10. *The Enzymic Synthesis and Degradation of Starch. Part XX.* The Disproportionating Enzyme (D-Enzyme) of the Potato.*

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It is shown that D-enzyme (*Nature*, 1953, **172**, 158) is different from phosphorylase, R-enzyme, and the amylases. It has not yet been possible to separate D-enzyme from Q-enzyme but it is demonstrated that the branching and the disproportionating activity are due to independent enzymes.

Evidence is presented which shows that D-enzyme is a transglycosylase, transferring two or more glucose units from a maltodextrin substrate to a suitable acceptor and that, in the transfer, only α-1 : 4-glucosidic links are synthesised.

The natural substrates for D-enzyme appear to be the maltodextrins, from maltotriose upwards; neither glucose nor, in all probability, maltose functions as donor substrate. The enzyme is not so selective with respect to the " accepting " molecule.

The anomalous position of maltose in this system is discussed.

IT has been shown (Part XVIII, *J.*, 1953, 1422) that Q-enzyme cannot effect branching of short amylose-type chains, the minimum chain length for rapid action being about 40 glucose units. In a further investigation of potato Q-enzyme we examined the effects of this enzyme preparation on the lower range of maltodextrins, *viz.*, on malto-triose, -tetraose, and -pentaose. In each case, products were synthesised which were stained brown-red by iodine (the substrates are achroic) and paper chromatograms of the digests revealed that

* Part XIX, *J.*, 1954, 4440.

sugars, including glucose, having R_F values higher and lower than that of the substrate had also been formed. Reference will be made later to the striking observation that maltose, the first member of the maltodextrin series, appears not to function as a substrate in this reaction. The iodine-staining products proved to be higher maltodextrins, *i.e.*, to consist of chains of α-1 : 4-linked glucose units only, as they were completely degraded by β-amylase. On β-amylolysis, the Q-enzyme digests became achroic and then contained only three sugars, identified by R_F values as glucose, maltose, and maltotriose. The same observations were made with crude potato juice as the enzyme source, although chromatographic analysis was complicated in this case by the presence of glucose, fructose, and sucrose in the juice.

It was clear that the enzyme system of the potato was capable of disproportionating short-chain linear dextrins into products of lower and higher molecular weight and it became necessary to determine whether this action was due to one of the known starch-metabolising enzymes, *i.e.*, phosphorylase, Q-enzyme, or R-enzyme, or to a hitherto undetected enzyme.

Non-identity of the Disproportionating Factor with Known Enzymes.—The Q-enzyme preparation used contained phosphorylase in small amount and, if a trace of mineral phosphate were also present, the phosphorylase would catalyse reversible degradation of the maltodextrin and formation of glucose 1-phosphate. Because the affinity of the enzyme for maltodextrins increases with the chain-length of the latter, the tendency in the presence of a limited amount of the Cori ester would be towards the synthesis of longer maltodextrin chains. Accordingly, amounts of the Q-enzyme preparation and of a potato phosphorylase equal with respect to phosphorylase activity were separately incubated with maltopentaose and the formation of iodine-staining material was measured. The results (Table 1) showed that the Q-preparation was much more efficient in synthesis than was the phosphorylase, whether or not mineral phosphate was added to the digests. It followed that the synthesis of iodine-staining dextrins by the Q-preparation cannot be ascribed entirely to the phosphorylase which it contains.

A fractionation of potato juice designed to lead to maximum recovery of Q-enzyme led also to maximum recovery of disproportionating activity. Inhibitors also failed to distinguish between the two types of activities : in experiments under the usual conditions (Bailey, Thomas, and Whelan, *Biochem. J.*, 1951, **49**, lvi) mercuric chloride caused roughly equal inhibition of both, and ammonium molybdate had no effect on either. It should be noted that R-enzyme is inhibited by molybdate (Part XVIII, *loc. cit.*) and consequently cannot be identical with the disproportionating enzyme. Despite the difficulty experienced in separating the branching (Q-)activity from the disproportionating (D)-activity evidence was obtained that two independent enzymes were involved. The temperature and pH optima of the two activities were determined on the same Q-preparation, the D-activity by measurement of the development of iodine-staining dextrins from maltopentaose and the Q-activity by the diminution in iodine-staining power of amylose. These measurements clearly distinguish between the two activities (see Figs. 1 and 2) and permit the inference that the disproportionating activity of potato juice is probably due to a hitherto undetected enzyme, for which provisionally the name, D-enzyme, is suggested.

