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Effect of Temperature on Starch Synthesis in Potato Tuber Tissue and in Amyloplasts

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ABSTRACT

A sharp temperature optimum is observed at 21.5°C when the incorporation of [^{14}C]sucrose into starch is measured with discs cut from developing tubers of potato (*Solanum tuberosum* L. cv Desirée). By contrast, increasing temperatures over the range 9 to 31°C only enhance release of ^{14}C to respiratory CO_2 and incorporation of ^{14}C into the ethanol-soluble fraction. By comparison, starch synthesis in discs from developing corms of cocoyam (*Colocasia esculenta* L. Schott) is increased by raising the temperature from 15 to 35°C. The significance of a relatively low temperature optimum for starch synthesis in potato is discussed in relation to the yield limitations imposed by continuously high soil temperatures. Amyloplasts isolated from protoplasts prepared from developing potato tubers contain activities of alkaline pyrophosphatase, NAD-dependent glyceraldehyde-3-phosphate dehydrogenase, fructose-1,6-bisphosphatase, and phosphoglucomutase in addition to ADP-glucose-pyrophosphorylase, starch phosphorylase and starch synthase. Cell-free amyloplasts released by thinly slicing developing potato tubers synthesize starch from [^{14}C]triose-phosphate generated from [^{14}C]fructose-1,6-bisphosphate in the reaction medium. This starch synthesis is inhibited by addition of 10 millimolar inorganic phosphate and requires amyloplast integrity, suggesting the operation of a triose-phosphate/inorganic phosphate exchange carrier at the amyloplast membrane. The temperature optimum at 21.5°C observed with tissue discs is not observed with amyloplasts.

The yield of potatoes in the warm tropics is limited by the continuously high soil temperatures (1). Growth of the potato tuber is closely linked to starch synthesis, and physiological studies with whole tubers still attached to the parent plant have indicated that higher temperatures could reduce yields by inhibiting starch synthesis in the tuber (17, 24, 28, 33). However, interpretation of the results of these studies has been complicated by the possible influence of a temperature-dependent redistribution of plant growth regulators, especially gibberellin (17, 28), and the sensitivity of starch synthesis *per se* to higher temperatures has remained unknown. Thus, the present work was carried out to determine the temperature optimum for starch synthesis in excised potato tuber tissue supplied with sucrose and in cell-free amyloplasts supplied with triose-P.

MATERIALS AND METHODS

Plant Material. Developing potato (*Solanum tuberosum* L.) tubers (weighing 20–30 g) of the main crop cultivar, Desirée, were taken from plants grown in Reading, either in the open ground during the summer or in a glass house with supplementary heating (15–20°C) and lighting (13 h daylength) during winter. Developing corms of cocoyam (*Colocasia esculenta* L. Schott) were taken from plants grown under natural lighting in a heated glasshouse. Tubers and corms were washed, surface sterilized, and peeled before use. In the glasshouses, plants were grown in potting compost contained in 10-L polythene tubs and watered daily.

Preparation of Discs and Measurement of Sucrose Uptake into Different Fractions. Potato tubers or cocoyam corms were sliced to 1 mm thickness, and discs 4 mm in diameter were cut from these slices under a medium containing 5 mM DTT and 33 mM PEG-4000. The discs were washed twice in fresh medium before 14 discs (containing about 10 mg starch) were added to each 10-mL vial. The incubation medium (0.5 mL per vial) contained 5 mM DTT, 33 mM PEG-4000, 25 mM bis-tris-HCl (bis[2-hydroxyethyl]iminotris[hydroxymethyl]methane) (pH 6.5), 25 mM KCl, and 150 mM [U-^{14}C]sucrose (specific activity 148 Bq/μmol). The vials were sealed with subaseal caps. When the $^{14}CO_2$ released by respiration was measured, the subaseal caps were fitted with paper filters (supplied by Gilson for their 5 mL automatic pipettes) which contained 200 μL of 3% (w/v) KOH. When the temperature was varied, the vials were placed on a copper temperature-gradient bar, one end of which was heated to 70°C and the other cooled to O°C. Clear plastic separated the bar into a number of zones, the temperatures of which were monitored continuously. After incubation for 7 h, incorporation of ^{14}C into the apparent free space (apoplast), CO_2, the ethanol-soluble fraction and starch was measured by modifications of procedures described by Bhullar and Jenner (2) as follows:

After incubation the suspension medium was transferred to clean vials which were capped with the appropriate subaseal and KOH-containing filter. These vials were incubated at 80°C, with shaking, for 10 min after an addition of 50 μL of 0.1 N HCl via a syringe had been made to release dissolved $^{14}CO_2$. The vials were cooled and the filters added directly to scintillation vials for counting. The discs were washed twice with 5 mL portions of ice-cold water for 30 s to remove adhering sucrose. The ^{14}C of the apparent free space was collected by shaking the discs twice in successive 5 mL portions of ice-cold water for 30 min each time. One mL aliquots of the pooled washes were taken for counting.

Discs then were rinsed with three successive 5 mL portions of ice-cold water before being transferred to 1 mL portions of 80% (v/v) ethanol maintained at 80°C. After 20 min the ethanol was removed and the extraction process was repeated twice. The extracts were pooled and the ^{14}C content of the ethanol-soluble fraction counted. Following ethanol extraction, the discs were autoclaved with 2 mL of water for 2 h at 121°C to gelatinize the starch. When cool, the starch was extracted with perchloric acid,

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precipitated with iodine, and reprecipitated, all essentially as described by Bhullar and Jenner (2). The starch then was hydrolyzed in 0.5 mL of 1 N HCl at 100°C for 30 min, neutralized with 1 N NaOH, and made up to 2 mL with H_2O before adding to the scintillation vial for counting. The total starch content of the perchloric acid extract of a sample of discs was determined using the anthrone reagent (4). Results are expressed on a mg total starch basis, which is convertible to g fresh weight assuming 18% of potato tuber fresh weight is starch (14).

