was then removed under reduced pressure. Benzene and ether were added and the solution was decanted from the pyridine hydrochloride. The benzene and ether were evaporated to leave a viscous residue. This residue was taken up in a hexane–ether mixture and filtered from further precipitated salts. Removal of the solvents under vacuum left 9.2 g. (78%) of the semi-solid tosylate which was used directly in the following reaction. Treatment of the 9.2 g. (0.0266 mole) of the tosylate with 2.26 g. (0.042 mole) of sodium methoxide in excess methanol for 84 hours at reflux and utilization of the procedure developed for the synthesis of DL-VII resulted in the formation of 3.88 g. (71%) of L-VII, b.p. 87–89° (0.4 mm.), $n^{25}D$ 1.5292, $[\alpha]^{25}D$ +56.4° (1.81% in $CHCl_3$).

*Anal.* Calcd. for $C_{12}H_{15}NO_2$: C, 70.21; H, 7.36. Found: C, 70.49; H, 7.48.

L-*threo*-3-Acetamido-4-methoxy-2-butanol Acetate (L-II).—The *threo*-L-5-methyl-2-phenyl-4-methoxymethyl-2-oxazoline (3.075 g., 0.015 mole) was treated with 40 ml. of 6 *N* hydrochloric acid at the reflux temperature for 6 hours, after which the product was separated from benzoic acid and acetylated as described for DL-II. Distillation gave 1.3 g. (43%) of L-II, b.p. 105–107° (0.3 mm.), m.p. 76.4–76.8°, $[\alpha]^{25}D$ +7.2° (1% solution in chloroform). The infrared spectrum of this compound in solution was identical and superimposable with the spectra of the DL-*threo*-isomer and with the D-*threo*-isomer obtained from Elaiomycin. A mixture melting point determination with the DL-*threo*-isomer VII melted at 70° while a determination with an equal amount of II from Elaiomycin raised the melting point to 79.8–80.2° indicating compound formation.

DETROIT 2, MICH.

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[CONTRIBUTION FROM THE NATIONAL INSTITUTE OF ARTHRITIS AND METABOLIC DISEASES, NATIONAL INSTITUTES OF HEALTH]

# Transglucosylation by a Mammalian Liver Enzyme

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The mechanism of action of a rat liver enzyme, termed transglucosylase, capable of transferring glucosyl residues from maltose, maltotriose or glycogen to glucose, maltose and higher homologs or to water has been studied. Glucose-$C^{14}$ is introduced by the enzyme into only the reducing terminal sugar of the oligosaccharides. It was shown that, whereas the transfer of glucosyl residues from one sugar to another is reversible, the delivery of glucosyl residues to water is irreversible. The enzymatic reaction is a slow one and does not require the participation of phosphate or other dialyzable co-factors.

The occurrence of transglycosylation reactions, resulting in the formation of oligosaccharides or polysaccharides by enzyme preparations from various plants, bacteria and molds has been shown to be widespread and has been extensively studied and well reviewed.[1,2] That such intermolecular transfer reactions may occur also in animal tissues is indicated by the progressive appearance of a series of oligosaccharides on incubation of rat liver homogenates with maltose[3] and the finding of significant amounts of such oligosaccharides in rat diaphragm[4] and normal liver.[5]

The mechanism generally proposed for such reactions involves the transfer of a glucosyl moiety from the non-reducing end of an oligosaccharide to the enzyme with the formation of a glucosyl enzyme complex. The glucosyl group is then pictured as being transferred either to a suitable carbohydrate acceptor or to water. Most, but not all[2] of the experimental observations with bacterial and plant enzyme preparations have been consistent with this concept. Studies on the nature and reversibility of the transglucosylation reaction

$$2 \text{ maltose} \rightleftharpoons \text{glucose} + \text{maltotriose} \qquad (1)$$

carried out by enzyme preparations from rat liver are here reported.

