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Use of Polyethylene Glycol in Isolation and Assay of Stable, Enzymically Active Starch Granules from Developing Wheat Endosperms

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ABSTRACT

A procedure using polyethylene glycol (PEG), molecular weight 1000, was developed for the isolation of starch granules from wheat endosperm. Immature endosperm tissue was cut repeatedly in 300 millimolar PEG 1000 and filtered through Miracloth. Centrifugation separated a pellet from a supernatant with inhibitory activity. The pellet contained several enzyme activities, including soluble and bound components of starch synthase, starch phosphorylase, and sucrose synthase activities. The starch phosphorylase activity was unaffected by several washings with 300 millimolar PEG 1000 but was lost when the granules were washed once without PEG or washed with sucrose, glycerol, or sorbitol (up to 30%, w/v). The fraction of starch synthase, remaining on the granules after a wash without PEG (the 'bound' activity) was not affected by the addition of 30% sorbitol to the wash buffer. This fraction became larger with grain development (0.2–0.7).

To obtain high activity, PEG was required not only during isolation of granules but also in the assay of both starch phosphorylase and starch synthase giving optimum activity at 225 to 255 millimolar. PEG reduced the requirement for glycogen as primer with soluble starch synthase. However, the 'bound' starch synthase activity was unaffected by PEG. PEG of different size were compared by their effects in the assay of starch granules: with increase in molecular size, the same effect was obtained at ever lower polymer concentration (w/v) down to a limit.

Treatment of granules with Triton X-100 did not affect their starch synthase activity, but it removed the capacity to incorporate label from UDP [^{14}C]G into non-starch polymers.

It is concluded that PEG, like some other active compounds (ethanol Na_3-citrate, and Ficoll) could mediate enzyme-primer interaction by exclusion.

As part of our continuing interest in identifying the factors determining the potential of wheat endosperm tissue to synthesize starch (9), it appeared desirable to investigate the potential of the smallest synthetic unit, the amyloplast. However, the isolation of intact, functional amyloplasts has not so far been successful to the extent of our purpose. The amyloplast fraction, isolated from soybean callus in a careful procedure by Macdonald and apRees (5), contained only 23.4% and 13.0% of the cell's starch synthase and starch phosphorylase, respectively. At the same time the authors stated: "As starch is confined to plastids, the enzyme that synthesizes starch is likely to be similarly confined." Starch synthase of wheat endosperm, as of some other tissues (3), has so far been isolated in 'bound' and 'soluble' fractions; the use of different primers in the assessment of the activities of these fractions prevents determination of their relative contributions to the total activity.

In the present approach, the amyloplast question has been side-stepped. The purpose of this paper is to show that by using PEG several enzyme activities, especially those of both soluble and bound starch synthase and of starch phosphorylase, can be assessed on the tissue's own starch granules and that PEG acts by maintaining or effecting the tissue's enzyme-starch granule (primer) interaction.

MATERIALS AND METHODS

Plant Materials. Plants of *Triticum aestivum* cv SUN9E were grown at 21/16°C (day 16 h/night 8 h) in natural daylight in the CSIRO Phytotron (CERES, Canberra). Some seasonal variation could not be avoided. Ears were taken approximately 15 d after anthesis to provide grains in the 45 to 55 mg fresh weight range. The grains were cut across at the basal end to remove the embryo. The starchy endosperm, uncontaminated by other tissue, was squeezed out by pressure exerted judiciously by the tip of the index finger and collected in the standard procedure, in PEG 1000 buffer (pH 7.4): 300 mM (30%, w/v) PEG, mol wt 1000 (PEG 1000), 25 mM Tes KOH (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT.

Starch Granule Isolation. Starch granules were freed from the endosperm cells by chopping with a modified electric knife with the blades replaced by two holders fitted with one-sided razor blades. The brei was filtered on ice through one layer of Miracloth to remove cell debris, with enough PEG 1000 buffer to give approximately 10 endosperm equivalents of granules per ml. The suspension was centrifuged at 4000*g* for 10 min at 2°C and the pellet carried usually through at least one more cycle of resuspension and centrifugation. The final suspension was in the range of 6 to 16 mg granules/ml. The dry weight of granule aliquots was obtained by washing them in 70% ethanol onto filters and drying the filters.