Finally, it should be mentioned that the Q-preparation was virtually free from α- and β-amylase. Thus in a period of action (on maltotetraose) during which the absorption value (A.V.; see *J.*, 1945, 924 for definition) attained a maximum value, the reducing power increased by only 1·3% of its initial value, a clear indication of the absence of hydrolysis. Moreover, the failure to detect maltose in quantity in the digests also indicates the absence of the amylases.

Action of D-Enzyme on Glucose and the Maltodextrins.—Since experiments designed to remove or inhibit Q-enzyme preferentially had failed, the investigations were pursued with the potato preparation of maximum D-activity which, as mentioned above, was also richest in Q-activity. It will be shown later that the Q-enzyme present in this preparation exerts no branching action during the disproportionation.

The action of D-enzyme on glucose and the maltodextrins was investigated by paper chromatography of the digests. There was no effect with glucose as substrate. With

maltose, small amounts of sugar having the R_F value of glucose were detected after 6 hours; in one experiment only, the chromatograms showed the presence of a saccharide having a R_F value slightly higher than that of maltose. Maltotriose was rapidly disproportionated, the products, named in relation to the observed R_F values, were maltopentaose, glucose (both showing strongly after 0·5 hr.), and a small amount of maltotetraose; thereafter, up to the final observations at 21 hours, all these products increased in amount and spots of lower and zero R_F values appeared. Only a trace of maltose was formed, and this only in the last stage of the reaction. Similar observations were made with maltotetraose and maltopentaose as substrates, glucose and a continuous series of maltodextrins of increasing molecular weight from maltotriose upwards being produced. Maltose constitutes a notable exception, only traces being formed.

The Mode of Action of D-Enzyme.—The simplest explanation of these phenomena is that the potato contains a transglycosylase (D-enzyme) which effects the transfer of glucose units either singly or severally from one maltodextrin substrate (the donor) to another

FIG. 1. *Temperature optima of D- and Q-enzymes at* pH 6·7. *D-Enzyme acting on maltohexaose* (○); *Q-enzyme acting on amylose* (●).

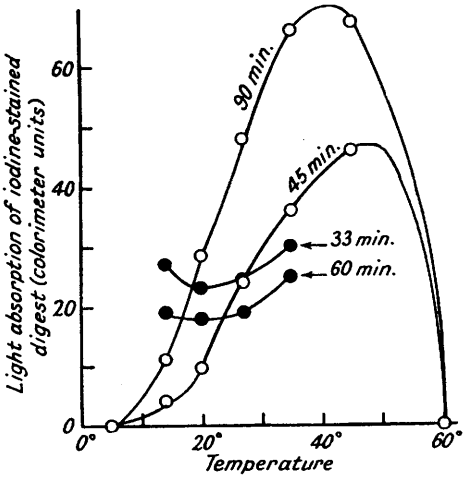
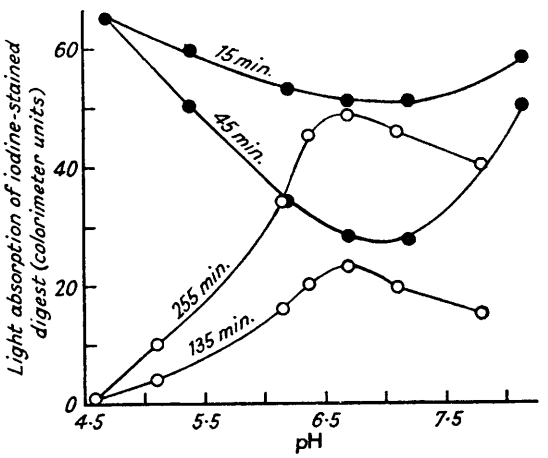


FIG. 2. pH *optima of D- and Q-enzymes at* 15°. *D-Enzyme acting on maltohexaose* (○); *Q-enzyme acting on amylose* (●).



(the acceptor). The indication that only α-1 : 4-links are synthesised in this transfer was strengthened on studying amylolysis of the products of the action of D-enzyme on maltodextrins. (*a*) Maltotetraose was treated successively with D-enzyme and salivary α-amylase and the products were fractionated on a charcoal–Celite column. Three sugars were isolated and identified by chromatographic behaviour on paper as glucose, maltose, and maltotriose, in a combined yield equivalent to 97%. These three sugars, and these alone, are to be expected as products of the α-amylolysis of mixtures of glucose and linear maltodextrins (Whelan and Roberts, *J.*, 1953, 1298). (*b*) It is known that the crystalline β-amylase of sweet potato rapidly degrades maltotetraose and higher maltodextrins but hydrolyses maltotriose only very slowly to give glucose and maltose (Whelan, Bailey, and Roberts, *J.*, 1953, 1293). Chromatographic comparison of the action of β-amylase on maltotriose with that of a mixture of D-enzyme and β-amylase revealed that, whereas β-amylase alone had a barely perceptible effect even after 17 hours, the mixture of enzymes had in 4 hours completely converted the maltotriose into maltose and glucose. This is explicable if D-enzyme converts maltotriose into a mixture of glucose and maltodextrins higher in the series than maltotriose; β-amylase rapidly hydrolyses the higher dextrins to maltose and maltotriose (Whelan and Roberts, *Biochem. J.*, 1954, 58), the latter product acting as further substrate for D-enzyme. The net result of the simultaneous action of D-enzyme and β-amylase is the quantitative conversion of maltotriose into maltose and glucose, as was found.

