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# STARCH SYNTHESIS IN ISOLATED *PHASEOLUS VULGARIS* CHLOROPLASTS

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**Key Word Index**—*Phaseolus vulgaris*, Leguminosae; bean, chloroplast, amylose-amylopectin, synthesis

**Abstract**—Isolated bean (*Phaseolus vulgaris*) chloroplasts were used to investigate the mode of synthesis of transitory amylose and amylopectin from ADP-glucose 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

## INTRODUCTION

THE BIOSYNTHESIS of an amylopectin-like polysaccharide was achieved 30 yr ago by incubating phosphorylase, Q-enzyme and glucose -1-P.[1] However, the manner in which amylose and amylopectin are synthesized is still a matter of controversy.

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.[2] Secondly, a hypothetical debranching enzyme is presumed to produce amylopectin from a phytoglycogen precursor.[3] Finally it has been suggested[4,5] 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.

In this study we have used isolated bean chloroplasts to follow the synthesis of amylose and amylopectin during transitory starch synthesis. This should approximate *in vivo* conditions and in this manner the problem of extrapolating results from isolated enzyme systems should be avoided. Evidence is presented supporting the hypothesis of amylopectin synthesis via branching of amylose. In addition evidence suggests that branched primer present in the preparation may act as a glucose receptor from ADP-glucose.

## RESULTS AND DISCUSSION

Experiments in which glucose-[1-$^{14}$C] was fed to intact wheat plants, indicated that amylopectin was formed from amylose in the endosperm.[6] To determine whether amylose

[1] HAWORTH, W N., PEAK, S and BOURNE, E J (1944) *Nature* **154**, 236
[2] WHELAN, W J. (1963) *Die Starke* **15**, 247.
[3] ERLANDER, S R (1958) *Enzymologia* **19**, 273
[4] GEDDES, R. and GREENWOOD, C T (1969) *Die Starke* **21**, 148
[5] GEDDES, R (1969) *Quarterly Reviews* **23**, 57.
[6] MCCONNELL, W B, MITRA, A K and PERLIN, A S (1958) *Can J Biochem Physiol* **36**, 958

or amylopectin is synthesized first in bean chloroplasts, from the immediate substrate ADP-glucose,[7,8] a pulse chase experiment was performed Starting with three incubations of known specific activity, one was diluted at 15 min with unlabelled substrate and run for a further 45 min to allow the incorporation of cold substrate. Of the two undiluted controls, one was incubated for 15 min and the other for 60 min. 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 1). During the same period a significant portion of this decrease in label was recovered in the amylopectin fraction Thus the evidence confirms the concept that linear amylose production precedes amylopectin production.

TABLE 1 FATE OF [$^{14}C$] GLUCOSE FROM ADPG AFTER INCORPORATION INTO STARCH

| Time of incubation (min) | | Distribution of radioactivity | | | Label in |
|---|---|---|---|---|---|
| ADPG-[$^{14}C$] | ADPG-[$^{12}C$] chase | Amylopectin | Amylose | Total | amylose |
| | | dpm | dpm | dpm | % |
| 60 | — | 11787 | 9806 | 21593 | 45 |
| 15 | – | 5043 | 3290 | 8333 | 39 |
| 15 | 45 | 8307 | 1219 | 9526 | 13 |

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 μmol (3 2 × $10^5$ dpm/μmol) for 15 min followed by addition of 3 2 μmol of ADP-[$^{12}C$] glucose, 60 min control same as above except for labelled addition, 15 min control same as above, without addition

Time course experiments also were carried out to investigate glucose incorporation from ADP-glucose into both amylose and amylopectin (Fig 1) 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-[14C] in amylose and amylopectin we did not observe this expected pattern (Fig 1). 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, was approximately linear 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 1) 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 chlorophyll initially, approx 68% of this starch

[7] PREISS, J, GHOSH, H P and WITTKOP J (1967) *Biochemistry of Chloroplasts* (GOODWIN, T W, ed), Vol II, pp 131, Academic Press, New York

[8] KOVACS, M I P and HILL, R D Unpublished observations

was amylopectin. It has been shown[9] that both amylose and amylopectin can accept glucose from ADP-glucose at similar rates using enzymes from spinach leaf. 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.

