A Polymer of Glucose and *N*-Acetylgalactosamine 1-Phosphate in the Wall of *Micrococcus* sp. A1

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1. The walls of *Micrococcus* sp. A1 contain about 43% of a phosphorylated polymer. It was extracted with cold trichloroacetic acid and purified by chromatography on DEAE-cellulose. 2. The polymer contained equimolar amounts of D-glucose, *N*-acetylgalactosamine and phosphate, and was readily hydrolysed under gentle acidic conditions to a phosphorylated disaccharide. 3. Chemical and enzymic degradation indicated that this was 3-*O*- α -D-glucopyranosyl-*N*-acetylgalactosamine with a phosphomonoester group at the 6-position on the glucose. 4. Related degradation of the polymer itself indicated that the repeating structure was the disaccharide with a phosphodiester residue joining the 1-position on galactosamine to the 6-position on glucose in a neighbouring unit. This polymer is thus another example of the increasing number of microbial wall polymers or teichoic acids possessing sugar 1-phosphate linkages.

Polysaccharides, teichoic acids and related polymers containing glycosyl 1-phosphate residues are now recognized as common components of the walls of micro-organisms (Archibald, Baddiley, Button, Heptinstall & Stafford, 1968b). They include phosphomannans (Wickerham & Burton, 1962) and phosphogalactans (Slodki, 1966) in yeasts, glycerol teichoic acids possessing *N*-acetylglucosamine 1-phosphate in the polymer chain (Archibald *et al.* 1968b; Archibald, Baddiley & Button, 1968a) in micrococci, and a polymer of *N*-acetylglucosamine 1-phosphate in micrococci (Archibald *et al.* 1968b). In a survey of wall polymers in micrococci (J. Baddiley, A. L. Davison & M. D. Partridge, unpublished work) several examples of this kind of polymer were observed, and the present paper describes a polymer of glucosyl-*N*-acetylgalactosamine 1-phosphate that is a major component of the walls of *Micrococcus* sp. A1; most of the structural features of the polymers have been established.

EXPERIMENTAL

Materials and methods

Materials. Calf intestinal phosphomonoesterase, wheat germ (acid) phosphatase and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. DEAE-cellulose was obtained from the Worthington Chemical Corp., Freehold, N.J., U.S.A. α -D-Glucosidase was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.

Growth of organism and preparation of walls. *Micrococcus* sp. A1 isolated from cheese oxidized glucose but not lactose or mannitol. This non-halotolerant strain produced acetoin and belonged to Baird-Parker subgroup 1 (Baird-Parker, 1965). Cocci were grown in 10-litre batches under forced aeration for 16 h at 37°C as described by Davison (1968) in a liquid medium with the following composition: Oxoid Nutrient Broth no. 2, 2.5%; Difco Yeast Extract, 0.5%; glucose (A.R.), 1.0%; K₂HPO₄, 0.5%; polypropylene glycol 2000 (Shell Chemicals U.K. Ltd., London S.E.1, U.K.).

Cells were harvested with a Sharples refrigerated centrifuge and washed with cold 0.85% NaCl solution. Disruption was carried out in aqueous suspension (30-50%) with no. 11 Ballotini beads in a MSK-Braun cell disintegrator for 2.5 min. Walls were recovered by centrifugation at 20 000g for 20 min in a refrigerated centrifuge. They were washed several times with 0.85% NaCl solution and then water and separated by centrifugation from unbroken cells (lower layer). Cleanliness of the wall preparation was confirmed by electron microscopy; it was stored in the freeze-dried state.

Extraction of polymer. Freeze-dried walls were suspended in 5% (w/v) trichloroacetic acid for 24 h at 4°C. The suspension was centrifuged at 20 000g for 20 min under refrigeration and to the supernatant was added 5 vol. of ethanol. After 24 h at 4°C the precipitate was collected by centrifugation. It was triturated with trichloroacetic acid solution, insoluble material was removed by centrifugation and the polymer was reprecipitated with ethanol. The product was washed successively with acetone, ethanol and ether and dried *in vacuo*.

Paper chromatography. Whatman no. 1, no. 4 and 3MM papers, previously washed with solvent A, were used for chromatography. The following solvent systems

were employed: *A*, propan-1-ol-aq. NH_3 (sp. gr. 0.88)-water (6:3:1, by vol.) descending; *B*, the same system as solvent *A* ascending; *C*, butan-1-ol-pyridine-water (6:4:3, by vol.) descending; *D*, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.) descending; *E*, methylCellosolve-ethylmethylketone-aq. 3M- NH_3 saturated with boric acid (7:2:3, by vol.) descending; *F*, butan-1-ol-acetic acid-water (4:1:1, by vol.) descending.

