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PHOSPHORYLASE ACTIVITY IN RELATION TO STARCH SYNTHESIS IN DEVELOPING *HORDEUM DISTICHUM* GRAIN

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Key Word Index—*Hordeum distichum*; Gramineae; barley; endosperm; phosphorylase; isoenzymes; gel electrophoresis; starch synthesis.

Abstract—Activity, control and primer requirements of starch phosphorylase in developing barley endosperm were investigated. Phosphorylase was detected in endosperm extracts from 3 days after anthesis. Unprimed activity was predominant between 2 and 10 days after anthesis, when it constituted 70–80% of total activity, but this proportion declined rapidly as the grain developed. The existence of at least 2 isoenzymes was indicated by studies of pH dependence and phosphate inhibition, and was further supported by acrylamide gel electrophoresis and column chromatography using DEAE-cellulose. The two isoenzymes which ere possibly both glyco proteins, appear in barley endosperm soon after anthesis. One appears capable of unprimed activity, and may be associated with the initiation of α-1,2 glucans, which then serve as primers for starch synthetase. This disappears by 13–15 days after anthesis. The other isoenzyme is capable of some unprimed activity but undergoes modification between 15 and 20 days after anthesis, resulting in the loss of unprimed activity. The relevance of the results to initiation of starch synthesis and to starch synthetase in amyloplasts is discussed.

INTRODUCTION

IT IS generally considered that starch synthesis in plants is catalysed mainly by ADPG*-starch transglucosylase (ADP glucose: α-1,4-glucan α-4 glucosyltransferase. E.C. 2.4.1.11., starch synthetase), and, to a lesser extent, by the related UDPG enzyme.[1,2] However, both of these enzymes require a short chain glucan primer. Leloir *et al.*[3] have shown that addition of maltosaccharides to a particulate starch synthetase system from developing *Phaseolus aureus* decreased the incorporation of labelled glucose into starch, longer chain maltosaccharides being preferentially synthesized. This suggests that transglucosylases are predominantly chain-lengthening enzymes. Thus the mechanism by which short chain glucans are initiated remains uncertain.

It has been reported[4] that phosphorylases (α-1,4-glucan: orthophosphate glucosyltransferase, E.C. 2.4.1.1.) from certain plant systems are capable of synthesizing an amylose-like glucan in the absence of added primer. Tsai and Nelson[5] isolated from developing maize endosperm two phosphorylase isoenzymes, one of which could utilize maltose as a primer, and also synthesize starch in a 'primer-free' system. In destarched chloroplasts

* *Abbreviations*: ADPG—ADP-glucose; cyclic AMP—3′5′-cyclic adenosine monophosphate; G1P—D-glucose-1-phosphate; G*1P—D-[U-^{14}C]-glucose-1-phosphate; G6P—D-glucose-6-phosphate; UDPG—UDP-glucose; UDPG*—uridine-5′-diphospho-[U-^{14}C]-glucose.

[1] DE FEKETE, M. A. R., LELOIR, L. F. and CARDINI, C. E. (1960) *Nature* **187**, 918.
[2] RECONDO, E. and LELOIR, L. F. (1961) *Biochem. Biophys. Res. Commun.* **6**, 85.
[3] LELOIR, L. F., DE FEKETE, M. A. R. and CARDINI, C. E. (1961) *J. Biol. Chem.* **236**, 636
[4] SLABNIK, E. and FRYDMAN, R. (1969) *Biochem. Biophys. Res. Commun.* **38**, 709.
[5] TSAI, C. Y. and NELSON, O. E. (1968) *Plant Physiol.* **43**, 103.

synthesis of a glucan polymer from GlP in the absence of added primer, and its incorporation into starch by transglucosylase enzymes have been demonstrated by Bird.[6]

Phosphorylase activity and control have therefore been investigated both in crude and partially purified extracts from developing barley endosperm, with and without added glucan primers.