Preparation of Amyloplasts from Protoplasts. Two-mm-thick slices of potato tuber were rinsed for 5 min under running water, and then plasmolysed in 100 mL of 0.6 M sorbitol shaken gently at 5°C for 1 h. Plasmolysed tissue was then added to a sterile Petri dish of 9 cm diameter, the bottom of which was covered with a 5 mm thick layer of 1% (v/v) agar containing 5% (w/v) insoluble PVP (Polyclar AT). The digestion medium (25 mL per Petri dish) contained 0.45 M sorbitol, 10 mM $CaCl_2$, 10 mM KCl, 100 units/mL penicillin, 50 μg/mL streptomycin, 1% (w/v) cellulase (Onozuka R-10), 0.1% (w/v) pectolyase Y-23 (R. W. Unwin & Co. Ltd. Hertfordshire, U.K.) and 25 mM Mes-NaOH (pH 5.5). Incubation and subsequent manipulation of the protoplasts was carried out in darkness or with a green safe light as normal light appeared to reduce protoplast stability. Following 12 h incubation at 25°C with gentle shaking on an orbital shaker, the digested material was filtered consecutively through nylon sieves of 300 and 180 μm mesh, and the protoplasts allowed to settle for 5 min. The supernatant was pipetted to waste and the protoplasts resuspended in a medium containing 0.45 M sorbitol, 10 mM $CaCl_2$, 10 mM KCl, 2 mM disodium EDTA, and 50 mM Tris Cl (pH 8.0). Protoplasts were allowed to settle, and after 5 min at room temperature, the supernatant was again discarded. The washed protoplasts were resuspended in a minimal volume of resuspension medium described above and added to a test tube containing 5 mL of resuspension medium to which 20% (w/v) Nycodenz (Nyegaard & Co., Oslo, Norway), 1 mg/mL BSA, and 5 mM DTT had been added. The final sorbitol concentration was reduced to 0.16 M to allow for the osmotic effect of Nycodenz. After 30 min at 4°C, the protoplasts remained suspended in the 20% Nycodenz, but the large starch grains had settled to the bottom. Less dense contaminants remained above the Nycodenz and were pipetted to waste. Four mL of the protoplast suspension was removed and diluted with 5 mL of resuspension medium to give a protoplast concentration of about 10^6 protoplasts/mL.

The purified protoplasts were lysed by drawing the suspension through a 64 μm nylon mesh secured at the end of a syringe, and the lysate mixed immediately with 1 mL of insoluble PVP that had been preequilibrated overnight with water. The lysate then was added to a layer of resuspension medium described above containing 20% Nycodenz and was allowed to settle for 30 min at 4°C. The top layer was pipetted off and termed the extraamyloplast fraction, and the bottom layer was termed the amyloplast fraction. For analysis of marker enzymes all fractions were treated with 0.1% (w/v) Triton X-100 and passed through 5 mL columns of preequilibrated Sephadez G-25.

Preparation of Cell-Free Amyloplasts. All operations were carried out at 0 to 4°C. The potato tubers were sliced into a 9 cm diameter Petri dish equipped with a plastic cover into which a stainless steel razor blade was set at an angle, so that slices about 1 mm thick passed immediately into 25 mL of collection medium in the dish (8). The bottom of the Petri dish was lined with a 5 mm thick layer of 1% (w/v) agar containing 5% (w/v) insoluble PVP. The collection medium contained 0.33 M sorbitol, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$, 5 mM disodium EDTA, 2 mg/mL BSA, 5 mM DTT, 5 mM KCl, 5 mM NaCl, 0.1 mM KH_2PO_4, and 100 mM glycylglycine (pH 7.8). The slices were removed, and the suspension was allowed to stand for 5 min. The upper layers of about 25 mL were pipetted off and allowed to stand for 1 h in a 50 mL glass tube of 23 mm internal diameter. The clear top half was carefully removed and 10 mL of the remainder (excluding any of the granules at the bottom of the tube) was retained and termed the cell-free amyloplast suspension.

Enzyme Assays. The volume of the reaction mixture for each assay was 1 mL unless otherwise stated. Given below are incubation temperatures and any features of the procedure and the reaction mixtures that differed essentially from those in the references cited:

Alcohol dehydrogenase (19), 20°C; nitrite reductase (13), 30°C; phosphoglucomutase (19), 20°C, 6-phosphogluconate dehydrogenase omitted; starch phosphorylase (25), 30°C; ADP-Glc pyrophosphorylase and UDP-Glc pyrophosphorylase (31), 30°C, assayed nonradioactively, glucose-1-P produced was assayed using a coupled enzyme system after stopping the reaction as in Levi and Preiss (18); fructose-1,6-biphosphatase (procedure A of Ref. 30), 20°C; NAD-dependent glyceraldehyde phosphate dehydrogenase (11), 20°C, 5 μmol DTT replaced cysteine and glutathione; soluble starch synthase (10) 30°C, 100 mM glycylglycine as buffer, DTT replaced GSH and the concentration of ADP-Glc increased to 2 mM, ADP produced was assayed enzymatically (26); sucrose synthase and invertase (32) 30°C, 5 mM DTT replaced mercaptoethanol; alkaline pyrophosphatase (9), 30°C.

The linearity of the assays with respect to time and extract concentration was determined using a crude extract prepared at 4°C as follows: A peeled potato tuber was homogenized in a medium (0.5 mL per g fresh weight tuber) containing 5 mM DTT, 1 mg/mL BSA, 0.1 g/mL insoluble PVP (Polyclar AT), and 0.5 M Tris-Cl (pH 7.5). The supernatant after centrifugation at 5000*g* for 30 min was passed through a 5 mL column of Sephadex G-25 equilibrated with homogenization medium from which the insoluble PVP had been omitted.