An alcohol precipitated powder obtained from the supernatant solution of rat liver homogenates was used as the source of enzyme activity. When an extract of the enzyme preparation in sodium acetate buffer of *p*H 5.0 was incubated at 37° with added maltose, liberation of glucose was observed, accompanied by the slower progressive accumulation of maltotriose and higher oligosaccharides in the medium. The synthetic reaction, with the accumulation of oligosaccharides, could be favored by the addition of glucose oxidase to the reaction mixture containing maltose and a dialyzed clear supernatant extract as the enzyme (Fig. 1). In the presence of boiled enzyme no reaction occurred. Glucose-$C^{14}$, introduced into the active reaction mixture, was incorporated into the maltose and oligosaccharides. Maltose-$C^{14}$ and maltotriose-$C^{14}$ so prepared were isolated and purified chromatographically (Fig. 2). Maltose-$C^{14}$ was characterized by conversion to its octaacetate, dilution with authentic maltose octaacetate and recrystalli-

TABLE I

RATE OF APPEARANCE OF GLUCOSE FROM A CRUDE TRANSGLUCOSYLASE PREPARATION WITH AND WITHOUT ADDED MALTOSE

| Time, hr. | Active enzyme[b] No added substrate, γ glucose/ml. | Active enzyme[b] 1000 γ maltose/ml., γ glucose/ml.[a] | Boiled enzyme 1000 γ maltose/ml., γ glucose/ml.[a] |
|---|---|---|---|
| 0 | 4.8 | 4.2 | 4.8 |
| 1 | 5.0 | 127 | 6.9 |
| 2 | 5.2 | 150 | 5.2 |
| 4 | 3.6 | 287 | 7.0 |
| 6 | .. | 326 | .. |
| 12 | 7.8 | 449 | 6.3 |
| 24 | 17.3 | 755 | 8.5 |
| Total on acid hydrolysis | 64[c] | 1074 | |

[a] Values corrected for 8 γ of glucose/ml. produced by glucose oxidase (Worthington Biochemical Corp.) under the same conditions from 1000 γ maltose/ml. [b] Five mg. enzyme powder per ml. of acetate buffer, *p*H 5.0. [c] In other experiments, when only the soluble portion of the dialyzed enzyme was used, there was no glucose precursor in the enzyme solution.

(1) E. J. Hehre, *Adv. Enz.*, **11**, 297 (1951).

(2) J. Edelman, *ibid.*, **17**, 189 (1956).

(3) K. V. Giri, A. Nagabhushanam, V. N. Nigam and B. Balavadi, *Science*, **121**, 898 (1955).

(4) A. Beloff-Chain, R. Catanzaro, E. B. Chain, I. Masi, F. Pocchiari and C. Rossi, *Proc. Roy. Soc. (London)*, **B143**, 481 (1955).

(5) W. H. Fishman and H.-G. Sie, THIS JOURNAL, **80**, 121 (1958).

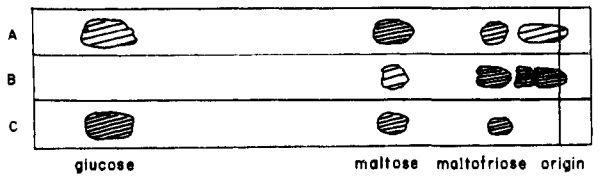


Fig. 1.—Products of the action of the transglucosylation enzyme on maltose for 2 days in the absence (A) and in the presence (B) of glucose oxidase. C gives the location of known compounds on a similarly developed strip. The relative intensity of the spots on the developed chromatograms after staining with benzidine spray is indicated by the cross hatching.

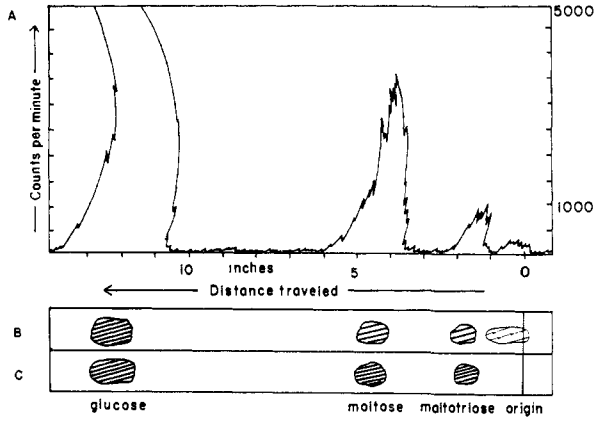


Fig. 2.—Separation of enzymatically prepared maltose-$C^{14}$ and maltotriose-$C^{14}$ from glucose-$C^{14}$. A is the type of record obtained with a portion of the reaction mixture developed on paper and counted with an automatic paper strip scanner coupled with an Esterline–Angus recorder. The strip (B) stained with benzidine spray, was compared with similarly developed strips containing known compounds.

