Multimolecular Substrate Reactions Catalyzed by Carbohydrases. *Aspergillus oryzae* α-Amylase Degradation of Maltooligosaccharides†

Jimmy D. Allen*,‡ and John A. Thoma

ABSTRACT: *Aspergillus oryzae* α-amylase degrades maltooligosaccharides by other pathways besides simple glycosidic bond scission. The utilization of the alternate pathways increases with the concentration of substrate implicating a multimolecular substrate mechanism. Reducing-end labeled and uniformly labeled maltooligosaccharides were used to elucidate these alternate degradation mechanisms. Condensation followed by hydrolysis is not a significant pathway. Transglycosylation is concluded to occur, but no single transglycosylation mechanism can account for all of the experimental data for maltotriose degradation. Rather, a combination of transglycosylations must be invoked.

It has become increasingly clear that the action pattern of carbohydrases is much more complex than was once suspected (Hehre et al., 1973, and references therein). Carbohydrases that are classified as hydrolases (Florkin & Stotz, 1973) have been shown to exhibit significant nonhydrolytic catalytic activity. Reactions such as condensations to form a glycosidic linkage (Abdullah & French, 1966, 1970; Hehre et al., 1969) and transfers to rearrange glycosidic bonds (Chipman, 1971; Robyt & French, 1970; Abdullah & French, 1970) have been shown to be catalyzed by carbohydrases such as lysozyme, α- and β-amylase, pullulanase, and glucoamylase. Despite these several reports of nonhydrolytic reactions, there have only been a few attempts (Robyt & French, 1970; Chipman, 1971) to relate the significance of these reactions to the degradation of carbohydrates.

Aspergillus oryzae (AO) α-amylase[1] is one of the more extensively studied amylases (e.g., Nitta et al., 1971; Omichi et al., 1972; Suetsugu et al., 1974; Allen & Thoma, 1976b). In addition to its amylase activity, AO amylase also exhibits glycosidase activity, hydrolyzing phenyl and *p*-nitrophenyl α-maltoside to maltose and the corresponding phenol (Matsubara et al., 1959). However, when an alternate alcohol acceptor is present, a fraction of the maltose is transferred to the alcohol (Matsubara, 1961). It was also observed that in the degradation of maltopentaose, a maltodextrin of chain length greater than five was formed (Okada et al., 1969). With amylose (Omichi & Matsushima, 1970) or glycogen (Takeshita & Hehre, 1975) as the substrate, transfer to maltitol and substituted glucosides was observed. AO amylase was also reported to catalyze the synthesis of maltose and maltotriose from α-D-glycopyranosyl fluoride (Hehre et al., 1971). It was recently reported that the products of degradation of maltotriose depend on substrate concentration (Suganuma et al., 1976). Despite reports of reactions other than unimolecular hydrolysis,[2] no general model for amylase degradation of carbohydrates has been presented.

Recently authors have begun to develop the theory of depolymerase action through subsite mapping (Chipman & Sharon, 1969; Hiromi, 1970; Allen & Thoma, 1976a). When refined models such as subsite mapping are applied to carbohydrases, complicating reactions such as multimolecular events and repetitive attack (Robyt & French, 1967) must be taken into account. For example, a subsite map for AO amylase was calculated from Michaelis parameters, $\tilde{K}_m$ and $\tilde{V}$, as a function of substrate chain length (Nitta et al., 1971). The study reported here and in the following paper (Allen & Thoma, 1978) reveals that the interpretation of $\tilde{K}_m$ and $\tilde{V}$ is complicated because the enzyme utilizes several reaction pathways simultaneously for substrate degradation. Hence, the Michaelis parameters are not a suitable probe of unimolecular events. We have calculated a new subsite map for AO amylase (Allen & Thoma, 1976b) using experimental parameters obtained under conditions where complicating reactions are minimized.

We report here a quantitative study of the action of AO amylase on reducing-end and uniformly ^{14}C labeled maltooligosaccharides. It is apparent from the products of degradation of the maltodextrin substrates that reactions other than simple unimolecular hydrolysis are major reaction pathways under certain experimental conditions. The products of the degradation of the maltodextrins are a function of the concentration of substrate, which is indicative of the involvement of more than one substrate molecule in the reaction mechanism (i.e., multimolecular). We have tested for possible bimolecular mechanisms in the degradation of maltotriose, maltotetraose, and maltipentaose. In the following paper (Allen & Thoma, 1978) we report the full scheme for degradation of maltotriose by AO amylase.

Experimental Procedure

Enzymes. Three-times-recrystallized *Aspergillus oryzae*

† From the Department of Chemistry, University of Arkansas, Fayetteville, Arkansas 72701. *Received June 20, 1977.* This work was supported in part by Corn Industries Research Association, by Agricultural Research Services, U.S. Department of Agriculture Grant 12-14-100-9908 (71), and by National Science Foundation Grant BMS-74-19838. A report of this work was presented at the 1976 American Society of Biological Chemists meeting (*Fed. Proc., Fed. Am. Soc. Exp. Biol. 35*, Abstr. 2019).

‡ Current address: Department of Chemistry, University of California, Santa Barbara, California 93106.

[1] Abbreviations used: AO amylase, *Aspergillus oryzae* α-amylase, 1,4-α-D-glucan glucanohydrolase (EC 3.2.1.1), frequently referred to as Taka-amylase A; G_n, maltodextrin of *n* glucosyl units; G_n*, a reducing-end ^{14}C-labeled maltodextrin of *n* glucosyl units; ○, D-glucopyranoside unit; ◌, reducing D-glucopyranoside unit; ●, ^{14}C-labeled D-glycopyranoside unit; ◉, reducing ^{14}C-labeled D-glucopyranoside unit; —, α-(1→4)-glycosidic bond.

