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The Mechanism of Carbohydrase Action

8. STRUCTURES OF THE MUSCLE-PHOSPHORYLASE LIMIT DEXTRINS OF GLYCOGEN AND AMYLOPECTIN

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Cori & Larner (1951) described the preparation from rabbit muscle of an enzyme splitting the α-1:6-bonds of glycogen and amylopectin. This enzyme, amylo-1:6-glucosidase, did not act on the native polysaccharides but on the muscle-phosphorylase limit dextrins (φ-dextrins), from which glucose was liberated, the dextrins then becoming susceptible to further phosphorolysis. They concluded that in the φ-dextrin the side chains (*A* chains; Peat, Whelan & Thomas, 1952) were reduced in length to single glucose units, and that the outer portions of the main chains (*B* chains) were five to six units long, an estimate which was revised to six or seven units by Larner, Illingworth, Cori & Cori (1952) (Fig. 1). The unequal attenuation of the *A* and *B* chains by muscle phosphorylase is in contrast with the effect of β-amylase, since the β-limit dextrin (β-dextrin) of amylopectin contains *A* and outer *B* chains of equal length (two or three units) (Peat, Whelan & Thomas, 1952; Summer & French, 1956). Accordingly the φ-dextrins of amylopectin and glycogen were reinvestigated to find a reason for these differences. The evidence suggests that the structure of the φ-dextrin proposed by Cori & Larner (1951) is incorrect, in that the *A* chain is not a single glucose unit and that there is no great disparity, if any, between the lengths of the attenuated *A* and outer *B* chains.

A preliminary account of this work has already been published (Walker & Whelan, 1959*a*).

MATERIALS AND METHODS

Substrates

Waxy-maize starch (amylopectin) was prepared from hand-sorted single-cross Tapicorn seed (Bear Hybrid Corn Co., Decatur, Ill., U.S.A.) as by Schoch (1957). Rabbit-liver glycogen was prepared as by Schlamovitz (1951). Oyster glycogen was a commercial specimen (British Drug Houses Ltd.). Some properties of these three polysaccharides are listed in Table 1.

Amylopectin φ- and β-dextrin were prepared by incubating enzyme and substrate, as indicated below, for 4 and 24 hr. respectively. The enzymes were inactivated (3 min. at 100°), protein was centrifuged away and the supernatant solutions were deionized with Bio-Deminrolit resin (The Permutit Co. Ltd.) in the carbonate form (Woolf, 1953) before being dialysed in cellophan under toluene against running distilled water at room temperature for 48 hr. Where necessary the non-diffusate was concentrated in a rotary vacuum evaporator.

Maltotriose was prepared as by Peat, Whelan & Kroll (1956). Maltose (T. Kerfoot Ltd., Vale of Bardsley, Lancs.) was purified on charcoal–Celite (Whelan, Bailey & Roberts, 1953) to remove a possible trisaccharide impurity. Glucose was AnalaR-grade.

The concentrations of oligo- and poly-saccharide solutions were estimated by acid hydrolysis to glucose (Pirt & Whelan, 1951).

Enzymes

R-Enzyme. The enzyme was prepared from broad beans as by Hobson, Whelan & Peat (1951), except that, at the stage of removal of other enzymes by their adsorption on starch, the suspension was shaken for 24 hr. rather than stirred. This resulted in a more consistent preparation.

Amylo-1:6-*glucosidase.* The method of Cori (1955) was used as far as step 4. The enzyme was active on amylopectin φ-dextrin.

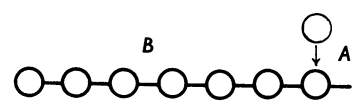

Fig. 1. Unit structure of muscle-phosphorylase limit dextrin (φ-dextrin) proposed by Cori & Larner (1951) and Larner, Illingworth, Cori & Cori (1952). ○, Glucose unit; —, α-1:4-link; ↓, α-1:6-link.

Table 1. *Properties of waxy-maize amylopectin, rabbit-liver glycogen and oyster glycogen*

	Average unit-chain length	Phosphorolysis (%)*	β-Amylolysis (%)†
Waxy-maize starch	24	43	53 (2)
Rabbit-liver glycogen	14	28	47 (21)
Oyster glycogen	11	20	39 (47)

* Conversion into glucose 1-phosphate.
† Conversion into maltose. Figures in parentheses indicate the time (hr.) taken to reach limiting conversion.

α-Amylase. Human salivary α-amylase was isolated as by Fischer & Stein (1954) and crystallized three times.

