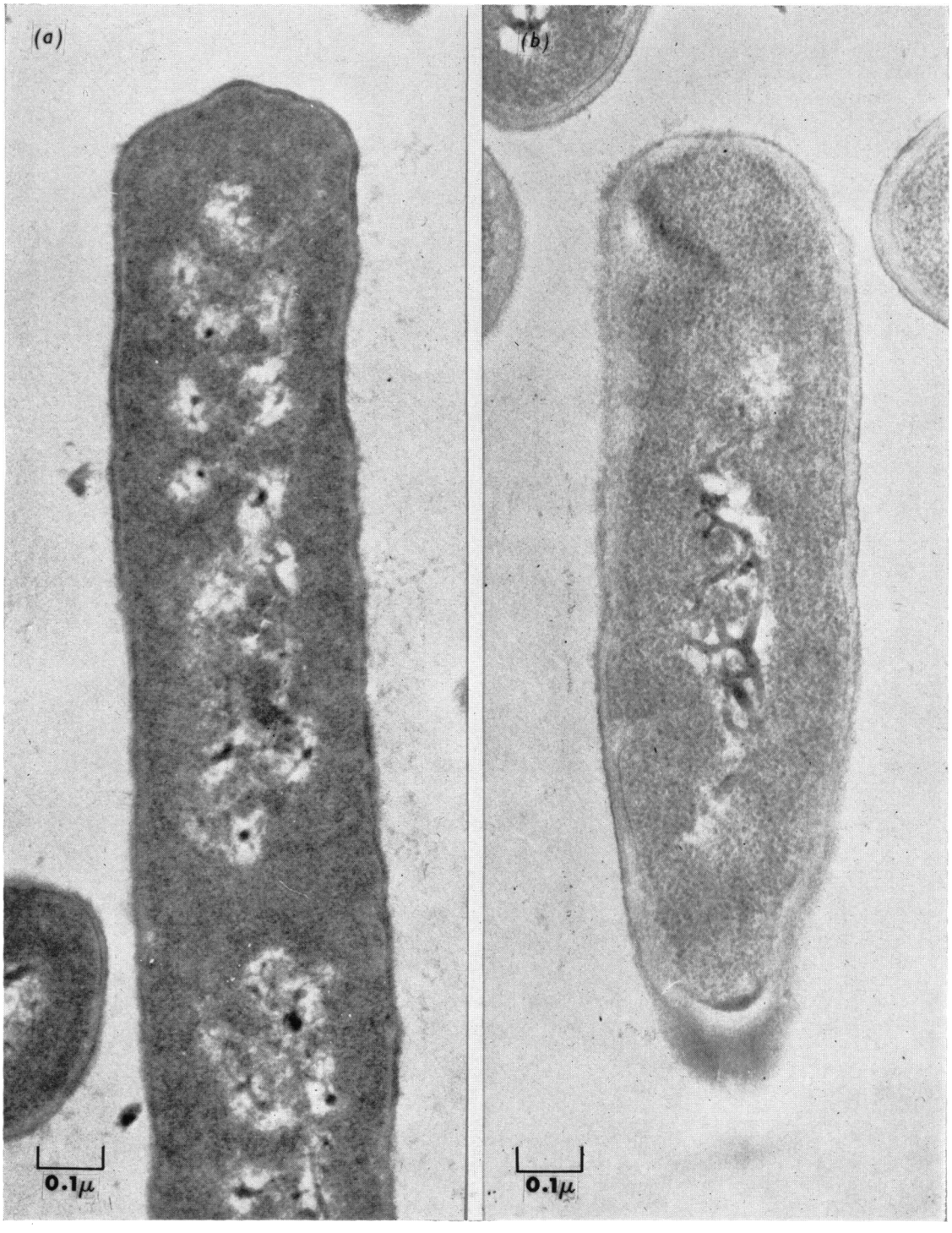
Structure of the thickened walls

Chemical analyses of thickened walls. The composition of walls isolated from *B. subtilis* cells after incubation respectively for 2.5, 30 and 180min in the complete wall medium described in the Methods section is shown in Table 2. The analytical values are very similar to those published by Hughes *et al.* (1968) for walls isolated from exponentially growing cells. No qualitative differences were found between the analyses of the different preparations. The constituents of mucopeptide, glucosamine, muramic acid, diaminopimelic acid, glutamic acid, alanine and ammonia accounted for approx. 31–33% of the dry weight of walls. This value gives an overestimate of the contribution of mucopeptide since a part of the alanine residues are present in the teichoic acid. In addition to D-alanine residues, the teichoic acid contains approximately equimolar amounts of glucose, glycerol and phosphorus, and galactosamine is present (Young, 1967; Hughes & Tanner, 1968).