[2] Unimolecular, bimolecular, and multimolecular are used to designate enzymic processes in which, respectively, one, two, or some indeterminate number greater than one molecule of substrate oligosaccharide participate in the reaction mechanism (Cleland, 1970; Robyt & French, 1970).

0006-2960/78/0417-2338$01.00/0 © 1978 American Chemical Society

α-amylase was a generous gift of Y. Nitta. The enzyme, which gave three bands of activity on polyacrylamide gel electrophoresis (Davis, 1964), was further purified on Sephadex A-50 (Toda & Akabori, 1963). The main peak of activity gave a single protein band on electrophoresis and gave a single component of $S_{20,buffer} = 4.2$ on ultracentrifugation (Allen, 1975b). A preparation of β-amylase (Marshall & Whelan, 1973), kindly supplied by J. Marshall, was free of maltase activity under the conditions used in this study. Cyclodextrin glucanotransferase (EC 2.4.1.19) was prepared as described (Allen, 1975b) from a *Bacillus macerans* culture kindly supplied by J. Robyt.

Assays. Reducing sugars (Dygert et al., 1965) and total carboyhdrate (Dubois et al., 1956) were determined as described.

For relative radioactivity determinations, the sample was dried on filter paper and the radioactivity counted in 5 mL of toluene cocktail (Thoma et al., 1970). For absolute specific activity determinations, the radioactivity of the aqueous sample was determined in Aquasol (New England Nuclear) and corrected for quenching.

Maltodextrin Oligosaccharides. Reducing-end labeled maltodextrin oligosaccharides were prepared by the action of cyclodextrin glucanotransferase on cyclohexaamylose (French et al., 1963) and D-[U-^{14}C]glucose (ICN, 30 μCi/mg; Amersham/Searle, 1.49 mCi/mg; Pazur, 1955). In a typical incubation 120 mg of cyclohexaamylose and 11 mg of D-[U-^{14}C]glucose were incubated with 7.5 units (Tilden & Hudson, 1942) of enzyme in 2 mL of 0.01 M sodium acetate, 5 mM $CaCl_2$, pH 4.8. A crystal of thymol was added and the mixture was incubated at 38 °C for 3 days before heating at 100 °C for 10 min. The mixture was concentrated and cyclohexane was employed to partially precipitate the cyclic dextrins (Thoma & Stewart, 1965). The oligosaccharides were fractionated on Whatman 3MM paper (Thoma et al., 1970) in water–ethanol (95%)–nitromethane (13:18:19, v/v) (Thoma & French, 1957) using multiple ascents (French & Wild, 1953) at room temperature. The radioactive maltodextrin oligosaccharides were visualized by autoradiography using Kodak No-Screen x-ray film. Cyclic dextrins were visualized with iodine vapors or with an $AgNO_3$–NaOH reagent (Trevelyn et al., 1950) after they had been hydrolyzed with AO α-amylase and glucoamylase (Sigma) (Allen, 1975b). The oligosaccharides were eluted and rechromatographed using the same solvent system or water–pyridine–1-butanol (19:36:45, v/v). Up to 15 ascents were employed to fractionate the larger oligosaccharides and the cyclic dextrins.

Unlabeled oligosaccharides were prepared in a similar manner on a larger scale and separated at 65 °C on a Bio-Gel P-2 (minus 400 mesh) 1.0 × 115 cm column (John et al., 1969). The purity of the oligosaccharides was confirmed by TLC (Weill & Hanke, 1962; Stahl & Kaltenbach, 1961) or by paper chromatography. When the oligosaccharides were not pure, they were rechromotographed on the same system or fractionated using preparative paper chromotography. Unlabeled maltotriose was also purchased from Sigma.

Uniformly labeled maltotriose was prepared from U-^{14}C-labeled tobacco leaf starch (1.5 mCi/mg, ICN) after debranching with *Pseudomonas* isoamylase (EC 3.2.1.68) (Harada et al., 1972), a generous gift of T. Harada. The starch (500 μCi) was dissolved in 1 mL of 0.05 M sodium acetate, pH 3.5, and 200 μL (0.16 unit) of enzyme was added. The mixture was incubated at 38 °C for 22 h, heated at 100 °C for 10 min, concentrated, and then fractionated by paper chromatography as described above. The absence of 1,6 linkages was confirmed by complete digestion with β-amylase.

Standard ^{14}C-labeled glucitol, maltitol, and maltotriitol were prepared by $NaBH_4$ reduction of the corresponding end-labeled sugars. All oligosaccharide solutions were stored at −15 °C and periodically repurified when degradation products became apparent on the autoradiograms.

Oligosaccharide Hydrolysis. The distribution of labeled products from the AO amylase hydrolysis of end-labeled or uniformly labeled maltodextrin oligosaccharides was determined as a function of the extent of reaction (Robyt & French, 1970), calculated as the ratio of radioactivity in products divided by total sample radioactivity. In a bimolecular process, where unlabeled substrate may be formed, the extent of reaction is less than the fraction of substrate reacted. The reactions were carried out at 25 °C in 0.08 M sodium acetate, pH 5.3, or in 0.05 M pyridine–acetic acid, pH 5.3. The two buffers were shown to result in the same products for maltooligosaccharide degradation (Allen, 1975b). Reactions were initiated by the addition of AO amylase to give an enzyme concentration which would result in ca. 50% disappearance of the original labeled substrate in 1 h. Aliquots were removed at appropriate time intervals. The aliquot size was chosen to give sufficient counts to be able to detect 0.5% hydrolysis at a single bond of the oligosaccharide. The aliquot was added to twice its volume of concentrated NH_4OH in a depression on a wax plate and immediately spotted, along with standards, on Whatman 3MM chromatography paper with hot air drying. For larger aliquots, the enzyme was denatured by heating at 100 °C for 10 min before the sample was concentrated and spotted. The chromatograms were irrigated a sufficient number of times to obtain good resolution of the oligosaccharides under investigation and were visualized by autoradiography. Using the resulting autoradiogram as a guide, the radioactive spots were cut out, and the radioactivity was determined in the toluene cocktail. When a particular product oligosaccharide was not present in sufficient quantities to darken the film, the standards were used as a guide to cut out the chromatogram.