Starch Synthesis by Cell-Free Amyloplasts. The reaction conditions were based on those previously used (12) to demonstrate starch synthesis in chloroplasts from exogenous triose-P. To 0.2 mL of a cell-free amyloplast suspension (0.02–0.04 mg starch, determined as in Ref. 4) were added, in 50 μL, 0.75 unit fructose-1,6-bisphosphate aldolase, 25 units triose-P isomerase (both enzymes desalted by passage through Sephadex G-25), 0.25 μmol oxaloacetate, 0.25 nmol fructose-2,6-bisphosphate, 25 nmol phosphoglyceric acid and, 16 μmol sorbitol. The oxaloacetate was added to help oxidize reduced pyridine nucleotide that would be generated if the ATP required for ADP-Glc synthesis was coupled to the oxidation of glyceraldehyde-3-P to phosphoglyceric acid. Reaction was started by the addition of 1.5 mM [U-^{14}C]fructose-1,6-bisP (12.2 kBq/μmol), incubation was continued for 90 min with uniform shaking (10 strokes/min (just sufficient to prevent particulate material settling in the reaction vessels, and the reaction stopped by the addition of 0.25 mL of ice-cold 2 N HCl. Commercial potato starch (10 mg) was added to assist the precipitation of the labeled starch. The precipitated starch was washed by resuspension in 1.0 mL of 1 N HCl and centrifugation (10,000*g* for 30 s) repeated twice. Finally, the pellet was resuspended in 0.5 mL of 2 N HCl and heated at 100°C for 30 min. The solution of hydrolyzed starch was then transferred to scintillation vials followed by two successive rinses with 0.75 mL of 0.5 N NaOH.

When ADP-Glc was the substrate, 50 μL, containing 0.25 μmol ADP-[U-^{14}C]Glc (4.625 KBq/μmol), 6.3 μmol potassium acetate, and 16 μmol sorbitol, were added to 200 μL of the cell-free amyloplast suspension (0.02–0.04 mg starch), and starch synthesis was determined as above.

Counting of Radioactivity. Samples were made up to 2 mL, and where necessary made alkaline with NaOH before addition of 10 mL of scintillant (Ecoscint, National Diagnostics, Aylesbury, U.K.). Quench curves were constructed and all samples

were left in the dark for 24 h prior to counting to allow chemiluminescence to decay.

RESULTS

Synthesis of Starch by Tissue Discs. The discs cut from developing potato tubers incorporated ^{14}C from sucrose into an ethanol-soluble fraction and into starch at constant rates for 7 h, with an initial lag period of nearly 1 h in the case of starch (Fig. 1). By contrast, when discs from dormant tubers were used as a control tissue (Fig. 1), incorporation of ^{14}C into the ethanol-soluble fraction did not continue after the first hour, and incorporation into starch proceeded at a rate about 5% of that observed with discs cut from developing tubers. The relatively slow incorporation of label into starch by the discs from dormant tubers may be attributable to a slow turnover of starch that can occur in mature tubers (14). At 21°C the rate of sucrose incorporation into starch by discs from developing tubers is equivalent to a daily growth rate of 0.014 g fresh weight/g tuber fresh weight.

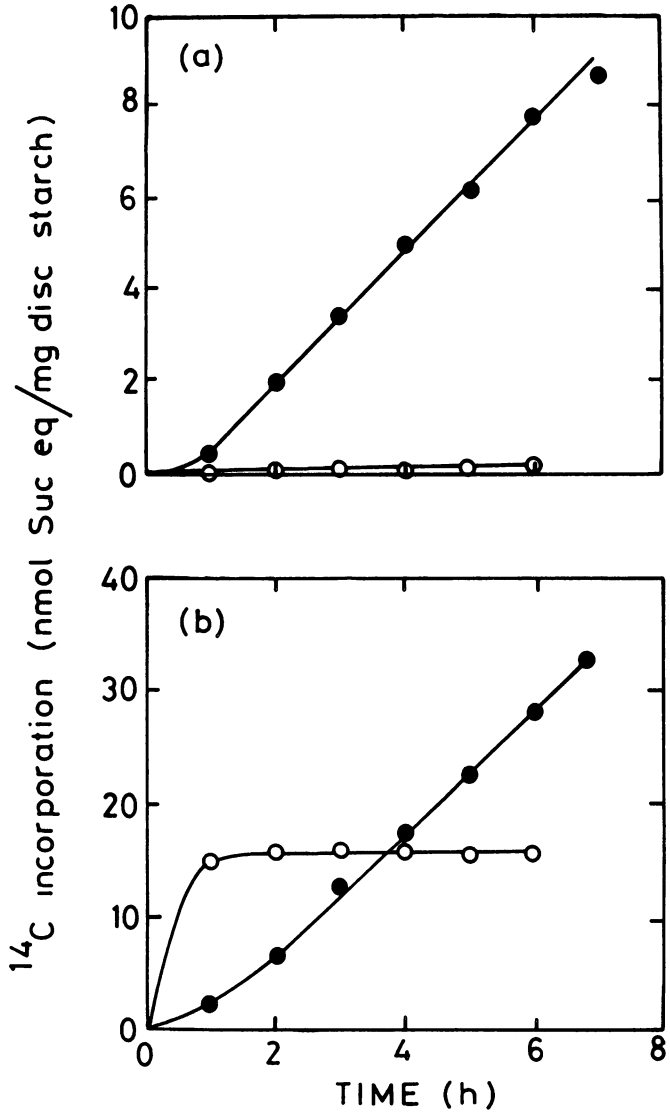

FIG. 1. Time-course of incorporation of ^{14}C from sucrose into (a) starch and (b) the ethanol-soluble fractions of discs cut from developing tubers (closed symbols) and dormant tubers (open symbols) of potato. The temperature was 20°C and sucrose was supplied at 100 mM.