Assay for Enzyme Activities. Starch granules were incubated in an assay mixture with a total volume of 1 ml at 25°C with a high shaking rate (approximately 150 rpm). The standard assay mixture contained a final PEG 1000 concentration of 255 mM (or 225 mM), Tes-KOH (16 mM), KCl (50 mM), $MgCl_2$ (5 mM) with or without ATP (2 mM), plus 250 μl starch granule suspension (or supernatant as specified) and 100 μl of the labeled substrates containing 1000 nmol of [^{14}C]glucose-1-P (G-1-P) (200 dpm/nmol) or 1000 nmol [^{14}C]adenosine diphosphoglucose (ADPG) (50 dpm/nmol), in 1 ml total volume. The reaction was stopped by adding 10 ml 70% ethanol and, after heating at 70°C for 20 min, the suspension was filtered through glass fiber filters (Whatman GF/C, 2.4 cm) and the filters washed three times

with 5 ml of 70% ethanol and three times with 5 ml of 20 mM K-phosphate (pH 6.4). Five ml 70% ethanol was poured into the Millipore filter device prior to the addition of the sample. Entrapment of label was reduced by substituting 50% for 70% ethanol in the processing steps following heating, whereas the phosphate wash was strictly required only with G-1-P label. In special checks, the filter method gave approximately equal values to and less variation than washing by centrifugation. With water-soluble primers, washing was limited to the use of 70% ethanol. After addition of 1 ml H_2O and 3 ml Triton X-100, the filters were vigorously stirred in 6 ml scintillation liquid of 0.6% (w/v) PPO in toluene and counted in a Beckman counter, model LS 6800. Correction was made for zero time controls (1–2 nmol). Data represent means of duplicate assays.

Sucrose synthase activity was assessed according to Chourey (1) in the sucrose cleavage direction yielding fructose which was measured with Nelson's reagent. Controls without UDP were included.

Starch Label. Air-dry filters with residues were transferred with 1.5 ml water to 15-ml centrifuge tubes and heated in a boiling water bath for 30 min. After cooling, 0.5 ml of 0.1 M acetate buffer (pH 5.0) and 250 μg amyloglucosidase were added, and the tubes incubated at 50°C for 1 h; then 10 ml 70% ethanol was added. The ethanol-soluble and insoluble fractions were separated by centrifugation; after evaporation, label was estimated as above.

Water Potential. The water potential of PEG solutions was measured by vapor pressure deficit with a Wescor dewpoint microvoltmeter.

Chemicals. D-[U-^{14}C]G-1-P (approximately 9 GBq/mmol) (potassium salt) and adenosine diphospho-D[U-^{14}C]glucose (approximately 9 GBq/mmol) (lithium salt) were from Amersham Australia Pty Ltd., Sydney. Amyloglucosidase (*Aspergillus niger*) and glycogen (rabbit liver) were from Boehringer Mannheim Australia Pty Ltd.; starch (wheat), amylose III (potato), amylopectin (potato), Ficoll 70, ADPG, ATP and G-1-P, PEG 200, 300, 400, 600, 1000, and 3350 from Sigma; PEG 1000, used initially, from Ajax Chemicals Australia; PEG 6000 from BDH Chemicals, Poole, England; and pure barley α-amylase was a gift from Dr. J. V. Jacobsen of this Division.

