but to a smaller extent. The possible significance of these findings is discussed.

4. The enzyme from *R. spheroides* is specific for Zn^{2+} ions. Of the salts tested as inhibitors only those with Cu^{2+} , Co^{2+} , Mn^{2+} and Ca^{2+} ions are active.

5. When ascorbic acid is present in the assay mixture ferrochelatase activity can be demonstrated in all the above extracts. Under these conditions Fe^{2+} ions competitively inhibit zinc protoporphyrin formation and Zn^{2+} ions are a non-competitive inhibitor of haem formation.

6. The specificity of the zinc-protoporphyrin chelatase towards other porphyrins has been tested. Unlike protoporphyrin some porphyrins readily form a zinc complex in the absence of enzyme.

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The Mechanism of Carbohydrase Action

10. ENZYMIC SYNTHESIS AND PROPERTIES OF 6- α -MALTOSYLGLUCOSE*

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The main polymeric linkages of amylopectin and glycogen are the chain-forming α -(1 $\rightarrow$ 4)-bond and the branch-forming α -(1 $\rightarrow$ 6)-bond. Partial acid hydrolysis of the polysaccharides yields glucose, maltose and isomaltose. Of the expected four trisaccharides [maltotriose, 4- α -isomaltosylglucose (panose), 6- α -maltosylglucose and 4,6-di- α -glucosylglucose], only maltotriose and panose have been isolated despite a thorough search for the isomers of panose (Edwards, 1955; see also Whelan,

1958). Isomaltotriose would also be formed if two (1 $\rightarrow$ 6)-bonds were in juxtaposition and was indeed isolated from glycogen by Wolfson & Thompson (1957). The question therefore arises whether the trisaccharides not detected are not formed, or are particularly labile to acid and so do not accumulate. A partial solution to the problem would be obtained by examination of the trisaccharides in question. Summer & French (1956) indicated that these compounds, 6- α -maltosylglucose and 4,6-di- α -glucosylglucose, could be synthesized by the coupling reaction between cyclomaltohexaose (Schardinger α -dextrin) and isomaltose, catalysed

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by *Bacillus macerans* transglycosylase. The yield is increased by subjecting the mixture of products to β -amylolysis.

The object of the present investigation was to synthesize these trisaccharides and to test their labilities towards acid. The aims were only partly realized, however, because only one of the sugars could be obtained. This was 6- α -maltosylglucose (I).

EXPERIMENTAL

General methods

Paper chromatograms were irrigated in ethyl acetate-pyridine-water (10:4:3, by vol.), and reducing sugars were located specifically with the aniline-diphenylamine-phosphoric acid spray (Harris & MacWilliam, 1954). Silver nitrate-sodium hydroxide (Trevelyan, Procter & Harrison, 1950) was used to detect reducing and non-reducing sugars. The transport medium for paper electrophoresis was sodium borate, pH 10 (Foster, 1953). Bio-Deminrolit ion-exchange resin (The Permutit Co. Ltd.) was used to remove electrolytes from sugar solutions and the anion-exchange component was in the CO_3^{2-} form (Woolf, 1953). Reducing powers were measured with a copper reagent (Shaffer & Hartmann, 1921). The concentrations of glucose polymers were determined by acid hydrolysis to glucose, as described by Pirt & Whelan (1951), except that the time of hydrolysis was extended to 6 hr. to ensure breakdown of the (1 $\rightarrow$ 6)-bonds. Fractionation of sugars on charcoal was carried out at pH 3 (Taylor & Whelan, 1962) by using 'activated charcoal for decolorising purposes' (British Drug Houses Ltd.) and Celite 535 (Johns-Manville Co. Ltd.). The use of human serum albumin and reduced glutathione to stabilize crystalline sweet-potato β -amylase is described by Walker & Whelan (1960). The activity of the enzyme is reported in the units defined by Hobson, Whelan & Peat (1950).