RESULTS

Phosphorylase Activity

Starch phosphorylase could be detected in barley endosperm extracts from as early as 3 days after anthesis (Fig. 1). Using soluble starch as a primer, maximum activity was attained around 22 days. Somewhat higher values, following the same developmental pattern, could be obtained with amylopectin, but soluble starch was used routinely because of its greater solubility. Unprimed activity was only slightly lower than total values during the first 10 days after anthesis, but remained considerably lower than total activity as development progressed. When unprimed activity, was expressed as a percentage of total activity, it became apparent that the highest proportion of unprimed activity occurred during the first

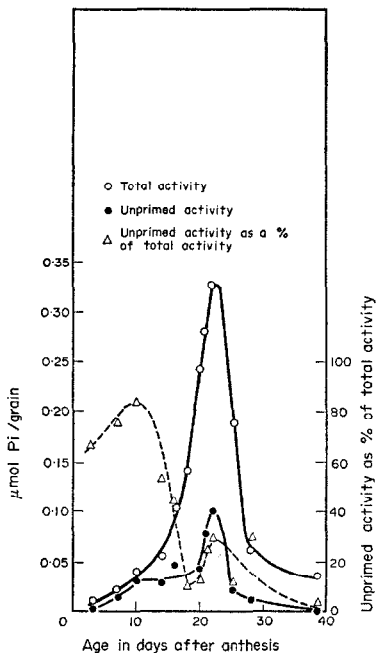


FIG. 1. PHOSPHORYLASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS DURING ENDOSPERM DEVELOPMENT.

Reaction mixtures contained: 15 μmol Tris-maleate pH 6·2; 10 μmol G1P; 0·2 ml soluble endosperm extract; 0·3 ml 2·5% soluble starch (for total activity). Mixtures were incubated at 25° for 2 hr and values corrected for controls without G1P and without endisperm extract.

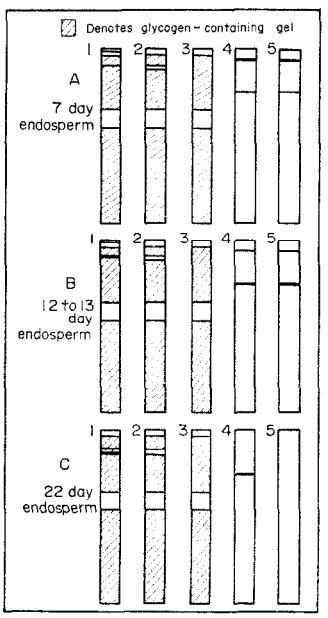


FIG. 2. PHOSPHORYLASE ACTIVITY AFTER ELECTROPHORESIS OF SOLUBLE ENDOSPERM EXTRACTS USING 7% POLYACRYLAMIDE GELS.

For conditions of electrophoresis, see text. Gels incubated overnight in: (1) 0·1 M citrate buffer, pH 5·0 + 0·025 M G1P; (2) 0·1 M citrate buffer, pH 5·0 + 0·1 M phosphate buffer pH 6·0; (3) 0·1 M citrate buffer; (4) 0·5% glycogen in citrate buffer + 0·025 M G1P; or 0·025 M G1P + 0·1 M citrate buffer.

[6] BIRD, I. F. (1969) Ph.D. Thesis, University of London.

10 days after anthesis, and declined during maturation. The increase in unprimed activity around 22 days (Fig. 1) was perhaps due to the accumulation in the endosperm extract of soluble oligosaccharides, sufficient to prime the phosphorylase reaction in the absence of added primer. This requires further investigation. Activity was chiefly located in the starchy endosperm, rather than in the aleurone layer. The enzyme appeared soluble, and traces of activity associated with the amyloplast fraction were readily removed by washing with buffer. There was no detectable increase in *free glucose* during the incubation indicating that the G1P was not being broken down by phosphatase activity.

Phosphorylase activity, measured in the direction of starch synthesis, was progressively inhibited by increasing concentrations of inorganic phosphate (Table 1). The extent of inhibition appeared dependent upon both the age of the endosperm and the presence or absence of primer. Primed activity was inhibited by increasing inorganic phosphate concentrations to a greater extent in 7- and 14-day extracts than in 22-day extracts. Conversely, unprimed activity was inhibited less in young grain than in older ones.