β-Amylase. The twice-crystallized sweet-potato enzyme was purchased from the Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Phosphorylases a *and* b. Phosphorylase *b* was prepared from rabbit muscle as by Fischer, Krebs & Kent (1958) and crystallized four times. It was converted into phosphorylase *a* (Fischer *et al.* 1958), which was also crystallized four times.

Enzyme assays

α-Amylase. The method of Schwimmer (1947) and Schwimmer & Balls (1949) for malt α-amylase was adapted for salivary α-amylase as by Walker & Whelan (1960).

β-Amylase. The activity was measured as by Hobson, Whelan & Peat (1950). Some difficulty was encountered with this enzyme owing to its instability. The suspension of crystals in ammonium sulphate supplied by the manufacturer (see above) was stable for several months in the refrigerator. Solutions made by diluting 0·1 ml. of suspension with 10 ml. of water (β_1 solution, 4000 units/ml.) lost about one-third of their activity in 1 day at 0°, and more dilute solutions (β_2 solution, 0·1 ml. of β_1 solutions with 1·9 ml. of water, 200 units/ml.) lost all activity in 1 hr. at 35°. This loss of activity was prevented by the addition of glutathione to 0·5 mM final concentration (Englard, Sorof & Singer, 1951). The solution then lost little activity during several days at 35°. When such solutions were diluted a further 20 times (β_3 solution, 10 units/ml.) the enzyme again became unstable, losing all activity in 4 hr. at 35°, and glutathione up to 5 mM-concentration was completely ineffective in preventing this. Freeze-dried human-serum albumin, in 0·05% concentration in β_3 solution, rendered the enzyme completely stable for 18 hr. at 35°. The albumin had no amylase activity. As a routine, therefore, 0·5 mM-glutathione was added to stock β_1 and β_2 solutions, which could then be stored for several weeks in the refrigerator. Serum albumin (0·05%) was added to all digests, irrespective of enzyme concentration, but no stabilizing agents were added to amylase-assay digests, for no loss of activity occurred during the 30 min. incubation period. In these digests the enzyme concentration is about 0·5 unit/ml. and the starch substrate presumably rendered the enzyme stable, for in the absence of starch there was an appreciable decay in activity in 30 min. The loss of activity in solution is not unexpected with low concentrations of a crystalline enzyme containing sulphydryl groups. On the other hand, in previous work with a several-times-crystallized enzyme prepared by Dr A. K. Balls (Balls, Walden & Thompson, 1948), it was not necessary to use stabilizing substances [see Table 3 in Peat, Pirt & Whelan (1952)].

Phosphorylase a. The method of Cori, Illingworth & Keller (1955) was used, except that adenylic acid was not added to the digest. Phosphorylase *a* prepared as by Fischer *et al.* (1958) is not activated by adenylic acid, in contrast with phosphorylase *a* prepared as by Cori *et al.* (1955).

Enzyme–substrate digestions

All digestions were at 35°, except where otherwise stated. Digests incubated overnight or longer were covered with toluene. Substrate-free controls were used to determine the reducing powers of the non-carbohydrate components. Enzymes were inactivated by heating for 3 min. at 100°. Reducing power was estimated with the Shaffer & Hartmann (1921) copper reagent. Glucose (60 mg./l.) was added to the reagent to compensate for loss of cuprous oxide by re-oxidation.

Action of R-enzyme. The digests contained 0·16% of polysaccharide, 55 mM-citric acid–NaOH buffer (pH 6·5) and R-enzyme (18 mg./ml.). For chromatographic examination the reaction was stopped after 3 hr. For determination of the increase in degree of β-amylolysis of the polysaccharide after debranching, the reaction was stopped after 24 hr. In control experiments with amylopectin under these conditions the increase in intensity of iodine stain consequent on R-enzyme action (Hobson *et al.* 1951) was complete after 24 hr. and no further change took place in the subsequent 24 hr. The extinction (680 mμ) of the stain at 24 hr. was 138% of its original value, having been 122% at 3 hr. Similarly, the degree of β-amylolysis of amylopectin β-dextrin rose from 0 to 98% after 22·5 hr. incubation with R-enzyme and remained at this value after 52·5 hr. When the debranching action was to be followed by β-amylolysis, the R-enzyme was inactivated and the digest adjusted to pH 5·5 with 0·2 M-sodium acetate–acetic acid buffer, pH 4·7.