The contribution of the teichoic acid to the dry weight of walls is calculated from Table 2 to be approx. 31–37%. The contents of galactosamine and phosphorus showed a significant decrease with time of incubation of the cells in wall medium before preparation of the walls (Table 2). Walls prepared from cells incubated for 3h in the glucose–amino acids–salts medium contained 35% of the galactos-

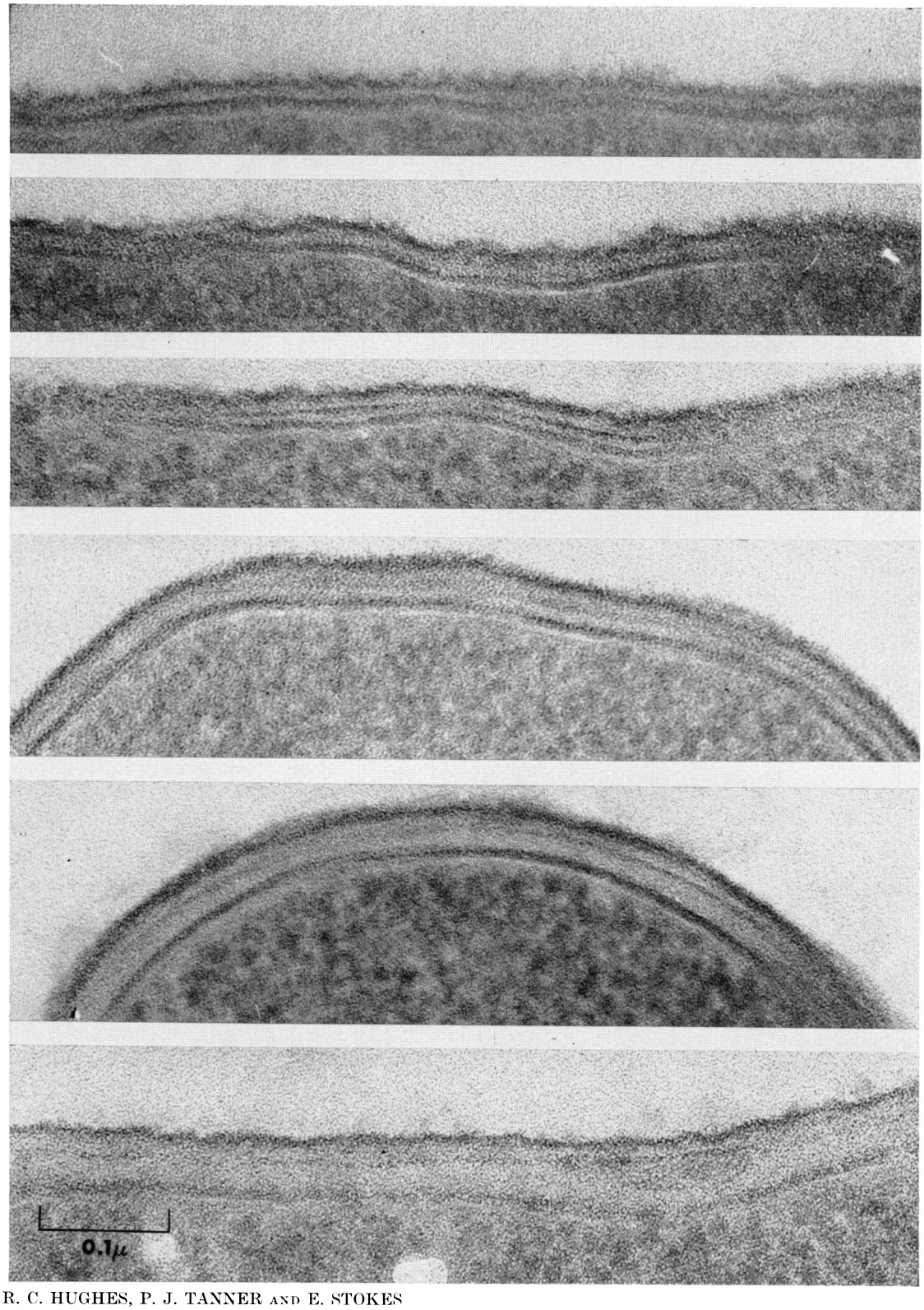
EXPLANATION OF PLATES 1 AND 2

Wall-thickening during incubation of *B. subtilis* 168 *trp* in an glucose–amino acids–salts mixture in the absence of tryptophan. Incubation at 35°C was carried out as described in Fig. 2 and the cells were removed for sectioning at (1*a*), 0min; (1*b*), 240min; (2) (from top to bottom) 0, 15, 30, 60, 120 and 240min. The appearance of the wall at 0 and 240min is given diagrammatically in Table 1.



