The Mechanism of Carbohydrase Action

4. THE MECHANISM OF D-ENZYME ACTION

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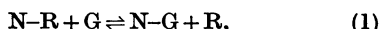
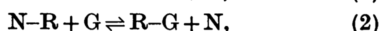
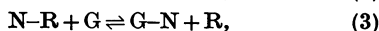
D-Enzyme is a disproportionating transglycosylase found in potatoes (Peat, Whelan & Rees, 1956). It acts on maltodextrins by transferring α-1:4-linkages (Peat, Whelan & Kroll, 1956). The smallest donor substrate on which it exerts a rapid action is maltotriose. There is some doubt whether it has any action at all on the lower homologue, maltose. Maltose can nevertheless act as an acceptor for transferred maltodextrinyl units, as can glucose and several mono- and di-saccharides, and also monosaccharide derivatives such as methyl α-glucoside (Peat, Whelan & Rees, 1956; Peat, Whelan & Jones, 1957). There are four ways in which a donor and acceptor substrate can be considered to react under the influence of D-enzyme. In the case where glucose (G) is the acceptor the reaction with the donor substrate (N–R) can be formulated as follows (R is the part of the molecule containing the free reducing-end group):



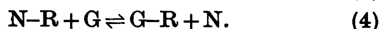
$$\text{N–R} + \text{G} \rightleftharpoons \text{N–G} + \text{R}, \quad (1)$$



$$\text{N–R} + \text{G} \rightleftharpoons \text{R–G} + \text{N}, \quad (2)$$



$$\text{N–R} + \text{G} \rightleftharpoons \text{G–N} + \text{R}, \quad (3)$$



$$\text{N–R} + \text{G} \rightleftharpoons \text{G–R} + \text{N}. \quad (4)$$

Peat *et al.* (1957) have already shown that reactions (3) and (4) do not take place, but their work left undecided the question whether the reaction followed eqn. (1) or eqn. (2). We now report evidence that D-enzyme acts according to eqn. (1).

EXPERIMENTAL AND RESULTS

D-Enzyme. The enzyme was prepared as by Peat *et al.* (1957).

Preparation of maltodextrins. A digest containing glucose (25·7 mg.), D-enzyme (50 mg.) and solubilized potato starch (20 mg. as free glucose; Hanes & Cattle, 1938) in 2 ml. was incubated under toluene for 17·5 hr. at 30°, and the enzyme was then inactivated by heat. The mixture was centrifuged, and portions of the supernatant solution were subjected to chromatography in propanol–ethyl acetate–water (6:1:3, by vol.) on Whatman no. 3MM paper ($18\frac{1}{4}$ in. × $22\frac{1}{2}$ in.) which had been soaked overnight in 1% acetic acid and then washed with water before drying. A portion (0·5 ml.) was spread along a 12 cm. line on one paper and a second portion (0·85 ml.) along an 18 cm. line on another. The papers were irrigated for 24 and 48 hr. respectively, control spots of a mixture of glucose and maltodextrins being used to locate the positions of the digest components by spraying with benzidine–trichloroacetic acid (Bacon & Edelman, 1951). The first paper was used to determine glucose, maltose and maltotriose, and the second to determine maltose–maltohexaose. The two papers were used because glucose and the maltodextrins up to maltohexaose could not be adequately separated on one paper. The digest components were cut out as strips from the papers and eluted with 1·5N-H_2SO_4 (25 ml.) by stirring in a beaker. The solution was filtered through sintered glass, and a 20 ml. portion was heated in a boiling-water bath for 2 hr., cooled, and neutralized with a predetermined volume of approx. 5N-NaOH. Glucose itself was not heated with acid. The reducing powers (as glucose) of 10 ml. portions were then determined with Somogyi (1945) reagent and the yield of each sugar was calculated, correction being made for loss of glucose on heating in acid (Pirt & Whelan, 1951). The yields, expressed as free glucose, were: glucose, 22·6 mg.; maltose, 2·79 mg.; malto-triose, 10·3 mg.; -tetraose, 5·13 mg.; -pentaose, 3·86 mg.; -hexaose, 2·36 mg. The total yield was 47·0 mg., as compared with 45·7 mg. of starch and glucose originally present.

Preparation of [^{14}C]*glucose.* [^{14}C]Glucose was prepared by acidic hydrolysis of uniformly labelled starch (0·1 mc; Radiochemical Centre, Amersham, Bucks). The experimental conditions, devised in experiments with inactive material, were as follows. The starch (about 100 mg.) was heated in 0·5N-H_2SO_4 (10 ml.) for 6 hr. at 100°, cooled and neutralized (NaOH). The solution was passed through charcoal–Celite (1:1, 20 g.) to remove oligosaccharides and then through Biodeminrolit resin (15 g., The Permutit Co. Ltd., London, W. 4) to remove salts. The resin had previously been stored in aqueous suspension overnight under a slight pressure of CO_2. This treatment is essential, otherwise serious losses of sugar will result (Woolf, 1953). The sugar was completely eluted by passing a total of 500 ml. of water successively through the two columns, most of it being in the fraction 250–500 ml. The recovery was 96% of chromatographically pure glucose.

