The Wall Content and Composition of *Bacillus subtilis* var. *niger* Grown in a Chemostat

By D. C. ELLWOOD

Microbiological Research Establishment, Porton Down, Salisbury, Wilts., U.K.

(Received 27 February 1970)

Bacillus subtilis var. *niger* was grown in a chemostat with various growth limitations and at various growth rates. The wall content and composition of the organism grown under these conditions were determined. The wall content, expressed as a percentage of the dry weight of organisms, varied with the growth rate. Analysis of wall samples showed that their composition also varied, particularly with respect to the phosphorus content. Wall samples extracted with trichloroacetic acid under carefully controlled conditions were found to contain various amounts of phosphorus, this being present as a glycerol phosphate polymer containing hexose (glucose and in some cases galactose), i.e. a teichoic acid. Teichoic acids were present in the walls of organisms grown under all conditions except when phosphorus limited growth. Then a different anionic polymer, composed of glucuronic acid and *N*-acetylgalactosamine (a teichuronic acid), was present. Under the specific growth conditions at pH 7.0 and 35°C in a chemostat, teichoic acid and teichuronic acid appeared to be mutually exclusive.

The walls of a number of *Bacillus* species have been studied chemically and a wide variation was found in their composition (see Ghuyssen, Strominger & Tipper, 1968). There is also evidence which suggests that wall composition may be subject to phenotypic variation; thus when walls were prepared from *Bacillus* organisms harvested at different stages of growth in batch cultures, it was found that the amounts of wall components varied (Young, 1965; Chin, Burger & Glaser, 1966).

We have studied the organism *Bacillus subtilis* var. *niger* A.T.C.C. 9372 because it can be grown in a simple salts medium and thus may easily be grown in a chemostat under a wide variety of growth limitations and growth rates. Therefore it is possible to investigate the wall composition of a specific organism grown in a specific way, something that is not feasible with batch cultures. We found that the composition of walls of this organism could vary enormously; thus under magnesium-limiting conditions they contained a glycerol teichoic acid, which was totally replaced by a teichuronic acid when the organism was grown under phosphorus limitation (Ellwood & Tempest, 1967; Tempest, Dicks & Ellwood, 1968). Further, by studying the rate of change of wall composition when the culture was changed from magnesium to phosphorus limitation and vice versa, it was possible to show that this was a phenotypic change (Tempest & Ellwood, 1968; Ellwood & Tempest, 1969).

In an attempt to specify and understand the reasons why the wall contained teichoic acid, teichuronic acid, or both, the effect of a wide variety of growth conditions on the wall of this organism was studied. The present paper describes a detailed quantitative study of the changes in wall composition of *B. subtilis* var. *niger*, grown under widely differing conditions and at different growth rates, in a chemostat. The results show that only phosphorus limitation produced organisms that contained teichuronic acid in their walls, whereas organisms grown under magnesium, potassium, nitrogen and sulphur limitations contained teichoic acid in various amounts in their walls. Further, the effect of increasing growth rate was to increase the amount of teichoic acid in the wall of magnesium-limited organisms and also to increase the amount of teichuronic acid in walls of organisms grown under phosphorus limitation. Some of these findings have been reported briefly (Ellwood & Tempest, 1968).

METHODS

Organism. *Bacillus subtilis* var. *niger* (A.T.C.C. 9372) was maintained by monthly subcultures on tryptic-meat-digest-agar slopes containing glucose (0.2%).

Growth conditions. Continuous cultures of *B. subtilis* were maintained in 0.5 litre 'Porton-type' chemostats (Herbert, Phipps & Tempest, 1965) with the temperature regulated at 35°C and the pH value controlled at 7.0 ± 0.1 . The magnesium-limited medium was that described by

Tempest, Dicks & Meers (1967); the potassium-limited and the phosphate-limited media were those described by Tempest *et al.* (1968). The nitrogen-, carbon- and sulphur-limited media were made up essentially as described by Tempest (1965) except that 1M-citric acid (40 ml) replaced the EDTA. With all media the pH value was adjusted in the growth vessel by the addition of alkali (2M-NH₃ or 2M-NaOH).