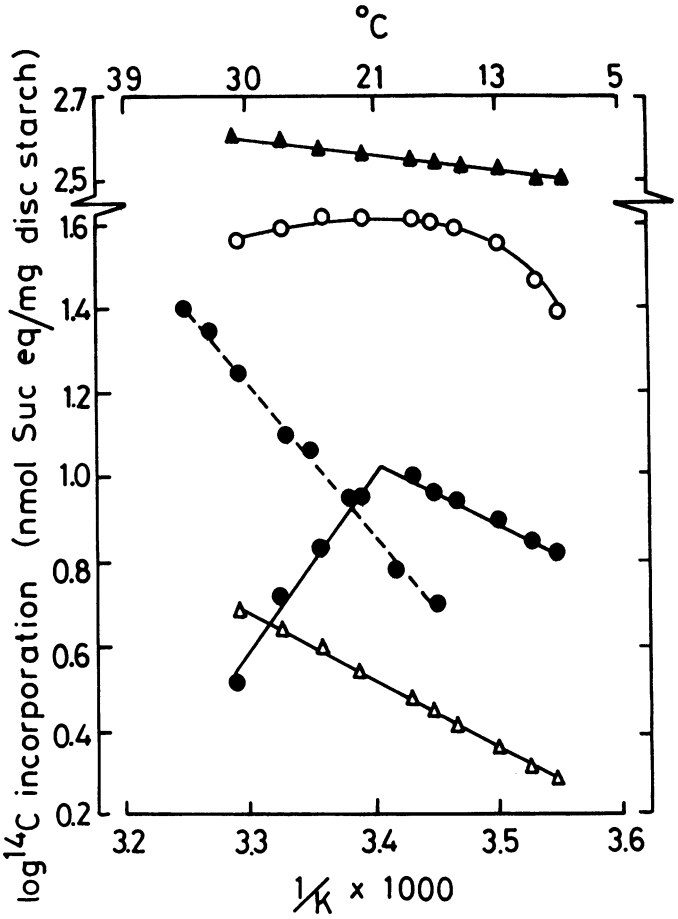

FIG. 2. Effect of temperature on the incorporation of ^{14}C from sucrose into various fractions prepared from discs of potato tubers (solid lines) and into the starch fraction of discs of cocoyam corms (broken line). CO_2 evolved (Δ), starch (●), apparent free space (▲), ethanol-soluble fraction (○).

This value is comparable to the average value for the recorded rate of tuber growth (7).

A previous study (21) of the incorporation of labeled sugars by tuber tissue discs over a similar time period and with similar sucrose concentrations gave rates of sucrose uptake of the same order as those observed in the present work, but incorporation into starch was relatively poor with ethanol-insoluble ^{14}C/ethanol-soluble ^{14}C ratios of about 0.05, which decreased with time (21). By comparison, these ratios in the present work were about 0.2 and remained constant with time (Fig. 1). A possible explanation for the relatively low incorporation of sucrose into starch in the previous work (21) is that the 0.1 M glycerol present in the uptake medium as osmoticum was converted to starch in preference to the labeled sucrose supplied. It is known (20) that suspension culture cells of soyabean readily convert glycerol to starch.

When the temperature of the uptake medium was varied from 9 to 31°C, starch synthesis showed a sharp optimum at about 21°C (mean of 10 experiments, 21.5°C ± 0.5). Incorporation of label into the ethanol-soluble fraction showed lower sensitivity to temperature with little decline above a broad optimum from 17 to 25°C. By contrast, incorporation of label into the apoplastic fraction and release of label as CO_2 both increased as the temperature was raised. These effects of temperature are illustrated in the form of conventional Arrhenius plots (Fig. 2).

In order to determine if the sharp break in the Arrhenius plot

Table I. *Activities of Marker Enzymes and Enzymes of Carbohydrate Metabolism in Lysates of Potato Tuber Protoplasts*

Lysates were prepared and fractionated as described in "Materials and Methods." Values are means of at least 3 experiments ± SE. Percentage activities in amyloplast fraction have been corrected in each experiment for contamination according to Klein (1986) using alcohol dehydrogenase as cytosolic marker.

Enzyme	Activity in Unfractionated Lysate	Percentage of Activity of Unfractionated Lysate Recovered in		Percentage Activities in Amyloplast Fraction Corrected for Cytosolic Contamination
		Amyloplast fraction	Amyloplast plus extraamyloplast	
	nmol/min/mL-lysate			
Alcohol dehydrogenase	25 ± 13.6	6.2 ± 2.7	97 ± 14	
UDP-Glc pyrophosphorylase	316 ± 179	7 ± 1.5	109 ± 21	0.9 ± 0.2
Sucrose synthase	157 ± 23	4.7 ± 1.7	106 ± 14	−1.4 ± 0.4
Alkaline invertase	65 ± 28	5.6 ± 2.8	101 ± 11	−0.9 ± 0.3
Phosphoglucomutase	107 ± 58	17 ± 3	107 ± 20	14 ± 5
Fru-1,6-bisphosphatase	1.8 ± 0.7	18 ± 3.4	103 ± 21	14.4 ± 4.9
NAD-Glyceraldehyde-3-P dehydrogenase	3.5 ± 3.1	23 ± 2.7	102 ± 19	21.4 ± 6.6
Alkaline pyrophosphatase	13 ± 5.2	26 ± 6	127 ± 11	30.5 ± 5.2
ADP-Glc pyrophosphorylase	11 ± 1.4	33 ± 1.3	105 ± 14	32.1 ± 3.5
Starch phosphorylase	6.0 ± 3.7	30 ± 7.1	110 ± 17	29.3 ± 4.8
Soluble starch synthase	6.4 ± 2.8	34 ± 5.1	99 ± 14	31.9 ± 6.2

Table II. *Latency of Marker Enzyme Activities in Amyloplasts Isolated from Protoplasts*

Enzymes were assayed in the presence or absence of 0.1% Triton X-100 which was used to lyse the amyloplasts. The difference in activity between the lysed and unlysed samples expressed as a percentage of the lysed is termed latency.

