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BIOSYNTHESIS OF STARCH IN BEAN CHLOROPLASTS

by

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ABSTRACT

Isolated bean, Phaseolus vulgaris (c.v. Kinghorn Special), chloroplasts were used to study the mode and regulation of starch synthesis. The synthesis of starch in the chloroplast was shown to have an absolute specificity for adenosine diphosphate glucose. Glucose incorporation was obtained from glucose-1-phosphate in the presence of adenosine 5'-triphosphate and was enhanced by 3-phosphoglyceric acid. No incorporation could be detected from UDP-glucose, sucrose or glucose-1-phosphate in the absence of adenosine 5'-triphosphate. 3-phosphoglyceric acid stimulated and inorganic phosphate inhibited the glucose transfer from glucose-1-phosphate and adenosine 5'-triphosphate. Thus all glucose seems to be incorporated via the adenosine diphosphate glucose pyrophosphorylase pathway. Both adenosine 5'-monophosphate and adenosine 5'-diphosphate inhibited starch synthesis from adenosine diphosphate glucose. Isolated chloroplasts lost their ability to synthesize starch when plants were kept in the dark for 18 hours regardless of the adenosine diphosphate glucose concentration. High concentration of citrate and bovine serum albumin or added amylose did not activate starch synthesis. The results suggest that in chloroplasts light regulation of the glucosyl transfer enzyme is an important factor in the control of starch synthesis.

Pulse chase experiments showed that labelled glucose in amylose decreased when chased with cold substrate as compared to controls. A significant portion of this decrease appeared in the amylopectin fraction indicating that amylopectin was formed from amylose. However, time course experiments showed that the rate of amylopectin synthesis is higher than that of amylose at the early stages of incubation, suggesting a certain degree of independent synthesis of the two fractions. High concentration of citrate increased the rate of amylopectin synthesis. The possibility of two pathways for amylopectin synthesis is discussed.

A method, using DMSO to extract starch from chloroplast suspensions, is reported. It is also demonstrated that the fractionation of starch into amylose and amylopectin by thymol and n-butanol is not as efficient as column chromatography.

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TABLE OF CONTENTS

PAGE

LIST OF FIGURES

LIST OF FIGURES - cont'd.

LIST OF TABLES

LIST OF ABBREVIATIONS

The following abbreviations are used in the present work:

DP	Degree of polymerization
G-1-P	α-D-glucose-1-phosphate
ADPG	adenosine-5'-diphosphate glucose
UDPG	uridine - " - " " "
ATP	adenosine- " -tri "
UTP	uridine - " - " "
ADP	adenosine- " -di "
AMP	adenosine- " -mono "
3-PGA	glycerate-3-phosphate
PEP	phosphoenolpyruvate
P_i	inorganic phosphate
PP_i	pyrophosphate
BSA	bovine serum albumin
Tris	tris (hydroxymethyl) aminomethane
Chl	chlorophyll
Phosphorylase	α-1,4-glucan:orthophosphate glucosyltransferase
ADP-glucose glucosyltransferase	ADP-glucose:α-1,4-glucan α-4-glucosyltransferase
ADP-glucose pyrophosphorylase	ATP:α-D-glucose-1-phosphate adenyl transferase
Q-enzyme	α-1,4-glucan:α-1,4-glucan
β-amylase	α-1,4-glucan maltohydrolase
Pullulanase	amylopectin 6-glucanohydrolase
Pyruvate kinase	ATP:pyruvate phosphotransferase

INTRODUCTION

The ultimate source of energy for all organisms is light, which is trapped by photosynthesis, and stored mostly in the form of carbohydrates. Starch represents the main reservoir of energy among carbohydrates. It is composed of two polymers amylose and amylopectin, both based on the same monomer, α-D-glucopyranose. Starch occurs in the form of discrete granules that are laid down inside small organelles, called plastids. Two types of plastids exist, amyloplasts and chloroplasts. The chief function of the former is to store starch. The chloroplasts, involved in photosynthesis, also synthesize "assimilation starch". However, this is a temporary product which remains in the plastids only as long as there is an excess of carbohydrate within the cell.

For many years, interest has centered on the enzymes involved in the hydrolysis of starch. It is only within the last two decades that enzymes of starch synthesis have been intensively investigated. The discovery of phosphorylase in potato tubers marked the beginning of progress in this area. The development of the concept of glucosyl transfer and the discovery of glucosyltransferases have been an important factor contributing to our modern views on pathways of biosynthesis of starch. Many aspects of starch biosynthesis are still poorly understood and await elucidation.

The majority of the information on the path of starch synthesis and the regulation of the enzymes involved is from experiments with isolated enzymes. A study using intact plastids should approximate *in vivo* conditions and in this manner the problem of extrapolating results from isolated enzyme systems should be avoided. Thus, this investigation attempted to study starch biosynthesis from glucose-1-P and ADP-glucose in isolated bean chloroplasts.

LITERATURE REVIEW

A. Early History

Starch is the most abundant reserve polysaccharide in the plant kingdom, and has been in the diet and of use to man since the dawn of civilization. Its early history and chemistry has been documented in numerous reviews (1,2,3,4,5). The practical use of starchy material for cementing papyrus strips has been dated at 3500 to 400 B.C. Perhaps the earliest report on starch preparation was written about 170 B.C. by Cato. Although starch was known to the ancients, glycogen was not discovered until about the middle of the nineteenth century. The basic observations of the microscopic and chemical properties of the starch granule also were made over 100 years ago. Leeuwenhoek previously observed starch granules with his microscope in 1719. Kirchoff obtained a sugar by acid hydrolysis of potato and wheat starches which was not as sweet as cane sugar. It was demonstrated by Saussure by dilute sulfuric acid hydrolysis that 100 parts of potato starch yielded 110 parts of sugar and that the sugar obtained was identical to grape sugar (glucose). Biot and Persoz followed the acid hydrolysis polarimetrically, precipitated the products of partial hydrolysis with alcohol and named them dextrins from their signs of rotation. They also noted that these products of incomplete hydrolysis gave a blue color with iodine. About 50 years later in 1882 the empirical

formula of D-glucose was established as $C_6H_{12}O_6$ by Salomon. He also concluded from his calculations on weight increase by acid hydrolysis that the molecular formula of starch must be $C_6H_{12}O_5$.

B. Chemical Properties of Starch

The existence of starch as a polymer was established when its low freezing point depression in aqueous solution was demonstrated by Brown and Morris in 1889 (6). The formula of starch as $(C_6H_{10}O_5)n$ was developed when Staudinger (7) and Carothers (8) demonstrated its covalent nature.

The introduction of paper partition, adsorption and ion exchange chromatography made possible the fractionation of the products of hydrolysis. Haworth and Machemer (9) demonstrated the existence of (1-4) and (1-6) glucosidic linkages in the starch molecules by methylation and subsequent hydrolysis. The configuration of (1-6) linkage was shown by the isolation of isomaltose (6-0-α-D-glucopyranosyl-D-glucose) by Wolfrom and O'Neill (10). Reducing and non-reducing end groups were determined by Hirst and his collaborators (11,12) with periodate oxidation.

It has long been recognized that starch is composed of two fractions possessing different physical properties. The two fractions were designated by Maquenne and Roux (13) as amylose and amylopectin. However complete clarification came only in the nineteen forties through the work of Schoch (14, 15) by the selective precipitation of amylose with polar

organic molecules. This was further improved by efficient disruption of the structure of granular starch with dimethylsulfoxide (16,17).

Generally, granules consist of about 70 to 90% amylopectin and only 10 to 30% amylose (18). Some starches, however, such as waxy corn or glutinous rice consist of less than 2% amylose (19). On the other hand starch of amylomaize and wrinkled pea contains about 50 and 80% amylose respectively (20).

Hollo et al. (21) found that sweet corn endosperm contains a water soluble polysaccharide (phytoglycogen) with a chemical structure indistinguishable from that of animal glycogen.

Haworth et al. (22) first suggested branching in unfractionated starch as early as 1937. Mayer et al. in 1940 (23, 24) proposed the linear and branched structure for amylose and amylopectin respectively. Their conclusions were based on the complete saccharification of amylose, and the formation of β-limit dextrin from amylopectin by β-amylase. Mayer et al. (25,26) showed that methylated amylose on hydrolysis yielded mainly (99.7%) 2,3,6-tri-O-methyl-D-glucose. The remaining (0.3%) tetramethylglucose, formed from non-reducing end groups, indicates a minimum polymerization of 300 glucose units. However, on methylation of amylopectin and separation of the methylated sugars after complete hydrolysis, they obtained approximately 96% trimethylglucose and 4% tetramethylglucose; a value corresponding to an average chain

length of about 27 glucose units. Since the molecular weight of amylopectin is in the 10^5 - 10^6 range they proposed a highly ramified structure for amylopectin (25,26). In addition the molecular sizes of amylose and amylopectin were confirmed by the method of periodate oxidation (10).

Since malt α-amylase mostly hydrolyzes amylose into six membered dextrins, Hanes (27) proposed a helical chain conformation for amylose with six glucose units per turn. Rundle et al. (28) using X-ray diffraction analysis of iodine-amylose complexes showed that one molecule of iodine is arranged linearly along the centre of each six membered unit providing further support for a six membered helical structure for amylose. Therefore they suggested that the blue coloring of amylose with iodine is due to the entrance of I_2 into this gyre, causing resonance in the helical conformation of the amylose molecule. A three fold helix has also been proposed from the X-ray diffraction diagrams obtained by Kreger (29). Recently Kainuma and French (30) proposed a model for potato B-starch which employs intertwined or double helices.

Various models are proposed in which the unit chains of approximately 25 glucose residues in the branched amylopectin may be arranged. These include the "laminated" structure by Haworth, Hirst and Isherwood (22), the "herring-bone" structure by Staudinger and Husemann (31), the "tree-like" structure by Meyer (32) and a revised tree-like structure by Whelan and his collaborators (33).

The above models were based on the recognition that amylopectin consists of three different types of chain, A, B and C, except the herring-bone where B chains are excluded. The outer most A chains are linked to the inner B chains which are linked to the C chain carrying the reducing end group. On the basis of enzymic degradations the tree-like structure is favored. In this model Meyer (32) proposed equal numbers for A and B unit chains. However Gunja-Smith et al. (33) have shown that only half of the B chains carry A chains.

Starch of higher plants is deposited as granular particles in organelles called plastids. Von Mohl in 1837 was the first to observe starch granules in the chloroplast (34). Sachs (35) demonstrated that these starch granules were the direct product of the assimilation of CO_2 in the light. Badenhuizen (36) and Salema and Badenhuizen (37) described two types of plastids containing starch. First, the chloroplasts, with transistory starch granules which remain small and whose shape is controlled by the space available. Secondly, the achlorophylous amyloplasts, found predominately in tubers and seeds and producing large reserve starch granules with genetically controlled shape.

Nägeli (38) intensively studied the formation of the layer arrangement of starch granules, and after examining over 1,200 different kinds of starches, stated that plants may be classified into natural groups according to the structure of their starches. Meyer (39) proposed that layers arise from diurnal variation in the biosynthesis of starch.

This was supported by Bakhuyzen (40) who showed that constant external factors during wheat growth produced starch granules without shell structure. However, Roberts and Proctor (41) showed that tubers from potato plants grown under constant light and temperature contain starch granules with a layer arrangement identical to that of granules formed under normal field conditions. Buttrose (42) compared the formation of layers in potato and wheat granules and concluded that in potato the shell formation (18.5 hour course and 2 hour fine cycle) is controlled by an endogenous mechanism, whereas in wheat (24 hour cycle) it is controlled by the environment.

Badenhuizen and Dutton (43) using potato tubers and Yoshida et al. (44) using beans demonstrated apposition growth of starch granules. That is carbohydrate supply within the cell but external to the granule is essential for the gradual addition of starch layers.

There is no evidence regarding the initiation of starch granules. However, formation of a coacervate from starch material which forms a gel and then solidifies to give the granule, has been suggested by Badenhuizen (45) and Erlander (46). Geddes and Greenwood (47) suggested that the nucleus or coacervate of starch material becomes surrounded by a protein monolayer which contains the starch synthesizing enzymes and remains associated with the expanding granule. This would also explain the apposition growth of the granule.

The relationship between the chemical composition of the starch granule and its shell structure has been studied by

many investigators. After the discovery of amylose and amylopectin (38), Nägeli's classical micellar theory was interpreted by Meyer et al. (23) and Frey-Wyssling (48). They suggested that the shells of the granules resulted from the radial arrangement of the mixed amylose and amylopectin crystals. Although the radial arrangement has been confirmed (49), the necessity for the mixed crystals of amylose and amylopectin was disproved by Buttrose (50). He demonstrated that waxy starch, consisting almost entirely of amylopectin, shows a distinct layered structure.

Katz and Derksen (51) using X-ray diffraction analysis observed two distinct X-ray patterns for starch granules, A and B, characteristic for cereal and tuber starches respectively. They also showed that higher temperatures converted B pattern starch to A pattern starch. Hizukuri and Nikuni (52) noted that mixtures of A and B type starches show different types of patterns depending on their ratio and that some mixtures show a C pattern, which is characteristic for bean and sweet potato starches. They concluded that A and B are basic types and C is an intermediate.

C. Enzymes Involved in Starch Metabolism

1. Enzymes of starch hydrolysis

The synthesis and breakdown of starch and glycogen are fundamental processes occurring in most living organisms. Probably the first enzymic conversion of starch was used by Abu Mansur (53), an Arabian pharmacologist, at about 975 A.D.

He used saliva to convert starch to "artificial honey" which was used for treating wounds. Hydrolyzing enzymes which convert starch to reducing sugars were detected over a century ago in various plants (54). Balls et al. (55) first obtained β-amylase in crystalline form from sweet corn. Beta-amylase is an exoenzyme, which hydrolyzes only the α-(1 → 4) glucosidic linkages and is unable to bypass α-(1 → 6) linkages.

Alpha-amylase, also present in many plants, is of importance for the conversion of starch to reducing sugars during seed germination. It was first crystallized from germinating barley endosperm by Swimmer and Balls (56). This enzyme attacks both amylose and amylopectin randomly throughout the molecule by the fission of α-(1 → 4) linkages.

R-enzyme which splits the α-(1 → 6) branch points was discovered and isolated from bean leaves by Hobson et al. (57). They also showed that the R-enzyme only partially debranches amylopectin. Yokobayashi et al. (58) discovered isoamylase; the first enzyme which totally debranches amylopectin and glycogen, but is unable to remove A chains with only 2 glucose units from β-limit dextrins. Gunja-Smith et al. (33) used this property to construct a revised model for the structure of amylopectin previously proposed by Meyer et al. (32).

Peat et al. (59) discovered D-enzyme in potato, which catalyzes neither net synthesis nor net breakdown of α-(1 → 4) glucosidic linkages, but merely catalyzes a reversible shuttle of these linkages from one chain to another. Hence

the name "disproportionation" or D-enzyme. This enzyme can split only the α-(1 → 4) linkages and transfer the portion of the chain having the non-reducing end (60,61). It was also shown that D-enzyme does not act on maltose. However, it does reversibly synthesize maltopentaose and maltoheptaose from two molecules of maltotriose and maltotetraose respectively with the liberation of one glucose molecule in each case. Since phosphorylase is unable to shorten maltodextrin chains to fewer than four glucose units (62), it was suggested that one of the functions of D-enzyme is to synthesize substrate for phosphorylase during breakdown of starch (59).

2. Enzymes of starch biosynthesis

Until about 1935 only hydrolyzing enzymes were known to degrade oligo- and polysaccharides, and it was assumed that these enzymes under favorable conditions were involved in the process of synthesis. Studies on the in vitro biosynthesis of polysaccharides began in 1936 when Cori and Cori (63) found an enzyme, in rabbit skeletal muscle, which degraded glycogen and which was of an entirely different nature than amylase. They showed that inorganic phosphate was required for the reaction and that the product was α-D-glucose-1-phosphate. This enzyme, termed phosphorylase, was also found to reverse the reaction and form glycogen, in the presence of a primer from glucose-1-P (Cori ester) in the following manner (64);

$$\alpha\text{-D-glucose-1-P} + (\text{D-glucose})_n \rightleftharpoons (\text{D-glucose})_{n+1} + P_i$$

Subsequently, Hanes (65) found similar enzymic activity in higher plants (peas, potatoes) which synthesized starch instead of glycogen. Doudoroff et al. (66) developed the concept of transglucosylation which is considered as the main mechanism for the biosynthesis of oligo- and polysaccharides. The enzyme was first crystallized from potato by Baum and Gilbert (67). In the catabolic reaction phosphorylase breaks down starch and glycogen from the non-reducing end of the molecule, attacking only α-(1 $\rightarrow$ 4) linkages, and is not able to bypass the α-(1 $\rightarrow$ 6) branch points.

