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SIGNIFICANCE OF MALTODEXTRINS IN PHOTOSYNTHETIC TISSUE.

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SIGNIFICANCE OF MALTODEXTRINS IN PHOTOSYNTHETIC TISSUE

by

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I. INTRODUCTION

The maltodextrins comprise the homologous series of amylose-related compounds ranging from maltose and the higher maltooligosaccharides to compounds which are large enough to be considered amylose. The D-glucose units of maltodextrins are linked by α-1,4-glucosidic bonds. Maltose contains two glucose units, maltotriose contains three, maltotetraose has four, and so forth. Past the range of 15-20 glucose units, maltodextrins become amylose molecules, because the chromatographic means [thin layer (1), paper (2), or charcoal column (3)] of isolating individual molecular species fail. Beyond this indefinite limit, any preparation of amylose molecules has an average degree of polymerization.

Maltodextrins are produced by the action of amylose on amylase. The existence of maltodextrins in organs of starch storage of plants has been attributed to amylase action. However, the early appearence and distribution of these compounds during photosynthesis in radioactive carbon dioxide brought question to this scheme. It was the purpose of this investigation to study the maltodextrins in photosynthetic tissue in order to determine their function in the cell. The basic question was whether they were leaf starch hydrolysis products, leaf starch precursors, a combination of these, or neither. Of course, in order to understand the metabolism of the maltodextrins, the specific enzymes in photosynthetic tissue which catalyze the processes should be understood.

In order to approach an answer to the question, the radioactive maltodextrins from three sources of photosynthetic tissue were studied. The quantities of individual maltodextrins in the tissues and the changes

in patterns of labelling of the maltodextrins during the course of photosynthesis were investigated. The products of action by the carbohydrases in these photosynthetic tissues on various well-characterized substrates were also studied.

Throughout this thesis, the following abbreviations have been used: maltose, G_2; maltotriose, G_3; maltotetraose, G_4; maltopentaose, G_5; maltohexaose, G_6; maltoheptaose, G_7; maltooctaose, G_8; maltononaose, G_9; maltodecaose, G_{10}; maltoundecaose, G_{11}; a maltodextrin molecule of n glucose units as G_n; adenosine-5'-diphosphoglucose, ADPG; uridine-5'-diphosphoglucose, UDPG; glucose-1-phosphate, G-1-P; glucose-6-phosphate, G-6-P; fructose-6-phosphate, F-6-P; fructose-1,6-diphosphate, FDP; adenosine triphosphate, ATP; adenosine diphosphate, ADP; uridine triphosphate, UTP; uridine diphosphate, UDP; inorganic phosphate, P_i; pyrophosphate, PP_i; radioactive adenosine diphosphoglucose labelled in the glucose unit, ADPG*; a maltotriose molecule labelled specifically in the reducing glucose unit, O-O-$\emptyset^*$; and a maltopentaose molecule labelled specifically in the nonreducing glucose unit, $\overset{*}{O}$-O-O-O-$\emptyset$.

II. REVIEW OF LITERATURE

A. Photosynthetic Carbohydrates

One-hundred years ago the German plant physiologist Sachs, gave evidence for the relationship of light to the fixation of carbon dioxide into starch (4). Sachs recognized that starch was deposited in the chlorophyll-containing particles of leaf tissue now known as chloroplasts. He postulated that photosynthesis converted all carbon into starch and that from starch the essential carbon compounds of plant cells were formed. However, the use of isotopes has shown much about the metabolism of carbon compounds in photosynthesis, and so Sachs' postulate has been modified. Photosynthetic carbohydrates,which are now considered primary products of photosynthesis. include among others phosphorylated and free monosaccharides, sucrose, maltose, trehalose, raffinose, starch, and inulin.

Particularly important has been the elucidation of the photosynthetic carbon reduction cycle by Calvin and his co-workers at the University of California (5). This cycle has been widely accepted as a starting point of a number of secondary biosynthetic pathways, only one of which is the formation of polysaccharides. Figure 1 summarizes some of the reactions leading to the formation of oligo- and polysaccharides in leaves during photosynthesis. Noteworthy in this scheme is the central position of the nucleotide diphosphate sugars. Evidence is gathering for their universal function as donor for synthesis of polysaccharides (6). However, on the periphery of the scheme are found exceptions. Transglycosylases such as stachyose synthetase (7), D-enzyme (8), and fructosyltransferase (9) are

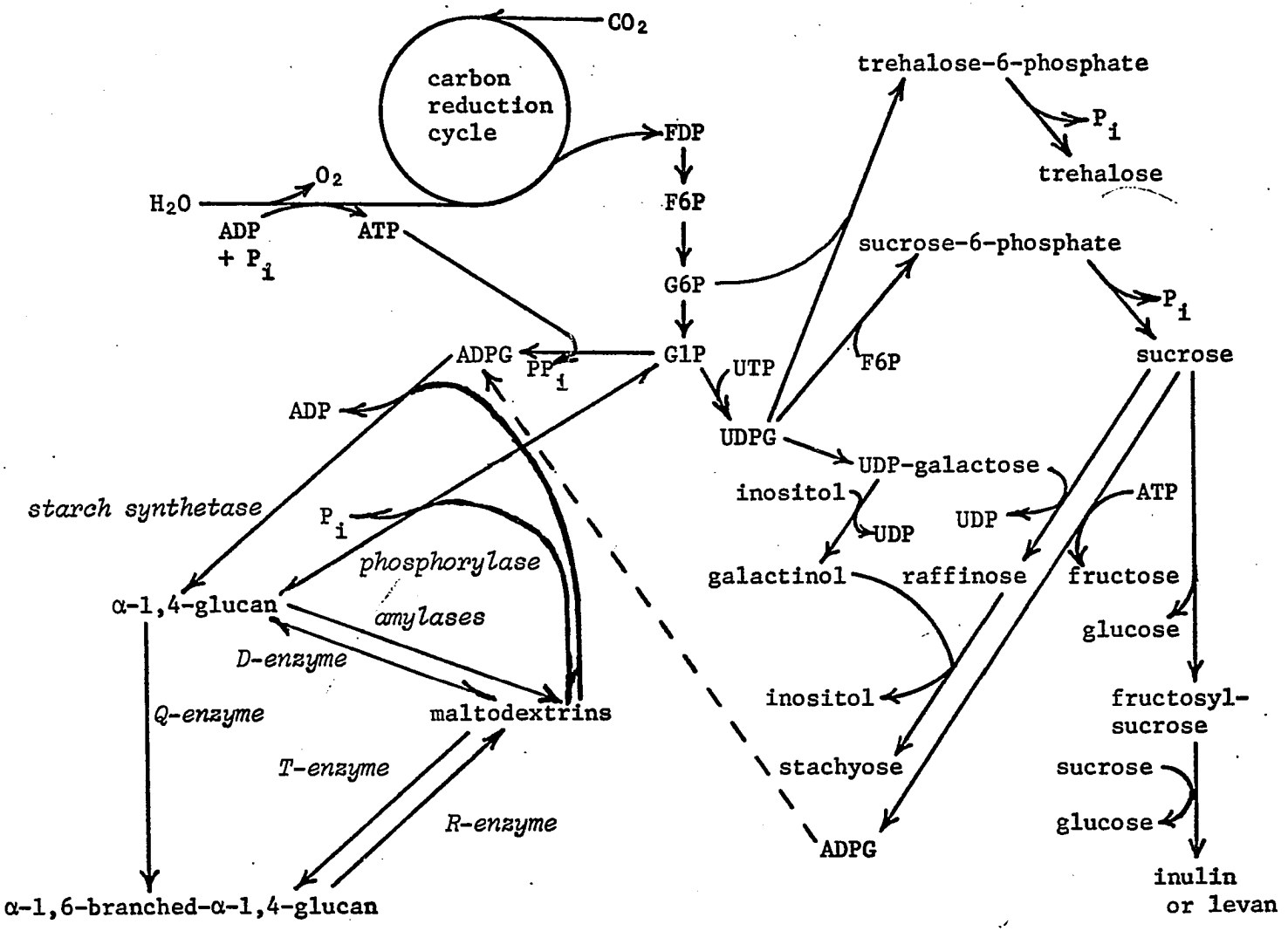

Figure 1. Scheme of possible pathways for the synthesis of photosynthetic carbohydrates

capable of forming and modifying the structure of oligosaccharides.

In *Chlorella* (10) as well as *Scenedesmus* and other plants (11), uridine diphosphoglucose (UDPG) and adenosine diphosphoglucose (ADPG) are labelled even after a few seconds of photosynthesis in radioactive carbon dioxide. The labelling kinetics of these compounds in *Chlorella* are compared with the labelling of sucrose and starch in Figure 2 (7).

Although UDPG and ADPG can both function as glucosyl donors in starch synthesis (11), it appears that ADPG is required for starch synthesis in leaf chloroplasts (12, 13, 14) and *Chlorella* (15). The conversion of UDPG and fructose to sucrose and the conversion of UDPG and fructose-6-phosphate to sucrose-6-phosphate has been demonstrated in sugar beet leaf homogenate (16), sugar cane chloroplasts (17), sugar beet roots (18), and artichoke tubers (19).

In photosynthesis in radioactive carbon dioxide, sucrose was observed to be the first free sugar thatwas labelled in beans (20) and *Chlorella* (21). After 30 seconds of photosynthesis, sucrose from *Chlorella* was found to have a higher labelling in the fructose than in the glucose, but after 90 seconds both moieties were equally labelled. Putnam and Hassid (22) showed that leaf homogenates could quickly distribute radioactivity in glucose between the pools of fructose, glucose and sucrose. Porter and May (23) extended this observation to include starch. The pathway for the synthesis of sucrose and starch apparently includes a step in which hexose units, whether free or as an intermediate, become equilibrated.

Starch biosynthesis in plant tissue after administration of exogenous sugars has been studied extensively. Saposchinikoff (24) found that starch was formed from sucrose in darkness in the leaves of *Astropara wallichii*

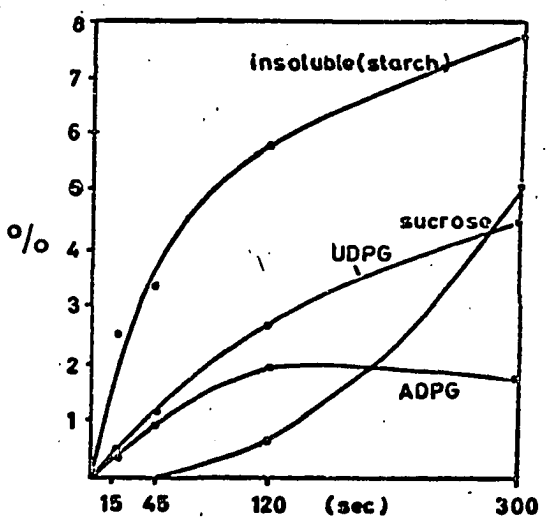

Figure 2. Kinetics of labelling of starch, sucrose, UDPG, and ADPG in *Chlorella* during photosynthesis in $^{14}CO_2$ (7)

and *Nicotiana tabacum*. Czapek in (25) reported the deposition of starch in leaves floating on solutions of glucose, fructose, galactose, mannose, and maltose. Spoehr (26) noted that leaves of *Zea mays* were able to synthesize starch from glucose and sucrose. Pallas (27) studied the formation of starch in guard cells of 31 different species by floating epidermal peels on sugar solutions. Glucose, glucose-1-phosphate, ribose, xylose, fructose, galactose, mannose, sucrose, maltose, and raffinose were effective in one or more species but only glucose-1-phosphate and sucrose were effective with a wide range of species. Reynolds, Porter and Martin (28) floated tobacco leaf disks on a 5% solution of reducing end labelled maltose. The starch, glucose and maltose after 24 hours had the same specific activity as the added maltose; that of fructose and sucrose was slightly lower. Sucrose was labelled equally in both hexose units, and starch was equally labelled in every glucose unit, as analyzed after combined action of α- and β-amylase on the starch. The distribution of label in the maltose was unchanged. It seems apparent that exogenous sugars can be phosphorylated and can undergo interconversions to sugar nucleotides for incorporation into polysaccharide.

B. Maltodextrins in Photosynthetic Tissue

In a survey of the products of short term photosynthesis in plants of nine phyla, Norris, Norris and Calvin (29) found that maltose contained 0.7 to 1% of the radioactivity in the 80% ethanol fraction of the photosynthetic tissue of barley, juniper, squash, soybean, kalanchoe, tomato, ivy, and avacado. Hexose monophosophates (4-10%) were detected in all of the above and UDPG (1-5%) in all except barley. The level of sucrose in

the tissues varied between 40 and 75% of the total radioactivity in the 80% ethanol extract.

In maple leaves, Nishida (30) found that within five minutes after initiating photosynthesis in $^{14}CO_2$ maltose contained 20% of total activity. Sucrose followed the same pattern reaching 35% of total activity, and the insoluble material claimed 45% of the total activity after five minutes. Maltose represented a higher percentage of total activity than starch after thirty seconds of photosynthesis in $^{14}CO_2$. Starch then surpassed the radioactivity of maltose and had three times the activity of maltose at sixty seconds. Maltose continued to increase in radioactivity in a linear fashion for more than two minutes. This evidence indicated that maltose was not formed by hydrolysis of starch but by a direct pathway from hexose phosphates.

Kandler *et al.* (31) has also presented evidence that maltose is formed directly from sugar phosphates. In several experiments where the leaves of different plants were incubated for a long time in light with $^{14}CO_2$, label in maltose could always be found (32). After pulse labelling (a short period of photosynthesis in $^{14}CO_2$ followed by photosynthesis in the atmosphere), the kinetics of labelling of maltose were similar to those of sugar phosphates and not at all similar to those of sucrose or starch (33). Maltose behaved as a typical intermediate. The labelling of the two glucose moieties of maltose was unequal very shortly after a pulse of $^{14}CO_2$ during steady state photosynthesis, but became equal within two minutes because of its apparently rapid rate of turnover.[1]

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[1]Kandler, O. Botanisches Institut des Universitaet, Munick, Germany. On the biosynthesis of maltose. Private communication. 1967.

The intermediate nature of maltose leads to the question: to what compound(s) is the activity in maltose passed during its rapid turnover? Kandler (7) has suggested that maltose is donor to transglycosylase activity to yet unknown acceptors as in Equation 1:

$$O\text{-}\emptyset + X \rightleftharpoons O\text{-}X + \emptyset. \qquad 1$$

Ghosh and Preiss (12) and Frydman and Cardini (33) have reported that maltose will serve as an acceptor for glucosyl transfer from ADPG when incubated with starch synthetase from spinach chloroplasts and sweet corn endosperm respectively. The reaction with maltose produces maltotriose as indicated in Equation 2:

$$O\text{-}\emptyset + ADPG^{*} \longrightarrow O\overset{*}{-}O\text{-}\emptyset + ADP. \qquad 2$$

Radioautograms of Bassham and Calvin have shown labelled maltodextrins following photosynthesis in $^{14}CO_2$ in *Chlorella* and *Scenedesmus* (5). On the two-dimensional chromatograms, compounds form what appears to be a homologous series extending from the origin to sucrose. The chromatograms and radioautograms were prepared in the Berkeley laboratory by a Dutch scientific visitor, Karl Louwrier, in search for the hypothesized keto acid intermediate of the carboxydismutase reaction.[2] The chromatograms were of the 20% methanol extract of *Chlorella pyrenoidosa* (1/2% v/v suspension) subjected to $^{14}CO_2$ for about 14 minutes. The algae were killed in a final volume of 80% methanol at -20°C. After 12-28 hours in 80% methanol, this extract was removed and the residue then extracted with 20% methanol. The 20% methanol extract was concentrated and chroma-

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[2]Bassham, J. A. University of California, Berkeley, California. Letter to Dexter French. Private communication. 1960.

tographed in two dimensions (30 hours each) in descending fashion--first in 72% freshly distilled phenol and 28% distilled water (by weight) and secondly in a n-butanol-propionic acid-water solvent prepared just before use by mixing equal parts of a solution of n-butanol (1246 ml n-butanol + 84 ml water) and of a solution of propionic acid (620 ml propionic acid + 790 ml water). The chromatograms were exposed to X-ray film for 1-2 weeks. R. W. Youngquist and D. French established these compounds to be members of the maltodextrin series (34). The radioactive compounds were cut from the chromatograms, eluted, subjected to one-dimensional chromatography and compared in mobility with the series of oligosaccharides resulting from starch hydrolysis, the maltodextrins. The most rapidly moving compound retained on the two-dimensional descending chromatogram compared with maltohexaose and the slowest resolved compound to maltoundecaose, G_{11}. Hydrolysis of maltohexaose and maltoheptaose with crystalline sweet potato β-amylase for ten minutes at 37°C yielded G_2 and G_4 from maltohexaose and G_2 and G_3 from maltoheptaose clearly indicating these compounds were linear α-1,4-linked dextrins.

Jensen and Bassham[3] have recently found maltodextrins in extracts of isolated spinach chloroplasts after photosynthesis in $^{14}CO_2$. The fixation of carbon dioxide is dependent on the structural integrity of the chloroplasts, and so the chloroplasts must be handled very gently during their isolation. By quickly centrifuging samples taken during photosynthesis, it was learned that oligosaccharides, fructose-6-phosphate, sedoheptulose-

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[3]Jensen, R. G. Department of Chemistry, University of Arizona, Tuscon. Seminar at Iowa State University. Private communication. 1966.

7-phosphate and ribulose-1,5-diphosphate remained within the intact chloroplast, while 3-phosphoglyceric acid, glyceraldehyde-3-phosphate, dihydroxyacetone phosphate, pentose phosphates, fructose-1,6-diphosphate, and glycolic acid leaked out (35).

Heber reported tri-, tetra-, and pentasaccharides which were present in the chloroplasts but not in the cytoplasm of leaves of *Nitella*, *Valonia*, and *Chara* (36). The maltodextrin series was also apparent in Kandler's published radioautograms of two-dimensional chromtograms of extracts of *Heliosperma alpestre*, *Hypnodendron* species, *Selaginella caulescens*, *Angiopteris* species, and *Buddleia davidii* (32).

Fisher (37) estimated the specific activity of the glucose from water-soluble dextrins and from starch after hydrolyzing these fractions from labelled soybean leaves with amyloglucosidase, and found them to be equal. Since the specific activity of the dextrin was equal to the specific activity of starch, the two appeared to be closely related metabolically.

Gibbs studied the distribution of C-14 atoms in glucose molecules from the dextrins and starch of sunflower leaf following short periods of photosynthesis in $^{14}CO_2$ (38). The labelling of dextrin glucose was virtually uniform even after the shortest photosynthetic periods, whereas the glucose derived from starch was initially labelled more strongly in carbon atoms 3 and 4. Only after several minutes did glucose from starch become uniformly labelled. The evidence suggested a more rapid turnover of the dextrins than of starch. The dextrins did not appear to be products of the hydrolysis of starch.

Fisher (37) fround that dextrin material represented 85-90% of the

ethanol-insoluble, water-soluble radioactive material in soybean leaves after pulse labelling with $^{14}CO_2$ during photosynthesis. Shown in Figure 3 are the carbon-14 kinetics of ethanol-insoluble materials from such an experiment. This figure was reproduced from Fisher's thesis. The dextrin material was shown to contain the radioactive maltodextrin series.

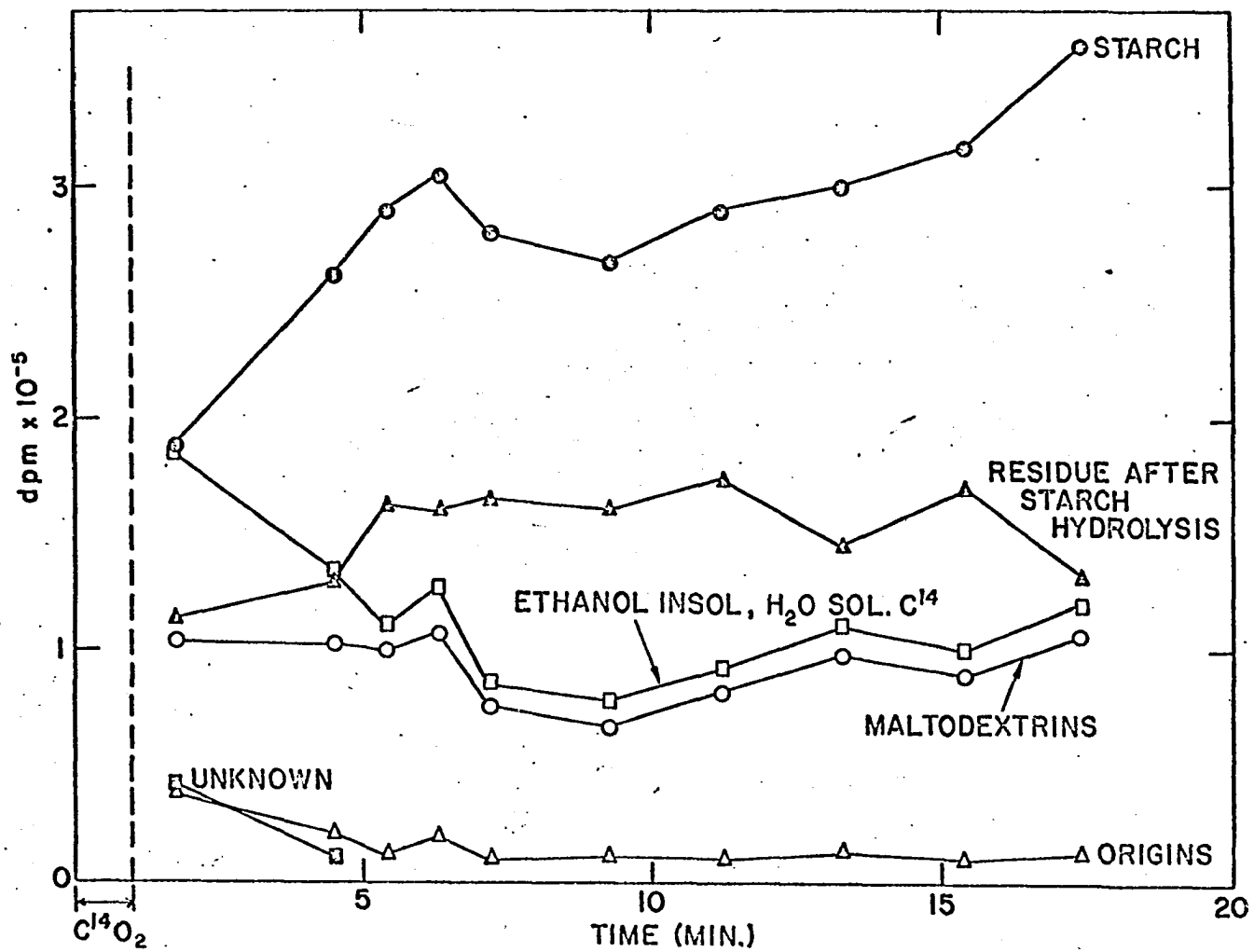

Figure 3. Kinetics of labelling of soybean leaf carbohydrates following pulse of $^{14}CO_2$ to the leaf (37)

C. Plant Enzymes which Metabolize Maltodextrins

The levels of various enzymatic activities localized in isolated chloroplasts have been assayed. Enzymes of the carbon reduction cycle are found in the chloroplast while enzymes for glycolysis and respiration are not (39). However, of the enzymes which are functional in polysaccharide synthesis and degradation, only starch synthetase, sucrose and sucrose-6-phosphate synthetases, ADPG and UDPG pyrophosphorylases, phosphoglucomutase, glucose phosphate isomerase, and sucrose-6-phosphate phosphatase have definitely been localized to the chloroplast (40). The localization of amylases in the leaf is not clear (39) and phosphorylase had been reported both absent and present in chloroplasts (40, 43, 44, 45, 46).

In this light, enzymes which could relate to the metabolism of maltodextrins in plant tissue will be reviewed. These enzymes are summarized in Table 1.

Table 1. Properties of trans-α-glucosylases and amylases from plant sources

enzyme	glycosyl donor	glycosyl acceptor	reversibility
phosphorylase	G-1-P	maltodextrin DP > 4 amylose, or amylopectin	+
starch synthetase	ADPG or UDPG	maltodextrin DP ⩾ 2 amylose, or amylopectin	-
D-enzyme	maltodextrin DP > 3	glucose or maltodextrin DP ⩾ 3, amylose, amylopectin	+
Q-enzyme	amylose or amylopectin	amylose or amylopectin	-
T-enzyme	maltose	glucose, isomaltose, panose	+
R-enzyme	amylopectin	H_2O	-
α-amylase	maltodextrins, amylose, or amylopectin	H_2O	-
β-amylase	maltodextrins, amylose, amylopectin	H_2O	-

1. Phosphorylase

The reaction of phosphorylase is

$$G\text{-}1\text{-}P + G_n \rightleftharpoons P_i + G_{n+1}. \qquad 3$$

Under conditions of synthesis, the ratio of inorganic phosphate to glucose-1-phosphate is less than the equilibrium value (10.8 at pH 5; 6.7 at pH 6; and 3.1 at pH 7)(41). Phosphorylase preparations of varying degrees of purity have been obtained from many plant tissues, including peas, maize, barley, broad beans, jack beans, sugar beet and potato (42). Phosphorylase activity has also been reported in leaf tissue, but there are conflicting reports about its localization in leaf cells. From some histochemical observation, Yin (43, 44) concluded that phosphorylase was located exclusively in the plastids of both higher and lower green plants. However, Stocking (45) could detect neither the liberation of phosphate nor the formation of iodine staining polysaccharide when leaf plastids were incubated with buffered glucose-1-phosphate, whereas phosphorylase activity was evident in the cytoplasm of those cells. Later (46), Stocking reported that at least half of the leaf cell phosphorylase was associated with chloroplasts, which had been isolated in nonaqueous media. This report was based on the liberation of inorganic phosphate from glucose-1-phosphate. In 1965, Stocking reported there was no incorporation of radioactivity from C-14 glucose-1-phosphate into soluble starch by the proteins from tobacco leaf chloroplasts isolated by the non-aqueous technique (40).