In another context this experiment is of value inasmuch as it suggests that the slow action of a crystalline β-amylase preparation on maltotriose could be due to its containing a trace of D-enzyme and that enzymically pure β-amylase does not attack maltotriose.

The ultimate result of the action of D-enzyme on a maltodextrin is the establishment of an equilibrated mixture of maltodextrins (excluding maltose) and glucose. Thus, when maltohexaose was digested with D-enzyme, iodine-staining products were formed and the A.V. of the digest rose to a value which remained constant during the period of the experiment (18 hours; see Fig. 3). This experiment incidentally confirms the views that D-enzyme is not a hydrolytic enzyme and that the D-preparation used did not contain α- or β-amylase. Further, it shows that the average chain length of the maltodextrin products of D-enzyme action varies directly with the initial concentration of the maltohexaose substrate since the addition of more maltohexaose to a digest in thich the A.V. had reached a constant value caused a further increase in intensity of staining. It also follows that the apparent cessation of action is not due to inactivation of the enzyme.

The Rôle of Glucose.—There were indications that, in the transglycosylation, glucose cannot act as a donor (*i.e.*, is not itself " transferred " by the enzyme to an acceptor) but can " accept " glycosyl radicals, and it is to be expected therefore that its addition to an equilibrated digest would, as a result of transfer to the added glucose, diminish the average

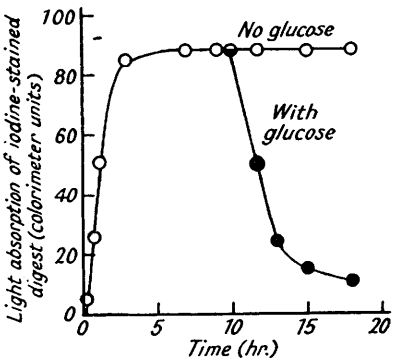


FIG. 3. *The effect of glucose on the equilibrated system D-enzyme + maltohexaose. The digest was divided at* 10 *hr. and glucose added to one portion. Incubation was at* 20°.

chain-length of the maltodextrins present. This inference was confirmed by experiment. When glucose was added to a digest of maltohexaose by D-enzyme which had attained equilibrium, the digest became achroic (Fig. 3). Paper-chromatographic comparison with a portion of the digest to which glucose had not been added revealed that the glucose-treated digest contained a much higher proportion of dextrins of low degree of polymerisation (DP). It was established that some of the added glucose was incorporated into the maltodextrins formed, by a repetition of this experiment with uniformly labelled [^{14}C]-glucose. Determination of the radioactivity associated with the sugar spots on a paper chromatogram showed each of the dextrins to be appreciably radioactive. A control experiment demonstrated that a non-enzymic exchange of radioactivity had not occurred.

Since the addition of glucose caused a fall in the average degree of polymerisation of the maltodextrins at equilibrium, it was to be expected that the removal of glucose from the equilibrated digest would have the reverse effect and increase the average chain length. Attempts to remove glucose with notatin failed owing to the presence of amylases in the two available specimens of glucose oxidase, but experimental confirmation was obtained in another way. Maltohexaose was incubated with D-enzyme, glucose and other products of low molecular weight were removed by dialysis, and the non-dialysable maltodextrins were isolated. This dextrin mixture was stained red by iodine, did not migrate on a paper chromatogram, and yielded maltose and maltotriose only when treated with β-amylase. Measurements of reducing power showed its average chain length to be 12 units. When this synthetic 12-unit dextrin was again digested with D-enzyme, the iodine stain doubled

in intensity and changed from red to purple, indicating the formation of yet longer-chain dextrins. It is to be remembered that our D-enzyme preparation is in fact a purified Q-enzyme fraction of potato juice, and in the experiment just described there was a suggestion that the Q-enzyme might have exerted some branching action on the 12-unit dextrin, inasmuch as a constant maximum A.V. was not maintained. This matter can be decided only when a D-enzyme free from Q-enzyme has been prepared and studies are proceeding with this object in view.