It was shown by Cori and Cori (68) that the reaction equilibrium is not affected by the concentration of $(\text{D-glucose})_n$ within wide limits and therefore depends on the ratio of glucose-1-phosphate to inorganic phosphate. Hanes (65) calculated the equilibrium constant to be K = 3.1 at pH 7. Since the ΔF° of hydrolysis of the glucosidic linkage and the phosphate ester linkage are -4300 and -4800 respectively, the overall reaction is readily reversible (70). However, Ewart et al. (69) found a high P_i/glucose-1-P ratio in the living bark of black locust and concluded that starch phosphorylase is responsible for the degradation of starch rather that its synthesis. Stocking (70) could not detect phosphorylase in chloroplasts, where starch synthesis occurs, using histochemical techniques. Also Mommaerts et al. (71), Larner and Villar-Palasi (72) and Schmid et al. (73) reported that skeletal muscle having enzyme defects involving the inability to break down glycogen lacks phosphorylase, although

there is excessive glycogen deposition in the tissues.

In spite of the arguments against phosphorylase as a synthesizing enzyme, there is some indication for a direct involvement in starch and glycogen synthesis. Illingworth et al. (74) demonstrated de novo synthesis of amylose in the complete absence of primer by muscle and potato phosphorylase. Tsai and Nelson (75) have obtained four phosphorylases from maize endosperm and two of these apparently do not require a primer for synthesizing amylose like polysaccharides. They concluded that phosphorylase may be involved in the de novo formation of starch. Slabnik and Frydman (76) have also demonstrated the presence of a phosphorylase in potato tubers that can catalyze polysaccharide synthesis in the absence of primer. However, Abdullah et al. (77) and Kamogawa et al. (78) have shown, from different tissues, that either the phosphorylase preparation or the substrate, glucose-1-P conceivably could contain sufficient amount of oligosaccharide contaminant to initiate polyglucan synthesis.

The Nobel prize winning discovery by Leloir and Cardini in 1957 (79) of a new enzymic mechanism of glycogen biosynthesis involving uridine diphosphate glucose (UDP-glucose) and UDP-glucose-α-1,4-glucan α-4-glucosyltransferase initiated a new era in polysaccharide biochemistry. The involvement of the UDP-glucose glucosyltransferase in starch synthesis and its association with bean and potato starch granules was also shown by Leloir's group (80,81). Subsequently the enzyme's

presence in numerous other plants was demonstrated (82-84). Leloir et al. (81) studied the reaction mechanism and showed the linkage synthesized is α-(1 → 4). The reaction is as follows:

$$\text{UDP-glucose} + \text{primer} \longrightarrow \text{UDP} + \text{glucosylprimer}$$

Since the ΔF^{o} of hydrolysis of UDP-glucose is -7600 cal. and that of the α-(1 → 4) linkage of glucose polymer is -4300 cal. the overall reaction favors synthesis. (K'eq being 250 at pH 7.5 in contrast to K'eq = 3 for the phosphorylase system) (85).

Recondo and Leloir (86) using chemically synthesized ADP-glucose reported that ADP-glucose is about ten times more efficient as a glucose donor than UDP-glucose in corn endosperm. They found that formation of starch from UDP-glucose was inhibited by ADP-glucose, ATP, ADP and AMP whereas starch formation from ADP-glucose was not inhibited by UDP-glucose, UTP, UDP or UMP. Similar results were reported by Frydman (83), Akazawa et al. (87) using starch granules of rice, potato tubers and sprouts, seeds of wrinkled pea, red and white corn and waxy corn. However, Leloir (88) has suggested that probably both nucleotide sugars are involved in equal portions because, although UDP-glucose reacts more slowly, its concentration is approximately five to ten times higher than ADP-glucose in tubers and seeds.

Turner (89) followed the levels of both UDP-glucose pyrophosphorylase and ADP-glucose pyrophosphorylase during

the growth of wheat grain. He found that although the activity of UDP-glucose pyrophosphorylase was much higher than the activity of ADP-glucose pyrophosphorylase, the onset of rapid starch formation was associated with a sharp rise in the latter's activity. He suggested that ADP-glucose is the main substrate for starch synthesis in wheat grain.

A soluble enzyme that synthesizes phytoglycogen from UDP-glucose and ADP-glucose was reported by Frydman and Cardini (90). This enzyme was isolated from sweet corn endosperm, required primer for activity and was present in the 100,000 x g supernatant of the kernel extract. Later they (91) demonstrated that while amylopectin, phytoglycogen and malto-oligosaccharides were glucosyl acceptors for the enzyme, amylose, starch and starch granules were ineffective as primer.

In some plants, particularly cereals, photosynthetically fixed carbohydrates are translocated in the form of sucrose (92). De Fekete and Cardini (93) have obtained a transfer of ^{14}C from sucrose to starch by an enzyme preparation from corn endosperm. In their report, evidence is given for the dominant role of ADP-glucose in sucrose-starch conversion. Moreover, they proposed two sequences for glucose incorporation into starch.

Sucrose $\rightleftharpoons$ ADP-glucose or UDP-glucose $\longrightarrow$ starch

Sucrose $\rightleftharpoons$ UDP-glucose $\rightleftharpoons$ glucose-1-P $\rightleftharpoons$ ADP-glucose $\rightarrow$ starch

They have postulated that UDP-glucose may have a primary role in the initial step of sucrose-starch transformation. Similar

results were obtained by Murata et al. (87,94) with enzyme systems of ripening rice grains. From Km determinations, they concluded that sucrose breakdown proceeds through the reverse of UDP-glucose-sucrose transglucosylation rather than through ADP-glucose-sucrose transglucosylation. They also pointed out that the formation of glucose-1-P from UDP-glucose is physiologically unfavourable since enzyme reactions releasing PP_i are considered irreversible due to the hydrolysis of PP_i (95).

In the chloroplast the dominant role of ADP-glucose as substrate in starch formation, known as "assimilation starch" (34), has been shown by Murata and Akazawa (96) using starch granules prepared from soybean leaf chloroplasts. They demonstrated that leaf starch synthetases are totally specific for ADP-glucose. Doi et al. (97) and Ghosh and Preiss (98) found a soluble enzyme system in spinach chloroplasts which transfers glucose only from ADP-glucose or deoxy-ADP-glucose to a primer to form α-(1 → 4) glucosyl linkages. Frydman and Cardini (99) also reported the presence of ADP-glucose glucosyltransferase in tobacco leaves.

There have been several attempts to demonstrate de novo starch synthesis. Gahan and Conrad (100) obtained an ADP-glucose-α-1,4-glucan α-4-glucosyltransferase from extracts of Aerobacter aerogenes that synthesized glycogen without added primer. Preiss and his collaborators demonstrated the presence of multiple forms of soluble ADP-glucose glucosyltranserase from spinach leaves (101,102), waxy maize endosperm

(103) and potato tubers (104). These forms had many properties in common, including high affinity for ADP-glucose, pH optima and the use of α-(1 $\rightarrow$ 4) glucans as primers.

Also it was shown that one of the transferases did not require primer and that unprimed activity was stimulated over 1,000 fold by BSA and high concentration of citrate. They suggested that transferases not requiring a primer, are responsible for de novo starch synthesis. However Tsai et al. (105) could not detect the presence of ADP-glucose pyrophosphorylase and soluble starch synthetase in maize kernels between 8 and 10 days after pollination when some starch was being synthesized. These authors proposed that starch synthesis in the early stages proceeds by a different pathway than is functional later. On the other hand, Ozbun et al. (106) demonstrated that ADP-glucose pyrophosphorylase and soluble ADP-glucose glucosyltransferase are present at all stages of starch synthesis and can therefore account for the amount of starch synthesized. It is interesting to note that Krisman (107) presented evidence for the involvement of protein or protein bound oligosaccharides in the initiation of glycogen (the animal counterpart of amylopectin) synthesis from UDP-glucose. Nevertheless, there is substantial evidence for the presence of oligosaccharides as contaminants either in the substrate or in the enzyme preparations (77,78, 108). As such, de novo starch synthesis remains a matter of controversy.

The conversion of linear chains of α-(1 → 4) linked D-glucose residues into branched chains with α-(1 → 6)-D-glucosidic linkages is catalyzed by branching enzymes. An enzyme involved in the formation of α-(1 → 6) linkages was first demonstrated in muscle by Cori and Cori (109). They called it "branching factor" and showed that it synthesized glycogen from glucose-1-P in combination with phosphorylase. The plant branching enzyme was first found in potato and named Q-enzyme by Haworth et al. (110). Subsequently, it was crystallized from the same plant by Gilbert and Patrick (111). It was shown by Barker et al. (112) that in vitro, the potato branching enzyme converts amylose, formed by incubating phosphorylase and glucose-1-P, into amylopectin-like molecules. The product resembled natural amylopectin in terms of average chain length, extent of β-amylolysis, iodine staining and its solubility. However, according to Fuwa (113) the phosphorylase / Q-enzyme ratio is not associated with the varied ratios of amylose to amylopectin in waxy and non-waxy maize starch. This was confirmed by Aimi and Murakami (114) using rice. Drummond (115) and Drummond et al. (116) compared the length distribution of unit chains in synthetic and natural amylopectins, obtained by debranching with pullulanase and fractionating on Sephadex G-50. Neither amylopectin synthesized from amylose by Q-enzyme nor amylopectin synthesized by the combined action of Q-enzyme and phosphorylase on α-glucose-1-P were similar to the natural amylopectin. They concluded that synthesis of amylopectin in vivo

operates by a different mechanism, and that amylopectin may be synthesized by the simultaneous action of the chain lengthening and chain branching enzymes. Previously it was shown (117) that glycogen, formed by incubating UDP-glucose glucosyltransferase UDP-glucose and Q-enzyme, more nearly resembled natural glycogen than glycogen synthesized with phosphorylase, glucose-1-P and Q-enzyme. Thus it was concluded that phosphorylase has no major involvement in glycogenesis.

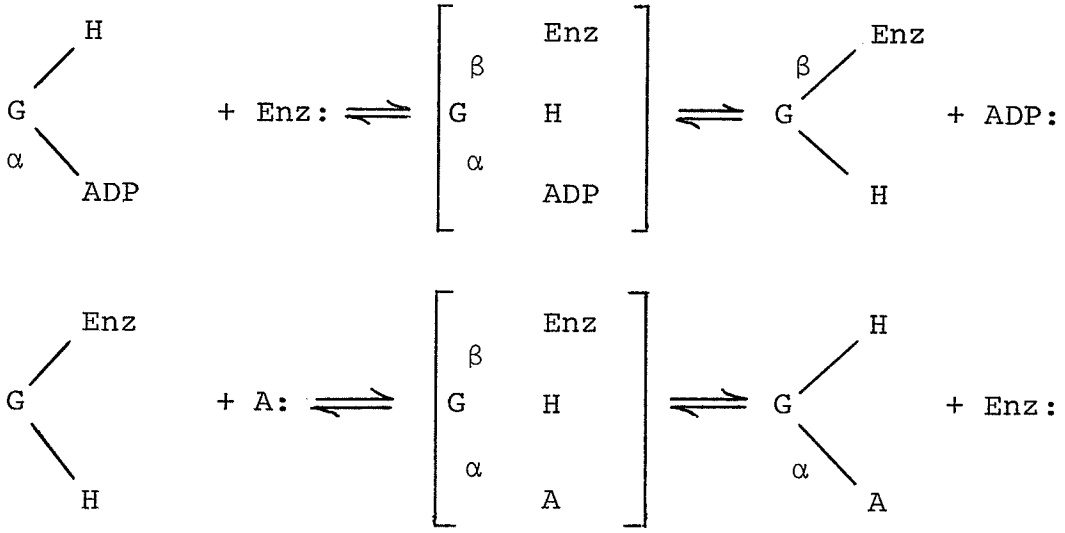
An enzyme preparation from sweet corn was shown (118) to act differently on amylose and amylopectin (phytoglycogen). Since the reactions have different pH and temperature optima, and since citrate activates only the amylose reaction, it was concluded that sweet corn contained two branching enzymes. Subsequently, the presence of the two enzymes was confirmed (119) by their separation on Sephadex columns. On the other hand it was shown that unlike sweet corn, potato contains only one Q-enzyme which catalyzes both branching of amylose and the introduction of additional branch points into amylopectin (115,116). The transfer of branched chain segments by the same enzyme was also suggested. These reports confirm Lavintman's suggestion (118) that genotypes, lacking phytoglycogen, contain only one type of branching activity.

Drummond (115) and Drummond et al. (116) demonstrated by the simultaneous action of Q-enzyme and pullulanase on amylose of DP 260 that the lower limit for efficient transglucosylation is DP 35-40. They also examined the mode of

chain transfer of Q-enzyme on amylose by incubating Q-enzyme and a mixture of amylose of DP 260 and 48. Chromatography on Sephadex columns revealed that inter-chain transfer occurred. However, intra-chain transfer can not be precluded.

3. Biosynthesis of nucleotide sugars

Nucleoside diphosphate glucoses are important intermediates in starch biosynthesis of higher plants. UDP-glucose was the first nucleotide sugar to be isolated by Leloir (120) in 1951. Its structure was established through the work of Kenner et al. (121) and Leloir and Cardini (122). A theory of transglucosylation from UDP-glucose postulated by Koshland (123) is based on the Walden inversion. Since nucleotide sugars are α-glucosides, he proposed a double inversion to explain the retention of α-(1 → 4) polyglucan synthesis.



It should be noted that in the synthesis of α-(1 → 4) polyglucans a glucosyl acceptor (A) is required for the catalysis of the reaction.

Natural ADP-glucose, identical to synthetic ADP-glucose (121), was identified first in Chlorella pyrenoidosa extracts (124). Subsequently it was isolated from sweet corn by Recondo et al. (125) and from rice grains by Murata et al. (126).

The first enzymic synthesis of UDP-glucose was demonstrated by Munch - Peterson et al. (127) with yeast extract. They showed that the enzyme catalyzes UDP-glucose formation from glucose-1-P and UTP with the simultaneous liberation of pyrophosphate, derived from the two terminal phosphates of UTP.

$$\text{UTP} + \alpha\text{-glucose-1-P} \longrightarrow \text{UDP-glucose} + PP_i$$

Since the equilibrium constant approximates unity it was postulated that the reaction is freely reversible. However, the reaction may be driven to the right due to the hydrolysis of the liberated pyrophosphate (95).

Pyrophosphorylases are widely distributed in nature. Enzymic synthesis of ADP-glucose by ADP-glucose pyrophosphorylase has been demonstrated by Espada (128) with wheat flour.

The presence of the enzyme was also reported in rice grains (126,87) spinach chloroplasts (96,129) and starchy maize endosperm (130,131). Preiss et al. (132) demonstrated

the presence of two different ADP-glucose pyrophosphorylases in starch maize and in shrunken-2 mutant maize seeds. One was present in the embryo tissue, the other was in the endosperm, and their kinetic properties were quite different.

D. Regulation of Starch Biosynthesis

Starch of higher plants and algae, and glycogen of mammals and bacteria can be considered as end products of energy metabolism. Starch of chloroplasts, resulting from photosynthetic carbon assimilation appears to have a higher turnover compared to starch of storage organs such as tubers, roots, and grains. The specific factors governing the biosynthesis of starch and its physical and chemical nature, are a matter of controversy.

The type and site of control is different for mammalian and plant starch biosynthesis (133). Leloir et al. (134) found that glucose-6-P stimulated UDP-glucose glucosyltransferase of rat liver. This is in contrast to what is observed in algal and plant systems, where the activity of α-(1 $\rightarrow$ 4) glucan synthetase is not stimulated by glucose-6-P or any other glycolytic intermediates (83,126,98). Ghosh and Preiss (98) have shown that ADP is a competitive inhibitor for ADP-glucose transglucosylase in spinach chloroplasts. Since the activity of ADP-glucose pyrophosphorylase is lower than the transglucosylase activity, they concluded that synthesis of ADP-glucose is most likely the limiting reaction in the synthesis of starch in chloroplasts.