R. C. HUGHES, P. J. TANNER AND E. STOKES

(*Facing p.* 164)



R. C. HUGHES, P. J. TANNER AND E. STOKES

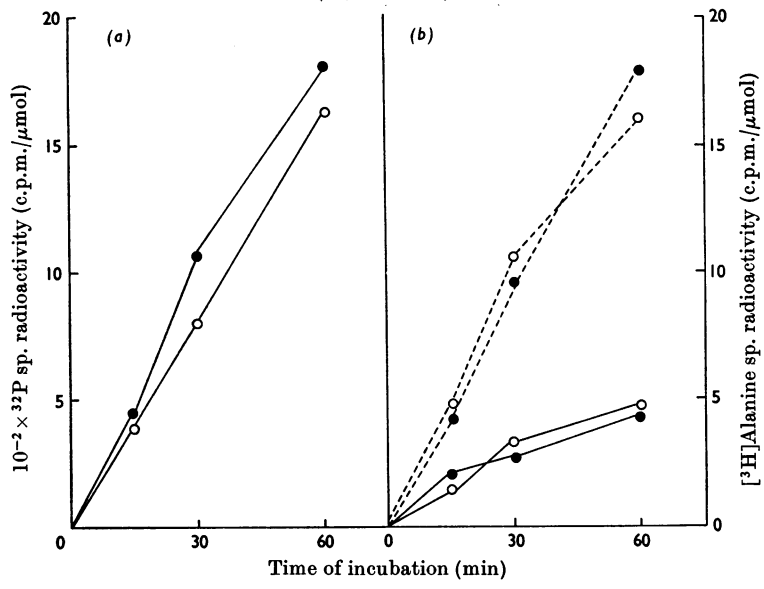


Fig. 3. Synthesis of teichoic acid in the wall medium and the effect of chloramphenicol on wall synthesis. Samples (200ml) of *B. subtilis* 168 *trp* cells suspended in 0.1M-KH_2PO_4–NaOH buffer, pH7 (200ml), at 2mg dry wt. of bacteria/ml, were added to flasks containing a glucose–amino acids–salts mixture plus [^{3}H]alanine and [^{32}P]P_i. Chloramphenicol (50μg/ml) was present in three flasks. The suspensions were shaken at 35°C and flasks with and without the inhibitor were removed at 15, 30 and 60min for preparation of cell walls. Teichoic acid and mucopeptide fractions were isolated by alkaline extraction of walls and the specific radioactivities of [^{32}P]phosphorus in teichoic acid and of [^{3}H]alanine in both fractions were measured as described in the text. (*a*) Incorporation of ^{32}P into teichoic acid in the absence (●) and presence (○) of chloramphenicol. (*b*) Incorporation of [^{3}H]alanine into teichoic acid (——) and mucopeptide (– – – –) in the absence (●) and presence (○) of chloramphenicol.

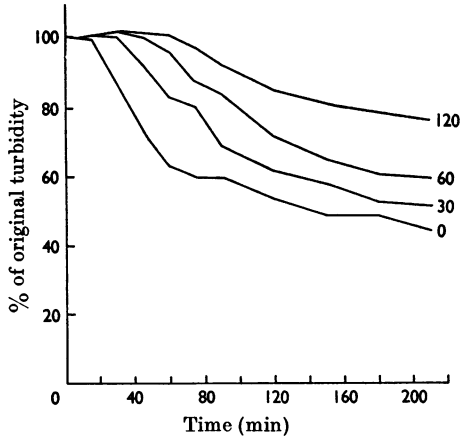


Fig. 4. Stability of cells incubated for various times in wall medium. *B. subtilis* 168 *trp* cells (2mg dry wt./ml) in 0.1M-KH_2PO_4–NaOH buffer, pH7 (200ml), were added to a glucose–amino acids–salts mixture and kept at 35°C. After 0, 30, 60 and 120min, with shaking, portions (20ml) of the mixture were removed. The cells were harvested by centrifugation, washed and suspended in 0.1M-phosphate buffer, pH7 (20ml). The turbidity was measured at intervals during incubation at 35°C.

Table 2. *Analysis of thickened cell walls*

Cells of *B. subtilis* 168 *trp* growing exponentially in casein hydrolysate medium were harvested and resuspended in KH_2PO_4–NaOH buffer, pH7.0, at approx. 2mg dry wt. of bacteria/ml. Measured volumes (800ml) of the suspension were added to flasks containing wall medium and incubated at 35°C. Flasks were taken from the shaker after 2.5, 30 and 180min of incubation and cell walls were prepared and analysed as described in the Methods section. Values refer to dry material.

Compound	Time of incubation in wall medium (min) 2.5	30	180
	μmol/100mg		
Diaminopimelic acid	39.2	41.0	42.0
Glutamic acid	31.6	34.6	43.2
Alanine	103.3	98.5	95.1
Glucosamine	32.2	37.1	30.5
Muramic acid	20.6	22.9	20.2
Ammonia	40.3	39.7	39.5
Phosphorus	125	116	107
Hexose	96	103	93
Galactosamine	14.5	7.1	5.0