Plant phosphorylases collectively differ from muscle and liver phosphorylases in having no requirement for adenylic acid. Potato phosphorylase has been crystallized (47) and the smallest efficient primer shown to

be maltotetraose; larger maltodextrins show increasing priming efficiency (48). Whelan and Bailey (48) showed that amylose synthesis proceeded by a multi-chain mechanism, in which all primer molecules were increased at approximately the same rate. Illingworth, Brown, and Cori (49) have discussed the redistribution of glucose units by muscle phosphorylase at the equilibrium concentration of G-1-P and P_i. By incubating enzyme with maltoheptaose and small amounts of either inorganic phosphate or glucose-1-phosphate under conditions which did not favor net degradation or synthesis, maltodextrin from G_4 to greater than G_{15} were produced.

Early workers considered phosphorylase to be the agent for starch synthesis in plants. The ratio of inorganic phosphate to glucose-1-phosphate was determined in the living bark of black locust (50). The ratio of 20-40 was so much greater than the equilibrium value that it was thought that *in vivo* conditions favor degradation rather than synthesis of starch by phosphorylase.

2. Starch synthetase

In 1960, de Fekete *et al.* reported that UDPG was a precursor for starch synthesis in potato (51). In 1964, the same workers found that ADPG was a better substrate than UDPG by ten times (52). Starch synthetase was initially isolated in particulate form, bound to the starch granules (53, 54, 55, 56, 14), but was later found in soluble form (11, 12, 13, 14, 15, 33, 57). Frydman and Cardini (57) have recently reported solubilization of the trapped particulate enzyme by swelling of the granule with 7 *M* urea. Detailed characterization of the enzyme has not been reported.

The particulate form of the enzyme from most sources has shown

synthesizing capacities when incubated with either UDPG or ADPG. However, Murata reported that UDPG was totally inactive in granular enzyme preparation from leaves (14). A preparation from waxy maize by Frydman (53) exhibited the specificity toward substrates noted in Table 2. The values in Table 2 show that ADPG is more effective than UDPG with respect to donor specificity. Addition of maltotriose to the incubation mixture decreased the radioactivity incorporation into starch. Other maltodextrins, glucose and isomaltose had the same effect, but glucose and isomaltose had the same effect, but glucose and isomaltose gave essentially no products. With maltotriose serving as acceptor, the products were mainly maltotetraose with some maltopentaose and maltohexaose.

Table 2. Donor specificity of particulate enzyme from waxy maize starch (53)

incubation of enzyme with	9000 cpm UDPG*		900 cpm ADPG*	
cpm in fraction	starch	oligo.	starch	oligo.
nucleotide	140	30	100	12
nucleotide + G_3	120	393	48	723
control	0	26	0	13

The results of other experiments, which have been reported in the literature, have indicated that G_2, G_3 and G_4 were better acceptors for the particulate enzyme than G_5, G_6 or G_7.

Data in Table 3 for both experiments showed a lower incorporation of radioactivity into the oligosaccharide fraction when G_5, G_6, and G_7 were added. The apparently better priming by the smaller maltodextrins may be quite the opposite. If G_5 were a better primer than G_4, the radioactivity in the oligosaccharide fraction might be lower because the maltopentaose

had elongated sufficiently with synthetase to be precipitated with the starch fraction.

Table 3. Acceptor specificity of granular synthetases

Addition of one mole of	5 mg mung bean prep. 0.53 µmole UDPG - C^{14}(55)		4 mg potato tuber prep. 0.25 µmole UDPG - C^{14}(54)	
	cpm in starch	cpm in oligo.	cpm in starch	cpm in oligo.
none t=0	0	48	---	---
none	700	64	570	15
glucose	304	58	---	---
G_2	610	775	325	350
G_3	284	930	350	400
G_4	370	897	300	468
G_5	304	634	400	370
G_6	---	---	475	235
G_7	---	---	575	192

Incorporation into starch was again lowered by the presence of maltodextrins. This result is contradictory with the latest data presented by Murata and Akazawa (58). The maltodextrins, G_2, G_3, and G_4, had a stimulatory effect on the incorporation of ^{14}C-glucose from ADPG* into the starch granules of particulate sweet potato starch synthetase. These workers also found a stimulatory effect of potassium ions on the particulate starch synthetase.

Starch synthetase in soluble form generally utilized amylopectin, glycogen, and maltodextrins as primers (33, 57). From some sources, however, the enzyme utilized amylose and inactive starch granules (12, 13, 15, 57). The *Chlorella* and the soluble leaf enzymes utilize only ADPG and deoxy-ADPG as glucose donors (11, 12, 13, 14). Completely inactive was UDPG as a donor with soluble leaf enzymes. Deoxy-ADPG had less than one-

half the activity of ADPG and when mixed, deoxy-ADPG was a competitive inhibitor of ADPG as were AMP, ADP, and ATP. The Michaelis constant for ADPG has been determined to be 2.72×10^{-4} *M* ADPG at pH 9.0 with *Chlorella pyrenoidosa* synthetase, and 1.8×10^{-4} *M* at pH 8.5 with spinach chloroplast synthetase.

For the chloroplast enzyme, preferred acceptor specificity was amylose > amylopectin > swollen starch granules > M_3 > glycogen > M_2 (12). *Chlorella* synthetase exhibited identical specificities (15) but the soluble synthetase from sweet corn preferred phytoglycogen > amylopectin > animal glycogen > maltodextrins (33). Maltodextrins inhibited incorporation of glucose from ADPG into glycogen, but glycogen did not inhibit incorporation into maltodextrins. Table 4a shows some results published by Frydman and Cardini (33) on the acceptor specificity of the soluble preparation from sweet corn.

Table 4a. Acceptor specificity of soluble synthetase from sweet corn (33)

Acceptor	Amount Added µmole	mg	ADP Formed mµmoles
G_1	2		0
G_2	2		7
G_3	2		22
G_4	2		60
G_5	2		36
amylose		1	0
starch granules		1	0
soluble starch		1	8
amylopectin		1	30
phytoglycogen		1	42

Of possible significance in Table 4a is the apparent maximum in the activity of the enzyme with G_4 as substrate. Similar discontinuities in the apparent activity between G_4 and G_5 are also seen in the granular

synthetases in Table 3. The apparent maximum activity with maltotetraose as substrate may perhaps be related to the specificity of the enzymes for maltodextrin acceptors. Fredrick (59) has noted the evolutionary tie which quite possibly existed between phosphorylase, branching enzymes, and glycogen synthetase. Phosphorylase and glycogen synthetase have similar acceptor specificities (maltotetraose is the minimum efficient primer) (60) Coupled with branching enzyme, they both synthesize highly branched polysaccharide. The evidence in Tables 3 and 4a may extend the relationship to starch synthetase, because of the apparent preferred specificity of this enzyme for G_4.

3. D-enzyme

The disproportionating enzyme (D-enzyme) is a transglucosylase capable of transferring two or more glucose units from a maltodextrin or starch donor to synthesize an α-1,4-glucosidic linkage with a suitable acceptor. A typical reaction,

$$G_3 + G_3 \rightleftharpoons G_5 + G_1, \qquad 4$$

shows the transfer of a maltosyl unit. The reaction was shown to be freely reversible, and at equilibrium, a series of maltodextrins from G_3 to G_6 was produced (61). If glucose was removed from the equilibrium mixture (as by hexokinase and ATP), iodine staining amylose was produced.

Maltotriose and higher maltodextrins, amylose, and the outer chains of amylopectin could function as donor substrates, but glucose and maltose were inactive. Glucose was the most efficient acceptor (62). Of nearly fifty sugars and sugar derivatives tested, only nine had appreciable acceptor activity: methyl-α-D-glucoside (37% that of equimolar glucose),

methyl-β-D-glucoside (12%), D-mannose (13%), L-sorbose (10%), methyl-α-L-sorboside (5%), D-xylose (10%), maltose (11%), sucrose (6%), leucrose (34%) and α-α-trehalose (10%).

Maltose was never produced in significant quantities from the action of D-enzyme. From this, Whelan specified which maltodextrin linkages were susceptible to and immune from action of D-enzyme (63). The non-transferable linkages were the single terminal linkage at the non-reducing end and the linkage penultimate to the reducing end.

The mechanism of D-enzyme action was shown by using radioactive glucose as the acceptor substrate (64). The maltodextrins formed in the reaction between starch and C-14 glucose were found to be radioactive only in the reducing-end glucose. The mechanism was given as

$$\phi^* + 0\text{-}0\text{-}0\text{-}0\text{-}\cdots\cdots \rightleftharpoons 0\text{-}0\text{-}\phi^* + 0\text{-}0\text{-}\cdots\cdots \qquad 5$$

Transfer is effected by breaking and forming α-1,4-glucosidic linkages. In this respect, the action of D-enzyme is similar to the transglucosylation reaction of amylomaltase from *Escherichia coli* (65) and *Bacillus macerans* (66).

D-enzyme has been isolated from potato and broad bean (42). Enzymes with similar properties to D-enzyme have been reported in germinated green gram (*Phaseolus radiatus*) (67), and minced algae (*Cladophora rupestris*, *Lamarinaria digitata*, and *Rhodymenia palmata*) (68). The green gram and three algae extracts produced small amounts of glucose, maltotriose, and maltotetraose when incubated with chromatographically pure maltose.

4. Q-enzyme

Q-enzyme has been obtained from green gram, broad bean and wrinkled pea as amorphous preparations. It has been crystallized from potato juice (42). Q-enzyme is a transglucosylase, which causes the scission of α-1,4-linked linear chains and the reunion of these fragments to the main chain with α-1,6-linkages. Amylose can be converted to glycogen-like polysaccharides by means of this enzyme. The smallest size of linear molecule susceptible to rapid branching action has been established to be DP = 40 (42), however action has been noted on amylose of average DP = 28. The use of radioactive maltose, maltotriose, maltotetraose and maltopentaose yielded radioactive amylopectin and so these molecules act as acceptors for the transfer while glucose, sucrose, fructose, galactose, cellotriose, lactose, inulin, xylan, dextran, or α- and β- Schardinger dextrins did not (42).

Hers and Verhue (69) allowed liver branching enzyme to react with non-reducing end labelled glycogen. Debranching of the product with pullulanase (70) and chromatography showed that after action of the liver branching enzyme, the radioactive branches were maltohexaose and maltoheptaose alone.

Branching enzyme from rabbit muscle exhibited similar specificity as the oligosaccharyl fragment preferentially moved during branching was 7 glucose units long (71). The specificity of this enzyme was reflected by the marked inhibition of the branching reaction by G_7. G_6, G_8 and G_9 had much less effect and G_1-G_5 had none. The K_m of the enzyme for "liver amylopectin" was 3×10^{-6} *M* (as end group), and the reaction was stimulated by citrate and by α-G-1-P.

Frederick has also studied the properties of branching enzymes from algae, *Oscillatoria princeps*, *Rhodymenia pertusa*, and *Spierogyra setiformis* (72). The action of the branching enzymes varied in all three algae. In the green alga (*Spirogyrus*) the branching enzymes (three isozymes) acted only on amylose and formed a moderately branched polysaccharide; all three were inactive on amylopectin. Two branching isozymes of *Oscillatoria* formed a branched material with a red iodine color (maximum absorption at 550 nm). This polyglucan was formed from either amylose or amylopectin. One isozyme of *Rhodymenia* acted like the *Oscillatoria* isozymes, but two other isozymes of *Rhodymenia* acted identically to the three isozymes of *Spirogyra* in giving a polysaccharide with an absorption maximum of its iodine complex at 580 nm. The branching enzymes of these algae are distinct but related. The more primitive the alga, the more highly branched was the polysaccharide.

The *Spirogyra* is of the same order as Chlorophyta, *Chlorella* and *Scenedesmus*, and these algae synthesize a type of starch which compares with that of higher plants in being a mixture of approximately 25% amylose and 75% amylopectin. The starch of these green algae stains blue with iodine-iodide solutions. Starch accumulates in the chloroplast of Chlorophyta around an amorphous mass of protein, called the pyrenoid. Starch is deposited around the pyrenoid in structures resembling flakes when observed in the light microscope. As the size of the resulting granule grows, some of the flakes are shed off and become dispersed about the stroma of the chloroplast.[5]

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[5]Dodd, J. Iowa State University. Algology: Botany 500. Private communication. Fall, 1968.

5. T-enzyme

A second enzyme capable of preforming the same α-1,4- to α-1,6-glucosidic bond transformations as Q-enzyme has been named T-enzyme. T-enzyme acts on much simpler substrates than Q-enzyme, for instance maltose,

2 maltose ⟶ panose and glucose. 6

With a potato preparation, the following reactions were also catalyzed (73).

2 panose ⟶ 6^2-α-isomaltosylmaltose + maltose 7

2 isomaltose ⟶ isomaltotriose + glucose 8

2 isomaltotriose ⟶ isomaltotetraose + isomaltose 9

Oligosaccharides synthesized from maltose by the minced seaweed extract of *Cladophora rupestris* (74) were panose, maltotriose, 6^3-α-glucosylmaltotriose, and maltotetraose. Maltose and maltotriose appeared to be acceptors whereas glucose and panose did not. Manners also noted α-1,4- to α-1,4-glucosidic transformations with maltose in extracts of *Cladophora rupestris* (68). The distribution of oligosaccharides above quite likely resulted from the combined activities of T- and "D type"-enzymes.

6. R-enzyme

R-enzyme has been obtained from broad beans, potatoes, and barley malt (42). The original R-enzyme preparations contained an α-limit dextrinase activity, which was considered characteristic of R-enzyme. The enzymes have been separated (75). Purified R-enzyme cleaved α-1,6-branch points from amylopectin and β-limit dextrin, but not from glycogen

or α-limit dextrin (5-8 glucose units). Purified α-limit dextrinase will hydrolyze isomaltose, isomaltotriose, or dextran, nor will it remove α-1,6-glucose stubs from maltodextrins. The pentasaccharide product resulting from α-amylolysis of amylopectin or glycogen is the smallest substrate for the α-limit dextrinase. The minimum number of glucose units in the side chain appears to be two, and so the minimum number of glucose units in the base chain must be three (76).

7. Amylases

β-Amylase is abundant in seeds and other parts of the higher plants. Crystalline β-amylases have been obtained from sweet potato, barley malt, and wheat. This enzyme is very specific for the second α-1,4-linkage from the nonreducing ends of polyglucans. If the substrate contains only α-1,4-glucosidic bonds and is of even degree of polymerization, it is converted completely into maltose by β-amylase. A molecule containing an odd number of glucose units will be converted to maltose and maltotriose, the maltotriose coming from the reducing end of the chain. Maltotriose is attacked slowly by β-amylase to give glucose (from the reducing end) and maltose.

α-Amylases catalyze the hydrolysis of α-1,4-glucosidic linkages in amylose, amylopectin, and glycogen in a more random manner than β-amylase. Branching points (α-1,6 linkages) in starch are unattacked, and such branch points protect some of the adjacent α-1,4 linkages from attack. Chain ends are attacked only with great difficulty (76). α-Amylases from various sources have been shown to yield distinct sets of hydrolysis products (77). The properties of α-amylases from plants have been

summarized by Greenwood and Milne (78). Action of α-amylases from oats, rye, and wheat on amylose produced larger amounts of maltose and maltohexaose than other hydrolysis products at the achroic limit. In digests of soya bean and broad bean α-amylase with amylose, the yield of maltotriose was equal to that of maltose at the achroic point. Maltohexaose was still the preponderant hydrolysis product, and slightly more glucose resulted than in the cereal α-amylase digests (78).

III. MATERIALS AND METHODS

A. Plant Material

1. Algae

Scenedesmus obliquus (strain No. 72, Indiana University Algae Collection, Bloomington) was grown in shaker flasks under the illumination of 300 Watt General Electric Reflector Spot Lamps. The flasks were maintained in a temperature-controlled water bath at 25°C. Nutrient solution consisted per liter of

12 ml 1 *M* KNO_3

10 ml 1 *M* $MgSO_4$

8 ml 1 *M* KH_2PO_4 + 0.08 *M* K_2HOP_4 (pH 5.7)

1 ml 0.1 *M* $Ca(NO_3)_2$

2 ml Sequestrene NaFe: 13% Fe (38.46 g/1)

1 ml micronutrient solution which contained per liter

1.56 ml H_3PO_4 (85%)

1.55 g $MnSO_4 \cdot H_2O$

0.10 g $ZnCl_2$

0.08 g $CuSO_4 \cdot 5\ H_2O$

0.02 g $H_2MoO_4 \cdot H_2O$

After approximately two weeks of growth under the given conditions, the cultures were harvested by centrifugation.

Chlorella pyrenoidosa (Emerson strain No. 395, Indiana University Algae Collection, Bloomington, Indiana) was grown in continuous culture in 15 liters of mineral nutrients (above) under the constant illumination of ten 24 inch Daylight fluorescent bulbs. Stirring by a four inch

magnetic stirring bar and aeration with 4% CO_2 in air (Matheson, Inc., Joliet, Illinois) kept the algae in suspension. The temperature was regulated at 25°C. Approximately 15 g of cells were harvested every four days by centrifuging 12 liters through a Sharples laboratory Super-centrifuge. Fresh medium was added to the carboy through a Seitz filter under positive pressure.

2. Soybean leaves

The soybean plants (*Glycine max*, var. Hawkeye) used in these studies were grown in soil in a Scherer growth chamber under 280 foot-candles of light supplied by Gro-Lux fluorescent bulbs in a cycle of 14 hours of light at 29°C and 10 hours of dark at 18°C. Experiments were conducted with plants between 26 and 30 days old. Since only the first trifoliate leaf was exposed to $^{14}CO_2$ in the labelling experiments, the shoot of all plants was excised just above the node of the first trifoliate leaf before the experiments. The wounds were sealed with lanolin, and the trifoliate leaves of the six plants were sealed into a rectangular, Plexiglass feeding chamber which had a removable top and three notches on each side for the petioles. The plants were left in this experimental situation for two hours before feeding. Light was provided in these experiments by a bank of two 24-inch Gro-Lux bulbs which were adjusted to produce 280 foot-candles of light at the leaf surface. The $^{14}CO_2$ was generated from high specific activity barium carbonate (Oak Ridge National Laboratories, Oak Ridge, Tennessee) with perchloric acid in a generating chamber and was circulated through a four-liter flask and the feeding chamber by a rotary peristaltic action pump. Simultaneous labelling of the six plants lasted

three minutes at which time the hose to the feeding chamber was clamped, the top removed from the chamber, and the $^{14}CO_2$ swept away in the hood. Extensive details of the feeding procedures have been recorded by Fisher (37). The investigator is indebted to D. B. Fisher for furnishing the soybean leaf tissue used in these investigations.

3. Spinach chloroplasts

Chloroplasts were isolated in pyrophosphate buffer (79) from fresh spinach which was purchased at a local grocery store during the winter months. Leaves were washed and the main veins were removed. About 50 g of chilled laminae were homogenized in a Waring blender for 3-5 seconds in 200 ml of semi-frozen buffer. The buffer, which was prepared just before use, contained 0.33 *M* sorbitol
0.005 *M* $MgCl_2$
0.010 *M* $Na_4P_2O_7$
0.004 *M* L-ascorbic acid.
Ascorbic acid was added after adjustment of the icy buffer to pH 6.6.

The macerate was squeezed through two layers of cheesecloth and then filtered through eight layers of cheesecloth into 50 ml centrifuge tubes. The chloroplasts were centrifuged at 0°C from rest to 1000 × g to rest in approximately 90 seconds. The supernatant was discarded and the pellet was suspended in approximately 5 ml of ice cold solution containing
0.33 *M* sorbitol
0.001 *M* $MgCl_2$
0.001 *M* $MnCl_2$
0.002 *M* Ethylenedinitrilotetra acetic acid (EDTA)
0.050 *M* N-2-Hydroxyethyl piperazine-N'-2-ethane-sulfonic acid (HEPES).

The pH of this buffer was adjusted to 7.6 at 20°C. The pellet was washed and centrifuged as above three times in this buffer. Photosynthesis was also conducted in this buffer. Chlorophyll content was determined spectrophotometrically by adding 1.0 ml of chloroplast suspension to 4.0 ml acetone (80). Absorption of light at 665 nm and 649 nm on a Beckman DU provided values for equation 8.

$$\frac{\text{mg total chlorophyll}}{\text{liter}} = 6.45\ (A_{665}) + 17.72\ (A_{649}) \qquad 8$$

B. Photosynthetic Feeding Procedures

1. Algae

Eight ml of a 0.5% suspension of cells in distilled water was placed in a "lollypop" culture flask between two 300 watt General Electric Reflector Spot Lamps (5). Large flat culture bottles containing water separated the lollypop from the lamps and kept the temperature of the suspension at less than 30°C during the feeding experiment. Carbon dioxide, 4% in air, for 90 minutes and then air for 5 minutes were bubbled through the suspension under full illumination before radioactive sodium bicarbonate was added. The closed system was shaken and after 16 minutes of photosynthesis, the suspension was drained into 300 ml of boiling methanol. The 80% methanol solution was boiled for 20 minutes at pH 5 to remove excess $^{14}CO_2$ from solution, to inactivate enzymes, and to extract pigments, amino acids, organic acids, and sugars from the algae.

2. Spinach chloroplasts

Photosynthesis by the isolated chloroplasts followed the procedure of Bassham, Kirk, and Jensen (35). The chloroplasts were suspended in 4-5 ml

of pH 7.6 buffer. Potassium bicarbonate and sorbitol were added so that the suspension would remain 0.01 *M* in bicarbonate and 0.33 *M* in sorbitol after addition of radioactive bicarbonate. The chloroplast suspension was placed in a 3.3 centimeter diameter, 24/25 standard taper, round-bottom flask with side-arm (Thomas, Philadelphia) and stoppered with a polyethylene standard taper stopper. The chloroplasts were kept suspended by the motion of a wrist-action shaker at setting of 2. The bottom of the flask was suspended in water, which was kept at 20°C by circulating water from a thermostated bath through the interspace of a double walled 600 ml beaker. The beaker rested on a bank of six 24-inch daylight fluorescent bulbs. Photosynthesis was conducted for 15-20 minutes before injection of a small volume of high specific activity C-14 sodium bicarbonate into the suspension from a hypodermic syringe by means of eight inches of one millimeter polyethylene tubing which was threaded through the side-arm of the flask. Since the end of the tubing was on the bottom of the flask, samples could be removed at intervals by the same means. The 0.4 ml samples were immediately injected into 1.6 ml of absolute methanol at 4°C and mixed. After centrifugation, the green supernatant was pipeted from the white pellet. Both were stored at -15°C until prepared for chromatography.

C. Paper Chromatography

1. Photosynthetic products

For the analysis of the products of photosynthesis in the alcoholic extracts in algae and chloroplasts, two-dimensional descending paper chromatography, as described by Bassham and Kirk (81) was employed. On

separate pieces of Whatman No. 1 chromatography paper, which had been washed with water, was spotted 0.2 ml of each sample from a photosynthetic feeding experiment. Descending chromatography in the machine direction of the paper was with the solvent of 84 parts liquified phenol, 16 parts water, and 1 part acetic acid. The solvent in the second direction was made by mixing equal volumes of A and B:

A. n-butyl alcohol: water, 370: 25 (v/v)

B. propionic acid: water, 180: 220 (v/v)

Forty-eight hours of chromatography in each direction gave maximum separation of the phosphorylated photosynthetic intermediates and maltodextrins while retaining glucose and aspartic acid on the chromatogram. Since the levels of radioactivity in the maltodextrins were frequently low, the irrigated chromatograms were pressed against Kodak No-Screen X-ray film for as long as one month for radioautography.

2. Maltodextrins

To obtain the neutral maltodextrins free from amino acids, organic phosphates and other ionic materials, the alcoholic extracts were deionized with Amberlite MB-3 mixed bed ion exchange resin. This resin has been shown to tenaciously adsorb maltodextrins, but this effect could be reduced by treating the resin with glucose before using.[6] Following deionization, the samples could be chromatographed more rapidly in solvents more applicable to carbohydrates. Separation of maltodextrins was accomplished on water-washed Whatman No. 1 or Whatman 3MM chromatography paper.

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[6]Kainuma, K. Iowa State University, Ames, Iowa. Private communication. November 1968.

High temperature ascending chromatography was conducted in closed stainless steel tanks at 65°C (2). Solvents used were: (A) 6 parts n-butyl alcohol, 4 parts pyridine, and 4 parts water, or (B) 70% n-propyl alcohol and 30% water. Multiple ascents were required for resolution of those maltodextrins greater than maltohexaose. Also, a solvent of 65% n-propyl alcohol was found useful for ascending and descending chromatography of maltodextrins at 35°-40°C on washed Whatman 3MM paper.

The length of time that the irrigated chromatograms were radioautographed was dependent on the level of radioactivity on the chromatogram. Generally, a chromatogram where the weakest spot registered 1000 cpm when counted with a Geiger-Mueller tube made a radioautogram which could be developed after 24 hours of exposure. Weaker activities required correspondingly longer exposures. Kodak No-Screen X-ray film was kept against the chromatograms either in standard X-ray cassetts or under a 13" × 17" × ½" piece of plywood weighted with bricks during the exposure period. The X-ray film was developed 1.5-3.0 minutes in Kodak X-ray Developer, stopped 5 seconds in 4% (v/v) acetic acid, fixed 15 minutes in Kodak X-ray Fixer, and washed one hour in running water. All operations were carried out under a red safe-light.

Chromatograms of amylase digests were developed by dipping in silver nitrate and alkali reagents (82). The chromatograms were dipped briefly in silver nitrate-acetone solution (2.5 g silver nitrate, 5 ml water, 500 ml acetone) and dried completely. The chromatograms were then soaked in alkali solution (4 g sodium hydroxide, 10 ml water, 1000 ml methyl alcohol) until the sugars were revealed. Dipping in fixer (130 g sodium thiosulfate, 12.6 g sodium metabisulfate, 1000 ml water) lightened the background before

the chromatograms were washed for thirty minutes in running water.