RESULTS

PEG during Isolation. Starch granules isolated from wheat endosperm in the standard PEG 1000 buffer showed considerable improvement in enzyme activity due to the inclusion of PEG in the medium. When incubated with 1 mM radiolabeled ADPG or G-1-P for 30 min, they converted more substrate into a 70% ethanol insoluble fraction (Table I). In another experiment the activity of such granules, when tested with 0.5 mM G-1-P, was constant through several cycles of centrifugation and resuspension in PEG 1000 buffer (20 nmol after the first cycle, 22 nmol after the second, and 22 nmol after the third cycle). However, when the pellet was resuspended in buffer without PEG twice, and then resuspended in PEG 1000 buffer again, little activity remained, *viz.* 2 nmol in 30 min. In one set of experiments, activity with G-1-P was lost from the granules after one wash with 200, 400, 600, and 800 mM sorbitol or with 30% (w/v) sorbitol, 30% (w/v) glycerol, or 30% (w/v) sucrose. It is to be noted that the decrease in water potential due to 300 mM PEG 1000 solution, the molar concentration used during granule isolation, exceeds by 3 to 4 times that of an ideal solution (12). Kept in 300 mM PEG 1000, activity was undiminished when assayed 4 h later but was diminished by 30 to 40% after storage overnight at 4°C. Activity was linear for 30 min when the granule preparation was incubated with 1 mM radiolabeled G-1-P, with and without 2 mM ATP, or with 1 mM radiolabeled ADPG (Fig. 1). Most of the label in the 70% ethanol insoluble product obtained under the three assay conditions was soluble in 60% ethanol after incubation with pure amyloglucosidase (98, 100, and 88%, respectively, being solubilized). The product from the first assay condition (*i.e.* with G-1-P) was also digested with barley α-amylase. The radioactivity in the digest when analyzed by paper chromatography (10), co-chromatographed with glucose, maltose, maltotriose, and higher polymers. These results are consistent with the product being starch. While the activity with ADPG may be ascribed to starch synthase, that with G-1-P to starch phosphorylase, the extra activity observed in the presence of G-1-P and ATP could involve ADPG pyrophosphorylase plus starch synthase. No activity was observed in the presence of 1 mM radiolabeled glucose and 2 mM ATP.

In order to check on possible gross interference with starch accumulation in the assay by glycosidase action or a possible role of PEG in repressing such action, granules, prelabeled by incubation with radioactive ADPG, were washed and reincubated in the absence of ADPG. The washing was done four times with PEG 1000 buffer for one subsample (A) and for another (B) three times with PEG 1000 buffer and the fourth time in buffer without PEG. Reincubation was done at 25°C and for (A) at 230 mM PEG 1000 and for (B) in the absence of PEG. Results in dpm were: at zero time for (A) 5029 SE 67 and for (B) 4884 (one entry) and after 60 min for (A) 4641 SE 153 and for (B) 4660 SE 81. The results indicate that the loss of label due to glycosidase

Table I. *Comparison of Isolation Media for Enzyme Activity in Starch Granules*

Isolation of granules was carried out with (a) PEG 1000 buffer, (b) the same buffer without PEG (No PEG), and (c) substitution of PEG with 300 mM sucrose (S No PEG). Assay was for 30 min at 25°C in the presence of 255 mM PEG 1000 for a and, in its absence, for b and c. Dry weight of starch granules in each assay was 4.1 mg.

Enzyme Isolated in	Incorporation of	
	[^{14}C]G-1-P (1 mM)	[^{14}C]ADPG (1 mM)
	nmol	
(a) PEG 1000 buffer (300 mM PEG)	23	169
(b) No PEG buffer	0	32
(c) S No PEG buffer (300 mM sucrose)	0	34

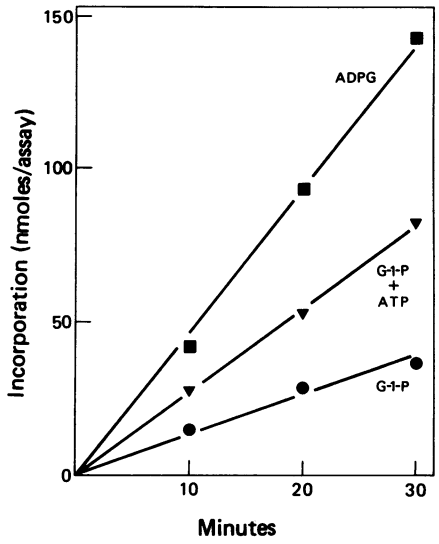

FIG. 1. Time course of incorporation of [^{14}C]ADPG (1 mM) and [^{14}C] G-1-P (1 mM) into starch granules, isolated in 300 mM PEG, in the standard assay. ATP, where added, was 2 mM.

activity was less than 10% in 1 h and not statistically significant.