zation to constant specific activity. Location of glucose-$C^{14}$ within the maltose-$C^{14}$ was studied by oxidation and by conversion to its 1-phenylflavazole. Acid hydrolysis of maltobionic acid formed from the maltose-$C^{14}$ gave radioactive gluconic acid and glucose free of detectable radioactivity (Fig. 3). Hydrolysis of the 1-phenylflavazole of maltose-$C^{14}$ produced radioactive glucose flavazole and unlabeled glucose (Fig. 4). The 1-phenylflavazole of maltotriose-$C^{14}$ was also prepared, purified and hydrolyzed and $C^{14}$ was found to have been exclusively in the reducing sugar residue. The action of the mammalian liver enzyme in the transferral of glucosyl groups to carbohydrate acceptors is very much slower under the conditions studied than is the transferral to water. In Table I is given a typical example of the rate at which free glucose is produced from maltose. More glucose is formed than can be accounted for by equation 1 and an equilibrium is not reached.

The steps involved in the enzymatic reactions may be represented as

$$\text{glucosyl glucose}^* + \text{enzyme} \rightleftharpoons \text{glucosyl enzyme} + \text{glucose}^* \quad (2)$$

$$\text{glucosyl enzyme} + \text{glucosyl glucose}^* \rightleftharpoons \text{glucosyl-glucosyl-glucose}^* + \text{enzyme} \quad (3)$$

$$\text{glucosyl enzyme} + H_2O \longrightarrow \text{glucose} + \text{enzyme} \quad (4)$$

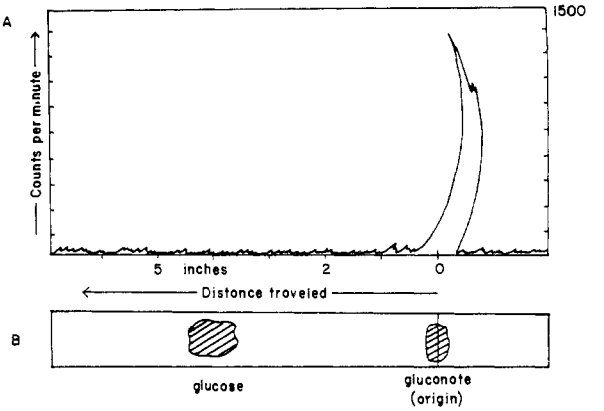


Fig. 3.—Glucose and gluconic acid from hydrolysis of maltobionic acid obtained from enzymatically prepared maltose-$C^{14}$. The products were separated by development on a paper strip for 24 hr. with *n*-butanol:pyridine:water:benzene (5:3:3:0.45). A is the record made from the strip by an Esterline–Angus recorder coupled to an automatic paper strip counter. B is the same strip stained with ammoniacal $AgNO_3$ containing NaOH.

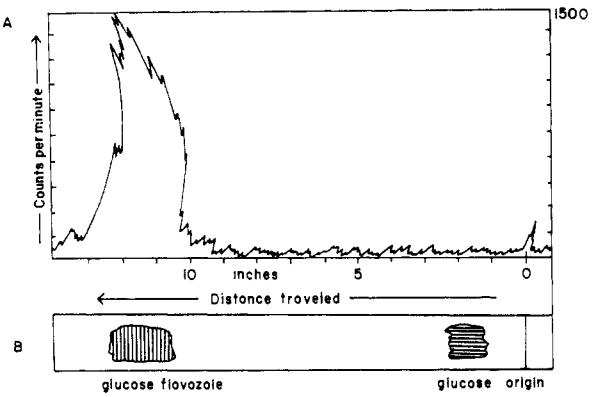


Fig. 4.—Hydrolysis products of the 1-phenylflavazole derived from enzymatically prepared maltose-$C^{14}$. A is the record of the radioactivity distribution and B gives the location of the products on the same paper strip.