Growth procedure. Chemostat cultures were established by inoculating the medium in the growth chamber with about 100 ml of a broth culture of *B. subtilis* (grown for 6 h in an Erlenmeyer flask on a reciprocating shaker). The dilution rate was set immediately at approx. 0.3 h⁻¹ and the culture was left to equilibrate overnight. The following day the dilution rate was adjusted to the required value and the culture was left to equilibrate for a further 2 days. Regular additions of small volumes (0.1–0.2 ml) of sterile PPG-2000 antifoam (Shell Chemical Co. Ltd., London S.E.1, U.K.) were used to suppress foaming of the culture. Large samples (1–2 litres) were collected over a period of time (usually overnight) from the overflow tube into a receiver surrounded by ice. Organisms were collected by centrifugation, washed once with deionized water and broken as described below; where necessary they were stored as frozen pastes at –20°C. When changing from one limitation to another 2 days were allowed for equilibration, and no run was allowed to continue for more than 3 weeks. When necessary the chemostat culture was restarted with a broth culture prepared from a stock spore suspension.

Preparation of walls. The organisms collected as described above were disrupted in a Braun M.S.K. homogenizer in a quantitative manner essentially as described for *Aerobacter aerogenes* by Tempest & Ellwood (1969). Shaking for 10 min at the fastest rate generally caused more than 99% disruption (as judged by microscopic examination). The temperature was kept between 0°C and 5°C throughout the disruption period by using liquid CO₂. The walls were heated (100°C for 10 min) to prevent any subsequent autolytic degradation of the walls. The walls were collected by centrifugation (10000g for 30 min) and washed three times with 0.85% NaCl and three times with water, all operations being carried out quantitatively. The dry weights of duplicate samples agreed to within ±2%, as did the determinations on samples from different batches (see Table 1). Each sample was checked for homogeneity by examination in the electron microscope.

Treatment of walls with enzymes. Walls (100 mg) were incubated in 0.05M-tris-HCl buffer, pH 7.2 (10 ml), with ribonuclease (1 mg), deoxyribonuclease (1 mg) and MgSO₄·7H₂O (5 mg) at 37°C for 5 h. The walls were sedimented and washed in a quantitative fashion as described above. Walls (100 mg) were incubated in 0.05M-tris-HCl buffer, pH 8.5 (10 ml), with trypsin (1 mg) at 37°C for 17 h. The treated walls were recovered quantitatively.

Extraction of wall samples with trichloroacetic acid at 4°C and at 37°C. The wall samples (100 mg) were suspended in 10% (w/v) trichloroacetic acid (10 ml) in a small stoppered Erlenmeyer flask and stirred slowly with a magnetic stirrer for 48 h in a cold-room at 4°C. The temperature of the wall suspension did not rise above 8°C provided that the stirring was kept at a low rate. It was found by controlled experiments that slight stirring and

carefully keeping to the above conditions were necessary to obtain reproducible results. The wall residue was removed by centrifugation (10000g for 30 min) and washed twice by centrifugation (10000g for 30 min) with water, freeze-dried and weighed. The supernatant and washings were combined, extracted five times with ether (5 ml) to remove the trichloroacetic acid, freeze-dried and weighed. Suspensions of walls (100 mg) in 10% (w/v) trichloroacetic acid (10 ml) were extracted as described above but at 37°C for 18 h. The temperature of the suspension itself rose to 45–47°C. The residue was removed by centrifugation, washed and recovered as before. The supernatant was poured into 5 vol. of ice-cold acetone and kept at 4°C overnight. The precipitate that formed was separated by centrifugation (3000g for 30 min), washed with cold acetone, dissolved in water, freeze-dried and weighed.