Glucose added to a digest of the synthetic 12-unit dextrin by D-enzyme had its usual A.V.-lowering effect and the digest became achroic. A comparison was made with the effect of D-enzyme on amylopectin, the outer chains of which average 12 glucose units in length. D-Enzyme lowered the A.V. of amylopectin even before glucose was added, but glucose addition markedly accelerated the rate of fall of A.V. This action on amylopectin could not have been due to the debranching action of R-enzyme contained in the D(Q)-preparation since the effect persisted in the presence of molybdate (cf. Part XIV, *J.*, 1951, 1451; Part XIX, *J.*, 1954, 4440). It may be that the fall of A.V. observed before the addition of glucose was due to further branching of the outer chains of amylopectin by Q-enzyme, but the acceleration of the fall of A.V. by glucose addition rather suggests that it is the D-enzyme which is responsible for the phenomenon and that when glucose is present it acts as acceptor for fragments of the outer chains of amylopectin transferred by D-enzyme. This view is supported by the observation that maltotriose was formed from amylopectin when glucose was present but not in its absence.

Other Acceptors and Donors.—That saccharides other than glucose can function as acceptors is indicated by the observation that methyl α-D-glucoside, maltose, mannose, and xylose each caused a diminution in the A.V. of a digest of maltopentaose with D-enzyme (at equilibrium), the efficiencies of these sugars relative to glucose (=100) being 44, 20, 17, and 15 respectively. On the other hand, fructose, sucrose, and αα-trehalose are not acceptors, having no influence on the iodine-staining power of the digest.

The natural substrates for D-enzyme appear to be the maltodextrins, from maltotriose upwards. The power to act as donors (*i.e.*, as substrates for D-enzyme) of 16 sugars and sugar derivatives was tested and of these only one was attacked by D-enzyme. The full list is given in the Experimental section but they included glucose, methyl α-D-glucoside, maltose, *iso*maltose, and cellobiose. The exception was panose (6-*O*-α-D-glucosylmaltose). When this trisaccharide was treated with D-enzyme, it gave rise to two well-defined spots on a paper chromatogram, one moving in the pentasaccharide region, the other as a disaccharide. A trace of (?)glucose was also present.

The Rôle of Maltose.—It has already been mentioned that maltose, although it can act (inefficiently) as an acceptor, appears not to function as a donor in the transglycosylation catalysed by D-enzyme. Further, maltose is not produced, except in traces, when D-enzyme acts on the higher dextrins. It could be argued that D-enzyme does liberate maltose as a residue but that the latter is so efficient an acceptor that it does not accumulate in sufficient quantity to be detected. This is contradicted however by the observations (i) that maltose has only one-fifth of the efficiency of glucose in lowering the A.V. of D-treated maltopentaose, and (ii) that when an artificial mixture of maltose with higher dextrins was incubated with D-enzyme the maltose did not disappear.

It might further be argued that maltose is in fact a donor substrate for D-enzyme action but that the position of equilibrium is almost entirely on the maltose side. The only conceivable action of D-enzyme on maltose would be to convert it into maltotriose and glucose, the former being further disproportionated by D-enzyme. The addition of β-amylase to such a digest should, by degrading the maltotriose and higher dextrins, disturb the equilibrium in the direction of further disproportionation of the maltose. The net result of this sequence of reactions would be to convert the maltose into glucose. When, however, maltose was incubated with D-enzyme and β-amylase no such conversion into glucose occurred. It must be concluded that maltose cannot function as a donor in the D-enzyme system.

Discussion.—It appears from the foregoing that D-enzyme catalyses a transglycosylation, the substrate on which it acts (the donor) being a linear chain of glucose units joined

by α-1 : 4-linkages (exemplified by the maltodextrins). The enzyme shows marked specificity within the group of maltodextrins, a minimum chain-length of 3 being necessary in the substrate. Maltose appears not to be a donor. D-Enzyme, by the scission of an α-1 : 4-link, splits a maltodextrin chain into two fragments and transfers one of the fragments to an appropriate acceptor which, in the first stage of the reaction, is a molecule of the maltodextrin substrate. The enzyme is not so selective with respect to the acceptor molecule, glucose and maltose as well as the higher maltodextrins and other sugars being capable of functioning in this way. In every case however the junction effected between the transferred glycosyl radical and the acceptor molecule is by an α-1 : 4-glucosidic link. The net result when the enzyme acts on a maltodextrin is to produce a range of products of higher and lower molecular weight. The conclusion that the α-1 : 4-link is the only linkage synthesised in the transfer is based largely but not entirely on paper chromatographic evidence but it is completely confirmed by the work reported in the following paper. In essentials therefore the mode of operation of D-enzyme is the simultaneous breaking and making of α-1 : 4-linkages. The spread of molecular weight when equilibrium has been attained is directly related to the degree of polymerisation of the initial substrate. Thus maltotriose gives products which are stained orange by iodine (and then only when in high concentration) whereas maltotetraose and higher maltodextrins as substrates yield equilibrium mixtures of progressively greater intensities of staining. One explanation of this relation between degree of polymerisation of the initial substrate and the upper limit of chain length of the maltodextrins synthesised might lie in the assumption that equilibrium is reached when glucose is liberated (as a residue) as fast as it disappears (by accepting transferred glycosyl radicals). Since D-enzyme has no hydrolytic action the only source of glucose is the *reducing* end group of the maltodextrin substrate and the proportion of such end groups varies inversely with the degree of polymerisation of the substrate. It follows that an equilibrium concentration of glucose will be attained with less disproportionation of a short- than of a long-chain substrate and the average chain-length of the products will be smaller.