Preparation of [^{14}C]*maltodextrins.* The dextrins were prepared by incubating soluble starch (100 mg.), [^{14}C]-glucose (97 mg.) and D-enzyme (200 mg.) in 8 ml. of solution for 17 hr. at 30°, under toluene. After heat-inactivation of the enzyme, the digest was passed successively through charcoal–Celite (to adsorb maltodextrins) and Biodeminrolit resin (to remove buffer salts), as in the preparation of [^{14}C]glucose. The charcoal column was then eluted with 50% (v/v) aqueous ethanol (300 ml.) to desorb the maltodextrins. The eluate was evaporated to dryness under reduced pressure and distributed between three sheets of Whatman no. 3MM paper which were irrigated for 50 hr. in the propanol–ethyl acetate–water solvent. The di-,

tri- and tetra-saccharide fractions were located by monitoring the paper with a Panax 5054 ratemeter (Panax Equipment Ltd., Mitcham, Surrey). The higher saccharides were located by radioautography with Ilford Industrial F X-ray film. The sugars were eluted by perfusing the paper strips with water, as in chromatography, about 20 ml. of solution being collected from each strip. The solutions were evaporated to dryness under reduced pressure and the sugars dissolved in hot 80% (v/v) methanol. The solutions were filtered to remove paper fibre, and re-evaporated.

Position of radioactive labelling in maltotriose

[^{14}C]Maltotriose (16 mg.), which was chromatographically pure, was dissolved in water (0·8 ml.) and sodium borohydride (20 mg., 0·1 ml.) was added. After the solution had stood for 20 hr. at room temperature 3N-H_2SO_4 was added until there was no further effervescence (0·15 ml.). One portion (0·45 ml.) was mixed with 3N-H_2SO_4 (0·056 ml.; final acid normality, 0·33) and heated at 100° for 30 min. in order partly to hydrolyse the maltotri-itol. A second portion was mixed with 3N-H_2SO_4 (0·090 ml.; final acid normality, 0·5) and heated for 4 hr. at 100° to hydrolyse the sugar alcohol completely. Each hydrolysate and the remainder of the maltotri-itol were passed through Biodeminrolit resin (2 g.) to remove electrolyte. The eluates were evaporated to dryness and methanol (5 × 10 ml.) was distilled off the sugar residue in order to remove boric acid (Zill, Khym & Cheniae, 1953), which is not retained by the resin when in the carbonate form. The products were examined by paper chromatography in butanol–pyridine–water (6:4:3, by vol.) (*A*), and in ethyl acetate–acetic acid–saturated aqueous boric acid (9:1:1, by vol.) (*B*) (W. R. Rees & T. R. Reynolds, in preparation). Reducing sugars were located with benzidine–trichloroacetic acid and reducing and non-reducing sugars with either periodate–benzidine (Cifonelli & Smith, 1954) or $AgNO_3$–NaOH (Trevelyan, Procter & Harrison, 1950). The following information, obtained in control experiments, will explain how the results of chromatography were obtained and interpreted. In solvent *A* there is no perceptible separation of maltotriose from maltotri-itol, or of maltose from maltitol. Glucose and sorbitol separate sufficiently well for them to be recognized as discrete spots, sorbitol having the lower R_F value. The R_F values of glucose, maltose and maltotriose are 0·37, 0·26 and 0·19 respectively, so that it was convenient to examine the partial hydrolysate of maltotri-itol in this solvent. In solvent *B*, however, the movement of the di- and tri-saccharides and their alcohols is so slow relative to glucose and sorbitol that the last two substances are eluted from the paper before the oligosaccharides have moved sufficiently from the origin. The great advantage of solvent *B* is that it achieves a clear-cut separation of a sugar and its corresponding alcohol. The following are typical results. After 22 hr. separation: sorbitol, 31·5 cm. movement; glucose 16·4 cm. After 36 hr.: maltitol, 8·5 cm.; maltose, 5·5 cm. After 110 hr.: maltotri-itol, 9·5 cm.; maltotriose, 7·3 cm. The solvent mixture is very volatile, and, while the relative rates of movement are reproducible, the absolute rates are not. When the two solvents were used the following observations were made. (*a*) The maltotri-itol was chromatographically pure (solvents *A* and *B*), and therefore reduction by borohydride was complete (cf. Whelan & Morgan, 1955). (*b*) The partial hydrolysate contained radioactive material in the regions corresponding to maltotriose + tri-itol, maltose + maltitol and sorbitol; glucose was inactive (solvent *A*). There was no sign of any disaccharide fraction other than maltose + maltitol. The original trisaccharide therefore contained only 1:4-linked glucose units. (*c*) The portion that had been hydrolysed for 4 hr. contained sorbitol (radioactive), glucose (inactive) and a trace of radioactive material in the disaccharide region (solvent *B*), which either represented incompletely hydrolysed material or was an acid-reversion product.