Analytical methods. Phosphorus was determined by the method of Chen, Toribara & Warner (1956). Amino compounds were determined by the method of Rosen (1957). Uronic acid was determined by reaction with conc. H₂SO₄ and carbazole (Dische, 1947). Total carbohydrate was determined by the phenol method of Dubois, Gilles, Hamilton, Rebers & Smith (1956). D-Glucose and D-galactosamine were determined with the Glucostat and Galactostat reagents from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Protein was determined by my colleague Dr H. E. Wade with an automated procedure based on the method of Lowry, Rosebrough, Farr & Randall (1951).

Chromatographic methods. The polymer and wall samples were hydrolysed by heating in a sealed tube (100°C for 3 h) with 2M-HCl. The HCl was removed by repeated evaporation *in vacuo* over NaOH pellets and P₂O₅. The hydrolysates were chromatographed on Whatman no. 1 paper with the solvents of Ellwood, Kelemen & Baddiley (1963) and those of Janczura, Perkins & Rogers (1961). Components were identified by comparison with authentic samples whenever possible.

Materials. Ribonuclease, deoxyribonuclease and trypsin were purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

RESULTS AND DISCUSSION

In a chemostat, the environment in which bacteria are grown, and the growth rate, can be varied extensively. When organisms are grown slowly, they are on average smaller than when they are grown at a faster rate (Herbert, 1958) and there is a corresponding increase in the surface/volume ratio. Thus the wall content of organisms expressed as a percentage of the bacterial mass would be expected to increase the slower they were grown. By using a reproducible method for determining wall content it was shown that bacterial size, growth rate and wall content were correlated in this way in the Gram-negative organism *A. aerogenes* (Tempest & Ellwood, 1969). The same method for estimating the wall content of bacteria was used in the present study of the Gram-positive organism *B. subtilis* var. *niger*. The wall contents of this

Table 1. *Cell wall content of B. subtilis var. niger when grown under various conditions in a chemostat*

Walls were prepared by disruption of the organisms with the Braun M.S.K. homogenizer as described in the text. Duplicate determinations are given and in some cases determinations are given for two different batches.

Growth limitation	Dilution rate (h ⁻¹)	Amount of wall (g/100 g of dried organisms)
Magnesium	0.1	27.1; 26.7
Magnesium	0.2	23.2; 23.9
Magnesium	0.3	18.7; 18.2 19.0; 19.7
Magnesium	0.3	17.8; 18.0*
Magnesium	0.3	18.0; 17.4†
Carbon (glucose)	0.3	12.0; 13.2 13.0; 13.7
Potassium	0.3	15.5; 15.3 15.8; 16.4
Nitrogen	0.3	21.0; 21.0
Sulphur	0.3	21.0; 21.0
Phosphorus	0.05	36.8; 37.7
Phosphorus	0.1	22.1; 23.1
Phosphorus	0.2	20.8; 21.1 19.7; 20.4
Phosphorus	0.3	17.0; 17.5

* Treated with deoxyribonuclease and ribonuclease.

† Treated with trypsin.

organism grown in a chemostat culture under conditions of magnesium and phosphorus limitation at different dilution rates are given in Table 1. Again, in general, the lower the imposed growth rate the larger the amount of wall; indeed, when the organism was grown at a dilution rate 0.05 h⁻¹ under phosphorus-limiting conditions the wall accounted for about 40% of the bacterial mass. Also included in Table 1 are the results for the wall content of *B. subtilis* var. *niger* grown at a dilution rate 0.3 h⁻¹ in a chemostat under conditions of magnesium, potassium, nitrogen, carbon (glucose), sulphur and phosphorus limitation. The wall content of these organisms varied slightly, carbon-limited organisms having the least amount. Treatment of isolated wall samples with ribonuclease and deoxyribonuclease did not release more than 0.5% of the wall weight from any sample, showing that the walls were not contaminated with intracellular material. This was confirmed by examination in the electron microscope, when all samples were found to be 'clean' wall preparations.