De Fekete and Cardini (93) have postulated a control for the level of ADP-glucose in sweet corn endosperm by the enzyme ADP-glucose pyrophosphorylase. Ghosh and Preiss (129) have shown that ADP-glucose pyrophosphorylase was stimulated by a number of glycolytic intermediates in spinach chloroplast. The highest stimulation (about 50 fold) was obtained by 3-PGA, and to a lesser extent by fructose-6-P (10 to 30 fold). Sanwal et al. (135) reported that the concentration of 3-PGA required for 50% maximal activation was 7 uM for the barley enzyme and 370 uM for the sorghum leaf enzyme. Kinetic studies have indicated that 3-PGA increased the apparent affinity of the enzyme for ATP and glucose-1-P, and increased the V_{max} for ADP-glucose synthesis (133,136). Similar results were obtained by Dickinson and Preiss (130) with enzyme preparations from maize endosperm. They also showed that the hyperbolic 3-PGA saturation curve became sigmoidal in the presence of the inhibitor, inorganic phosphate. Since this is characteristic for allosteric enzymes (137,138) they suggested that regulation of starch biosynthesis in non photosynthetic tissue also occurs at the level of ADP-glucose synthesis.

Inorganic phosphate was shown to be an effective inhibitor of ADP-glucose pyrophosphorylase in a number of plants and algae (129,135,133). There is a great variation in phosphate concentration required for inhibition. Fifty percent inhibition of the barley and sorghum leaf enzymes required a concentration of 20 and 200 uM phosphate respectively (135).

However, in all cases phosphate inhibition could be antagonized by 3-PGA. Inhibition of ADP-glucose pyrophosphorylase from spinach chloroplasts by phosphate concentration of 0.5 mM was reversed by 1 mM 3-PGA (133).

Therefore Ghosh and Preiss (129) concluded that during CO_2 fixation the accumulation of 3-PGA increases the concentration of ADP-glucose by stimulation of the ADP-glucose pyrophosphorylase, thereby resulting in an increase in starch synthesis which would then be at the pyrophosphorylase level rather than, as in glycogen, at the transglucosidase level (139).

Ghosh and Preiss (136) using spinach chloroplasts obtained a 60 fold increase in ADP-glucose synthesis when the ratio of 3-PGA to glucose-1-P was 2. On the other hand, Dickinson and Preiss (131) showed that ADP-glucose pyrophosphorylase activity of maize endosperm was stimulated about 5 fold when the activator concentration was 20 times that of glucose-1-P.

Farineau (140) found that the physiological concentration of inorganic phosphate in maize leaves is higher than the level of 3-PGA. In the light the concentration of inorganic phosphate was approximately 3 to 3.5 umoles per mg chlorophyll, while the 3-PGA concentration was 0.15 to 0.20 umoles. Santarius and Heber (141) found that inorganic phosphate decreased rapidly (ca. 30%) in chloroplasts from dark to light, and slowly increased in a subsequent dark period. They also investigated changes in the concentration of pyruvate, AMP, ADP and ATP and concluded that the controlling

factor in the inhibition of glycolysis by light is the ratio of ATP to total adenylates rather than a drop in orthophosphate. Further they showed that spinach chloroplasts, equipped with sufficient adenylate kinase, convert AMP rapidly to ADP. Heber (142) did not find significant fluctuations in 3-PGA levels between light to dark transitions. This was verified by Bassham (143) who in addition, showed that the concentration of ATP was at least as high in the dark as in the light.

Mechanisms controlling the fine structure of starch granules, as well as the amount and chain lengths of synthesized amylose and amylopectin are not clearly understood. Several theories have been developed to explain how amylose and amylopectin are synthesized.

Whelan (144) proposed a compartment theory whereby amylose and amylopectin are synthesized independently. The semipermeable membrane which separates the two processes is composed of amylopectin. While amylose is synthesized by ADP-glucose, amylopectin is formed by the concurrent action of phosphorylase and Q-enzymes. According to this theory the action of Q-enzyme is limited to amylose, formed by starch synthetase, due to the semipermeable effect of amylopectin. He suggested that, if the starch synthetase becomes surrounded by amylopectin, Q-enzyme is unable to penetrate to the amylose, whereas small molecules such as ADP-glucose can pass through. Electron-microscopic examination has provided evidence for membranes in starch granules (145). However, Frydman (83) found ADP-glucose trans-

glycosylase activity in sweet corn, composed almost entirely of amylopectin. Moreover, it has been shown that amylose itself contains limited branching (146) and that this branching increases with growth (147).

Erlander (148) proposed that amylopectin is produced from phytoglycogen by a hypothetical debranching enzyme. He also postulated that plant glycogen is formed by the conversion of sucrose into starch.

Geddes and Greenwood (47) and Geddes (149) have suggested that during peaks of starch production the Q-enzyme is saturated around the granule. Consequently, not all α-(1 → 4) chains are converted into amylopectin. In this case a certain amount of starch is deposited as amylose depending on the overall rate of starch synthesis. With this theory they provide an explanation for the relative increase in amylose, compared to amylopectin, which occurs with increase in granule size and with time of growth.

Previous experiments, in which glucose-1-^{14}C was fed to intact wheat plants, indicated that amylopectin was formed from amylose in the endosperm (150).

MATERIALS AND METHODS

Plant Material

Beans, Phaseolus vulgaris (c.v. Kinghorn Special) grown on soil for 2-3 weeks on a regime of 16 hours daylight, were used as a source of chloroplasts.

Chemicals

ADPG, UDPG, G-1-P, ATP, UTP, 3-PGA, ADP, AMP, sucrose and amylose were purchased from Sigma Chemical Company. ^{14}C labelled chemicals were purchased from New England Nuclear Corporation.

Isolation of Chloroplasts

About 70 gm of fully grown primary leaves were harvested in the morning (after 4 hours illumination) and homogenized in a chilled Waring Blendor for 30 seconds at maximum speed. Approximately 3 ml of chilled homogenizing solution was used for each gram (fresh weight) of leaves. The solution consisted of 0.5 M tris-HCl (ph 8.4), 0.33 M sorbitol, 0.001 M $MgCl_2$ and 0.2% BSA. For incubations at pH 6.8, 0.5 M tris-maleate (pH 6.8) was used in place of 0.5 M tris-HCl. The slurry was filtered through two layers of fine nylon cloth. The filtrate was centrifuged for 3 min. at 50 x g to remove the debris. The supernatant was centrifuged for 2 min. at 800 x g to collect the first crude plastid pellets. The pellet contained two layers; the bottom layer containing starch and the remaining debris, and the top layer containing

the plastids. The chloroplasts were separated from the bottom layer with fresh homogenizing medium and the differential centrifugation was repeated. The resulting pellet was stored in concentrated form (about 5 mg chlorophyll/ml) at 4^{o} for up to 1 hr. Before being incubated the pellet was resuspended in isolating medium so as to give the required chlorophyll concentration. About 75-80% of the plastids were highly refractive (Class I) under phase contrast microscopy.

Where the effect of dark treatment was tested the isolation of chloroplasts and subsequent incubations were carried out under a low intensity green safe-light.

Chlorophyll Determination

Chlorophyll concentration was determined spectrophotometrically in an 80% acetone extract of chloroplasts using the method of Mackinney (151).

Assay of Glucose Incorporation

Incorporation from ADP-glucose. Assay A. The reaction mixture contained 75 nmoles of ADP-glucose -^{14}C (400 dpm/nmole), 82.5 umoles sorbitol, 125 umoles tris-HCl buffer (pH 8.4), 0.25 umoles $MgCl_2$, 0.2% BSA and chloroplasts (1 mg chlorophyll/ml) in a final volume of 0.25 ml. The incubations were carried out in glass centrifuge tubes in a water bath at 30 C. The reaction was terminated by adding 3 ml of 80% methanol.

Incorporation from ADP-glucose. Assay B. Reaction mixture contained 900 nmoles ADP-glucose -^{14}C (3.2×10^5 dpm/

umole), 495 umoles sorbitol, 750 umoles tris-HCl buffer (pH 8.4), 1.5 umoles $MgCl_2$, 0.2% BSA and chloroplasts (1.5 mg chl/ml) in a final volume of 1.5 ml. The changes in the amount of substrate and other additives are listed in the text. The incubations were carried out under conditions described in Assay A. The reaction was terminated by adding 5 ml of 80% methanol.

Incorporation from glucose-1-P. Assay C. The reaction mixture contained 250 nmoles of glucose-^{14}C-1-P (1200 dpm/nmole), 0.5 umoles of ATP or UTP, 5 umoles of 3-PGA, 165 umoles of sorbitol, 250 umoles of tris-HCl buffer (pH 8.4), 0.5 umoles of $MgCl_2$, 0.2% BSA and chloroplasts (2 mg chlorophyll/ml) in a final volume of 0.5 ml. The incubations were carried out and terminated under conditions described in Assay A. Where chloroplasts were illuminated, incubation was in a water bath at 30 C at a light intensity of 100,000 lux.

Extraction of Starch

A. After the termination of reactions, 3 mg of carrier starch was added to each tube, followed by centrifugation for 5 min. at 1000 x g. The pellet was washed twice with 3 ml of 80% acetone and four times with 3 ml of 0.5 M acetic acid-methanol (1:1). In the last washing no radioactivity or sugar was detectable in the supernatant. From the remaining pellet, starch was solubilized with 1.5 ml of dimethylsulfoxide at 55 C with occasional mixing, then centrifuged for 20 min. at

10,000 x g. The extraction was repeated twice and the dimethylsulfoxide containing the starch was combined. Starch was precipitated overnight with 2 volumes of ethanol. A small amount of saturated NaCl solution was added to aid flocculation. The precipitated starch was washed with ethanol, the wet precipitate solubilized in 1 ml of boiling 1 N HCl and hydrolyzed in a boiling water bath for 2 hours. An aliquot was taken and mixed with aquasol to measure the radioactivity with a liquid scintillation counter.

B. For incubations carried out to study synthesis of amylose and amylopectin (Assay B), the extraction procedure was slightly modified. Carrier starch (4 mg) was added to each tube after the reaction was terminated. The suspension was centrifuged for 5 min. at 1000 x g and the pellet was washed three times with 6 ml of 80% acetone and four times with 6 ml of 0.5 M acetic acid-methanol (1:1). The washing procedure was stopped when no radioactivity or sugar was detectable in the supernatant. Starch was solubilized from the pellet with 2 ml of DMSO at 55 C with occasional mixing, and centrifuged for 20 min. at 10,000 x g. The extraction was repeated twice and the extracts were combined. Starch was precipitated over-night with 2 volumes of ETOH and 50 ul of saturated NaCl solution. The precipitate was washed with ethanol and the air dried starch was resolubilized in 0.5 ml of DMSO at 35 C. After solubilization the volume was made up to 1.5 ml with a solution of 0.1 M NaCl, and 0.005 M EDTA. An aliquot (0.1 ml) was removed for carbohydrate and radio-

activity determination and 1 ml for column chromatography.

Carbohydrate Determination

Total starch was determined by the phenol-H_2SO_4 test according to Hodge and Hofreiter (152). When amylose concentration was measured by amperometric titration the method of Williams was adapted (153).

Fractionation of Starch Components

1. Chromatography of starch

The method was essentially similar to that described by Metheson (154) with slight modifications. One ml of starch suspension [15 mg in DMSO-eluent (1:3)] was applied to a column of 4% sepharose gel (Pharmacia sepharose 4B-200, approximate exclusion limit for polysaccharides 5 x 10^6 daltons). The column was eluted with a solution containing 0.1 M NaCl, 0.005 M EDTA and 0.02% NaN_3. The effluent solution was fractionated into 7 ml portions and about 40 fractions were collected. In order to identify amylose and amylopectin an aliquot was removed from each fraction, mixed with 0.02% iodine and 0.2% KI in 0.5 N HCl solution and the absorption read at 560 and 600 nm corresponding to amylopectin and amylose respectively. Recovery of amylose and amylopectin was 90 to 95% as tested by amperometric titration (153) and the phenol-sulfuric acid test. No amylose was detected in the amylopectin fraction. The radioactivity of each fraction was measured with a liquid scintillation counter.

2. Differential precipitation of amylose with thymol and n-butanol

About 15 mg of ethanol precipitated and washed DMSO-soluble starch, extracted from chloroplast suspensions, was redissolved in 4 ml of DMSO then precipitated again with ethanol and sodium chloride as described previously. The starch was then washed with ethanol and, while still moist, was solubilized in 2 ml of boiling water with vigorous mixing. After complete solubilization the starch solution was cooled to room temperature and adjusted to 6 ml in graduated centrifuge tubes. An aliquot (0.5 ml) was removed for total starch and radioactivity determinations. The tubes were then placed in a 60 C water bath and 0.1 ml of thymol solution (10% in 95% ethanol) was added with mixing. After complete stirring the tubes were covered with glass marbles and cooled slowly in an insulated 60 C sand bath. It was cooled to room temperature in about 24 hours. The tubes were left in the sand bath for approximately 24 hours then transferred to a cold room (4 C) for an additional 24 hours. The amylose-thymol complex was obtained by centrifugation at 10,000 x g for 30 minutes. The supernatant containing the amylopectin was decanted and saved. The amylose-thymol complex was resolubilized in 3 ml of boiling water and after solubilization with vigorous mixing 0.7 ml of n-butanol was added to each tube. The amylose suspension was stirred, covered with glass marbles and placed in an 80 C sand bath. After the sand was cooled to room temperature, in about 24 hours, the tubes were

transferred and kept in a cold room (4 C) for 10 hours. The amylose-n-butanol complex was collected by centrifugation at 15,000 x g for 60 minutes at 4 C. If the amylose was to be used for column chromatography, it was solubilized as described in Section B of the extraction of starch; otherwise, it was hydrolyzed in 1 N HCl for total carbohydrate and radioactivity determinations. The supernatant (mother liquor) either was saved separately or was combined with the thymol supernatant. If the branched fractions were chromatographed on a column they were first heated in a drying oven to drive off the thymol and n-butanol and the remaining fractions were freeze-dried. The dried amylopectin was then solubilized as described above. If only carbohydrate concentration and radioactivity was determined the branched fractions were hydrolyzed in 1 N HCl.

Hydrolysis of Starch by Pullulanase and β-amylase

The starch, precipitated by ethanol from DMSO, was solubilized with 1 ml of boiling sodium acetate buffer (0.1 M), pH 5.5, and after cooling to room temperature the solution was adjusted to 1 ml. Aliquots (0.2 ml) were taken to determine total carbohydrate and radioactivity. An additional 75 ul was used for thin-layer chromatography. The remaining solution was incubated at 37 C after adding 50 ug of β-amylase and 100 ug of pullulanase. The incubations were carried out with continuous shaking for 18 hours. The reaction was stopped by transferring the tubes into a boiling water bath

for 5 minutes. The samples were dried, then solubilized in 0.3 ml of 70% DMSO and 75 ul was used for thin-layer chromatography.

The residues remaining after DMSO extraction, were suspended in 1 ml of boiling sodium acetate buffer, mixed and centrifuged. An aliquot (75 ul) of the supernatant was used for chromatography. The pellet was resuspended in the remaining supernatant and incubated with β-amylase and pullulanase as described previously. After stopping the reaction, the solution containing the residue was centrifuged at 2,000 x g for 10 minutes and the supernatant was saved. The centrifugation was repeated with water and the combined supernatants were concentrated in a desiccator at room temperature to 0.5 ml. An aliquot of 75 ul was used for thin-layer chromatography.

Thin-Layer Chromatography

The enzyme digest was fractionated by ascending thin-layer chromatography using 20 cm x 40 cm glass plates, coated with a 0.5 mm layer of Kieselguhr G absorbent. The solution was applied in a 2 cm narrow band. The plate was developed sequentially twice in two solvents, containing n-butanol-pyridine-water. The plate was first developed to a height of approximately 28 cm, using the above solvent system (6:4:3). The plate was then air-dried and developed to a height of 35 cm with a solvent composition of 13:4:3. After developing and drying the plates, bands were located with iodine vapour

and the maltose was identified by co-chromatography. The bands were scraped into a test tube, hydrolyzed with 1 N HCl then washed into a graduated centrifuge tube. The volume was made up to 3 ml from which 2.5 ml was used to measure the radioactivity.

