The Structure of an Acylated Inositol Mannoside in the Lipids of Propionic Acid Bacteria

By N. SHAW AND F. DINGLINGER

Microbiological Chemistry Research Laboratory, Department of Organic Chemistry,
University of Newcastle upon Tyne, NE1 7RU

(Received 6 January 1969)

1. Lipids were extracted from five strains of *Propionibacterium* with chloroform-methanol mixtures and fractionated by chromatography on silicic acid. 2. All five extracts contained a glycolipid composed of fatty acids, inositol and mannose in the molar proportions 2:1:1. 3. Hydrolysis of the glycolipid with alkali gave a mixture of fatty acids and *O*- α -D-mannopyranosyl-(1 $\rightarrow$ 2)-myoinositol. 4. Analysis of the fatty acids by g.l.c. showed that they were predominantly straight- and branched-chain isomers of pentadecanoic acid and heptadecanoic acid. 5. The location and distribution of the fatty acid residues in the molecule was established by periodate oxidation studies and mass spectrometry. The structure of the major glycolipid is 1-*O*-pentadecanoyl-2-*O*-(6-*O*-heptadecanoyl- α -D-mannopyranosyl)myoinositol. 6. The glycolipids are located in the membrane; the cell walls are devoid of lipid. 7. Possible functions of the glycolipid are discussed.

In a preliminary note we reported the isolation from several strains of propionic acid bacteria of a new glycolipid, which we suggested was an acylated derivative of *O*- α -D-mannopyranosyl-(1 $\rightarrow$ 2)-myoinositol (Shaw & Dinglinger, 1968). Independently, Prottey & Ballou (1968) isolated a similar glycolipid from *Propionibacterium shermanii* A.T.C.C. 9614, and proposed the structure 1-*O*-acyl-2-*O*-(6-*O*-acyl- α -D-mannopyranosyl)myoinositol. The results described in the present paper are in agreement with this structure and further studies by g.l.c. and mass spectrometry have enabled the major glycolipid to be identified as 1-*O*-pentadecanoyl-2-*O*-(6-*O*-heptadecanoyl- α -D-mannopyranosyl)myoinositol.

EXPERIMENTAL

Materials. Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden), Mallinckrodt 100-mesh silicic acid from Kodak Ltd. (Kirkby, Lancs.), and Merck Kieselgel G (nach Stahl) for t.l.c. from Anderman and Co. Ltd. (London S.E.1). The strains of *P. shermanii* and *P. arabinosum* K7 were kindly provided by Professor K. Bernhauer, Technischen Hochschule, Stuttgart, Germany. A sample of *O*- α -D-mannopyranosyl-(1 $\rightarrow$ 2)-myoinositol isolated from mycobacteria was a gift from Dr E. Vilkas, C.N.R.S., Gif-sur-Yvette, Seine-et-Oise, France.

Analytical methods. Hexoses were determined by the phenol-H₂SO₄ method of Dubois, Gilles, Hamilton, Rebers & Smith (1956) and fatty acid esters were determined by the method of Rapport & Alonzo (1955). Mannose and inositol were determined by g.l.c. by the method of Crowell

& Burnett (1967). Periodate was measured spectrophotometrically by the method of Dixon & Lipkin (1954).

Paper chromatography. Paper chromatography of sugars, polyols and glycosides was carried out on Whatman no. 1 paper by the descending method in the solvent systems butan-1-ol-pyridine-water (6:4:3, by vol.) (Jeanes, Wise & Dimler, 1951) and butan-1-ol-ethanol-water-aq. NH₃ (sp. gr. 0.88) (40:10:49:1, by vol., organic phase) (Foster, Horton & Stacey, 1957). Compounds were detected by the periodate-Schiff reagents for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956) and the alkaline AgNO₃ reagent for sugars and polyols (Trevelyan, Procter & Harrison, 1950).

Thin-layer chromatography. Lipids were examined by t.l.c. in the solvent system chloroform-methanol-water (65:25:4, by vol.) (Wagner, Horhammer & Wolff, 1961). Components were detected by reagents specific for phosphorus (Dittmer & Lester, 1964), α -glycols (Shaw, 1968a) and amino compounds (Marinetti, 1962).

Growth of organisms. *P. shermanii* PZ3, *P. shermanii* St33, *P. arabinosum* K7, *P. arabinosum* N.C.I.B. 5958 and *P. freudenreichii* N.C.I.B. 5959 were grown by the method of Bernhauer, Becher & Wilharm (1959). After incubation for 5-6 days, 10l. batches were harvested in a Sharples centrifuge. The cells were washed twice in 0.9% NaCl and then freeze-dried. The yield of dry cells was in the range 13-16 g./l. of culture.