*Action of amylo-*1:6*-glucosidase.* The enzyme solution (0·1 ml.) was incubated separately with maltotriose, maltose and glucose (10 mg., 0·5 ml.) for 24 hr. at room temperature. After the enzyme had been inactivated the digests were applied to paper for chromatography.

α-Amylolyses. Polysaccharides were hydrolysed with two concentrations of crystalline salivary α-amylase, as by Walker & Whelan (1960). The concentrations of the digest components were 0·4% of polysaccharide, 0·1 M-NaCl, 30 mM-citric acid–NaOH buffer (pH 6·5) and enzyme (0·25 or 0·0025 unit/ml.). The reducing power of the digests became constant after 48 hr.

β-Amylolyses. The digests contained 0·4% of polysaccharide, 0·5 mM-glutathione, 0·05% of serum albumin and 30 mM-sodium acetate–acetic acid buffer (pH 4·8). With debranched polysaccharide as substrate, the enzyme concentration was 10 units/ml. Digestion was complete within 4 hr. and hydrolysis of maltotriose during this time was negligible.

β-Amylolysis of native polysaccharides was carried out as described above but with 1000 units of enzyme/ml. The time taken to achieve limiting β-amylolysis varied with the type of polysaccharide, amylopectin or glycogen (see Table 1).

Phosphorolyses. The crystalline suspension of phosphorylase *a* in 30 mM-cysteine hydrochloride–40 mM-sodium glycerophosphate buffer (pH 6·8) was dissolved in the same buffer (0·2 ml. of suspension/10 ml. of buffer). The digestion mixtures contained 0·4% of polysaccharide, 80 mM-sodium phosphate (pH 6·8) and enzyme (520 units/ml.). The glucose 1-phosphate formed was freed from inorganic phosphate as by Liddle & Manners (1957) and estimated by hydrolysis as by Whelan & Bailey (1954), with Allen's (1940) colorimetric method. The degree of phosphorolysis attained a constant value at 4 hr., remaining unchanged during the subsequent 20 hr.

Paper chromatography

The paper was Whatman no. 3, previously washed in 1% acetic acid and distilled water. Descending chromatograms were irrigated with ethyl acetate–pyridine–water

(10:4:3, by vol.). The spray reagents were silver nitrate–NaOH (Trevelyan, Procter & Harrison, 1950) or aniline–diphenylamine–phosphoric acid (Harris & MacWilliam, 1954). Digests containing citrate buffer were deionized with Bio-Deminrolit resin (see above) before being applied to paper. Other digests were applied directly.

RESULTS AND DISCUSSION

Action of R-enzyme on φ-dextrin

R-Enzyme does not hydrolyse single α-glucose units joined through 1:6-links to starch molecules (Roberts, 1953; Whelan, 1953). The smallest side chain that can be detached is maltose. R-Enzyme should not therefore detach the *A* chains from φ-dextrin, if these are glucose units as postulated by Cori & Larner (1951). The only action of R-enzyme should be to split 1:6-links joining *B* chains to each other. Waxy-maize amylopectin φ-dextrin was treated with R-enzyme and then with β-amylase in order to estimate the extent of debranching. The yield of maltose from the Cori-type φ-dextrin should be 30%, according to calculations from the model φ-dextrin molecule constructed by Larner *et al.* (1952), assuming the average unit-chain length of the φ-dextrin to be 14. [The original amylopectin had chain length 24 and underwent 43% conversion into glucose 1-phosphate during phosphorolysis (Table 1).] The actual conversion into maltose after R-enzyme/β-amylase action was 79%, compared with 86% after the successive actions of R-enzyme and β-amylase on the original amylopectin and 92% from the β-limit dextrin (cf. Peat, Whelan & Thomas, 1956).

Action of α-amylase on φ-dextrin

When human salivary α-amylase attacks amylopectin and glycogen the reaction can be stopped at either of two stages, depending on the concentration of enzyme (Walker & Whelan, 1960). With a low concentration of enzyme the products are maltose, maltotriose and α-limit dextrins consisting of maltodextrins joined to each other through the α-1:6-branch links of the parent polysaccharide. The smallest of these α-limit dextrins is a pentasaccharide (Fig. 2*a*) (Bines & Whelan, 1960). With a 100-fold greater concentration of enzyme the maltotriose is hydrolysed to maltose and glucose and the α-limit dextrins are correspondingly attenuated, the smallest now being a tetrasaccharide (Nordin & French, 1958). This contains a glucose stub of the type postulated for φ-dextrin (Fig. 2*b*). If therefore these stubs are pre-existent in φ-dextrin, then the low concentration of α-amylase, which by itself cannot form the glucose stub, should nevertheless liberate the tetrasaccharide. The φ-dextrins of amylopectin, rabbit-liver glycogen and oyster glycogen were repeatedly examined by paper chromatography after α-amylolysis to this degree and the pentasaccharide was always the smallest α-limit dextrin (Fig. 3). When the φ-dextrins were treated with the high concentration of α-amylase the tetrasaccharide appeared (Fig. 3).