Coupling reaction

The cyclohexane complex of cyclomaltotetraose (30 g., equivalent to 25.8 g. of carbohydrate; French, Pulley & Whelan, 1963) was boiled in water until cyclohexane had been removed and the dextrin had dissolved. The solution was evaporated to dryness under reduced pressure and isomaltose (5 g.; Taylor & Whelan, 1963) was added. The sugars were dissolved in 75 ml. of *B. macerans*-transglycosylase solution (French *et al.* 1963) containing 7.5 units of enzyme/ml. (Tilden & Hudson, 1942). The solution was passed through a Seitz filter into a sterilized flask and the filter washed with water (5 ml.). The digest was kept at room temperature for 6 days. A white precipitate was formed during the first overnight period. The solution was then decanted from the solid and boiled

for a few minutes to inactivate the enzyme. To remove cyclodextrin, *sym*-tetrachloroethane (about 5 ml.) was added and emulsified with the cooled solution. On standing overnight a precipitate formed and was removed by filtration. The clear solution was diluted to 100 ml. with water and the carbohydrate content determined. The concentration, as glucose, was 176.5 mg./ml.

β -Amylolysis and fractionation of the products of the coupling reaction

To the solution of coupling reaction products (98.5 ml.) were added, in order, 0.2M-sodium acetate-acetic acid buffer, pH 4.7 (3 ml.), 1% (w/v) human serum albumin (5 ml.), 10 mM-reduced glutathione (5 ml.) and crystalline sweet-potato β -amylase (Worthington Biochemical Corp., Freehold, N.J., U.S.A.) (1 ml.; 4100 units). The digest was incubated for 212 hr. at 35° under toluene and reducing power was determined on 0.1 ml. portions. The reducing power attained a constant value of 57.5% apparent conversion into maltose between 20 and 212 hr. The enzyme was then inactivated by heating the digest in a boiling-water bath for 5 min.

The digest was adsorbed on a column (8 cm. diam.) of charcoal-Celite (1:1) (1500 g.). The effluent from the column was passed through a 2 cm. cell in an ETL-NPL automatic polarimeter mark 143A (Ericsson Telephones Ltd., Nottingham). A white-light source with a 589 m μ interference filter and a 2 cm. continuous-flow cell gave useful signals corresponding to optical rotations in the range 0-0.5 angular degrees. The output from the polarimeter was fed to a chart recorder so that a record of the optical rotation of the effluent was obtained. The effluent then passed to a fraction collector (100 ml. tubes). 10 mM-Formic acid (3.7 l.) was passed through the column. As expected, no sugar (monosaccharide) was desorbed. 7.5% (v/v) Ethanol in aq. 10 mM-formic acid was then applied. Isomaltose (1.67 g.) and maltose (10.9 g.) were eluted successively and appeared, respectively, in the fractions emerging when 4.8-6.4 l. and 6.4-12.9 l. of ethanolic eluting medium had been applied to the column. The amounts of the two sugars were determined by combining the appropriate fractions and measuring the optical rotation (4 dm. tube). 15% (v/v) Ethanol in aq. 10 mM-formic acid was applied to the column and the trisaccharide fraction emerged over the period when 5.5-7.8 l. of eluting medium had been applied. Then 25% (v/v) propanol in aq. 10 mM-formic acid was used to elute the remaining sugar (tetrasaccharide) which emerged in the fraction 5-7 l. The tri- and tetra-saccharide fractions were evaporated to about 500 ml. under reduced pressure. Residual formic acid was removed with De-Acidite E (CO_3^{2-} form) resin (The Permutit Co. Ltd.), and the solution evaporated to dryness. The syrups were dissolved in aq. 80% (v/v) methanol

(50 ml.), boiled with decolorizing charcoal, filtered and evaporated to dryness. The trisaccharide fraction weighed 2.52 g. and the tetrasaccharide 1.54 g.