Product Ratio. The product ratio is the ratio of the radioactivity due to a particular product divided by the total radioactivity of the products of an end-labeled oligosaccharide degradation. The term bond cleavage frequency has been used (Allen & Thoma, 1976a) when referring to the relative rates of unimolecular hydrolysis of the bonds in an oligosaccharide. It was shown (Allen, 1975a) that the product ratios for an *n*-mer are best evaluated by plotting (demonstrated in Figure 1) the radioactivity of each product divided by the total radioactivity of the sample vs. the total radioactivity of the products divided by the total radioactivity of the sample. The initial slope of each plot is the initial product ratio of that product. The zero-time sample background influences only the intercept of such a plot; hence subtraction of a sample background is unnecessary and merely propagates error throughout the measurements (Allen, 1975a). A curvilinear plot is indicative of either secondary attack on the initial hydrolysis products or a shift in mechanism resulting in different products.

Test For Label Redistribution. The labeled maltose produced from the AO amylase degradation of reducing-end labeled maltotriose at 40 mM initial concentration was examined for the presence of ^{14}C-labeled glucose in the nonreducing end. The maltose was separated from other degradation products by paper chromatography, visualized by autoradiography, and eluted from the chromatography paper with water. The maltose was reduced by reaction with a ten times molar excess of $NaBH_4$ for 24 h before the reaction mixture was neutralized with acetic acid and concentrated. The sample was fractionated, along with standards of maltose and maltitol, by elec-

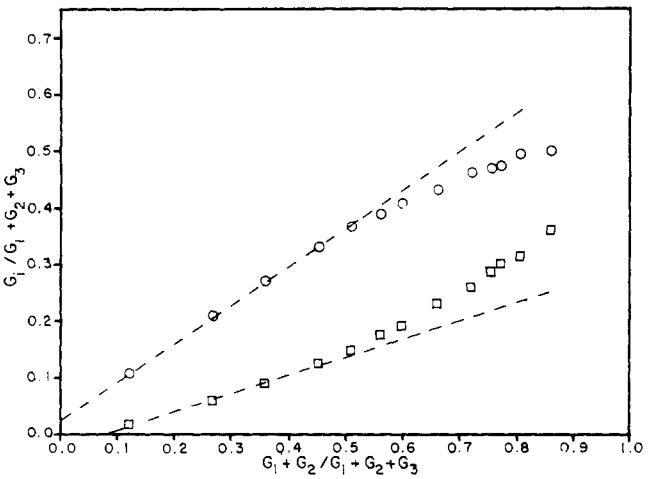

FIGURE 1: Shift in product ratio in maltotriose degradation as a function of the extent of reaction. The ratio of (□) labeled glucose and (○) labeled maltose to total labeled oligosaccharides is plotted vs. the extent of reaction for G_3* at an initial concentration of 40 mM (see Experimental Procedure for rationale of this type of plot). For unimolecular hydrolysis, in the absence of secondary attach on products, the plot is rectilinear and the slope is the bond cleavage frequency. The slopes of the broken lines are the initial product ratios determined by a least-squares line through the first four points.

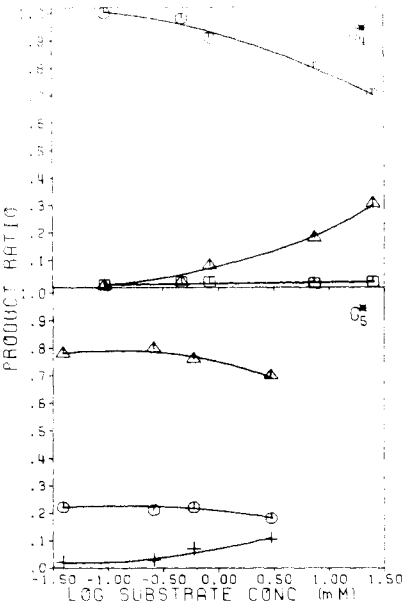

FIGURE 2: The effect of concentration on maltotetraose and maltopentaose product ratios. The initial ratio for oligosaccharides smaller than the substrate oligosaccharide from G_4* and G_5* degradation is plotted for (□) glucose; (○) maltose; (△) maltotriose; and (+) maltotetraose as a function of the initial substrate concentration. In G_5* hydrolysis, the production of G_1* was insignificant. The plotted values were determined from the initial slope of bond cleavage frequency plots as described in Experimental Procedure. In the degradation of G_4* at an initial concentration of 7.5 and 25 mM and of G_5* at 3.1 mM, oligosaccharides larger than substrate were also observed (Figure 3).

trophoresis on Whatman no. 1 paper with a Pherograph, Mini 68 (Brinkman), in 0.05 M sodium borate (Frahn & Mills, 1959). An autoradiogram was prepared, and the maltitol was eluted and hydrolyzed with 0.5 N H_2SO_4 for 4 h at 100 °C before neutralization with 1 N NaOH. The sample was desalted on Bio-Gel P-2 and Sephadex G-10 to give 97% recovery of the radioactivity. The concentrated sample was then subjected to paper electrophoresis, along with standards. Radioactive products were visualized with autoradiography, and a portion of the electrochromatogram was visualized with the $AgNO_3$–NaOH reagent with the NaOH made 4.5% in pentaerythritol (Frahn & Mills, 1959). The remaining part of the chromatographed sample was cut out using the autoradiogram and the stained portion as a guide, and the radioactivity was counted.