3. Sorbitol and glucose

Reduction of the small quantities of photosynthetic maltose with 1.5 mg of sodium borohydride in 0.1 ml of water at room temperature was complete overnight. Hydrolysis of the resulting glucosylsorbitol to glucose and sorbitol was complete in 4 hours at 100°C in 2.5% hydrochloric acid. The digests were reduced in volume under reduced pressure and spotted on unwashed Whatman 3MM chromatography paper.

Walker and Whelan (64) reported a mixture of 9 parts of ethyl acetate, 1 part acetic acid, and 1 part saturated boric acid to be a very good solvent for the separation of reduced from unreduced maltodextrins. This irrigant, in descending chromatography on unwashed Whatman 3MM paper, moved the reduced maltodextrins approximately twice as rapidly as it moved the corresponding unreduced compounds. Twelve hours of chromatography were required to separate glucose and sorbitol, and 30 hours to separate maltose and maltitol.

4. Two-dimensional chromatography interspersed with β-amylase treatment

A technique for looking at the distribution of radioactivity in the glucose units of maltodextrin molecules was given by French *et al.* (83). The procedure was modified for this work as follows: deionized extracts of soybean leaves or spinach chloroplasts were spotted at one corner of individual pieces of chromatography paper. After chromatography of the maltodextrins in a suitable solvent, the chromatograms were dried and then dipped in a suspension of β-amylase containing 0.05 ml of sweet potato β-amylase (Worthington Biochemical Corporation, Freehold, New Jersey) in 200

ml of cold water (which was made approximately pH 4 with a crystal of mercaptoacetic acid) and 800 ml of acetone at -15°C. The chromatograms were hung for 3-4 minutes in the air while the acetone evaporated and then for 90 minutes in a moist chamber at 35-40°C. After enzymatic reaction, the chromatogram was again dried and then chromatographed at 90° to the first direction so that the hydrolysis products were separated in a line directly above the maltodextrin, as shown in Figure 4. Maltodextrins having an even number of glucose units were converted solely to maltose by the action of β-amylase, and the odd-numbered maltodextrins were converted to glucose, maltose and maltotriose.

In the action of β-amylase, hydrolysis of maltotriose and maltotetraose are slow compared to the hydrolysis of higher maltodextrins. By limiting the incubation time for hydrolysis, the odd-numbered maltodextrins were converted only to maltose and maltotriose--the maltotriose coming from the three glucose units at the reducing end of the molecule. The radioactive areas were located by radioautography. By simply cutting the maltose and maltotriose spots out of the chromatogram and counting their radioactivity, the distribution of radioactivity in the glucose units of the odd-numbered maltodextrins was estimated. When the distribution of radioactivity is equal in all glucose units of the molecule, the ratio of activity of maltose to maltotriose, G_2/G_3, for the odd-numbered maltodextrins would be:

maltopentaose	2/3	0.67
maltoheptaose	4/3	1.33
maltononaose	6/3	2.00

A ratio greater than the equal-distribution ratio would indicate more

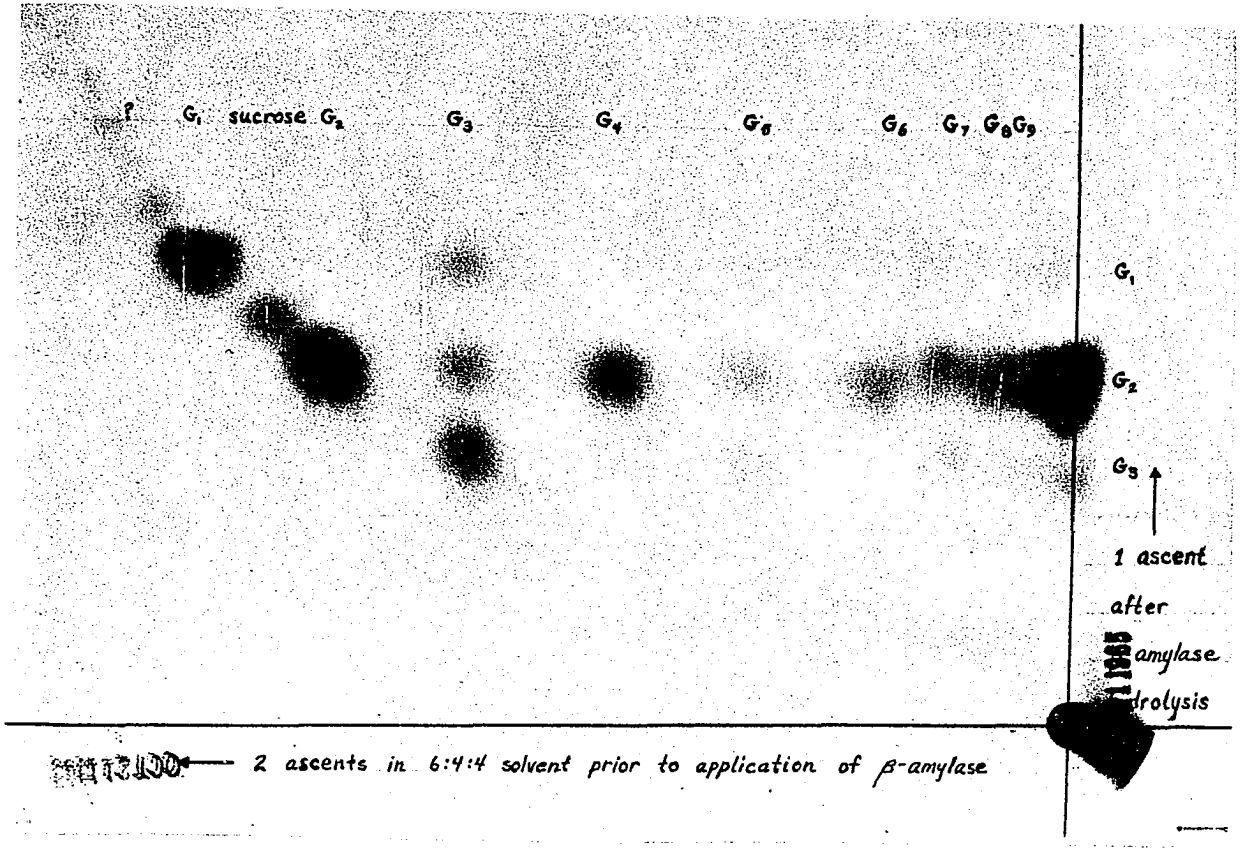


Figure 4. Two-dimensional paper chromatography of soybean ^{14}C-maltodextrins interspersed with treatment by β-amylase on the paper

radioactivity in the non-reducing end than in the reducing end of the molecule, and of course, a ratio less than those above would indicate relatively more activity in the reducing end of the odd-numbered maltodextrin.

The relative distribution of radioactivity in maltotriose can also be estimated by counting the radioactive maltose and glucose spots following β-amylolysis on paper. Equal distribution of radioactivity in maltotriose yields a G_2/G_1 ratio of 2.0. Values greater than 2.0 indicate distribution of radioactivity in favor of the non-reducing end and values less than 2.0 indicate higher relative distribution in the reducing end of maltotriose.

The hydrolysis of maltotriose by β-amylase was also carried out in test tubes after elution of the maltotriose from paper chromatograms. The β-amylase used for this was prepared by adding 0.025 ml of Worthington β-amylase to 3.0 ml of 0.005 *M* acetate buffer at pH 4.8. Each sample of maltotriose was incubated with 0.1 ml of the above solution for 13 hours at 38°C. The digests were evaporated to near dryness and spotted on washed Whatman No. 1 paper for ascending chromatography and radioautography.

D. Analytical Procedures

1. Scintillation counting

One of two scintillation solutions were used depending upon the nature of the radioactive material. The naphthalene-dioxane scintillation solution was used when counting aqueous solutions. This cocktail contained 4.0 g of 2,5-diphenyl oxazole (PPO), 0.2 g of 2,2-p-phenylenebis (5-phenyloxazole) (POPOP), 100 ml of methyl alcohol, 20 ml of ethylene glycol, and 60.0 g of

naphthalene diluted to 1 liter with dioxane (84).

The toluene scintillation solution contained 4.0 g PPO and 0.2 g POPOP per liter of toluene, and was used for counting radioactivity on paper, in non-aqueous solution, and in material which had been solubilized with Hyamine base (Nuclear Chicago, Inc., Des Plaines, Illinois) (85).

By utilizing the automatic external standard on the Packard Tri-Carb Scintillation Spectrometer, efficiency of counting could be obtained when counting homogeneous samples. Quenching curves were prepared for both solvents with nitromethane and standard ^{14}C-toluene. Maximum efficiency for unquenched toluene solution was 86% and that for the naphthalene-dioxane solution was 58%.

2. Carbohydrate determinations

Total carbohydrate was determined by the phenol-sulfuric acid method (86). This determination has been shown to be independent of structure for glucose and glucose polymers in the range of 1-100 micrograms of glucose (87). Samples in 1 ml of water were mixed with 0.1 ml of liquified phenol and 5.0 ml concentrated sulfuric acid. After the tubes cooled, the amber color was determined in a Klett colorimeter with a No. 54 (green) filter.

The reducing values were determined by two methods. Most sensitive was the Park-Johnson ferric-ferrocyanide method (88). Samples containing 1-9 micrograms of glucose in 1-3 ml of water were mixed with 1 ml of A (0.5 g potassium ferricyanide per liter of water) and 1 ml of B (5.3 g sodium carbonate and 0.65 g potassium cyanide per liter of water). After heating in boiling water for 15 minutes, 5 ml of C (1.5 g ferric ammonium

sulfate and 0.1 g sodium lauryl sulfate per liter of 0.005 *N* sulfuric acid) was added. Blue color developed in 15 minutes and was determined in a Klett colorimeter with a No. 66 (red) filter.

The reducing value was also determined by the method of Nelson (89) in the range of 10-100 micrograms of glucose. To 0.5 ml of sample in a Folin-Wu tube was added 1.0 ml of the alkaline-copper reagnet. This reagent contained 1.0 ml of A and 25 ml of B.

A. 30 g cupric sulfate pentahydrate per 200 ml of water with 4 drops of concentrated sulfuric acid.
B. 25 g sodium carbonate, 25 g sodium, potassium tartrate, 20 g sodium bicarbonate, and 200 g anhydrous sodium sulfate per liter of water.
C. 25 g ammonium molybdate, 450 ml of water and 21 ml of concentrated sulfuric acid. To this was added with stirring 25 ml of water in which 3 g sodium molybdate was dissolved.

The solution in the tube was mixed and heated 20 minutes in boiling water. After cooling, 1.0 ml of C was added and mixed with the evolution of carbon dioxide. Green color developed while standing for 10 minutes, and after diluting to 25 ml with water, the color was determined with a No. 54 (green) filter on the Klett colorimeter.

Starch-iodine blue color was measured as per Robyt and Whelan (77). The procedure was as follows: 0.1 ml of 1% soluble starch digest was mixed with 5 ml of iodine solution. This iodine solution was prepared by diluting 1 ml of a stock solution (2 mg iodine plus 20.0 mg potassium iodide per ml) solution to 100 ml and adding 1 ml of concentrated hydrochloric acid. The blue color was determined on the Klett colorimeter using a No. 66 (red) filter. The blank was iodine solution.

E. Starch Synthetase Preparation

The unit of starch synthetase activity was defined as the incorporation of one micromole of glucose into waxy maize starch in fifteen minutes at 37°C (15). The reaction solution contained

0.010 ml 1% waxy maize starch

0.010 ml 0.015 *M* ADP-^{14}C-glucose (0.00115 μc/μmole) (International Chemical and Nuclear Corporation, City of Industry, California)

0.050 ml reaction buffer (pH 9.0 at 37°C) containing

0.5 *M* glycine

0.1 *M* EDTA

0.02 *M* KCl

0.005 *M* dithiothreitol

At zero time, 0.050 ml of enzyme was mixed with the reaction solution in 10 × 75 mm test tubes. After 15 minutes at 37°C, 0.20 ml of 95% ethanol was added, mixed, and the precipitated starch and protein were centrifuged at 10,000 × g for 10 minutes. The precipitate was washed three times with 50% ethanol (w/w) and finally dissolved in 0.10 ml of 0.05 *N* sodium hydroxide. The starch solutions were transferred quantitatively to scintillation vials and counted in 10 ml of the dioxane-naphthalene scintillation solution.

A procedure for the isolation of soluble starch synthetase from *Chlorella* has been published by Preiss and Greenberg (15). The procedure was adapted and extended in this study in an attempt to attain higher purification of the enzyme for use in kinetic and binding studies with

maltodextrins.

Chlorella were grown as described in part A of this section. It was found that frozen cells yielded starch synthetase activity as well as did freshly harvested algae, and so if there was not an immediate need for enzyme, the *Chlorella* cells were frozen for later use. The cells were centrifuged in a Sharples centrifuge and washed twice by suspending the cells in buffer followed by centrifugation. The buffer used in all steps of the enzyme purification contained 0.5 *M* Tris-HCl (Fisher Primary Standard THAM, Fisher Scientific Company, Fair Lawn, New Jersey) and 0.001 *M* EDTA (Baker Chemical Company, Phillipsburg, New Jersey) and 0.001 *M* dithiothreitol (Calbiochem, Los Angeles, California). Purification began with suspension of fifteen to twenty grams of cells in approximately 100 ml buffer and disruption of the cells in the French press at 20,000 psi. The milieu was centrifuged for thirty minutes at 30,000 × g and the supernatant of this was centrifuged at 105,000 × g in a preparative ultracentrifuge for four hours.

The ammonium sulfate fraction between 25% and 40% saturation contained most transferase activity. Thirty-three ml of neutralized saturated ammonium sulfate solution (Mann Assayed Special Enzyme Grade, Mann Research Laboratories, Inc., New York, N. Y.) was added with stirring to 100 ml of 105,000 × g supernatant. The resulting precipitate was discarded and 34 ml of saturated ammonium sulfate solution was added to the supernatant. The precipitate resulting from this fractionation was dissolved in minimal buffer and dialyzed for two to three hours in one liter of buffer. The enzyme solution was assayed, and for each unit of transferase activity, 10 mg of calcium phosphate gel (90), was added to the solution with

stirring. The starch synthetase activity was adsorbed on the gel as supernatant lost approximately eightly percent of the original activity. After suspending and centrifuging the gel three times in 0.05 *M* sodium phosphate (pH 6.8), ninety percent of the absorbed activity was recovered. The protein was precipitated by adding an equal volume of saturated ammonium sulfate solution, centrifuged, and again dissolved in 2 ml of buffer. The enzyme was applied by means of a sample loop to a 2.5 × 45 cm *Pharmacia* column which was assembled with two flow adaptors for upward flow chromatography (91). A flow rate of 8 ml hour^{-1} through the Bio-gel P-300 (92) from a pressure head of 10 cm eluted the transferase activity from the column in 1.2 ml fractions as shown in the elution diagram in Figure 5. The starch synthetase active fractions were pooled and concentrated by ammonium sulfate precipitation.

Table 4b shows the purification obtained by the fractionation steps described above. Unless otherwise stated, the enzyme was used in this state of purity for studying the reaction of the maltodextrins with starch synthetase.

Table 4b. Purification of starch synthetase from *Chlorella*

fraction	total ml	total units	total* mg protein	units mg	% recovery
102,000 × g supernatant	100	5880	---	---	---
25-40% $(NH_4)_2SO_4$ ppt.	17	4030	71	57	68.7
$Ca_3(PO_4)_2$ gel eluate	2	333	2.4	135	5.7
Bio-Gel P-300 eluate	1	61.6	0.22	285	1.0

*Protein was determined against a standard curve of bovine serum albumin (Grade A, Calbiochem, Los Angeles, California) according to the Folin-Lowry procedure (93).

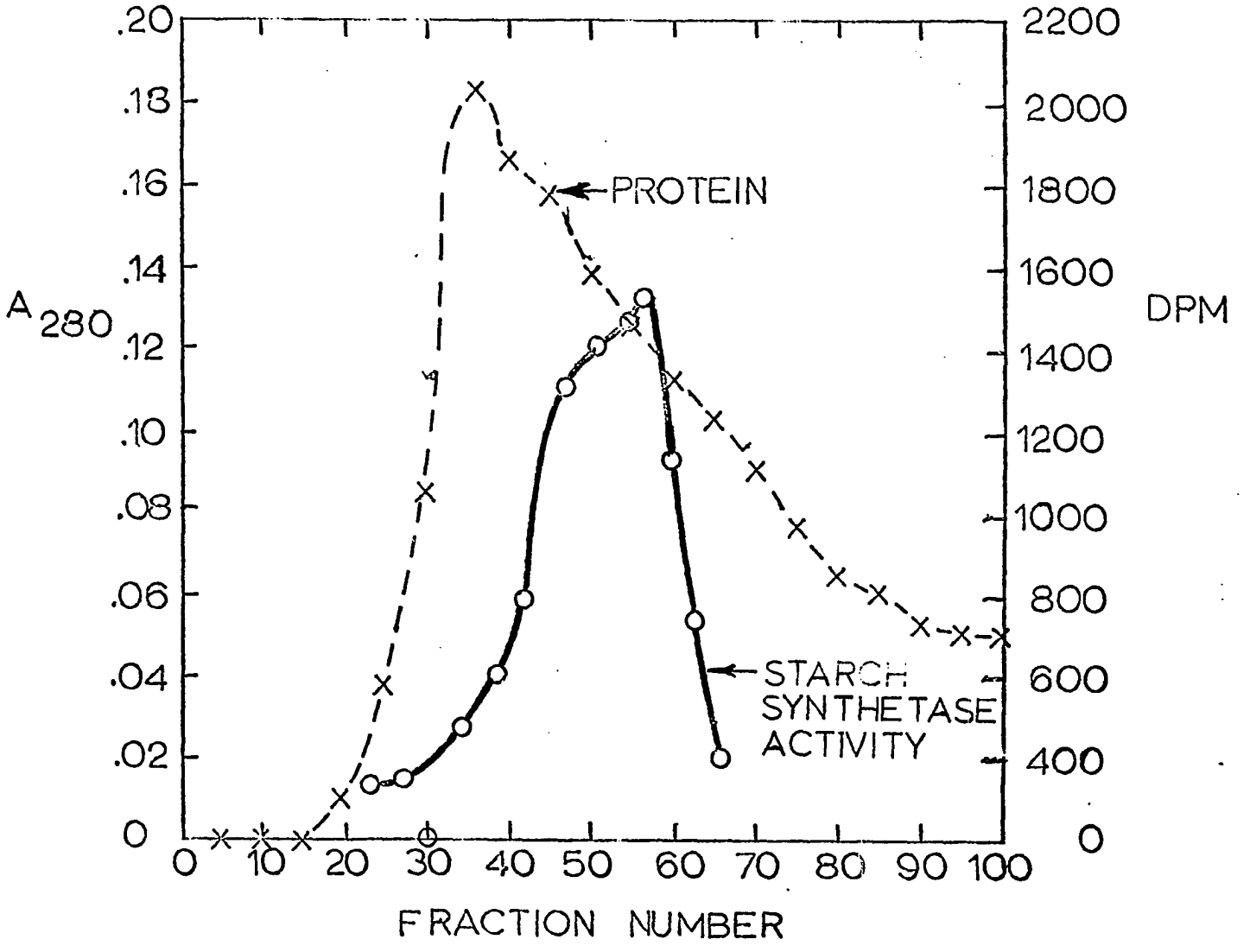


Figure 5. Elution diagram of *Chlorella* proteins and starch synthetase activity from Bio-gel P-300 column Each fraction contained 12. ml

When stored at 4°C with thymol as preservative, preparations were active for four to five weeks with loss of only 50% of their original activity. Kornfeld (94) reported that freezing a 10% saturated ammonium sulfate solution of rabbit muscle UDPG-glycogen transglucosylase aided the purification of that enzyme. The enzyme could be stored in this way indefinitely without loss of activity. However, freezing under such conditions destroyed the activity of the *Chlorella* starch synthetase. It was noted that room temperatures, sustained high pH, and the absence of reducing agents also inactivated the enzyme.

The starch synthetase preparations contained amylolytic activity. Data in Table 5 compares the amylase activities of enzyme fractions with 1% soluble starch in two buffers--one optimal for amylase activity and the other for starch synthetase activity. The change in reducing values of aliquots of the digests after sixteen hours indicated that the conditions of the standard assay eliminated the amylase activity. However, in digests of purified starch synthetase preparations with radioactive maltodextrins in the pH 9.0 buffer system, hydrolysis products from the substrate were noted.

Table 5. Amylase activity in starch synthetase fractions

fraction	0.2 *M* sodium glycerol phosphate + 0.02 *M* calcium acetate (pH 6.9)	starch synthetase reaction buffer (pH 9.0)
blank	40 µg G_2	35 µg G_2
$(NH_4)_2$ SO_4 fraction	700	98
30 ml fraction (Figure 5)	40	30
60 ml fraction (Figure 5)	182	30

IV. EXPERIMENTS AND RESULTS

A. Distribution of Maltodextrins in Green Algae

The maltodextrins of photosynthetic tissue, which were first identified as such by French (32), were from photosynthesis experiments conducted at Berkeley on the alga, *Chlorella pyrenoidosa*. This work has been reviewed in detail in the review of literature. Subsequent work in this laboratory has identified the maltodextrin series in radioactive extracts of *Scenedesmus obliquus*, an alga of the same order as *Chlorella* (Chlorophyta).

After feeding C-14 sodium bicarbonate to *Scenedesmus* during photosynthesis, the following fractionation of the radioactive extract was conducted. The 80% methanol solution was centrifuged and decanted, and the residue was refluxed 20 minutes in 40 ml of 20% aqueous methanol. After centrifugation and decantation, the material was suspended in 10 ml of distilled water and autoclaved for 15 minutes. This fraction was designated H_2O A. After centrifugation, the residue (0.2 ml wet packed volume) was dried and counted. The 80% methanol fraction was reduced under diminished pressure to dryness and extracted three successive times with acetone followed by 25 ml of 80% methanol (designated 80%). The 20% methanol fraction was reduced to 20 ml and the green lipophyllic residue resulting from concentration was redissolved in two successive washings of acetone followed by 10 ml of 20% methanol. This fraction (20%) was then passed through a column of carbonated MB-3 mixed bed ion-exchange resin at 1 drop/5 seconds and washed with 60 ml of 20% methanol at 1 drop/2 seconds. The deionized 20% methanol extract was designated 20% D.

Aliquots of these fractions were counted on copper planchets in Q-gas (1.3% butane, 98.7% helium, Matheson, Inc., Joliet, Illinois) with a windowless gas-flow detector in a Nuclear-Chicago Model D-47 sample changer coupled with a Nuclear Chicago Model 161A Sealing Unit (Nuclear Chicago Corp., Des Plaines, Illinois.)

Table 6 shows the distribution of radioactivity in the extraction fractionation. Of the 105 microcuries of sodium bicarbonate which were injected into the suspension, 10.4 microcuries were utilized in the 16 minutes of photosynthesis. Only 17.5% of this activity (1.8 microcuries) was found in neutral soluble compounds (20% D + H_2O A).

Table 6. Extraction of radioactivity from *Scenedesmus obliquus* following photosynthesis in $^{14}CO_2$

Extract	Quantity counted	cpm in fraction	Total dpm 10^{-5}	Percent of total dpm
80%	0.75/75 ml	7,550	151.0	65.4
20%	0.4/40 ml	2,420	48.4	21.0
20% D	1.0/100 ml	1,750	35.0*	15.1*
H_2O A	0.1/10.2 ml	270	5.5	2.4
residue	total	130,000	26.0	11.3
*not included in total			230.9	100.1

Chromatography and radioautography revealed that both the H_2O A and the 20% D extracts contained maltodextrins from maltose to maltodecaose. The spots corresponding to maltodextrins were cut from the chromatogram and the radioactivity in the papers was counted in the toluene scintillation solution. The data in Table 7 show that only 2.0-2.5% of the radioactivity (0.36 microcurie) appeared in the chromatographically resolved

maltodextrins (G_9 and smaller). The remainder of the activity was either non-resolved (G_9 and larger) or stayed at the origin. After 16 minutes of photosynthesis, maltose contained the lowest level of activity. Maltotriose and maltotetraose contained approximately twice the activity of the other maltodextrins.

Table 7. Distribution of maltodextrins from *Scenedesmus obliquus* following photosynthesis in $^{14}CO_2$

	H_2O A extract		20% D extract	
	cpm	% of total	cpm	% of total
G_2	171	0.9	1076	1.0
G_3	534	2.7	2529	3.4
G_4	633	3.2	4331	4.1
G_5	174	0.9	1871	1.8
G_6	146	0.7	1569	1.5
G_7	222	1.1	2670	2.5
G_8	275	1.4	2227	2.1
G_9	251	1.3	2804	2.7
G_{10} & up	802	4.1	10,550	10.1
Origin	16,408	83.6	67,600	64.6

Glucose was not seen in either extract, but sucrose and fructose were apparent in the 20% methanol extract. Also appearing in the 20% methanol extract were compounds with R_f values between those of maltotetraose and maltopentaose, and between maltopentaose and maltohexaose. These two compounds were thought to be α-1,4-glucose oligosaccharides with α-1,6 branches, similar to those resulting from the hydrolysis of amylopectin or glycogen.

B. Carbohydrases of Green Algae

1. Amylolytic activity

In the purification of starch synthetase from *Chlorella* as described in Materials and Methods, contamination by amylase activity was always noted. Analysis of the products of amylase activity in the initial fractions of synthetase purification showed extensive release of maltose, maltotriose, and maltotetraose after incubation with 1% waxy maize starch. The change in the reducing value of digests incubated with initial fractions from two preparations of starch synthetase are given in Table 8.