Examination of the trisaccharide

Purity and specific optical rotation. The trisaccharide fraction migrated as a single zone when examined by paper chromatography and electrophoresis. During electrophoresis it migrated at the same rate as isomaltose. A solution of the sugar (7.75 mg./ml.) was prepared and a 1 ml. portion hydrolysed in acid to determine the concentration. The carbohydrate content of the product was 82.5%. All subsequent quantitative determinations of sugar properties were based on concentrations determined by the acid-hydrolysis method. The reducing power of the trisaccharide was 101.8% of that of panose and 90.8% of that of glucose (molar basis). The sugar had $[\alpha]_D + 159^\circ$ (c 2.1 in water). The *undecabenzoylate* was prepared from 82 mg. of sugar as by Turvey & Whelan (1957) and was crystallized to constant m.p. (yield, 83%). It had m.p. 139–141° and $[\alpha]_D + 112^\circ$ in chloroform (Found: C, 69.1; H, 4.68. $C_{95}H_{76}O_{27}$ requires C, 69.2; H, 4.61%).

Fragmentation of the reduced trisaccharide. The sugar (53.9 mg.) was reduced with sodium borohydride (100 mg.) in water (2.5 ml.) for 18 hr. at room temperature. The excess of borohydride was destroyed by the dropwise addition of 3*N*-acetic acid until effervescence ceased, and the solution was diluted to 5 ml. A portion (0.65 ml.) was treated with Bio-Deminrolit ion-exchange resin and then methanol was repeatedly evaporated from the dried solid, to remove boric acid. The sugar alcohol was hydrolysed in water (2 ml.) containing a few particles of Zeo-Karb 215 (H^+ form) resin (The Permutit Co. Ltd.) in a boiling-water bath for 2 hr. Paper chromatography of the partial hydrolysate revealed substances behaving as unchanged alcohol, isomaltitol and glucose. Sorbitol was presumably present also but its R_F value is the same as glucose. A further portion of the sugar (100 mg.) was reduced, acetylated with sodium acetate-acetic anhydride and the syrupy acetate dissolved in 5 ml. of acetic anhydride-conc. sulphuric acid (100:9, v/v). After being incubated at 35° for 17 hr., the solution was poured into ice-water and stored at 0° for 18 hr. to hydrolyse acetic anhydride. The sugar acetates were extracted with chloroform and after evaporation treated with 5% (v/v) triethylamine in aq. 50% (v/v) ethanol for 3 hr. at 60°, evaporated and left in 0.1*N*-sodium hydroxide for 10 min. at 0°, the deacetylated sugars being finally deionized with Bio-Deminrolit resin. Paper chromatography of the mixture now revealed unchanged alcohol, isomaltitol (trace), maltose and glucose (plus sorbitol).

Oxidation of the reduced trisaccharide: (a) Oxidation with concentrated periodate. The sugar alcohol (25.2 mg. in 2.5 ml., prepared as above) was added to a mixture of sodium acetate-hydrochloric acid buffer, pH 1.03 (8 ml.), 0.2*M*-sodium metaperiodate (5 ml.) and diluted to 25 ml. with water (Parrish & Whelan, 1959). A control was prepared from the reagents together with 50 mg. of decomposed sodium borohydride (2.5 ml.). The solutions were incubated at 50° in the dark, and at 2, 4 and 6 hr. were sampled for determination of periodate uptake (1 ml.) (Peat, Whelan & Roberts, 1957) and formaldehyde released (0.1 ml.) (Parrish & Whelan, 1961). The expected consumption of periodate was 7 mol.prop. and of formalde-

hyde released was 1 mol.prop. The respective determinations (mol.prop.) were: 2 hr., 6.8 and 0.96; 4 hr., 8.3 and 1.01; 6 hr., 8.0 and 1.01.

(b) Oxidation with dilute periodate. The sugar alcohol (16.0 mg. in 1.5 ml.) was diluted with water (about 450 ml.) containing 0.04*M*-sodium metaperiodate (5 ml.) and diluted to 500 ml. with water. A control solution containing decomposed sodium borohydride (30 mg.) in place of sugar solution was also prepared. The digests were incubated at room temperature in the dark and samples (25 ml. and 2.5 ml. respectively) removed after 1 and 2 hr. for determination of periodate uptake and release of formaldehyde. The calculated values were 4 mol.prop. of periodate and 1 mol.prop. of formaldehyde. The measured values (mol.prop.) were respectively: 1 hr., 4.3 and 1.02; 2 hr., 4.2 and 1.02.