Glucose-$C^{14}$, represented by glucose*, would be introduced into the reducing sugar moiety of maltose by the reversal of reaction 2 and into maltotriose by reaction 3. Further continuing reactions in which maltotriose-$C^{14}$, etc., act as receptor substrates would result in the synthesis of higher oligosaccharides labeled only in the terminal reducing sugar. Reaction 3 is reversible as shown by the data in Table II. Enzymatically prepared maltotriose-$C^{14}$, having radioactivity exclusively in the terminal reducing glucose residue, was used as the sole substrate with a dialyzed soluble preparation of the liver enzyme. The appearance of other compounds and their changing radioactivity was followed as a function of time. As the maltotriose was utilized, maltose-$C^{14}$ progressively accumulated, presumably by the reversal of reaction 3. The initial glucose formed by reactions 3 and 4 was not radioactive. Glucose-$C^{14}$ appearance lagged well behind the maltose-$C^{14}$ and probably resulted from the secondary reaction of the maltose with the enzyme.

TABLE II

MALTOTRIOSE-$C^{14}$[a] AS SUBSTRATE FOR TRANSGLUCOSYLASE[b]

| Hr. of incubation | C.p.m.[c] in glucose | C.p.m. in maltose | C.p.m. in maltotriose | C.p.m. in higher oligosaccharides |
|---|---|---|---|---|
| 0 | 0 | 0 | 1000 | 0 |
| 1 | 0 | 40 | 840 | 0 |
| 2 | 0 | 50 | 760 | |
| 4 | 10 | 70 | 740 | |
| 6 | 30 | 150 | 720 | |
| 24 | 40 | 260 | 460 | 110 |

[a] Enzymatically prepared, labeled exclusively in the reducing glucose residue. [b] 0.15 ml. of a soluble dialyzed extract equivalent to 10 mg. of the liver enzyme preparation, in acetate buffer, *p*H 5.0. [c] The values for counts per min. above background here given represent the activity at the height of the peak recorded for each compound when counted on comparable paper strip chromatograms. Twenty λ aliquots were used at each time interval.

That reaction 4 is irreversible is shown by several facts. Reversibility in the presence of glucose-$C^{14}$ would, in conjunction with reactions 2 and 3, have resulted in the introduction of glucose-$C^{14}$ into the non-reducing as well as the reducing glucose moieties of maltose and maltotriose. This has not been observed. Also, with the enzyme and glucose alone, no maltose or other oligosaccharides were formed. Free glucose may act as an acceptor but not as a donor of glucosyl groups. Part of the hydrolytic action may have been due to another type of maltase reaction which may be represented by

$$\text{maltose} + H_2O \longrightarrow 2\ \text{glucose} \qquad (5)$$

which would be indistinguishable from reaction 2 plus reaction 4. If such a reaction is occurring, it must be irreversible. The absence of α-glucosidase activity of the sort exhibited by brewer's yeast[6] was established by the finding that methyl α-glucoside is not hydrolyzed by the liver enzyme preparation at *p*H 7.0 under the same conditions which result in relatively rapid hydrolysis of maltose.

The finding of $C^{14}$ exclusively in the reducing sugar residue as a result of the action of the liver transglucosylase is similar to the findings of French, *et al.*,[7] in which *Bacillus macerans* amylase was found to catalyze the formation from cycloamylose and glycose-$C^{14}$ of a series of amyl-oligosaccharides labeled only in the reducing terminal sugar. The transfer reaction is dissimilar to the transgalactosylation recently reported by Pazur, *et al.*,[8] in which the hydrolytic step was found to be reversible, resulting in the introduction of free galactose-$C^{14}$ into galactosyl glucose.

The mammalian liver transglucosylation enzyme has many properties similar to those of the amylomaltase of *E. coli*.[9] It has approximately the same activity in acetate buffer as in phosphate buffer, acts well at both *p*H 5.0 and 7.0, the activity is retained after dialysis, and there is no action on glucose 1-phosphate, sucrose or methyl α-glucoside. Its mode of action may, however, be different, in view of the observation of Porter and Reynolds[10] that amylomaltase from *E. coli* permitted the incorporation of glucose-$C^{14}$ into both moieties of maltose.