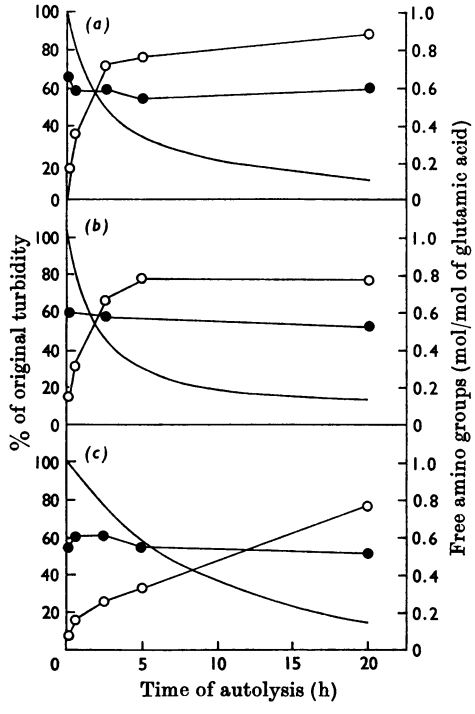


Fig. 5. Autolysis of thickened cell walls. Walls (4mg/ml), prepared from cells incubated in the glucose–amino acids–salts medium for (*a*) 2.5min, (*b*) 30min and (*c*) 180min (Table 2) were shaken in 0.05M-sodium borate buffer, pH8.9, at 35°C. Samples (0.2ml) were allowed to react with fluorodinitrobenzene and the proportions of mono-DNP-diaminopimelic acid (●) and DNP-alanine (○) present in the mucopeptide fractions were determined. The decrease in turbidity of the wall suspensions is shown by the continuous line.

amine present in walls isolated from cells incubated for a few minutes only.

Autolysis of thickened walls. Samples (approx. 10mg) of the walls (Table 2) were suspended in 0.05M-sodium borate buffer, pH8.9, at 4mg/ml. The suspensions were shaken at 35°C. The turbidity of the suspensions and contents of free amino groups in the mucopeptide fractions were then determined (Fig. 5.) In each case autolysis of the walls was relatively rapid with a 50% decrease in turbidity within about 5h. There was an increase in the content of *N*-terminal alanine without release of any other *N*-terminal groups within the limits of experimental error. At the end of autolysis approximately 0.8mol of *N*-terminal alanine/mol of glutamic acid was detected for each cell-wall preparation, indicating hydrolysis of *N*-acetylmuramyl-L-alanine linkages by amidase.

Fractionation of autolysates of cell walls on DEAE-cellulose. Samples (116, 87 and 106mg respectively) of walls prepared from cells incubated in wall medium for 2.5, 30 and 180min were allowed to autolyse overnight in dilute ammonium carbonate solution. The walls became almost completely soluble and a part of the solubilized material was fractionated by chromatography on DEAE-cellulose (Hughes, 1970). Almost identical elution profiles were obtained, and a typical one is shown in Fig. 6. A large ninhydrin-positive peak, A1, was eluted with 0.01M-pyridine–acetate buffer, pH5.1. No glucose, phosphorus or hexosamine material was present. Diaminopimelic acid, glutamic acid and alanine were detected in the approximate molar ratios 1:1:3 (Table 3). This fraction contained the peptides produced by hydrolysis of the amide linkages between L-alanine and *N*-acetylmuramic acid residues in mucopeptide. Fraction A1 accounted for 73, 75 and 67% respectively of the total diaminopimelic acid recovered from the three columns. The majority of the remaining diaminopimelic acid was found in fraction A2 (Table 3). This material contained glucosamine and muramic acid in addition to the amino acids and was presumably a mucopeptide in which amide linkages had not been hydrolysed by amidase. The oligosaccharides produced by the action of the enzyme on mucopeptide were found in fraction A3. Fraction A3 contained approximately equimolar amounts of glucosamine and muramic acid, and accounted for approx. 60% of the total glucosamine or muramic acid equivalents recovered from the columns. The remaining oligosaccharides were present in a complex fraction, A4, containing phosphorus, glucose and galactosamine. Amino acids were almost completely absent in fraction A4, which represents a complex of the teichoic acid and a part of the oligosaccharides of mucopeptide (Young *et al.* 1964). Since the fraction contained very little alanine it was concluded that the ester-linked alanine of teichoic acid was removed during autolysis of the walls in alkaline solution. This is also indicated by the large molar excess of alanine to diaminopimelic acid in the peptide fraction A1.

Chromatography of fraction A1 on Sephadex G-25. The peptides present in the A1 fraction from DEAE-cellulose were separated by chromatography on Sephadex G-25 (Hughes, 1970). Samples of the A1 fractions (Table 3) isolated from the walls of cells incubated in wall medium for 2.5, 30 and 180min and containing 7.6, 5.8, and 7.6μmol of diaminopimelic acid equivalents respectively were applied separately to columns (2cm × 140cm) of Sephadex G-25 and eluted with 0.1 M-pyridine–acetate buffer, pH5.1. Three peaks were detected with the ninhydrin reagent and the appropriate fractions were pooled and analysed. The recovery of diaminopimelic acid from the chromatography of the different A1 fractions was 75–85%. Peak P3, eluted last from Sephadex G-25, contained alanine as