The anomalous position of maltose needs further investigation and this is proceeding. This sugar, although the first member of the maltodextrin series, cannot function as a donor, *i.e.*, its α-1 : 4-link is not attacked by the enzyme. Nevertheless a maltosyl radical can be transferred to an appropriate acceptor as is shown by the observation that the first products of the action of D-enzyme on maltotriose to be detected are glucose and maltopentaose. There is a suggestion here that the smallest glycosyl radical which can be transferred by D-enzyme contains two glucose units and that maltose fails to function as a donor because scission of the α-1 : 4-link would provide a radical for transfer consisting of a single glucose residue. If the usual assumption is made that the enzyme attacks from the non-reducing end of a maltodextrin chain, this statement is equivalent to saying that the terminal linkage at the non-reducing end is not susceptible to scission by D-enzyme.

It is more difficult to explain the non-appearance of maltose as a residual fragment in the transglycosylation process. One "explanation" would lie in the assumption that, for a reason at present unknown, the penultimate link at the *reducing* end of a maltodextrin chain is not susceptible to scission by D-enzyme; maltose could not then be liberated as a residue.

Comparison of D-Enzyme with Other Transglucosylases.—Two other enzymes are known which redistribute α-1 : 4-glucosidic linkages and D-enzyme is distinguishable from each. The amylase of *Bacillus macerans* has long been known to effect the synthesis of *cyclo*dextrins from starch. French, Levine, Norberg, Nordin, Pazur, and Wild (*J. Amer. Chem. Soc.*, 1954, **76**, 2387) have shown that the enzyme also catalyses the transfer of glycosyl radicals from these *cyclo*dextrins and from maltodextrins to acceptor molecules which include glucose, methyl α-D-glucoside, maltose, cellobiose, sucrose, and maltobionic acid. Maltose is, however, converted into maltotriose and glucose by this enzyme which is clearly less specific in its requirements than is D-enzyme.

Amylomaltase (Monod and Torriani, *Ann. Inst. Pasteur*, 1950, **78**, 65) is also distinguished from D-enzyme by the fact that it catalyses the synthesis of amylose-type polysaccharides from maltose, a reaction in which, as with D-enzyme, the amount of glucose

present controls the position of equilibrium. Xylose and mannose can also function as acceptors, as has been found to be the case with D-enzyme. It as evident that with both D-enzyme and amylomaltase a high degree of specificity with respect to the constitution and configuration of the " accepting " sugar unit is not observed.

Experimental

Concentrations of digest components are final values.

Methods.—Reducing powers were measured by the Somogyi copper reagent (*J. Biol Chem.*, 1945, **160**, 61) and when necessary the sugar solutions were deproteinised with Somogyi's reagents (*ibid.*, p. 69). Unless otherwise stated, iodine-staining was carried out by mixing 0·2 ml. of digest with 6 ml. of 0·005% iodine solution in 0·02% aqueous potassium iodide, the intensity of colour (A.V.) being measured in cells of 1·3 cm. diameter in an E. E. L. colorimeter with a No. 404 filter (wavelength of peak transmission = 510 mμ). Inorganic phosphate and phosphorylase activity determinations were made as in Part X (*J.*, 1950, 3566).

Paper-chromatographic fractionations of sugars were carried out on Whatman No. 54 paper with propan-1-ol–ethyl acetate–water (6 : 1 : 3, v/v) as irrigating solvent. When necessary, salts and protein were precipitated by mixing the sugar solution with 4 volumes of ethanol before applying the supernatant liquid to the paper. The positions of the sugars were located with benzidine–trichloroacetic acid (Bacon and Edelman, *Biochem. J.*, 1951, **48**, 114) or, when non-reducing carbohydrates were present, with silver nitrate–sodium hydroxide (Trevelyan, Proctor, and Harrison, *Nature*, 1950, **166**, 444).