Glycogen, as well as maltose, is effective as a source of glucosyl groups for transferral (Table III). The possibility that this action is secondary to the formation of free maltose by the action of an amylase is difficult to eliminate. However, when a clear supernatant extract of the enzyme preparation, dialyzed exhaustively against water, and entirely free of all amylase activity by the usual starch iodide test[11] is used, glycogen is just as effective a donor substrate as when a crude suspension is used. The fact that the incorporation of glucose-$C^{14}$ into maltose appears to be greater with glycogen than with maltose as substrate is no doubt due to greater dilution of the newly formed maltose-$C^{14}$ in the latter case and is not significant. A sample of purified maltose-$C^{14}$, isolated from the reaction of glucose-$C^{14}$ and glycogen with such an enzyme preparation, was converted to its flavazole, which was then hydrolyzed. The $C^{14}$ was found exclusively in the residue which had been the reducing sugar moiety of the maltose. It would thus appear that terminal glucosyl residues of glycogen are transferred to free glucose.

TABLE III

TRANSGLUCOSYLATION

| Composition of incubation mixture | 1 Day incubation C.p.m.[e] in maltose | 1 Day incubation C.p.m. in maltotriose | 2 Day incubation C.p.m. in maltose | 2 Day incubation C.p.m. in maltotriose | 4 Day incubation C.p.m. in maltose | 4 Day incubation C.p.m. in maltotriose |
|---|---|---|---|---|---|---|
| Enzyme[a] Glucose-$C^{14}$[b] | 0 | 0 | 0 | 0 | 0 | 0 |
| Enzyme Glucose-$C^{14}$ Maltose[c] | 130 | 10 | 330 | 20 | 660 | 120 |
| Enzyme Glucose-$C^{14}$ Sucrose[c] | 0 | 0 | 0 | 0 | 0 | 0 |
| Enzyme Glucose-$C^{14}$ Glycogen[d] | 160 | 50 | 360 | 90 | 850 | 140 |
| Enzyme Glucose-$C^{14}$ 1-phosphate[f] alone or with glucose or maltose | 0 | 0 | 0 | 0 | 0 | 0 |

[a] The enzyme solution contained 10 mg. of the alcohol-precipitated powder per ml. of 0.02 *M* sodium acetate buffer of *p*H 5.0 and had been dialyzed for 3 hr. against the buffer. [b] Ten mg. uniformly labeled glucose-$C^{14}$ (0.83 μc./mg.) per ml. of reaction mixture. [c] Twenty mg. per ml. [d] Twenty mg. of rat liver glycogen per ml. [e] The values for counts per min. above background here given represent the activity at the height of the peak recorded for each compound when counted on comparable paper strip chromatographs. The glucose spot on each chromatogram in which glucose-$C^{14}$ was used was about 6000 c.p.m. in this experiment. [f] The potassium salt of glucose-$C^{14}$-1-$PO_4$, prepared from starch $C^{14}$ by the action of phosphorylase, contained 26,300 c.p.m./milliatom C. The height of the glucose-1-$PO_4$ spot on the chromatograms was about 3000 c.p.m. No glucose-$C^{14}$ was formed from glucose-$C^{14}$-1-$PO_4$.

(6) J. Leibowitz and S. Hestrin, *Biochem. J.*, **36**, 772 (1942).

(7) D. French, M. L. Levine, E. Norberg, P. Nordin, J. H. Pazur and G. M. Wild, THIS JOURNAL, **76**, 2387 (1954).

(8) J. H. Pazur, J. M. Marsh and C. L. Tiptou, *ibid.*, **80**, 1433 (1958).

(9) J. Monod and A. M. Torriani, *Ann. Inst. Pasteur*, **78**, 65 (1950); *Compt. rend. acad.*, **227**, 240 (1948).

(10) Cited in ref. 2; T. Reynolds, "The Metabolism of Maltose by Tobacco Leaf Tissue," M.Sc. Thesis, University of London.

(11) G. T. Cori and J. Larner, *J. Biol. Chem.*, **188**, 17 (1951).

Glucose 1-phosphate was found to be completely inactive both as a donor and as an acceptor of glucosyl groups. No significant difference in transglucosylation activity with maltose or glycogen was observed when phosphate buffer was substituted for acetate buffer.

Thus a mammalian enzyme exists which is capable of a slow, simple transfer of glucosyl groups from maltose and oligosaccharides and possibly from glycogen to glucose, maltose and higher homologs, with the possible formation of seeds for new polysaccharide molecules. Neither phosphate nor phosphorylated intermediates are involved in these reactions.