Products of debranched φ-dextrin

The treatment of amylopectin φ-dextrin with R-enzyme yielded substances behaving chromatographically as maltodextrins, the smallest being maltotetraose (Fig. 3). R-Enzyme would not, of

Fig. 2. α-Limit dextrins from glycogen and amylopectin. ⊘, Reducing glucose unit; other symbols are as identified in Fig. 1.

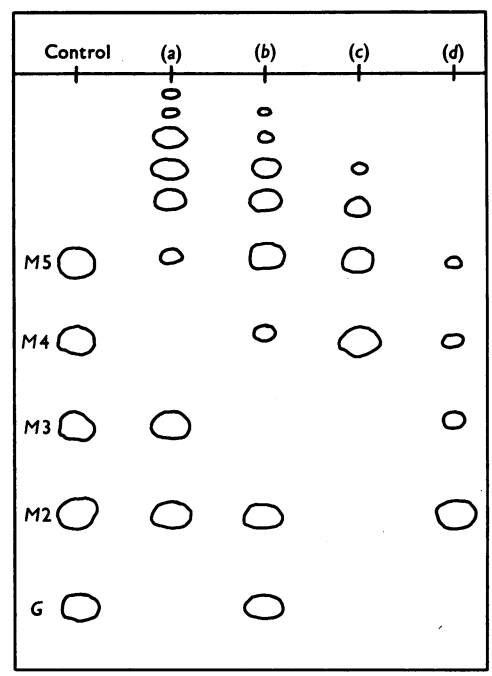

Fig. 3. (*a*) Paper-chromatographic fractionation of the products of action of dilute salivary α-amylase on the φ-dextrins of amylopectin, rabbit-liver and oyster glycogens. (*b*) Products of the action of concentrated α-amylase on the same three substrates. (*c*) Products of the action of R-enzyme on amylopectin φ-dextrin. (*d*) Products of the action of R-enzyme on amylopectin φβ-dextrin. Control substances are glucose (*G*), maltose (*M* 2), maltotriose (*M* 3), etc.

course, liberate glucose stubs if these were present (see above), but since the parent amylopectin with R-enzyme yielded no low-molecular-weight maltodextrins, the maltodextrins formed from the ϕ-dextrin were not preformed in the starch and clearly represented the residues left by phosphorolytic degradation. The smallest maltodextrin formed from the Cori ϕ-dextrin could not be smaller than maltoheptaose (Fig. 1). The ϕ-dextrin was treated with β-amylase and freed from maltose. The resulting $\phi\beta$-dextrin treated with R-enzyme now yielded maltose as a major component, with somewhat smaller amounts of maltotriose and higher maltodextrins (Fig. 3).

Structure of ϕ-dextrin

The foregoing evidence is consistent with an A-chain residue of the ϕ-dextrin at least four units long, and two units long in the $\phi\beta$-dextrin. It does not give direct information about the length of the outer portion of the B chain but this can be deduced from the action of β-amylase on the ϕ-dextrin. Hestrin (1949) found that about 1 mol.-prop. of maltose was liberated by β-amylase per unit chain of the ϕ-dextrin. Cori & Larner (1951) concluded that since the A chains were single glucose units the maltose must arise solely from the B chains and, assuming equal numbers of A and B chains, in the proportion of 2 mol.prop./chain. The A chain in the new formula (Fig. 4) would be attacked by β-amylase, losing 1 mol.prop. of maltose. The B chain must therefore be shorter than was supposed and could be four units long, losing 1 mol.prop. of maltose on β-amylolysis. The attenuation of the A chain and the outer B chain to four units by muscle phosphorylase is analogous to the similar attenuation of linear chains by the potato enzyme (Whelan & Bailey, 1954). Fig. 4 is a tentative picture of the average unit structure of glycogen and amylopectin muscle-phosphorylase limit dextrins. Further evidence for the picture is being sought. If it is correct then the calculation of the degree of multiple branching of amylopectin and glycogen from the degrees of phosphorolysis and β-amylolysis, as suggested by Liddle & Manners (1957), is not possible.