Enzymes	Activity		Latency
	Unlysed	Lysed	
	nmol/min/mL amyloplast suspension		%
Experiment 1			
Alkaline pyrophosphatase	16.5	22.5	27
Experiment 2			
Alkaline pyrophosphatase	33.3	45.8	27
Nitrite reductase	32.4	39.4	18
ADP-Glc pyrophosphorylase	0.23	0.27	16
Soluble starch synthase	1.56	1.86	16
Experiment 3			
Alkaline pyrophosphatase	25	35	29
Nitrite reductase	19.5	26	25

Table III. *Latency of Enzyme Activities in Cell-free Amyloplasts*

Amyloplasts were isolated from sliced potato tissue. Latency was determined for various enzymes as described in Table II.

Enzyme	Activity		Latency
	Unlysed	Lysed	
	nmol/min/mL amyloplast suspension		%
Experiment 1			
Alkaline pyrophosphatase	7.5	16	53
Alcohol dehydrogenase	23.0	20	
Experiment 2			
Alkaline pyrophosphatase	2.4	5.4	56
Soluble starch synthase	0.06	0.12	50
ADP-Glc pyrophosphorylase	0.13	0.33	61
Starch phosphorylase	0.33	0.6	45
NAD-Glyceraldehyde-3-P dehydrogenase	9.4	12.3	23
Alcohol dehydrogenase	16.7	16	
UDP-Glc pyrophosphorylase	217	213	

was a feature of starch synthesis in discs of storage tissue in general, we repeated the experiment with discs cut from corms of the cocoyam, a crop adapted to the warm humid tropics. As shown in Figure 2, the rate of starch synthesis with discs of cocoyam showed a continuous increase as the temperature was raised from 15 to 35°C. Thus, it is concluded that the break in the Arrhenius plot with potato discs (Fig. 2) is related to the particular temperature requirements of starch synthesis in potato tubers.

Two of the enzymes involved in starch synthesis in potato tubers, ADP-Glc-pyrophosphorylase and starch synthase, have been shown previously to have temperature optima *in vitro* above 35°C (6, 15). We have confirmed that starch synthase from the potato tubers used in the present work shows a strictly linear response to temperature (Arrhenius plot) over the range 12 to 37°C when assayed *in vitro* as described in "Materials and Methods" (data not shown).

Enzymes of the Amyloplasts. In order to locate the biochemical step(s) responsible for limiting starch synthesis at temperatures >21.5°C, we have examined starch synthesis in amyloplasts isolated from developing potato tubers.

The form in which assimilate is taken up by amyloplasts has not been identified, although there are indications that amyloplasts resemble chloroplasts in taking up assimilate in the form of triose-P in exchange for Pi (19). As a preliminary to our studies of starch synthesis in isolated potato tuber amyloplasts, we have determined if they are enzymatically equipped for the conversion of triose-P to starch. In our determination of the enzyme complement of potato tuber amyloplasts, we have isolated them from lysed protoplasts and followed the experimental approaches of MacDonald and ap Rees (19), who worked with amyloplasts of suspension cultures of soybean, and Echeverria *et al.* (5), who worked with amyloplasts from developing maize endosperm.

Preliminary experiments indicated that starch granules released from lysed protoplasts sedimented through a layer of suspension medium containing 20% (w/v) Nycodenz at 1 g. When this rapidly sedimenting fraction was assayed for enzymes

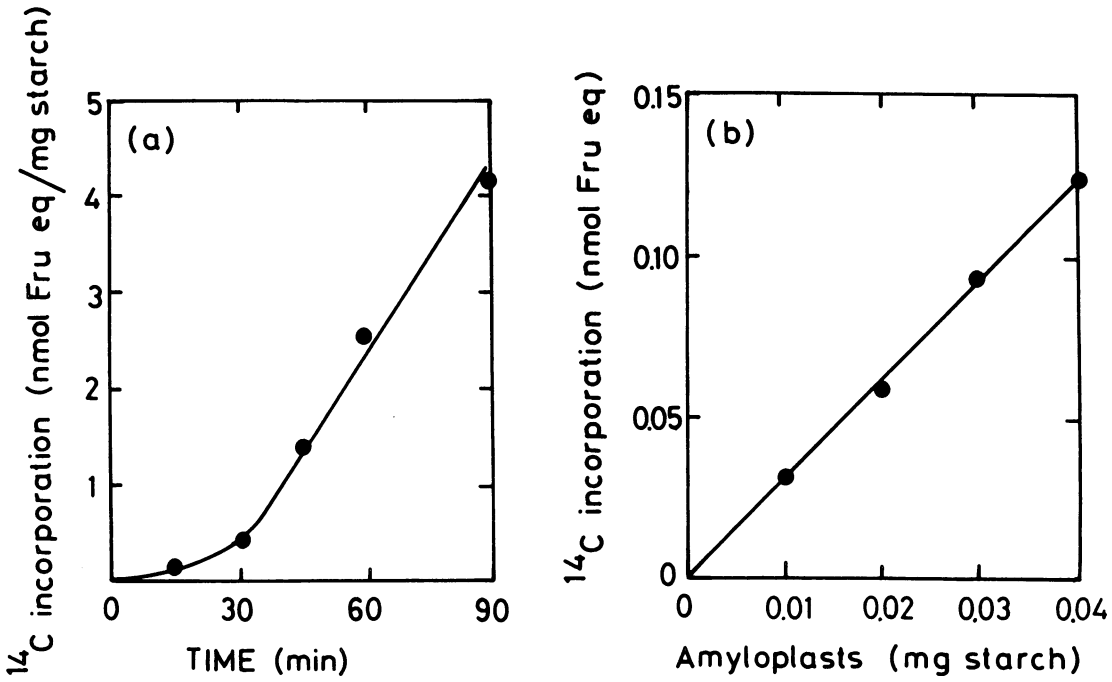

FIG. 3. Synthesis of starch by cell-free amyloplasts obtained from potato tuber. The temperature was 21°C. a, Time-course with 0.03 mg starch present; b, effect of amyloplast concentration, reaction time of 90 min.