## Experimental

**Enzyme Preparations.**—Adult white rats, fasted for 24 hr. to deplete their livers of glycogen, were killed by a blow on the head and allowed to bleed freely from the neck vessels. The livers were perfused with cold isotonic KCl to remove blood and were homogenized with 2 volumes of cold distilled water in an Omni-mixer for 3 min. The *p*H of the homogenate was adjusted to 5.8 with very dilute HCl and the precipitated material removed by centrifugation for 10 min. at about 4000 × *g*. Ethanol was added to the supernatant solution at 0° until the alcohol content reached a concentration of 80% by volume, the mixture was centrifuged, the precipitate dried with acetone and ether and the resulting tan powder stored in a desiccator at 4°.

In some preparations the adjustment of *p*H was omitted before centrifugation and the crude enzyme powders obtained were found to have essentially the same transglucosylase activity but to contain more extraneous material. The crude enzyme preparations, suspended in buffer, exhibited no amylase activity against starch when tested in acetate buffer, *p*H 5.0, but did show slight amylase activity at *p*H 7.0. Attempts were made to eliminate this amylase activity by adsorption on corn starch from the crude supernatant solutions after adjustment to *p*H 7.0.[11] This procedure was not found to decrease the amylase activity and had the additional disadvantage of introducing into the enzyme preparation appreciable quantities of oligosaccharides, formed during the starch treatment, which subsequently could serve as substrate for the transglucosylase. All detectable amylase activity could be eliminated by exhaustive dialysis against water of the clear supernatant portion of buffer extracts of the enzyme preparations. Attempts were made to concentrate the transglucosylase activity by fractional precipitation with ethanol. Fractions precipitating at 50% ethanol concentration were found to have approximately the same activity as those precipitating between 50 and 80% ethanol.

**Rate of Appearance of Glucose.**—Glucose was determined by oxidation with glucose oxidase and coupling of the $H_2O_2$ produced with *o*-dianisidine in the presence of peroxidase.[12] The color produced was read at 390 mμ. It was found that the glucose oxidase preparations used (Worthington Biochemical Corp.) exhibited some maltase activity (Fig. 5). The rate of production of glucose from maltose by the glucose oxidase preparation was relatively slow, however, when compared with the rate of oxidation of glucose. The method could be used to analyze for glucose in the presence of large amounts of maltose by stopping the reaction after 10 min. and making a correction for the hydrolysis of maltose. For example a sample containing 500 γ of maltose consistently yielded about 6 γ of glucose in 10 min.

A solution containing 5 mg. of the transglucosylase preparation per ml. of acetate buffer at *p*H 5.0 was incubated at 37° with and without the addition of 1000 γ of maltose per ml. A portion of the reaction mixture, heated for 5 min. at 100°, was also incubated as a control. Aliquots were removed for glucose determinations at various time intervals. Glucose was determined by the glucose oxidase method with corrections made for maltose hydrolysis during the analysis. The results are given in Table I. Glucose was not formed in the absence of active enzyme. Added maltose resulted in the continual slow liberation of glucose.

**Transglucosylation activity** was assayed by carrying out the enzymatic reaction at 37° in the presence of glucose-$C^{14}$ or glucose-$C^{14}$-1-phosphate with and without various added carbohydrate substrates. A typical experiment is given in Table III. After various time intervals of incubation, 10 λ aliquots of the reaction mixture were deposited on paper as spots of uniform circumference and developed for either one or two days by descending chromatography using ethyl acetate:acetic acid:water (3:1:3) or butanol:ethanol:water (3:2:1) as solvent. $C^{14}$ on the resulting chromatograms was located by scanning with a modified Forro automatic paper strip scanner, coupled to an Esterline–Angus recorder.[13] The values given represent the activity at the height of the peak recorded for each compound. The compounds were identified by comparison with strips containing the known compounds, developed at the same time and stained with benzidine.[14] With glucose-$C^{14}$ alone or with added sucrose no maltose is formed. With maltose or glycogen, glucose-$C^{14}$ is progressively incorporated into maltose and maltotriose. Glucose-$C^{14}$ 1-phosphate is not split to glucose-$C^{14}$ nor incorporated into maltose either alone or in the presence of added glucose or maltose.