Wall samples were analysed for protein, phosphorus, total hexose, glucuronic acid and galactosamine by the techniques described in the Methods section (Table 2). Small but significant amounts of protein are seemingly associated with the wall as shown by the presence of non-mucopeptide amino acids in the wall samples. This protein could not be removed by further washing with 0.85% sodium chloride solution or with 0.1 M-potassium phosphate buffer, pH 7.0, and thus it must be firmly attached to, or be an integral part of, the wall structure.

Treatment of the wall preparations with trypsin only removed a small amount of this protein, showing that it was resistant to attack by trypsin. The protein present was determined with the Folin-Ciocalteu reagent by the procedure of Lowry *et al.* (1951) (with bovine serum albumin as a standard), and therefore the results may not be a true reflexion of the amount of protein present. However, the range of results (8–13% of wall weight) are in agreement with previously reported results (8–10% of wall weight) for *B. licheniformis* N.T.C.C. 6346 (Janczura *et al.* 1961). The amounts of protein associated with the wall varied slightly with the various conditions of growth, but even under conditions of nitrogen and carbon limitation there was still protein present, suggesting that this material had a functional role.

A variation in the amount of phosphorus in the different wall preparations was apparent (Table 2). When *B. subtilis* var. *niger* was grown under magnesium, potassium, carbon, nitrogen and sulphur limitation, i.e. with an excess of phosphorus in the medium, the wall contained a relatively large amount of phosphorus [3.9–6.9% (w/w) of the wall]. The large amounts of phosphorus were also paralleled by the large amounts of hexose found in the walls of the organisms grown under these five limitations. Magnesium limitation resulted in the largest amounts of wall-bound phosphorus when the organisms were grown at dilution rate 0.3 h⁻¹. Even at the lowest growth rate (dilution rate 0.1 h⁻¹) magnesium-limited organisms had more phosphorus in their walls than did organisms grown

Table 2. *Analysis of walls of B. subtilis var. niger when grown in a chemostat*

Walls were prepared as described in the text. Total hexose, glucuronic acid and phosphorus determinations (after acid digestion) were carried out on unhydrolysed suspensions of bacterial walls (5 mg/ml of water). Galactosamine was determined enzymically on samples that had been hydrolysed with 4 M-HCl (at 100°C for 4 h in a sealed tube) and neutralized with NaOH. Glucose was also determined enzymically on wall preparations that had been hydrolysed with 0.5 M-H₂SO₄ (at 100°C for 3 h in a sealed tube) and neutralized with NaOH. The glucose determinations are given in parentheses. —, Not determined.

Growth limitation	Dilution rate (h ⁻¹)	Wall composition (g/100 g of wall)				
		Protein	Phosphorus	Hexose	Glucuronic acid	Galactosamine
Magnesium	0.1	10.0	4.8	24 (22)	<3.0	<3.0
Magnesium	0.2	9.0	6.0	28 (26)	<3.0	<3.0
Magnesium	0.3	12.5	6.9	32 (28)	<3.0	<3.0
Magnesium, treated with trypsin	0.3	11.5	6.8	32	—	—
Magnesium, after being phosphorus-limited	0.3	—	6.0	28	<3.0	<3.0
Magnesium, after being phosphorus-limited	0.2	—	5.0	26	<3.0	<3.0
Carbon (glucose)	0.3	10.0	3.9	22 (18)	<3.0	<3.0
Potassium	0.3	10.5	4.3	20 (20)	<3.0	<3.0
Nitrogen	0.3	11.5	4.8	23 (22)	<3.0	<3.0
Sulphur	0.3	12.5	4.9	23 (21)	<3.0	<3.0
Phosphorus	0.05	—	0.3	<2.0	10.0	11.0
Phosphorus	0.1	—	0.2	<2.0	19.1	14.0
Phosphorus	0.2	13.5	0.4	<2.0	24.0	17.0
Phosphorus	0.3	10.0	0.5	<2.0	25.0	17.0
Phosphorus, after being magnesium-limited	0.2	—	0.6	<2.0	19.0	—
Phosphorus, after being magnesium-limited	0.3	—	0.8	<2.0	21.0	—