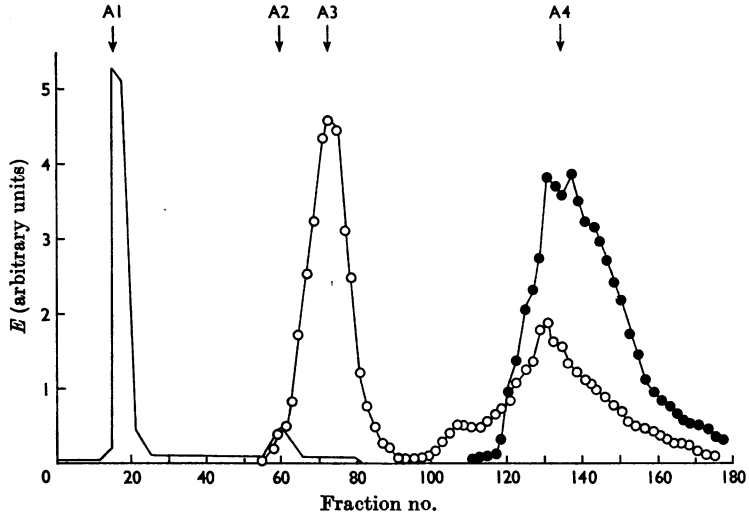


Fig. 6. Chromatography on DEAE-cellulose of autolysed walls. Walls (85mg) prepared from *B. subtilis* 168 *trp* incubated in the glucose–amino acids–salts medium for 180min were autolysed and the soluble products were separated on the column. Initial elution was with 0.01M-pyridine–acetate buffer, pH5.1 (150ml), followed by a gradient made by running 5M-pyridine–acetate buffer, pH5.1, into a mixing flask containing 1 litre of the dilute buffer. Fractions (5.0ml) were collected and analysed for glucose (●), free amino groups (——), and after acid hydrolysis for total hexosamines (○). The fractions that were made are indicated by the arrows.

Table 3. *Analyses of fractions obtained from autolysates of* B. subtilis *cell walls of different thickness by chromatography on DEAE-cellulose*

Samples (93, 70 and 85mg respectively) of the wall preparations from Table 2 after autolysis were separated on DEAE-cellulose. The elution profile obtained with one wall autolysate is shown in Fig. 6 and similar results were found with the other preparations. Fractions isolated from the columns were analysed as described in the Methods section.

Time in wall medium ...	Total content (μmol)											
	2.5 min				30min				180min			
Fraction ...	A1	A2	A3	A4	A1	A2	A3	A4	A1	A2	A3	A4
Diaminopimelic acid	19.1	6.2	0.6	0.3	14.4	4.5	0.6	0.2	19.0	8.3	1.1	0.6
Glutamic acid	19.0	7.0	0.9	0.3	15.6	3.9	0.2	0.2	18.7	8.1	2.3	0.5
Alanine	59.3	10.4	2.1	0.5	39.0	6.6	0.8	0.3	55.9	16.9	4.2	0.9
Glucosamine	0	4.6	15.4	6.6	0	2.9	9.4	3.1	0	4.0	14.3	5.0
Muramic acid	0	3.5	11.6	4.4	0	1.7	8.5	2.7	0	2.8	10.0	3.5
Galactosamine	0	0	0	7.8	0	0	0	2.9	0	0	0	2.5
Phosphorus	0	0	0	83	0	0	0	59	0	0	0	76

shown by chromatography in solvents (*B*) and (*C*) and by electrophoresis at pH3.6 in solvent (*F*). A trace of lysine was also present, the origin of which is obscure, but it may have arisen, together with the alanine, from alkaline hydrolysis of ester-linked amino acids present in teichoic acids.

Chromatography of fraction P2 in solvent (*A*) gave a major compound with a mobility relative to diaminopimelic acid (R_{Dap}) of 1.12. A minor component had R_{Dap} 1.28. The peptide fractions contained equimolar amounts of diaminopimelic acid, glutamic acid and L-alanine. The major peptide was indistinguishable chromatographically and electrophoretically from a peptide isolated, by methods identical with those described in the present paper, from autolysates of walls prepared from *B. subtilis* 168 *trp* growing exponentially in casein hydrolysate. This peptide has been shown to be the monomer peptide, L-Ala–D-Glu–Dap (Hughes *et al.* 1968; Hughes, 1970).

Fraction P1 was shown to contain a dimer heptapeptide composed of two monomer tripeptides linked together through a residue of D-alanine from a carboxy-terminal diaminopimelic acid residue to

a free amino group in a second diaminopimelic acid molecule. The material present in fraction P1 contained approximately equimolar amounts of L-alanine, glutamic acid and diaminopimelic acid. In addition, approx. 0.5 mol of D-alanine was present. Chromatography in solvent (*A*) showed the presence of a major component (R_{Dap} 0.78) and traces of another component (R_{Dap} 0.63). The major component was chromatographically and electrophoretically identical with a heptapeptide of established structure isolated from *B. subtilis* 168 *trp* walls of normal thickness (Hughes *et al.* 1968; Hughes, 1970).