The enzyme is very stable. After being incubated in solution for 12 days at 37°, a solution of the enzyme was dialyzed and glucose-$C^{14}$ and maltose re-added. Further incubation showed that the transglucosylation activity was retained with little loss.

**Characterization of the Maltose Produced by the Transglucosylation Reaction.**—A sample of radioactive disaccharide was prepared by the action of the enzyme on nonisotopic maltose in the presence of glucose-$C^{14}$ and was separated from other components of the reaction mixture by descending paper chromatography using ethyl acetate:acetic acid:water (3:1:3). The radioactive area of the paper which corresponded to known maltose was eluted and redeveloped with butanol:ethanol:water (3:2:1). The maltose area was again eluted and the resulting material, estimated to contain about 10 mg., was diluted in solution with about 50 mg. of known maltose, dried and converted to maltose octaacetate.[15] The crude derivative was diluted with about 50 mg. of known maltose octaacetate and carefully purified by three recrystallizations from aqueous ethanol. Forty-three mg. of maltose octaacetate was obtained, m.p. 156–157°. The specific activity was 226 c.p.m. per mg., counted as $BaCO_3$ after wet combustion.

Thirty-seven mg. of this three-times recrystallized material was further twice recrystallized and the specific activity was found to be unchanged. Thirty-two mg. was recovered, m.p. 156–157°; specific activity, 222 c.p.m. per mg.

The enzymatically synthesized oligosaccharides exhibited $R_g$ values identical with those of the maltose series and significantly different from those of the isomaltose series which could readily be distinguished in the solvents employed for chromatography (Table IV).

**Conversion of Maltotriose-$C^{14}$ to Maltose-$C^{14}$ and Glucose.**—A sample of purified, enzymatically prepared maltotriose-$C^{14}$, having a total of about 7000 c.p.m. at the highest point of the peak recorded, was added to 0.15 ml. of a concentrated dialyzed enzyme solution equivalent to the soluble extract of 10 mg. of the enzyme preparation in acetate buffer at *p*H 5.0. The mixture was incubated at 37°. A 20 λ aliquot was taken at each time interval, deposited uniformly on paper and developed for 2 days with ethyl acetate:acetic acid:water (3:1:3). Each strip was counted and the compounds were then located by spraying with benzidine spray and compared with known compounds similarly developed. The $C^{14}$ results are given in Table II. Initially the only staining spot was maltotriose. With the passage of time the quantity of maltotriose decreased and spots corresponding to maltose, glucose and higher oligosaccharides appeared and became progressively more prominent.

**Location of $C^{14}$ in Enzymatically Prepared Maltose-$C^{14}$—Oxidation Method.**—A solution of 20 mg. of the crude enzyme preparation, 20 mg. of maltose and 10 mg. of glucose-

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(13) F. Eisenberg, Jr., and I. G. Leder, *Anal. Chem.*, in press.

(14) W. J. Whelan, J. M. Bailey and P. J. P. Roberts, *J. Chem. Soc.*, 1293 (1953).

(15) K. Freudenberg, H. v. Hochstetter and H. Engels, *Ber.*, **58**, 666 (1925).

TABLE IV

$R_g$ VALUES—DESCENDING PAPER CHROMATOGRAPHY WITH ETHANOL:ACETIC ACID:WATER (3:1:3)

| | |
|---|---|
| Maltose | 0.37 |
| Isomaltose[a] | .27 |
| Maltotriose[b] | .14 |
| Isomaltotriose[a] | .08 |

[a] Isolated from partially hydrolyzed dextran. [b] Isolated from partially hydrolyzed amylose.

$R_f$ VALUES—DESCENDING PAPER CHROMATOGRAPHY WITH BUTANOL:ETHANOL:WATER (3:2:1) (v./v.)

| | |
|---|---|
| Maltotriose[c] | 0.017 |
| Maltose[c] | .043 |
| Glucose | .124 |
| Maltotriose flavazole | .34 |
| Maltose flavazole | .59 |
| Glucose flavazole | .84 |

[c] Values calculated from $R_{glucose}$ values obtained from chromatograms run for longer periods of time.

