

Biosynthesis of Starch in Chloroplasts¹

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Summary. The enzymic synthesis of ADP-glucose and UDP-glucose by chloroplastic pyrophosphorylase of bean and rice leaves has been demonstrated by paper chromatographic techniques. In both tissues, the activity of UDP-glucose-pyrophosphorylase was much higher than ADP-glucose-pyrophosphorylase. Glycerate-3-phosphate, phosphoenolpyruvate and fructose-1,6-diphosphate did not stimulate ADP-glucose formation by a pyrophosphorylation reaction. The major metabolic pathway for UDP-glucose utilization appears to be the synthesis of either sucrose or sucrose-P. On the other hand, a specific precursor role of ADP-glucose for synthesizing chloroplast starch by the ADP-glucose-starch transglucosylase reaction is supported by the coupled enzyme system of ADP-glucose-pyrophosphorylase and transglucosylase, isolated from chloroplasts. None of the glycolytic intermediates stimulated the glucose transfer in the enzyme sequence of reaction system employed.

Sucrose is generally considered to be a major carbon assimilation product in chloroplasts of photosynthesizing plant organ. This product is eventually transported in the form of non-reducing oligosaccharides to other plant parts (1, 2, 7). On the other hand, in the chloroplasts of some dicotyledons and photosynthetic green algae, prominent starch formation has been demonstrated (1, 2). Starch of this specific character is conventionally called assimilation starch, which should more appropriately be termed as chloroplast starch. In contrast to reserve starch, chloroplast starch exists in transitory form and is readily utilized as an energy source in the metabolism of leaf tissues. This is observed in the diurnal pattern of its synthesis and breakdown (32).

Early studies on the synthesis of chloroplast starch received much attention, in particular, the role of chloroplastic phosphorylase (1, 19, 29). However, the current view on starch biosynthesis, through the nucleotide sugar pathway, has led us to reexamine the enzymic mechanism of the process. Murata and Akazawa (21) and Ghosh and Preiss (11, 12) have reported the specific role of ADP-glucose starch transglucosylase in starch biosynthesis under conditions where the UDP-glucose pathway was totally inactive. Since sucrose and sucrose-P synthesis in both leaf tissues and chloroplasts involving UDP-glucose has been reported by several workers (3, 4, 6, 8, 14, 20), the interplay of the 2 different nucleotide sugars is

of interest from the view of the photosynthetic carbon metabolism. This paper deals with the enzymic synthesis of ADP-glucose and UDP-glucose. Starch synthetase associated with the chloroplast starch, which operates chiefly through ADP-glucose pathway, was also studied.

Materials and Methods

Growing of Plants. Both bean and rice were grown in soil in a greenhouse for about 14 to 15 days. The bean was at fifth and sixth leaf stage and rice at third to fourth leaf stage.

Isolation of Chloroplasts Preparation. Freeze-dried chloroplasts were prepared using the non-aqueous method of Stocking (30).

Preparation of the Soluble Enzyme. A) Bean leaves (60 g) were homogenized in a Waring Blendor with 210 ml of 50 mM phosphate buffer (pH 7.0) containing 0.5% iso-ascorbate. After squeezing the homogenate through cheese cloth (8 layers), the filtrate was centrifuged at $15,000 \times g$ for 20 minutes. The solution was adjusted to pH 5.0 with 1 N acetic acid and centrifuged again at $15,000 \times g$ for 10 minutes. The resulting precipitate was dissolved in 60 ml of 10 mM K-phosphate buffer (pH 7.0), made to 30% saturation by adding crystalline $(\text{NH}_4)_2\text{SO}_4$, and centrifuged at $10,000 \times g$ for 10 minutes to remove most of the soluble protein (fraction-I-protein). The supernatant fraction was made to 55% saturation by adding crystalline $(\text{NH}_4)_2\text{SO}_4$, and centrifuged at $10,000 \times g$ for 10 minutes. The precipitate was dissolved in 1.0 ml of distilled water, dialyzed against water for about 2 hours in a cold room (2°), and then used as an enzyme source.