Materials.—*Maltodextrins.* These were prepared from potato amylose as by Whelan, Bailey, and Roberts (*J.*, 1953, 1293). The maltose used in the following experiments was material prepared in this way since commercial specimens contained a small amount of a trisaccharide. The concentrations of maltodextrins given refer to weights of products stored *in vacuo* over phosphoric oxide and usually containing at least 95% of carbohydrate.

Enzymes. α-Amylase was prepared from human saliva as by Whelan and Roberts (*J.*, 1953, 1298). The specimen used had a slight maltotriase activity. β-Amylase was the crystalline sweet-potato enzyme kindly provided by Dr. A. K. Balls. Potato phosphorylase was prepared as by Whelan and Bailey (*Biochem. J.*, 1954, **58**, 560), R-enzyme as in Part XIX (*J.*, 1954, 4440).

(*a*) Potato juice. Potatoes (300 g.) were peeled and sliced and either ground or minced with rapid incorporation of charcoal (10 g.) which had previously been boiled with water. It was important to produce an intimate mixture of potato pulp and charcoal as quickly as possible. The juice was expressed through muslin and centrifuged. The thick colloidal suspension of charcoal was removed from the supernatant solution by filtration through a silver-plated pressure filter and a series of two, or sometimes three, graded filters (KS, EK, and EK special, John Carlson Ltd., Ashton-under-Lyne, Lancs. The resulting juice was pale amber and did not darken over several days, but became red and eventually black when added to a dilute catechol solution.

(*b*) D-Enzyme. Ethanol (76·2 ml.) was added to potato juice (400 ml.) during 10 min. with stirring. The temperature, initially −2°, was allowed to fall to −5° as soon as sufficient alcohol was present to prevent freezing. This 16% ethanol precipitate was discarded on the centrifuge. Ethanol (43·4 ml.) was added at −5° and the 16—23% precipitate removed, dissolved in 0·01M-citrate buffer (pH 6·0; 400 ml.), and cooled to −2°, and the 16% and the 16—23% precipitate removed as above. The latter precipitate was dissolved in buffer (25 ml.), and the solution stored at 0°. The solution retained sufficient activity to be used up to 4 days from its preparation.

Proof that the Disproportionating Activity of the D-Enzyme Preparation is not due to Phosphorylase.—Phosphorylase solution (350 mg. of freeze-dried enzyme in 3·5 ml. of water) and D-enzyme solution were analysed for their inorganic phosphate contents; 1 ml. of each solution contained no detectable phosphate. The method is capable of detecting 5 μg. of phosphorus. The phosphorylase activities of each enzyme solution were determined, and two digests were prepared each containing 0·02M-maltopentaose and 0·038M-citrate buffer (pH 6·0). In addition one contained phosphorylase solution in 235-fold overall dilution; the other contained D-enzyme in 1·3-fold dilution. In this way both solutions contained equal phosphorylase activities. Portions (0·3 ml. each) of the digests were stained with iodine at intervals during incubation at 17°. The digests were each divided into two portions after 13 hr.; then one portion (1·2 ml.)

was mixed with potassium dihydrogen phosphate solution (0·2 ml., equiv. to 0·2 mg. of phosphorus) before continuing the incubation. The results are tabulated.

Comparison of D-enzyme and phosphorylase.

Time of incubation (hr.)	A.V. (510 mμ) D-Enzyme	D-Enzyme + phosphate	Phosphorylase	Phosphorylase + phosphate
3	4·8	—	0·0	—
4	6·6	—	0·0	—
7	12·1	—	0·0	—
8·5	15·2	—	0·0	—
13·25	—	18·3	0·0	0·0
19	29·0	20·8	0·3	0·0
31	36·5	25·5	3·3	2·1
60	43·5	31·3	9·4	6·5
80	45·5	34·0	14·2	10·0

Optimum Temperatures and pH *Values of D- and Q-Enzyme.*—(a) *D-Enzyme.* The digests of optimum pH contained D-enzyme solution in two-fold dilution, 0·017M-maltohexaose, and Michaelis's veronal buffer (*Biochem. Z.*, 1931, **234**, 139) in three-fold dilution. Portions of each digest were stained with iodine after 2¼ and 4¼ hr. The values of A.V. (510 mμ) are plotted in Fig. 1. The digests at optimum temperature contained D-enzyme and substrate as above and 0·067M-citrate buffer (pH 6·7). After incubation at various temperatures for 45 and 90 min. portions were stained with iodine. The values of A.V. (510 mμ) are plotted in Fig. 2.