Acid hydrolysis of panose and 6- α -maltosylglucose. Solutions of panose (20.0 mg.) and 6- α -maltosylglucose (20.0 mg.) in 0.3*N*-sulphuric acid (10 ml.) were heated in a boiling-water bath. At intervals portions (about 1.2 ml.) were removed, cooled rapidly, and 1 ml. portions were taken, neutralized, and their reducing powers determined. The results are given in Fig. 1.

Properties of the tetrasaccharide fraction

Paper chromatography of the fraction eluted from the charcoal column after 6- α -maltosylglucose showed it to contain a substance with the R_F value expected of an α -glucose tetrasaccharide, together with traces of 6- α -maltosylglucose and sugars of lower R_F values than the tetrasaccharide. The whole fraction (1.54 g.) was dissolved in 100 ml. of water and treated at 35° with β -amylase (5740 units) as described above. After 25 and 48 hr. the reducing power of the digest had increased to 118% of its initial value. The enzyme was then inactivated and a portion of the digest examined by paper chromatography. Maltose and isomaltose were present in approximately equal amounts and the tetrasaccharide had disappeared.

RESULTS

Synthesis of oligosaccharides. The transfer reaction between cyclomaltohexaose and isomaltose was carried out, unchanged cyclodextrin was removed and the products were degraded with β -amylase. At the termination of the reaction the mixture was fractionated on charcoal-Celite and there were obtained isomaltose, maltose, a trisaccharide fraction and a tetrasaccharide fraction.

Structure of the trisaccharide. The trisaccharide fraction was examined by paper electrophoresis in borate buffer, pH 10, and migrated as a single zone at the same rate as isomaltose. 4,6-Di- α -glucosylglucose could not therefore have been present for this should migrate with maltose, at only about half the rate of isomaltose. The trisaccharide was therefore presumably 6- α -maltosylglucose. This was proved as follows.

The sugar had $[\alpha]_D + 159^\circ$. Its glycosidic linkages were therefore both in the α -configuration. Panose has $[\alpha]_D + 154^\circ$ and maltotriose $+ 160^\circ$. The

sugar was reduced to the corresponding alcohol with sodium borohydride. Partial acid hydrolysis of the alcohol should give maltose and isomaltitol as intermediates. Paper chromatography of a partial aqueous acid hydrolysate revealed isomaltitol, but only a very small amount of maltose was seen. Acetolysis, which causes a preferential splitting of (1 $\rightarrow$ 6)-glucosidic bonds over the bonds involving secondary hydroxyl groups (Goldstein & Whelan, 1962), however, yielded an easily detected amount of maltose.

Quantitative structural information was obtained by measurement of periodate consumed and formaldehyde formed on complete oxidation of the alcohol and on preferential oxidation of the polyol end group (Clancy & Whelan, 1959). The results were in agreement with expectation (see the Experimental section). A crystalline undecabenzate of the trisaccharide was obtained as a characterizing derivative. The sugar itself could not be crystallized.

Structure of the tetrasaccharide. The tetrasaccharide was completely degraded to maltose and isomaltose on further treatment with β -amylase. One of the three tetrasaccharides that might be formed in the coupling reaction, 6¹- α -maltosylmaltose, is resistant to β -amylase (Summer & French, 1956) and therefore could not have been present. The other tetrasaccharides are 6- α -maltotriosylglucose and 6¹- α -glucosylmaltotriose. The second of these sugars would be formed by the transfer of two glucose units to the reducing glucose unit of isomaltose. Since the trisaccharide formed by the transfer of one glucose unit to the reducing end of isomaltose was not found (see above) it may be assumed that 6¹- α -glucosylmaltotriose was also absent. The tetrasaccharide was therefore probably 6- α -maltotriosylglucose. Its relatively slow hydrolysis by β -amylase indicates that it behaves like maltotriose and not maltotetraose (Whelan, Bailey & Roberts, 1953).