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B) Rice leaves (60 g) were first frozen with dry ice, then ground with the same buffer as that used for extracting soluble enzyme of bean leaves. Subsequent steps were essentially the same as that employed for bean leaves.

Preparation of Chloroplast Starch. Bean leaves (50 g) were harvested at midday and immediately homogenized to obtain the starch granules as reported previously (21). Greenish white starch samples were used for the enzyme assay.

Enzyme Assay. A) ADP-glucose and UDP-glucose pyrophosphorylase. Reaction mixture contained (in μ moles): glycine buffer (pH 8.5), 5.0; $MgSO_4$, 0.1; EDTA, 0.25; NaF, 2.5; GSH, 0.5; ATP, 0.84 or UTP, 0.54; glucose- ^{14}C -1-P, 0.5 ($2.7 \times 10^{-2} \mu c$, 5.9×10^4 cpm), either chloroplasts preparation, 1.0 mg (equiv. 0.01 mg chlorophyll and 0.28 mg protein) or soluble enzyme, 50 μl (ca 10.2 mg protein/ml), and H_2O , 50 μl in a total volume of 90 μl . The reaction was continued for 60 minutes at 37°. After adding 2.0 ml of 80% (V/V) ethanol, the whole reaction mixture was centrifuged, and the residue was washed twice with ethanol. The whole extract was evaporated in vacuo at 2°, and dissolved in 100 μl of water. One half of the final eluate was applied to an acid-washed Whatman No. 1 paper and chromatographed as reported previously (22, 23). The radioactivity was determined in a Packard liquid scintillation counter.

B) ADP-glucose and UDP-glucose starch transglucosylase. The reaction mixture contained (in μ moles): glycine buffer (pH 8.5), 5.0; $MgSO_4$, 1.0; EDTA, 0.25; ADP-glucose- ^{14}C , 0.16 ($1.2 \times 10^{-2} \mu c$, 2.7×10^4 cpm), or UDP-glucose- ^{14}C , 0.16 ($8.2 \times 10^{-3} \mu c$, 1.8×10^4 cpm); chloroplast starch granules, 5.0 mg; and H_2O , 50 μl in a total volume of 90 μl . The method of assaying ^{14}C -incorporation into starch was the same as that reported previously (22, 23).

C) Coupling system of pyrophosphorylase and transglucosylase. The composition of the reaction mixture was essentially the same as that for the pyrophosphorylase system, except that either soluble enzyme preparation (50 μl) or chloroplasts (1.0 mg) was added, in addition to the chloroplast starch sample (5.0 mg).

D) Sucrose-synthetase. The soluble enzyme as explained above (A) was used for the sucrose synthesis experiment in the following reaction mixture (in μ moles): tris buffer (pH 7.5), 30; $MgSO_4$, 0.9; NaF, 20; fructose or fructose-6-P, 1.0; UDP-glucose or ADP-glucose, 0.25; and enzyme preparation, 20 μl in a total volume of 130 μl . At the end of incubation, the sucrose formation was assayed by the resorcinol method of Roe (26).

Ion Exchange Column Chromatography. Dowex-1 anion exchange column chromatography was carried out to analyze the formation of sucrose-P produced in the following reaction mixture (in μ moles): tris buffer (pH 7.4), 400; NaF, 400;

$MgSO_4$, 9.0; UDP-glucose- ^{14}C , 2.2 ($1.9 \times 10^{-2} \mu c$, 4.7×10^4 cpm); fructose-6-P, 100; enzyme preparation, 200 μl in a total volume of 1.26 ml. The mixture was incubated for 60 minutes at 37°. At the end of this incubation period, the reaction mixture was treated by the method used previously and applied to a column of Dowex-1 anion exchange resin (23). Carrier hexose-P (2-4 mg) were added to the eluate, and aliquots used for the measurement of radioactivity of sucrose (26) and total sugar (9).