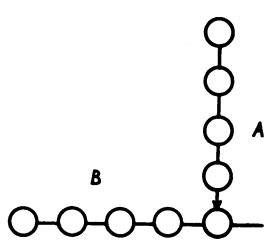

Fig. 4. Unit structure of muscle-phosphorylase limit dextrin proposed by the present authors. Symbols are as identified in Fig. 1.

Debranching action of amylo-1:6-glucosidase

If there are no glucose stubs in ϕ-dextrin then it is not readily apparent why amylo-1:6-glucosidase liberates glucose from the dextrin. An explanation advanced by Whelan (1958) requires that the preparations of amylo-1:6-glucosidase from rabbit muscle are contaminated with a starch-disproportionating enzyme of the type already known to exist in the potato (Peat, Whelan & Rees, 1953, 1956). This is D-enzyme, which is believed to be implicated in potato-starch metabolism (Walker & Whelan, 1959*b*). A similar enzyme is known in rat liver (Giri, Nagabhushanam, Nigam & Belavadi, 1955; Petrova, 1958; Stetten, 1959). D-Enzyme reversibly transfers segments of starch chains to other chains or to acceptors such as glucose and maltose (Peat, Whelan & Jones, 1957). Whelan (1958) suggested that a rabbit D-enzyme could act reversibly on the true ϕ-dextrin (Fig. 4) and convert it into the Cori–Larner molecule (Fig. 1). Only then could the glucosidase split off glucose. Other structures would also be formed by the D-enzyme, but the glucosidase could act only in the particular case where a glucose stub is exposed.

This hypothesis was examined as follows. Amylo-1:6-glucosidase from rabbit muscle was allowed to act on glucose, maltose and maltotriose. It had no action on glucose but maltose was converted into substances migrating on paper with glucose and maltotriose, and maltotriose into glucose, maltose, maltotetraose and higher oligosaccharides.

Support for this explanation of the action of amylo-1:6-glucosidase comes from the observation by Cori & Larner (1951) of a slow action of the enzyme on amylopectin β-limit dextrin. This dextrin contains no glucose stubs of the type postulated in ϕ-dextrin, and the A chains are two or three units long (see above). Therefore the glucosidase should have no action. That it has an action is at variance with the Cori–Larner hypothesis, but is explicable if the enzyme preparation contains a transferase capable of exposing glucose stubs.

The heterogeneity of the amylo-1:6-glucosidase preparation was further revealed in experiments on isomaltose carried out with Mr M. Abdullah. Cori & Larner (1951) reported that amylo-1:6-glucosidase attacks this sugar slowly and regarded the result as proving that the glucosidase attacks the 1:6-links of ϕ-dextrin. We purified the glucosidase as by Cori (1955) and the ammonium sulphate precipitate and supernatant solution at step 2 of the procedure was tested for hydrolytic activity towards isomaltose. The precipitate, said to contain 80% of the glucosidase (Cori, 1955), split

ϕ-dextrin. It was only very weakly active towards isomaltose, whereas the supernatant solution was strongly active. Even if pure amylo-1:6-glucosidase proves to have a slight isomaltose-splitting activity, there is certainly another enzyme in rabbit muscle with much more of this activity; indeed, an enzyme of this kind was found in rabbit muscle by Lukomskaia (1956). Moreover, the corresponding amylopectin-debranching enzyme of plants, R-enzyme, does not attack isomaltose (Hobson *et al.* 1951).

SUMMARY

1. The structures of the limit dextrins produced by the action of rabbit-muscle phosphorylase on glycogen and amylopectin were examined by enzymic methods. The formula previously assigned by Cori & Larner (1951) to the outer chains of the dextrins is incorrect. The side chains are not single glucose units but are probably four units long. Correspondingly the outer portions of the main chains are shorter than was supposed.

2. A preparation of amylo-1:6-glucosidase, the enzyme which splits the branch links in the phosphorylase limit dextrins, proved to be contaminated with a transglycosylase, acting on maltodextrins. The presence of this second enzyme may account for the formation of glucose when the amylo-1:6-glucosidase acts on phosphorylase limit dextrins.

3. The splitting of isomaltose by crude muscle extracts is largely, if not entirely, mediated by an enzyme distinct from amylo-1:6-glucosidase.

4. The instability of weak solutions of crystalline β-amylase was investigated and overcome by the addition of glutathione and serum albumin.