Determination of the Packed Volume of Chloroplasts

The determination is based on the selective permeability of the chloroplasts for inulin (155) and phenylalanine (156). Inulin-^{3}H was used as a non-penetrating, and phenylalanine-^{14}C as a freely penetrating solute. The packed volume of the chloroplasts was calulated in microliters per milligram chlorophyll from the relationship between the specific activities of the two solutes in the supernatant and in the pellet. Values obtained by the three different reaction mixtures were compared.

Two ml of plastid suspension (0.5 mg chlorophyll/ml was added to 50 ul of the following reaction mixtures:

Inulin-Phenylalanine incubation:	100 ul	Inu	- ^{3}H	
	35 "	Pha	- ^{14}C	(0.9 nmoles)
	25 "	ATP		(1,125 nmoles)
	40 "	H_2O		
Inulin-glucose-1-P incubation:	100 ul	Inu	- ^{3}H	
	40 "	G-1-P	- ^{14}C	(0.9 nmoles)
	25 "	ATP		(1,125 nmoles)
	35 "	H_2O		
Inulin-ADP-glucose incubation:	100 ul	Inu	- ^{3}H	
	40 "	ADPG	- ^{14}C	(0.9 nmoles)
	25 "	ATP		(1,125 nmoles)
	35 "	H_2O		

Samples were incubated for 3 minutes at room temperature, then centrifuged at 700 x g for 3 minutes. After centrifugation 200 ul of the supernatant was transferred into a graduated centrifuge tube. The remaining supernatant was syphoned off and the tube wall was wiped taking some of the pellet. The pellet was resuspended in 2 ml of H_2O and 50 ul used for chlorophyll determination. To both, the resuspended pellet and the 200 ul supernatant, 0.5 ml of 20% $HClO_4$ (v/v) was added to precipitate the chlorophyll and protein. After 5 minutes, 0.25 ml of 5 M K_2CO_3 was added. The precipitated $KClO_4$ and pellet was centrifuged down and the clear supernatant was used for radioactivity determination. Both ^{14}C and ^{3}H were counted simultaneously in aquasol with a liquid scintillation counter.

RESULTS AND DISCUSSION

Recovery and Characterization of Polyglucans

In order to study the biosynthesis of starch, using intact chloroplasts, a dependable method is required for the recovery of synthesized polyglucans. Glucose-1-P and ADP-glucose are substrates not only for starch but may also be precursors for other metabolites in the chloroplasts. It is therefore critical that the experimental methods differentiate between starch and other plastid constituents (glycoproteins, glycolipids, other oligo- and polysaccharides, etc.). In addition it is important to have a quantitative and reproducible method for starch extraction.

The completeness of starch solubilization and extraction by the procedure used in the experiments was tested by measuring the recovery of added starch granules. Starch prepared from bean seeds was added to chloroplast suspensions and its recovery was determined by the phenol-H_2SO_4 test. It can be seen from Table I that starch was satisfactorily solubilized by DMSO. The chloroplast suspension was determined to contain 0.15 mg starch. When cold bean starch was added to the plastid suspension and subsequently extracted the recovery was approximately 100%.

However, when chloroplasts were incubated with ADP-glucose-^{14}C, followed by starch extraction, a large amount of radioactivity was left in the residue (Table II). While

TABLE I

Recovery of starch from chloroplast suspensions

Chloroplast suspension (0.5 mg chl/ml)	Starch added	Starch recovered	Recovery
ml	mg	mg	%
0.0	1.32	1.31	99
0.5	-	0.15	
0.5	1.32	1.49	101

TABLE II

Radioactivity in the DMSO extract and its residue

Reaction mixture: A) glucose-1-P assay: 0.5 ml plastid suspension (2.3 mg chlorophyll/ml); 250 nmoles glucose-1-P (5.34×10^5 dpm); 500 nmoles ATP. B) ADP-glucose assay: 0.5 ml plastid suspension (mg chlorophyll/ml); 300 nmoles ADP-glucose-^{14}C (1.02×10^5 dpm).

		DMSO extract		HCl extract		
Substrate	added 3-PGA mM	dpm	nmoles glu incorporated/ mg chl/hr	dpm	nmoles glu incorporated/ mg chl/hr	DMSO/ HCl
glucose-1-P	10	1616	0.76	2568	1.20	0.63
glucose-1-P	0	91	0.04	2438	1.14	0.03
ADP-glucose	0	6341	37.36	2385	14.06	2.66

the extraction procedure with added cold starch gave almost 100% recovery this does not exclude the possibility of synthesized starch-^{14}C being present in the residue in an unextractable form. This radioactivity was successfully extracted with 1 N HCl in a boiling water bath for 2 hours. It is important to note that 3-PGA increased the radioactivity only in DMSO extracts from glucose-1-P and not in the HCl extract. Since 3-PGA stimulates ADP-glucose pyrophosphorylase (133) it is quite probable that the radioactivity in the HCl extracts does not represent starch. Preiss suggested that ADP-glucose is involved only in starch synthesis in the chloroplasts (157). However, according to the high radioactivity of the HCl extracts glucose-1-P and ADP-glucose are probably substrates for other reactions as well.

Further confirmation that all the starch was being recovered, was obtained by chromatographing DMSO extracts and their residues prior to and after incubation with β-amylase and pullulanase. Beta-amylase, releasing maltose, will degrade amylose and the outer chains of amylopectin to within 2 to 3 glucose units of any α-(1 → 6) branch points. Pullulanase specifically cleaves the α-(1 → 6) linkages. As it can be seen from Table III, all the labelled starch, extracted with DMSO, was hydrolyzed by β-amylase and pullulanase into maltose and malto-oligosaccharides to $\overline{DP}$ higher than 2. This indicates that the extract was starch. On the other hand, radioactivity could not be extracted from the

TABLE III

Thin-layer chromatography of β-amylase and pullalanase treated DMSO extracts of chloroplasts and chloroplast residues

Fraction	Enzymes added[1]	Total activity applied to TLC	R_M values of radioactive bands[2]				Total activity recovered
			origin	22-55	85-107	107-123	
			dpm				
DMSO extract	-	365	357	0	0	0	357
"	+	935	73	314	526	0	913
Residue and radioactive starch	+	318	0	23	270	0	293

1
+ addition of β-amylase and pullulanase;
- no additions.

2
Maltose = 100.

residues incubated under the same conditions with β-amylase and pullulanase.

To ascertain whether or not any starch remaining in the residue could be hydrolyzed by the enzyme treatment, radioactive starch was added to the residue. Results similar to those obtained with the DMSO extracts were observed.

These results indicate that DMSO extraction of starch is a reliable technique for following starch synthesis in chloroplasts from ^{14}C-labelled substrates.

Permeability of Chloroplasts to Glucose-1-P and ADP-Glucose

To study and compare starch synthesis from different substrates in intact chloroplasts, some information on the permeability of the chloroplast membrane to these substrates is useful. Several methods have been discussed for determining the rate of transport of small molecules and ions in cells and subcellular organelles (155,156,158). Using the known permeability of chloroplasts to inulin (155) and phenylalanine (156) an attempt was made to study the permeability of the chloroplast membrane to glucose-1-P and ADP-glucose. The method is based on the osmotic behavior of the chloroplasts.

The results of these experiments are shown in Table IV. When inulin and phenylalanine were used, the packed volume of chloroplasts was 11 ul/mg chlorophyll. However, negative values were obtained with both glucose-1-P and ADP-glucose. There are two possible explanations for this. Either glucose-

TABLE IV

Internal volumes of the chloroplasts available to glucose-1-P and ADP-glucose as determined by membrane permeability

	Inside volume of chloroplasts	
	Not hydrolyzed	Hydrolyzed
	ul/mg chlorophyll	
Inulin + Phenylalanine	11	11
Inulin + glucose-1-P	- 9	3
Inulin + ADP-glucose	-32	26

1-P and ADP-glucose were incorporated into insoluble materials or the chloroplast membrane is more permeable to inulin than to glucose-1-P and ADP-glucose. Since it is accepted that inulin is a nonpenetrating solute to the chloroplast (155) the negative values were in all probability due to a certain degree of incorporation of glucose-1-P and ADP-glucose into starch and other compounds during or subsequent to centrifugation. Starch is not soluble; hence the negative value. It should be noted that after acid hydrolysis, the inside volume using ADP-glucose was much higher than that obtained with phenylalanine and glucose-1-P. This is in keeping with the results obtained earlier where a higher rate of incorporation was observed with ADP-glucose in both the DMSO extract and the 1 N HCl hydrolysate.

It is clear that a reliable estimate of the internal space available to the substrates can not be obtained under these conditions. If the metabolites enter into the chloroplasts and synthesis proceeds, equilibrium will not be attained, since there would be a concentration gradient (glucose-1-P and ADP-glucose) across the membrane controlled by the rate of synthesis. However, taking into account the incorporation of label into starch, the data in Table IV do suggest a transfer of ADP-glucose across the membrane and at least some transport of glucose-1-P.

The Effect of Substrates, Regulators and Light on the Rate of Starch Synthesis

Rate of Starch Synthesis from Glucose-1-P and ADP-Glucose

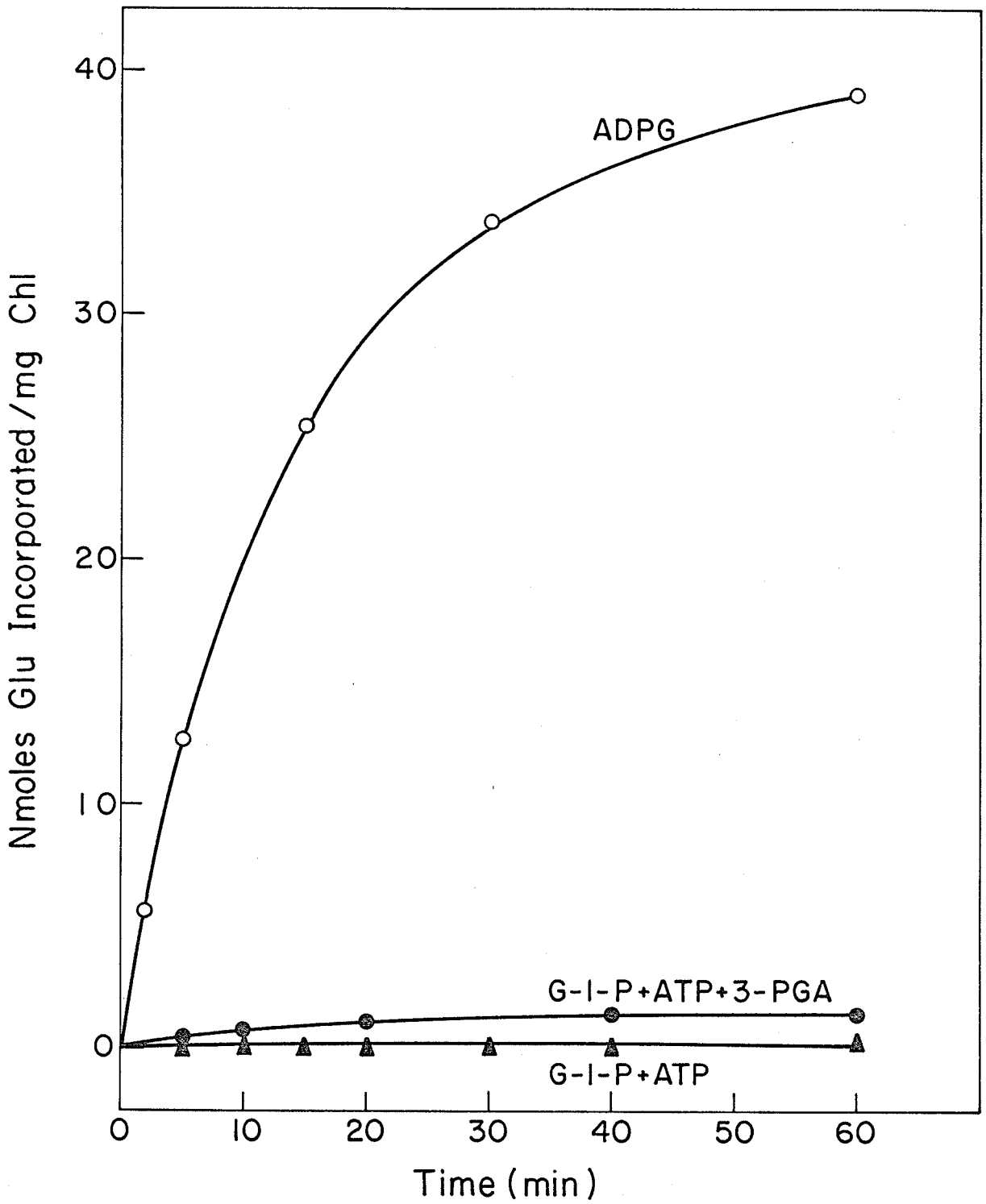
To establish the conditions for following the incorporation of labelled glucose into starch in the intact chloroplast, a time course study of glucose-^{14}C incorporation into starch from a number of ^{14}C-labelled substrates was performed.

Although incorporation was obtained from glucose-1-P in the presence of ATP, the amount was less than 1% of the maximum incorporation from ADP-glucose (Fig. 1). Addition of 3-PGA and ATP increased the incorporation from glucose-1-P. These results indicate that glucose-1-P alone is incapable of directly providing glucose for starch synthesis.

The effects of 3-PGA and ATP on the incorporation of glucose-1-P suggest that these compounds act at the level of ADP-glucose-pyrophosphorylase. This is further indicated by the fact that ADP-glucose acts as an efficient donor of glucose for starch synthesis in these chloroplasts. Assuming that all the glucose-1-P incorporated into starch in the presence of ATP and 3-PGA proceeds via the phosphorylase action, less than 2% of the starch synthesizing capacity can be accounted for by the phosphorylase system. No detectable incorporation into starch was obtained with UDP-glucose, sucrose or glucose-1-P in the absence of ATP.

Figure 1

Time course of glucose incorporation into starch from ADP-glucose and glucose-1-P.


40
30
20
10
0
Nmoles Glu Incorporated / mg Chl
ADPG
G-1-P+ATP+3-PGA
G-1-P+ATP
0
10
20
30
40
50
60
Time (min)

When isolations and incubations were carried out at the pH optimum of phosphorylase (6.8) no incorporation of glucose into starch was detected from glucose-1-P either alone or in the presence of ATP and 3-PGA. However 16.8 nmoles glucose/mg chlorophyll/hr was incorporated into starch from ADP-glucose (about one half the activity at pH 8.4). When the chloroplast suspensions were illuminated during incubation the rate of starch synthesis from glucose-1-P was increased but the effect was small as compared to the effect of 3-PGA (Table V).

It is interesting to note that whole chloroplasts are required to show incorporation from glucose-1-P in the presence of ATP and 3-PGA. Thus it is difficult to argue that lack of glucose incorporation from glucose-1-P in intact chloroplasts is due to the inability of glucose-1-P to penetrate chloroplast membrane. The effect noted in this instance may indicate that compartmentation of metabolites in the chloroplast occurs to facilitate synthesis. This is substantiated by the increase in the ADP-glucose level for 1/2 maximum rate from 3.37×10^{-4} for intact chloroplasts to 4.3×10^{-3} for lyzed chloroplasts (Fig. 2). Thus it is suggested that phosphorylase is not the enzyme responsible for the major portion of starch synthesized in the chloroplasts. However, an involvement of phosphorylase in the initiation of starch synthesis cannot be ruled out.