$C^{14}$ (0.83 μc./mg., uniformly labeled, Nuclear Instrument & Chemical Corp.) in 1 ml. of 0.02 *M* sodium acetate buffer of *p*H 5.0 was incubated for 2 days at 37°. Maltose-$C^{14}$ was isolated from the supernatant solution of the resulting mixture by elution of the appropriate area of a paper chromatogram after 6 days of development by descending paper chromatography with butanol:ethanol:water (3:2:1) (Fig. 2). The maltose was converted to maltobionic acid-$C^{14}$ by oxidation with $I_2$ in KOH-methanolic solution.[16] The potassium salt of the bionic acid was separated from traces of unoxidized maltose-$C^{14}$ and any possible contaminating glucose by descending paper chromatography for 2 days with pyridine:butanol:water:benzene (5:3:3:0.45).[17] The radioactive bionic acid, after elution from the paper, was hydrolyzed to glucose and gluconic acid by refluxing for 3 hr. in a very small volume of dilute HCl. The solution was made slightly alkaline with NaOH and the glucose and Na gluconate were separated by paper chromatography. Staining with $AgNO_3$–$NH_3$–NaOH revealed approximately equal amounts of glucose and gluconate. The glucose was entirely free of radioactivity while the gluconic acid contained all of the $C^{14}$ which had been incorporated enzymatically into the maltose (Fig. 3).

**Flavazole Method.**—Glucose flavazole, maltose flavazole and maltotriose flavazole were prepared by condensation of authentic samples of these sugars with *o*-phenylenediamine and phenylhydrazine in aqueous acetic acid.[18] It was found that small quantities of these compounds could readily be separated from each other, from the free sugars and from an excess of reagents and by-products of the synthesis by an 18 hr. development by descending chromatography using butanol:ethanol:water (3:2:1). *o*-Phenylenediamine, phenylhydrazine and aniline migrated with the solvent front while the sugars and flavazoles exhibited the $R_f$ values given in Table IV. For the synthesis of the flavazoles of the radioactive sugars a micro modification was used in which 2 to 4 mg. of sugars and appropriate quantities of reagents in a total volume of about 0.1 ml. were heated together in small sealed tubes at 100° for 8 hr. A 3.6-mg. portion of maltose-$C^{14}$ prepared enzymatically from glucose-$C^{14}$ and maltose and purified chromatographically, was converted to its flavazole, which was in turn chromatographed on paper. This purified maltose flavazole, eluted with acetic acid, was hydrolyzed by heating at 100° for 8 hr. with 0.1 ml. 4 *N* HCl in a sealed tube. The resulting hydrolysis mixture was evaporated to dryness, redissolved in hot propanol, and the products separated by paper chromatography using butanol:ethanol:water (3:2:1). Equivalent amounts of glucose and glucose flavazole were obtained. The glucose was free of radioactivity while the glucose flavazole contained all of the radioactivity initially present in the maltose-$C^{14}$ flavazole (Fig. 4).

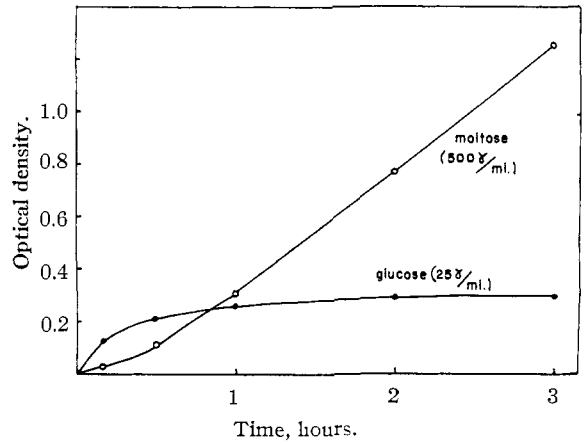


Fig. 5.—Action of glucose oxidase preparation (Worthington Biochemical Corp.) on glucose and maltose. Absorbancy (λ mμ) developed by treatment of the enzyme digests with *o*-diamisidine is plotted against the time of the enzymic reaction.

**Location of $C^{14}$ in Maltotriose.**—A sample of purified enzymatically prepared maltotriose-$C^{14}$ was converted to its 1-phenylflavazole.[19] The maltotriose flavazole, separated by paper chromatography, was eluted and hydrolyzed with HCl. All of the $C^{14}$ was found in the glucose flavazole derived from the terminal reducing glucose residue, while the free glucose, derived from the non-reducing portion of the oligosaccharide, was free of radioactivity.

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