Table IV. *Effect of Amyloplast Lysis on the Incorporation of ^{14}C into Starch*

The ^{14}C labeled substrates were supplied to cell-free amyloplasts which were incubated in the presence of 0.1% (v/v) Triton X-100 or the concentration of sorbitol was reduced to 65 mM to give a hypotonic medium. The temperature was 21°C. Values are means of duplicate samples.

Treatment	Radiolabeled Substrate	^{14}C Incorporated	Percent of Control
		nmol hexose eq/mg amyloplast starch	
Experiment 1			
Control	Fru-1,6-bisP	1.94	100
+ Triton X-100	Fru-1,6-bisP	0.36	19
Hypotonic medium	Fru-1,6-bisP	0.48	25
Experiment 2			
Control	Fru-1,6-bisP	2.40	100
+ Triton X-100	Fru-1,6-bisP	0.38	16
Hypotonic medium	Fru-1,6-bisP	0.45	19
Experiment 3			
Control	Fru-1,6-bisP	2.85	100
+ Triton X-100	Fru-1,6-bisP	0.44	15
Control	ADP-Glc	17.7	100
+ Triton X-100	ADP-Glc	42.2	240

characteristic of the amyloplastic and cytosolic cell compartments (Table I), it was found to be relatively enriched in amyloplastic enzymes and relatively lacking in cytosolic enzymes. Thus, the enzymes directly involved in starch metabolism, ADP-Glc-pyrophosphorylase, starch phosphorylase and starch synthase were well represented in the rapidly sedimenting fraction, while alkaline invertase and the enzymes of sucrose metabolism, UDP-Glc-pyrophosphorylase and sucrose synthase, remained largely above the layer of 20% Nycodenz. In Table I we have corrected for contamination of the amyloplasts by cytosolic enzymes according to Klein (16) using alcohol dehydrogenase as cytosolic marker (27). This procedure gives a distribution of about 30% for the three amyloplast marker enzymes in the rapidly sedimenting fraction (Table I). From this value we infer that this fraction contains some 30% of the total amyloplast content of the protoplasts, and we term it the amyloplast fraction. The same procedure applied to phosphoglucomutase, fructose-1,6-bisphosphatase, and glyceraldehyde-3-P dehydrogenase (chosen as representative enzymes of the pathway leading from triose-P to glucose-1-P) show that the amyloplast fraction contains some 14 to 21% of their activity in the protoplasts (Table I). Therefore, they are likely to be components of the amyloplast stroma as well as being represented in the cytosol. The percentage distribution of alkaline pyrophosphatase (Table I) indicates that it is located in the amyloplast (19).

The enzymes, phosphoglucomutase, fructose-1,6-bisphosphatase, and NAD-dependent glyceraldehyde-3-P dehydrogenase, have not been found in other plant cell organelles such as mitochondria and peroxisomes (27). Moreover, these organelles are unlikely to have penetrated a layer of 20% Nycodenz at 1 *g*. Therefore, we have not been concerned to determine the presence of these organelles in the amyloplast and extraamyloplast fractions.

Table V. *Effect of Pi on the Incorporation of ^{14}C Triose-P into Starch by Cell-free Amyloplasts*

Potassium dihydrogen phosphate (pH 7.8) was added as indicated at 10 mM. The temperature was 21°C. Values are means of duplicate samples.

Experiment	Additions	^{14}C Incorporation	Percent of Control
		nmol Fru eq/mg amyloplast starch	
1	Control	4.05	100
	Pi	2.70	67
2	Control	2.25	100
	Pi	1.80	80

Table VI. *Effect of Temperature on the Incorporation of ^{14}C into Starch by Cell-free Amyloplasts*

Values are means of duplicate samples.

Temperature	^{14}C Incorporated		
	Experiment 1	Experiment 2	Experiment 3
°C	*nmol Fru eq/mg amyloplast starch*		
11	1.50	1.95	1.11
21	1.95	2.70	1.50
31	3.15	3.25	2.70

The sedimentation of the amyloplast enzymes into the layer of 20% Nycodenz suggested that they were associated with the dense, rapidly sedimenting starch granules. In order to demonstrate that these enzymes were contained within the amyloplast membrane, we determined the latency of these enzymes as revealed by the stimulation of their activity when the physiological barrier of the amyloplast membrane(s) is destroyed by the addition of the detergent Triton X-100 (5, 19). Stimulation depends upon the inability of substrate and/or coenzymes, added to the suspension medium, to reach the enzyme located within the amyloplast membrane(s). The results presented in Table II show that 16 to 29% stimulation was obtained on addition of Triton X-100. Thus, we estimate that about one-fifth of the amyloplasts remained intact during the assays. Similar determinations had revealed 44% of the amyloplasts to be intact (starch synthase as indicator) in an unfractionated lysate of protoplasts of cultured soybean cells (19), and 47% (nitrite reductase as indicator) and 66% (ADP-Glc-pyrophosphorylase as indicator) of the amyloplasts isolated from developing maize endosperm to be intact (5).