Table 8. Amylase activity in enzyme fractions of *Chlorella*: Increase in reducing value as micrograms of apparent maltose

fraction	0 hours	7 hours	19 hours
A: Calcium phosphate gel supernatant from 5 day old preparation	89	101	119
B: Ammonium sulfate precipitate	89	117	330
C: Calcium phosphate gel supernatant	89	110	236

Also, a chromatogram on which was spotted with the deionized digests of the fractions tested in Table 8 after forty-two hours of digestion. The chromatographic analysis showed no hydrolysis products from the 5 day old preparation (sample A, Table 8). However, the activity which was seen in both B and C of Table 8 was revealed chromatographically with G_2, G_3, and G_4 as the preponderant products; G_3 and G_4 were darker than G_2. The activity of the enzymes producing this distribution of products may be responsible for the identical distribution of radioactivity following

photosynthesis in $^{14}CO_2$.

Upon further purification of the enzymes, however, such distribution of products could not be produced upon incubation with waxy maize starch. The enzymes of algae were carried through the steps of ammonium sulfate precipitation and chromatography on Bio-gel P-300 (Bio-Rad Laboratories, Richmond, California) as described in Materials and Methods. Incubation of this enzyme fraction with 0.5% waxy maize starch resulted in increasing reducing value with time as seen in Table 9. At the end of 72 hours of

Table 9. Amylase activity in purified enzyme fraction of *Chlorella*: Increase in reducing value expressed as micrograms apparent maltose

Hours	µg G_2
0	4.5
0.25	7
1.5	7
11	10
48	43
72	87

digestion, the digest was deionized with Amberlite MB-3. A chromatogram showed large quantities of glucose plus a spot with mobility slightly greater than G_3, which may have been panose. Small amounts of maltose, isomaltose, and maltotriose were also observed. The activity was clearly not the same as that demonstrated in more impure fractions of starch synthetase.

2. Reactions of maltodextrins with starch synthetase

The digests for studying the reaction properties of the maltodextrins

contained 3 units of starch synthetase activity, 3 micromoles of ADPG (dipotassium·4 H_2O salt, B grade, Calbiochem, Los Angeles, California), 100 micromoles of glycine (pH 9.0), 20 micromoles of ethylene diamine tetraacetic acid (EDTA), 4 micromoles of potassium chloride, and 1 micromole of dithiothreitol in a total of 0.5 milliliter. Aliquots of 0.1 milliliter were withdrawn at five time intervals and were placed immediately in 45-50 mg of Amberlite MB-3 beads in 10 × 75 millimeter test tubes which were kept in an ice bath. Within 1-3 hours, the deionized solutions were spotted on Whatman No. 1 chromatography paper, and after chromatography, the paper was cut into strips--each strip containing the separated maltodextrins from one sample. The strips were analyzed on the Packard Chromatogram Scanner. When the positions of the radioactive area were located, these were cut from the strips and counted on the paper in toluene scintillation solution.

The substrates for these studies were maltodextrins prepared from an *Aspergillus oryzae* α-amylase digest with recrystallized amylose ("Superlose", Stein-Hall Mgf. Co.). The digest was chromatographed on a charcoal-celite column (4), and the individual maltodextrins were purified by ascending chromatography on Whatman No. 17 chromatography paper. Radioactive maltodextrins were purified by ascending chromatography from radioactive soybean leaf extracts. These leaves underwent photosynthesis in $^{14}CO_2$ was for several hours, and so the maltodextrins were presumably uniformily labelled. After combining the cold and radioactive maltodextrins, concentrations of these solutions were determined by the phenol-sulfuric acid method (86, 87).

When maltotriose and maltotetraose were incubated with starch

synthetase, the results in Table 10 were obtained. The loss of substrate was linear with time for both G_3 and G_4, but the increase in transferase products was non-linear over the long incubation period. The accumulation of maltose in both digests revealed the amylase contamination in the enzyme preparation. Hydrolysis of the substrates and products may have accounted for non-linearity of product curves as well as the fact that the products were also being used as substrates for starch synthetase. Multiple products were formed more noticeably with G_3 than G_4 as substrate. Maltopentaose was the predominant product at 450 minutes from both G_3 and G_4 digests. Glucose was not detected in either digest by the radiochromatogram scanner.

Incubation of maltotriose, maltotetraose, maltopentaose, and maltohexaose with 1.5 units of starch synthetase for only 15 minutes gave only the maltodextrin of one more glucose unit as product. No hydrolysis products were noted. In the five samples withdrawn in the 15 minutes period, the percent of product for each digest increased with time but the data was scattered and so linear initial rates could not be deduced for comparison of the relative rates of reaction of G_3, G_4, G_5 and G_6.

Maltose was found to be ineffective as a primer for *Chlorella* starch synthetase. In two experiments pure radioactive maltose (0.05 *M*) was incubated with 6.0 and 13.3 units of starch synthetase for 450 and 220 minutes respectively. No reaction at all was detected by chromatography of the digest mixtures. This result differs from reports of starch synthetase activity toward maltose in spinach chloroplasts (12) and corn endosperm (33). Perhaps there are differences in specificity of the enzymes from these sources, but contamination of maltose by maltotriose,

Table 10. Products of action of starch synthetase on radioactive maltotriose and maltotetraose

minutes	10	30	60	90	450
substrate: 0.0023 *M* G_3					
G_2	0.4	0.8	1.1	3.1	3.8
G_3	95.8	92.9	88.6	78.1	60.6
G_4	2.6	4.4	6.5	8.5	11.5
G_5	1.9	1.8	3.4	3.3	18.4
G_6	0	0	0.4	0.5	2.6
substrate: 0.0053 *M* G_4					
G_2	0	0.2	0.6	0.9	2.7
G_3	0	trace	trace	trace	trace
G_4	98.3	95.0	89.1	89.3	73.1
G_5	1.8	3.7	8.8	8.7	23.5
G_6	0	0	trace	trace	trace

which can serve as primer for starch synthetase, would also give maltose apparent priming ability. The pure maltose used in the experiments described here was isolated from β-amylase digestion of a commercial cross-linked dextrin, and therefore did not contain other oligosaccharides.

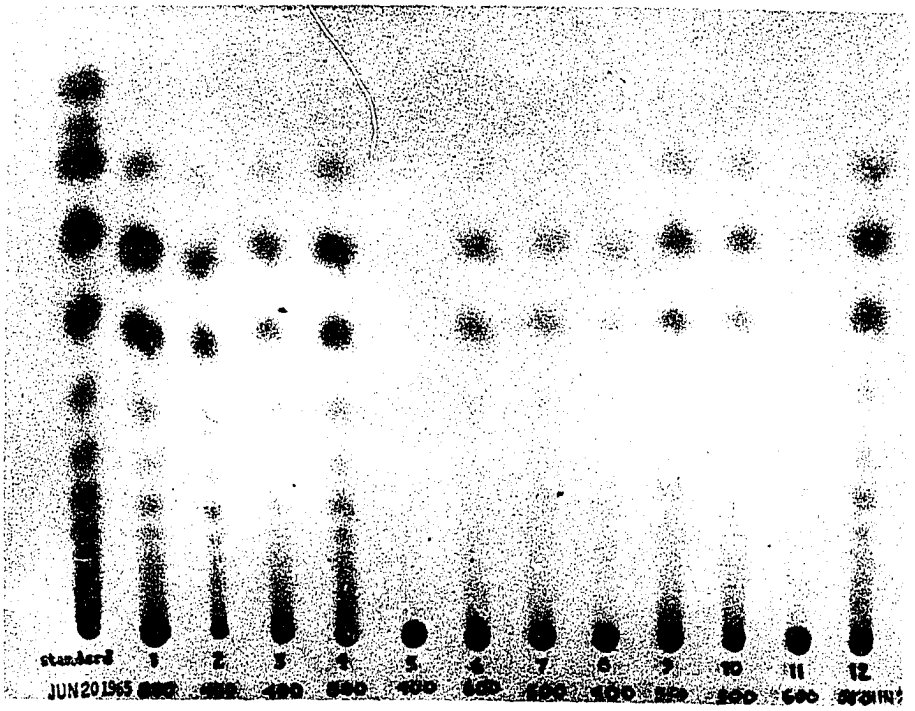
The purification of starch synthetase was initiated to determine by kinetic analysis the rates of reaction and the Michaelis constants of the maltodextrins. It was hoped these parameters would help to understand the discontinuity in the distribution of radioactivity between G_4 and G_5 that was seen following photosynthesis in $^{14}CO_2$. However, the technical difficulties of assay and the amylase contaminant in the starch synthetase preparation plagued the study and so kinetics were not begun. This point remains open except for the evidence of the discontinuity in reaction of granular synthetase with maltodextrins as has been discussed earlier.

C. Distribution of Maltodextrins in Soybean Leaf Tissue

The observation of maltodextrins in extracts of soybean leaf tissue has been discussed in the review of literature, and a graph of the maltodextrin and starch labelling kinetics from soybean leaves has been presented in Figure 3. For Figure 3, Fisher (37) conducted pulse labelling for 1 minute and took ten samples during the ensuing 16 minutes. In the experiment to be discussed here, samples were taken at each of the intervals of 11, 25, 50, 75, and 125 minutes after a three-minute pulse exposure to $^{14}CO_2$. The samples were the trifoliate leaf from a separate plant, *i.e.* six soybean plants, each with one trifoliate, were fed all at once. Hence, observations about the metabolism of the maltodextrins from this experiment are of doubtful significance. In preparation for chromatography, the samples were extracted three times in boiling 80% aqueous alcohol. The leaves were ground in a mortar with a few grams of acid-washed sea sand, and the resulting powder was dried. Duplicate samples of approximately 20 mg were shaken with 2.0 ml of water at 40°C for six hours. The residue material in each tube was then centrifuged and washed with approximately 1 ml of water three times. A small portion of each of the combined aqueous extracts was spotted on Whatman No. 1 paper (Figure 6). The remainders of the extracts were hydrolyzed with salivary α-amylase for 18 hours at 40°C, and aliquots of each digest were deionized and also spotted on Whatman No. 1 paper (Figure 7). The chromatograms in Figure 6 and 7 were obtained after ascending chromatography and radioautography for 6 days. Extracts 1 and 2 are duplicate samples from the plant killed at eleven minutes after the pulse of $^{14}CO_2$; 3 and 4 are duplicates of the 25 minute sample, and so on. Approximately

Figure 6. Water extracts of soybean leaf following photosynthesis in $^{14}CO_2$

Figure 7. Salivary α-amylase digests of water extracts of soybean leaf following photosynthesis in $^{14}CO_2$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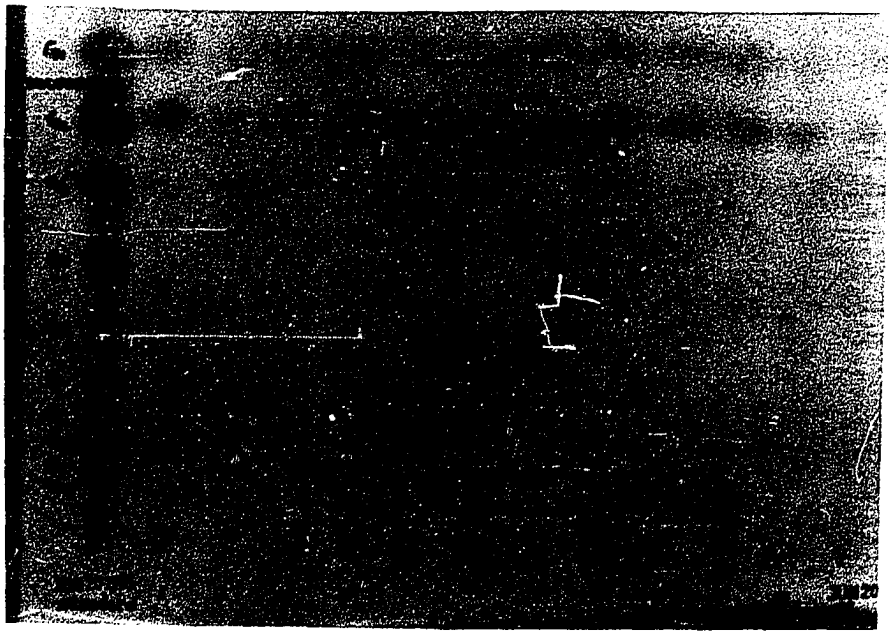


equal amounts of radioactivity in each sample were spotted.

It appears from Figure 6 that supposedly identical extraction techniques did not remove the same compounds from the sample--for example compare 5 with 6 and 11 with 12. This may be a consequence, however, of preparation of the samples for chromatography. It has been observed that in concentration of samples for chromatography, reduction of volume to dryness has seemingly polymerized the maltodextrins. If samples 5 and 11 were taken to dryness, it was not noted, however.

Figure 7 shows the radioautogram of the salivary α-amylase digests of the extracts. Essentially all of the material, including that too large to move from the origins of Figure 6, was converted to glucose and maltose as a result of the extensive hydrolysis. Exception to this are small amounts of branched dextrins, probably B_4, B_5, and B_6. These branched dextrins from soybean leaves have been noted as well by Fisher (37).

It is seen in Figure 6 that maltotriose and maltotetraose appeared to contain more activity than the other members of the maltodextrin series throughout the sampling period. This result may be compared with the distribution of radioactive algal maltodextrins. It also appears that the level of activity among the higher homologs, that is from G_5 up, is greater in samples 1-4 than in later samples. This observation was verified when the radioactive spots were cut from the chromatogram and counted on paper. This data is plotted in Figure 8 as time versus percent of the total radioactivity which was found in each maltodextrin spot after chromatography.

Figure 8 shows the curves for G_2, G_5, G_6, G_7, and G_8.

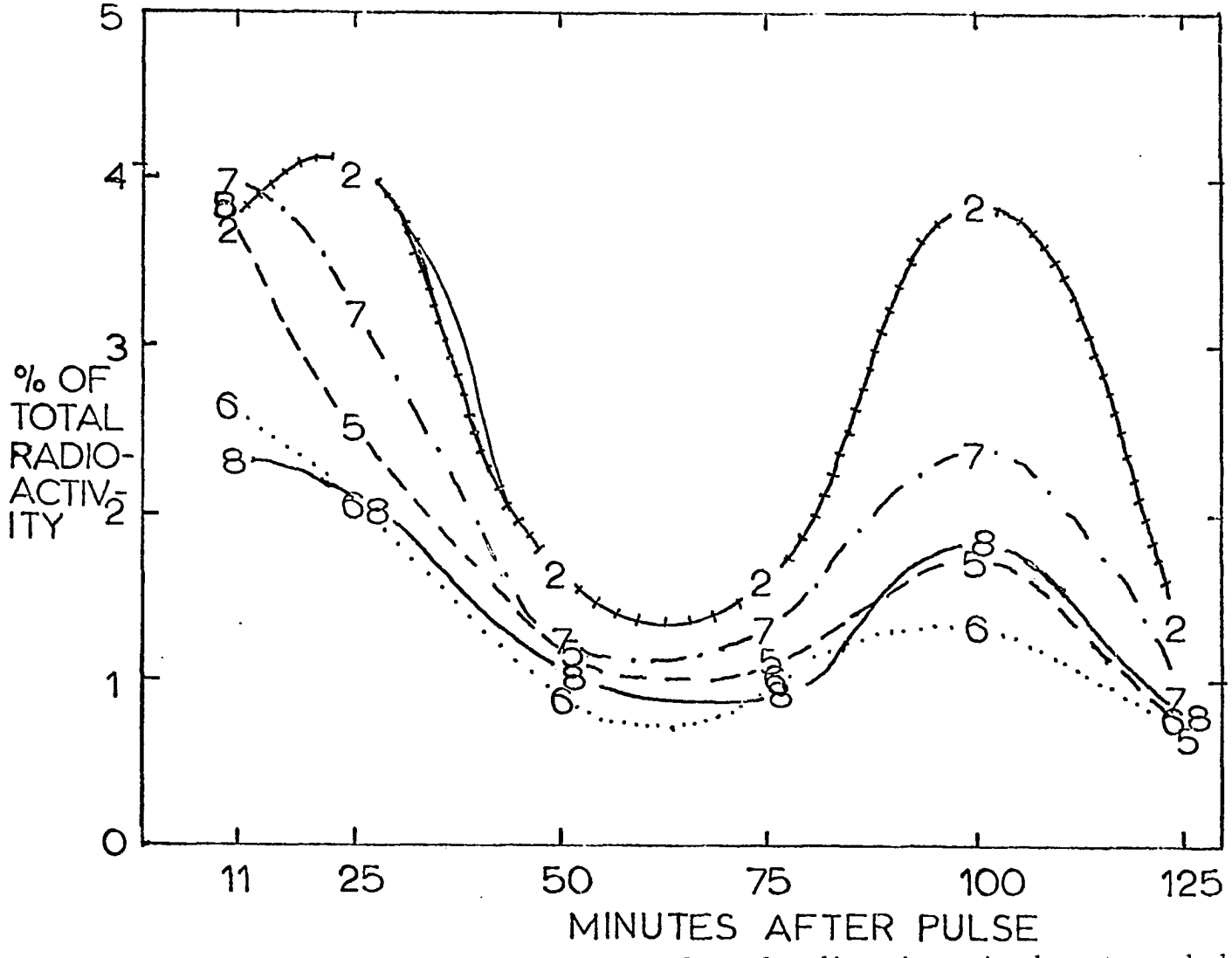


Figure 8. Activities of G_2, G_5, G_6, G_7, G_8: Percent of total radioactivity in chromatographed aliquot of sample The numerals indicate experimental points

indeed a decrease in the percent of activity in maltose and the higher maltodextrins with time. The increase in radioactivity of all these curves at 100 minutes is likely a "sampling" error due to the nature of metabolism of that plant. There is nothing to say that the 11- and 25-minute plants were not also of the same nature, but the point to make is the comparison of these curves with those in Figure 9. The labelling kinetics of G_3 and G_4 appear to be distinct from maltose, the higher homologs, and those compounds which did not move from the origin of the chromatogram. The curves of G_3 and G_4 are identical to each other and their activities are at least 5 times that of the higher homologs at all points during the sampling. These results suggest that, at least in part, G_3 and G_4 are metabolically independent of the other members of the maltodextrin family.

Figure 9 also shows the percent of radioactivity in the sample aliquots which upon chromatography did not move from the origin. This fraction would include starch-like oligosaccharides containing perhaps 15 or more glucose units. It is interesting that these oligosaccharides are metabolically active. At 125 minutes, a portion of the oligosaccharide chains which were labelled in the pulse of radioactivity to the plants is metabolized. The "mirror-image" type curves of the G_3-G_4 activity and of the origin activity may be significant in that these maltodextrins may be products of the hydrolysis of these large oligosaccharides which did not move from the origin. The metabolic source of G_3 and G_4 in soybean plants may be very different from the metabolic source of G_2 and higher maltodextrins.

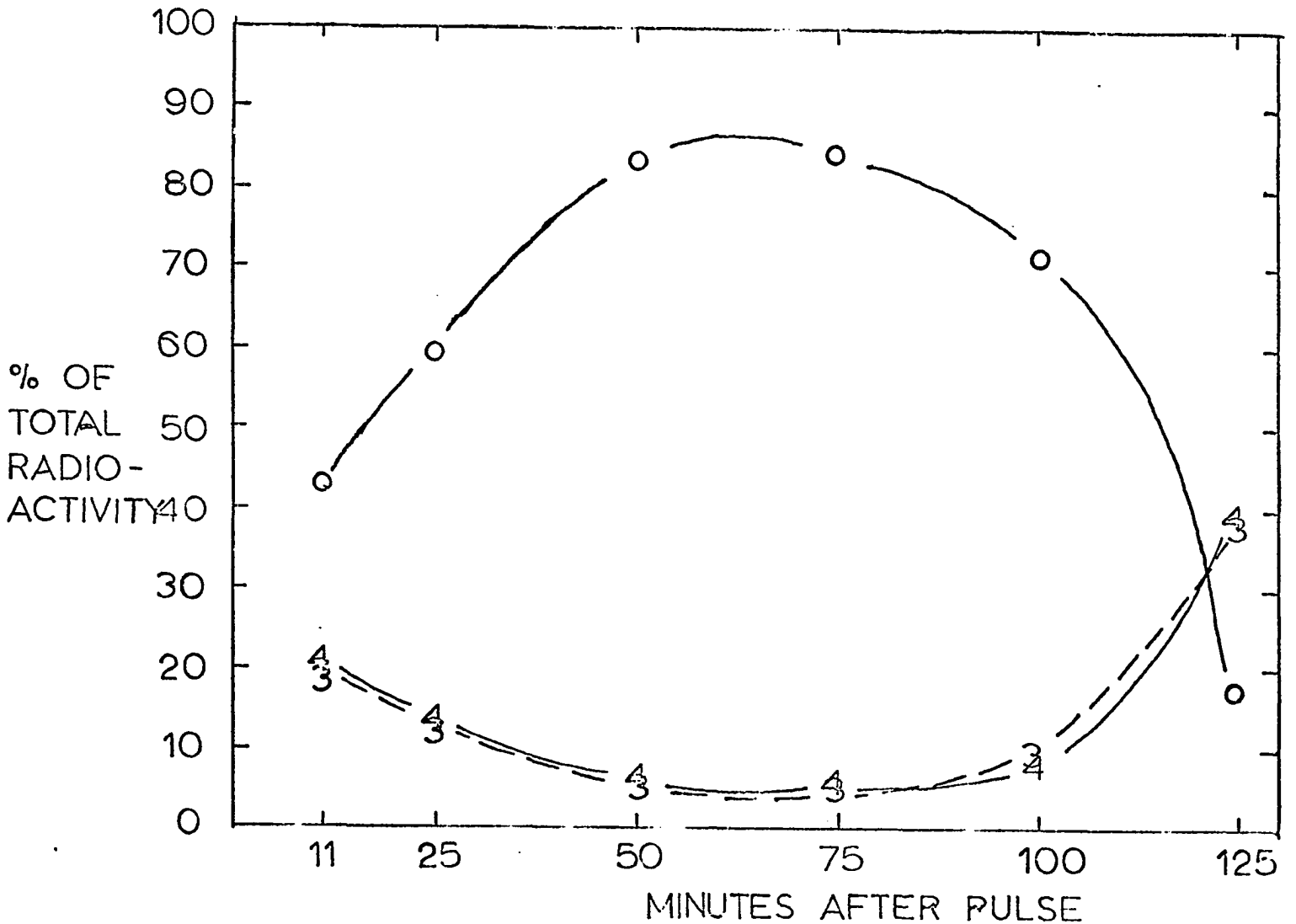


Figure 9. Activities of G_3 (3), G_4 (4) and of the material which did not move from the origin (0): Percent of total radioactivity in chromatographed aliquot of sample. The numerals indicate experimental points.

D. Carbohydrases of Soybean Leaf Tissue

The fact that maltodextrins were always found in soybean leaf tissue where the total activity in G_3 and G_4 after photosynthesis in $^{14}CO_2$ was greater than the activity in homologs larger than G_4, and the fact that the labelling kinetics of G_3 and G_4 was different from that in larger homologs, led to the thought that G_3 and G_4 might be hydrolysis products of leaf starch. Consequently, a series of experiments were conducted with the enzymes of soybean leaves.

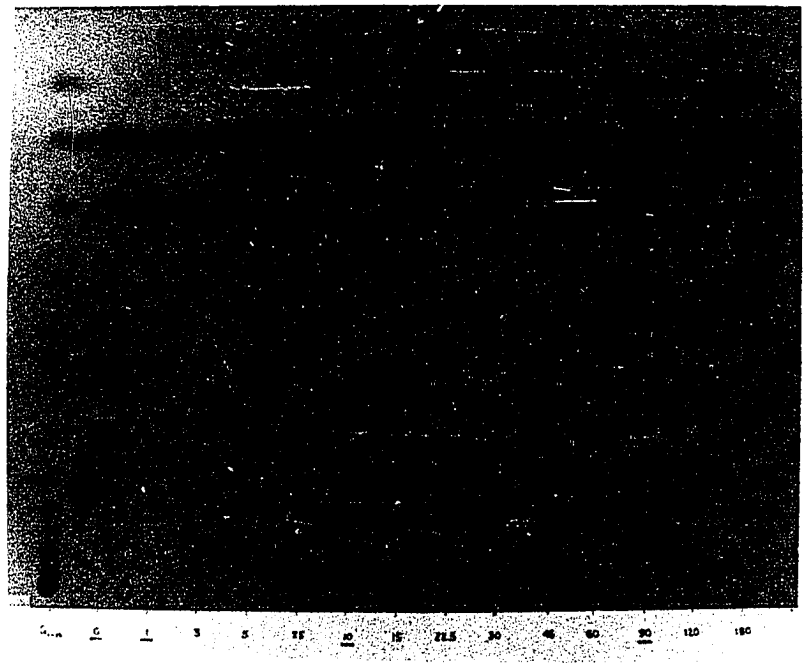
In the first of these, the youngest mature soybean trifoliate leaf from a plant grown 28 days was homogenized in a Waring blender in 50 ml of distilled water. Ten ml of the green, glass wool-filtered homogenate was incubated with 4.5 mg of radioactive maltoheptaose in a volume of 15 ml. Samples of 1 ml were withdrawn at times up to three hours and boiled in 80% ethanol. The alcoholic solutions were evaporated to approximately 0.3 ml, extracted with Skelly F, and the clear solutions were spotted for chromatography.