Isolation of the glycolipids

The cells were stirred with twice their volume of chloroform-methanol (2:1, v/v) for 3 hr. at room temperature. This process was repeated twice and the combined extracts were evaporated to dryness *in vacuo*. The residue from *P. arabinosum* K7 was a mixture of a gum and a

white crystalline material, which was removed by extraction with methanol: the nature of this product was not investigated. The crude lipid extracts were dissolved in chloroform-methanol-water (40:20:3, by vol.) and purified by passage through a column of Sephadex G-25 (Wells & Dittmer, 1963). Analysis of the lipids by t.l.c. showed the presence in all five extracts of a component, R_F 0.55, that gave a negative reaction to the phospholipid reagent, but with the periodate-Schiff reagents gave a slow-developing blue-grey colour typical of glycolipids. The lipid extracts were fractionated into neutral, glycolipid and phospholipid components by chromatography on silicic acid by the method of Vorbeck & Marinetti (1965). The fractions eluted with acetone contained all the glycolipid together with traces of phospholipids. These were removed by repeating the fractionation procedure, giving the glycolipid as a white waxy solid. In *P. shermanii* the glycolipid represented some 40% of the total lipid, whereas in the other strains it was much less. The yield of total pure lipid was approx. 3% of the cell dry weight in all five strains.

Examination of the glycolipids from P. shermanii PZ3

Acid hydrolysis. The glycolipid (2mg.) was hydrolysed in 2M-HCl at 100° for 3hr. The hydrolysate was evaporated to dryness *in vacuo* over KOH and the residue dissolved in the minimum quantity of water and examined by paper chromatography. Mannose and inositol were the only products detected; glycerol was absent.

Analysis. A sample of the acid hydrolysate was reduced with NaBH₄ and the resulting mixture of mannitol and inositol was converted into their acetate derivatives and then analysed by g.l.c. (Crowell & Burnett, 1967). A mannose:inositol molar ratio 0.95:1 was obtained. The molar ratio of fatty acid to hexose was 1.85:1. (The ratios given are averaged over three determinations.)

Alkaline hydrolysis. The glycolipid (2mg.) was deacylated (Marinetti, 1962) and the water-soluble product examined by paper chromatography. A single non-reducing glycoside was produced (with R_{glucose} 0.18 in the system butan-1-ol-pyridine-water), which with the periodate-Schiff reagents gave an initial yellow colour that subsequently turned to blue-grey.

The glycolipid (160mg.) was heated at 100° for 1hr. with 0.01M-NaOH (40ml.) in an atmosphere of N₂, and a homogeneous solution was obtained. The solution was cooled, adjusted to pH 1 with 2M-HCl and extracted with light petroleum (b.p. 60–80°) (3 × 40ml.). The extracts were combined, washed with water and dried over Na₂SO₄. The drying agent was removed by filtration and the solvent evaporated *in vacuo*, giving a residue of fatty acids (79mg.). These were converted into their methyl esters and analysed by g.l.c. by the method of Brundish, Shaw & Baddiley (1965). The results are given in Table 1. The aqueous solution from the alkaline hydrolysis was deionized by passage through columns (10ml.) of Dowex 1 (OH⁻ form) and Dowex 50 (H⁺ form) resins and the eluate was evaporated to dryness. The residue was dissolved in a mixture of acetic anhydride (2ml.) and pyridine (2ml.) and left at room temperature for 1hr. The solvents were removed by evaporation *in vacuo* and the residue was recrystallized three times from ethanol, yielding a crystalline acetate derivative, m.p. 173–174° and $[\alpha]_D + 21^\circ$. The m.p. was undepressed on admixture with an authentic sample of the

nona-acetate of *O*- α -D-mannopyranosyl-(1→2)-myoinositol, and their i.r. spectra were identical. The derivative was also identical with the acetate of the glycoside isolated by Prottey & Ballou (1968) from *P. shermanii* A.T.C.C. 9614.