TABLE 1. EFFECT OF INORGANIC PHOSPHATE AND ADENOSINE NUCLEOTIDES ON PHOSPHORYLASE ACTIVITY

Addition (final concn) (M)		Activity as a % of activity in untreated extract*					
		Primed			Unprimed		
		Age in days after anthesis					
		7	14	22	7	14	22
Soluble endosperm extract	2×10^{-3} Pi†	92	96	85	91	81	71
	2×10^{-2} Pi	80	71	83	67	67	52
	2×10^{-1} Pi	16	28	64	17	2	0
	10^{-4} cyclic AMP	—	90	—	—	63	—
	10^{-4} AMP	—	47	—	—	68	—
	10^{-4} ATP	—	40	—	—	26	—
Filtered homogenate	70^{-2} cyclic AMP	100	—	—	75	—	—

* Reaction conditions as for Fig. 1. Each result is the average of three determinations.
† Abbreviation: Pi, inorganic phosphate.

At a final concentration of 10^{-4} M, cyclic AMP, AMP and ATP were inhibitory to both primed and unprimed phosphorylase in 14 day endosperm extracts. No significant effects could be induced by increasing the concentration of cyclic AMP to 10^{-2} M (Table 1).

A fairly broad spectrum of pH dependence of both primed and unprimed activity was obtained with young grain, maximum activity shifting from pH 6 in 7-day endosperm extracts to pH 7 with 14-day extracts. With 22-day extracts a sharp peak of primed activity occurred around pH 7, while unprimed activity remained relatively unchanged between pH 6 and 7.

Incorporation of Glucose into Starch

Soluble extracts from young endosperm incorporated [^{14}C]-glucose from G*1P into a water insoluble fraction which pelleted with carrier starch. In the presence of UDPG, glucose from G*1P was incorporated by 2–3 day endosperm extracts (Table 2). In older grain, UDPG appeared to exert an inhibitory influence. The progressive incorporation of glucose with time was investigated using 5-day extracts. Addition of UDPG to the reaction

mixture had little effect during the first 10–12 hr of incubation, but a significant reduction in glucose incorporation became apparent as the incubation period was prolonged to 24 hr. Considerable incorporation, apparently unaffected by UDPG, was obtained with 16-day extracts.

TABLE 2. INCORPORATION OF [^{14}C]-GLUCOSE FROM G*1P INTO STARCH, IN ABSENCE OF ADDED PRIMER

Glucosyl donor	Age in days after anthesis	cpm grain					
		3 hr		12 hr		24 hr	
		+ UDPG	− UDPG	+ UDPG	− UDPG	+ UDPG	− UDPG
1.0 μCi G*1P	2–3			74	0		
	3–4			754	11		
	5–6 (all dissected immediately)	200	250	350	250	1500	6900
0·5 μCi G*1P + 0·1 μmol G1P	5–7 (dissected immediately)					1408	2042
	5–7 (left in H_2O overnight)					2276	779
0·5 μCi G*1P + 0·1 μmol G1P	16 (dissected immediately)					3072	3239

Reaction mixtures contained: 0·2 ml soluble endosperm extract; 15 μmol Tris-maleate, pH 6·2; 1·0 or 0·5 μCi G*1P; 0·1 μmol G1P carrier where specified; 0·6 μmol unlabelled UDPG where indicated. Incubation at 37°. All values corrected for background counts and for controls (see text).

Incorporation of [^{14}C]-glucose from UDPG* into starch by transglucosylase activity was reduced when unlabelled G1P was included in the reaction mixture for both 12- and 24-hr incubations with 5–7 day soluble endosperm extracts (Table 3). No incorporation could be detected by 2–3 day extracts over 4, 12 or 24 hr.

In some cases barley ears 5–7 days after anthesis were allowed to stand in H_2O overnight before endosperm extracts were prepared. It was then observed that increased incorporation of [^{14}C]-glucose into starch was obtained from either G*1P or UDPG* when both glucosyl donors were present (Tables 2 and 3).