Reconstitution Experiments. Restoration of activity lost from starch granules centrifuged in the absence of PEG was attempted by recombining pellets and supernatant (Table II). The first PEG 1000 pellets (P_1) and the supernatant (S_1) were retained. One of the PEG 1000 pellets was resuspended in buffer without PEG (No PEG) and, after centrifugation, the supernatant (S_2) was retained and the corresponding pellet was finally resuspended in PEG 1000 buffer (P_2). In the assays comparing combinations of P_2 with S_1 or S_2, the PEG concentration of the standard assay was maintained. It is seen that P_2 as compared with the P_1 preparation had lost 90% of its starch phosphorylase activity. However, 25% of the starch synthase was retained in the P_2 starch granules. In another experiment starch synthase retention appeared to be independent of the osmotic value of the No PEG buffer used to wash P_1. Washes with No PEG buffer containing no or 10% or 30% (w/v) sorbitol gave 24, 24, and 22% retention, respectively. This fraction must represent the bound starch synthase. No restoration of activity followed addition of S_1 to P_2 but substantial restoration followed addition of S_2 to P_2 (Table II, exp. a). This demonstrates that during centrifugation in buffer without PEG, the enzyme is not inactivated, but merely detached from the granule. The fact that the addition of S_1 to P_2 did not stimulate activity at all could suggest that, in PEG buffer, all the cell's starch phosphorylase and starch synthase activities were associated with the starch granule. In another experiment to check on this point an exogenous primer, 500 μg/assay glycogen (rabbit liver), 1 mM ADPG and 210 mM PEG 1000 was used (see below): S_1 converted <1 nmol and S_2 161 nmol. However, it was found that S_1 contained an inhibitory agent. As shown in Table II, experiment b, activity of a whole homogenate (WH) was considerably lower than that of P_1. Addition of S_1 to P_1 reduced the activity to that of WH, but boiling of S_1 removed completely the inhibition with starch synthase and substantially with starch phosphorylase. The 'bound' starch synthase activity of P_2 was not inhibited by S_1.

Table II. *Reconstitution of Enzyme Activity to Buffer-Washed Starch Granules*

Whole homogenate (WH) was obtained in PEG 1000 buffer; centrifugation yielded pellet (P_1) and supernatant (S_1); S_1 (boiled) was kept for 15 min on boiling water; Pellet (P_2) and supernatant (S_2) obtained by resuspending P_1 in buffer without PEG 1000. Incubation for 30 min in presence of 255 mM PEG 1000.

Enzyme	Incorporation of	
	G-1-P (1 mM)	ADPG (1 mM)
	nmol	
Exp. a		
P_1	36	143
P_2	4	36
$P_2 + S_1$	2	34
$P_2 + S_2$	23	92
Exp. b		
WH	1	36
P_1	17	101
P_2	1	23
$P_1 + S_1$	1	37
$P_1 + S_1$ (boiled)	10	96
$P_2 + S_1$	1	22

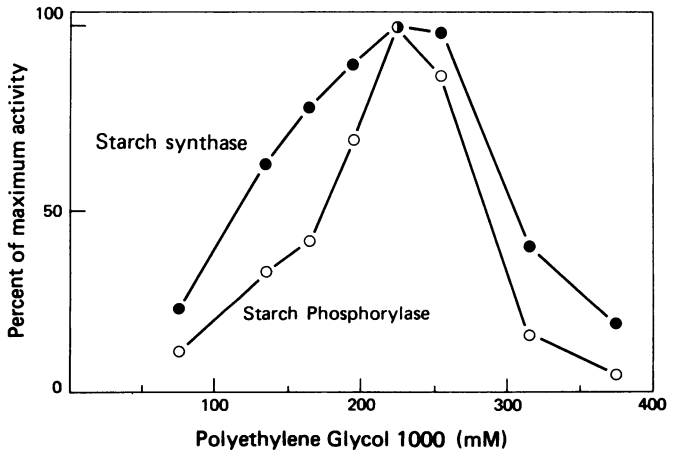

FIG. 2. Effect of PEG 1000 on incorporation of [^{14}C]ADPG (1 mM, ●) and of [^{14}C]G-1-P (1 mM, ○) into starch granules incubated for 30 min. Maximum incorporation with ADPG (76 nmol) and with G-1-P (26.6 nmol).