Oxidation with periodate. The glycolipid (16mg.) was dissolved in ethanol (7ml.) and oxidized with NaIO₄ (100mg. in 3ml. of water) in the dark. Determination of periodate consumption showed that the reaction was complete after 72hr., when 5mol.prop. of periodate had been reduced. Excess of periodate was destroyed by the addition of a solution of ethylene glycol (3ml. of 1% in water); water was added (10ml.) and the solution was deionized by passage through columns of Dowex 1 (OH⁻ form) and Dowex 50 (H⁺ form) resins. The eluate was evaporated to a volume of about 2ml.; NaBH₄ (25mg.) was added and the solution left overnight. Excess of NaBH₄ was destroyed by the addition of several drops of acetic acid, and the solution was passed through a column of Dowex 50 (H⁺ form) resin. The eluate was evaporated to dryness, and boric acid was removed by evaporation with three successive portions (3ml.) of methanol on a steam bath. The residue was hydrolysed for 3hr. in 2M-HCl at 100°, and the hydrolysate was examined by paper chromatography: ethylene glycol, glycerol and erythritol were detected.

Mass spectra. The glycolipid (10mg.) was dissolved in acetic anhydride (1ml.) and pyridine (1ml.) and left at room temperature for 1hr. The solvents were then removed *in vacuo*, leaving a gummy residue, which solidified on standing. No hydroxyl absorption band was present in the i.r. spectrum. The mass spectrum of this material was obtained on an A.E.I. MS9 mass spectrometer with a source temperature of 250°. The results are described in the Results and Discussion section. Spectra of the nona-acetate of *O*- α -D-mannopyranosyl-(1→2)-myoinositol and hexa-acetylmyoinositol were also determined for comparison.

Examination of the glycolipids from P. shermanii St33, P. arabinosum K7, P. arabinosum N.C.I.B. 5958 and P. freudenreichii N.C.I.B. 5959

Degradations similar to those described above were carried out on the glycolipids from these strains of propionic acid bacteria. The same crystalline acetate derivative of *O*- α -D-mannopyranosyl-(1→2)-myoinositol was isolated from each, and the mass spectra of the peracetylated glycolipids were very similar.

Table 1. *Analysis of the fatty acids from the
glycolipids of propionic acid bacteria*

The glycolipids were hydrolysed by 0.01M-NaOH. The analysis was by g.l.c. (Brundish, Shaw & Baddiley, 1965) of the methyl esters.

Fatty acid	Composition (%, w/w)
unidentified	2
C _{15:0}	10
C _{15:0} , branched	40
C _{16:0}	2
C _{17:0}	30
C _{17:0} , branched	15
C _{18:0}	Trace

Distribution of the lipids in the cell

A suspension of *P. shermanii* PZ 3 cells (1 ml. of water/g. wet wt. of cells) was disrupted with Ballotini no. 11 beads in a MSK Braun homogenizer at 4000 rev./min. The glass beads were removed by filtration and the suspension was centrifuged at 10000 *g* for 15 min. to sediment the cell walls. The supernatant was then centrifuged at 35000 *g* for 60 min. to sediment the membrane fragments. The remaining supernatant containing the cytoplasm was freeze-dried. Both wall and membrane fractions were washed several times in 0.9% NaCl and then freeze-dried. The three fractions were then extracted with chloroform-methanol (2:1, v/v) to remove any lipid. The purified cell walls contained no lipid. The membrane fraction contained approx. 20% of lipid of similar composition, as judged by t.l.c., to that extracted from whole cells.

RESULTS AND DISCUSSION

The purified lipids of *P. shermanii* PZ3, *P. shermanii* St33, *P. freudenreichii* N.C.I.B. 5959, *P. arabinosum* N.C.I.B. 5958 and *P. arabinosum* K7 represent approx. 3% of the cell dry weight. All strains contained a glycolipid that was readily purified by chromatography on silicic acid. The concentration of the glycolipid varied in the different strains: *P. freudenreichii* and *P. arabinosum* contained the smallest amounts, whereas in *P. shermanii* it represented about 40% of the total lipid. In view of the larger amounts available, initial structural studies were carried out on the glycolipid from *P. shermanii*.