The labeled maltotriose produced from AO amylase degradation of reducing-end labeled maltotetraose at 25 mM initial concentration was examined for label redistribution. The maltotriose, isolated from the other degradation products by paper chromatography, was digested with β-amylase until 97% of the maltotriose had been hydrolyzed. The products were separated by paper chromatography, detected by autoradiography, and quantitated by radioactivity counting.

Results

Maltotriose Degradation. The products of AO amylase degradation of reducing-end labeled maltotriose were found to depend on the concentration of maltotriose (see Figure 4 of the following paper, Allen & Thoma, 1978). At initial maltotriose concentrations of 0.06–0.4 mM, the only significant labeled product is glucose. As the starting concentration of maltotriose is raised, labeled maltose becomes evident, and above 16 mM maltotriose, labeled maltose is the predominant product. At the highest concentration examined (500 mM), maltose constituted 80% of the labeled product. These observations indicate the participation of more than one substrate molecule in the degradation reaction at high substrate concentrations.

As shown in Figure 1, at high initial maltotriose concentrations the product ratio depends on the extent of reaction. At low initial substrate concentrations the product ratio is constant throughout the reaction (not shown) indicating that the shift of product ratio at high substrate concentration is not due to a secondary attack on the product maltose by the enzyme. The change in observed product ratio is apparently due to a shift in reaction mechanism as the maltotriose is consumed.

When uniformly labeled maltotriose at an initial concentration of 0.019 mM is degraded by AO amylase, the initial mole ratio of glucose to maltose is 1.06 consistent with simple scission of a bond. However, when the initial maltotriose concentration is raised to 40 mM this ratio becomes 0.55, showing that maltose is the predominate product of maltotriose degradation. Deviation of this ratio from unity is inconsistent with unimolecular hydrolysis.

Labeled maltose, produced from degradation of reducing-end labeled maltotriose at high concentration, was examined to determine if the label had been preserved in the reducing glucosyl residue or if it had been scrambled by some multimolecular mechanism so that label also appeared in the nonreducing glucosyl residue of the molecule. The maltose from an AO amylase digest was reduced with $NaBH_4$, separated from unreduced maltose, and acid hydrolyzed, and the hydrolysate products were fractionated. If there were label redistribution, both glucose and glucitol, produced from the hydrolysis of the maltitol would contain ^{14}C. There were 14 000 cpm in the glucitol but no detectable radioactivity present above background for glucose, showing the absence of label redistribution.

Maltotetraose and Maltopentaose Degradation. As shown in Figure 2, the product ratios from reducing-end labeled maltotetraose and maltopentaose degradation were observed to be dependent on the concentration of the respective oligosaccharide in the reaction mixture. For maltotetraose at the lowest concentration examined, the only observed labeled

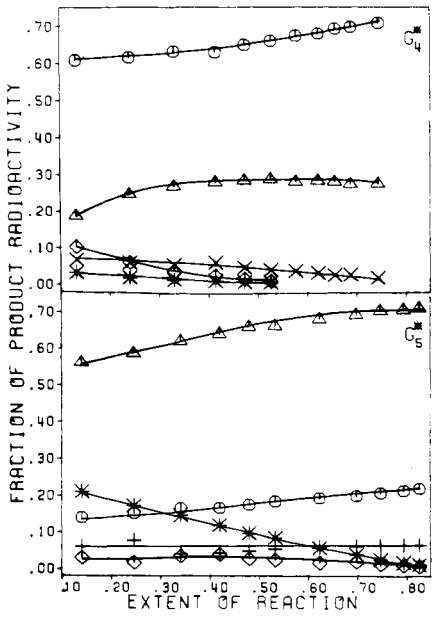

FIGURE 3: Radioactivity distribution of the products of maltotetraose and maltopentaose degradation at high initial substrate concentration. G_4* and G_5* at an initial concentration of 25 mM and 3.1 mM, respectively, were degraded by AO amylase. (○) G_2*; (△) G_3*; (+) G_4*; (X) G_5*; (◇) G_6*; (*) G_7*. The production of glucose was insignificant.