Compounds were detected by the following spray reagents: (a) periodate-Schiff for α -glycols (Baddiley, Buchanan, Handschumacher & Prescott, 1956); (b) molybdate for phosphates (Hanes & Isherwood, 1949); (c) ninhydrin for amino compounds (Consden & Gordon, 1948); (d) alkaline AgNO_3 for reducing compounds (Trevelyan, Procter & Harrison, 1950); (e) aniline phthalate for pentoses and hexoses (Partridge, 1949); (f) Morgan-Elson for amino sugars and *N*-acetyl derivatives (Partridge, 1948).

Analytical methods. Phosphate was determined by the method of Chen, Toribara & Warner (1956), glucose by Glucostat, reducing sugars by the method of Park & Johnson (1949), amino compounds by the method of Rosen (1957), glucose 6-phosphate by the use of glucose 6-phosphate dehydrogenase (Horecker & Wood, 1957), periodate oxidation by the method of Dixon & Lipkin (1954) with the correction for iodate absorption (Aspinall & Ferrier, 1957), and hexose by the phenol- H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956).

Chromatography on DEAE-cellulose. The material extracted from walls was purified by ion-exchange chromatography. A solution of the polymer (100mg) in water was applied to a column (15mm $\times$ 300mm) of DEAE-cellulose (CO_3^{2-} form) prepared by the procedure described by Tener, Khorana, Markham & Pol (1958). An ammonium carbonate gradient (0–0.5M) was used for elution and fractions (2.5ml) were collected automatically; phosphorus content and extinction at 260nm were measured on samples of eluate. Material that absorbed light at 260nm was eluted rapidly (fractions 0–20), and nearly all of the phosphate on the column, representing the polymer, was eluted as a sharp band (fractions 55–70). The appropriate fractions were combined and freeze-dried.

The material had $[\alpha]_D +44^\circ$ (c 0.9 in water) and on i.r. spectroscopy in a KBr disc gave absorption bands at 3400cm^{-1} and 1645 and 1555cm^{-1} , corresponding to hydroxyl and amide groups respectively. It contained 6.5% of phosphorus, and examination on the amino acid analyser revealed only traces of wall amino acids.

Examination of the polymer

Hydrolysis with 2M-hydrochloric acid. A sample (3mg) of the polymer was hydrolysed in 2M-HCl (0.3ml) for 3h at 100°C in a sealed tube. Acid was removed *in vacuo* over KOH and the residue dissolved in a little water. Chromatography in solvents *B* and *C* showed the products glucose, galactosamine and a glucose phosphate with $R_{\text{glycerol 1-phosphate}}$ 1.1 in solvent *B*. A larger sample was hydrolysed and run as a band in solvent *B*. Two bands were formed; one that had moved 188mm from the origin reacted with reagents *c* and *d*, and rechromatography in solvent *C* gave spots corresponding to glucose and galactosamine. The identity of the galactosamine was confirmed by ninhydrin degradation to lyxose, indistinguishable from

authentic lyxose in solvent *F* (Stoffyn & Jeanloz, 1954). The other band had moved 56mm from the origin and contained phosphate. Material was eluted with water and treated with intestinal phosphomonoesterase in 0.1M-ammonium carbonate buffer (10mg of enzyme/ml) at pH 9.3 for 10h at 37°C . Chromatography of products in solvents *C* and *D* showed that all of the phosphate had been hydrolysed to inorganic phosphate and glucose. Chromatography of the glucose phosphate in solvent *E* during 2 days alongside authentic glucose 1-phosphate and glucose 6-phosphate showed that the product ran coincidentally with the 6-phosphate. All of the phosphorus of this product analysed as glucose 6-phosphate in the quantitative procedure with glucose 6-phosphate dehydrogenase.

Quantitative analysis of an acid hydrolysate indicated that the polymer contained aminohexose, hexose and phosphate in the proportions 1.1:1.2:1.0. All of the hexose was D-glucose (Glucostat).

Hydrolysis with 0.1M-hydrochloric acid. The polymer (8mg) was hydrolysed with 0.1M-HCl (1.5ml) at 100°C for 30min, and the solution neutralized and evaporated *in vacuo* over KOH. Only one product was detected after chromatography in solvent *B* and this gave reactions for a reducing sugar phosphate. The product was eluted with water and treated with the phosphatase as described above. All of the phosphorus was converted into inorganic phosphate, and acid hydrolysis of the dephosphorylated material gave glucose and galactosamine. The identity of the latter product was confirmed by ninhydrin degradation to lyxose.