Reagents. All the chemical reagents used were commercial products, and ADP-glucose- ^{14}C , UDP-glucose- ^{14}C , and glucose- ^{14}C -1-P were purchased from International Nuclear Corporation, California.

Results and Discussion

Synthesis of ADP-glucose and UDP-glucose. Enzymic synthesis of ADP-glucose and UDP-

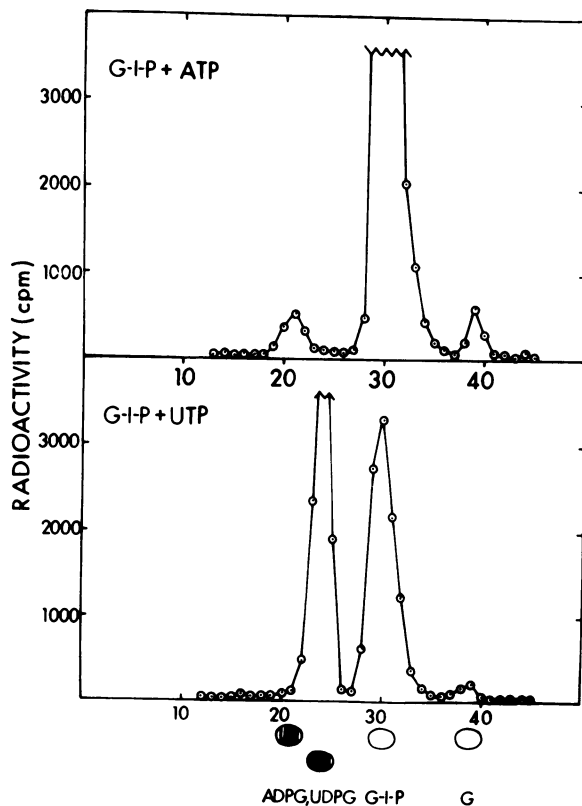


FIG. 1. ADP-glucose and UDP-glucose synthesis by rice chloroplasts. Reaction mixture contained (in μ moles): glycine buffer (pH 8.5), 5.0; $MgSO_4$, 1.0; EDTA, 0.25; GSH, 0.5; ATP, 0.84 or UTP, 0.54; glucose- ^{14}C -1-P, 0.5 ($2.7 \times 10^{-2} \mu c$, 5.9×10^4 cpm); chloroplast preparation 1.0 mg; and H_2O , 50 μl in a total volume of 90 μl . Reaction was continued for 60 minutes at 37°, and the 80% (V/V) ethanol eluate of the reactant was analyzed by paper chromatography. Abbreviations used are: ADPG, ADP-glucose; UDPG, UDP-glucose; G-1-P, glucose-1-P; and G, glucose.

glucose by pyrophosphorylase residing in chloroplast preparations of rice leaves was demonstrated by paper chromatography (fig 1). Essentially identical paper chromatogram was obtained by the bean chloroplast preparation, but the enzyme activity of the bean plant was much higher. There exist discrepancies in the literature concerning the localization of UDP-glucose-pyrophosphorylase in chloroplasts. Opposed to the initial notion on the localization of UDP-glucose-pyrophosphorylase outside chloroplasts (28), recent experiments of Bird et al. (3) have shown the presence of enzyme in chloroplasts isolated from tobacco leaves by non-aqueous method. The specific activity (20 $m\mu$ moles UDP-glucose formed/mg protein/60 minutes) of the soluble enzyme preparation used in our present experiment showed nearly the same value (24 $m\mu$ moles UDP-glucose formed/mg protein/60 minutes) of the whole chloroplasts preparation obtained by the non-aqueous method, supporting a confined localization of the enzyme in chloroplasts. This finding is indeed in accordance with the results of Bird et al. (3). Whole chloroplasts and soluble enzyme preparation of both rice and bean system showed a marked activity of the glycolytic trans-