A portion of this same hydrolysate, representing about 0·1 mg. of original maltotriose, was fractionated in solvent *B*. The zones corresponding to glucose and sorbitol were cut out and eluted with water. After evaporation to dryness the boric acid was removed as before and the residue dissolved in water (1 ml.). Portions (0·35 ml. each) were transfered to polythene planchets (1 cm. diam.) and the radioactivity was measured with an Ekco N530C scaler (Ekco Electronics Ltd., Southend-on-Sea). The radioactivity of the sorbitol, expressed as counts/100 sec., corrected for background and coincidence, was 871. That in the glucose fraction was 2. These results, taken with the fact that there was twice as much glucose present as sorbitol, show that the radioactivity in the original maltotriose was centred exclusively in the reducing-end glucose unit.

Position of radioactive labelling in the other maltodextrins

The di-, tetra-, penta- and hexa-saccharide fractions were examined by paper chromatography in solvent *A*. They were chromatographically pure except for the disaccharide fraction, which was resolved into two zones, one moving with maltose and the other (*X*) with isomaltose. The radioactivity associated with the zones was in the approximate ratio 4:1. The two components were separated by chromatography of the whole disaccharide fraction on thick filter paper. Each of the sugars maltose, *X*, malto-tetraose, -pentaose and -hexaose was then examined for the position of radioactive labelling, as for maltotriose, i.e. by the sequence of operations

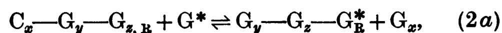
(i) reduction with borohydride, (ii) acidic hydrolysis, (iii) deionization and removal of boric acid as methyl borate, (iv) chromatographic separation in solvent *B*. The glucose and sorbitol zones were not, however, eluted, the radioactivity being detected qualitatively by radioautography. With maltose, maltotetraose, -pentaose and -hexaose the radioactivity was present solely in the derived sorbitol. With disaccharide *X*, however, both the glucose and sorbitol zones were radioactive although the latter zone appeared to have several times more radioactivity than the glucose.

DISCUSSION

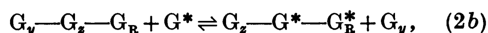
The reaction between starch and glucose, catalysed by D-enzyme, leads to the production of maltodextrins in excellent yields. From the total amount of maltodextrins obtained it would seem that almost the whole of the starch had been utilized in their production. This could not happen unless the α-1:6-branch linkages of the amylopectin component, representing about 75% of the starch, had been broken. It was expected that this would take place since the D-enzyme preparation is known also to contain the debranching R-enzyme (Peat, Whelan & Hobson, 1951), the presence of which can be recognized by the increase in intensity of iodine stain which takes place when the D-enzyme preparation is incubated with starch alone. The method of preparing maltodextrins as now described is an advance on a previously published method (Peat, Whelan & Kroll, 1956) since this latter technique involved the prior production of maltotriose, by α-amylolysis of starch, for use as the substrate from which D-enzyme produced other maltodextrins.

When starch and [^{14}C]glucose are incubated with D-enzyme the maltodextrin products are labelled with radioactivity exclusively in the reducing-end glucose unit. Such dextrins have previously been prepared by the reaction between [^{14}C]glucose and cyclohexa-amylose, catalysed by *Bacillus macerans* amylase (Pazur, 1955; Pazur & Budovich, 1956). The donor substrate and enzyme used in the present work are much more accessible.

End-labelled maltodextrins provide a means of studying the pattern of breakdown effected by starch-metabolizing enzymes. They have also been used to determine the effect of position within the maltodextrin molecule on the rate at which acid hydrolyses α-1:4-glucosidic linkages (G. J. Walker & W. J. Whelan, in preparation). As regards D-enzyme itself, the finding that the maltodextrin products of its action are labelled only at the reducing end proves that it acts according to eqn. (1) and not in the manner expressed by eqns. (2), (3) or (4). That eqn. (3) or (4) cannot hold is self-evident and confirms the earlier findings of Peat *et al.* (1957). With eqn. (2) a single transfer between an inactive maltodextrin substrate and radioactive glucose would yield a polymer labelled only at the reducing end, but a second participation of this molecule in such a transfer would cause radioactivity to appear elsewhere in the molecule (eqns. 2*a*, 2*b*):



$$C_x—G_y—G_{z,R} + G^* \rightleftharpoons G_y—G_z—G^*_R + G_x, \quad (2a)$$



$$G_y—G_z—G_R + G^* \rightleftharpoons G_z—G^*—G^*_R + G_y, \quad (2b)$$

(G^* = [^{14}C]glucoses; G_R denotes reducing-end group).