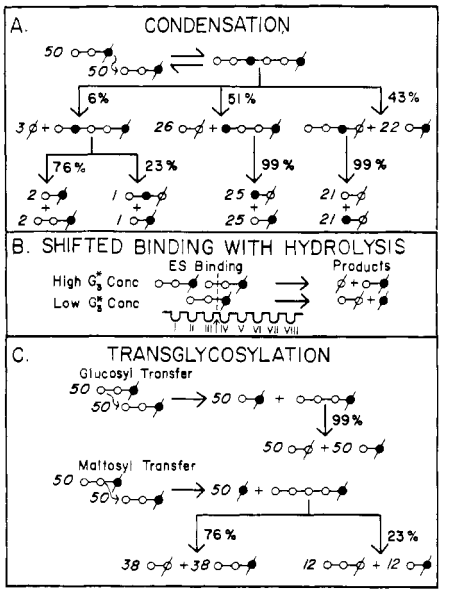

FIGURE 4: Possible bimolecular maltotriose degradation pathways. The numbers next to the arrows are the percent of a particular intermediate degraded to give the indicated products; some minor degradation pathways are not shown. The numbers beside the products are mole percents of original substrate, that is the moles of a particular product formed from 100 mol of substrate maltotriose. Oligosaccharides larger than trimer were not detected in maltotriose hydrolysates and are, consequently, treated as transient intermediates. The unimolecular bond cleavage frequencies for G_4* and G_5* are unaltered by the presence of 40 mM maltotriose (Allen, 1975b); therefore these sugars bind to the enzyme in the same way as in the absence of maltotriose and are assumed to be fragmented in the same manner as in unimolecular hydrolysis (Allen & Thoma, 1976b) to a chain length of three or less. However, when G_4* and G_5* are degraded in the presence of high maltotriose concentration, the fate of the unlabeled nonreducing end of the molecule cannot be detected and the possibility of subsequent transfers exists. (A) Condensation. The reverse of condensation to yield two G_3* does not result in a net reaction and, hence, is not included in the reaction scheme. The percent distributions of products (including some minor pathways not illustrated) are: 2 ○, <1 ●, 31 ○—○, 33 ○—●) 31 ●—○, <1 ○—○—○, 2 ○—○—●, <1 ○—●—○. (B) Shifted two-one binding with hydrolysis. (U) subsite on the enzyme; (↑) catalytic site; the broken line shows the position of bond cleavage. The binding region of AO amylase is comprised of eight subsites as shown (Allen & Thoma, 1976b). (C) Transglycosylation. The percent product distribution from glucosyl transfer is 33 ○—○, 67 ○—● and from maltosyl transfer is 34 ●, 26 ○—○, 8 ○—●, 8 ○—○—○, 26 ○—○—●. The supplementary material gives a detailed analysis of maltosyl transfer (see paragraph concerning supplementary material at the end of this paper).

product is maltose; whereas, at the highest concentration examined, maltotriose constitutes 30% of the labeled products. In the degradation of reducing-end labeled maltopentaose, as the concentration is increased more maltotetraose is formed at the expense of maltotriose production (Figure 2).

In the degradation of both maltotetraose and maltopentaose at high starting concentrations, oligosaccharides were observed on the chromatogram that were of greater chain length than the substrate sugar. These oligosaccharides were quantitated as shown in Figure 3 along with the typical product maltodextrins as a function of the extent of the reaction. It can be seen that these larger multimolecular reaction products are more prominant at the early stages of the degradation and are consumed as the reaction progresses.

For the oligosaccharides of chain length seven through ten (Allen & Thoma, 1976b), no concentration dependency of product ratios was observed over the range examined: G_6 (0.016–1.9 mM), G_7 (0.0083–0.9 mM), and G_8–G_{10} (ca. 0.008–0.010 mM). However, when maltodecaose was hydrolyzed at the higher concentration, larger sugars were faintly visible on the autoradiogram.

The labeled maltotriose produced from hydrolysis of reducing-end labeled maltotetraose at 25 mM starting concentration was examined for the occurrence of a label in other than the reducing end of the molecule. Since β-amylase hydrolyzes maltose units from the nonreducing end of a maltodextrin, hydrolysis of maltotriose will yield maltose from the nonreducing end and glucose from the reducing end. When the maltotriose was carried to 97% hydrolysis, there was 74 000 cpm in the glucose and no detectable radioactivity above background in the maltose, showing the absence of label redistribution.

Discussion

It is apparent from the concentration dependence of the product ratio, from the shift in product ratio as a function of the extent of reaction, from the formation of oligosaccharides larger than substrate, and from the products of hydrolysis of uniformly labeled maltotriose that AO amylase does not degrade substrate exclusively by simple unimolecular hydrolysis. The utilization of alternate degradation mechanisms depends on the concentration of substrate molecules and is, therefore, a multimolecular process. At least three multimolecular degradation mechanisms (illustrated in Figure 4 for maltotriose) have to be considered (Robyt & French, 1970).

1. The *condensation* mechanism (Figure 4A) involves polymerization of two molecules of substrate to form a new glycosidic bond with water elimination. The resulting sugar can then undergo hydrolysis to smaller sugars. Condensation products have been observed with carbohydrases such as porcine pancreatic α-amylase (Robyt & French, 1970; Hehre et al., 1969), β-amylase, glucoamylase (Hehre et al., 1969), and pullulanase (Abdullah & French, 1966, 1970).

2. *Shifted two-one binding* (Figure 4B) is based on the subsite model for a depolymerase (Allen & Thoma, 1976a,b) where the binding region of the enzyme is envisioned to be composed of an array of subsites that are complementary to and bind glucosyl units. This model permits two substrate

molecules to bind simultaneously to one enzyme molecule to give a two-one complex. Depending on the relative binding affinities of the subsites, the second substrate molecule may bind independently of the first substrate molecules or may bind in such a way as to shift the first substrate into a new set of subsites exposing a different bond to cleavage. A two-one complex has been reported for lysozyme (Holler et al., 1974) where two chitohexoses bind nonproductively (i.e., no susceptible bonds exposed to the catalytic amino acids). In addition, shifted two-one binding, where the second molecule of substrate causes a shift from nonproductive to productive binding, was proposed for lysozyme based on kinetic observations (Rupley & Gates, 1967). However, Chipman et al. (1968) have suggested that this kinetic observation can be explained by a transglycosylation mechanism. Hence, although two-one binding has been observed, *shifted* two-one binding has not been convincingly shown to occur.