DISCUSSION

Susceptibility of 6- α -maltosylglucose to acid hydrolysis. 6- α -Maltosylglucose and panose behaved in identical fashion (Fig. 1) when submitted to acid hydrolysis under conditions used to fragment glucose polysaccharides (e.g. Peat, Whelan & Edwards, 1955). If 6- α -maltosylglucose is liberated during acid hydrolysis of glycogen or amylopectin, its chance of survival must therefore be equal to that of panose. It must be concluded that steric or energetic factors operate in such a way as to favour the formation of panose from the polysaccharides. A solution of the problem requires a knowledge of the relative labilities of the (1 $\rightarrow$ 4)-bonds in the vicinity of the glycogen and amylopectin (1 $\rightarrow$ 6)-branch linkages.

Bouveng, Kiessling, Lindberg & McKay (1963) reported the isolation of 6- α -maltosylglucose and panose from a partial acid hydrolysate of the linear α -glucan, pullulan [α -maltotriose polymerized through (1 $\rightarrow$ 6)-bonds (Bender & Wallenfels, 1961)]. The relative yields were in the ratio 2:3. The calculated ratio in terms of the structure of pullulan and the equal acid-lability of the trisaccharides reported above is 1:1. Therefore, even in a linear structure capable of yielding both sugars a preferential formation of panose takes place.

Action pattern of Bacillus macerans transglycosylase. The products of the coupling reaction between cyclomaltohexaose and isomaltose were not those expected on the basis of the work of Summer & French (1956), who found products resulting from the coupling of glucose units to either and both of the glucose units in isomaltose. They found also that both of the unsubstituted C-4-hydroxyl groups in the trisaccharide panose act as receptors for transferred glucose units, a finding confirmed by D. French, E. E. Smith & W. J. Whelan (unpublished results). The experimental conditions for the isomaltose-coupling reaction used here were not those used by Summer & French (1956), where the relative amount of isomaltose to cyclomaltohexaose was five times as great as the amount we used. The number of glucose units available for transfer to each molecule of isomaltose was therefore greater in the present experiment and this probably accounts in part for the deposition from solution of a synthetic polysaccharide during the digestion period (see the Experimental section). Experiments in progress may shed some light on the above divergent observations, but it is at least clear that the transglycosylase is able to couple glucose units to the non-reducing glucose moiety of isomaltose much more readily than to the reducing moiety.

Fig. 1. Relative rates of hydrolysis of 6- α -maltosylglucose (●) and 4- α -isomaltosylglucose (panose) (○) in 0.3N-sulphuric acid at 100°.

SUMMARY

1. The coupling reaction between cyclomaltose and isomaltose, catalysed by *Bacillus macerans* transglycosylase, was carried out and the products were degraded with β -amylase.

2. A trisaccharide, proved to be 6- α -maltosylglucose, was isolated as a β -amylase-resistant end product. A tetrasaccharide, believed to be 6- α -maltotriosylglucose, was also isolated. This was slowly hydrolysed by β -amylase.

3. The rate of hydrolysis of 6- α -maltosylglucose in hot aqueous acid is the same as of the isomeric trisaccharide panose (4- α -isomaltosylglucose).

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Plasma Glucose, Non-Esterified Fatty Acid and Insulin Concentrations in Hypothalamic-Hyperphagic Rats

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Hyperphagia may be induced in rats by electrolytic lesions in or near the ventromedial nuclei of the hypothalamus (Hetherington & Ranson, 1940), although the mechanism of the appetite disturbance is unknown. There is much evidence in favour of the suggestion by Mayer & Bates (1952) that food intake normally varies in inverse proportion to the peripheral arteriovenous difference in the concentration of glucose, which to a certain extent reflects the uptake of glucose by muscle, and it has been proposed that specialized 'glucose receptors' provide the hypothalamic regulatory centres with information about this rate of uptake. If this highly specific signal to the hypothalamus provides

the regulatory mechanism then other changes in metabolism can only affect appetite indirectly by modifying the rate of uptake of glucose (Mayer, 1955).

As rats with acute lesions of the hypothalamus become fat their intake of food gradually returns to normal (Kennedy, 1950, 1953), and our first object was to find out whether obesity modified carbohydrate metabolism in any way that might explain its inhibitory effect on appetite.

The metabolism of glucose and that of non-esterified fatty acids are interrelated in such a way that in the mammal either one may decrease the oxidation of the other (Randle, Garland, Hales &