at the faster rate in other nutrient-limiting environments. No glucuronic acid or galactosamine was found in the wall samples prepared from organisms grown under nutrient-limiting conditions with an excess of phosphorus in the medium. However, as shown previously with this organism (Tempest *et al.* 1968), the amount of phosphorus in the wall of organisms grown under conditions of phosphorus limitation was very low and there was only a slight increase when the organisms were grown at faster rates. The low amounts of phosphorus were also reflected in the virtual absence of hexose in the wall, but glucuronic acid and galactosamine were both found in abundance. There was an increase in the amounts of these two last-named compounds when the organism was grown at faster rates with phosphorus limiting growth. It was noticed that, when chemostat cultures were inoculated with organisms from a broth culture and made magnesium-limiting directly (dilution rate 0.3 h⁻¹), then when equilibrium was reached (1–2 days) the wall phosphorus content was 6.9%. However, when the initial culture was phosphorus-limited and conditions were then changed so that magnesium was limiting, the steady-state wall phosphorus content

was found to be only 6.0%. A similar discrepancy was found in the wall phosphorus content of organisms grown under phosphorus limitation. At dilution rate 0.3 h⁻¹, organisms that initially started as phosphorus-limited growing at a steady state had a wall phosphorus content of 0.6%, whereas if they had been initially magnesium-limited and they were then grown under phosphorus-limited conditions the wall phosphorus content at equilibrium was 0.8%. These differences, though small, were reproducible. It is known that the chemostat is a highly selective method of growth (Powell, 1958), and these variations may result from selective pressures on the organisms when grown in a chemostat.

The extraction of phosphorus with trichloroacetic acid (10%, w/w) was studied as a function of temperature. When the extraction was carried out in a cold-room at 4°C with the stirring rate kept low the actual temperature of the extraction process was 8 ± 1°C. These conditions allowed reproducible measurements to be made of the amount of phosphorus extracted from the wall in a given time. The results of these extraction experiments with trichloroacetic acid carried out for a standard time

Table 3. *Extraction of phosphorus from walls of B. subtilis var. niger when grown in a chemostat*

The cell walls were extracted with 10% (w/v) trichloroacetic acid.

Growth limitation	Dilution rate (h ⁻¹)	% of P in wall	Extraction for 48 h in cold-room at 4°C		Extraction for 24 h in hot-room at 37°C	
			% of P in wall after extraction	P extracted as % of total P	% of P in wall after extraction*	P extracted as % of total P
Magnesium	0.1	4.8	1.9	60	0.7	85
Magnesium	0.2	6.0	2.2	63	0.8	87
Magnesium	0.3	6.9	4.3	38	1.0	85
Carbon (glucose)	0.3	3.9	1.6	59	0.4	90
Potassium	0.3	4.3	3.0	30	0.5	88
Nitrogen	0.3	4.8	2.1	56	0.5	90
Sulphur	0.3	4.9	2.0	57	0.5	90

* Chromatography of hydrolysates of these residues showed the absence of teichoic acid components.

of 24h on the wall samples prepared from *B. subtilis* var. *niger* grown in different growth-limiting environments are given in Table 3. The results fall into two groups; in most cases about 60% of the total wall phosphorus was extracted, but with walls prepared from potassium-limited organisms only 30% of the wall phosphorus was extracted. Possibly this difference in extractability of wall phosphorus may reflect a difference in the way the phosphorus is bound to the other wall components.