The monomer and dimer peptides isolated from the thickened walls were positively charged during electrophoresis at pH 3.6 and were neutral at pH 6.5 because of the presence of amide groups. The amide groups are substituted on carboxyl groups of diaminopimelic acid residues (Warth & Strominger, 1968).

The dimer peptide fractions P1 accounted for 65, 63 and 66% respectively of the total diaminopimelic acid residues recovered from the Sephadex columns. Therefore, on a molar basis, each A1 fraction (Table 3) is calculated to contain approx. 1 mol of monomer tripeptide/mol of dimer heptapeptide. This average value is very similar to that (1.2) reported previously for the peptides isolated from an autolysate of walls prepared from cells growing exponentially on casein hydrolysate (Hughes *et al.* 1968; Hughes, 1970).

Purification of the oligosaccharides from mucopeptide. Fractions A3 isolated from DEAE-cellulose chromatography of cell-wall autolysate (Fig. 6) contained almost equimolar amounts of glucosamine and muramic acid (Table 3). In addition, small amounts of amino acids were present. Samples of the A3 fractions (Table 3) (containing 7.5, 5.1 and 4.6 μmol of glucosamine equivalents respectively) were passed through columns (0.84 cm × 30 cm) of Dowex 50 (H^+ form) and the oligosaccharides were eluted with water. The purified oligosaccharides migrated towards the anode during electrophoresis at pH 3.6 and behaved similarly to the oligosaccharide fraction prepared from the walls of exponentially growing cells (Hughes, 1970). During chromatography in solvent (*E*) the oligosaccharide fractions moved as elongated spots with a mobility relative to *N*-acetylglucosamine (R_{NAG}) of approx. 0.43–0.52.

Chain lengths of the purified oligosaccharides. The reducing power of the oligosaccharide fractions was determined by the method of Park & Johnson (1949), with *N*-acetylglucosamine as a standard. The number-average chain lengths were calculated to be 9, 13 and 13 hexosamine residues respectively for the three fractions. These values are similar to the result obtained by Young *et al.* (1964) for a similar fraction isolated from *B. subtilis* 168 *trp* walls of normal thickness. The identity of the hexosamine carrying the free reducing group was not examined directly in this work but it has been shown that only reducing end groups of glucosamine are present in the oligosaccharides obtained from exponentially growing cells of *B. subtilis* 168 *trp* (Hughes, 1970). No reducing groups of muramic acid could be detected.

The number-average chain lengths of the oligosaccharide fractions purified from thickened walls were also determined by measurement of the amounts of *N*-acetylglucosamine released from non-reducing terminals with exo-β-*N*-acetylglucosaminidase. Values of 10, 11 and 9 hexosamine units respectively were obtained, in reasonable agreement with the estimates derived from reducing-power determinations. A value of 10 hexosamine residues was obtained by enzyme assay for a polysaccharide fraction of mucopeptide isolated from exponentially growing cells (Hughes, 1970).

DISCUSSION

The results described in this paper show that *B. subtilis* 168 *trp*, in common with a number of other organisms (Shockman, 1965) continues to synthesize cell-wall material when protein synthesis is blocked either by deprivation of the essential amino acid or by the addition of chloramphenicol (50 μg/ml). Synthesis of mucopeptide and teichoic acid continued linearly for about 1 h and then stopped. The reason for this limited synthesis is not known.

Anraku & Landman (1968) reported that [^{32}P]P_i incorporation into teichoic acid is strongly inhibited by chloramphenicol. Experimentally their procedures differed greatly from those we have used. A mutant strain of *B. subtilis* 168 requiring both thymine and tryptophan was starved of the amino acid for 4 h. It was then transferred to a growth medium containing ^{32}P, tryptophan, thymine and hydrolysed casein. ^{32}P incorporation into teichoic acid continued for at least 6 h and the incorporation was blocked by chloramphenicol. It is likely that the starved cells had synthesized a limited amount of wall material during 4 h and were in the plateau region shown in Fig. 2. Mucopeptide synthesis was not measured during the second incubation in the presence or absence of chloramphenicol, but presumably would also be dependent on renewed protein synthesis.

As pointed out by Shockman (1965), the additional cell-wall substance could be accumulated in a wall of greater thickness or of greater density. The amount of continued synthesis of mucopeptide and teichoic acid observed during incubation of *B. subtilis* in the wall medium appeared to correlate reasonably well with the extent of cell-wall thicken-