Chromatography of endosperm extracts after incubation with either G*1P or UDPG* demonstrated the accumulation in the reaction mixture of a labelled product which remained immobile in the solvent system used. A series of labelled substances lying between the origin and the maltose reference spot could also be observed; these were especially pronounced in incubations containing both G*1P + UDPG or G1P + UDPG*. These spots were no longer visible, and the proportion of immobile material was significantly reduced, when the reaction mixture was incubated with β-amylase before chromatography (Table 4). A concomitant increase in labelled maltose was observed. The apparent larger amount of activity in the maltose after treatment is due to its release from oligosaccharides

TABLE 3. INCORPORATION OF [^{14}C]-GLUCOSE FROM UDPG* INTO STARCH IN ABSENCE OF ADDED PRIMER, BY 5–7 DAY ENDOSPERM EXTRACTS

Length of incubation (hr)	Fraction	Glucosyl donor	cpm/grain + G1P	cpm/grain − G1P
12	Endosperm homogenate	0·1 μCi UDPG	66	119
24	Soluble endosperm extract	0·1 μCi UDPG	41	268
24	Soluble endosperm extract	0·05 μCi UDPG* + 0·3 μmol UDPG	18	51
12	Soluble endosperm extract left in H_2O overnight	0·1 μCi UDPG	184	82

Reaction conditions as for table 2, with 0·1 or 0·5 μCi UDPG* as labelled glucosyl donor; 0·3 μmol UDPG carrier; 0·1 μmol unlabelled G1P where indicated. All values corrected for background and controls (see text).

as well as the non-mobile polymer. The immobile material is thus identified as a long chain α- 1,4 glucan, and the slow moving components as maltosaccharides. Glucans synthesized from G*1P alone gave rise to a much higher proportion of maltose than material synthesized from UDPG* alone. Incubations containing both G1P and UDPG produced approximately equivalent amounts of free maltose.

TABLE 4. RADIOACTIVITY OF SPOTS ELUTED FROM CHROMATOGRAM SHOWING EFFECT OF β-AMYLASE ON NEWLY SYNTHESISED GLUCAN

Glucosyl donors	β-Amylase treatment	cpm/grain At origin	cpm/grain At maltose spot
G*1P + UDPG	Before	3440	498
	After	1747	8313
G1P + UDPG*	Before	635	482
	After	194	1597
G*1P	Before	2411	384
	After	1810	11 858
UDPG*	Before	1281	388
	After	1043	646

Reaction mixtures contained: 0·5 μCi G*1P + 0·1 μmol G1P carrier; 0·6 μmol UDPG where indicated: or 0·1 μCi UDPG*; 0·1 μmol G1P where indicated. Incubated at 37° for 24 hr, then treated with 0·1 ml β-amylase (1 mg/1 ml) for 1·5 hr at 23°. All values corrected for background radiation.

Pretreatment of Endosperm Extract with Amylases

Both primed and unprimed phosphorylase activity increased considerably after incubation of 7- and 15-day endosperm extracts with glucoamylase (Table 5). The extent of increase was reduced by dialysis but remained significant. No significant change in activity was noted after preincubation with β-amylase. A similar activation occurred with 50% saturated

$(NH_4)_2SO_4$ fractions pre-incubated with glucoamylase. In these fractions unprimed activity, although present initially, was abolished during incubation with boiled glucoamylase, suggesting an increased instability of the enzyme after $(NH_4)_2SO_4$ treatment.

Incorporation of [^{14}C]-glucose from G*1P into starch was similarly increased by glucoamylase pretreatment, the activation being more pronounced in the absence of UDPG (Table 5).

TABLE 5. PHOSPHORYLASE ACTIVITY IN SOLUBLE ENDOSPERM EXTRACTS PRETREATED WITH GLUCOAMYLASE

Enzyme source	Glucosyl donor	Treatment	μmol Pi/10^3 grain ± s.d. Primed Not dialysed	Primed After dialysed	Unprimed Not dialysed	Unprimed After dialysed
7-day soluble endosperm extract	G1P	+ Glucoamylase	57 ± 3	28 ± 3	50 ± 3	28 ± 8
		Control..	30 ± 7	10 ± 1	15 ± 1	6 ± 2
15-day soluble endosperm extract	G1P	+ Glucoamylase	80 ± 10	45 ± 9	75 ± 3	33 ± 2
		Control	15 ± 4	15 ± 5	16 ± 4	9 ± 3
			μmol Pi/mg Protein			
50% sat. $(NH_4)_2SO_4$ ppt. of 7-day extract	G1P	+ Glucoamylase	7·94	4·72	6·83	4·44
		Control	0·88	0·55	0	0
			Incorporation of [^{14}C]-glucose into starch without primer cpm/grain			
			+UDPG		−UDPG	
3-day soluble endosperm extract	0·25 μCi G*1P + 0·5 μmol G1P	+ Glucoamylase	333		367	
		Control	130		—	
5-day soluble endosperm extract	0·25 μCi G*1P + 0·5 μmol G1P	+ Glucoamylase untreated	863		3650	
			257		293	
5-day soluble endosperm extract	0·5 μCi G*1P + 0·1 μmol G1P	+ Glucoamylase untreated	2583		6359	
			319		432	