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REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Balls, A. K., Walden, M. K. & Thompson, R. R. (1948). *J. biol. Chem.* **173**, 9.
Bines, B. J. & Whelan, W. J. (1960). *Biochem. J.* **76**, 253
Cori, G. T. (1955). In *Methods in Enzymology*, vol. 1, p. 211. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Cori, G. T., Illingworth, B. & Keller, P. J. (1955). In *Methods in Enzymology*, vol. 1, p. 200. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Cori, G. T. & Larner, J. (1951). *J. biol. Chem.* **188**, 17.
Englard, S., Sorof, S. & Singer, T. P. (1951). *J. biol. Chem.* **189**, 217.
Fischer, E. H., Krebs, E. G. & Kent, A. D. (1958). In *Biochemical Preparations*, vol. 6, p. 68. Ed. by Vestling, C. S. New York: John Wiley and Sons, Inc.
Fischer, E. H. & Stein, E. (1954). *Arch. Sci., Genève*, **7**, 131.
Giri, K. V., Nagabhushanam, A., Nigam, V. N. & Belavadi, B. (1955). *Science*, **121**, 898.
Harris, G. & MacWilliam, I. C. (1954). *Chem. & Ind.* p. 249.
Hestrin, S. (1949). *J. biol. Chem.* **179**, 943.
Hobson, P. N., Whelan, W. J. & Peat, S. (1950). *J. chem. Soc.* p. 3566.
Hobson, P. N., Whelan, W. J. & Peat, S. (1951). *J. chem. Soc.* p. 1451.
Larner, J., Illingworth, B., Cori, G. T. & Cori, C. F. (1952). *J. biol. Chem.* **199**, 641.
Liddle, A. M. & Manners, D. J. (1957). *J. chem. Soc.* p. 4708.
Lukomskaia, I. S. (1956). *Dokl. Akad. Nauk S.S.S.R.*, **109**, 358.
Nordin, P. & French, D. (1958). *J. Amer. chem. Soc.* **80**, 1445.
Peat, S., Pirt, S. J. & Whelan, W. J. (1952). *J. chem. Soc.* p. 705.
Peat, S., Whelan, W. J. & Jones, G. (1957). *J. chem. Soc.* p. 2490.
Peat, S., Whelan, W. J. & Kroll, G. W. F. (1956). *J. chem. Soc.* p. 53.
Peat, S., Whelan, W. J. & Rees, W. R. (1953). *Nature, Lond.*, **172**, 158.
Peat, S., Whelan, W. J. & Rees, W. R. (1956). *J. chem. Soc.* p. 46.
Peat, S., Whelan, W. J. & Thomas, G. J. (1952). *J. chem. Soc.* p. 4546.
Peat, S., Whelan, W. J. & Thomas, G. J. (1956). *J. chem. Soc.* p. 3025.
Petrova, A. N. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, 4–123.
Pirt, S. J. & Whelan, W. J. (1951). *J. Sci. Fd Agric.* **2**, 224.
Roberts, P. J. P. (1953). Ph.D. Thesis: University of Wales.
Schlamovitz, M. (1951). *J. biol. Chem.* **188**, 145.
Schoch, T. J. (1957). In *Methods in Enzymology*, vol. 3, p. 5. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Schwimmer, S. (1947). *Cereal Chem.* **24**, 315.
Schwimmer, S. & Balls, A. K. (1949). *J. biol. Chem.* **179**, 1063.
Shaffer, P. A. & Hartmann, A. F. (1921). *J. biol. Chem.* **45**, 365.
Stetten, M. (1959). *J. Amer. chem. Soc.* **81**, 1437.
Summer, R. & French, D. (1956). *J. biol. Chem.* **222**, 469.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Walker, G. J. & Whelan, W. J. (1959*a*). *Biochem. J.* **73**, 20P.
Walker, G. J. & Whelan, W. J. (1959*b*). *Nature, Lond.*, **183**, 46.
Walker, G. J. & Whelan, W. J. (1960). *Biochem. J.* **76**, 257.
Whelan, W. J. (1953). *Symp. biochem. Soc.* **11**, 17.
Whelan, W. J. (1958). In *Lectures on the Scientific Basis of Medicine*, 1956–57, vol. 6, p. 246. London: Athlone Press.
Whelan, W. J. & Bailey, J. M. (1954). *Biochem. J.* **58**, 560.
Whelan, W. J., Bailey, J. M. & Roberts, P. J. P. (1953). *J. chem. Soc.* p. 1293.
Woolf, L. I. (1953). *Nature, Lond.*, **171**, 841.