ing seen in the electron microscope. There was an approximately two- to three-fold increase in both wall thickness and the amount of mucopeptide measured chemically. The absolute values given for cell-wall thickness (Table 1) must be treated with some caution because of the techniques necessarily employed to prepare the sections for microscopy. Undoubtedly, shrinkage or distortion of the walls goes on during this process (Wyatt, 1970). Nevertheless the change in appearance of the walls shown in Plates 1 and 2 is striking. The trilaminar appearance of the walls of the early samples was replaced by a five-layered structure in extended areas of the thickened walls. The middle layer (*e*, Table 1), staining darkly with uranyl acetate, was consistently found in the sections of walls of abnormal thickness. There was very little change (Table 1) in the thickness of the inner (*c*) and outer (*a*) darkly stained layers of the walls of the various samples, and the major change was an increase in thickness of the middle translucent layer that in the thickened walls was divided by the darkly stained layer (*e*) referred to above. The thickness of the translucent zone (*f*) on the outer side of the thickened walls had approximately the same dimensions (50–60 Å) as the middle translucent layer (*b*) of normal wall (50–70 Å). The inner, lightly stained layer (*d*) increased in thickness markedly during wall thickening. For example, the thickness of this layer (*d*) increased from 30 Å at 30 min (the earliest time at which a median darkly stained line could be made out) to 100–150 Å in the walls of cells incubated for 240 min in wall medium. Further work is required to identify the material that was synthesized during the thickening process and the significance of the differential staining of the walls. It is not known if the new material was laid down between the old wall and the cytoplasmic membrane. Presumably the most recently formed wall substance would be adjacent to the membrane containing the synthetic enzymes. Mucopeptide subunits must be transferred from a lipid intermediate in the membrane to acceptor groups in pre-formed strands of mucopeptide in the wall, and adjacent to the membrane (Strominger, 1969). Subsequently the wall substance formed must grow away from the membrane.

There was no clear indication of localized wall thickening in the limited number of sections examined, and obviously further work is required including serial sectioning of cells with thickened walls. However, similar provisional results were obtained by Landman and colleagues (Miller *et al.* 1967; Anraku & Landman, 1968) who observed an approximately twofold uniform thickening of walls of *B. subtilis* incubated in the presence of chloramphenicol. Studies *in vivo* (Chung, Hawirko & Isaac, 1964; Chung, 1967) have shown that growth in *Bacillus cereus* and *Bacillus megaterium* proceeds by the localized addition of new material to the existing wall. The insertion of newly synthesized material takes place in particular near the point of incipient cell division (Cole, 1965). Possibly, wall thickening in *B. subtilis* involves a similar mechanism in which the new wall substance is inserted at multiple but specific sites over the cell surface (Shockman, 1965). If the number of sites were sufficiently large the overall effect would appear to be uniform thickening around the cell. Delocalization of wall synthesis was also reported in *B. cereus* incubated in a medium containing chloramphenicol and examined by radioautography (Chung, 1967).

Mucopeptide and teichoic acid synthesis in the glucose–amino acids–salts medium take place in the absence of appreciable protein and RNA synthesis. The synthesis of DNA is less rigorously regulated in these conditions (Copeland, 1969). Controlled wall growth presumably is fully integrated with protein and nucleic acid synthesis in the normal cell. For example, autolytic enzymes have been repeatedly implicated in the proper laying-down of wall material during growth. The walls of *B. subtilis* contain a lytic system that includes an amidase and a glycosidase (Young, 1966; Brown & Young, 1970), the continued synthesis or activation (Pooley & Shockman, 1969) of which may be essential for an orderly deposition of new wall material at proper sites in the preformed wall. Wall synthesis in synchronized cultures of *B. subtilis* was found to take place only near the time of cell division (Dadd & Paulson, 1968) indicating a strong repression of wall synthesis during part of the cell cycle. The system controlling the enzymes responsible for wall synthesis may be sensitive to an inhibition of protein synthesis. For example, inhibition of the production of repressor molecules of finite half-life could conceivably affect the normal deposition of wall material in precise locations around the cell. The initial rate of mucopeptide synthesis in cells incubated in wall medium was very similar (Fig. 2) to that expected for cells growing exponentially in casein hydrolysate medium. Therefore the overall rate of mucopeptide synthesis may be controlled in part by the availability of substrates, e.g. nucleotide precursors at the sites of biosynthesis.

The thickened walls rapidly lysed on incubation in alkaline solution, showing that the autolytic amidase was fully active. Presumably no synthesis of new amidase molecules was possible in the wall medium. The complement of amidase originally present in the cells taken from cultures growing exponentially, when the activity of the enzyme is at a maximum (Young, 1966), was sufficient to solubilize the thickened walls. The walls prepared from cells incubated in wall medium for 3 h autolysed more slowly than the other wall preparations (Fig.

5). This may be due to the decreased activity of the enzyme on the thickened walls, as found in starved cells of *Strep. faecalis* (Pooley & Shockman, 1969), or alternatively to a decay of enzyme activity during the long incubation in wall medium. The products of autolysis of the walls of different thickness appeared to be very similar. In each case about 80% of the theoretical number of *N*-acetylmuramic acid–L-alanine amide linkages of mucopeptide were hydrolysed by the enzyme. This value is very similar to the result obtained with walls prepared from *B. subtilis* cells growing exponentially in casein hydrolysate medium (Hughes, 1970).