The rate of transfer of glucose from ADP-glucose was sigmoidal when determined as a function of chlorophyll con-

TABLE V

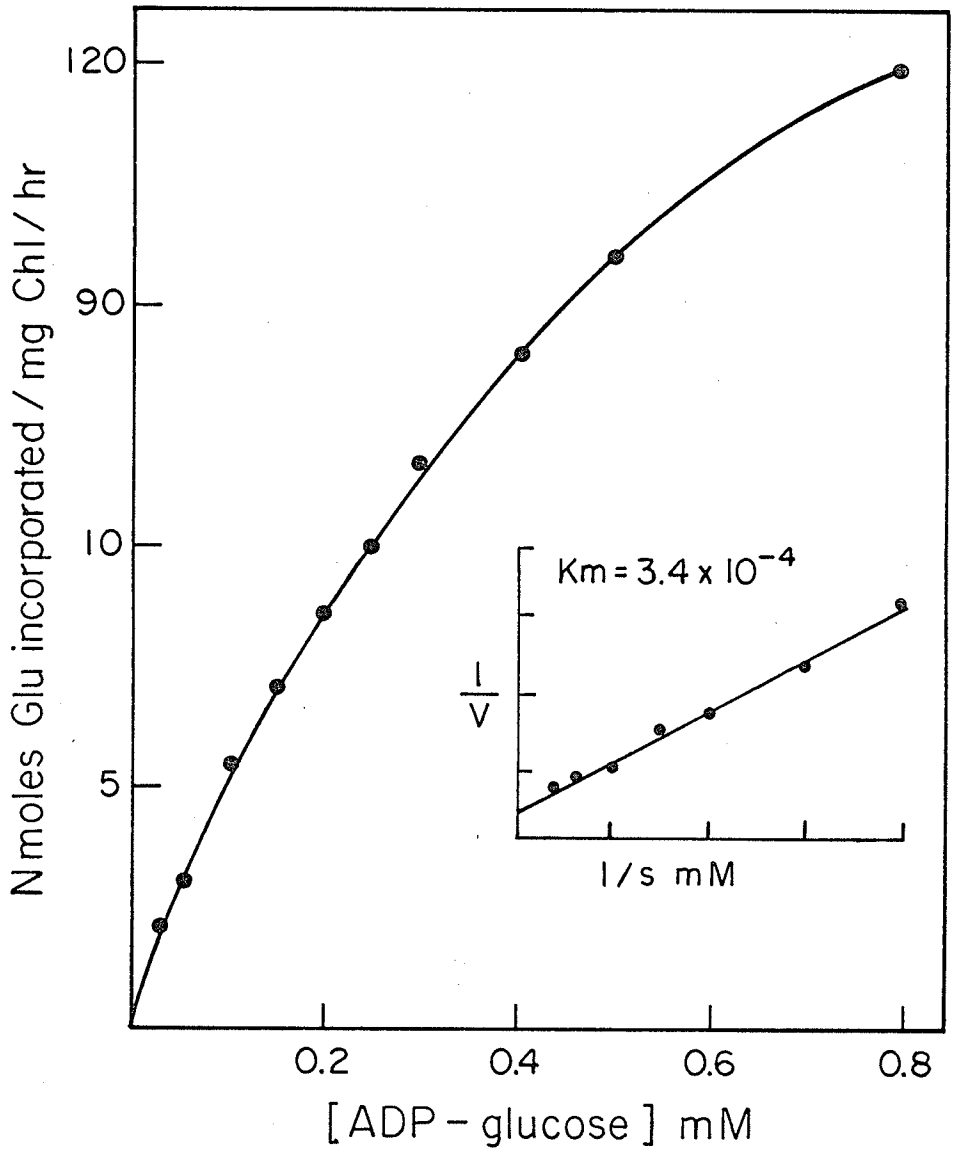
Effect of light and 3-PGA on glucose incorporation into starch from glucose-1-P

Incubations were carried out for 45 minutes.

	Glucose incorporated	
3-PGA	Dark	Light
mM	nmoles/mg chl/hr	
0	0.03	0.06
10	1.77	2.13

Figure 2

Rate of glucose incorporation from ADP-glucose into starch as a function of ADP-glucose concentration in intact chloroplasts. Incubations were carried out for 10 minutes.


120
90
10
5
N moles Glu incorporated / mg Chl / hr
0.2
0.4
0.6
0.8
[ADP - glucose] mM
Km = 3.4 x 10⁻⁴
1/V
1/s mM

centration (Fig. 3). However, between concentrations of 0.4 to 1.2 mg/ml it was linear. The 20 to 25% broken chloroplasts in the preparation could account for the fact that the linear portion of the curve does not extrapolate to zero chlorophyll concentration.

It has been demonstrated that bovine serum albumin (BSA) stimulates photophosphorylation in chloroplasts (159) and it is thought that this stimulation is due to its ability to bind uncouplers such as unsaturated fatty acids and phenols. Also, Ghosh and Preiss have shown (101,103) that unprimed starch synthesis by ADP-glucose glucosyltransferase, isolated from spinach leaves and waxy maize, is stimulated by BSA in the presence of high citrate concentration. It is not known how BSA stimulates starch synthesis. Experiments were conducted in order to see if a similar effect occurs on glucose incorporation into starch from ADP-glucose by BSA in intact chloroplasts. Figure 4 demonstrates that the presence of BSA affected the rate of glucose transfer from ADP-glucose. The highest stimulation by BSA was obtained at concentrations of 1.5 to 2.5 mg/ml chloroplast suspension. Above these concentration the stimulatory effect decreased.

Effect of Pi, ATP and 3-PGA on Starch Synthesis from Glucose-1-P

As mentioned previously little or no synthesis of starch could be demonstrated from glucose-1-P in the absence of ATP. Thus it appeared that synthesis occurred via ADP-glucose

Figure 3

Rate of glucose incorporation from ADP-glucose into starch as a function of chlorophyll concentration. Incubations were carried out for 30 minutes.

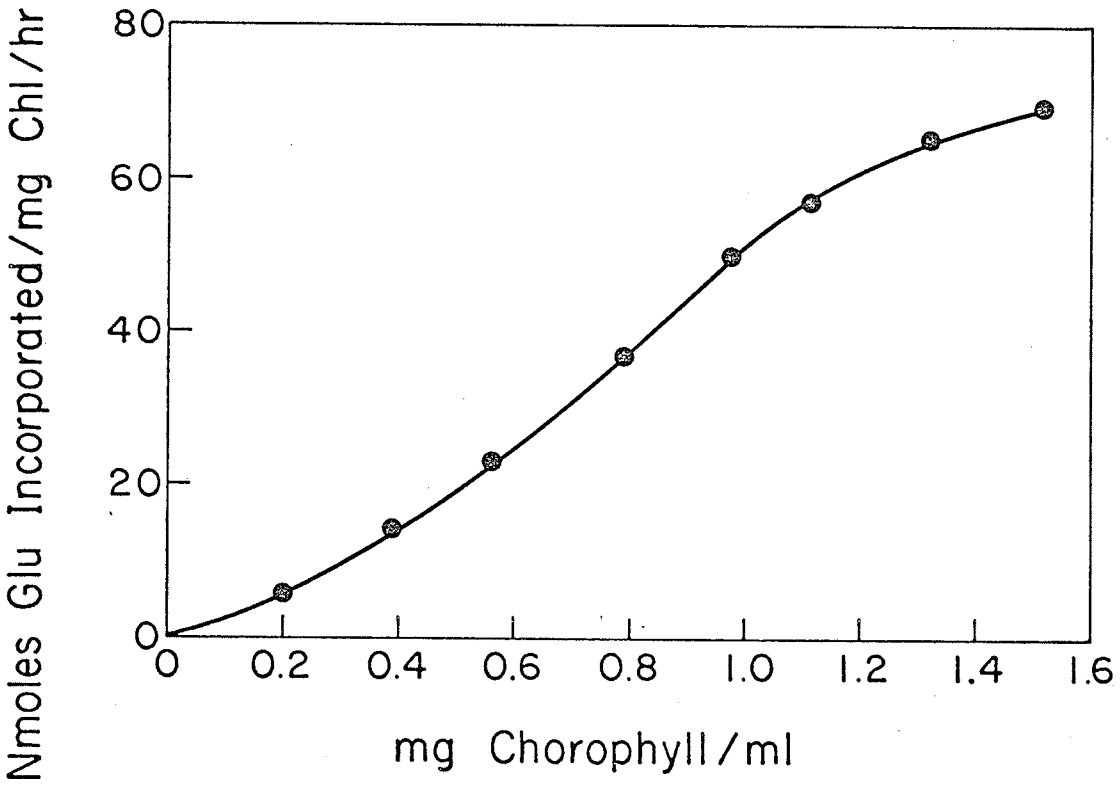
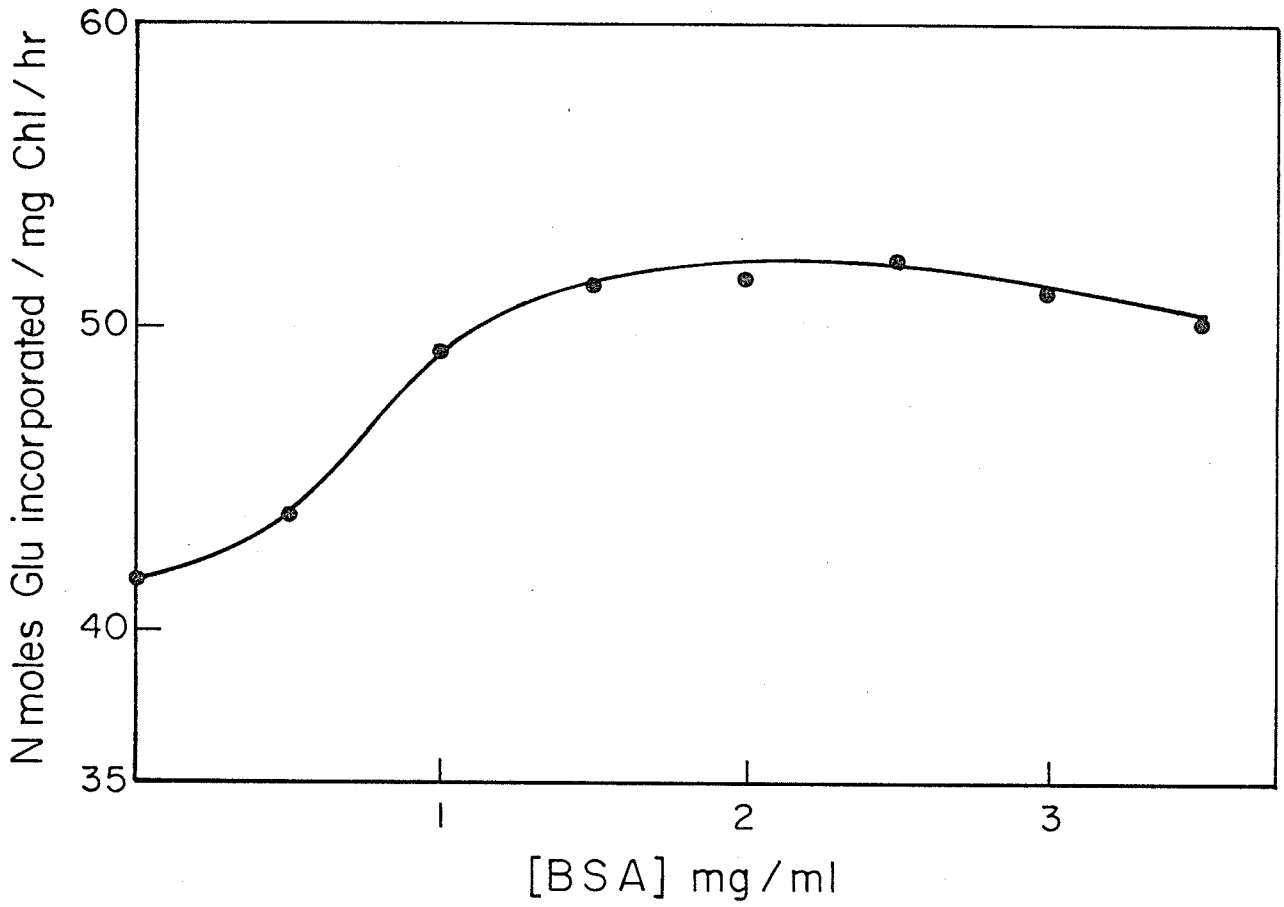

Nmoles Glu Incorporated/mg Chl/hr
80
60
40
20
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
mg Chorophyll/ml

Figure 4

Rate of glucose incorporation from ADP-glucose into starch as a function of BSA concentration. Incubations were carried out for 30 minutes.


60
50
40
35
N moles Glu incorporated / mg Chl / hr
1
2
3
[BSA] mg / ml

pyrophosphorylase. Preiss and coworkers have shown that phosphate and 3-PGA are major regulators for ADP-glucose pyrophosphorylase (133). The effect of ATP concentration on glucose incorporation from glucose-1-P was studied in the presence or absence of 3-PGA (Fig. 5). In the absence of the effector (3-PGA) the rate of synthesis was insignificant. In the presence of 10 mM 3-PGA the synthesis from glucose-1-P increased with increasing ATP concentrations up to 1 mM ATP. Incorporation from glucose-1-P in the presence of 1 mM ATP increased with increasing 3-PGA concentration (Fig. 6) with the effect levelling off at 10 mM 3-PGA. Phosphate (5 mM) strongly inhibited this stimulation up to about 10 mM 3-PGA. Beyond this point the inhibition was slightly overcome by 3-PGA. Inhibition of starch synthesis by a phosphate concentration of 0.5 mM was shown to be reversed or antagonized by 1 mM 3-PGA (133). Farineau has found that the physiological concentration of inorganic phosphate in maize leaves is higher than the level of 3-PGA (140). For example, in the light the concentration is approximately 3 to 3.5 umoles of phosphate per mg chlorophyll; whereas the 3-PGA concentration is 0.15 to 0.20 umoles per mg chlorophyll. Using 2.5 umoles of inorganic phosphate per mg chlorophyll in our experiments we were unable to overcome the inhibition of starch synthesis by phosphate with 3-PGA concentrations as high as 14 umoles per mg chlorophyll. In addition Bassham (143) has shown that the 3-PGA concentration does not change appreciably on light to dark transitions. Thus control of

Figure 5

Rate of glucose incorporation from glucose-1-P into starch as a function of ATP concentration in the presence or absence of 3-PGA. Incubations were carried out for 60 minutes.