Alkali hydrolysis yielded a non-reducing glycoside and a mixture of fatty acids, predominantly pentadecanoic acid and heptadecanoic acid (Table 1). This composition is similar to that previously found in the total cellular lipids from *P. freudenreichii* (Laneelle & Asselineau, 1968) and *P. shermanii* (Moss, Dowell, Lewis & Schekter, 1967). Acid hydrolysates of the glycoside contained inositol and mannose; glycerol was absent. Analysis of the glycolipid gave the molar proportions of fatty acids:mannose:inositol as 2:1:1, suggesting that it was a diacyl inositol mannoside. Acetylation of the glycoside yielded a crystalline acetate derivative whose physical constants corresponded to those of the derivative of *O*- α -D-mannopyranosyl-(1 $\rightarrow$ 2)-myoinositol, which was isolated as a degradation product from the phosphatidylmyo-inositol mannosides of mycobacteria (Vilkas, 1960; Ballou & Lee, 1964), and it seemed probable that the glycoside had the same structure. This was confirmed by comparison with authentic material kindly supplied by Dr E. Vilkas.

Evidence for the location of the two acyl residues was obtained from periodate oxidation studies. The glycolipid consumed 5 mol. prop. of periodate, and after treatment of the oxidation product with sodium borohydride, followed by acid

hydrolysis, glycerol and erythritol only were detected. The absence of a pentitol, hexitol or inositol as products of this reaction excludes a structure with both acyl residues attached to the inositol. Only two structures, in which the acyl residues are attached either to the 4- and 6-positions of the mannose or to the 6-position of the mannose and the 1-position of the inositol, are consistent with these results (Scheme 1). However, the reaction of the glycolipid with the periodate-Schiff reagents suggested that at least one acyl residue was attached to the inositol, as it did not give the yellow colour characteristic of a substituted malondialdehyde residue (Hardy & Buchanan, 1963) which would be produced from a mono-substituted inositol. Both the deacylated glycolipid and phosphatidylmyo-inositol give this reaction. An examination of the mass spectrum of the peracetylated glycolipid conclusively established that (I) represents the structure of the glycolipid. The mass spectrum showed a series of three molecular ions of very low intensity, but more intense peaks were found at *M*-60, produced by the loss of a molecule of acetic acid. These peaks, *m/e* 1024, 1052 and 1080, correspond to the loss of acetic acid from compound (II) where *R*=*R'*=C₁₅, *R*=C₁₅, *R'*=C₁₇ (or vice versa), and *R*=*R'*=C₁₇ acyl residues respectively. The relative intensities of these peaks were approx. 1:3:1, showing that the major component (60%) was the glycolipid containing one C₁₅ and one C₁₇ fatty acyl residue. A characteristic feature of the mass spectra of peracetylated hexapyranosides (Biemann, DeJongh & Schnoes, 1963) is a peak at *m/e* 311 produced by cleavage of the glycosidic bond and formation of the oxonium ion (III). Further fragmentation by loss of molecules of acetic acid and ketene gives peaks at *m/e* 271, 229, 211 and 169. Cleavage of the glycosidic linkage in compound (II) would yield the oxonium ion (IV) with *m/e* 513 where *R*=C₁₅, and 541 where *R*=C₁₇; both peaks were observed in the spectrum. The intensity of the peak at *m/e* 541 (IV, *R*=C₁₇) was five times greater than the peak at *m/e* 513 (IV, *R*=C₁₅). Since the intensities of the molecular ions have established that the glycolipids containing two C₁₅ residues and two C₁₇ residues are present in minor but approximately equimolar amounts, the ratio of the intensities at *m/e* 541 and 513 is a measure of the proportions of compound (II, *R*=C₁₇; *R'*=C₁₅) and compound (II, *R*=C₁₅; *R'*=C₁₇) respectively. Thus the fatty acids are not randomly distributed between the two sites of acylation in the molecule, but the major glycolipid has the structure 1-*O*-pentadecanoyl-2-*O*-(6-*O*-heptadecanoyl- α -D-mannopyranosyl)myo-inositol. Further peaks were observed in the mass spectrum at *m/e* 271, 229, 211 and 169 from fragmentation of the oxonium

Scheme 1. Degradative sequence establishing two possible structures for the glycolipid. R, Fatty acid residue.

ion (IV) and also at m/e 43 ($\text{CH}_3\cdot\text{CO}$), 225 (C_{15}) and 253 (C_{17}), the latter two confirming the identification of the fatty acid components.

Similar degradations to those described above were carried out on the glycolipids from the other strains of propionic acid bacteria. The same crystalline acetate derivative of *O*- α -D-mannopyranosyl-(1 $\rightarrow$ 2)-myoinositol was isolated from each, and the mass spectra of the peracetylated glycolipids were very similar to that of the peracetylated glycolipid from *P. shermanii* PZ3. The preparation of cell fractions has established that the glycolipid, together with the other lipid components, is located in the membrane. The cell walls of propionic acid bacteria, like those of most Gram-positive bacteria, are devoid of lipid.