Reaction conditions as for Fig. 1 and Table 3. Endosperm extract (2 ml) pretreated with 0·2 ml glucoamylase (50 mg/ml) for 2 hr at 30°. (Control, boiled glucoamylase.)

Electrophoresis

After fractionation on glycogen-containing gels 7- and 12—13-day soluble endosperm extracts showed two bands of synthetic phosphorylase activity, (1) R_f 0·01, and (2) R_f 0·13 (Fig. 2 A1, B1). Component (1) was more pronounced in 7-day extracts while (2) predominated in 12–13 day extracts. With 22-day extracts only (2) was visible, split into 2 bands, R_f 0·11 and 0·13 (Fig. 2, C1).

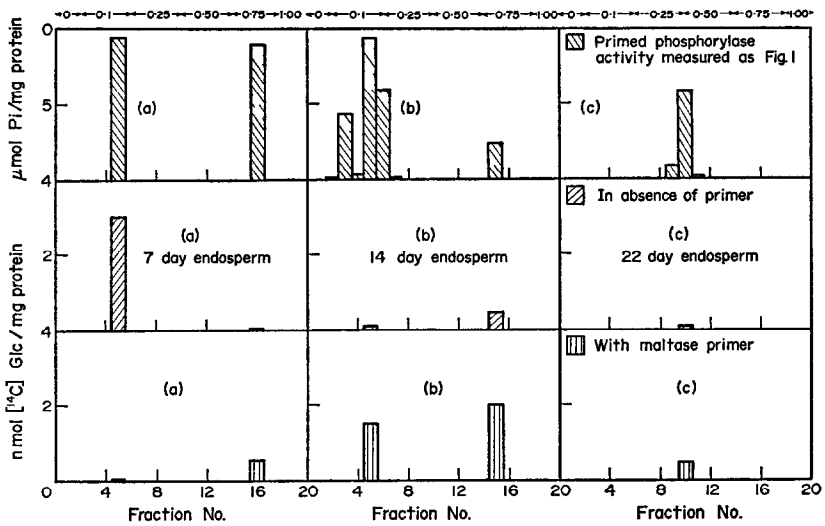
Substitution of inorganic phosphate for G1P in the incubation mixture resulted in colourless degradative bands of the same R_fs (Fig. 2, A2, B2, C2). Colourless amylase bands (R_f 0·028 and 0·42), which remained unchanged from 7 to 22 days, were distinguishable

from phosphorylase bands by omitting inorganic phosphate and G1P from the incubation mixture, in which case only amylase bands were visible (Fig. 2, A3, B3, C3).

Glycogen-free gels incubated in glycogen + G1P showed similar zoning, except that fractions migrated slightly faster and were less well defined (Fig. 2, A4, B4, C4). Dark bands occurred at R_f 0·045 and 0·23 with 7- and 12–13-day extracts, and at R_f 0·23 with 22-day extracts. If glycogen was completely omitted synthetic bands could still be distinguished, (1) being more prominent in 7-day extracts, and (2) in 12–13 day extracts. No bands were distinguishable with 22-day extracts (Fig. 2, A5, B5, C5).

DEAE-cellulose Chromatography

Extracts from 4-day endosperm could be resolved into 2 peaks of phosphorylase activity, (1) (fraction 4), and (2) (fraction 16) (Fig. 3). Only (1) could synthesize an insoluble glucan polymer from G*1P in the absence of added primer, although (2) showed slight activity with maltose. Twelve-day extracts gave 3 active peaks, 2 of which were very close and probably corresponded to (1), while the third peak corresponded to phosphorylase (2). Incorporation of glucose into an insoluble glucan polymer was negligible in all fractions (except fraction 15) in the absence of added primer, but was increased by the addition of maltose.