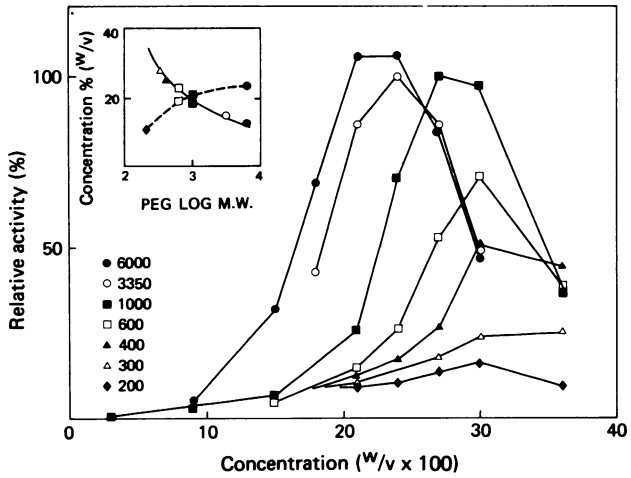

FIG. 3. Effect of PEG 200, 300, 400, 600, 1000, 3350, and 6000 on incorporation of [^{14}C]G-1-P (0.5 mM) into starch granules incubated for 30 min. In addition to the PEG indicated on the abscissa, each assay contained 3% (w/v) PEG 1000. Relative activity is with respect to the maximum found with PEG 1000 (84 and 86 nmol, two experiments combined). Inset: concentration (%, as derived by interpolation) at which the different PEG achieved 20% activity, as a function of mol wt. Broken line denotes concentration at which the different PEG reduced the water potential by 1.5 MPa.

Activity bound to the granules, as in P_2, appeared to be bound tightly. In another experiment, in which P_2 gave 36% of the starch synthase activity of P_1, the activity dropped only to 31% after five more washes in the absence of PEG. The percentage, bound, appeared to be a developmental attribute: comparing the P_2/P_1 ratios of starch synthase activities of grains, obtained 11, 17, and 23 d after anthesis, gave respectively: 0.23, 0.45, and 0.70.

PEG in the Granule Assay. An important factor in the establishment of high activity appeared to be the concentration of PEG 1000 in the assay mixture. As shown in the experiment of Figure 2, a sharp optimum was found at 225 to 255 mM PEG 1000 for both the starch synthase and starch phosphorylase activities. When the bound starch synthase was assayed in the absence and presence of 180 mM PEG 1000, no clear difference in activity (51 and 58 nmol, respectively) was found. PEG, therefore, does not seem to interfere with the reaction itself. In another set of two experiments (Fig. 3), the effectiveness of a series of PEG from mol wt 200 to 6000 was compared in the starch phosphorylase assay. In this case, 100-μl aliquots of granules, prepared and suspended in 300 mM PEG 1000—and thereby providing 30 mM (3% w/v) of PEG 1000 to each assay—were incubated with increasing amount (w/v) of the specific

PEG. The results show that, with increase in mol wt, the maximum itself increased up to the value of PEG 1000 and stayed there up to mol wt 6000, and that with increasing mol wt the same activity was obtained at ever lower concentration, expressed on a weight basis (Fig. 3, inset). Since at any given concentration (w/v) the total number of monomer-subunits in the solution is the same independent of polymer size, the results suggest that with increase in molecular size the activity per PEG-monomer increases, *i.e.* up to a limit. In contrast, to obtain the same reduction in water potential with different PEG, the regression between concentration (w/v) and mol wt appeared to be positive (Fig. 3, inset), but also with the features of a limit value. In this case, at the lower end of the mol wt scale, the effect of molecule number outweighs the effect of subunit number.

PEG and Other Agents with Exogenous Primer. PEG appeared to mediate the binding of soluble starch synthase to exogenous primer. With amylose (potato) high activity was found at lower PEG 1000 concentration than with the natural starch granule (Figs. 2 and 4). However, the effective concentration range was wider. Also other agents were found to enhance activity: ethanol, trisodium citrate, and especially Ficoll, (a polysucrose of mol wt 70,000) but not sucrose (Fig. 4). Hardly any activity was found in the absence of amylose notwithstanding near optimal Na_3-citrate concentration. With trisodium citrate in the assay, Ozbun *et al.* (7) observed 'unprimed activity,' i.e. starch synthase activity without added α-glucan polymer as primer but with BSA added instead, perhaps aiding precipitation of the product. Therefore a check was made here on the possibility of an artefactual result.