Independently, Prottey & Ballou (1968) isolated a glycolipid of similar structure from *P. shermanii* A.T.C.C. 9614. They obtained additional evidence for the location of the fatty acids by methylation

analysis, but did not completely identify the fatty acids or establish their distribution in the molecule.

Glycolipids of the diglycosyl diglyceride type are widely distributed in Gram-positive bacteria of the Order Eubacteriales, and their structure may have taxonomic significance. Although the glycolipid from propionic acid bacteria is of a different type, its presence in all strains so far examined further supports the proposition that members of the same genus contain similar glycolipids (Shaw & Baddiley, 1968). The structure of the glycolipid, which is the first non-phosphorylated inositol-containing lipid to be isolated from bacteria, resembles that of the 6,6'-diacyl- $\alpha\alpha'$ -trehalose ('cord factor') isolated from mycobacteria (Lederer, 1967). A taxonomic relationship between the genera *Mycobacterium* and *Propionibacterium* was suggested on the basis of morphological and physiological similarities by Brennan & Ballou (1968). Further support for this close relationship was provided by the isolation

(I)

(II)

(III)

(IV)

R, R', Fatty acid residues; Ac, acetyl.

from *P. shermanii* (Brennan & Ballou, 1968) and from *P. freudenreichii* (Laneelle & Asselineau, 1968) of a phosphatidylmyoinositol monomannoside; similar phospholipids are widely distributed in *Mycobacteria* (Lederer, 1967). These reports led us to suggest in our preliminary note (Shaw & Dinglinger, 1968) that the glycolipid may be involved in the biosynthesis of phosphatidylmyoinositol mannosides. This route would involve transfer of the phosphatidic acid residue of CDP-diglyceride to the acylated inositol mannoside, giving initially a diacyl phosphatidylmyoinositol monomannoside (Scheme 2). It is significant that such compounds have been isolated from *Mycobacteria*, although the location of the two additional acyl residues has not been established (Pangborn & McKinney, 1966). No similar glycolipid has yet been isolated from *Mycobacteria*, although, if present, it may not accumulate in large amounts. Prottey & Ballou (1968) suggested that the glycolipid may be formed in the cell by the action of a phospholipase D on a diacyl phosphatidylmyoinositol monomannoside. This seems unlikely, since an equimolar quantity of phosphatidic acid would be formed in this reaction and this product was not observed either by Prottey & Ballou (1968) or by ourselves. The absolute configuration of the glycolipid remains to be established. As Prottey & Ballou (1968) pointed out, if a structural relationship exists between the glycolipid and phospholipid, the fatty acid esterified to the inositol should be attached to the 1-D- or 3-L-position, since the

phosphatidyl residue in all the phosphoinositides so far investigated is attached to the 3-D- or 1-L-position.

An inositol mannoside of unknown structure was isolated from yeast by Tanner (1967) and, in view of the recognition of the function of galactinol [*O*- α -D-galactopyranosyl-(1 $\rightarrow$ 1)-myoinositol] as a donor of galactosyl residues in the biosynthesis of stachyose in seeds of *Phaseolus vulgaris* (Tanner & Kandler, 1968), it was suggested that the mannosyl inositol may also serve as a mannose donor in the biosynthesis of yeast mannans. A similar function for the glycolipid would seem unlikely in view of the presence of an acyl residue on the mannose. The molecular architecture of bacterial membranes is still a matter for conjecture (Salton, 1967), but most model structures so far proposed are based on the aggregation of 'subunits' composed of protein and phospholipid. Such models have so far neglected the role of glycolipids, since their widespread distribution has only recently been established. It is reasonable to suppose that in many bacteria, especially those where they are present in large concentrations, e.g. *Mycoplasma laidlawii* B (Shaw, Smith & Koostra, 1968), *Microbacterium lacticum* (Shaw, 1968b), and *Propionibacterium*, these subunits contain quantities of glycolipids as structural components. An examination of the conformation of diglycosyl diglycerides from molecular models led Brundish, Shaw & Baddiley (1967) to suggest that the hydrophilic parts of the glycolipids form pores in the membrane through which small ions

Scheme 2. Possible route for the biosynthesis of the glycolipid and its utilization as an intermediate in the biosynthesis of diacyl phosphatidylmyoinositol monomannoside. R, R', R'' and R''', Fatty acid residues.

and molecules may pass. A similar conformation can be adopted by a diacyl inositol mannoside and it may carry out a similar function.