(b) *Q-Enzyme.* Digests of optimum pH contained 0·1% of amylose, D-enzyme in five-fold dilution, and veronal buffer in 1·66-fold dilution. Portions (0·1 ml.) of the digests were stained for measurement of A.V. (680 mμ) after 15 and 45 minutes' incubation at 15°. The digests at optimum temperature contained enzyme and amylose as above and 0·12M-citrate buffer (pH 6·7). Portions (0·1 ml.) of the digests were stained after incubation for 33 and 60 min. The results are given in Figs. 1 and 2.

Action of D-Enzyme on Glucose and Maltodextrins.—0·12M-Solutions of glucose and maltodextrins through to maltopentaose were prepared in D-enzyme solution containing 0·05M-citrate buffer (pH 7·0). Incubation was at 18° and at intervals portions of the digests were removed for fractionation on paper.

Successive Actions of D-Enzyme and α-Amylase on Maltotetraose.—The digest (50 ml.) contained D-enzyme in 3·33-fold dilution, maltotetraose ($4·45 \times 10^{-4}$ mole), and 0·048M-acetate buffer (pH 7·0) and was incubated at room temperature. Measurements of A.V. (510 mμ) of iodine stain after 0, 175, and 260 min. indicated that the intensity had increased from zero to a very nearly constant value of 10 colorimeter units. Measurements of reducing power were also made at these times on deproteinised portions of the digest. The reducing equivalents of 0·2 ml. portions of digest were, respectively, 2·31, 2·34, and 2·37 ml. of 0·005N-thiosulphate. At 260 min. the whole digest was heated at 100° for 3 min., then cooled and filtered, and a portion (27·5 ml.) was treated with α-amylase solution (3·6 ml.; 150 mg.) at 35° under toluene. After 12 hours' incubation the enzyme was inactivated by heat, a portion (1 ml.) removed for paper chromatography, and the remainder, equivalent to $2·37 \times 10^{-4}$ mole of original maltotetraose, fractionated on charcoal–Celite as decribed by Whelan *et al.* (*loc. cit.*). Only three sugars were eluted. These were chromatographically identical with glucose, maltose, and maltotriose. Paper-chromatographic fractionation of the whole digest similarly revealed only these sugars. The amounts of the sugars eluted were calculated by summation of the optical rotations of the fractions, using the $[\alpha]_D$ values reported by Whelan *et al.* (*loc. cit.*). These amounts were $1·56 \times 10^{-4}$, $2·38 \times 10^{-4}$, and $1·00 \times 10^{-4}$ mole, respectively, equivalent to $2·30 \times 10^{-4}$ mole of maltotetraose, *i.e.*, 97% of the material fractionated on the column.

Simultaneous Actions of β-Amylase and D-Enzyme on Maltotriose.—Two digests (3 ml. each) contained 0·033M-maltotriose, 0·083M-citrate buffer (pH 7·0), and crystalline β-amylase (*ca.* 20 units). In addition, one digest contained D-enzyme in 2·5-fold dilution. After incubation for 4 and 17 hr. at room temperature portions of the digests were removed for fractionation on paper. The results are described in the Discussion section.

Effect of Addition of Glucose to the D-Enzyme–Maltohexaose System when in Equilibrium.—The digest (3 ml.) contained 0·017M-maltohexaose, 0·083M-citrate buffer (pH 6·0), and D-enzyme in two-fold dilution. Iodine-staining was carried out as usual during 10 hours' incubation at 20°;

a portion of the digest (0·9 ml.) was then added to glucose (30 mg.), and iodine staining carried out on both portions of the digest. The results are shown in Fig. 3.

Possible Alternative Substrates for D-Enzyme.—Potato juice (2 ml.) was added to 50 mg. of each of the following sugars and the digests were incubated for 18 hr. at 18°; whereafter portions were inactivated by heat and fractionated on paper: D-glucose, D-fructose, L-sorbose, D-rhamnose, D-ribose, D-sorbitol, DL-erythritol, D-mannitol, sucrose, *iso*maltose, lactose, melibiose, αα-trehalose, cellobiose, methyl α-D-glucoside, and panose. For results see p. 48.