The fragility of amyloplasts containing large starch granules had been noted previously (5, 19, 23). For the present purpose, retention of amyloplast intactness and biosynthetic capability were of greater importance than freedom from contamination by extraamyloplast enzymes. Therefore, in subsequent experiments, we used amyloplasts released by slicing thinly developing potato tubers. The more rapidly sedimenting amyloplasts were rejected, and those that sedimented between 5 and 60 min were used without further purification. These we term cell-free amyloplasts. The percentage intactness of these cell-free amyloplasts (Table III) was at least twice that of the amyloplasts isolated from the protoplasts. Activities of the enzymes likely to be confined to amyloplasts were approximately doubled by the addition of Triton X-100 to the cell-free amyloplasts, whereas activities of the enzymes of cytosolic origin were unaffected by the Triton X-100 treatment. Activity of glyceraldehyde-3-P dehydrogenase, probably present in both cell compartments, showed an intermediate latency (Table III). When the cell-free amyloplasts were treated with trypsin essentially as described (19) in order to inactivate amyloplast enzymes that had escaped from broken amyloplasts before the addition of Triton, no consistent improvement in the percentage latency was observed (data not shown).

Starch Synthesis by Cell-free Amyloplasts. A demonstration of triose-P uptake into the potato amyloplasts was precluded by the difficulty of retaining amyloplast integrity when amyloplasts are separated from the suspending medium. However, having established that the potato amyloplasts, like those of soybean suspension culture cells (19) were enzymatically equipped to convert triose-P to starch, we supplied the cell-free amyloplasts with [^{14}C]triose-P and measured the incorporation of label into starch. The [^{14}C]triose-P was generated in the reaction mixture from [^{14}C]fructose-1,6-bisP by the activity of excess aldolase. In order to inhibit activity of cytosolic fructose-1, 6-bisphosphatase, fructose-2,6-bisP was added. Enzymic assays by the methods of Bergmeyer (22) revealed that under the conditions employed for starch synthesis the levels of triose-P (predominantly as dihydroxyacetone-P) and fructose-1,6-bisP were maintained at about 0.6 and 0.8 mM, respectively, during the incubation period.

The formation of starch by the cell-free amyloplasts was constant with time after an initial lag period (Fig. 3a), and the amount of starch formed was proportional to the concentration of amyloplasts (Fig. 3b). When the amyloplasts were lysed, either by the addition of Triton X-100 to the reaction medium or by the omission of osmoticum, starch synthesis was reduced by about 80% (Table IV). Therefore, the major part of the starch synthesized depended on amyloplast intactness. In agreement with this interpretation, starch synthesis with [^{14}C]ADP-Glc as substrate was stimulated by amyloplast lysis (Table IV). If triose-P enters amyloplasts in exchange for Pi, then the addition of Pi to the suspending medium would be expected to inhibit triose-P uptake and thus slow starch synthesis. This effect is shown in Table V where the addition of 10 mM Pi has resulted in a significant decrease in the rate of starch synthesis.

When measured at 21°C, the rate of starch synthesis expressed on a mg total starch basis by the cell-free amyloplasts was similar to the rate observed with the potato tissue discs.

Having established that starch synthesis in the cell-free amyloplasts was qualitatively and quantitatively representative of that observed in tissue discs, the effect of temperature was examined. The results (Table VI) show that starch synthesis by the cell-free amyloplasts was increased by temperature increments from 11 to 21°C and from 21 to 31°C. Thus, the inhibition observed with tissue discs at temperatures >21.5°C was not observed with cell-free amyloplasts.

DISCUSSION

The present observation of a sharp temperature optimum at 21.5°C for starch synthesis in potato tuber tissue is in good agreement with Burton's conclusion (3) that the optimum temperature for tuber growth with adequate light is 22°C. A temperature optimum at 21.5°C also helps to explain a variety of previous findings which were made with whole tubers: reduced quantity of starch granules per cell when soil temperatures were raised from the range 7 to 10°C to the range 24 to 27°C (33); reduced tuber yields and starch contents when soil temperatures were raised from 20 to 32°C (24); reduced partitioning of assimilate from sugars to starch when tuber temperatures were raised from 16 to 28°C (28) and from 20 to 30°C (17); and increased yields when maximum soil temperatures were reduced from 30 to 23°C (1). It will be of interest to determine whether any of the heat-tolerant varieties show a temperature optimum for starch synthesis higher than that found in Desirée.

The present finding that cell-free amyloplasts show no decline in the rate of starch synthesis when the temperature is raised from 21 to 31°C suggests that assimilate transport into the amyloplast and subsequent steps in starch biosynthesis are not responsible for the inhibition of starch synthesis observed when

the temperature of the tissue is raised above 21°C. However, this conclusion remains tentative until more is known about starch synthesis in isolated amyloplasts and about the time course of inhibition at the higher temperatures. We note that in the developing wheat endosperm inhibition of starch synthesis by temperatures above the optimum of 30°C is attributable to the sensitivity of starch synthase to a pretreatment at high temperature (29). It will be of interest to determine whether enzymes of starch synthesis show lower activities when isolated from potato tubers pretreated at temperatures >21.5°C.

The Pi-sensitive incorporation of ^{14}C from triose-P to starch that we observed with the intact, cell-free amyloplasts provides additional evidence for the existence on the amyloplast membrane of a triose-P/Pi exchange carrier resembling that of chloroplasts. However, the amyloplasts will need to be purified further before we can exclude the possibility that ^{14}C from the fructose-1,6-bisP supplied entered the amyloplasts as a metabolite other than triose-P.

In physiological terms, the potato plant is sink-limited, and the driving force for tuber enlargement appears to be starch synthesis (14). Therefore, the identification of a temperature optimum at 21.5°C for tuber starch synthesis has obvious implications for the productivity of the potato where soil temperatures are high.