The similarity in the analytical results of the various wall preparations (Table 2) and of the fragments of autolysis (Table 3) indicates that wall synthesis taking place during the thickening process is similar to that going on in normal wall growth, apart, of course, from the difference in the topological distribution of the newly synthesized material. The changes in the contents of phosphorus, galactosamine and the other constituents of the thickening walls were similar to the effects on cell-wall composition produced by changing the medium of cultivation and to the changes occurring in the course of the regular growth cycle (Young, 1965). The extent of cross-linking between the peptide side chains of mucopeptide in thickened walls was similar to that in walls of normal thickness isolated from cells grown in casein hydrolysate medium. The polysaccharide strands of *B. subtilis* mucopeptide are relatively short (about 10 hexosamine units) and do not appear to lengthen in the thickening process. The chain lengths of the isolated polysaccharide strands of mucopeptide are presumably controlled to some extent by the action of the autolytic glycosidase, an endo-β-*N*-acetylglucosaminidase, as discussed elsewhere (Hughes, 1970). However, the activity of this enzyme *in vitro* was minimized by preparing walls in the cold and conducting the autolysis of isolated walls in alkaline solution where the glycosidase is virtually inactive (Brown & Young, 1970). Further, endo-β-*N*-acetylglucosaminidase activity could not form non-reducing end groups of *N*-acetylglucosamine in the polysaccharide strands. The values obtained for the number-average chain lengths by a determination of these residues with exo-β-*N*-acetylglucosaminidase would be independent of the extent of degradation during isolation.

We thank Dr H. J. Rogers and Mr I. D. J. Burdett for useful discussion.

REFERENCES

Anraku, N. & Landman, O. (1968). *J. Bact.* **95**, 1813.
Bayer, M. E. & Shockman, G. D. (1963). *Bact. Proc.* p. 27.
Brown, W. C. & Young, F. E. (1970). *Biochem. biophys. Res. Commun.* **38**, 564.
Chung, K. L. (1967). *Can. J. Microbiol.* **13**, 341.
Chung, K. L., Hawirko, R. Z. & Isaac, P. K. (1964). *Can. J. Microbiol.* **10**, 43.
Cole, R. M. (1965). *Bact. Rev.* **29**, 326.
Copeland, J. C. (1969). *J. Bact.* **99**, 730.
Dadd, A. H. & Paulson, R. J. L. (1968). *J. gen. Microbiol.* **53**, 111.
Findlay, J. & Levvy, G. A. (1960). *Biochem. J.* **77**, 170.
Giesbrecht, P. & Ruska, H. (1968). *Klin. Wschr.* **46**, 579.
Hancock, R. & Park, J. T. (1958). *Nature, Lond.*, **181**, 1050.
Hash, J. H. & Davies, M. C. (1962). *Science, N.Y.*, **138**, 828.
Hughes, R. C. (1968). *Biochem. J.* **106**, 41.
Hughes, R. C. (1970). *Biochem. J.* **119**, 849.
Hughes, R. C., Pavlik, J. G., Rogers, H. J. & Tanner, P. J. (1968). *Nature, Lond.*, **219**, 642.
Hughes, R. C. & Tanner, P. J. (1968). *Biochem. biophys. Res. Commun.* **33**, 22.
Mandelstam, J. & Rogers, H. J. (1958). *Nature, Lond.*, **181**, 956.
Mandelstam, J. & Rogers, H. J. (1959). *Biochem. J.* **72**, 654.
Miller, I. L., Zsigray, R. M. & Landman, O. E. (1967). *J. gen. Microbiol.* **49**, 513.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Pooley, H. M. & Shockman, G. D. (1969). *J. Bact.* **100**, 617.
Reissig, J. L., Strominger, J. L. & Leloir, L. F. (1955). *J. biol. Chem.* **217**, 959.
Rogers, H. J. (1967). *Biochem. J.* **103**, 90.
Ryter, A. & Kellenberger, E. (1958). *Z. Naturf.* **13b**, 597.
Shockman, G. D. (1965). *Bact. Rev.* **29**, 345.
Strominger, J. L. (1969). In *Inhibitors: Tools in Cell Research*, p. 187. Ed. by Bucher, Th. & Sies, H. Berlin, Heidelberg and New York: Springer-Verlag.
Warth, A. D. & Strominger, J. L. (1968). *Bact. Proc.* p. 64.
Work, E. (1957). *Biochem. J.* **67**, 416.
Wyatt, P. J. (1970). *Nature, Lond.*, **226**, 277.
Young, F. E. (1965). *Nature, Lond.*, **207**, 104.
Young, F. E. (1966). *J. biol. Chem.* **241**, 3462.
Young, F. E. (1967). *Proc. natn. Acad. Sci. U.S.A.* **58**, 2377.
Young, F. E., Spizizen, J. & Crawford, I. P. (1963). *J. biol. Chem.* **238**, 3119.
Young, F. E., Tipper, D. J. & Strominger, J. L. (1964). *J. biol. Chem.* **239**, PC 360.