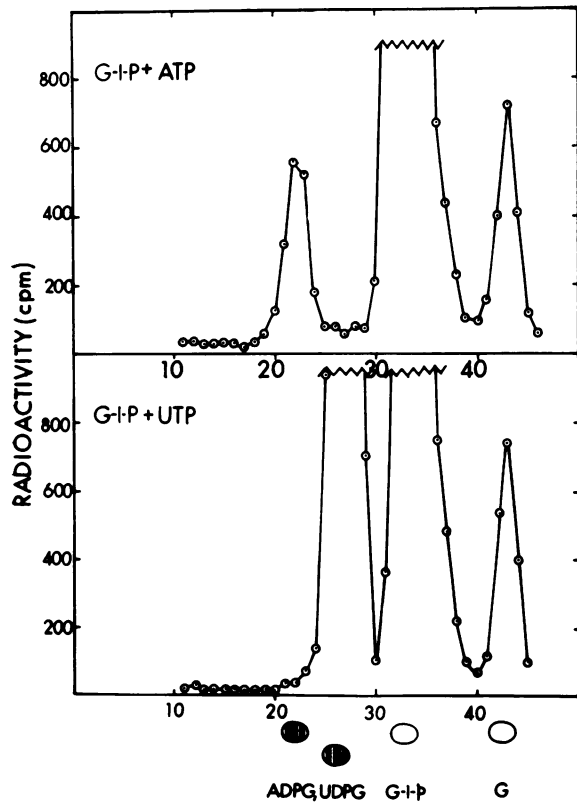


FIG. 2. ADPG and UDPG synthesis by soluble pyrophosphorylase of rice leaves. Experimental condition was the same as that of figure 1, except 50 μ l of soluble enzyme preparation of rice leaves was used. Same abbreviations as that for figure 1 were employed.

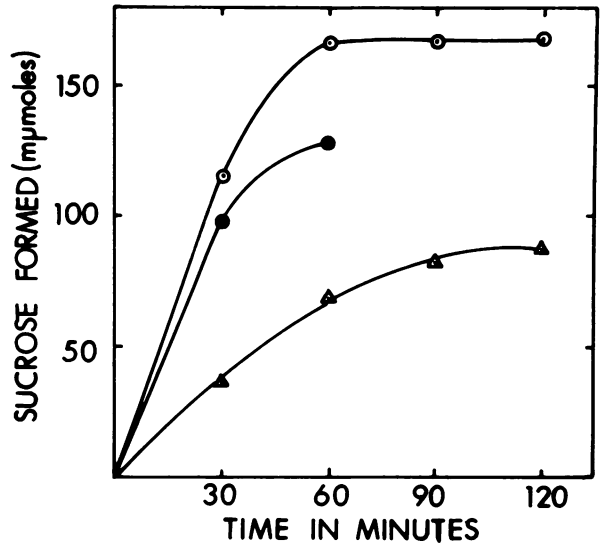


FIG. 3. Sucrose and sucrose-P synthesis by soluble enzyme of rice leaves. Reaction mixture contained (in μ moles): tris buffer (pH 7.5), 30; $MgSO_4$, 0.9; NaF, 20; fructose or fructose-6-P, 1.0; UDP-glucose or ADP-glucose, 0.25; and soluble enzyme preparation, 20 μ l in a total volume of 130 μ l. Fructose + UDP-glucose, \circ — \circ ; fructose-6-P + UDP-glucose, \bullet — \bullet ; and fructose + ADP-glucose, \triangle — \triangle . Sucrose was assayed by the method of Roe (23).

formation of glucose-1-P to glucose-6-P and fructose-6-P. This transformation was much less in the soluble enzyme system, however. The presence of phosphoglucomutase and phosphohexoisomerase in chloroplasts has been reported by Bird et al. (3).

It has been claimed by Preiss and his associates (11, 24, 27) that some glycolytic intermediates, including glycerate-3-P, P-enolpyruvate, fructose-1-6-P, stimulate chloroplastic and bacterial ADP-glucose-pyrophosphorylase. We tested these compounds without any positive effect, regardless of the varied concentration ranges of the compounds (0.5–5.0 mM) and various incubation time intervals (15–180 min).