3. *Transglycosylation* (Figure 4C) is the transfer of a glycosyl group from a substrate donor to an acceptor other than water. Transglycosylation mechanisms are well documented for lysozyme (Rupley & Gates, 1967; Chipman et al., 1968; Chipman, 1971). Robyt & French (1970) reported that porcine pancreatic α-amylase degrades maltotetraose, in part, through maltotriosyl transfer, and the products of transglycosylation have been observed for pullulanase (Abdullah & French, 1970).

The multimolecular pathway for degradation by AO α-amylase may be any one or any combination of these three mechanisms. For example, shifted two-one binding could either be followed by hydrolysis or by transfer. We will consider each one of these mechanisms in light of the experimental results and attempt to arrive at the mechanism for AO amylase degradation of maltooligosaccharides.

Maltotriose Degradation. Uniformly labeled maltotriose at low concentration (0.019 mM) gave approximately equal molar amounts of glucose and maltose, the results expected from unimolecular scission of a single bond in the maltotriose. The product ratio for reducing-end labeled maltotriose became constant below 0.4 mM initial concentration and gave glucose as the sole labeled product. From these observations, we conclude that under unimolecular conditions the only measurable bond cleavage occurs at the reducing-end glycosidic bond of maltotriose. Consequently, any labeled maltose in the hydrolysate arose from the multimolecular event.

The condensation of two maltotriose molecules will yield maltohexaose as shown in Figure 4A. AO amylase hydrolyzes reducing-end labeled maltohexaose under unimolecular conditions to give 67% reducing-end labeled maltotriose. Hence, by the principle of microscopic reversibility, the condensation mechanism is certainly feasible. The distinguishing feature of the condensation mechanism as shown in Figure 4A is that the label from the reducing-end glucosyl unit of the substrate will appear approximately equally distributed between the reducing and nonreducing glycosyl residues of product maltose. If condensation were a contributing pathway of maltotriose degradation, label redistribution would be evident in the maltose formed. A test for label redistribution, which would have detected a label in the nonreducing end had it constituted more than 1% of the labeled maltose, was negative. We reject the condensation mechanism as contributing to degradation of maltotriose. This is in contrast to a recent report that this enzyme does catalyze some condensation (Suganuma et al., 1976).

Two-one shifted binding followed by hydrolysis of maltotriose is shown in Figure 4B. The energetics of binding strongly favor binding so as to expose the reducing-end glycosidic bond to the catalytic amino acids (Allen & Thoma, 1976b). However, at higher concentrations the binding of a second maltotriose might cause a shift in the binding so that the nonreducing-end glycosidic bond is subject to cleavage. This shift in binding would be manifested as an increase in production of labeled maltose and would account for the trend observed in product ratios as a function of concentration. However, this mechanism predicts that hydrolysis of uniformly labeled maltotriose will result in equal molar amounts of labeled glucose and labeled maltose which is in opposition to the experimental results. We must reject shifted binding with hydrolysis as the sole multimolecular degradation pathway, but we cannot eliminate it as a contributing mechanism.

As shown in Figure 4C, transglycosylation in maltotriose degradation can lead to either transfer of a glycosyl or a maltosyl residue from the nonreducing end of a donor maltotriose to the nonreducing end of acceptor maltotriose. Hydrolysis and transfer are mechanistically similar, differing in acceptor for the glycosyl residue. Hence, we expect the products of hydrolysis and transfer to be consistent with a single substrate fragmentation pattern as was observed with lysozyme (Rupley & Gates, 1967). Maltotriose fragments almost exclusively to yield an enzyme-maltosyl intermediate as evidenced by the production of labeled glucose from reducing-end labeled maltotriose, so that we would expect maltosyl transfer to dominate glycosyl transfer. In addition, an analysis of the experiments where uniformly labeled and reducing-end labeled maltotriose were degraded at the same concentration, detailed in the supplementary material (see paragraph concerning supplementary material at end of this paper), reveals that the multimolecular degradation mechanism produces a glucose to maltose ratio greater than 0.37. Since glycosyl transfer does not produce any glucose (Figure 4C), it alone cannot account for the data.

The multimolecular mechanism must account for all of the labeled maltose observed at the highest substrate concentrations studied (500 mM) where $[G_2^*]/([G_1^*] + [G_2^*]) = 0.8$, but Figure 4C reveals that, even if all maltotriose were shunted through the maltosyl transfer pathway, this ratio would never exceed 0.2. Hence, maltosyl transfer does not produce sufficient labeled maltose to account for the observed ratio. The maltosyl transfer pathway also predicts that the products of uniformly labeled maltotriose degradation will be equal molar amounts of labeled glucose and maltose, which does not agree with the experimental results. Consequently, maltosyl transfer is not the only multimolecular mechanism operative, but we cannot reject it as a contributing mechanism.

We are forced to conclude that no single bimolecular mechanism can account for all of the experimental results. We have eliminated condensation as a contributing mechanism so that the multimolecular pathways must be some combination of transglycosylation and possibly shifted binding. In the following paper (Allen & Thoma, 1978) we will quantitate the extent to which these various pathways contribute to maltotriose degradation.

Maltotetraose Degradation. As shown in Figure 2, at low concentrations, G_4^* is cleaved exclusively at the middle glycosyl bond to give labeled maltose, but at the higher concentrations the production of labeled maltotriose is increased at the expense of labeled maltose. Figure 3 shows that, in the degradation of end-labeled maltotetraose at high initial concentrations, there is an initial buildup of oligosaccharides larger than the substrate. As the hydrolysis progresses the concentrations of maltohexaose and maltoheptaose become negligible, but the concentration of maltopentaose is still significant at the later stage of the reaction.