When the extraction with trichloroacetic acid was carried out in a hot-room at 37°C the temperature of the extraction mixture rose to about 45°C. Under these conditions nearly 90% of the wall phosphorus was released from all the wall samples except those grown in phosphorus-limited environments. The amount of phosphorus left in the wall residue after warm-trichloroacetic acid extraction varied from 0.4 to 1.0% of the wall weight for the different wall samples. Chromatographic analysis of hydrolysates of these residues showed that this residual phosphorus was not associated with teichoic acid; it could be present as muramic acid 6-phosphate residues found in the mucopeptide component of walls of Gram-positive bacteria (Liu & Gotschlich, 1967). Those residues that were examined in the electron microscope showed no loss of wall integrity, indicating that this extraction procedure had not disrupted the wall.

The composition of acid hydrolysates of trichloroacetic acid extracts prepared at 37°C were examined by paper chromatography (Table 4). There were large amounts of glycerol monophosphates, traces of glycerol, glycerol diphosphates and traces of some mucopeptide components in all wall samples except those from organisms grown under phosphorus-limiting conditions. The results showed that, when *B. subtilis* var. *niger* was grown with an

excess of phosphorus in the medium, then a glycerol teichoic acid formed part of the wall structure of the organism. Glucose was the sugar found in all teichoic acid preparations, but in the preparations from carbon-limited and magnesium-limited organisms (dilution rate 0.3 h⁻¹) galactose also was found.

The teichoic acid preparations from different wall samples all had a hexose/phosphorus ratio about 1:1 and they contained little or no ester-bound alanine. When corrected for non-teichoic acid phosphorus (i.e. the phosphorus remaining after extraction with trichloroacetic acid for 48 h at 37°C) the hexose/phosphorus ratio in the unextracted wall was also about 1:1 in all the wall samples that contained teichoic acid.

Glucuronic acid and galactosamine were found only in the trichloroacetic acid extracts from walls of organisms grown under conditions of phosphorus limitation, and suggested the presence of teichuronic acid in these walls. Attempts to remove all the teichuronic acid from these walls by increasing the extraction time or temperature gave preparations of teichuronic acid heavily contaminated with mucopeptide components, and examination of the residue by electron microscopy showed that the rigid cell-wall shape had been lost.

The results show that when *B. subtilis* var. *niger* was grown under a variety of growth-limiting conditions in a chemostat (pH 7.0 and 37°C) the wall invariably contained an anionic polymer. This was a teichoic acid when the organisms were grown under conditions of phosphorus excess, but when phosphate became growth-limiting then teichoic acid was no longer synthesized but a teichuronic acid was produced in its place. Further, as these two polymers did not occur simultaneously in the wall, the possibility exists that they may be mutually exclusive in organisms grown in these nutrient-limiting environments.

Table 4. Products of acid hydrolysis of trichloroacetic acid extracts (at 35°C) of *B. subtilis* var. *niger* (identified by paper chromatography)

Growth limitation	Dilution rate (h ⁻¹)	Glycerol	Glucose	Galactose	Glycerol mono-phosphate	Glycerol diphosphate	Alanine	Glucuronic acid	Galactos-amine	Mucopolysaccharide components
Magnesium	0.1	Trace	+	-	+	Trace	Trace	-	-	Trace
Magnesium	0.2	Trace	+	-	+	Trace	Trace	-	-	Trace
Magnesium	0.3	Trace	+	+	+	Trace	Trace	-	-	Trace
Carbon (glucose)	0.3	-	+	+	+	-	Trace	-	-	Trace
Potassium	0.3	Trace	+	-	+	Trace	Trace	-	-	Trace
Nitrogen	0.3	Trace	+	-	+	Trace	Trace	-	-	Trace
Sulphur	0.3	Trace	+	-	+	Trace	Trace	-	-	Trace
Phosphorus	0.05	-	-	-	-	-	-	+	+	+
Phosphorus	0.1	-	-	-	-	-	-	+	+	+
Phosphorus	0.2	-	-	-	-	-	-	+	+	+

* Unknown sugar also present.