The disaccharide products of the reaction between starch and [^{14}C]glucose call for comment. One of these was maltose, labelled only in the reducing-end unit. It is doubtful whether maltose is a true product of D-enzyme action. The amounts of maltose formed when D-enzyme acts on other maltodextrins or, as in the present case, on starch plus glucose, are always small by comparison with other products, and it may well be that traces of amylolytic activity in the enzyme preparation are responsible. It is not yet known what is the structure of the second disaccharide (*X*), which was formed in even smaller amounts than maltose and has not previously been detected. Its paper-chromatographic and -ionophoretic behaviour was like that of isomaltose and gentiobiose. The fact that it was radioactive showed it to be a product of an enzyme-catalysed reaction and not a contaminant of the enzyme preparation. That it was labelled in both sugar units, although unequally, points to a direct polymerization of glucose, such as has already been observed with α- and β-glucosidases (Peat, Whelan & Hinson, 1952, 1955). It has now been shown (unpublished experiments) that the D-enzyme preparation does in fact slowly catalyse the dimerization of glucose and the identification of the product is in progress.

SUMMARY

1. The reaction between starch and glucose, catalysed by D-enzyme, is a convenient means of preparing maltodextrins.

2. When [^{14}C]glucose is used in this reaction the maltodextrin products are radioactive, the activity being confined solely to the reducing-end glucose unit.

3. The proof of the position of radioactive labelling in the maltodextrins has enabled a precise formulation to be given to the manner in which D-enzyme transfers glycosyl radicals between donor and acceptor substrates.

4. An unidentified disaccharide is a minor product of the reaction between starch, glucose and D-enzyme, and may have arisen by enzymic polymerization of glucose.

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Toxicity of Autoxidized Squalene and Linoleic Acid, and of Simpler Peroxides, in Relation to Toxicity of Radiation

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Dam & Granados (1945) found that in rats deficient in vitamin E there was an increase in organic peroxide-like material (hereinafter abbreviated to 'organic peroxide'). They suggested that it resulted from the well-known chain reaction by which unsaturated fats, rubber, etc. 'autoxidize' in air to form peroxides. Though this interpretation is not the only possible one it was the first plausible indication that autoxidation could occur *in vivo* as well as *in vitro*, and was consistent with the known 'antioxidant' properties of vitamin E (for reviews see Mattill, 1947; Holman, 1954). According to the accepted mechanism chain autoxidation is initiated by free radicals having a high content of chemical free energy, and may be terminated by formation of low-energy free radicals such as semiquinones, as happens in the presence of vitamin E and other antioxidants. In absence of antioxidants one high-energy free radical can give rise to several hundred molecules of organic peroxide before the chain is terminated by mutual reaction of two free radicals. In many chemical systems the ions produced by 'ionizing radiations' (X-rays, etc.) give rise to high-energy free radicals, e.g. OH from water (Weiss, 1944). Thus X-rays would be expected to induce autoxidation, and Mead (1952) has found *in vitro* that they do, though the conditions seem to be critical because V. J. Horgan & J. St L. Philpot (unpublished work) failed to repeat the experiment. The question whether X-rays induce autoxidation *in vivo* as well as *in vitro* was provisionally answered in the affirmative by Horgan & Philpot (1953, 1954*a*, *b*), who extracted mice that had received a lethal dose (950 r.) of X-rays. With various methods of peroxide estimation, they sometimes found a significant increase ($P < 0{\cdot}01$; apparent mean chain length about 80) as compared with unirradiated mice. The technique was, however, unreliable and is still being developed. Dubouloz & Dumas (1954) found that in rat skin, 0·1 mm. thick, 5000 r. of X-rays produced 2×10^{-9} and 20 000 r. produced $1{\cdot}3 \times 10^{-8}$ mole/cm.^{2} of peroxide (for references from 1950 onwards see Dubouloz & Dumas, 1954). These figures correspond to mean chain lengths of about 14 and 23 respectively; but it is not clear what fraction of this peroxide was in the living part of the skin. Thus there is some evidence that X-rays can initiate autoxidation *in vivo*, though the observed peroxide could be formed in some other way.

There are not many substances in living matter with a structure suitable for chain autoxidation. The most suitable are the 'methylene-interrupted' [m, $(m+3n)$ diene and polyene] fatty acids, linoleic, linolenic and arachidonic, i.e. the essential fatty acids. Other possibilities are the monoenes, notably oleic acid, the carotenoids [conjugated, i.e. m, $(m+2n)$ polyenes] and squalene [m, $(m+4n)$ polyene]. Though all these substances might be