Synthesis of Sucrose and Sucrose-P. In an attempt to examine the role of UDP-glucose, as seen in the preceding experiment, the sucrose-synthesizing system was tested. There is much debate concerning the site of sucrose synthesis in chloroplasts (3, 14, 16, 31). Several investigations favor the idea that there is enzymic synthesis of sucrose and sucrose-P in chloroplasts. Our analysis clearly indicates the presence of UDP-glucose-sucrose (sucrose-P) transglucosylation, and also ADP-glucose-sucrose transglucosylation, reactions in rice leaf tissues (fig 3). The production of sucrose-P was rigorously established by ion-exchange column chromatography (fig 4), although it cannot be ascertained from this study alone whether fructose or fructose-6-P reacts predomi-

nantly in intact leaf tissues. It is probable that sucrose-P is enzymically hydrolyzed to free sucrose by a specific sucrose-P phosphatase (15). The nature of the minor radioactive compound elutable

earlier than sucrose-P is not known, although this fraction has often been detected in ^{14}C -feeding experiments (23).

Starch Synthesis. Results of the preferential utilization of ADP-glucose by starch synthetase, associated with the chloroplast starch granules, conforms with our preliminary experiment (21) (table I). Many later workers obtained similar results using soluble enzyme preparations from chloroplasts of bean and spinach leaves (10, 11, 13). However, none of them has tested the coupled enzyme system of pyrophosphorylase and transglucosylase. This coupling reaction system is indeed considered to be essential to demonstrate the role of ADP-glucose as an intermediate in the starch synthesis. Murata and Akazawa (21) have speculated that photosynthetically produced ATP could be readily utilized for the starch synthesis by transforming glucose-1-P, one of the initial photosynthetic sugar phosphates, to ADP-glucose. Results shown in table I support this presumption. The reaction system consisting of both ADP-glucose-pyrophosphorylase (soluble enzyme) and ADP-glucose-starch transglucosylase (chloroplast starch granules) was the only efficient one for starch synthesis (Expt. II-IV), notwithstanding that the rate of ADP-glucose formation was lower than that of UDP-glucose. However, glycerate-3-P, P-enolpyruvate and fructose-1, 6-P, did not exhibit any stimulating effect for the glucose transfer from glucose-1-P via ADP-glucose. Recent elegant study of Ghosh and Preiss (13) has shown the stimula-

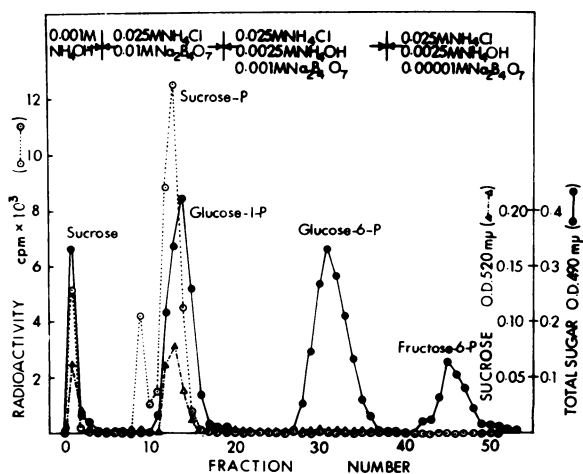


FIG. 4. Ion exchange column chromatographic separation of sucrose-P. Reaction mixture contained (in μmoles): tris buffer (pH 7.4), 400; NaF, 400; MgSO_4 , 9.0; UDP-glucose- ^{14}C , 2.2 ($1.9 \times 10^{-2} \mu\text{c}$, 4.7×10^{-4} cpm); fructose-6-P, 100; soluble enzyme preparation of rice leaves, 200 μl in a total volume of 1.26 ml. Reaction was continued for 60 minutes at 37° . Afterwards, de-ionized reactant was deionized and applied to a column of Dowex-1 \times 8 (Cl $^-$ form) (0.8×7.0 cm); eluted by the stepwise method as indicated in the figure.