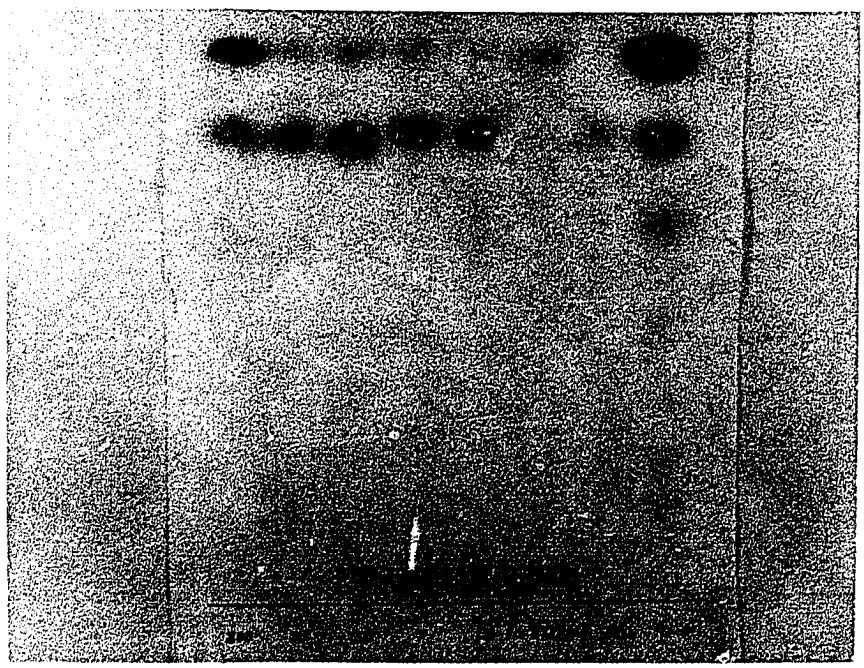
After radioautography for two weeks, the picture in Figure 10 resulted. Part of the samples taken at 0, 1, 10, and 90 minutes were lost and have been underlined in Figure 10 for that reason. The radioautogram clearly showed amylase activity yielding only maltose and maltotriose with glucose increasing with time. The zero and one minute samples showed G_7, G_5, G_3 and G_2 more definitely than G_6, G_4, and G_1 which certainly is an indication of β-amylase activity, although an α-amylase would give somewhat similar results.

Two spots which were resistant to the action of the leaf enzymes

Figure 10. Incubation of soybean leaf milieu with radioactive maltoheptaose

Figure 11. Incubation of soybean leaf milieu with pure maltose


0
1
3
5
15
30
90
120
180



remained in the vicinity of, but not identical with, maltoheptaose. These spots were cut out and eluted from the paper with water. One-half of the eluate was incubated with 200 ml of crude salivary α-amylase in 0.02 sodium glycerol phosphate at pH 6.8, and the other half with 225 ml of Worthington β-amylase (Worthington Biochemical Corporation, Freehold, New Jersey) at pH 4.8 in 0.2 *M* acetate buffer. These quantities of enzymes and the incubation periods at 40°C of one hour for the α-amylase and 12 hours for the β-amylase brought constant reducing values to 1% soluble starch solutions incubated under the same conditions. The digests were deionized and spotted on Whatman No. 1 paper for 3 ascents in the 6:4:4 butanol:pyridine:water solvent. After radioautography it was apparent that β-amylase hydrolysis of the resistant compounds was slight as only a trace of glucose and maltose were observed. The α-amylase digestion produced glucose, maltose, and maltotriose along with compounds having mobilities similar to G_4, G_5 and G_6. These latter three compounds and one of the resistant spots were probably branched oligosaccharides. The other resistant spot did not chromatograph as a discreet spot as the other oligosaccharide did, but spread a bit more. Possibly it was not an oligosaccharide. The activity of neither spot decreased appreciably in the hydrolysis by either enzyme. The compound having mobility slightly behind G_4 contained the highest amount of activity. Since the branched oligosaccharide was β-amylase resistant but not α-amylase resistant, the leaf enzyme which hydrolyzed G_7 was probably β-amylase.

The second experiment with enzymes of chopped soybean leaves was conducted with pure maltose as the substrate. The leaf enzyme solution was prepared as above. Ten ml of glass wool filtered extract was incuba-

ted with 50 mg of maltose in a total volume of 15 ml. Samples of 1 ml, containing 8 mg of maltose, were taken during a five hour period and boiled in 80% alcohol solutions to stop the reactions. The white flocculate was centrifuged and the supernatant was extracted with Skelly F after evaporation to less than 1 ml. The samples, a maltodextrin series standard, substrate, and a plant extract control were spotted on Whatman 3 MM paper and chromatographed twice in the pyridine solvent (3). The chromatogram, shown in Figure 11, was developed in silver nitrate-alkali dip for detection of reducing compounds (82). The results in Figure 11 show maltose activity since glucose increased and maltose decreased with time. There is a possibility that the apparent maltose activity may be due to the β-amylase hydrolysis of maltotriose which is formed by disproportionating enzymes similar to those enzymes discussed in the review of literature as being like the D-enzyme of potato. The reaction catalyzed by the hypothetical enzyme would be

$$\text{O-}\phi + \text{O-}\phi \rightleftharpoons \text{O-O-}\phi + \phi \qquad 10$$

In a third experiment, chloroplasts were isolated from soybean leaves following a procedure of Ghosh and Preiss (12) for isolation of spinach chloroplasts. The chloroplasts were homogenized for fifteen seconds in a solution containing 0.5 *M* sucrose, 0.1 *M* phosphate buffer (pH 7.4), 0.01 *M* EDTA, and 0.005 *M* glutathione. Following filtration through four layers of cheese cloth and centrifugation at 100 × g for one minute, the supernatant was centrifuged at 1000 × g for ten minutes to yield the pellet containing chloroplasts. Under the phase contrast microscope, the yield was estimated at 80% intact chloroplasts versus 20% cell debris after two

washings and centrifugations in the homogenizing media. The pellet was then suspended in 0.02 *M* phosphate buffer at pH 6.8 to burst the limiting membrane of the chloroplasts. This preparation was incubated with 0.1% soluble starch and since the reducing values of aliquots of the digest did not change in forty-eight hours, it was concluded that no amylases were present in the chloroplasts. These chloroplast preparations did show enzymatic activity whenincubated with ADPG and oligosaccharides as estimated by the production of ADP. This experiment was not repeated, and the negative results are not conclusive, but the results from it indicate that starch in chloroplasts is mobilized by phosphorolysis rather than by amylolysis. Also indicated is compartmentalization of the amylases outside of the chloroplast and its starch synthesizing machinery.

The products of debranching of soybean leaf starch by the action of pullulanase (70) were also studied. The course of the reaction was followed by increasing reducing values as well as by increasing blue values. No reaction other than the hydrolysis of the α-1,6 linkages appeared to be involved. Chromatographic analysis showed first the production of G_1, G_2, G_6, G_7, and larger maltodextrins. G_5 appeared after 10 minutes of digestion, G_4 appeared after 25 minutes and finally G_3 appeared after 100 minutes. This work indicated that G_3 and G_4 were not products of debranching of soybean leaf starch until after the higher homologs were produced. *In vivo* debranching of soybean leaf starch is probably not responsible for the higher levels of G_3 and G_4 found following photosynthesis.

E. Maltodextrins of Spinach Chloroplasts

1. Distribution of maltodextrins during steady state photosynthesis

Spinach chloroplasts were prepared and photosynthesis was conducted as described in Materials and Methods. The chloroplast suspension containing 0.1 *M* $KHCO_3$ was exposed to light for 20 minutes before the addition of high specific activity $NaH^{14}CO_3$ to the 4 ml of suspension. Thirteen micromoles (520 microcuries) of $NaH^{14}CO_3$ in 0.2 ml were added. Presumably the 20 minutes of photosynthesis before addition of label was sufficient for the maltodextrin metabolism to come to steady state, because it will be shown by the results of this experiment, that the C-14 kinetic curves of the photosynthetic compounds became linear in approximately 15 minutes after the injection of label.

In this experiment photosynthesis was allowed to continue for 45 minutes. Twenty 0.20 ml samples were taken during this period; they were taken especially rapidly during the first 20 minutes in order to capture the initial processes of maltodextrin metabolism in photosynthesis. After deionization of the samples, only that fraction of the radioactivity which was fixed in soluble, neutral compounds, remained in solution. They were chromatographed and radioautographed.

Figure 12 shows the ascending chromatography of samples taken during this experiment. The W-shaped spot cochromatographed with sucrose, and the heavy one beneath it was similarly shown to be maltose. Glucose and fructose appeared above sucrose. The compounds in the 0.72 minute sample were not identified.

The higher maltodextrins, those not resolved on the chromatogram,

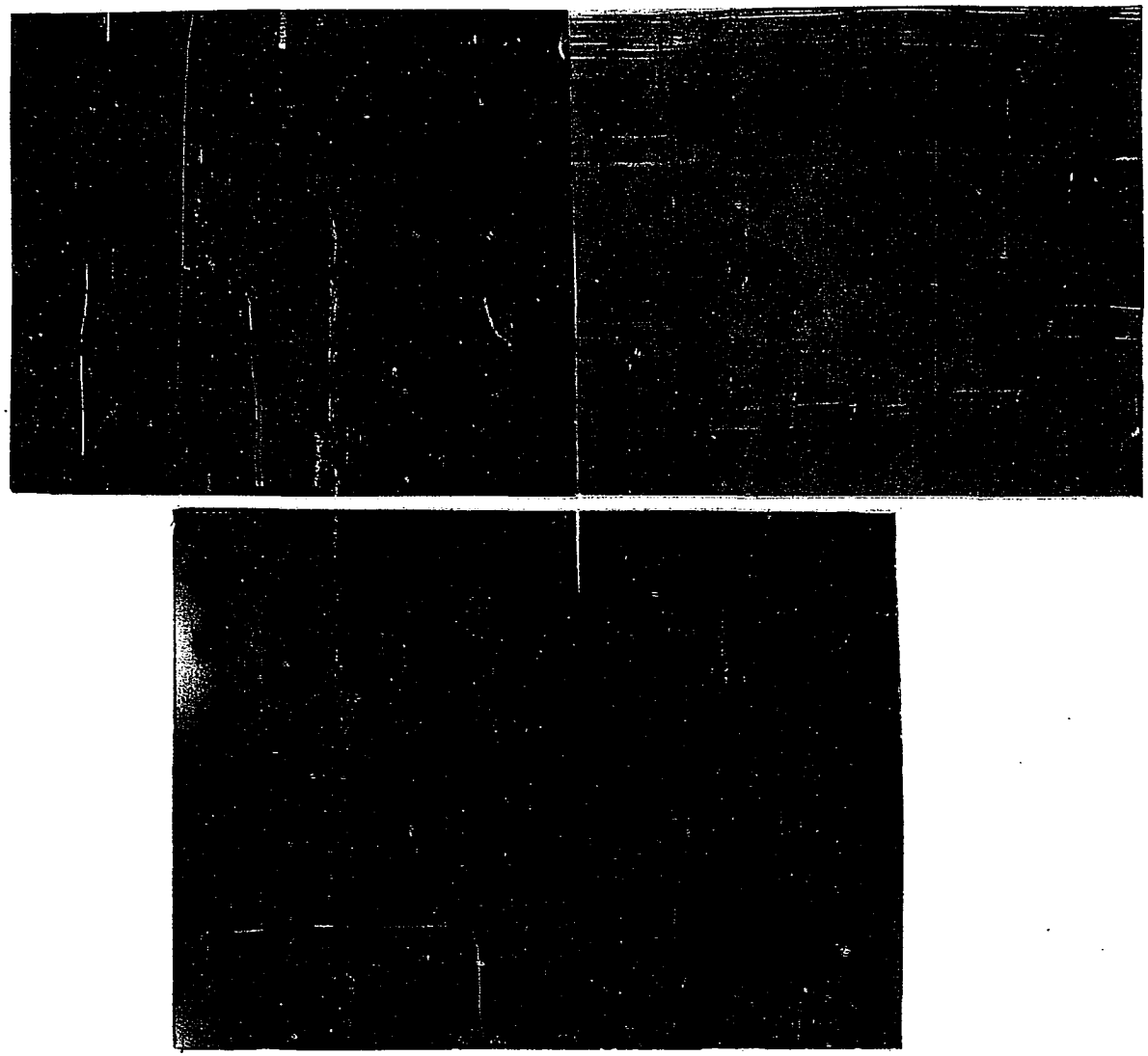


Figure 12. Chromatography of spinach chloroplast extracts taken during steady state photosynthesis

were labelled more rapidly than those which were resolved. Maltose and sucrose were labelled as rapidly as the higher maltodextrins but continued to acquire label throughout the 45 minute sampling period, whereas the level of radioactivity in the maltodextrins reached a plateau after about fifteen minutes. The patterns of labelling are clarified in Figure 13, 14, and 15 where the radioactivities in the various compounds are plotted against photosynthesis time in labelled bicarbontate. In Figure 13, the labelling curves of fructose, glucose and the non-resolved maltodextrins are compared, and in Figure 14, the patterns of sucrose and maltose are shown. All curves became linear at approximately fifteen minutes and so a steady state in carbohydrate metabolism appeared to be attained in that time. Likewise, the labelling patterns of the maltodextrins in Figure 15 appeared to be in steady state at between fifteen and twenty minutes. There was great similarity in the curves for the non-resolved maltodextrins and the the maltodextrins, G_5 through G_8. Maltotriose and maltotetraose had higher radioactivity values after fifteen minutes than the other maltodextrins, and so the metabolism of these two compounds may have been different.

2. Distribution of carbohydrates during photosynthesis with light-dark transition

The spinach purchased for this experiment was of a different variety than the spinach used in the steady state experiment. The leaves used here were relatively flat compared to the curled and crinkled leaves used previously. This experiment was conducted in the same manner except that the light were turned off at 6.75 minutes after injection of labelled bicarbonate. It was reasoned that without photosynthesis, the production

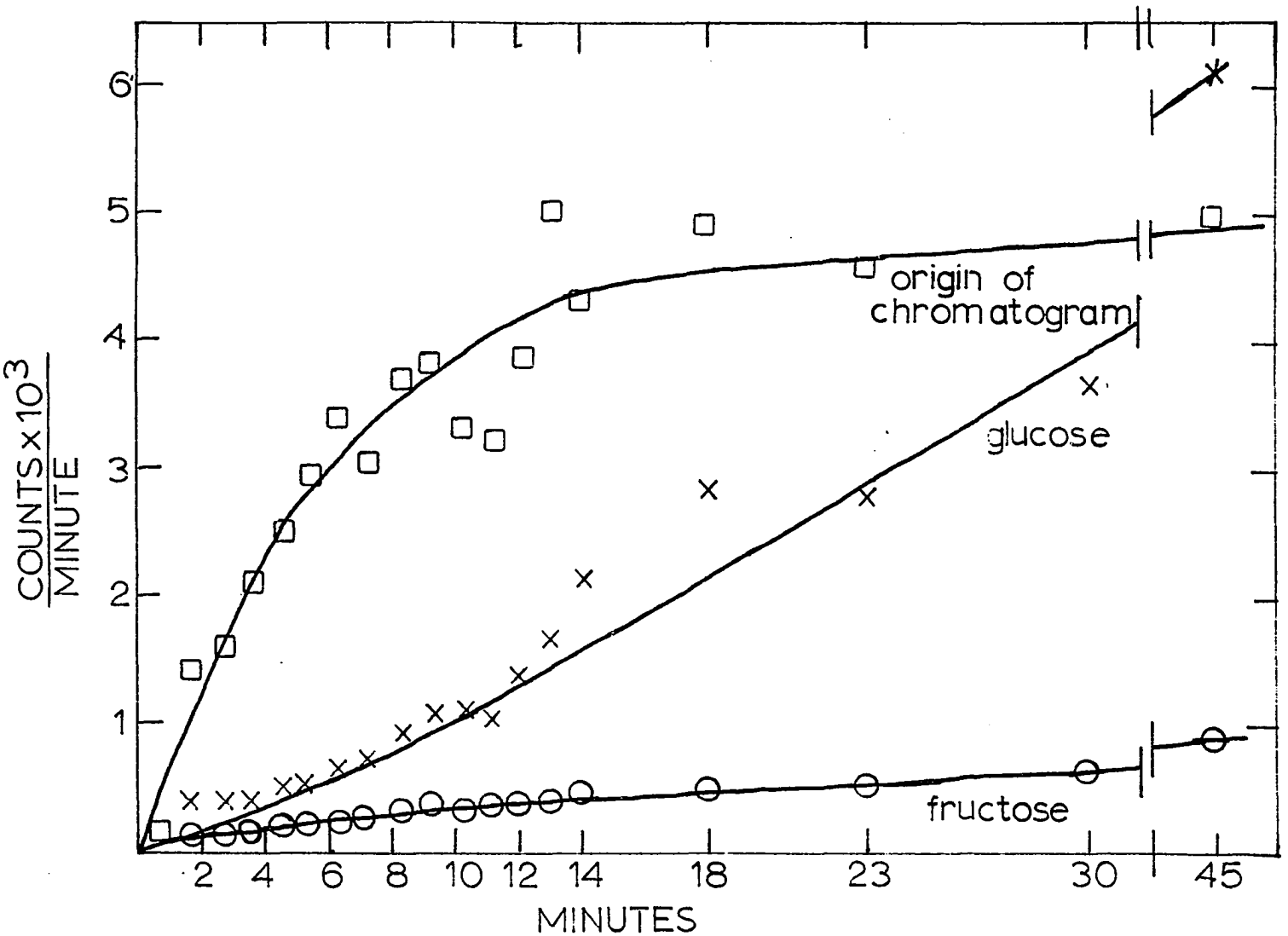


Figure 13. ^{14}C-kinetics of glucose, fructose, and unresolved maltodextrins during steady state photosynthesis

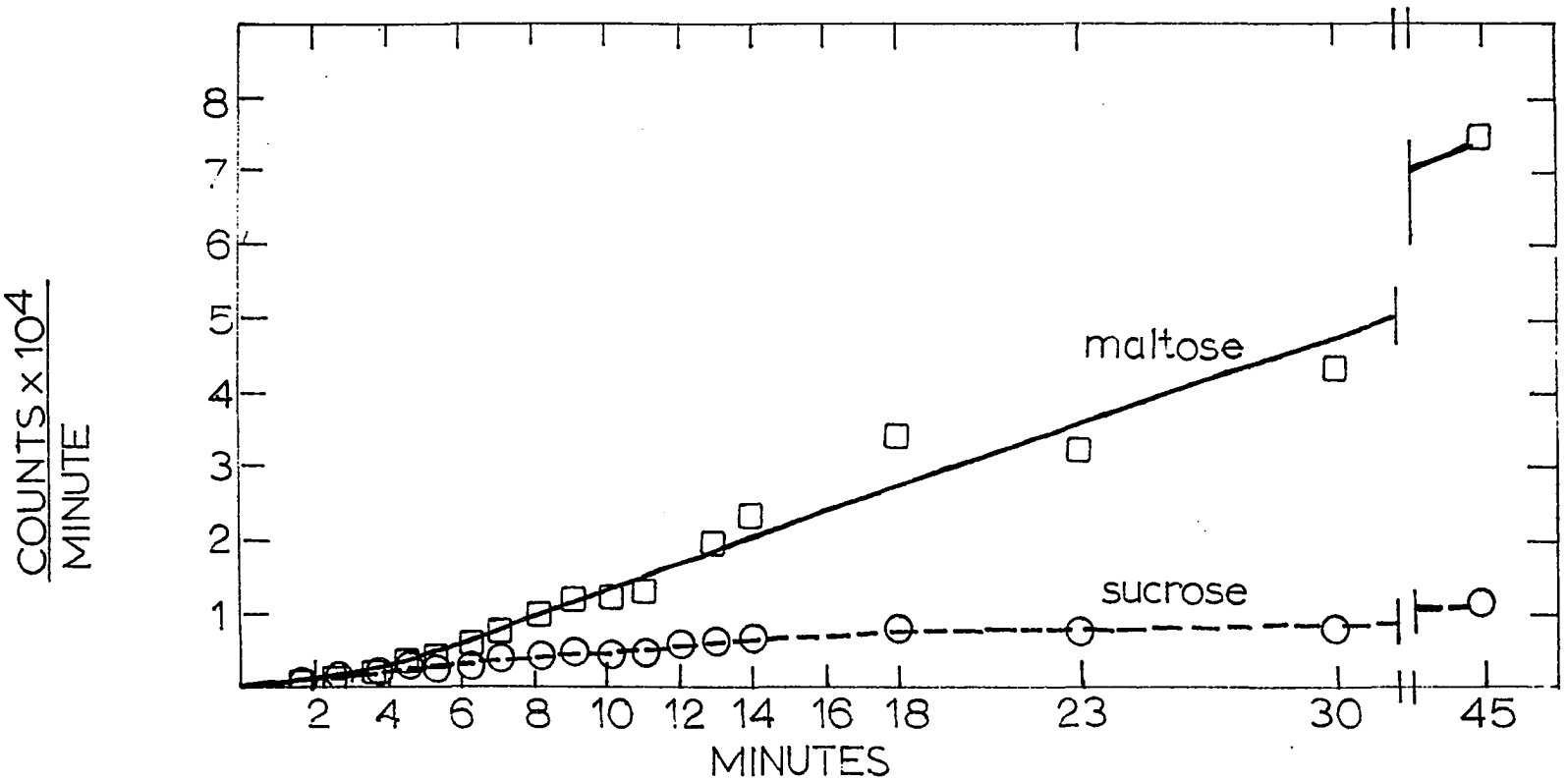


Figure 14. ^{14}C-kinetics of sucrose and maltose during steady state photosynthesis

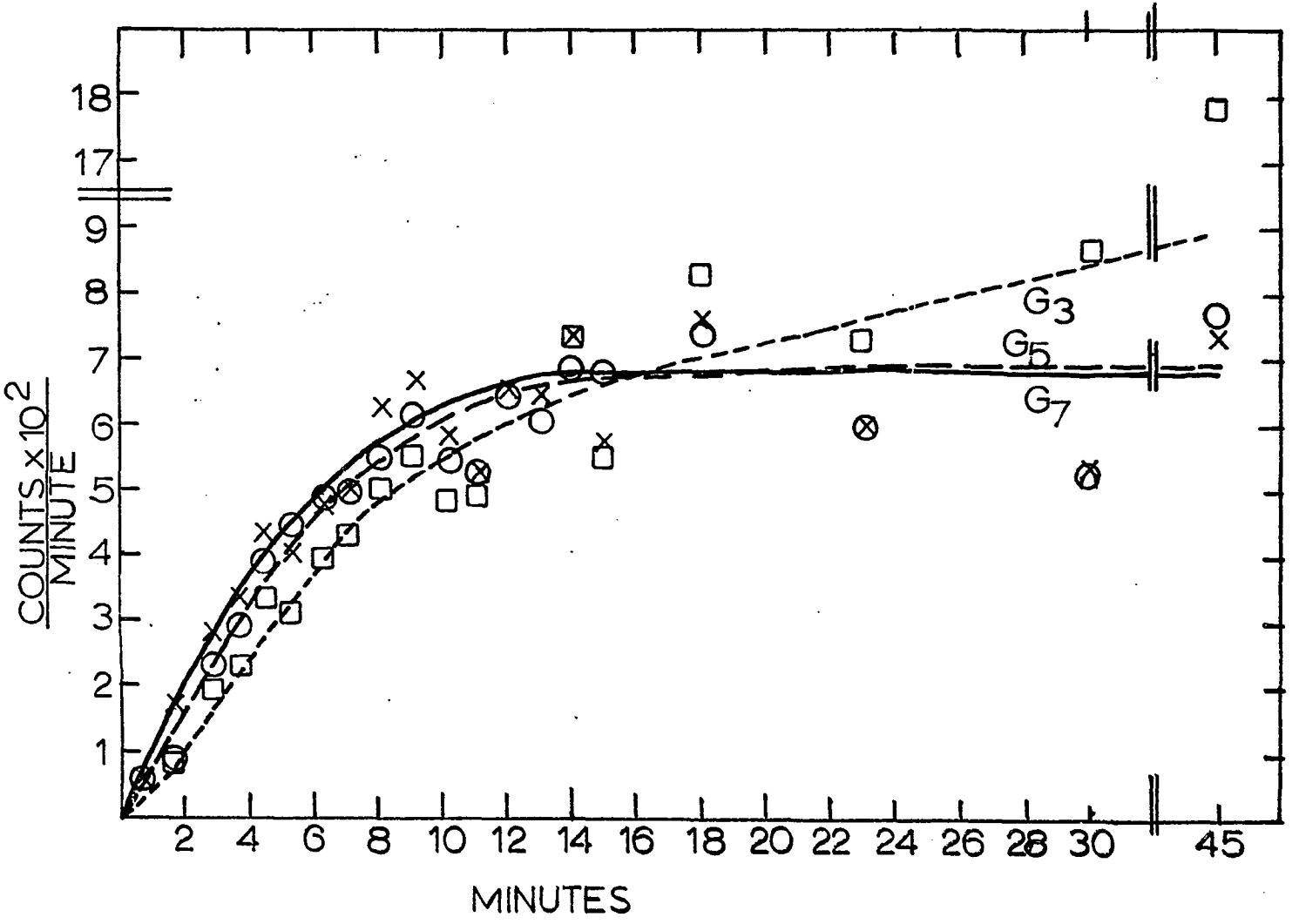


Figure 15. ^{14}C-kinetics of G_3, G_5, and G_7 during steady state photosynthesis

of ATP in the chloroplast would stop, and the synthesis of polysaccharides with nucleotide sugars would also stop because the pool of ADPG would diminish.

Thirteen 0.4 ml samples were taken for 20 minutes after addition of label; they were taken rapidly just before and after the light-dark transition. Figure 16 shows the ascending chromatography of the samples taken during this experiment. These chromatograms are similar to those shown in Figure 12 for the steady state experiment. The higher maltodextrins were labelled more rapidly than G_3, G_4, G_5 and G_6. Maltose appeared very early and continued to accumulate throughout the sampling period. These and other features of maltodextrin metabolism were common to the two species of spinach.