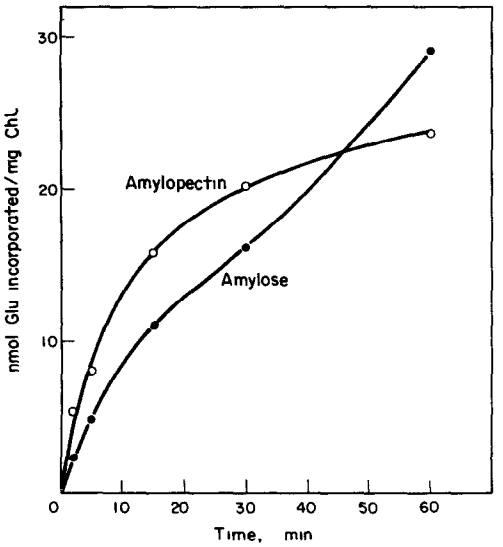


FIG 1 TIME COURSE OF THE GLUCOSE (glu) INCORPORATION INTO AMYLOSE AND AMYLOPECTIN

It has been shown that starch synthesis in isolated chloroplasts was enhanced by high concentrations of citrate and BSA.[8] It has also been shown that one of the two branching enzymes in sweet corn was stimulated by citrate.[10] 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 2. The ratio of amylopectin to amylose was affected during the early stages of incubations. Citrate increased the label in the amylopectin possibly at the expense of amylose. At a later stage, a similar shift was not observed, possibly due to product inhibition by amylopectin.

The passage of label from amylose to amylopectin demonstrated in the pulse-chase experiment, indicates that amylopectin synthesis is probably not compartmented. It is concluded that in the chloroplasts amylopectin can by synthesized by the action of branching enzyme using amylose as substrate formed by ADP-glucose-α-1,4-glucan α-4-glucosyltransferase. Moreover, since starch containing amylose contains 5–10% intermediate

[9] OZBUN, J L, HAWKER, J S and PREISS, J (1972) *Biochem. J* **126**, 953
[10] LAVINTMAN, N (1966) *Arch Biochem Biophys* **116**, 1

TABLE 2. EFFECT OF HIGH CITRATE CONCENTRATION ON GLUCOSE INCORPORATION IN TO AMYLOPECTIN AND AMYLOSE

| Time of incubation | Citrate (0·1 M) | Glucose incorporated (nmol/mg chlorophyll) Amylopectin | Amylose | Total | Increase | Amylopectin amylose ratio |
|---|---|---|---|---|---|---|
| min | | | | | % | |
| 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 |

Reaction mixture and assay conditions were as described in the Experimental section

material, the strict independence of pathways is unlikely [11–13]. However considering our time course experiment and the fact that both amylose and amylopectin can accept glucose molecules from ADP-glucose,[9] the possibility of a certain degree of independent synthesis of amylopectin cannot be excluded. Formation of amylopectin from amylose by Q-enzymes, isolated from plants and its stimulation by citrate has been demonstrated *in vitro*.[10] Furthermore, additional branching enzyme stimulated glycogen production from ADP-glucose in *Streptococcus mitis*[14] which suggests that the branching reaction is a limiting step. It is possible therefore that the increase of the overall starch synthesis resulted from a citrate stimulation of the branching enzyme.

The results presented appear to suggest that amylopectin may be synthesized by two pathways, directly from ADP-glucose and via amylose.

## EXPERIMENTAL

*Plant material.* Chloroplasts were isolated from beans, *Phaseolus vulgaris* (cv. Kinghorn Special) which were grown in soil until the primary leaves were fully developed (2–3 weeks).