Hydrolysis with formic acid. The polymer (20mg) was hydrolysed with aq. 66% (v/v) formic acid (0.6ml) at 100°C for 15min. Acid was removed *in vacuo* and a few drops of dilute aq. NH_3 were added to the residue to neutralize traces of acid; ammonium formate was then removed by evaporation *in vacuo*. A single reducing sugar phosphate was produced; it is described below as the 'chemical repeating unit' of the polymer.

Glucosyl-*N*-acetylgalactosamine. The 'chemical repeating unit' above was dephosphorylated with the wheat germ acid phosphatase. The material from the hydrolysis with formic acid was dissolved in 0.1M-ammonium acetate buffer, pH 4.8, with the enzyme at a concentration of 1mg/ml. After evaporation to dryness *in vacuo* the residue was dissolved in a little water and purified by paper chromatography in solvent *C*.

The glucosyl-*N*-acetylgalactosamine had R_{glucose} 0.77 and $R_{\text{glucosamine}}$ 0.91 in solvent *C*. It reacted readily with the Morgan-Elson spray reagents.

Action of alkali on the 'chemical repeating unit'. A sample was treated with 2M-NaOH solution at 100°C for 2h. After removal of alkali by passage through a short column of Dowex 50 (NH_4^+ form) resin, the material was chromatographed in solvent *A*; glucometasaccharinic acid, galactometasaccharinic acid and maltose that had been treated with alkali (isosaccharinic acid) were examined simultaneously. The reaction mixture gave two spots (spray reagent *a*) with $R_{\text{glucometasaccharinic acid}}$ 1.0 and 0.21; it was noted that glucometasaccharinic acid and galactometasaccharinic acid did not separate in this solvent, but they were well separated from the isosaccharinic acid. Electrophoresis (3V/mm) of the mixture on paper soaked in pyridine-acetic acid buffer, pH 6.5, alongside authentic glucometasaccharinic acid and its

6-phosphate (obtained by the action of alkali on glucose 6-phosphate) confirmed that the mixture contained a metasaccharinic acid and a metasaccharinic acid phosphate; they migrated respectively 163 and 235mm towards the anode.

Action of alkali on glucosyl-N-acetyl-galactosamine. The disaccharide was treated with 2M-NaOH at 100°C for 2 h. After removal of alkali by passage through a short column of Dowex 50 (NH₄⁺ form) resin the products were examined by chromatography in solvent *A*. Only one product, R_{glycerol} 0.56, rapidly giving a purple colour with spray reagent *a*, was observed. This colour reaction is characteristic of compounds that give formaldehyde on oxidation with periodate. The identity of the product with metasaccharinic acid was shown by co-chromatography with an authentic sample; glucoisosaccharinic acid, prepared by the action of alkali on maltose, had R_{glycerol} 0.58 in solvent *A*, and gave a yellow colour with the periodate-Schiff spray reagent.

Action of alkali on the polymer. The polymer reacted with alkali giving the same products that were obtained from the action of alkali on the 'chemical repeating unit'. Experimental conditions and the identification of metasaccharinic acid and its 6-phosphate were as given above.

Oxidation of the polymer with periodate. The polymer (10mg) was mixed with 20mM-NaIO₄ (5ml) at room temperature in the dark. Samples (50 μ l) were removed at intervals and diluted to 10ml, and the periodate that had been reduced was determined spectrophotometrically. Oxidation was almost complete after 22 h and no further oxidation was observed after 46 h. The polymer reduced 1.95 mol of periodate/mol of phosphate.

A sample (20mg) of the polymer was oxidized with 20mM-NaIO₄ (10ml) in the dark at room temperature for 80 h. Excess of periodate was destroyed by the addition of 20mM-ethylene glycol solution (10ml), and after 2 h the resulting solution was mixed with a 1% solution (3ml) of freshly distilled *NN*-dimethylhydrazine that had been adjusted to pH 6. The solution was kept at 37°C overnight and coloured products were removed by extraction with chloroform. Salts were removed by passage through a short column of Dowex 50 (NH₄⁺ form) resin followed by evaporation to dryness *in vacuo*. Products were examined by paper chromatography in solvent *B*. Two phosphates, $R_{\text{glycerol 1-phosphate}}$ 1.03 and 0.65, were detected. These were isolated by band chromatography on the remaining solution; the faster-running product, on dephosphorylation with the alkali phosphatase under conditions described above, yielded about 10% of its phosphorus as inorganic phosphate, whereas the slower-running product was completely dephosphorylated by the enzyme. Hydrolysis of both phosphates with 2M-HCl gave inorganic phosphate and galactosamine. Glucose residues in the polymer had been completely destroyed.