Figure 17 clearly shows the effect of darkness on the labelling patterns of sucrose and the higher non-resolved maltodextrins. The pattern of labelling of glucose and fructose was unaffected and Figure 18 shows the same held for maltose, however glucose-1-phosphate was affected. The cessation of photosynthesis also affected the labelling of the resolved maltodextrins, as seen in Figure 19. Counts in maltotriose (counts in maltotetraose were nearly identical), maltopentaose, maltoheptaose, and maltononaose are plotted against time. The even-numbered maltodextrins were not plotted for clarity of the graph. The patterns of G_5, G_7, and G_9 were again similar to that of the higher unresolved maltodextrins, and the pattern of G_3 (and G_4) was again somewhat different. The level of activities of each of the resolved maltodextrins was approximately equal during the 6.75 minutes of photosynthesis. Darkness immediately decreased the activity in all of these maltodextrins. After

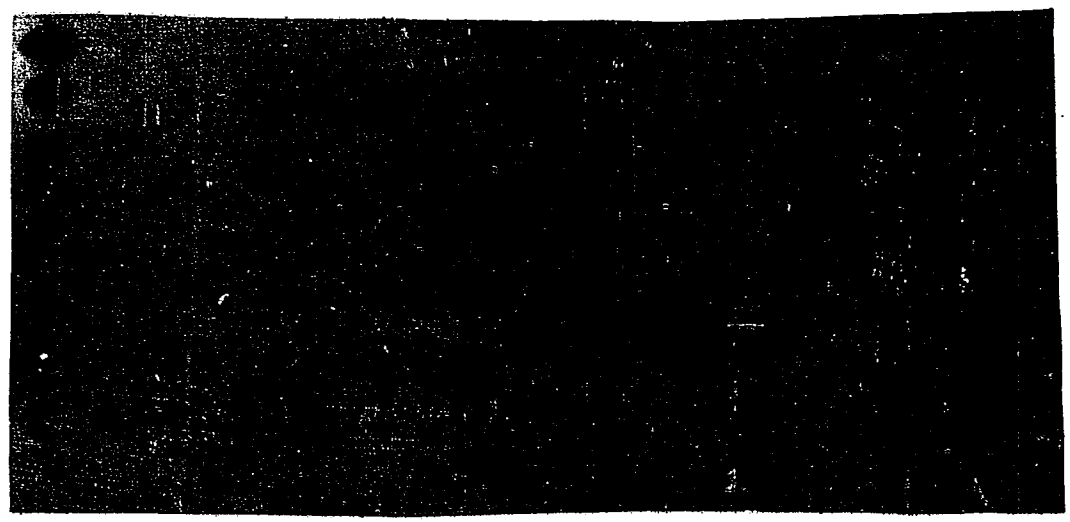


Figure 16. Chromatography of maltodextrins from photosynthesis with a light-dark transition

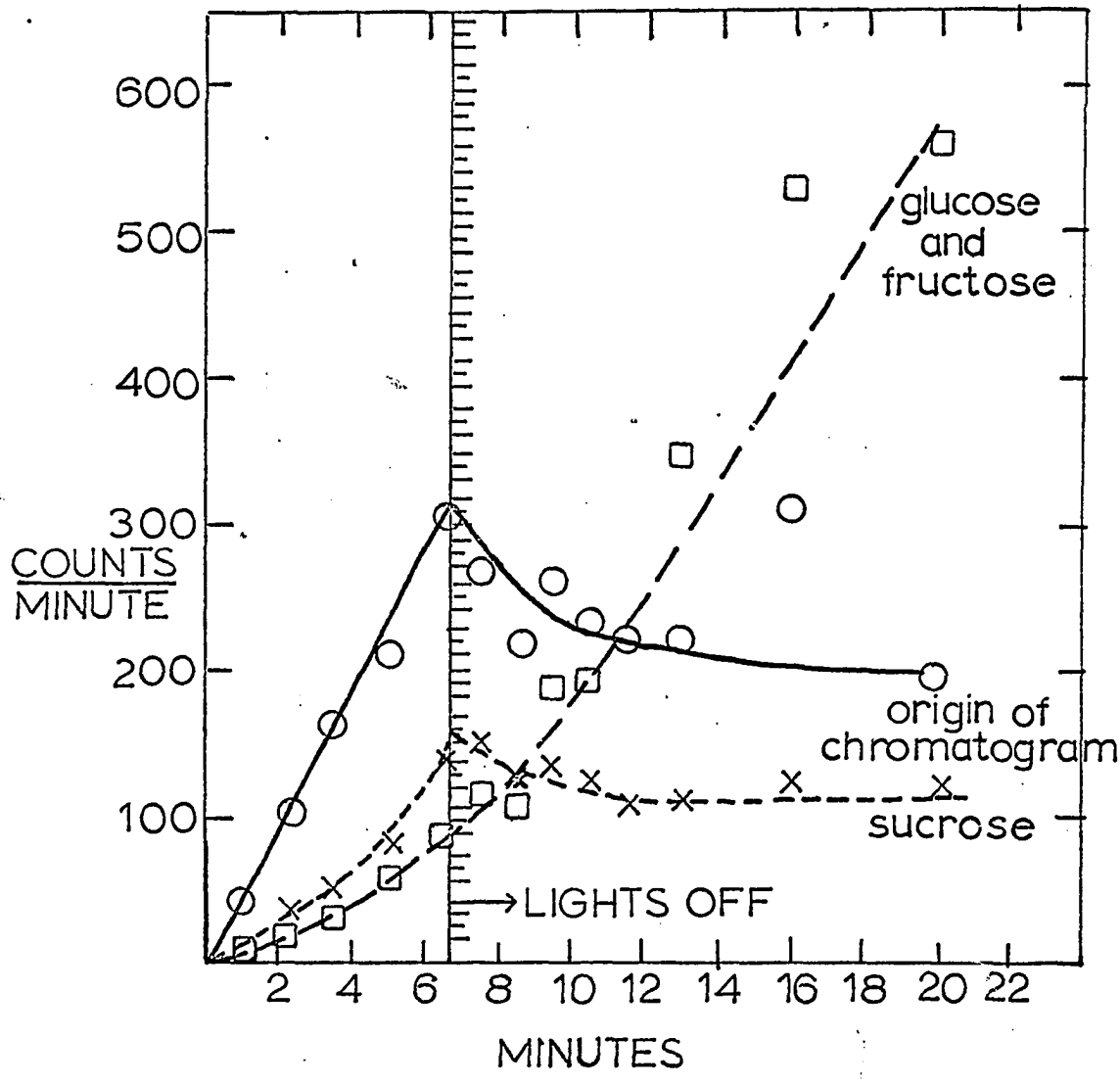


Figure 17. ^{14}C-kinetics of glucose, fructose, sucrose, and unresolved maltodextrins during photosynthesis with light-dark transition

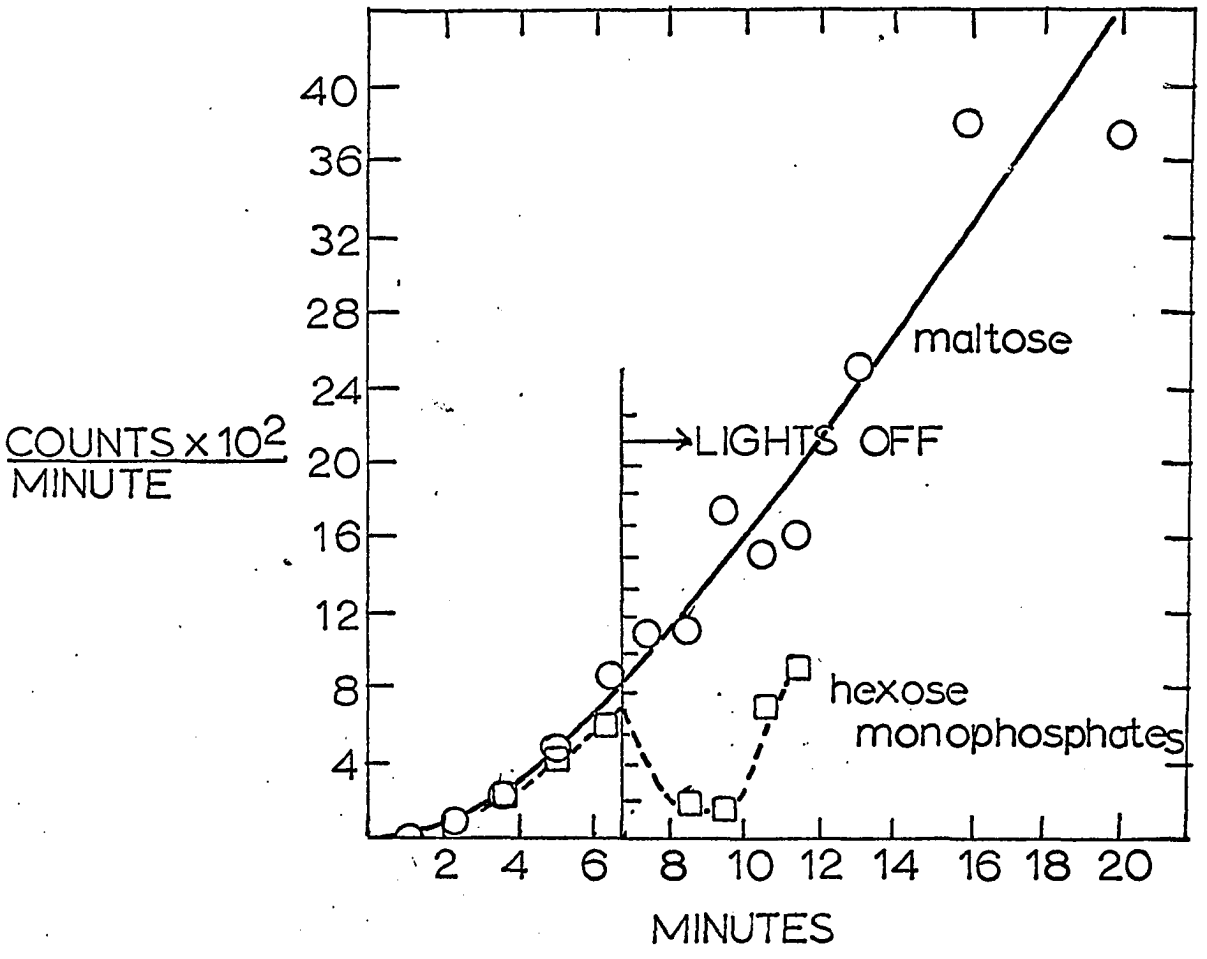


Figure 18. ^{14}C-kinetics of maltose and the hexose monophosphates

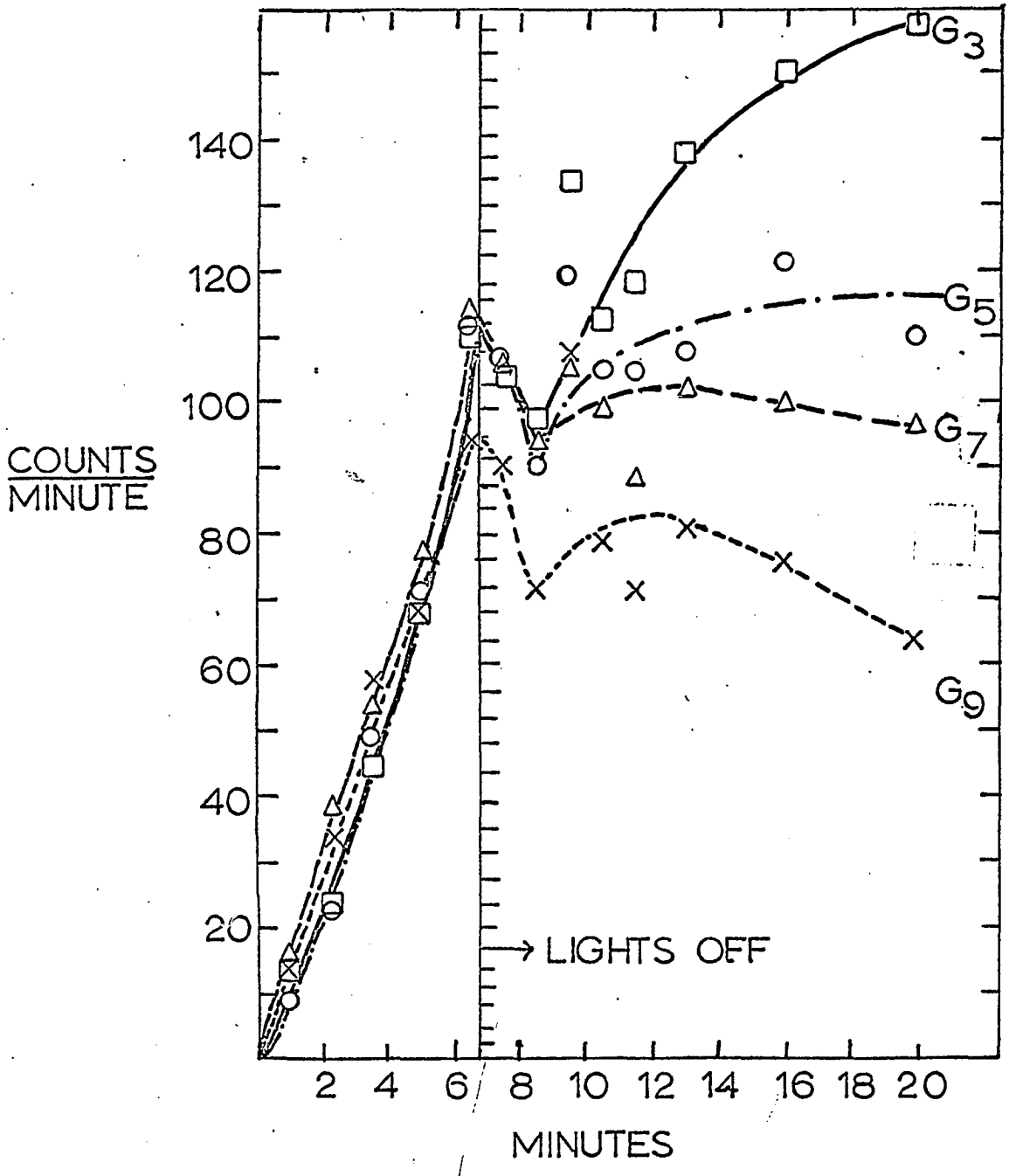


Figure 19. ^{14}C-kinetics of G_3, G_5, G_7 and G_9 during photosynthesis with light-dark transition

approximately 5 minutes, G_3 and G_5 increased again while G_7 and G_9 gradually decreased.

3. Distribution of radioactivity in maltose

The maltose spots were cut from the chromatograms of the second experiment (photosynthesis with light-dark transition) and eluted with water. Reduction of the maltose with sodium borohydride, hydrolysis with acid, chromatography, radioautography and counting of the radioactive glucose and sorbitol on the paper in the toluene scintillation solution allowed the distribution of radioactivity in the molecule to be determined. Maltose from all thirteen samples was analyzed; some were analyzed twice.

The ratio of glucose to sorbitol from these determinations is plotted against time in Figure 20. Glucose represents the nonreducing end and sorbitol the reducing end of the original maltose. The ratios decrease with time until 10-12 minutes of photosynthesis have passed when they level at a ratio value of 1.5. Uniformly labelled maltose would have a ratio value of 1. Initially, the nonreducing end of maltose was labelled much more highly than the reducing end, and at the value of 1.5, the nonreducing glucose is still 50% more radioactive than the reducing glucose. The break in the ratio curve is centered on the light-dark transition. Stopping of the light reaction of photosynthesis may have caused this effect. A comparison with the steady state experiment would have been valuable, but those samples were unfortunately destroyed. As discussed in the review of literature, Kandler privately related that maltose became uniformly labelled shortly after introduction of labelled carbon dioxide to plant leaves.

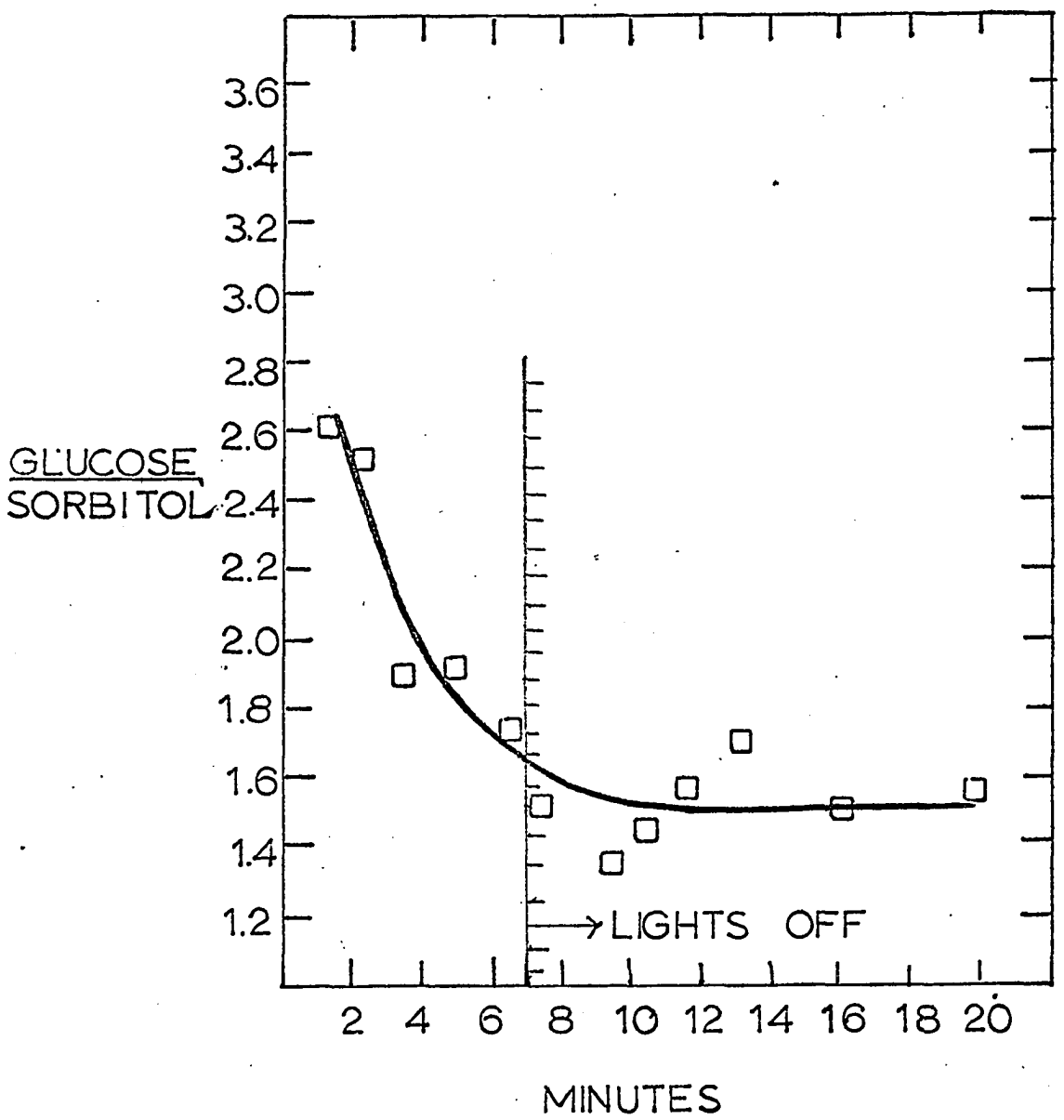


Figure 20. Distribution of radioactivity in maltose from photosynthesis with a light-dark transition

4. Distribution of radioactivity in maltotriose

The distribution of radioactivity was determined on the maltotriose from both experiments. After elution from the chromatograms, the maltotriose was hydrolyzed specifically by β-amylase.

$$O\text{-}O\text{-}\overset{*}{\emptyset}^{*} \longrightarrow O\text{-}\emptyset^{*} + \emptyset^{*} \qquad 11$$

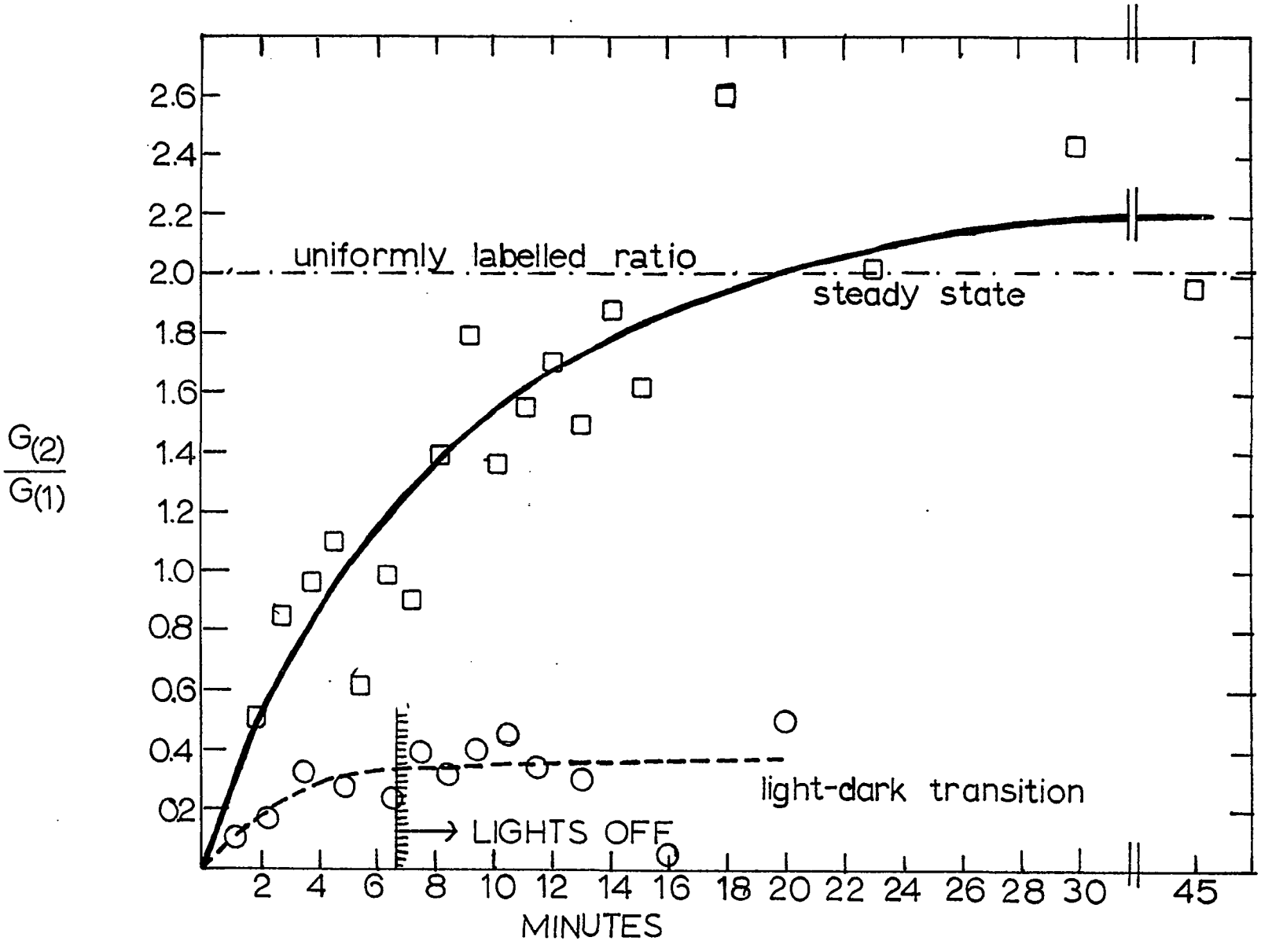
Following ascending chromatography and radioautography, the radioactive glucose and maltose spots were cut from the chromatogram and counted in toluene scintillation solution.

The ratio of maltose to glucose is plotted in Figure 21 as a function of time. Initially, the glucose is more highly labelled than maltose. In fact, the curves obtained from both experiments extrapolate to zero in which case maltose would have no label at zero time, and the reducing end of the molecule would have acquired label almost immediately after injection of radioactivity in the system. It appears from these observations that the labelling of maltotriose is very specific.

The differences between the curves of the first and second experiments may have been a result of the light-dark transition after 6.75 minutes of steady state photosynthesis in the second experiment. However, since the shapes of the two curves are similar, and the initial slopes of the two curves are unequal, the differences were more likely physiological differences in the chloroplasts of the two preparations. The extent of labelling is apparently dependent upon the ability of the chloroplast to fix carbon dioxide, and the fixation of carbon dioxide is dependent on the structural integrity of the chloroplasts (96).

The complete distribution of labelling in maltotriose from the steady

Figure 21. Distribution of radioactivity in maltotriose


$\frac{G_{(2)}}{G_{(1)}}$
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
uniformly labelled ratio
steady state
light-dark transition
LIGHTS OFF
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 45
MINUTES

state experiment was determined. The maltose, which had been hydrolyzed from maltotriose by β-amylase, was eluted, reduced with sodium borohydride, hydrolyzed by acid, chromatographed and radioautographed. The radioactivity in the resulting glucose and sorbitol was analyzed. The glucose corresponded to the nonreducing end and the sorbitol to the second glucose unit of the original maltotriose. The fraction of radioactivity in the three glucose units of the molecule is plotted versus time in Figure 22. Two minutes after injecting label into the system, 65% of the radioactivity was found in the reducing glucose. At twelve minutes, the same fraction of radioactivity (37%) was found in the reducing glucose as in the middle glucose; and from that time on the label in the middle glucose was greater than that in the reducing glucose which was greater than that in the nonreducing glucose. In the course of G_3 metabolism, cold G_3 was first replaced by G_3 which was reducing end labelled. As time passed radioactivity also found its way into other precursors so that the molecules of the G_3 pool gradually became labelled in the middle position and finally in the nonreducing position.