TABLE I: Predicted Distribution of Labeled Products from Reducing-End Labeled Maltotetraose and Maltopentaose Transglycosylations.[a]

	% label distribution in product			
	●*	O—●*	O—O—●*	O—O—O—●*
Maltotetraose transglycosylations				
Glucosyl O—O—O—● / O—O—O—O—●	0	12	88	
Maltosyl O—O—O—● / O—O—O—O—●	0	63	37	
Maltotriosyl O—O—O—● / O—O—O—O—●	55	9	36	
Maltopentaose transglycosylations				
Glucosyl O—O—O—O—● / O—O—O—O—O—●	0	7	34	59
Maltosyl O—O—O—O—● / O—O—O—O—O—●	0	5	84	11
Maltotriosyl O—O—O—O—● / O—O—O—O—O—●	0	67	24	9
Maltotetraosyl O—O—O—O—● / O—O—O—O—O—●	61	5	25	9

[a] The percent distributions of labeled products are predicted, assuming unimolecular hydrolysis of all oligosaccharides formed larger than the substrate, analogous to the analysis for maltotriose (Figure 4C).

A condensation mechanism would account for the increase in labeled maltotriose at higher substrate concentrations. An analysis of a reducing-end labeled maltotetraose condensation mechanism, analogous to that in Figure 4A for maltotriose, predicts that the labeled maltotriose produced will have the following label distribution: 0.6 O—O—●*, 0.29 O—●—φ, and 0.11 ●—O—φ. Since no maltotriose is formed by unimolecular hydrolysis of maltotetraose (Figure 2), any maltotriose formed is a consequence of the multimolecular mechanism. If condensation played a significant role in the degradation of maltotetraose, label redistribution would be evident. A test for label redistribution, with a sensitivity such that redistribution constituting greater than 1% would have been detected, was negative. We conclude that condensation is not significant in maltotetraose degradation. Shifted binding with hydrolysis could account for the concentration dependence of product ratios but not for the occurrence of oligosaccharides larger than maltotetraose in the digest.

We conclude that transglycosylation is operative in the degradation of maltotetraose. Since the initial transfer products G_5*, G_6*, and G_7* are all present in the reaction mixture, all three transfer mechanisms probably contribute to some extent. As shown in Table I, all of the transfer reactions predict the formation of maltotriose, which is consistent with the experimental results. However, maltotriosyl transfer cannot be a major pathway since a greater increase in glucose than in maltotriose is predicted, which is not observed. The most favorable binding mode for maltotetraose will result in the formation of the enzyme–maltosyl intermediate (Allen & Thoma, 1976b) to favor maltosyl transfer. However, the maximum possible maltotriose produced by maltosyl transfer, even if all maltotetraose were degraded by this pathway, is 37% of total labeled products (Table I). Figure 2 shows that, at the highest maltotetraose concentration examined, approximately 30% of the labeled product has become maltotriose. Hence, it is unlikely that maltosyl transfer is the only significant bimolecular pathway. Glucosyl transfer produces maltotriose as 88% of the labeled products; consequently, the glucosyl pathway is a more likely candidate to account for the trend observed in Figure 2 for maltotriose production. In addition, Figure 3 shows that the first product of glucosyl transfer, G_5*, is present in measurable concentration throughout the reaction. We surmise that glucosyl transfer is probably the most significant transglycosylation reaction in maltotetraose degradation.

Maltopentaose Degradation. As with G_4*, G_5* exhibits a concentration dependence of product ratios (Figure 2) and oligosaccharides larger than the original substrate are observed in the digests (Figure 3). Since the condensation mechanism is insignificant in maltotriose and maltotetraose degradation, we assume that it is insignificant for maltopentaose degradation as well. Again we reject the two-one shifted complex as the only bimolecular mechanism because of the formation of oligosaccharides larger than substrate.

The feasible transglycosylations are shown in Table I along with the predicted distribution of labeled products. We rule out maltotetraosyl transfer because significant amounts of glucose are not detectable in the hydrolysate. The observed increase in G_4* is predicted by any one of the three remaining transfer reactions. The most likely candidate is glucosyl transfer since the upper limit of formation of G_4* by maltosyl and maltotriosyl transfer is 11% and 9%, respectively, and, at the highest substrate concentration analyzed, G_4* amounts to 10% of total products.

In general, we conclude that the utilization of multimolecular pathways for the degradation of carbohydrates depends on the substrate concentration and chain length. For AO amylase degradation of maltotriose, maltotetraose, and maltopentaose the most prominent multimolecular degradation mechanisms are transglycosylations. In addition, as we will show in the following paper (Allen & Thoma, 1978), shifted two-one binding plays a role in the maltotriose degradation.

In order to ascertain with certainty that a carbohydrate is free of multimolecular reaction catalysis, careful analysis is essential. As demonstrated with maltotriose in this study, the presence of a measurable amount of oligosaccharides larger than substrate is not a necessity in multimolecular reactions and simply depends on the relative rate constants (Chipman, 1971). It is also interesting to note that, if maltosyl transfer had

been the sole bimolecular mechanism, uniformily labeled maltotriose degradation would have given equal molar amounts of labeled glucose and maltose (Figure 4C), not detecting the bimolecular reaction. However, any of the bimolecular mechanisms would have been detected by examining the product ratios of end-labeled substrates as a function of substrate concentration.

As more detailed information regarding the action of carbohydrases becomes available, the distinction among the classification of these enzymes becomes less distinct (Hehre et al., 1973). Although the *Aspergillus oryzae* enzyme studied here is classified an an α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1) it acts as an α-glucosidase (α-D-glucoside glucohydrolase, EC 3.2.1.20) (Nitta et al., 1968), and we have not shown that the enzyme acts as a 4-α-glucanotransferase (1,4-α-D-glucan:1,4-α-D-glucan 4-α-glycosyltransferase, EC 2.4.1.25).