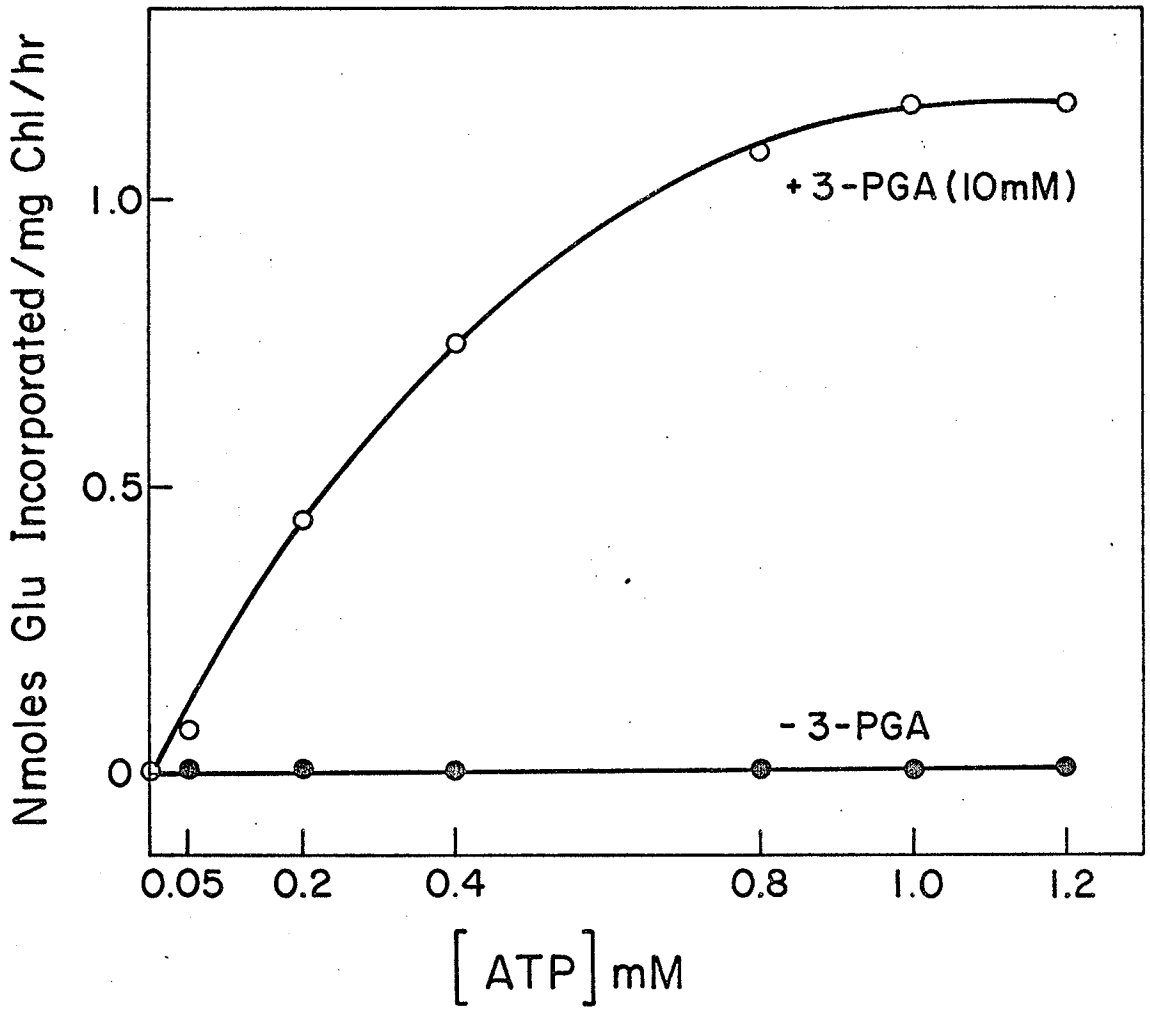
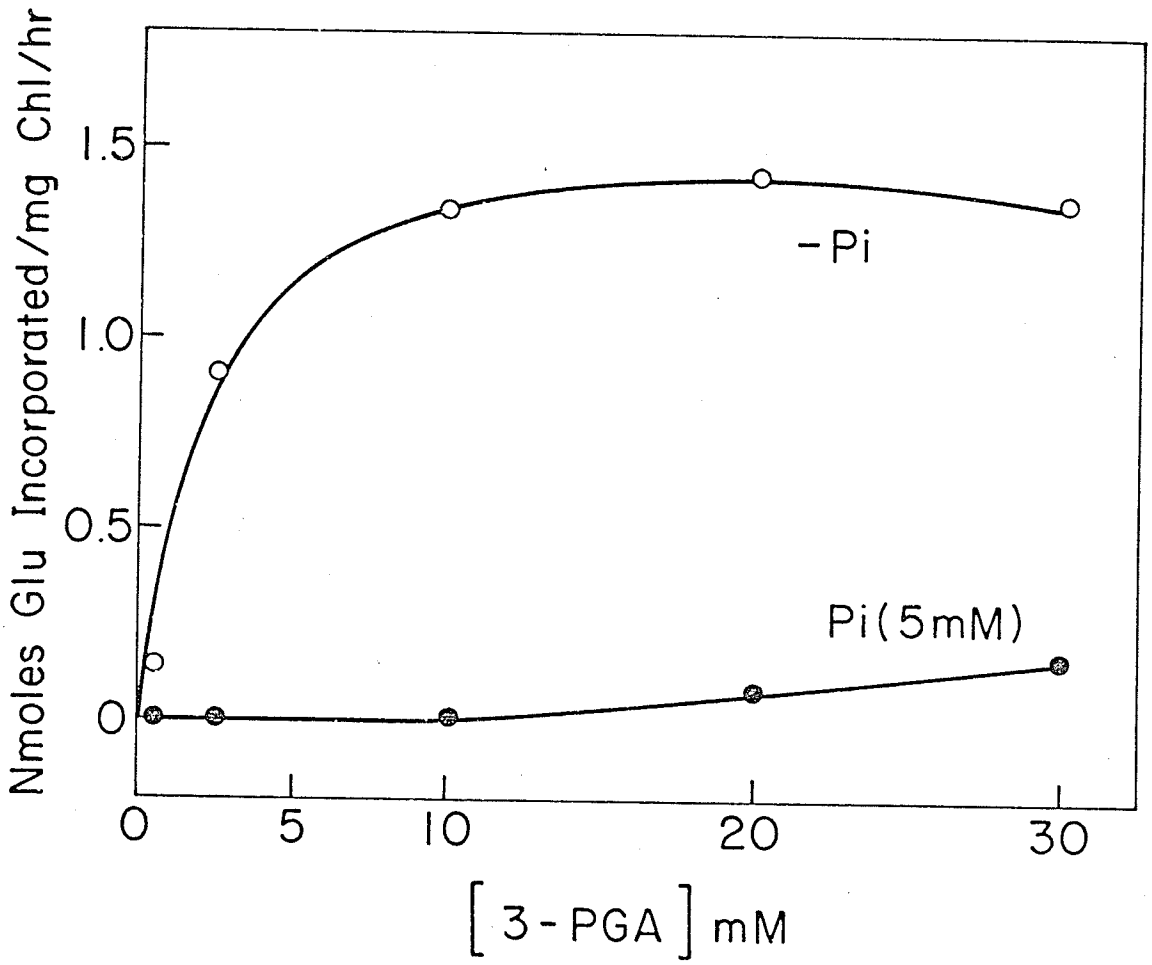

Nmoles Glu Incorporated/mg Chl/hr
1.0
0.5
0
+3-PGA (10mM)
-3-PGA
0.05
0.2
0.4
0.8
1.0
1.2
[ATP] mM

Figure 6

Rate of glucose incorporation from glucose-1-P into starch as a function of 3-PGA concentration in the presence or absence of inorganic phosphate.
Incubations were carried out for 60 minutes in the presence of 1 mM ATP.


Nmoles Glu Incorporated/mg Chl/hr
1.5
1.0
0.5
0
-Pi
Pi(5mM)
0
5
10
20
30
[3-PGA] mM

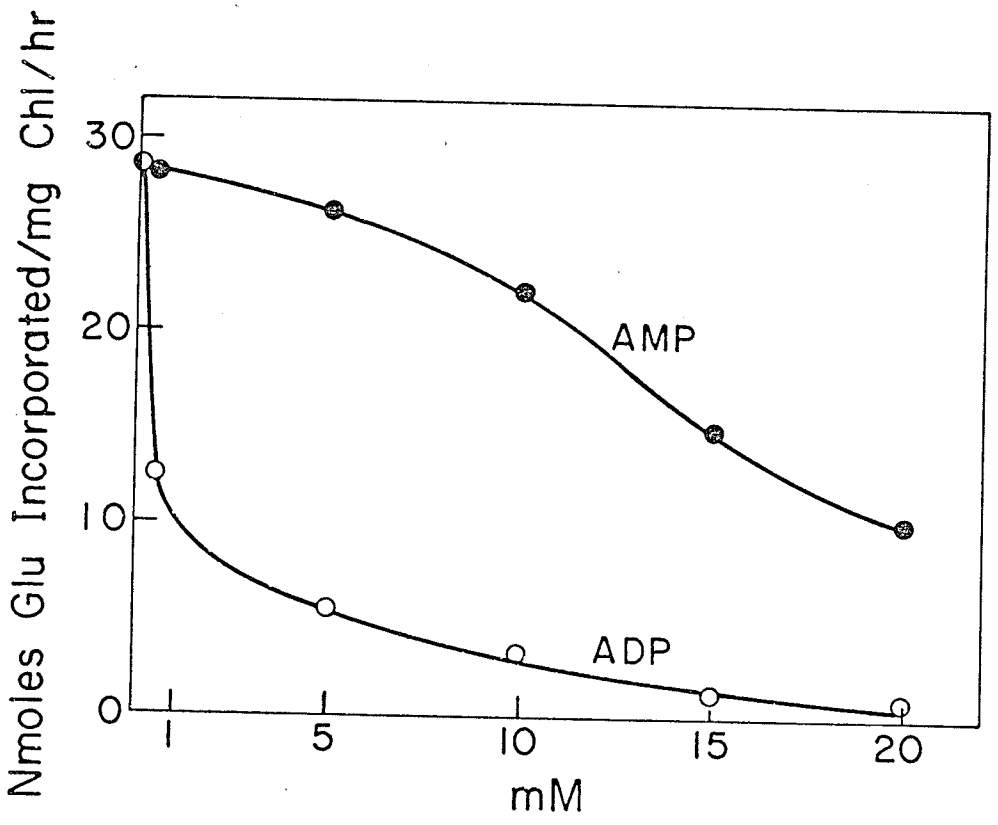
starch synthesis via ADP-glucose pyrophosphorylase requires either a compartmentation of metabolites or large fluctuations in inorganic phosphate concentration within the chloroplasts. Moreover Santarius and Heber (141) measured the concentrations of inorganic phosphate, AMP, ADP, ATP and pyruvate in chloroplasts of different plants, and concluded that the shutting off of glycolysis is controlled by the ratio of ATP, ADP and AMP rather than the fluctuation in the concentration of inorganic phosphate.

Effect of AMP, ADP and Organic Acids on Starch Synthesis from ADP-glucose

Both AMP and ADP inhibited the incorporation of glucose into starch when isolated chloroplasts were incubated with ADP-glucose (Fig. 7). AMP concentrations of 5 and 20 mM resulted in an inhibition of 10 and 65% respectively; whereas ADP of 5 and 20 mM resulted in an inhibition of 80 and 98% respectively. Since, it was shown that chloroplasts have high adenylate kinase activity (141), AMP may be converted to ADP during incubation. Furthermore, Bassham (143) has stated that the concentration of ATP does not change from light to dark transition, since the termination of ATP production by photosynthetic phosphorylation is compensated by oxidative phosphorylation in the mitochondria. Consequently, it is suggested that ADP plays a major role in the regulation of starch synthesis in the chloroplast.

Figure 7

Effect of ADP and AMP on glucose incorporation from ADP-glucose. Incubations were carried out for 30 minutes.


Nmoles Glu Incorporated/mg Chl/hr
30
20
10
0
AMP
ADP
1
5
10
15
20
mM

Previously it was reported that starch synthetase, isolated from spinach chloroplasts, was stimulated by acetate and to a greater extent by citrate (101,103). To investigate the effect, on starch synthetase, of increasing number of carboxylic groups of these anions, incubations were carried out with malate as well as acetate and citrate in the presence or absence of BSA (Table VI). However, in intact chloroplasts the individual effects of acetate, malate and citrate were all essentially similar, both with and without BSA. The effect of the citrate appeared to be near saturation at 0.1 M (Fig. 8).

Starch Synthesis During Light to Dark and Dark to Light Transitions

It has been known for some time that starch granules in the chloroplasts, are synthesized in the light (36), and may disappear in the dark (160). While photoregulation of many enzymes has been reported to date (161), little is known of the light regulation of ADP-glucose glucosyltransferase. Thus the effect of light starvation on the ability of chloroplasts to incorporate glucose from ADP-glucose was investigated. Chloroplasts isolated from plants grown in darkness for eighteen hours contained no starch. Furthermore they were unable to synthesize starch from ADP-glucose (Fig. 9). Upon illumination of these plants the isolated chloroplasts regained the ability to synthesize starch. A lag period of approximately 30 minutes occurred before appreciable starch

TABLE VI

Effect of acetate, malate and citrate on glucose incorporation into starch from ADP in the presence and absence of BSA

Chloroplasts were assayed for 30 minutes. The acetate, malate and citrate were added as their potassium salts.

Additions		Glucose incorporated	
		No BSA	2 mg/BSA/ml
		nmoles/mg chl/hr	
	None	10.4	17.0
0.01M	Acetate	13.8	26.0
0.10M	"	15.6	29.0
0.01M	Malate	15.2	28.0
0.10M	"	17.2	29.0
0.01M	Citrate	14.0	24.0
0.10M	"	15.0	35.2

Figure 8

Rate of glucose incorporation from ADP-glucose into starch as a function of potassium citrate concentration.
Incubations were carried out for 30 minutes.

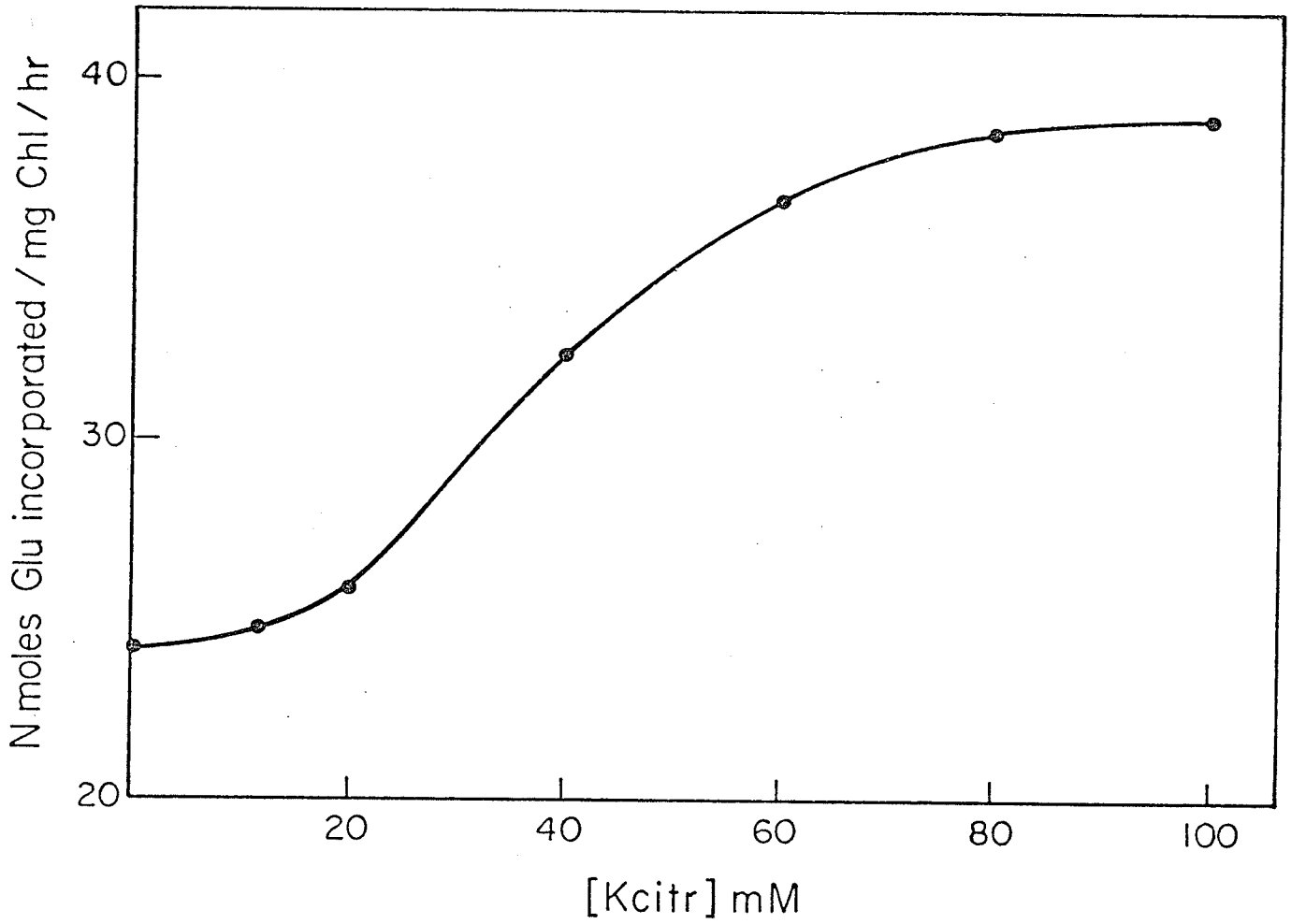
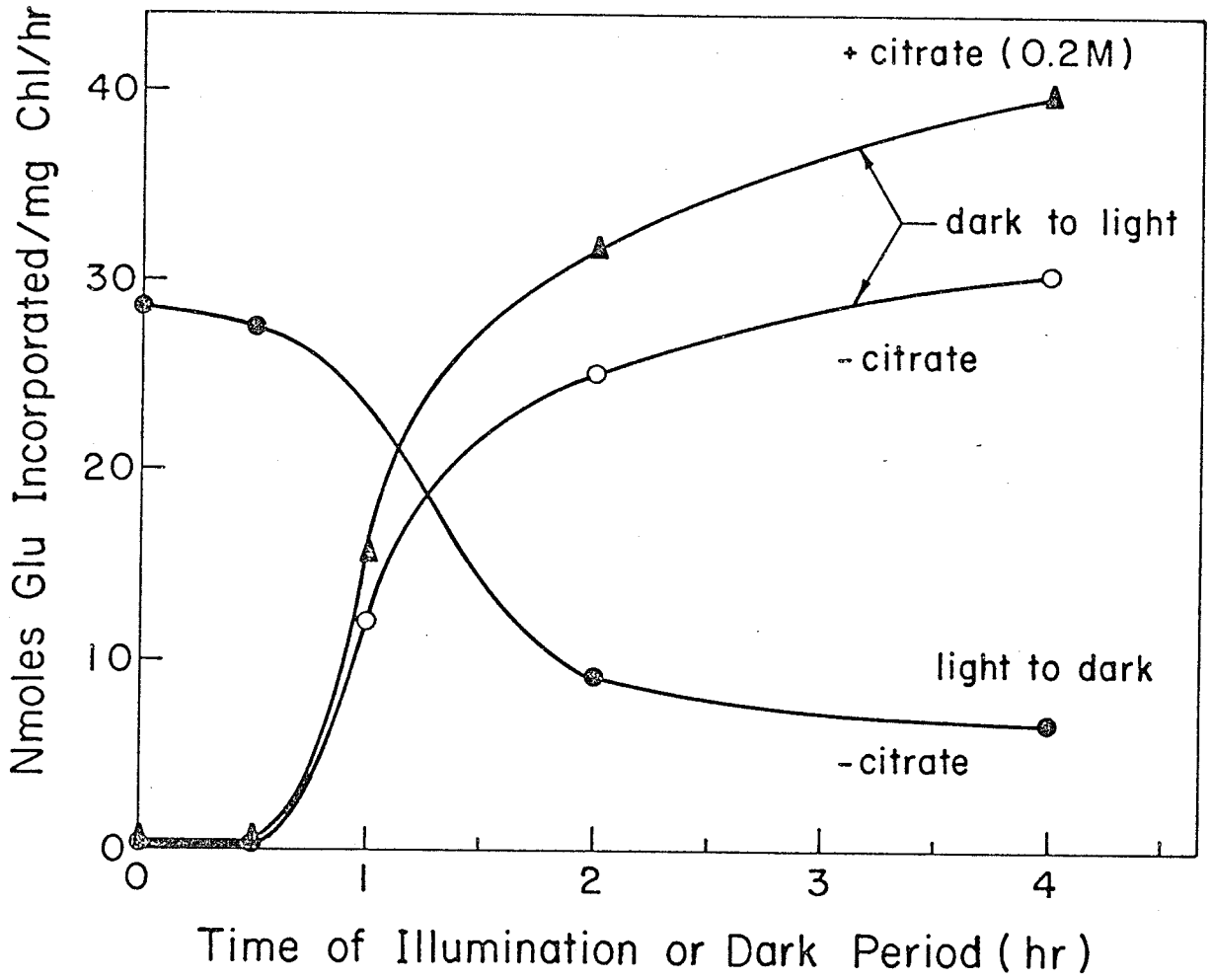