Degradation of the polymer with periodate, borohydride and acid. A sample (10mg) of the polymer was oxidized with 0.1M-NaIO₄ (2ml) in the dark at room temperature for 30 h. NaBH₄ (20mg) was added and the solution was kept overnight. Excess of borohydride was destroyed by the dropwise addition of dilute aq. acetic acid to pH 6. The resulting solution was passed through a short column of Dowex 50 (H⁺ form) resin and the eluate was evaporated to dryness *in vacuo*. The residue was evaporated five times with methanol, and then hydrolysed in 2M-HCl

(2ml) at 100°C for 3 h. Products were isolated by preparative chromatography in solvent *B*. The glycerol phosphates did not separate from each other, had indistinguishable chromatographic properties from authentic glycerol 1-phosphate and were dephosphorylated to glycerol by the alkali phosphatase. The other product was galactosamine; it was indistinguishable from an authentic sample in solvent *C* and was degraded to lyxose with ninhydrin.

Apparent chain length of polymer. The polymer (2.2mg) in water (2ml) was incubated at 37°C for 12 h with a solution (0.33ml) of intestinal phosphomonoesterase (10mg/ml) in 0.1M-ammonium carbonate. The amount of inorganic phosphate in the solution was determined and compared with the total phosphate. The apparent chain length, i.e. total phosphate/inorganic phosphate ratio, was about 6.

Action of glucosidases on glucosyl-N-acetyl-galactosamine. A sample (2mg) of the disaccharide was dissolved in a few drops of water and an aq. 2% (w/v) solution (0.2ml) of α -D-glucosidase was added. After incubation for 15 h at 37°C the solution was evaporated to dryness *in vacuo* and the residue was examined by paper chromatography in solvent *C* (reagent *d* and development with steaming). Control experiments with methyl α -D-glucoside were carried out. The disaccharide gave glucose and *N*-acetyl-galactosamine, identified by cochromatography with authentic materials, and the methyl glucoside gave glucose. Similar experiments with β -D-glucosidase brought about no hydrolysis of the glycosides.

RESULTS AND DISCUSSION

The walls of *Micrococcus* sp. A1 contain 2.8% of phosphorus, whereas the extracted polymer, which is the only known phosphorylated compound in the wall, contains 6.5% of phosphorus. Thus the polymer comprises about 43% of the wall. It was extracted with cold dilute trichloroacetic acid, but, as acid-labile sugar 1-phosphate linkages occur in the polymer chain, it is likely that the conditions of extraction caused partial degradation of the material. The precipitated polymer contained small quantities of wall amino acids and other impurities that could be removed by chromatography on DEAE-cellulose.

The purified polymer did not contain amino acids, and on hydrolysis with 2M-hydrochloric acid it gave glucose, a glucose phosphate, galactosamine and inorganic phosphate. Quantitative analysis indicated a molecule comprising D-glucose, galactosamine and phosphate in the proportions 1:1:1. The galactosamine was characterized chromatographically and by degradation with ninhydrin to lyxose; the glucose phosphate was identified as glucose 6-phosphate by chromatography and by the use of glucose 6-phosphate dehydrogenase. The occurrence in the polymer of a linkage between phosphate and a non-reducing position on glucose was thereby established, but, although position 6 on glucose was

likely, that was not certain at this stage because the acidic conditions required to give the glucose phosphate might have caused phosphate migration.

The presence of sugar 1-phosphate linkages in the polymer was established by its ready hydrolysis under gentle acidic conditions. With 0.1M-hydrochloric acid at 100°C for 30 min, or better with 66% formic acid for 15 min, it was completely hydrolysed to a 'chemical repeating unit' containing the same components in unaltered proportions as the original polymer. Whereas most of the phosphorus in the original polymer occurred as phosphodiester and was unaffected by a phosphomonoesterase, in the repeating unit it was all present as phosphomonoester and was completely hydrolysed to inorganic phosphate by the phosphatase. The dephosphorylated product was a reducing disaccharide and gave D-glucose and galactosamine on acid hydrolysis. Although it was unaffected by a β -D-glucosidase, it was readily and completely hydrolysed by an α -D-glucosidase to glucose and *N*-acetylglucosamine.