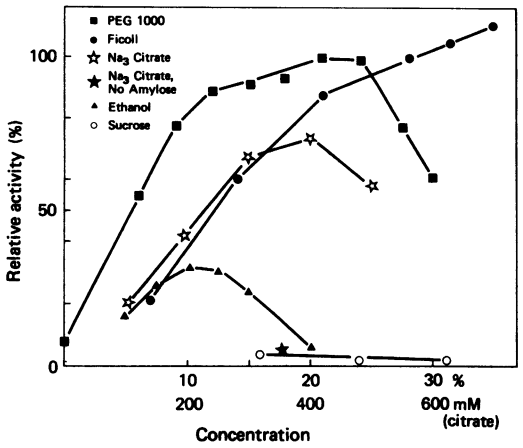

FIG. 4. Effect of PEG 1000, ethanol, trisodium citrate, Ficoll 70, and sucrose on incorporation of [^{14}C]ADPG (1 mM) with soluble starch synthase (fraction S_2) in presence of 250 μg amylose (potato) in 30 min. Relative activity is with respect to maximum found with PEG 1000 (96–115 nmol in four combined experiments). An entry without amylose at 350 mM citrate is included. Per cent is (w/v), but for ethanol (v/v).

Table III. *Effect of Amylose in Assay of Soluble Starch Synthase*

Pellet (P_1) obtained in PEG 1000 buffer with 1.25% Triton X-100. Supernatant with soluble enzyme, obtained by resuspending P_1 in buffer without PEG. Incubation with 0.5 mM [^{14}C]ADPG for 30 min in presence of agents and with and without amylose (potato) (250 μg/assay).

Agent	Incorporation		
	No amylose	Amylose for last 30 s	Amylose for full 30 min
		nmol	
PEG 1000, 21% (w/v)	11	11	79
NA_3-citrate, 400 mM	2	1	75
Ficoll 70, 25% (w/v)	1	2	75

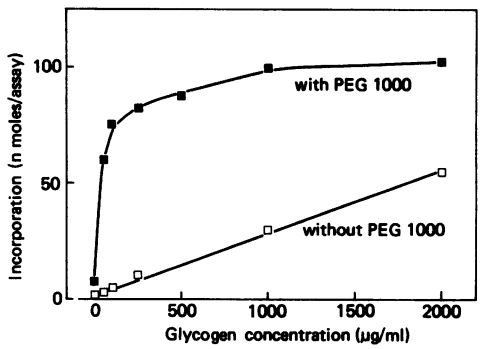

FIG. 5. Effect of glycogen concentration in assay of soluble starch synthase in presence and absence of 210 mM PEG 1000. Incubation was with [^{14}C]ADPG (0.5 mM) for 30 min at 25°C.

Table IV. *Effect of Triton X-100 Treatment of Starch Granules on Activity with ADPG and UDPG*

Pellets of starch granules obtained from PEG 1000 buffer with and without 1.25% (w/v) Triton X-100 were both resuspended in PEG 1000 buffer and assayed in the presence of 255 mM PEG 1000

	Incorporation			
	Control granules		Triton-treated granules	
	ADPG (1 mM)	UDPG (1 mM)	ADPG (1 mM)	UDPG (1 mM)
	nmol			
Exp. 1	130		138	
Exp. 2	92	72	111	7
Exp. 3	100	65	89	7

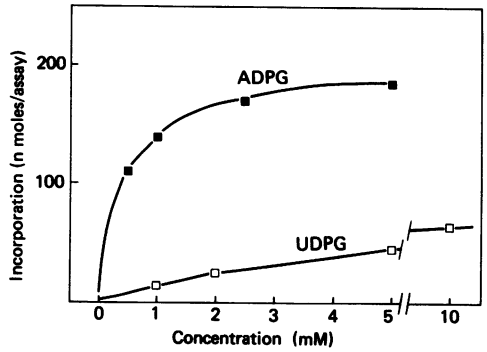

FIG. 6. Effect of [^{14}C]ADPG and of [^{14}C]UDPG concentration on incorporation into Triton X-100-washed starch granules. Incubation was with 255 mM PEG 1000 for 30 min at 25°C.

In particular, the question was considered whether amylose merely acted as a precipitating agent at termination of the assay. This was achieved by comparing assays with amylose present either over the full 30 min, or present only over the last 30 s or not at all. During processing, only 70% ethanol washes were applied. The results show that for activity to occur amylose must be present over the full incubation period, and that amylose did not act by enhancing precipitation (Table III). Only with PEG was some unprimed activity (14%) observed. On another occasion, higher unprimed activity with PEG was observed, but it was very low in the experiment of Figure 5.