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LITERATURE CITED

1. AWAN AB 1964 Influence of mulch on soil moisture, soil temperature and yield of potatoes. Am Potato J 41: 337–339
2. BHULLAR SS, CF JENNER 1986 Effect of temperature on the conversion of sugar to starch in the developing wheat endosperm. Aust J Plant Physiol 13: 605–615
3. BURTON WG 1981 Challenges for stress physiology in potato. Am Potato J 58: 3–14
4. CLEGG KM 1956 The application of the anthrone reagent to the estimation of starch in cereals. J Sci Food Agric 7: 40–44
5. ECHEVERRIA E, C BOYER, KC LIU, J SHANNON 1985 Isolation of amyloplasts from developing maize endosperm. Plant Physiol 77: 513–519
6. FRYDMAN RB, CE CARDINI 1966 Studies on the biosynthesis of starch. I. Isolation and properties of the soluble adenosine diphosphate glucose : starch glucosyltransferase of *Solanum tuberosum*. Arch Biochem Biophys 116: 9–18
7. GRAY D 1973 The growth of individual tubers. Potato Res 16: 80–84
8. GROB K, P MATILE 1979 Vacuolar localization of glucosinolates in horseradish root cells. Plant Sci Lett 14: 327–335
9. GROSS P, T AP REES 1986 Alkaline inorganic pyrophosphatase and starch synthesis in amyloplasts. Planta 167: 140–145
10. HAWKER JS, JL OSBUND, J PREISS 1972 Unprimed starch synthesis by soluble ADP-Glc-starch glucosyltransferase from potato tubers. Phytochemistry 11: 1287–1293
11. HEBER U, NG PON, M HEBER 1963 Localization of carboxydismutase and triosephosphate dehydrogenase in chloroplasts. Plant Physiol 38: 355–360
12. HELDT HW, CJ CHON, D MARONDE, A HEROLD, ZS STANKOVIC, DA WALKER, A KRAMINER, MR KIRK, U HEBER 1977 Role of orthophosphate and other factors in the regulation of starch formation in leaves and isolated chloroplasts. Plant Physiol 59: 1146–1155
13. HUCKELSBY DP, MJ DALLING, RH HAGEMAN 1972 Some properties of two forms of nitrite reductase from corn (*Zea mays* L.) Planta 104: 220–233
14. JENNER CF 1982 Storage of starch. *In* Encyclopedia of Plant Physiology (New Series) Vol 13A. Springer-Verlag, Berlin pp 700–747
15. KENNEDY MGH, FA ISHERWOOD 1975 The effect of temperature on adenosine diphosphate glucose pyrophosphorylase from *Solanum tuberosum*. Phytochemistry 14: 111–112
16. KLEIN U 1986 Compartmentation of glycolysis and oxidative pentose phosphate pathway in *Chlamydomonas reinhardii*. Planta 167: 81–86
17. KRAUSS A, H MARSCHNER 1984 Growth rate and carbohydrate metabolism of tubers exposed to high temperatures. Potato Res 27: 297–303
18. LEVI C, J PREISS 1978 Amylopectin degradation in pea chloroplast extracts. Plant Physiol 6: 218–220
19. MACDONALD FD, T AP REES 1983 Enzymic properties of amyloplasts from suspension cultures of soyabean. Biochim Biophys Acta 755: 81–89
20. MACDONALD FD, T AP REES 1983 Labelling of carbohydrates by ^{14}C glycerol supplied to suspension cultures of soyabean. Phytochemistry 22: 1141–1143
21. MARES DJ, H MARSCHNER 1980 Assimilate conversion in potato tubers in relation to starch deposition and cell growth. Ber Dtsch Bot Ges 93: 299–313
22. MICHAL G 1984 D-Fructose-1,6-bisphosphate, dihydroxyacetone phosphate and D-glyceraldehyde-3-Phosphate. *In* Methods of Enzymatic Analysis, H U Bergmeyer, ed, Ed 3, Vol 6. Verlag GmbH, Weinheim, pp 342–350
23. MOORE TS, H BEEVERS 1974 Isolation and characterization of organelles from soyabean suspension cultures. Plant Physiol 53: 261–265
24. NAKASEKO K, K YOSHIDA, M YOSHIDA 1970 Physio-ecological studies in potatoes. IV. Influence of soil temperature on growth and yield. Mem Fac Agric Hokkaido Univ 7: 287–293
25. OKITA TW, E GREENBERG, DN KUHN, J PREISS 1979 Subcellular localization of the starch degradative and biosynthetic enzymes of spinach leaves. Plant Physiol 64: 187–192
26. PONTIS HG, LF LELOIR 1963 Measurements of UDP-enzyme systems. Methods Biochem Anal 10: 107–136
27. QUAIL CA 1979 Plant cell fractionation. Annu Rev Plant Physiol 30: 425–484
28. RANDENI G, K CAESAR 1986 Effect of soil temperature on the carbohydrate status in the potato plant (*S. tuberosum* L.). J Agron Crop Sci 156: 217–224
29. RIJVEN AHGC 1986 Heat inactivation of starch synthase in wheat endosperm tissue. Plant Physiol 81: 448–453
30. ROSA L 1981 The rapid activation in vitro of the chloroplast fructose-1,6-biphosphatase followed using a new assay procedure. FEBS Lett 134: 151–154
31. SOWOKINOS JR, J PREISS 1982 Pyrophosphorylases in *Solanum tuberosum* L. III. Purification, physical and catalytic properties of ADP-Glc pyrophosphorylase in potatoes. Plant Physiol 69: 1459–1466
32. SOWOKINOS JR, EC LULAI, JA KNOPER 1985 Translucent tissue defects in *Solanum tuberosum* L. I. Alterations in amyloplast membrane integrity, enzyme activities, sugars, and starch content. Plant Physiol 78: 489–494
33. YAMAGUCHI M, H TIMM, AR SPURR 1964 Effect of soil temperature on growth and nutrition of potato plants and tuberization, composition and periderm structure of tubers. Proc Am Soc Hortic Sci 84: 412–423