Further details of the structure of the disaccharide and its phosphate were obtained from a study of their behaviour towards alkali. The disaccharide gave only metasaccharinic acid, whereas the phosphate gave a mixture of metasaccharinic acid and metasaccharinic acid 6-phosphate; the latter compound was indistinguishable from that obtained by the action of alkali on glucose 6-phosphate. These results suggest that the disaccharide is α -D-glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglucosamine, and that the phosphate substituent is at position 6 on glucose. The structure of the repeating unit is given

as (I). It is known that 3-substituted sugars are readily converted by alkali into metasaccharinic acids through β -elimination and rearrangement (Kenner & Richards, 1954), and that 2-acetamido-2-deoxy sugars give the same saccharinic acids with alkali as do their parent sugars (Bemiller & Whistler, 1962). The disaccharide would first undergo a β -elimination to give glucose and galactometasaccharinic acid, and further action of alkali on the glucose would give glucometasaccharinic acid. The repeating unit (I) would be degraded similarly to a mixture of galactometasaccharinic acid and glucometasaccharinic acid 6-phosphate. Under the conditions used for chromatography, glucometasaccharinic acid and galactometasaccharinic acid did not separate from each other, and it was therefore not possible to establish unambiguously which metasaccharinic acids had been formed by the alkali degradation.

The proposed structures for the repeating unit and the disaccharide are supported by their behaviour towards the Morgan-Elson reagent. In both cases chromogen was formed readily in alkali at room temperature, and was also formed under neutral conditions (Knox & Hall, 1965). This very ready formation of chromophore under the alkaline conditions is typical of 3-*O*-substituted sugars, which are known to react more rapidly than do their parent *N*-acetylhexosamines (Kuhn, Gauhe & Baer, 1954); 4-*O*-substituted *N*-acetylglucosamine derivatives are relatively stable. The conclusion that *N*-acetylglucosamine bears a substituent at its 3-position is confirmed by the behaviour of a closely related disaccharide isolated by degradation of carboxyl-reduced chondroitin (Wolfson & Juliano, 1960). This disaccharide was given the structure β -glucosyl-(1 $\rightarrow$ 3)-*N*-acetylglucosamine, thereby differing from that from the micrococcus only in the anomeric configuration of the glycosidic linkage. It is also characterized by its extreme alkali-lability and ready production of colour in the Morgan-Elson reaction; the same disaccharide was also isolated by gentle acid hydrolysis of the specific polysaccharide from *Lactobacillus casei*, serological group C (Knox & Hall, 1965).

The behaviour of the polymer towards oxidation with periodate shows that the repeating unit (I) is in fact a repeating structure in the polymer, and that the linkage between the units is through the phosphate at position 6 on glucose joined to position 1 on *N*-acetylglucosamine of a neighbouring unit, as shown in structure (II). Oxidation occurred readily and smoothly with the reduction of 2 mol of periodate/mol of phosphate. In this oxidation the glucose was destroyed and the *N*-acetylglucosamine was unaffected; it follows that the *N*-acetylglucosamine is substituted at position 3 or 4. Confirmation that the phosphodiester linkages in

the polymer are at position 6 on glucose was obtained by oxidation with periodate, reduction of the product with borohydride, followed by acid hydrolysis to glycerol phosphate(s) (Smith degradation). Oxidation must have occurred between positions 3 and 4, and 4 and 5 in glucose residues.

Oxidation of the polymer with periodate, followed by treatment of the product with *NN*-dimethylhydrazine was used to isolate *N*-acetylgalactosamine 1-phosphate. This modification of the Barry degradation (Barry, 1943) has been applied successfully to teichoic acids and related polymers containing phosphodiester linkages (cf. Dixon, Buchanan & Baddiley, 1966). In the present case the dialdehyde residues formed in the polymer by oxidation of glucose units are destroyed by α -elimination. The product from the polymer (II) should be *N*-acetylgalactosamine 1-phosphate, but under the conditions employed two phosphates were formed. Both yielded galactosamine and inorganic phosphate on acid hydrolysis. One was readily hydrolysed to *N*-acetylgalactosamine and inorganic phosphate by a phosphomonoesterase, and this is presumably the 1-phosphate. The other yielded only about 10% of its phosphate with the enzyme, and we believe that it is a product of incomplete degradation. In neither case was sufficient material obtained for optical-rotation studies to establish the configuration of the glycosyl linkage; however, the biosynthetic studies referred to below in connexion with the nature of the biological repeating unit suggest that the sugar 1-phosphate residues in the polymer originate by transfer from a nucleoside diphosphate sugar and so must possess an α -configuration.