N moles Glu incorporated / mg Chl / hr
40
30
20
20
40
60
80
100
[Kcitr] mM

Figure 9

Effect of the light-dark transition on the rate of glucose incorporation from ADP-glucose.
Incubations were carried out for 30 minutes. For light to dark plants were illuminated for 4 hours then subjected to darkness prior to chloroplast isolation. For dark to light plants were subjected to 18 hours dark period then illuminated prior to chloroplast isolation.


+ citrate (0.2M)
dark to light
- citrate
light to dark
- citrate
Nmoles Glu Incorporated/mg Chl/hr
0
10
20
30
40
0
1
2
3
4
Time of Illumination or Dark Period (hr)

synthesis was evident. After 4 hours illumination, the plants completely regained their ability to synthesize starch from ADP-glucose. At this time the chloroplasts contained 0.31 mg starch/mg chlorophyll. Illuminated plants transferred to darkness lose their ability to synthesize starch from ADP-glucose after 4 hours darkness. As with the dark to light transition there is a 30 minute lag period before the loss becomes evident.

Ghosh and Preiss have shown that ADP is a competitive inhibitor for ADP-glucose transglucosylase in spinach chloroplasts (98). Since the activity of ADP-glucose pyrophosphorylase was lower than the transglucosylase activity, they believe that the point of regulation of starch synthesis is at the glucose-1-P $\longrightarrow$ ADP-glucose step. If starch synthesis is controlled primarily by the concentration of ADP-glucose, then one would expect synthesis to continue in the dark, provided ADP-glucose was supplied. However in our experiments the addition of ADP-glucose did not stimulate starch synthesis. This would suggest that factors other than ADP-glucose concentration are involved.

Ozbun et al. (101,103) reported the existence of an enzyme in spinach chloroplasts and in waxy maize endosperm which was capable of synthesizing starch in the absence of added primer. This reaction was stimulated a thousand fold by high concentrations of citrate and BSA. However it can be seen from Figure 9 that added citrate had no effect on the ability of dark isolated chloroplasts to produce starch

from ADP-glucose even though synthesis was stimulated in chloroplasts from light treated plants.

Lack of primer was also not a factor, as addition of amylose had no effect on starch synthesis in the dark isolated chloroplasts. This was true for both intact and lyzed chloroplasts. On the other hand, additional primer did stimulate starch synthesis in chloroplasts obtained from illuminated plants (Table VII). The greater increase in the rate of stimulation in the case of lyzed chloroplasts is probably due to the elimination of a membrane effect. This would indicate that either the requirement of a primer is not the limiting factor or that some primer other than amylose is needed.

Inhibition by high concentrations of ADP could have been a factor since ADP is known to increase upon light to dark transitions (141,142,162,163). However, inhibition due to ADP was eliminated as a factor by preincubating chloroplasts with phosphoenolpyruvate and pyruvate kinase to generate ATP from ADP and thus lower the endogenous concentration of ADP. While the rate of glucose incorporation into starch from ADP-glucose was increased for both whole and lyzed light exposed chloroplasts (Table VIII), phosphoenolpyruvate and pyruvate kinase did not affect starch synthesis in dark conditioned chloroplasts. Moreover, the table indicates that while boiled pyruvate kinase did not effect the rate of synthesis, PEP increased glucose incorporation for both intact and lyzed chloroplasts. Although it is believed that chloroplasts

TABLE VII

Effect of amylose on starch synthesis in chloroplasts of light or dark treated plants

Plants, exposed to an 18 hour dark period were illuminated at 50,000 lux for 4 hours before harvesting.

Chloroplasts were incubated for 30 min in the presence or absence of primer (1 mg amylose per ml chloroplast suspension).

Condition of chloroplasts	Glucose Incorporated	
	No primer	primer
	nmoles/mg chl	
Intact	12.62	17.57
Lyzed	8.84	14.57

TABLE VIII

Effect of PEP and Pyruvate Kinase on starch synthesis from ADP-glucose in chloroplasts of light or dark treated plants

Plants, exposed to an 18 hour dark period, were illuminated at 50,000 lux for 4 hours before harvesting.
Incubations were carried out for 30 min. Chloroplasts were preincubated for 5 min with PEP and Pyruvate kinase.

Condition of chloroplasts	Glucose incorporated			
	Control	Boiled Pyruvate kinase	PEP + boiled kinase	PEP + Pyruvate kinase
	nmoles/mg chl			
Intact	30.9	29.9	40.6	49.9
Lyzed	10.6	12.7	23.8	28.6

contain little or no pyruvate kinase (164) the stimulation by PEP and boiled enzyme suggests the presence of endogenous pyruvate kinase in the plastid suspension.

The light regulation of starch synthesis seems to be more complex than previously reported (133). Since the synthesis of many plant enzymes is known to be photoregulated (161), it is quite possible that the lack of starch synthesis in the dark treated chloroplasts is due to a lack of enzyme synthesis.

Fractionation of Starch by Thymol-n-Butanol Selective Precipitation and Sepharose Column Chromatography

There are a number of methods available for starch fractionation. Most of these are based on the fact that amylose is selectively precipitable with polar organic molecules, such as n-amyl alcohol, n-propyl alcohol, n-butanol and thymol with which it forms an insoluble crystalline complex (15). Precipitation of total amylose with thymol and n-butanol after disrupting the starch granule with DMSO then precipitating with ethanol and solubilizing with boiling water was reported as an efficient method (165). However, Metheson (154), using preparative ultra-centrifugation and exclusion chromatography techniques, showed that complexing procedures incompletely separate amylose and amylopectin.

Using non-radioactive starch, isolated from bean seeds, thymol-n-butanol precipitation and sepharose column chromatography techniques were tested by amperometric titration (specific for amylose) and phenol-sulfuric acid test to determine their usefulness in a reproducible assay of the degree of branched versus unbranched starch polymers.

Table IX demonstrates that after sedimentation of the amylose-thymol complex by centrifugation, the supernatant fraction containing amylopectin also contains 1.64 mg of amylose, measured by amperometric titration. This contamination may be due to a decreased affinity of thymol for shorter amylose chains. Complex formation becomes ineffective toward

TABLE IX

Selective precipitation of amylose from bean starch

Fraction	Estimated starch	
	phenol-H_2SO_4	amperometric
	mg	mg
Thymol supernatant (amylopectin)	33.55	1.64[1]
n-Butanol supernatant (mother liquor)	3.95	-
Thymol-n-Butanol precipitate (amylose)	20.60	18.20
Total	58.10	19.84

1 5 mg of amylose added to conduct iodometric titration.

crystallization when the chain length of amylose is reduced to below a $\overline{DP}$ of 20 - 40 (166). The higher value for amylose, obtained by the phenol-H_2SO_4 test as compared to amperometric titration, suggests that amylopectin is present in the amylose-thymol complex (Table IX).

Fractions obtained by thymol-n-butanol precipitation were chromatographed on sepharose columns (Fig. 10). Amylose gave two peaks; the larger, representing 98% (17.9 mg) amylose according to amperometric titration and the smaller, representing 5.3% of the amylose according to phenol-H_2SO_4 test. The smaller peak would appear to indicate that it is a contamination in the amylose fraction obtained by thymol-n-butanol fractionation. The amylopectin fraction gave only one peak but tailed through the fractions where amylose normally eluted and contained 4.5% (1.51 mg) amylose of the total amylopectin fraction obtained from the thymol supernatant.

The results obtained above were duplicated when newly synthesized starch, obtained by incubating chloroplasts with ADP-glucose-^{14}C, was used (Fig. 11). Measurement of the radioactivity of the fractions resulted in two peaks which were identified as amylopectin and amylose by staining with iodine and measuring the absorbance at 560 and 600 nm respectively. The recovery of radioactivity was 97%.

Comparison of thymol-n-butanol selective precipitation and column chromatography indicated that the latter was more

Figure 10

Sepharose column chromatography of thymol and n-butanol fractions.

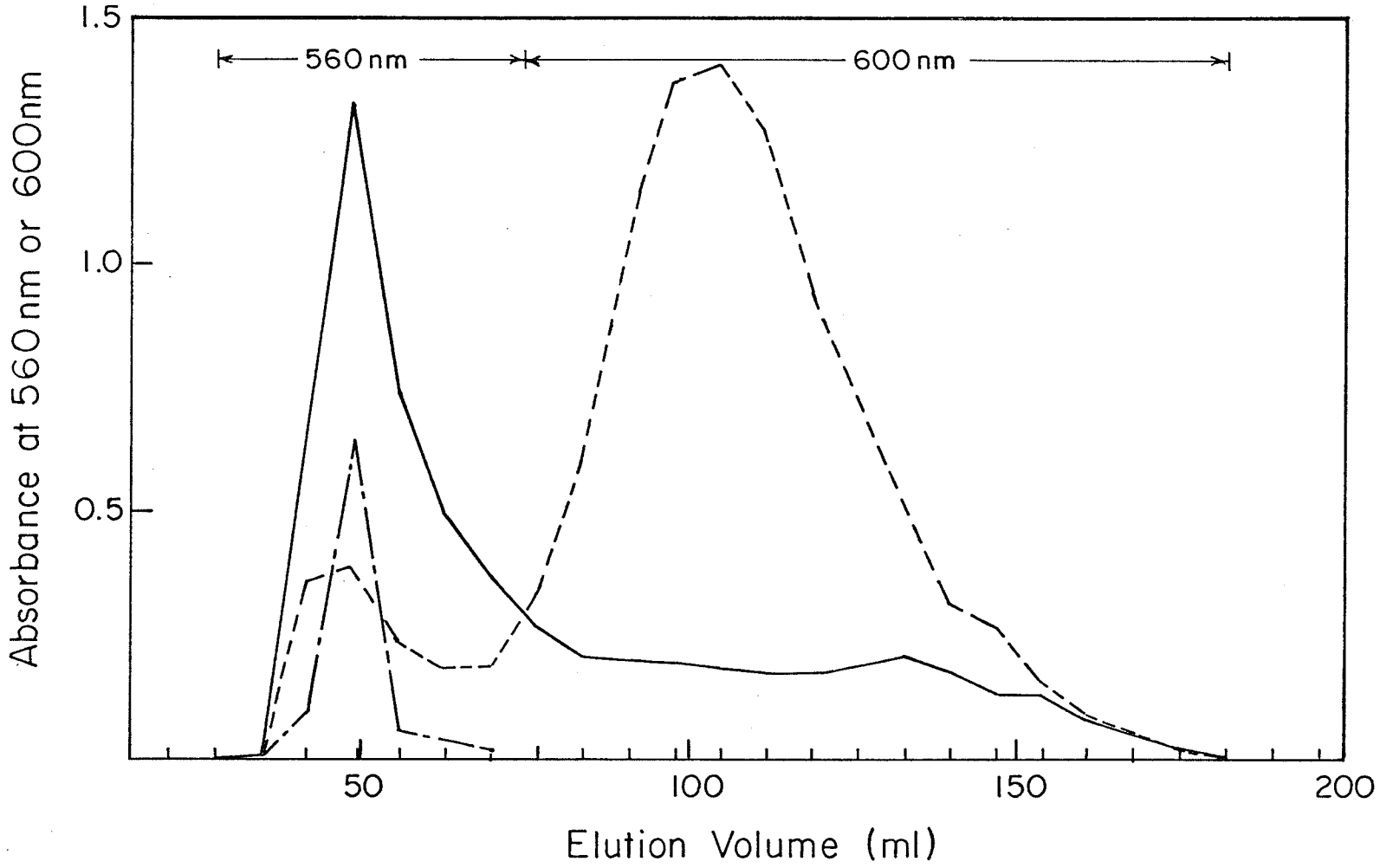
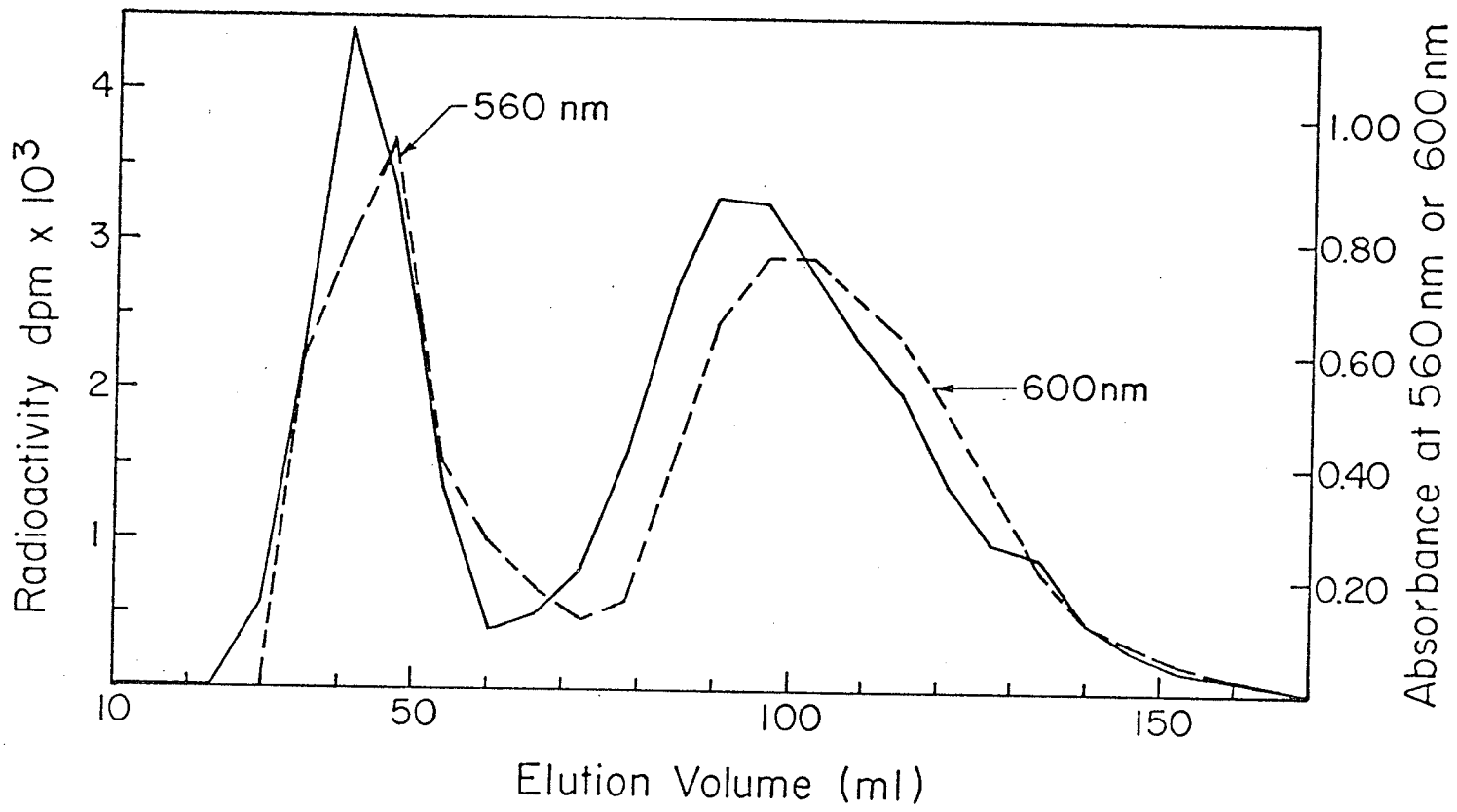

1.5
1.0
0.5
560 nm
600 nm
Absorbance at 560 nm or 600nm
50
100
150
200
Elution Volume (ml)

Figure 11

Fractionation of starch-^{14}C by sepharose colum chromatography. Solid line represents radioactivity; dashed line absorbance at 560 or 600 nm.