Note added in proof. Takayama & Goldman (1969) have demonstrated that in mycobacteria the phosphatidylmyoinositol dimannoside biosynthesized from GDP-[¹⁴C]mannose contains only one [¹⁴C]mannose residue attached to the 6-position of the inositol: the mannose attached to the 2-position is inactive. This result supports the proposal discussed above that the phosphatidylmyoinositol monomannoside is not formed by direct transfer of mannose to phosphatidylmyoinositol.

We thank Dr C. E. Ballou and Dr C. Prottey for communicating their results before publication and for a sample of the acetylated glycoside. F.D. thanks the Deutscher Akademischer Austauschdienst-Bad Godesburg for the award of a Kurzstipendium and Professor J. Baddiley, F.R.S., for the provision of laboratory facilities.

REFERENCES

- Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.
- Ballou, C. E. & Lee, Y. C. (1964). *Biochemistry*, **3**, 682.
- Bernhauer, K., Becher, E. & Wilharm, G. (1959). *Arch. Biochem. Biophys.* **83**, 248.
- Biemann, K., DeJongh, D. C. & Schnoes, H. K. (1963). *J. Amer. chem. Soc.* **85**, 1763.
- Brennan, P. & Ballou, C. E. (1968). *Biochem. biophys. Res. Commun.* **30**, 69.
- Brundish, D. E., Shaw, N. & Baddiley, J. (1965). *Biochem. J.* **97**, 158.
- Brundish, D. E., Shaw, N. & Baddiley, J. (1967). *Biochem. J.* **105**, 885.
- Crowell, E. P. & Burnett, B. B. (1967). *Analyt. Chem.* **39**, 121.
- Dittmer, J. C. & Lester, R. L. (1964). *J. Lipid Res.* **5**, 126.
- Dixon, J. S. & Lipkin, D. (1954). *Analyt. Chem.* **26**, 1092.
- Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, P. F. (1956). *Analyt. Chem.* **28**, 350.
- Foster, A. B., Horton, D. & Stacey, M. (1957). *J. chem. Soc.* p. 81.
- Hardy, F. E. & Buchanan, J. G. (1963). *J. chem. Soc.* p. 5881.
- Jeanes, A., Wise, C. S. & Dimler, R. J. (1951). *Analyt. Chem.* **23**, 415.
- Laneelle, M. A. & Asselineau, J. (1968). *C. R. Acad. Sci., Paris*, **266D**, 1901.
- Lederer, E. (1967). *Chem. Phys. Lipids*, **1**, 294.
- Marinetti, G. V. (1962). *J. Lipid Res.* **3**, 1.
- Moss, C. W., Dowell, V. R., Lewis, V. J. & Schekter, M. A. (1967). *J. Bact.* **94**, 1300.
- Pangborn, M. C. & McKinney, J. A. (1966). *J. Lipid Res.* **7**, 627.
- Prottey, C. E. & Ballou, C. E. (1968). *J. biol. Chem.* **243**, 6196.
- Rapport, M. M. & Alonzo, N. (1955). *J. biol. Chem.* **217**, 193.
- Salton, M. R. J. (1967). *Annu. Rev. Microbiol.* **21**, 417.

- Shaw, N. (1968a). *Biochim. biophys. Acta*, **164**, 435.
Shaw, N. (1968b). *Biochim. biophys. Acta*, **152**, 427.
Shaw, N. & Baddiley, J. (1968). *Nature, Lond.*, **217**, 142.
Shaw, N. & Dinglinger, F. (1968). *Biochem. J.* **109**, 700.
Shaw, N., Smith, P. F. & Kooststra, W. L. (1968). *Biochem. J.* **107**, 329.
Takayama, K. & Goldman, D. S. (1969). *Biochem. biophys. Acta*, **176**, 196.
Tanner, W. (1967). *Z. Pflanzenphysiol.* **57**, 474.
Tanner, W. & Kandler, O. (1968). *Europ. J. Biochem.* **4**, 233.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Vilkas, E. (1960). *Bull. Soc. Chim. biol., Paris*, **42**, 1005.
Vorbeck, M. L. & Marinetti, G. V. (1965). *Biochemistry*, **4**, 296.
Wagner, H., Horhammer, L. & Wolff, P. (1961). *Biochem. Z.* **334**, 175.
Wells, M. A. & Dittmer, J. C. (1963). *Biochemistry*, **2**, 1259.