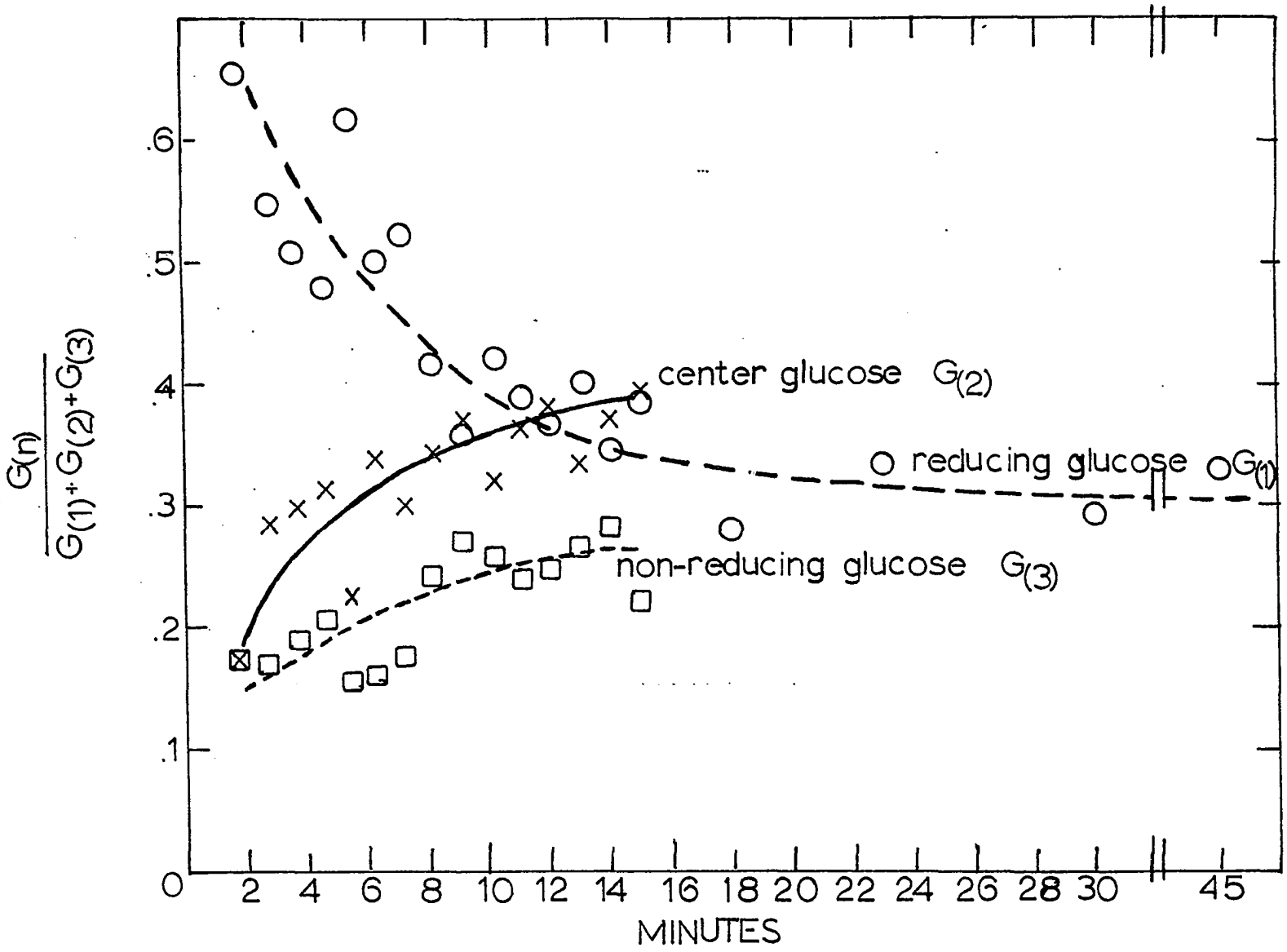
5. Distribution of radioactivity in maltotetraose

The analysis of the distribution of radioactivity in maltotetraose from the light-dark transition experiment was attempted by reducing the maltotetraose with sodium borohydride, and hydrolyzing the molecule with glucamylase according to the following equation:

$$\text{O-O-O-}\phi \xrightarrow{NaBH_4} \text{O-O-O-S} \longrightarrow 2\,\phi + \text{O-S} \qquad 12$$

The glucose, representing the nonreducing end, and maltitol, the reducing

Figure 22. Fractional distribution of radioactivity in maltotriose


$\frac{G(n)}{G(1)+G(2)+G(3)}$
.6
.5
.4
.3
.2
.1
0
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 45
MINUTES
center glucose $G_{(2)}$
reducing glucose $G_{(1)}$
non-reducing glucose $G_{(3)}$

end, were separated by ascending chromatography, radioautographed, cut from the chromatogram, and counted on the paper.

The scatter in the points was so great that a reliable trend in the labelling of maltotetraose during photosynthesis could not be reported. However, the values for the ratio of nonreducing end to the reducing end were all greater than the uniformly labelled ratio of 1, indicating that maltotetraose was labelled more strongly in the nonreducing end. This result was somewhat unexpected since the ^{14}C-kinetics of maltotriose and maltotetraose were identical (Figure 19) and since the distribution of radioactivity was in favor of the reducing end of maltotriose.

6. Distribution of radioactivity in maltopentaose, maltoheptaose and maltononaose

The distribution of radioactivity in G_5, G_7 and G_9 was determined by two-dimensional chromatography interspersed with β-amylase. This analysis was made on all samples from both photosynthesis experiments. The extent of hydrolysis by β-amylase was controlled so that no hydrolysis of maltotriose was detected.

The distribution of radioactivity in maltopentaose is plotted in Figure 23 as the ratio of maltose (nonreducing end) to maltotriose (reducing end). In both experiments, the nonreducing end was initially labelled. Similar results were obtained with maltoheptaose as seen in Figure 24 and in maltononaose, results of which are not presented. In each of the three cases, the curves from both experiments were similarly shaped and the ratios from the light-dark transition experiment were higher than those of the steady state experiment. The similarity of the curves

Figure 23. Distribution of radioactivity in maltopentasoe from steady state photosynthesis and photosynthesis with light-dark transition

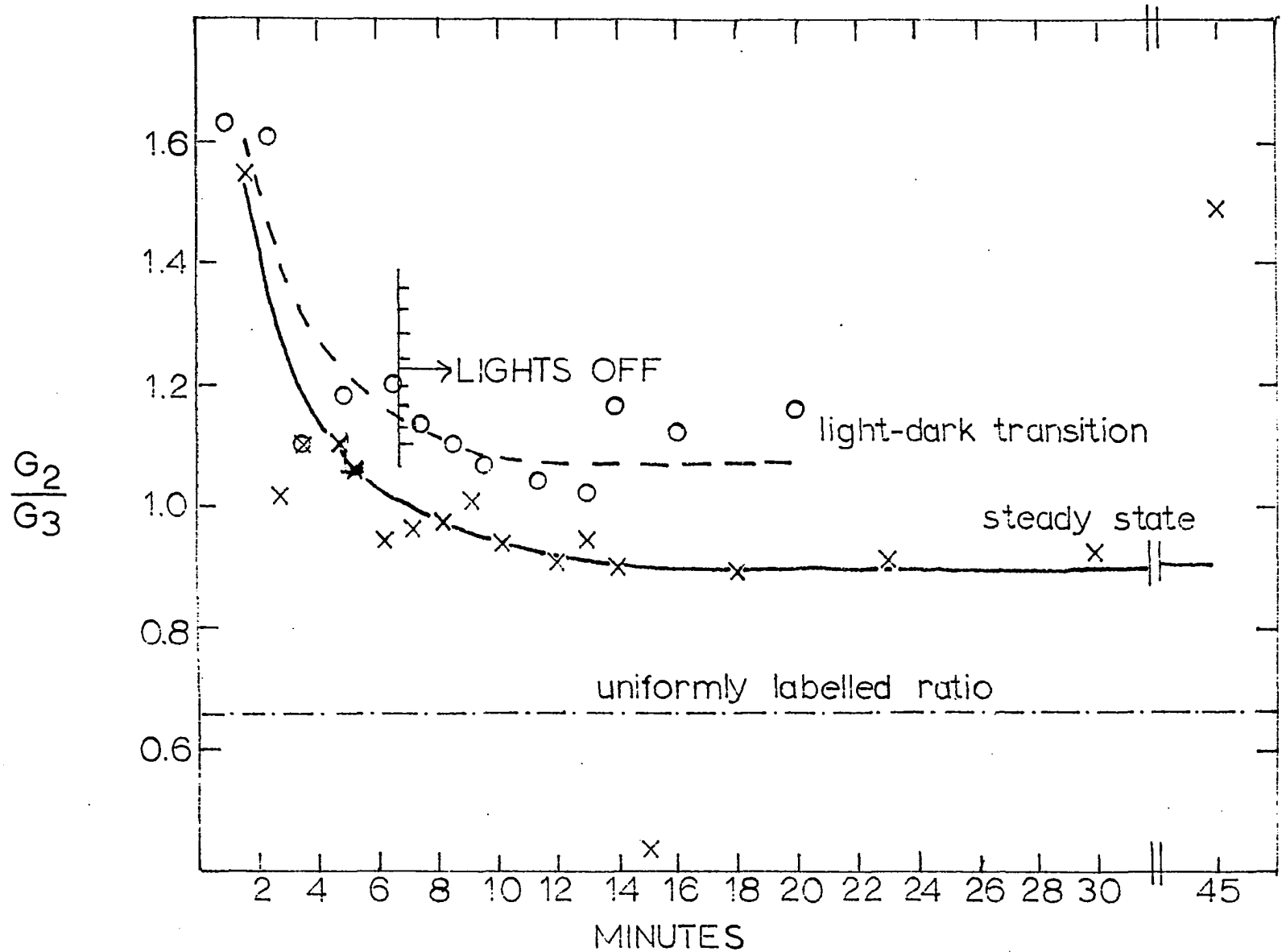
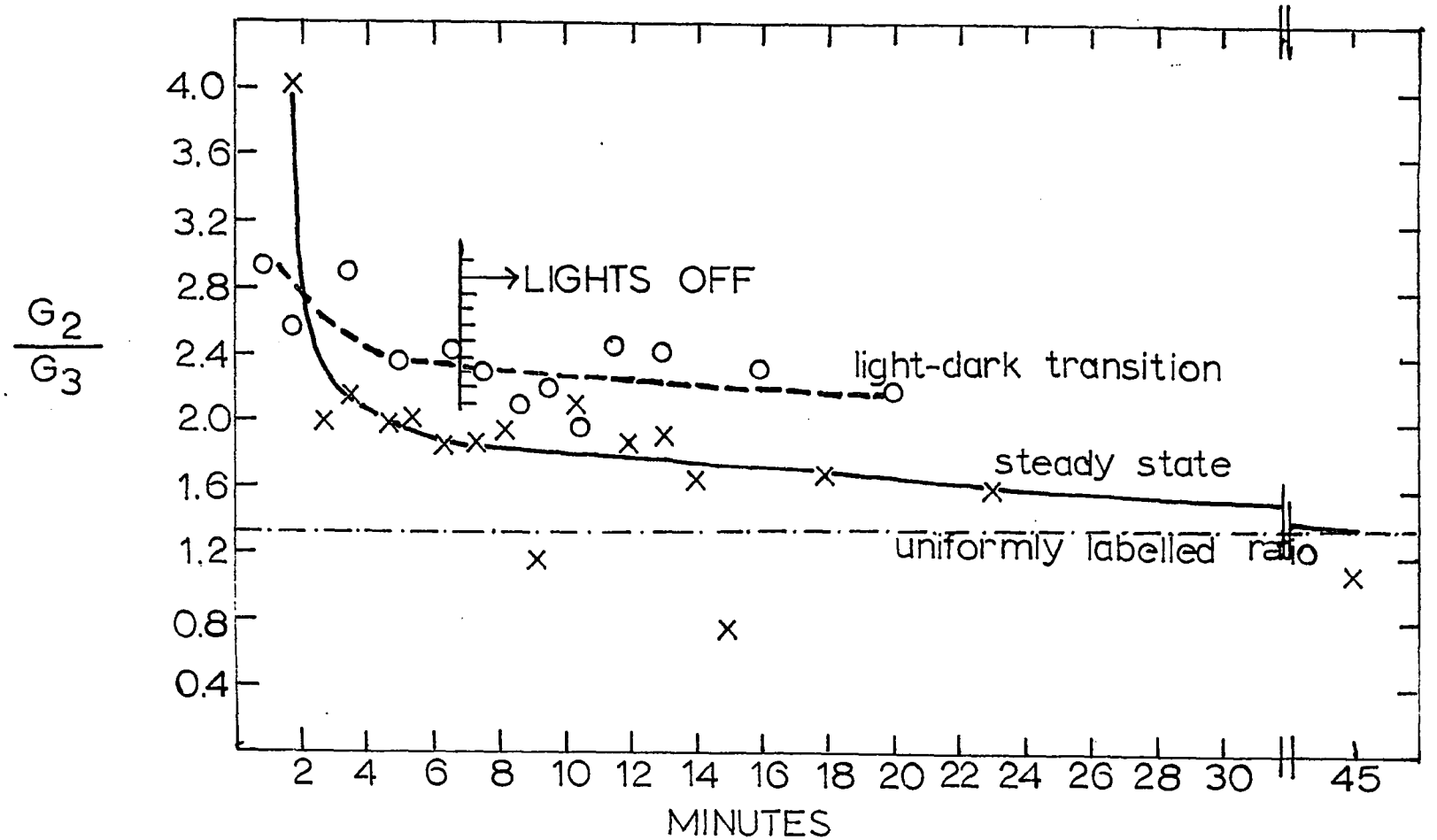

$\frac{G_2}{G_3}$
1.6
1.4
1.2
1.0
0.8
0.6
LIGHTS OFF
light-dark transition
steady state
uniformly labelled ratio
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 45
MINUTES

Figure 24. Distribution of radioactivity in maltoheptaose from steady state photosynthesis and photosynthesis with light-dark transition


$\frac{G_2}{G_3}$
4.0
3.6
3.2
2.8
2.4
2.0
1.6
1.2
0.8
0.4
LIGHTS OFF
light-dark transition
steady state
uniformly labelled ratio
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 45
MINUTES

indicated that the light-dark transition had no effect on the distribution of radioactivity in these molecules. Also noted, in all cases, was leveling off or only slight decline after 10-12 minutes of photosynthesis. These molecules never became uniformly labelled.

A more complete distribution in the maltopentaose molecules was determined. The maltotriose which resulted from the β-amylolysis of maltopentaose was eluted from the chromatography paper and hydrolyzed specifically with β-amylase. Chromatography and radioautography enabled the determination of radioactivity in the latter products shown here:

$$0\text{-}0\text{-}0\text{-}0\text{-}\phi \longrightarrow 0\text{-}\phi^{*} + 0\text{-}0\text{-}\phi \qquad (13)$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad \longrightarrow 0\text{-}\phi + \phi.$$

Thus, the maltose from the latter reaction (termed sandwich maltose) represented the second and third glucose units from the reducing end of the original maltopentaose. Of course, the glucose represented the reducing unit of the original maltopentaose.

The analysis of the maltopentaose from the light-dark transition experiment is given in Figure 25. Radioactivity is incorporated into maltopentaose first in the nonreducing end. After the curves leveled off, the nonreducing end contained 50% of the radioactivity in the molecule and most of the remainder is found in the sandwich maltose. The decline in the reducing glucose curve may only reflect the relative increase of radioactivity in the rest of the molecule, but may as well indicate dilution of the radioactivity originally found in the reducing glucose.

As seen in Figure 26, identical results to this were obtained from the same analysis of the maltopentaose from steady state photosynthesis. At 10 minutes, the curves for nonreducing maltose and sandwich maltose had leveled

Figure 25. Distribution of radioactivity in maltopentaose from photosynthesis with light-dark transition

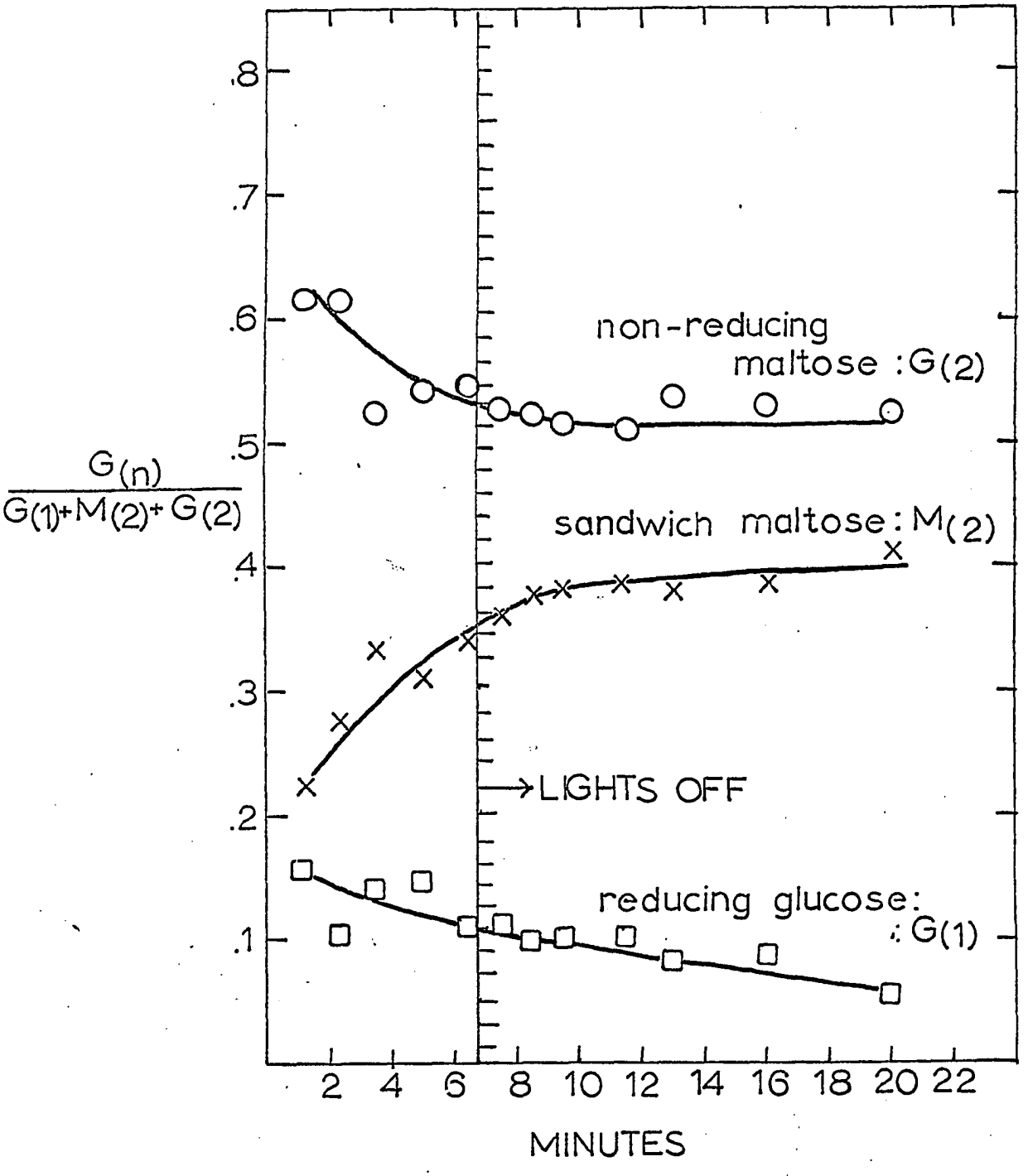
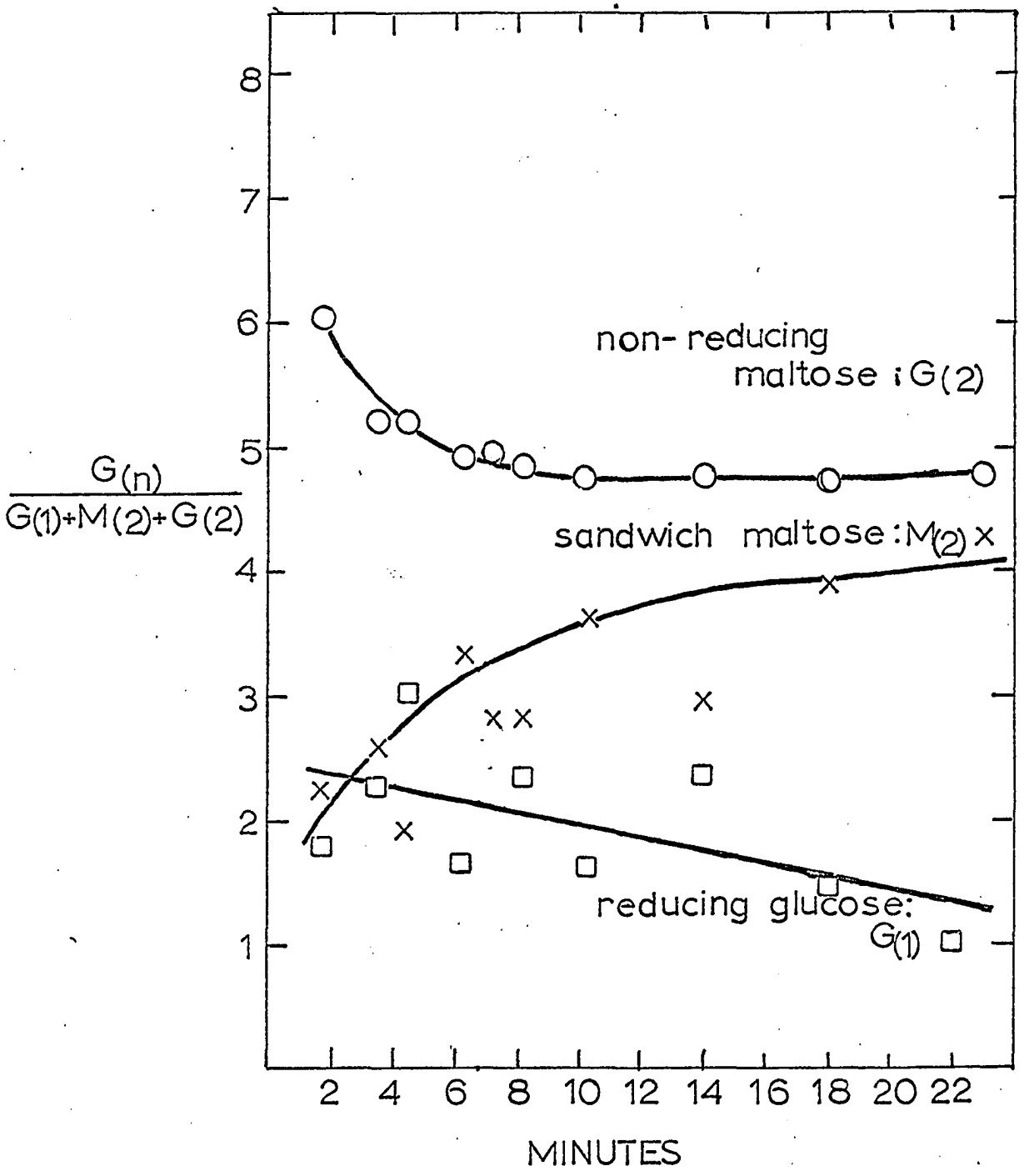

.8
.7
.6
.5
.4
.3
.2
.1
$\frac{G(n)}{G(1)+M(2)+G(2)}$
non-reducing
maltose : G(2)
sandwich maltose : M(2)
→LIGHTS OFF
reducing glucose:
: G(1)
2 4 6 8 10 12 14 16 18 20 22
MINUTES

Figure 26. Distribution of radioactivity in maltopentaose from steady state photosynthesis


$\frac{G(n)}{G(1)+M(2)+G(2)}$
8
7
6
5
4
3
2
1
non- reducing
maltose : G(2)
sandwich maltose : M(2)
reducing glucose:
G(1)
2 4 6 8 10 12 14 16 18 20 22
MINUTES

at 50% and 40% respectively, and the rates of change, which were reflected in the curves, were also very close in both experiments. The only difference was the higher initial level of radioactivity in the reducing glucose. These similarities definitely ruled out an effect by the light-dark transition on the redistribution of label in maltopentaose and most likely in the higher maltodextrins as well.

F. Carbohydrases of Spinach Chloroplasts

A number of very simple experiments were conducted in order to investigate the activity of carbohydrases in spinach chloroplasts. The chloroplasts were isolated as for photosynthesis experiments, and during isolation and washing they were kept intact by handling gently in buffers containing 0.33 *M* sorbitol. The chloroplasts were washed 4-5 times so that they would be essentially free of enzymes from the cytoplasm. They were then broken by simply suspending in buffer without the 0.33 *M* sorbitol. The rupture of the chloroplast membrane was verified by phase contract microscopy as shown by Jensen and Bassham (95). Digests contained 50 microliters of broken chloroplast suspension and 50 microliters of buffered 1% waxy maize starch, to which a relatively small volume of radioactive substrate was added. Samples of 10 microliters were removed periodically during the incubation and spotted directly on Whatman #1 paper. After ascending chromatography and radioautography various analyses were made.

From one preparation of chloroplasts, four such experiments were conducted. Glucose, reducing end labelled G_8, G-1-P, and ADPG were used as radioactive substrates. Approximately 0.5 mg of cold carrier substrate

was also added to each digest. All four were buffered at pH 7.0 with 20 *mM* sodium glycerol phosphate buffer. In these experiments, 10 microliter samples were taken at 0, 1, 6 and 17 hours. Figure 27 shows the radioautogram of the experiments with maltooctaose and glucose; Figure 28 shows the experiments with G-1-P and ADPG.

With radioactive maltooctaose as substrate, the product radioactivity was distributed from glucose to unresolved maltodextrins after 1 hour of incubation with broken chloroplasts. The appearance of the maltodextrins smaller than G_8 could be accounted for by amylases in the chloroplasts, especially since G_6, G_4, G_3 and G_2 were predominant products. The appearance of compounds larger than G_8 may have represented a process of disproportionation, and either D-enzyme or phosphorylase may be involved. After 6 hours the radioactivity was distributed primarily among G_2, G_3 and starch, and at 17 hours it was found almost exclusively in maltose and starch. The implications of the product distribution at the longer incubation times will be discussed later.