Radioactivity dpm x 10^3
4
3
2
1
560 nm
600nm
1.00
0.80
0.60
0.40
0.20
Absorbance at 560nm or 600nm
10
50
100
150
Elution Volume (ml)

efficient in the fractionation of starch. Therefore this method was adopted for all subsequent experiments.

Fate of Glucose-^{14}C from ADP-glucose After Incorporation into Starch

Experiments in which glucose-1-^{14}C was fed to intact wheat plants, indicated that amylopectin was formed from amylose in the endosperm (150). To determine whether amylose or amylopectin is synthesized first in bean chloroplasts, from the immediate substrate ADP-glucose (133), a pulse chase experiment was performed. Starting with three incubations of known specific activity, one was treated with ADP-glucose-^{12}C to dilute its specific activity then run for a further 45 min to allow the incorporation of unlabelled substrate. The remaining two incubations were reacted for 15 and 60 min without changing the specific activity of ^{14}C. It was possible to map the path of labelled glucose by comparing the distribution of ^{14}C in the three incubations. If amylose and amylopectin were synthesized independently, one would expect the ^{14}C ratio of amylose to amylopectin to remain constant in all incubations. If amylose was synthesized from amylopectin by a debranching enzyme, ^{14}C in amylopectin should decrease accompanied by an increase label in amylose in the diluted sample. The reverse would be true if amylose served as the substrate for amylopectin. This is, in fact, what we observed in our experiment. Chasing with cold substrate after 15 min resulted in a 26% decrease in labelled amylose as compared to the control (Table X). During the same period a significant portion of this decrease in label

TABLE X

Fate of glucose-^{14}C from ADP-glucose after incorporation into starch

Except for the concentration of ADP-^{14}C-glucose, reaction mixtures and assay conditions were as specified in the experimental section. Pulse chase: 0.9 umoles (3.2×10^5 dpm/umoles) for 15 min followed by addition of 3.2 umoles of ADP-^{12}C-glucose; 60 min control: same as above except ADP-^{14}C-glucose; 15 min control: same as above, without addition.

Time of incubation		Distribution of radioactivity			label in
ADPG-^{14}C	ADPG-^{12}C chase	amylopectin	amylose	total	amylose
min		dpm	dpm	dpm	%
15	-	5 043	3 290	8 333	39
60	-	11 787	9 806	21 593	43
15	45	8 307	1 219	9 526	13

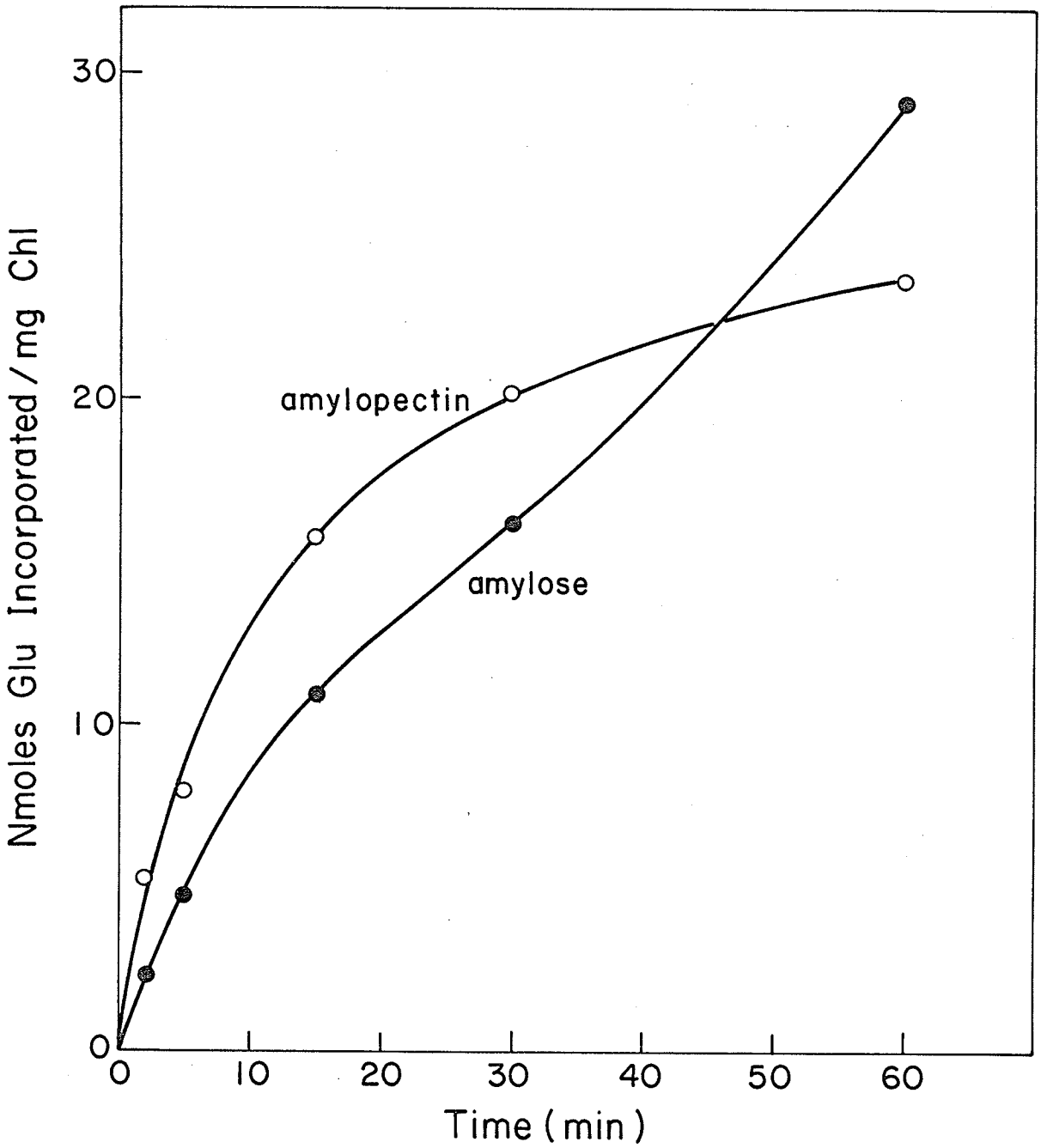
was recovered in the amylopectin fraction. Thus the evidence confirms the concept that linear amylose production precedes amylopectin production.

Time Course of Glucose Incorporation into Amylose and Amylopectin

Time course experiments were also carried out to investigate glucose incorporation from ADP-glucose into both amylose and amylopectin (Fig. 12) Initially a time course should give a pattern characteristic of a single precursor - product relationship. Thus if amylopectin is formed only from amylose via a branching reaction, label should first appear in amylose. However, following the time course of glucose-^{14}C in amylose and amylopectin we did not observe this expected pattern (Fig. 12). The incorporation into amylopectin increased rapidly up to about 15 min then remained relatively constant. By contrast the rate of incorporation into amylose, although initially less than that of amylopectin, continued to increase throughout the time period. Consequently, the ratio of amylose to amylopectin-^{14}C increased with time, so that after 30 min there was more label in the amylose fraction than in amylopectin. Our cold chase experiment (Table X) showed that this increase in labelled amylose could not have resulted from a debranching reaction. There are two possible explanations for the results observed. Firstly the chloroplasts used in these experiments contained 0.3 mg starch/mg chl. Approximately 68%

Figure 12

Time course of glucose incorporation into amylose and amylopectin from ADP-glucose.


30
20
10
0
0
10
20
30
40
50
60
Nmoles Glu Incorporated / mg Chl
Time (min)
amylopectin
amylose

of this starch was amylopectin. It has been shown (102) that both amylose and amylopectin can accept glucose from ADP-glucose at similar rates using enzymes from spinach leaves Thus, it is possible that the observed rapid incorporation into amylopectin was due to the presence of a greater proportion of amylopectin-like primer. A second explanation is based on product inhibition of Q-enzyme by amylopectin. Thus, at early time periods the turnover of amylose to amylopectin would be rapid. As amylopectin accumulated, inhibition of Q-enzyme would occur and the turnover of amylose would diminish. Either of the two explanations would account for the decrease in amylopectin synthesis at longer incubation times.

Effect of Citrate on the Rate of Amylose and Amylopectin Synthesis

It has been shown above that starch synthesis in isolated chloroplasts was enhanced by high concentrations of citrate in the presence of BSA (Table XI). It has also been shown that one of the two branching enzymes in sweet corn was stimulated by citrate (118). We conducted similar experiments in order to see if a similar shift, from amylose to amylopectin, occurs in isolated chloroplasts. The effect of high citrate concentration on glucose incorporation into amylose and amylopectin is shown in Table XI. While the increase in rate of total starch synthesis was similar for both 10 and 60 min incubations (17.5 and 18.6% respectively) the ratio of amylopectin to amylose was affected only during the early stages

TABLE XI

Effect of high citrate concentration on glucose incorporation into amylopectin and amylose

Reaction mixture and assay conditions were same as described in the experimental section. All reaction mixtures contained 0.2% BSA.

Time of incubation	citrate (0.1 M)	Glucose incorporated				amylopectin / amylose ratio
		amylopectin	amylose	total	increase	
min		nmoles/mg chl			%	
10	-	4.5	3.5	8.0	-	1.28
10	+	5.7	3.7	9.4	17.5	1.54
60	-	6.9	8.7	15.6	-	0.79
60	+	7.9	10.6	18.6	18.6	0.75

of incubation. Citrate increased the label only in the amylopectin in the 10 min incubations, thus increasing the ratio of amylopectin to amylose from 1.28 to 1.54. However in the 60 min incubations for both amylopectin and amylose a similar increase was observed, possibly due to product inhibition of the branching reaction by amylopectin.

GENERAL DISCUSSION

The biosynthesis of starch from nucleotide sugar was first demonstrated by Leloir et al. (81). It has also been shown that the glucosyltransferase in the chloroplast is totally specific for ADP-glucose (96). Since both ADP-glucose-α-1,4-glucan α-4-glucosyltransferase and phosphorylase are capable of synthesizing starch, there is still controversy as to which is of primary importance (99,106). However, most workers are of the opinion that phosphorylase is mainly involved in the degradation of starch rather than its synthesis because of the higher ratio of Pi to glucose-1-P in the cell (69).

The experiments reported here, using intact chloroplasts, could not demonstrate starch synthesis due to phosphorylase. The rate of glucose incorporation from glucose-1-P into starch was found to be effected by metabolites, known to regulate ADP-glucose pyrophosphorylase (93,129). Consequently, it is concluded that phosphorylase is not the enzyme responsible for starch synthesis in the chloroplasts, and that all the glucose from glucose-1-P appears to be incorporated via the ADP-glucose pyrophosphorylase pathway. However, since the initiation of unprimed starch synthesis is still not yet understood, the involvement of phosphorylase enzyme in the formation of primer cannot be ruled out.

Controversy exists concerning the control of starch synthesis in the plastid. Disagreement with respect to the

regulation of starch synthesis can be accorded to at least two facts. Firstly, the enzymes involved in starch synthesis are not fully understood. Secondly, the extrapolation of results obtained by *in vitro* experiments, to *in vivo* conditions is questionable.

While in mammalian systems the regulation of glycogen synthesis occurs at the level of the transferase reaction (139) it is believed that no regulatory phenomena exist for ADP-glucose glucosyltransferase in higher plants (133,157). It has been found that the enzyme catalyzing the synthesis of ADP-glucose from glucose-1-P and ATP can be activated by 3-PGA and inhibited by P_i. Consequently, it was proposed that lack of starch synthesis in the dark is due to a decreased amount of 3-PGA and an increased amount of P_i. However, it would appear that factors other than ADP-glucose concentration are also involved, since addition of ADP-glucose to intact chloroplasts, isolated from dark treated plants, did not overcome dark induced inhibition. In addition, lowering the concentration of ADP or providing amylose as primer did not eliminate this dark induced inhibition of starch synthesis.

The regulation of starch synthesis during light to dark or dark to light transitions appears to be more complex than has been proposed (133). It is quite possible that the lack of starch synthesis in the dark treated chloroplasts is due to a lack of enzyme synthesis. Evidence, supporting this

conclusion, has been presented by Salema and Badenhuizen (160).

Several theories have been proposed to explain how amylose and amylopectin are synthesized in vivo. Firstly, Whelan has proposed a compartment theory in which amylose and amylopectin are synthesized independently (144). Secondly, a hypothetical debranching enzyme is presumed to produce amylopectin from a phytoglycogen precursor (148). Finally it has been suggested (47,149) that during peaks of starch production the Q-enzyme is saturated around the granule. Consequently, not all α-(1 $\rightarrow$ 4) chains are converted into amylopectin. In this case a certain amount of starch is deposited as amylose depending on the overall rate of starch synthesis.

According to pulse-chase experiments, conducted in this research, the label passed from amylose to amylopectin. This indicates that amylopectin synthesis is probably not compartmented and confirms the concept that linear amylose synthesis precedes amylopectin production. Thus it is concluded that in the chloroplast amylopectin can be synthesized by the action of branching enzyme using amylose as substrate, formed by ADP-glucose-α-1,4 glucan α-4-glucosyltransferase. Moreover, the strict independence of pathways is unlikely, since starch containing amylose, also contains 5-10% intermediate material (147). However considering the time course experiment, reported here, and the fact that both amylose and amylopectin can accept glucose molecules from ADP-glucose (102),

the possibility of a certain degree of independent synthesis of amylopectin cannot be excluded.

Although, pulse-chase experiments did not indicate production of amylose by debranching of amylopectin, this possibility cannot be definitely eliminated and has in fact been suggested by Erlander (148). Bioenergetically this would be improbable.

Formation of amylopectin from amylose by Q-enzymes, isolated from plants and its stimulation by citrate has been demonstrated *in vitro* (118). Furthermore, additional branching enzyme stimulated glycogen production from ADP-glucose in *Streptococcus mitis* (167) which suggests that the branching reaction is a limiting step. It is possible therefore that the increase in the overall starch synthesis in isolated chloroplasts resulted from a citrate stimulation of the branching enzyme. It has been suggested that this stimulation results from a conformational change in the enzyme brought about by citrate and BSA (103).

The results of this research would suggest that at least three levels of control are involved in the regulation of starch synthesis in the chloroplast; (i) light regulated synthesis of the starch forming enzymes; (ii) regulation of glucosyltransferase by ADP, and; (iii) regulation of ADP-glucose pyrophosphorylase by inorganic phosphate and glycolytic intermediates. In addition, it is suggested that amylopectin may be synthesized by two pathways: directly from ADP-glucose and via amylose.

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