3. FRACTIONATION OF BARLEY ENDOSPERM PHOSPHORYLASE BY DEAE-CELLULOSE CHROMATOGRAPHY (for conditions of chromatography see text).
Incorporation of [^{14}C]-glucose from G*1P estimated as for Table 2—in absence of primer with maltose primer.

Only one active fraction was obtained from 22-day extracts: this was eluted in fractions 9 and 10, and did not appear to correspond to either (1) or (2) of 7- and 12-day extracts. This fraction was almost inactive in the absence of added primer, and only slightly activated by addition of maltose.

No carbohydrate could be detected in any fractions. The assay method used[7] could detect minimum glucose concentrations of 1 μg/ml.

[7] MORRIS, D. L. (1948) *Science* **107**, 254.

DISCUSSION

In barley endosperm, soluble phosphorylase could be detected several days before particulate starch synthetase, although periods of maximum activity of both enzymes coincided.[8] Unprimed activity was prominent between 2 and 10 days after anthesis, when it constituted 70–80% of total activity, but this proportion declined rapidly as starch synthetase increased.

The variation of inhibition by inorganic phosphate with both the age of the grain and the presence of primer suggested that two or more phosphorylase isoenzymes could be present in the endosperm, having different susceptibilities to inorganic phosphate, and different primer requirements. Changes in the relative concentrations of such isoenzymes may indicate a means of controlling the rate and direction of phosphorylase activity *in vivo*.

Indications of enzyme multiplicity could also be drawn from pH dependence measurements. The broad spectrum of activity with 7- and 14-day extracts suggested a mixture of protein, whereas the sharper activity maximum of 22-day extracts implied a single protein. The negative effects of adenosine nucleotides on enzyme activity supported previous evidence[5,9] that starch phosphorylase in higher plants does not consist of subunits activated by adenosine nucleotides in the same way as glycogen phosphorylase in animal systems.

Since it is probable that glucans synthesised by phosphorylase, either *de novo* or using small maltosaccharide precursors, may be available as primers for transglucosylase activity, then incubations with G1P and UDPG (or ADPG) should show incorporation of glucose from both glucosyl donors.[6] Incorporation from G*1P was accelerated by UDPG with 2–3 day endosperm, but inhibited with 5–7 day endosperm. ADPG and UDPG have been shown to inhibit starch and glycogen phosphorylase.[10,11] The lack of inhibition with 2–3, and 16-day extracts suggests that the effect may be due to competition between the enzymes for available primer. Assuming that 2–3 day endosperms contain no endogenous primer, synthesis of starch by phosphorylase will be extremely slow, and thus addition of UDPG (allowing elongation of labelled glucans) will significantly increase measurable incorporation from G*1P. By 5–7 days, limited quantities of endogenous primers in the endosperm will cause competition between the enzymes, but by 16 days, primer will no longer be limiting. Similarly incorporation from UDPG* will be reduced by the addition of G1P. Enhanced incorporation from G1P and UDPG together was obtained by Bird[6] using destarched chloroplasts, which presumably contained no endogenous primer, and corresponded to the situation with 2–3 day endosperm. Similar results could be induced by leaving barley ears in water overnight before preparing endosperm extracts, so that endogenous primers were used up in respiration.

The higher susceptibility of starch synthesised from G*1P alone to β-amylase attack indicates that phosphorylase synthesises *de novo* only straight chain glucans, while both amylopectin and amylose result from transglucosylase activity.[10,12,13]

Since starch phosphorylase can utilize shorter chain primers than muscle phosphorylase,[14] unprimed activity may be due to priming by endogenous maltosaccharides, or by carbohydrate contaminants of substrates. However, glucose was incorporated by soluble

[8] BAXTER, E. D. and DUFFUS, C. M. (1971) *Phytochemistry* **10,** 2641.
[9] GREEN, D. E. and STUMPF, P. K. (1942) *J. Biol. Chem.* **142,** 355.
[10] DE FEKETE, M. A. R. (1969) *Planta* **87,** 311.
[11] MADSEN, N. B. (1961) *Biochem. Biophys. Res. Commun.* **6,** 310.
[12] HANES, C. S. (1940) *Proc. Roy. Soc. (London)* **128 B,** 421.
[13] OZBUN, J. L., HAWKER, J. S. and PREISS, J. (1971) *Biochem. Biophys. Res. Commun.* **43,** 631.
[14] CORI, G. T., SWANSON, M. A. and CORI, C. F. (1945) *Federation Proc.* **4,** 234

endosperm extracts using only G*1P (purified by chromatography and therefore free from low MW contaminants) as substrate. Furthermore, Bird[6] found comparable rates of unprimed activity using purified or unpurified G1P.[15] Contamination of G1P does not, therefore appear significant.