Supplementary Material Available

A mathematical analysis of the amount of glucose formed by the AO amylase degradation of maltotriose (2 pages). Ordering information is given on any current masthead page.

References

Abdullah, M., & French, D. (1966) *Nature (London) 210,* 200.

Abdullah, M., & French, D. (1970) *Arch. Biochem. Biophys. 137,* 483.

Allen, J. D. (1975a) *Carbohydr. Res. 39,* 312.

Allen, J. D. (1975b) Ph.D. Thesis, University of Arkansas.

Allen, J. D., & Thoma, J. A. (1976a) *Biochem. J. 159,* 105.

Allen, J. D., & Thoma, J. A. (1976b) *Biochem. J. 159,* 121.

Allen, J. D., & Thoma, J. A. (1978) *Biochemistry 17* (following paper in this issue).

Brückner, J. (1955) *Biochem. J. 60,* 200.

Chipman, D. M. (1971) *Biochemistry 10,* 1714.

Chipman, D. M., & Sharon, N. (1969) *Science 165,* 454.

Chipman, D. M., Pollock, J. J., & Sharon, N. (1968) *J. Biol Chem. 243,* 487.

Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2,* 1–65.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121,* 404.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956) *Anal. Chem. 28,* 350.

Dygert, S., Li, L. H., Florida, D., & Thoma, J. A. (1965) *Anal Biochem. 13,* 367.

Florkin, M., & Stotz, E. H., Ed. (1973) *Compr. Biochem. 13* 212.

Frahn, J. L., & Mills, J. A. (1959) *Aust. J. Chem. 12,* 65.

French, D., & Wild, G. M. (1953) *J. Am. Chem. Soc. 75* 2612.

French, D., Pulley, A. O., & Whelan, W. J. (1963) *Staerk 8,* 280.

Harada, T., Misaki, A., Akai, H., Yokobayashi, K., & Sugimoto, K., (1972) *Biochim. Biophys. Acta 268,* 497.

Hehre, E. J., Okada, G., & Genghof, D. S. (1969) *Arch. Biochem. Biophys. 135,* 75.

Hehre, E. J., Genghof, D. S., & Okada, G. (1971) *Arch. Biochem. Biophys. 142,* 382.

Hehre, E. J., Okada, G., & Genghof, D. S. (1973) *Adv. Chem. Ser. No. 117,* 309–333.

Hiromi, K. (1970) *Biochem. Biophys. Res. Commun. 40,* 1.

Holler, E., Rupley, J. A., & Hess, G. P. (1974) *FEBS Lett. 40,* 25.

John, M., Trénel, G., & Dellweg, H. (1969) *J. Chromatogr. 42,* 476.

Marshall, J. J., & Whelan, W. J. (1973) *Anal. Biochem. 52,* 642.

Matsubara, S. (1961) *J. Biochem. (Tokyo) 49,* 226.

Matsubara, S., Ikenaka, T., & Akabori, S. (1959) *J. Biochem. (Tokyo) 46,* 425.

Nitta, Y., Hiromi, K., & Ono, S. (1968) *J. Biochem. (Tokyo) 63,* 632.

Nitta, Y., Mizushima, M., Hiromi, K., & Ono, S. (1971) *J. Biochem. (Tokyo), 69,* 567.

Okada, S., Kitahata, S., Higashihara, M., & Fukumoto, J. (1969) *Agric. Biol. Chem. 33,* 900.

Omichi, K., & Matsushima, Y. (1970) *J. Biochem. (Tokyo) 68,* 303.

Omichi, K., Ikenaka, T., & Matsushima, Y. (1972) *J. Biochem. (Tokyo) 72,* 665.

Pazur, J. H. (1955) *J. Am. Chem. Soc. 77,* 1015.

Robyt, J. F., & French, D. (1967) *Arch. Biochem. Biophys. 122,* 8.

Robyt, J. F., & French, D. (1970) *J. Biol. Chem. 245,* 3917.

Rupley, J. A., & Gates, V. (1967) *Proc. Natl. Acad. Sci. U.S.A. 57,* 496.

Stahl, E., & Kaltenbach, U. (1961) *J. Chromatogr. 5,* 351.

Suetsugu, N., Koyama, S., Takeo, K., & Kuge, T. (1974) *J. Biochem. (Tokyo) 76,* 56.

Suganuma, T., Ohnishi, M., Matsuno, R., & Hiromi, K. (1976) *J. Biochem. (Tokyo) 80,* 645.

Takeshita, M., & Hehre, E. J. (1975) *Arch. Biochem. Biophys. 169,* 627.

Thoma, J. A., & French, D. (1957) *Anal. Chem. 29,* 1645.

Thoma, J. A., & Stewart, L. (1965) in *Starch Chemistry and Technology* (Whistler, R. L., & Paschall, F. F., Eds.) pp 209–249, Academic Press, New York, N.Y.

Thoma, J. A., Brothers, C., & Spradlin, J. (1970) *Biochemistry 9,* 1768.

Tilden, E. B., & Hudson, C. S. (1942) *J. Bacteriol. 43,* 527.

Toda, H., & Akabori, S. (1963) *J. Biochem. (Tokyo) 53,* 102.

Trevelyn, W. E., Proctor, D. P., & Harrison, J. S. (1950) *Nature (London) 166,* 444.

Weill, C., & Hanke, P. (1962) *Anal. Chem. 34,* 1736.