For the comparison of different primers with soluble starch synthase again only 70% ethanol washes were used; this appeared to be a necessity with the highly water-soluble primers. Assayed at 210 mM PEG 1000 and 500 μg primer/assay, relative activities with amylose (potato), amylopectin, and glycogen (rabbit liver)

Table V. *Effect of Carbohydrate Polymers and PEG 1000 on Pelletability of Enzyme Activity*

Pellet (P_1) obtained in PEG 1000 buffer plus 1.25% (w/v) Triton X-100. Supernatant (S_2) obtained by resuspending P_1 in buffer without PEG. Supernatant (S_3) obtained by adding 25 mg starch (wheat) per ml or 25 mg cellulose powder (Whatman, standard grade per ml) to S_2 and centrifugation for 20 min at 16,000 *g* in the absence and presence of 300 mM PEG 1000. Incubation for 30 min in presence of 255 mM PEG 1000 with 250 μg amylose (potato) per assay

Enzyme	Incorporation of	
	G-1-P (1 mM)	ADPG (1 mM)
	nmol	
S_2	26	45
$S_{3(starch)}$	24	27
$S_{3(cellulose)}$	25	46
$S_{3(starch + PEG)}$	1	<1
$S_{3(cellulose + PEG)}$	2	18

were respectively 100, 116, and 158%. Results of an experiment with varying glycogen concentrations suggest that in the presence of PEG 1000 primer saturation is reached at a markedly lower concentration than in its absence (Fig. 5).

Triton Treatment. Although addition of Triton to assays with radiolabeled ADPG did not seem to affect incorporation at all, further analysis showed that other activities were affected. This was done in experiments in which aliquots of granules had been washed in PEG 1000 buffer with and without 1.25% Triton X-100. When these treatments were compared, it appeared that the Triton-treatment drastically reduced incorporation from UDPG, but not from ADPG, into the 70% ethanol-insoluble fraction (Table IV). Except for the control granules incubated with UDPG, amyloglucosidase solubilized 100% of the reaction products confirming that the product was a starch-like compound. In contrast, only 20% of the label from UDPG-treated control granules was solubilized by amyloglucosidase. Presumably, in that case label went mainly into β-glucans which are known to be synthesized from UDPG in a membrane-bound fraction (11). Starch synthesis from UDPG appeared to be less than one-tenth that from ADPG. However, this fraction increased with the concentration of the glycosyl donors and was approximately 35% at 10 mM (Fig. 6). This is in agreement with the early literature reviewed in Preiss and Levy (8).

Absolute Activities. An obvious way to describe absolute activities would be to express them on the basis of the amount of starch or dry weight of 70% ethanol-washed granules. However, a most important variable appears to be developmental stage. Starch granules, prepared according to the standard procedure from ears taken 12 days after anthesis, produced in the starch synthase assay 71 nmol mg^{-1} h^{-1} (0.3 mg mg^{-1} d^{-1}) but those prepared from ears taken 24 d after anthesis produced only 8 nmol mg^{-1} h^{-1} (0.03 mg mg^{-1} d^{-1}). For comparison, 0.12 mg mg^{-1} d^{-1}) may be considered a relatively high growth rate (dry weight) for intact grains in the ear (9). Since in wheat endosperm two granule classes are found, in a further analysis this variable also must be considered. This may be achieved with PEG.

A fraction of large granules, 6×10^5/mg dry weight, was obtained by centrifuging the original, Triton-treated suspension twice for 1 min at 160*g*. An optimum PEG 1000 response similar to the one of Figure 2 was found at 195 to 225 mM PEG 1000 for this preparation. It converted G-1-P and ADPG at 18 and 42 nmol/mg dry weight starch·h (or 0.07 and 0.16 mg mg^{-1} d^{-1}), respectively.

PEG Mechanism. Although it seems reasonable to presume that starch phosphorylase and starch synthase are amyloplast enzymes, the above experiments do not preclude the possibility that during isolation with 30% (w/v) PEG 1000 the enzymes are precipitated from the cytosol onto the starch granules in a 'salting-out' action. When Triton-treated granules (P_1) were resuspended in buffer without PEG and centrifuged, the enzyme activity in the resultant supernatant (S_2) became partly pelletable upon addition of abundant starch and wholly pelletable upon addition of both starch and 300 mM PEG 1000; with cellulose powder, however, PEG was less effective in completely pelleting the enzyme (Table V).