Contamination by soluble endogenous primers remains probable. The increase in primed activity after treatment of crude extracts with glucoamylase is probably due to breakdown of the starch primer, giving rise to more short-chain glucans. Increase in unprimed activity suggests that the crude extract contained a limited amount of maltosaccharides which could be broken down by glucoamylase, yielding a larger number of suitable primer chains. Shorter chains would be lost on dialysis, reducing the extent of activation, but longer chains, or possibly protein-bound glucans, would be retained. Persistence of glucoamylase-induced activation in 50% satd $(NH_4)_2SO_4$ fractions supports a hypothesis of protein-bound glucan primers.

Purification of endosperm extracts by electrophoresis and chromatography indicated the presence of multiple forms of phosphorylase. In each procedure two isoenzymes could be isolated from endosperms up to 15 days after anthesis. Unprimed activity was associated exclusively with phosphorylase (1) in very young endosperm, but as development proceeded this potentiality was acquired by phosphorylase (2). As (2) accumulated, phosphorylase (1) disappeared. By 22 days after anthesis only one isoenzyme, which required a glucan primer, persisted. The loss of unprimed activity may reflect modifications of the protein without alteration of electrophoretic mobility, since the isoenzyme detected in 22 day endosperm did not correspond to either phosphorylase (1) or (2) of younger endosperm, as separated by DEAE-cellulose chromatography.

Two phosphorylase isoenzymes, separable by acrylamide gel electrophoresis, have been identified[10] in spinach leaf extracts and immature *Vicia faba* cotyledons. Gerbrandy and Verleur,[16] who separated several phosphorylase isoenzymes from potatoes, found that different isoenzymes appeared in young and mature tubers, and suggested that certain isoenzymes were active mainly in the direction of synthesis, whilst others were concerned with starch degradation. No unprimed activity was detected[5] in maize endosperms fractionated by DEAE-cellulose until 12 days after anthesis but since incubation periods were only 30–45 min duration it is quite possible that low levels of unprimed activity in very young endosperm were not detected.

Maintenance of unprimed activity in young endosperm throughout purification procedures reduces the possibility of its being attributable entirely to contamination by endogenous maltosaccharides, and supports the hypothesis of a protein-bound glucan primer, possible attached to the phosphorylase enzyme. Concentrations of such a bound primer could well be too low to be detected in the eluate by the method used.[7] For example, Fukui and Kamogawa[17] found 0·2–0·6 glucose residues/mol enzyme protein in a crystalline preparation of potato phosphorylase. Removal of this carbohydrate by glucoamylase abolished unprimed activity. Fredrick[18] obtained similar results with algal phosphorylase, and suggested that the isoenzymes were glycoproteins.

The results of the present investigation are consistent with the appearance in the barley endosperm soon after anthesis of two phosphorylase isoenzymes, possibly both glycoproteins. Phosphorylase (1) appears capable of unprimed activity, and may be associated

[15] KAMOGAWA, A., FUKUI, T. and NIKUNI, Z. (1968) *J. Biochem.* **63**, 361.
[16] GERBRANDY, SJ. J. and VERLEUR, J. D. (1971) *Phytochemistry* **10**, 261.
[17] FUKUI, T. and KAMOGAWA, A. (1969) *J. Jap. Soc. Starch Sci.* **17**, 117.
[18] FREDRICK, J. F. (1971) *Physiol. Plant.* **25**, 32.

with the initiation of α-1,2-glucans, which then serve as primers for starch synthetase. This isoenzyme disappears after 13–15 days. Phosphorylase (2) is capable of some unprimed activity but undergoes modification between 15 and 20 days after anthesis (possibly by removal of the carbohydrate moiety), resulting in loss of unprimed activity.