Incorporation of [^{14}C]*Glucose into Maltodextrins.*—Radioactive starch from tobacco leaves (20 mg.) was hydrolysed for 2 hr. with sulphuric acid as described by Pirt and Whelan (*J. Sci. Food Agric.*, 1951, **2**, 224), neutralised with barium hydroxide, and evaporated to 0·05 ml. A digest containing 0·03M-maltopentaose, 0·05M-citrate buffer, and D-enzyme in two-fold dilution was incubated at pH 7·0 and 20° until the maximum intensity of iodine staining was attained; then a portion (1·2 ml.) equivalent to $0{\cdot}36 \times 10^{-4}$ mole of substrate was mixed with 0·4 ml. of the hydrolysed starch solution. After incubation for 55 hr. under toluene at 20° a portion (0·2 ml.) of the digest was treated with alcohol to remove salts and protein, and half the material transferred to a paper chromatogram for fractionation. An equivalent quantity of hydrolysed starch was fractionated on the same paper, and also an artificial mixture of maltodextrins and the hydrolysed starch. After development and location of the sugars the radioactivity associated with each of the sugar spots was determined by Dr. L. H. May. The total counts/min. in the two controls were 979 and 1059 respectively; 731 counts/min. were recovered from the digest. The results showed that no exchange of activity occurred between glucose and maltodextrins in the artificial mixture but that in the digest activity had disappeared from the glucose zone and was approximately accounted for by the appearance of activity in the zones associated with the maltodextrins.

Sugars as Acceptors in the D-Enzyme–Maltopentaose System in Equilibrium.—A digest (6·2 ml.) containing 0·097M-maltopentaose, 0·03M-citrate buffer (pH 7·0), and D-enzyme in 1·2-fold dilution was incubated at room temperature until a constant intensity of iodine staining was reached; then portions (0·5 ml.) were transferred to solutions (0·2 ml.) each containing 25 mg. of one of the following sugars: glucose, maltose, methyl α-D-glucoside, xylose, mannose, fructose, sucrose, and αα-trehalose. A further portion (0·5 ml.) was mixed with 0·2 ml. of water. Iodine staining was carried out after further incubation for 0·45, 6·45, and 19 hours. The results are described on p. 48.

Synthesis of a Non-dialysable Polysaccharide.—A digest (50 ml.) containing 0·01M-maltohexaose, 0·02M-citrate buffer (pH 7·0), and D-enzyme in 4·1-fold dilution was incubated at 18° until iodine staining indicated that equilibrium was attained (14 hr.). Measurements of reducing power of deproteinised samples after 0·1, 3, and 14 hr. gave the following values (ml. of 0·005N-thiosulphate per ml. of digest): 10·96, 10·43, and 10·04 respectively. The remainder of the digest was heated at 100° for 3 min., then dialysed against distilled water (4 × 1 l.) during 3 days, with continual stirring of the external solution. The impermeable material was filtered through a Seitz pad, to remove protein, and freeze-dried, yielding 78 mg. of a white product which did not migrate on a paper chromatogram.

Properties of the Synthetic Polysaccharide.—A solution of the polysaccharide of known concentration (method of Pirt and Whelan, *loc. cit.*) was treated with the Somogyi copper reagent (*loc. cit.*) at 100° for 1 and 1·5 hr. (cf. Whelan *et al.*, *loc. cit.*). Titration of the cuprous oxide after these times gave 0·005N-thiosulphate equivalents of 1·27 ml. and 1·34 ml. respectively per 1·94 mg. of polysaccharide [as $(C_6H_{10}O_5)_n$]. From the average of these two values it was calculated that the average degree of polymerisation of the polysaccharide was 12·1. β-Amylolysis at pH 4·8 followed by paper-chromatographic fractionation revealed only maltose and maltotriose. D-Enzyme in 3·5-fold dilution was incubated in 0·08M-citrate buffer (pH 7·0) with 0·005M-polysaccharide at 20° and iodine staining carried out at intervals. The following changes in intensity (colorimeter units) and colour were noted [the time of measurement (hr.) precedes the A.V. (510 mμ)]: 0, 63·2 (red); 0·25, 71·5 (red); 1, 83·0 (deep red); 2·25, 113·6 (purple); 5, 125·6 (violet); 21, 114·5 (purple).

Action of D-Enzyme on Amylopectin.—Two digests were prepared, containing 0·5% of waxy maize starch, 0·04M-citrate buffer (pH 7·0), and D-enzyme in 3·3-fold dilution; one digest contained 0·27M-glucose. Iodine staining was carried out at intervals during incubation at 20°. The results are given in Fig. 3. After 20 hr. portions of the digests were examined on a paper chromatogram. No sugars were detected in the enzyme + amylopectin digest, but the digest containing glucose contained maltotriose.

Simultaneous Actions of β-Amylase and D-Enzyme on Maltose.—These experiments were

carried out in the same way as when maltotriose was used as substrate; two digests were prepared, one containing only D-enzyme, the other containing D-enzyme and β-amylose. Paper chromatography of the digests showed that D-enzyme, neither by itself nor in combination with β-amylase, had any effect on the maltose other than to produce a trace of glucose after prolonged incubation (25 hr.).

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