The amounts of teichoic acid in the walls of this organism varied depending on the growth-limiting environment in which they had been cultured. The fact that when grown under carbon limitation *B. subtilis* still has teichoic acid in its wall indicates that this polymer plays some functional role; it is not simply a storage compound. The growth limitation that resulted in the largest amount of teichoic acid in the walls of this organism was magnesium limitation, suggesting that the teichoic acid could have a functional role in the uptake of this cation specifically or of bivalent cations in general, as suggested by Heptinstall, Archibald & Baddiley (1970).

The rate of growth also had an effect on the wall composition of *B. subtilis* var. *niger*. The faster the magnesium-limited organisms were grown, the more teichoic acid was found in the wall; up to 70% of the wall weight was teichoic acid when the organism was grown at a dilution rate 0.3 h⁻¹. The amount of teichuronic acid present in the wall of phosphorus-limited organisms also increased with increasing growth rate (Table 2).

The variation in wall composition of *B. subtilis* var. *niger* with the growth environment has been demonstrated unequivocally, and it is now clear that growing bacteria in controlled environments, in a chemostat, offers a new approach to the study of the molecular architecture of the bacterial wall.

I am deeply grateful to my colleague Dr D. W. Tempest for growing the bacteria and for many valuable discussions and criticisms of this work. I am also heavily indebted to Mr G. R. G. Moody for his skilled technical assistance, which made much of this work possible.

REFERENCES

- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
 Chin, T., Burger, M. M. & Glaser, L. (1966). *Archs Biochem. Biophys.* **116**, 358.
 Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
 Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
 Ellwood, D. C., Kelemen, M. V. & Baddiley, J. (1963). *Biochem. J.* **86**, 213.
 Ellwood, D. C. & Tempest, D. W. (1967). *Biochem. J.* **104**, 69 f.
 Ellwood, D. C. & Tempest, D. W. (1968). *Biochem. J.* **108**, 40 f.
 Ellwood, D. C. & Tempest, D. W. (1969). *Biochem. J.* **111**, 1.
 Ghuysen, J. M., Strominger, J. L. & Tipper, D. J. (1968). In *Comprehensive Biochemistry*, vol. 26, part A: *Extracellular and Supporting Structures*, p. 53. Ed. by Florkin, M. & Stotz, E. H. Amsterdam, London and New York: Elsevier Publishing Co.
 Heptinstall, S., Archibald, A. R. & Baddiley, J. (1970). *Nature, Lond.*, **225**, 519.

- Herbert, D. (1958). In *Recent Progress in Microbiology, 7th int. Congr. Microbiol.: Some Principles of Continuous Culture*, p. 381.
- Herbert, D., Phipps, P. J. & Tempest, D. W. (1965). *Lab. Pract.* **14**, 1150.
- Janczura, E., Perkins, H. R. & Rogers, H. J. (1961). *Biochem. J.* **80**, 82.
- Liu, T. Y. & Gotschlich, E. C. (1967). *J. biol. Chem.* **242**, 471.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Powell, E. O. (1958). *J. gen. Microbiol.* **18**, 259.
- Rosen, H. (1957). *Archs Biochem. Biophys.* **67**, 10.
- Tempest, D. W. (1965). *Biotechnol. & Bioengng*, **7**, 367.
- Tempest, D. W., Dicks, J. W. & Ellwood, D. C. (1968). *Biochem. J.* **106**, 237.
- Tempest, D. W., Dicks, J. W. & Meers, J. L. (1967). *J. gen. Microbiol.* **49**, 139.
- Tempest, D. W. & Ellwood, D. C. (1968). *Biochem. J.* **108**, 39P.
- Tempest, D. W. & Ellwood, D. C. (1969). *Biotechnol. & Bioengng*, **11**, 775.
- Young, F. E. (1965). *Nature, Lond.*, **207**, 104.