On the right-hand side of Figure 27 were spotted the samples from the incubation with radioactive glucose. The primary reaction was the conversion of glucose to starch. The only other reaction products which appeared were G_3, G_4 and a compound with an intermediate chromatographic mobility, perhaps panose, after 17 hours. These products could be accounted for by the action of D-enzyme (and perhaps T- or Q-enzyme) in the presence of waxy maize starch as donor, with radioactive glucose as acceptor.

In Figure 28 are shown the experiments using radioactive G1P (left) and ADPG (right). Other than the decomposition products of ADPG*, there

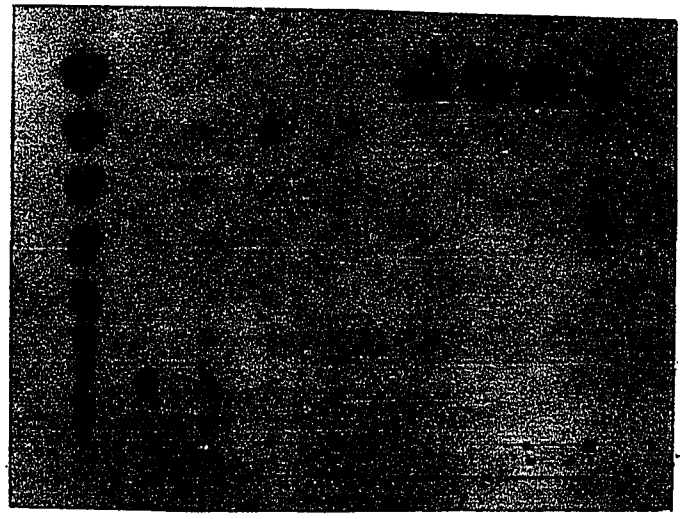


Figure 27. Incubation of maltooctaose and of glucose with broken spinach chloroplasts

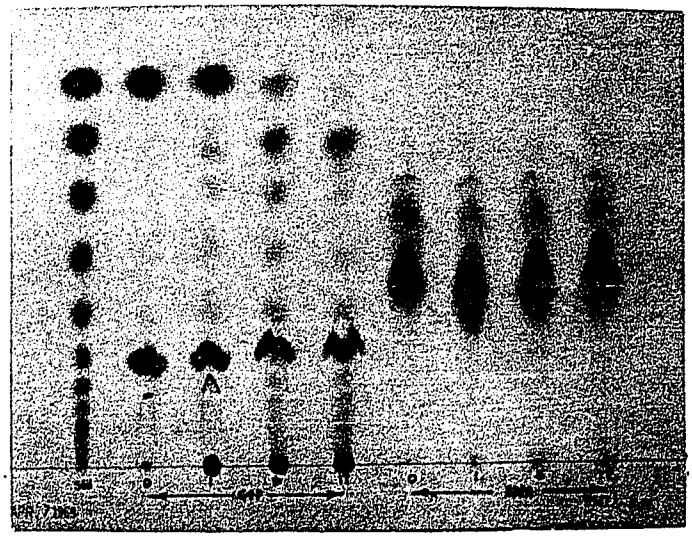


Figure 28. Incubation of G-1-P and of ADPG with broken spinach chloroplasts

was no evidence of incorporation of radioactivity into soluble products by starch synthetase from ADPG*, but a very small amount of radioactive starch appeared after 17 hours of incubation with the broken chloroplast suspension. The apparently slow reaction of starch synthetase in this instance may very well have been a result of the pH of the suspension. The activity of starch synthetase from spinach chloroplasts is reportedly low at pH 7.0 relative to its optimum pH of 9.0 (12, 13).

Contrasting with starch synthetase was the activity of phosphorylase on the left-hand side of Figure 28. At zero time it was obvious that the G-1-P was contaminated with glucose. (G-1-P has a mobility approximating that of G_6). The phosphorylase activity in this preparation of chloroplasts was so great that even in the two minutes required to spot the zero hour sample, radioactive G-1-P was added to endogenous G_4 primer giving a radioactive G_5 spot; radioactive starch was also observed. Other maltodextrins were apparent after 1 hour.

The samples from the G-1-P digest at 1, 6, and 17 hours exhibited a compound with a chromatographic mobility slightly less than that of maltose. This spot, which was also apparent after 17 hours of incubation with glucose in Figure 27, was not characterized but may have been isomaltose. If so, T-enzyme may be implicated in its formation.

Maltose was apparent after 6 hours and its increase was probably due to amylase action on labelled starch. The contaminating glucose had nearly disappeared at 6 hours and was completely gone at 17 hours. The fate of glucose in these digests will be discussed below.

A second series of experiments with a second preparation of spinach chloroplasts yielded similar conclusions. Carbohydrase activity was

investigated by incubations with reducing end labelled G_2 and G_3 and with glucose. These substrates were previously purified by chromatography, radioautography and elution. All conditions were the same as those in the previous set of experiments except that no carrier maltodextrins were added to the digests. In this experiment, samples were taken at 0, 1, 5 and 18.5 hours.

On the right half of the radioautogram in Figure 29 are shown the glucose digest samples. Besides glucose, the 1 and 5 hour samples contained radioactive compounds with mobilities similar to maltotriose, but these compounds were not identified. They could have arisen by D- or T-enzyme activity on glucose and waxy maize starch but also by the action of amylases on the radioactive starch.

Figure 30 shows the same chromatogram as in Figure 20, after being developed with silver nitrate. On the silver nitrate-developed chromatogram, the spots produced by sorbitol (which was used in the buffers during the isolation of the chloroplasts) coincide with glucose on the radioautogram. The 18.5 hour sample of the glucose digest showed a relatively high concentration of maltose. This would indicate amylase action on unlabelled waxy maize starch since maltose does not appear on the corresponding radioautogram.

Likewise, on the left hand portions of Figure 29 and 30 are the digest samples of G_2 with the broken chloroplast suspension. The radioautogram in Figure 29 shows a decrease in the labelling of maltose, the appearance of labelling in glucose, starch and maltodextrins. The glucose may have been converted to starch by phosphorylase as described below, and the maltose appeared to be hydrolyzed to glucose by a process

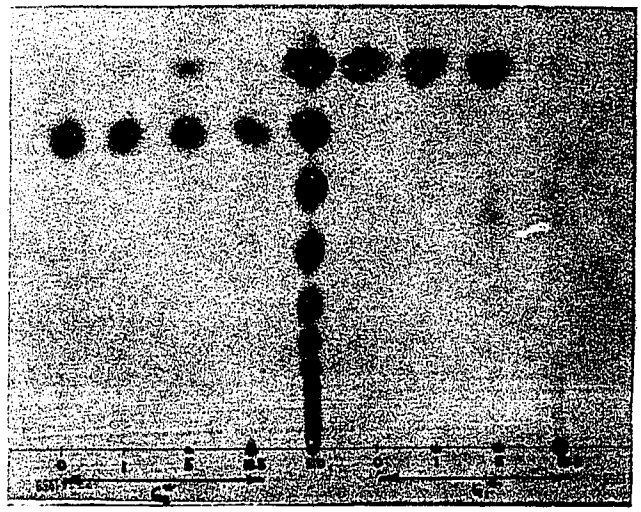


Figure 29. Incubation of maltose and of glucose with broken spinach chloroplasts: radioautogram

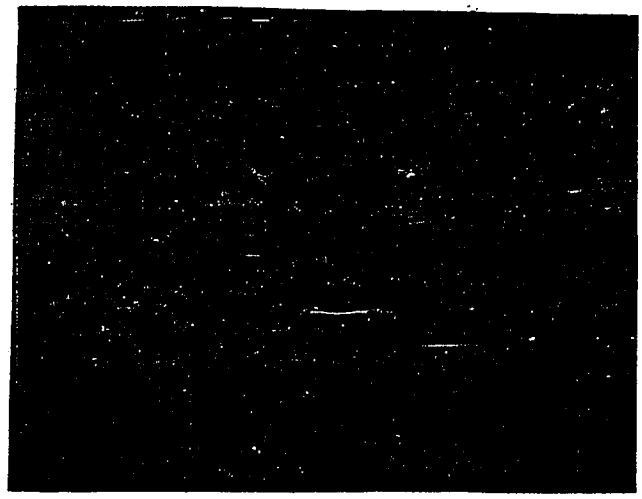


Figure 30. Incubation of maltose and of glucose with broken spinach chloroplasts: chromatogram

Same as Figure 29 except developed with silver nitrate

that is not understood. Amylase activity was again noted in the chromatogram in Figure 30, because the concentration of maltose increased during the incubation. Some of the higher maltodextrins were seen both in the radioautogram and the chromatogram.

The conversion of glucose to high molecular weight compounds or "starch" was observed in this experiment as well as in Figure 27, 28 and 29 above. The broken chloroplasts appear to phosphorylate glucose and to incorporate it into high molecular weight compounds in the digest mixture, *i.e.* waxy maize starch, endogenous starch granules and unresolved maltodextrins. The phosphorylation of glucose was thought to be involved because a small amount of radioactivity was found in the G-1-P spot of a radioautogram from an experiment where G-1-P and radioactive glucose were incubated with broken chloroplasts. This radioautogram is presented in Figure 31 and the G-1-P is noted slightly above the origin in the 5 and 18.5 hour samples. This spot was identified as G-1-P from the corresponding silver nitrate-developed chromatogram, because the radioactivity corresponded with the salt spot. Other silver nitrate-developed chromatograms without G-1-P do not show salt spots, *e.g.* Figure 30. Because the radioactivity in the G-1-P spot is so slight in Figure 31, a small amount of G-1-P is perhaps involved in the glucose-starch conversion. Only catalytic amounts of G-1-P would be necessary to disproportionate G_8 as seen in Figure 27. However, in order to convert all of the glucose to "starch", the equilibration of glucose and G-1-P as well as the equilibration of G-1-P and starch by phosphorylase would be necessary.

An incubation of reducing end-labelled G_3 is shown in Figure 31. It is apparent from the photograph that glucose and maltose are produced in

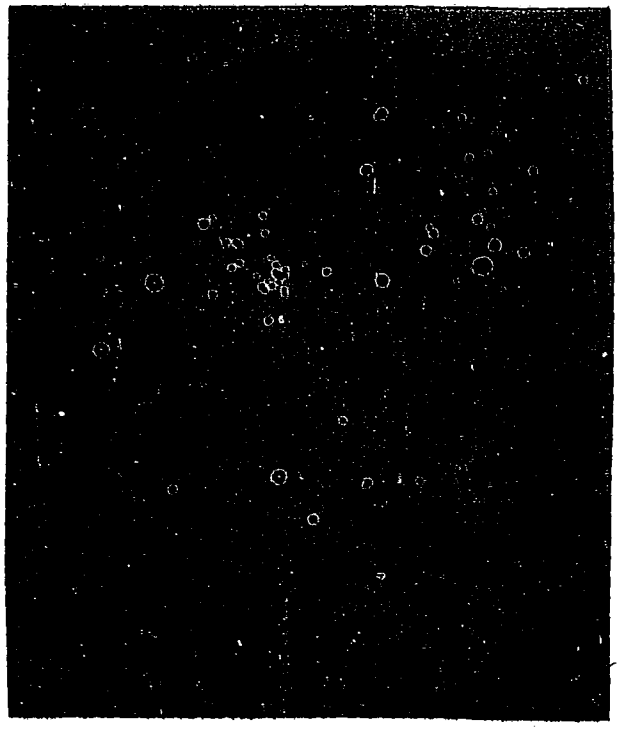


Figure 31. Incubation of glucose-1-phosphate and radioactive glucose with broken spinach chloroplasts

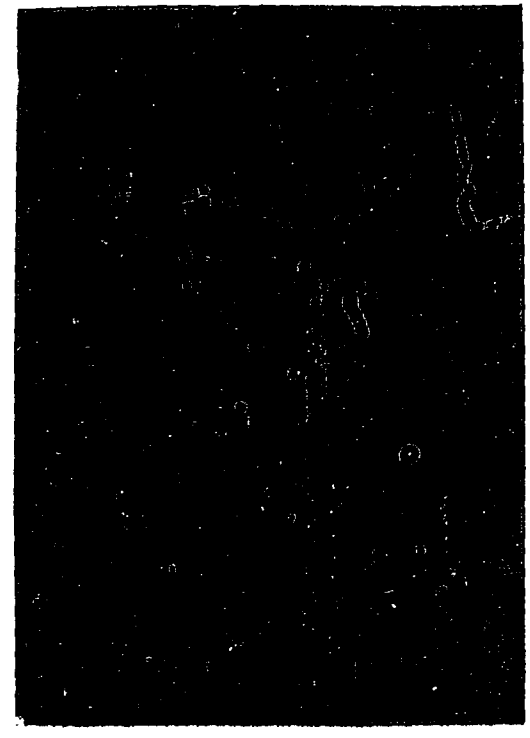


Figure 32. Incubation of maltotriose with broken spinach chloroplast

approximately equal quantities after 1 hour, but G_4 and G_5 were also apparent when looking directly at the radioautogram. The appearance of label in glucose, G_4 and G_5 would be expected from the action of D-enzyme. However, the production of maltose from reducing end-labelled G_3 becomes difficult to explain. There are four possible explanations: (1) maltose might be produced as a product of amylolysis of a D-enzyme reaction product, especially G_4; (2) there might be an unknown glucosyl-α-1,4-transglycosylase acting on maltotriose as a donor to give labelled G_2; (3) there could be an amylase capable of cleaving both α-1,4-linkages of G_3 producing labelled G_1 and G_2; or (4) perhaps an amyloglucosidase is present which is capable of cleaving not only G_3 to give labelled G_2 but also G_2 to give labelled glucose. Although an amyloglucosidase or maltase type of activity has not been reported in plant tissue, this author favors the last explanation because it also provides an answer for the hydrolysis of maltose to glucose as seen in Figures 27 and 29.

Clearly, the enzymatic mechanisms producing the maltodextrins in spinach chloroplasts are complex. No one enzyme can likely be assigned for the production of an individual maltodextrin from a particular set of substrates. However, these experiments have fairly certainly implicated the action of phosphorylase, amylase, and D-enzyme in the maltodextrin metabolism of spinach chloroplasts.

V. DISCUSSION OF RESULTS

A. Distribution of Maltodextrins in Photosynthetic Tissue

The maltodextrins have not been generally recognized as constituents of photosynthetic tissue (96). They are present in such minute concentrations that they were not observed in photosynthetic tissue until they were labelled during photosynthesis in $^{14}CO_2$. Radioactivity is sensitive enough that small amounts of these compounds are easily recognized. Fisher's work, (37) as well as this work, has shown that the maltodextrins as a group maintained a steady state concentration, whereas starch accumulated with time. In one feeding experiment with *Scenedesmus*, 17.5% of the radioactivity utilized in photosynthesis was found in non-ionic soluble compounds. Only 2-3% of these soluble compounds were chromatographically resolvable maltodextrins. According to these figures, this thesis is concerned with compounds which represent approximately 0.5% of algal constituents. The maltodextrins in algae as well as in other photosynthetic tissue, represent a small but significant part of the total carbohydrate metabolism.

Much of the research described in this thesis was motivated by the observation early in the research that G_2, $G_3$and G_4 showed higher labelling after photosynthesis in $^{14}CO_2$ than did other maltodextrins. In *Scenedesmus*, in soybean leaves, and in spinach chloroplasts, G_3 and G_4 always contained identical amounts of radioactivity. *Scenedesmus* exhibited 2-3 times more G_3 and G_4 than maltose or higher maltodextrins. In soybean leaves, the degree of labelling of G_3 and G_4 was twice that of maltose and 6-10 times that of the higher maltodextrins. During steady state photosynthesis by

isolated spinach chloroplasts, G_3 and G_4 possessed only a few percent more activity than the maltodextrins larger than maltotetraose. However, in this case maltose had ten times more radioactivity than G_3 and G_4.

Certain kinetic data also indicated an unique metabolism for G_3 and G_4. Following pulse labelling of soybean leaves with $^{14}CO_2$, the kinetics of G_3 and G_4 were definitely different from the kinetics of the other maltodextrins, and in fact exhibited a "mirror image" kinetic curve to the kinetic curve of the material which did not move from the origin of the chromatogram. This would indicate that G_3 and G_4 were produced at the cost of the unresolved maltodextrins.

A similar deduction came out of the spinach chloroplast experiment where photosynthesis was interrupted with a light-dark transition. As time passed after the lights where out, G_3 and G_4 accumulated while the higher maltodextrins diminished. From this information, it would seem possible to implicate amylases in the formation of G_2, G_3 and G_4.

B. Carbohydrases in Photosynthetic Tissue

Amylase activities were detected in *Chlorella*, soybean leaf tissue, and spinach chloroplasts. In *Chlorella* more than one type of amylase existed. Initial ammonium sulfate separations of *Chlorella* proteins gave enzyme fractions which showed extensive release of G_2, G_3 and G_4 after incubation with waxy maize starch. Upon further purification of the *Chlorella* enzymes by gel filtration, such a distribution of products was not obtained when incubated with waxy maize starch. These digests produced large quantities of glucose; maltodextrins were not apparent.

Soybean leaf homogenates incubated with impure maltoheptaose showed

amylase activity which yielded G_1, G_2 and G_3. Glucose increased with time and maltotriose decreased. In the initial samples from the maltoheptaose digest, G_5, G_3, and G_2 were the predominant hydrolysis products. Further work on branched contaminants from these digests indicated that β-amylase was the active agent in degrading the maltoheptaose.

In another experiment, soybean leaf homogenates were incubated with maltose. The results showed that glucose increased and maltose decreased with time. The apparent maltase activity in soybean leaf tissue may alternatively have been the action of D-enzyme in conjunction with β-amylase. Since the activity of β-amylase was seen to be great in the maltoheptaose experiment, and since D-enzyme activity was indicated by a trace of G_3 after 300 minutes of incubation with maltose, the combination of enzymes may have been active in converting maltose to glucose.

Amylases, D-enzyme and phosphorylase were identified in spinach chloroplasts. Starch synthetase in spinach chloroplasts was not demonstrated in this work but is well documented (12, 13). In two out of three papers, Stocking concluded that phosphorylase activity was not present in chloroplasts (40, 45, 46). However in this work, the activity of phosphorylase in spinach chloroplasts was certain. The phosphorylase activity was demonstrated in three preparations of chloroplasts.

From all three sources of photosynthetic tissue, the activities of amylases, D-enzyme, and starch synthetase have been demonstrated. The various enzymes have been demonstrated, however, in a way that cannot reflect their activity *in vivo*. Slight difference in the relative concentrations of substrates or in the pH values, markedly alter the action patterns of some of these carbohydrases. For example, in the broken

chloroplast experiments, starch synthetase was nearly inactive, presumably because the pH of the buffer was less than optimal. On the other hand, phosphorylase appeared to be very active in the formation of maltodextrins because sufficient G-1-P was added to drive the reaction in the direction of synthesis.

Finding the enzymes in photosynthetic tissue which catalyze reactions with maltodextrins is significant in itself. As these enzymes are acting on maltodextrins *in vivo*, the situation is very complex, because several enzymes are acting at once on the same compounds. At present, one can only state that certain carbohydrases are found in photosynthetic tissue. These enzymes must be taken into account in working out a more definitive picture of carbohydrate metabolism.

C. Significance of Maltodextrins in Photosynthetic Tissue

Maltose has been observed as a product of photosynthesis in many plant species (29, 32). During photosynthesis by leaves in $^{14}CO_2$, Kandler observed that the kinetics of maltose rose to a maximum and fell to a low level again in the first 10-15 minutes (7). Kandler also observed that in the course of photosynthesis the level of maltose immediately dropped when the lights were turned off.

These observations are at variance with maltose kinetics obtained from isolated spinach chloroplasts. During steady state photosynthesis, maltose did not rise and fall, as Kandler had observed in leaves, but continued to increase in a linear fashion for 45 minutes. A light-dark transition during photosynthesis did not alter the maltose kinetics; they were linear during seven minutes of photosynthesis and the 13 minutes of

darkness which followed.

The difference between the kinetics observed by Kandler and those of isolated spinach chloroplasts is very curious. The chloroplasts appear to be the site of maltose formation in both studies. In the leaf, maltose behaved as an intermediate; but in isolated chloroplasts, it simply accumulated. It is as if isolation of the chloroplast removed a potential for maltose utilization. Perhaps a more careful investigation of the enzymes involved with maltose metabolism will eventually explain the difference.

The kinetics of maltose during photosynthesis with a light-dark transition indicated that maltose was not a primary photosynthesis product. The label in maltose was initially in the nonreducing glucose moiety. The distribution of label was becoming more uniform until the lights were turned off; after that the distribution did not change. Perhaps maltose was the product of amylolysis of starch which was becoming labelled. For instance, β-amylase could cleave maltose from starch labelled in the non-reducing ends by ADPG* during photosynthesis. When photosynthesis was stopped by the light-dark transition, the distribution of label in non-reducing ends of starch molecules would be unchanged, and hence the distribution of label in the maltose resulting from amylolysis would also be unchanged. Since the presence of amylases in chloroplasts has been demonstrated, such an explanation is feasible. Furthermore, the varying degrees to which maltose was found in other photosynthetic tissues may be related to the amylolytic activities present.

The formation of maltotriose during photosynthesis is unique. The distribution of the label in the glucose units of G_3 was different from

the distribution in the other maltodextrins. Maltotriose was labelled initially in the reducing glucose moiety, and the other two glucose units accumulated radioactivity very rapidly. The unit at the nonreducing end was labelled last. Of the enzymes which were demonstrated in chloroplasts, the only mechanism which could explain such a distribution in G_3 is that of D-enzyme. The transfer of maltosyl moieties from starch to radioactive glucose would give the distribution initially noted in G_3.

$$\phi^{*} + 0\text{-}0\text{-}0\text{-}0\text{-}0\text{-}0\text{-}\cdots \rightleftharpoons 0\text{-}0\text{-}\phi^{*} + 0\text{-}0\text{-}0\text{-}0\text{-}\cdots \qquad 14$$

The kinetics of G_3 from the light-dark transition experiment show that the level of G_3 dropped immediately when the lights were turned off. Since some glucose is surely formed early in photosynthesis, its labelling would be decreased immediately when photosynthesis stopped.

However, the D-enzyme mechanism fails to meet the evidence of the distribution of label in G_3 as time passes. The labelling of the nonreducing end of starch would put radioactivity in the nonreducing end of G_3---not in the second glucose unit as observed.

$$\phi^{**} + 0^{*}\text{-}0\text{-}0\text{-}0\text{-}0\text{-}0\text{-}\cdots \rightleftharpoons 0^{*}\text{-}0\text{-}\phi^{**} + 0\text{-}0\text{-}0\text{-}0\text{-}\cdots \qquad 15$$

Since the G_3 kinetics show a rise in the level of G_3 above those levels of higher maltodextrins, it is possible that amylase products begin to cloud the picture of G_3 metabolism very early. It is once again apparent that the complex metabolism of the maltodextrins in chloroplasts and leaves cannot be defined.

The distribution of label in maltotetraose was not determined as completely as it was in the other maltodextrins. The data did indicate that the label was in the nonreducing end of G_4 throughout the photo-

synthesis experiment. However, every other piece of evidence has indicated that the metabolism of G_4 was parallel to that of G_3. In spite of this uncertainty, the author would tend to group G_3 and G_4 separately from maltose and from the higher maltodextrins for the purpose of discussing the metabolism of these compounds.

There is no question but that a major process in the formation of G_5, G_7 and G_9 is the addition of glucose units to the nonreducing end of primers. This deduction is easily extended to include G_6, G_8, G_{10} and larger maltodextrins. Addition to the nonreducing end of primers larger than G_3 implicates synthetase or phosphorylase in the formation of the higher maltodextrins. Evidence was presented for phosphorylase activity in spinach chloroplasts. However, it is generally felt that phosphorylase is the starch degrading agent and that starch synthetase is the synthesizing agent. The evidence could as well implicate starch synthetase in the formation of the higher maltodextrins, because the primer specificities of the two enzymes are probably very similar, although G_2 and G_3 have also been reported as primers for starch synthetase.

The processes which yield maltodextrins appear to be complex, as the maltodextrins occur by a combination of synthetic and degradative processes. The results of the experimental work in this thesis indicate that amylolytic and transglucosylation processes in chloroplasts yield G_2, G_3 and G_4, which in turn serve as substrates for synthetic processes that yield the higher homologs of the maltodextrins series.

The importance of the maltodextrins in photosynthetic tissue has been the question guiding this research. The amount of label in the chromatographically resolvable maltodextrins (G_2-G_{10}) represents only 2-3% of the

starch and oligosaccharides which are labelled during photosynthesis. Their existence is of interest, even though they do not represent major metabolites of plant cells. Their significance may be similar to that of sucrose and other oligosaccharides, in that they represent a storage product which is soluble and can be readily synthesized and degraded. The metabolism of the maltodextrins is closely related to that of starch, and another possible significant function to photosynthetic tissue is to provide primer for the starch synthetase and phosphorylase reactions.

VI. SUMMARY

The metabolism of maltodextrins in photosynthetic tissue was investigated. Plant sources included *Chlorella pyrenoidosa*, *Scenedesmus obliquus*, soybean leaves and spinach chloroplasts. The maltodextrins were detected in these tissues following photosynthesis in $^{14}CO_2$. In these tissues maltose, maltotriose and maltotetraose predominated over the higher homologs of the maltodextrins series.

Analysis of the distribution of label in the individual maltodextrins following photosynthesis by isolated spinach chloroplasts showed the following: maltose was initially labelled in the nonreducing moiety; maltotriose was initially labelled in the reducing glucose unit; and the maltodextrins larger than maltotetraose were initially labelled in the nonreducing ends.

Carbohydrases detected in spinach chloroplasts included amylase, phosphorylase and D-enzyme. The role of these enzymes and of starch synthetase in the formation of individual maltodextrins was investigated on the basis of the distribution of label. However, the processes which yield maltodextrins appear to be complex combinations of enzymatic processes. The results of the experimental work indicate that amylolytic and transglycosylation processes in chloroplasts yield maltose, maltotriose, and maltotetraose. These in turn serve as primers for starch synthetase and phosphorylase.

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