*Isolation of chloroplasts.* Fully grown primary leaves were harvested and homogenized in a chilled Waring Blender for 30 sec at max. speed. Approximately 3 ml of chilled homogenizing soln. was used for each g (fr. wt) of leaves. The soln. consisted of: 0·5 M Tris-HCl (pH 8·4), 0·33 M sorbitol, 0·001 M $MgCl_2$, and 0·2% BSA. The slurry was filtered through two layers of fine nylon cloth. The filtrate was centrifuged for 3 min at 50 *g* to remove the debris. The supernatant was centrifuged for 2 min at 800 *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 the homogenizing medium and the differential centrifugation was repeated. The resulting pellet was stored in cone. form (about 5 mg chlorophyll/ml) at 0° for up to 1 hr. Before the incubations the pellet was resuspended in isolating medium so as to give the required chlorophyll conc. About 75–80% of the plastids were highly refractive (Class I) under phase contrast microscopy. The chlorophyll concentration was estimated by the method of MacKinney.[15]

*Assay of glucose incorporation.* Reaction mixture contained 0·9 μmol ADP-glucose-[$^{14}$C] ($3·2 \times 10^5$ dpm/μmol), 495 μmol Sorbitol, 750 μmol Tris-HCl buffer (pH 8·4), 1·5 μmol $MgCl_2$, 0·2% BSA, and chloroplasts (1·5 mg chl/ml) in a final vol. of 1·5 ml. The changes in the amount of substrate and other additives are listed in the text. The incubations were carried out in glass centrifuge tubes in a $H_2O$ bath at 30°. The reaction was terminated by adding 5 ml of 80% MeOH.

*Extraction of starch*[8] 4 mg carrier starch was added to each tube after the reaction was terminated. The suspension was centrifuged for 5 min at 1000 *g* and the pellet was washed three times with 6 ml of 80% acetone and four times with 6 ml of 0·5 M HOAc–MeOH (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° with occasional mixing, and centrifuged for 20 min at $10^4$ *g*. The extraction was repeated twice and the extracts were combined. Starch was precipitated overnight with 2 vol. of EtOH and 50 μl of saturated NaCl. The ppt. was washed with EtOH and the air-dried starch was redissolved in 0·5 ml of DMSO at 35°.

[11] LANSKY, S., KOOL, M. and SCHOCH, T. J. (1949) *J. Am. Chem. Soc.* **71**, 4066.
[12] BANKS, W. and GREENWOOD, C. T. (1959) *J. Chem. Soc. (Lond.)* 3436.
[13] GEORGES, R., GREENWOOD, C. T. and MACKENZIE, S. (1965) *Carbohydr. Res.* **1**, 71.
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[15] MACKINNEY, G. (1941) *J. Biol. Chem.* **140**, 315.

After solubilization the vol was made up to 1 5 ml with a solution of 0 1 M NaCl, and 5 mM EDTA One hundred $\mu$l were removed for carbohydrate and radioactivity determination and 1 ml for column chromatography Carbohydrate concentration was estimated by the phenol-$H_2SO_4$ test [16]

*Chromatography of starch* The method was essentially similar to that described by Matheson[17] with slight modifications 1 ml of starch suspension (15 mg) was applied to a column of 4% sepharose gel (Pharmacia sepharose 4B-200, approximate exclusion limit average mol wt for polysaccharides $5 \times 10^6$) The column was eluted with a soln containing 0 1 M NaCl, 5 mM 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% of iodine and 0 2% KI in 0 5 N HCl soln and the absorption read at 560 and 625 nm corresponding to amylopectin and amylose respectively Recovery of amylose and amylopectin was 90 95% as tested by amperometric titration and the phenol $H_2SO_4$ test No amylose was detected in the amylopectin fraction The radioactivity of each fraction was measured with a liquid scintillation counter

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[17] MATHESON, N K (1971) *Phytochemistry* **10**, 3212