EXPERIMENTAL

Materials. Glucoamylase (E.C. 3.2.1.3. specific activity 2700 mg Glc/min/g at 55°, pH 4·5) and β-amylase (E.C. 3.2.1.2. specific activity 15 mg maltose/min/mg at 20°, pH 4·8) and most biochemicals and substrates including corn endosperm amylopectin and oyster glycogen were obtained from Sigma Chemical Co. (St. Louis, Mo. U.S.A.). Soluble starch was obtained from British Drug Houses (London), and radioactively labelled substrates from The Radiochemical Centre, (Amersham, Bucks.). The two row barley *Hordeum distichum* (L) Lam. cv. Maris Baldric, was used throughout, as described previously.[8] Soluble endosperm extracts were prepared by suspending the endosperm (including the aleurone layer) in Tris-maleate buffer (0·1 M, pH 6·2) and homogenizing by hand in an all-glass homogenizer. The filtered homogenate was centrifuged for 10 min at 10 000 *g*, 4°. The supernatant solution constituted the soluble endosperm extract. Pelleted material, which upon examination under the microscope (×1000) appeared to consist mainly of amyloplasts, was washed and resuspended in buffer, and constituted the insoluble endosperm extract. In older grain (20 days after anthesis) the aleurone layer could be scraped off the endosperm, and a soluble extract prepared as for the endosperm. In some cases the soluble endosperm extract was treated for 2 hr at 30° with glucoamylase (50 mg/ml soln) or β-amylase (2 mg/2 ml soln).

Phosphorylase assay. This was measured by the release of inorganic phosphate from G1P[19] in Tris-maleate buffer pH 6·2. Soluble starch or amylopectin was used as a primer. Inorganic phosphate was estimated according to Lowry and Lopez[20] using 2% ascorbic acid. All values were corrected for endogenous inorganic phosphate and non-enzymatic breakdown of G1P. Samples were also tested for any increase in free glucose after incubation. Total activity detected in the presence of added primer is referred to as 'primed activity', and activity with no added primer as 'unprimed activity'.

Incorporation of [^{14}C]-glucose into starch. After incubation of endosperm extracts with G*1P (specific activity 277 mCi/mmol) or UDPG* (233 mCi/mmol) at 37° for periods of up to 24 hr, solid starch was added as a carrier and insoluble material collected by centrifugation (10 min, 4°, 13 000 *g*), washed several times to remove unreacted material, and the radioactivity estimated in a Beckmann liquid scintillation system after resuspending the pellet in 0·5 ml boiling H_2O. Values were corrected for background radiation and controls with endosperm extract. Counting efficiency for ^{14}C was about 25%. Labelled products were separated by descending PC for 20 hr using a PrOH-EtOAc–H_2O (6:1:3) system,[21] and visualized by exposure to an X-ray film. In some cases product mixtures were treated with β-amylase (1 mg/ml) for 1·5 hr at 23° before chromatography.

Electrophoresis. This was carried out according to Rainer-Maurer[22] using gel system 1 with 7% polyacrylamide gels. Tris-glycine buffer, 0·1 M, pH 8·3, was used as reservoir buffer. A 0·4% soln of glycogen was polymerized with some gels to serve as a primer. Current (3 mA/tube) was passed for about 2 hr at room temp., using bromophenol blue as the front indicator. Gels were incubated overnight in a solution containing 3 ml each of 0·025 M G1P and 0·1 M citrate buffer, pH 5·0. With primer-free gels, glycogen was sometimes added to the incubation mixture. Gels were then stained in I_2–KI soln.

DEAE-cellulose chromatography. That fraction of the soluble endosperm extract precipitating at 50% $(NH_4)_2SO_4$ saturation was collected by centrifugation (20 min at 34 000 *g*, 4°), suspended in Tris-maleate buffer (0·01 M, pH 7·0), dialysed against the same buffer, and a 1-ml sample applied to a DEAE-cellulose column (1 × 20 cm) previously equilibrated with the same buffer.[5] The sample was eluted in a total vol of 110 ml by a linear discontinuous concentration gradient, from 0 to 1 M NaCl in Tris-maleate buffer, pH 7·0. 5·0 ml fractions were collected, dialysed overnight, and assayed for (a) phosphorylase activity, (b) incorporation of glucose into starch, (c) protein[23] and (d) carbohydrate content.[7]

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