The average apparent chain length of purified polymer that had been extracted from walls was determined by the action of a phosphomonoesterase. By comparing the amount of inorganic phosphate formed with the total phosphate it was concluded that the chains contained one phosphomonoester group in six repeating units, i.e. a chain length of 6. The phosphomonoester groups are presumably attached to glucose residues and represent ends of chains that have been broken during the acid extraction procedure. It is not known how chains terminate in the wall itself, but it seems unlikely that they would terminate in phosphate groups. From consideration of the probable route for the biosynthesis of this polymer it is likely that *N*-acetylgalactosamine 1-phosphate residues are transferred as intact units from nucleotide precursors through lipid intermediates into the polymer chain. This is the route for the synthesis of other wall polymers containing sugar 1-phosphate residues, and is probably a general route (for a review see Baddiley, 1970); preliminary studies (B. Starkey, I. C. Hancock & J. Baddiley, unpublished

work) on the biosynthesis of the polymer from *Micrococcus* sp. A1 agree with this generalization. Thus although the end of the polymer at its point of attachment to peptidoglycan in the wall may well bear a phosphate (as phosphodiester), it is unlikely that a phosphate would also occur at the other end of the chain. These biosynthetic considerations are important in defining the precise repeating structure of the polymer. The structure (II), in which the phosphate group is assigned to position 1 on *N*-acetylgalactosamine, probably represents the true biological repeating unit, whereas the structure (I) is a 'chemical repeating unit' formed by preferential hydrolysis of the most acid-labile linkages in the polymer chain. Thus, since the chains examined in the experiment with the phosphatase must terminate in phosphomonoester groups, they probably represent fragments of polymer.

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REFERENCES

- Archibald, A. R., Baddiley, J. & Button, D. (1968a). *Biochem. J.* **110**, 543.
Archibald, A. R., Baddiley, J., Button, D., Heptinstall, S. & Stafford, G. H. (1968b). *Nature, Lond.*, **219**, 855.
Aspinall, G. O. & Ferrier, R. J. (1957). *Chem. Ind.* p. 1216.
Baddiley, J. (1970). *Accounts chem. Res.* **3**, 98.
Baddiley, J., Buchanan, J. G., Handschumacher, R. E. & Prescott, J. F. (1956). *J. chem. Soc.* p. 2818.
Baird-Parker, A. C. (1965). *J. gen. Microbiol.* **38**, 363.
Barry, V. C. (1943). *Nature, Lond.*, **152**, 537.
Bemiller, J. N. & Whistler, R. L. (1962). *J. org. Chem.* **27**, 1161.
Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
Consden, R. & Gordon, A. H. (1948). *Nature, Lond.*, **162**, 180.
Davison, A. L. (1968). *Biochem. J.* **110**, 557.
Dixon, J. R., Buchanan, J. G. & Baddiley, J. (1966). *Biochem. J.* **100**, 507.
Dixon, J. S. & Lipkin, D. (1954). *Analyt. Chem.* **26**, 1092.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. & Smith, F. (1956). *Analyt. Chem.* **28**, 350.
Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
Horecker, B. L. & Wood, W. A. (1957). In *Methods in Enzymology*, vol. 3, p. 152. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kenner, J. & Richards, G. N. (1954). *J. chem. Soc.* p. 278.
Knox, K. W. & Hall, E. A. (1965). *Biochem. J.* **96**, 302.
Kuhn, R., Gauhe, A. & Baer, H. H. (1954). *Chem. Ber.* **87**, 1138.
Park, J. T. & Johnson, M. J. (1949). *J. biol. Chem.* **181**, 149.
Partridge, S. M. (1948). *Biochem. J.* **42**, 238.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.

- Rosen, H. (1957). *Archs Biochem. Biophys.* **67**, 10.
Slodki, M. E. (1966). *J. biol. Chem.* **241**, 2700.
Stoffyn, P. J. & Jeanloz, R. W. (1954). *J. Am. chem. Soc.* **76**, 561.
Tener, G. M., Khorana, H. G., Markham, R. & Pol, E. H. (1958). *J. Am. chem. Soc.* **80**, 6223.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 440.
Wickerham, L. J. & Burton, K. A. (1962). *Bact. Rev.* **26**, 382.
Wolfson, M. C. & Juliano, B. O. (1960). *J. Am. chem. Soc.* **82**, 1673.