Consistent with these results, it is not unexpected that a large, probably cytosolic (5) enzyme, sucrose synthase, coprecipitated in the presence of PEG 1000 buffer with the starch granules. The supernatant, S_2, obtained from such a pellet by washing in the absence of PEG, contained as much sucrose synthase activity as the extract prepared from endosperms in the absence of PEG (in the range of 260 to 330 nmol/endosperm in 10 min).

DISCUSSION

It is shown above that, by simply including PEG during isolation and assay of starch granules, starch phosphorylase and both soluble and bound starch synthase can be assessed on them. (However, due to the presence of an inhibitor, it was not conclusively shown that all the starch synthase and starch phosphorylase activity of the tissue became associated with the granules.) In the present procedure, the assessment applies to the cell's natural primer, its own starch. The separation between soluble and bound activity is avoided and thereby a source of confusion. Mengel and Judel (6) underestimated the contribution of soluble starch synthase during wheat grain development as in their assessment it amounted to a mere 10% of the total. By contrast, in the present study, a contribution of as much as 77% of activity on the starch granule was contributed by soluble starch synthase at 11 d after anthesis. For soluble starch synthase high activity may be recorded with glycogen as primer (7), but then with the different primers the relative contributions of the bound and soluble fractions to the total would become confusing. The differences between primers are not well understood.

On the basis that in the cell starch synthesizing enzymes would be confined to the amyloplast (5) it would follow that, during isolation, PEG acted as a suitable osmoticum preventing membrane damage. However, if the enzyme were to occur outside that organelle or in case these organelles did not persist intact in the cell, then the action of PEG could be understood on the basis of 'exclusion' (2) of the enzyme-protein from part of the solution onto some particle, as suggested by the data of Table V. *A priori*, the persistence of amyloplasts as intact organelles into the adult cell stage is not, or not always, to be taken for granted. The consistent loss of enzyme activity from granules after No PEG washes of high osmotic value, noted above, disagrees with PEG effecting enzyme retention on the granules by the maintenance of a semipermeable membrane. Likewise, Triton X-100, which destroys membranes, included in the PEG buffer wash did not cause removal of the enzymes from the granules. PEG has been used in the isolation of cell organelles and has been reported to have no deleterious effect (4).

Also in the gentlest of transitions from 30% (w/v) Ficoll 70 to 30% (w/v) sucrose, loss of starch synthase from granules occurred as high as when omitting sucrose (unpublished data). It is highly probable that after Triton treatment in the presence of PEG 1000, the 'soluble' starch synthase was retained on the starch granule by exclusion alone and that at the optimum PEG concentration the retention was quantitative. Sucrose synthase probably also pelleted with the granules by exclusion from the PEG 1000 solution. It is clear that the PEG granule preparation does not represent a pure system. It also retained a membranous fraction capable of incorporating label from [^{14}C]UDPG into non-starch, presumably into β-glucan (11) but this enzyme activ-

ity could be removed by Triton treatment. It is noteworthy that pelleting the homogenate freed the pellet from a heat-labile inhibitory agent (Table II, exp. b). This agent may well be a protein. Its size could prevent it from interacting with the bound starch synthase in the interior of the granule. Preliminary observations demonstrated the presence of β-amylase and of abundant protein in the supernatant (S_1), perhaps largely gliadin, which was found to dissolve into the PEG 1000 buffer. Treating S_1 for 5 and 10 min at 60°C destroyed its apparent β-amylase activity by, respectively, 57% and 83% and its inhibition of starch synthase activity by, respectively, 38% and 75%. β-Amylase activity could account for the inhibition of starch synthase observed (unpublished data).

The requirement for PEG, in the assay itself, as indicated by Figures 2, 3, and 4 should be of assistance in the evaluation of starch phosphorylase and starch synthase activities. This requirement can also be interpreted on the principle of exclusion, in this case effecting the necessary association of primer and enzyme. Results with glycogen as primer suggest that, in the presence of PEG, fully effective primer concentration is reached at markedly lower concentration than in its absence (Fig. 5). To reach an effective primer concentration *in vitro* with an insoluble primer such as starch, exclusion of enzyme onto the granule may well be a practical necessity. *In vivo*, in cells packed with starch granules, this problem may not arise. Alternatively, *in vivo* soluble starch synthase could be confined to the amyloplast stroma in close association with the primer. In a fuller evaluation, an effect of PEG on the K_m of the enzyme, independent of its effect on primer-enzyme association, would have to be considered as a possibility.

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