Table I. Incorporation of Glucose- ^{14}C into Starch

Concentration of substrates contained in reaction mixture was (in μmoles): ADP-glucose- ^{14}C , 0.185 (2.7×10^4 cpm); UDP-glucose- ^{14}C , 0.16 (1.8×10^4 cpm); glucose- ^{14}C -1-P, 0.5 (5.9×10^4 cpm); and either ATP, 0.5 or UTP, 0.5. Abbreviations used were: 3PGA, 3-phosphoglycerate; PEP, P-enolpyruvate; and FDP, fructose-1,6-diP.

Expt.	Glucose donor system	Enzyme source	Incubation time min	Radio-activity cpm	% glucose- ^{14}C incorporation into starch
I	ADP-glucose- ^{14}C	Granules of chloroplast starch (5.0 mg)	60	19,500	17.7
	UDP-glucose- ^{14}C	"	"	580	3.3
II	Glucose- ^{14}C -1-P+ATP	Granules of chloroplast starch (5.0 mg)	60	6200	10.5
	Glucose- ^{14}C -1-P+UTP	+ soluble enzyme (50 μl)	"	965	1.6
III	Glucose- ^{14}C -1-P+ATP	Granules of chloroplast starch (5.0 mg)	30	8000	13.6
	"	+ soluble enzyme (50 μl)	"	"	"
	"	"	60	10,700	18.1
	"	"	120	11,600	19.7
	"	+3PGA (2.5mM)	30	7450	12.6
IV	"	"	60	9800	16.6
	"	"	120	11,900	20.2
	Glucose- ^{14}C -1-P+ATP	Granules of chloroplast starch (5.0 mg)	60	8000	13.6
	"	+ soluble enzyme (50 μl)	"	"	"
	"	+3PGA (1mM)	"	9300	15.8
	"	" (2.5mM)	"	9800	16.6
	"	" (5.0mM)	"	8900	15.1
"	+PEP (2.5mM)	"	9600	16.3	
"	+FDP (2.5mM)	"	11,400	19.3	

tion of highly purified (260 fold) ADP-glucose-pyrophosphorylase of spinach leaf by 3-phosphoglycerate and other glycolytic intermediates and inferred the important regulatory role of the enzyme in the starch synthesis of leaf tissues. Reason of our failure to reconfirming their results will certainly be due to the use of crude enzyme in our system. It is our opinion that more thorough experimentation is needed to prove whether or not the point of control in the storage polysaccharide biosynthesis in plants is, unlike the case in the glycogen, at the level of ADP-glucose-pyrophosphorylase, instead of the transglucosylase. It should be pointed out in this regard that ADP-glucose is in fact utilized for the formation of sucrose (cf. fig 3).

It is interesting to recall that before the currently prevailing concept of the starch biosynthesis through the ADP-glucose pathway is established by the original work of Recondo and Leloir (25) in 1961, showing the much faster synthesis of starch from ADP-glucose than that from UDP-glucose, radioisotopic experiment of Kauss and Kandler (17) had shown the close correspondence of the ADP-glucose labelling and starch formation in photosynthesizing *Chlorella* cells. However, in the general scheme of starch biosynthesis in reserve organs, such as seeds and tubers, the interaction of UDP-glucose and ADP-glucose has been found to be rather complex, and their control mechanism remains to be studied (1). In fact, in agreement with a view presented by Leloir (18), we cannot ignore completely the UDP-glucose pathway of starch synthesis in ripening rice seeds (23). Our present experiments, however, have shown the specific, predominant role of ADP-glucose in starch synthesis of chloroplasts, whereas both ADP-glucose and UDP-glucose are synthesized by the respective pyrophosphorylase system residing in chloroplasts. Hence, we feel that the unique feature of the starch synthesizing system associated with the chloroplast starch is distinguishable from the one residing in cereal starch granules. It would be of value to study the biophysical characteristics of chloroplastic and reserve starch.

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