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STARCH SYNTHETASES FROM *VITIS VINIFERA* AND *ZEA MAYS*

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Key Word Index—*Vitis vinifera*; Vitaceae; grape; *Zea mays*; Gramineae; maize; starch synthesis; ADPglucose-starch glucosyltransferase; isoenzymes; unprimed starch synthesis; Q-enzyme; branching enzyme.

Abstract—ADPglucose: α-1,4-glucan α-4-glucosyltransferases (starch synthetases) from leaves of *Vitis vinifera* and leaves and kernels of *Zea mays* were chromatographed on DEAE-cellulose columns. One form of the enzyme was present in grape leaves having activity both in the presence and absence of primer. Two forms were present in both leaves and kernels of maize. The second peak of activity in maize leaves and the first peak in maize kernels synthesized a polyglucan in the absence of primer. A peak of branching enzyme (Q-enzyme) occurred between the two starch synthetase peaks with both tissues. When fractions containing starch synthetase and branching enzyme were added to the first leaf starch synthetase peak, up to 100-fold activation of the unprimed reaction occurred. Branching enzyme did not stimulate the unprimed activity of the first kernel peak and no branching enzyme could be detected in this peak. The unprimed product was a branched polyglucan with mainly α-1,4-links.

INTRODUCTION

MULTIPLE forms of ADPglucose: α-1,4-glucan α-4-glucosyltransferase (ADPG starch synthetase) have been obtained from spinach leaves and maize kernels by gradient elution from DEAE-cellulose columns[1,2] and from maize kernels by disc-gel electrophoresis.[3] One of the forms from each plant catalysed the synthesis of a glucan in the absence of added primer and this reaction was stimulated by branching enzyme (Q-enzyme).[4] Partially purified ADPG starch synthetase from *Escherichia coli* is stimulated 10 to 100-fold by branching enzyme and a partially purified enzyme from spinach leaves is stimulated 5- to 12-fold by branching enzyme (Preiss, personal communication).

The involvement of ADPG starch synthetase in the initiation and maintenance of starch synthesis in bacteria and higher plants has recently been proposed[4] while the participation of phosphorylase in the same roles has also been suggested.[5]

In contrast to grape leaves, the berries contained only traces of starch and low activities of ADPG starch synthetase and ADPG pyrophosphorylase while the activity of phosphorylase was relatively high.[6] Mesophyll and bundle sheath cells of maize leaves contained relatively high activities (compared to grape berries) of the three enzymes and

[1] OZBUN, J. L., HAWKER, J. S. and PREISS, J. (1972) *Biochem. J.* **126**, 953.
[2] OZBUN, J. L., HAWKER, J. S. and PREISS, J. (1971) *Plant Physiol.* **48**, 765.
[3] SCHIEFER, S., LEE, E. Y. C. and WHELAN, W. J. (1973) *FEBS Letters* **30**, 129.
[4] FOX, J., KENNEDY, L. D., HAWKER, J. S., OZBUN, J. L., GREENBERG, E., LAMMEL, C. and PREISS, J. (1973) *Annals N.Y. Acad. Sci.* **210**, 90.
[5] FRYDMAN, R. B., and SLABNIK, E. (1973) *Annals N.Y. Acad. Sci.* **210**, 153.
[6] DOWNTON, W. J. S. and HAWKER, J. S. (1973) *Phytochemistry* **12**, 1557.

the activities increased under growth conditions which caused higher rates of starch synthesis.[7]

To gain more information on ADPG starch synthetases in higher plants, results of DEAE-cellulose chromatography of extracts from grape and maize are compared with results obtained previously for other plants.[1,2,8] Multiple forms of starch synthetase have now been shown to occur in maize leaves as well as in maize kernels and branching enzyme stimulates the reaction in the absence of added primer (hereafter referred to as the unprimed reaction).

RESULTS

ADPG starch synthetase from grape leaves

Previously, high activities of ADPG starch synthetase were extracted from grape leaves in a medium containing sodium metabisulphite and bovine serum albumin. However, only 40% of the activity was soluble, the remainder precipitating with the disrupted chloroplast and starch pellet.[6] The use of a medium containing Carbowax 4000 and sodium diethyldithiocarbamate has now given a preparation in which 70% of the enzyme is soluble facilitating DEAE-cellulose chromatography. In mature leaves of grapevine very little activity was found unless Carbowax 4000 was used, presumably due to the presence of tannins and phenols.[6,9]

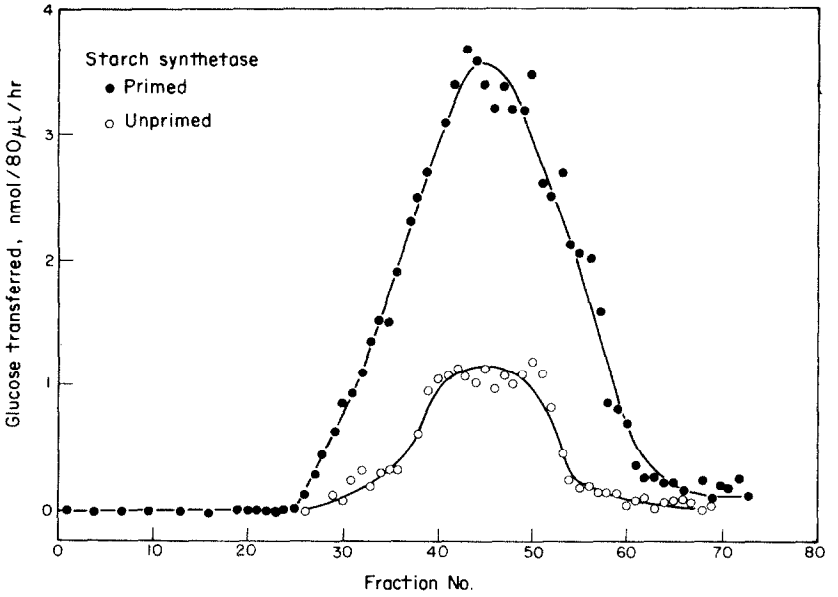

FIG. 1. ELUTION PATTERN FROM DEAE-CELLULOSE OF *Vitis vinifera* LEAF ADPG STARCH SYNTHETASE. Primed activity was assayed with potato starch.

Treatment of soluble extracts from grape leaves with $(NH_4)_2SO_4$ resulted in the loss of most of the activity and hence soluble extract was dialysed and applied to the DEAE-cellulose column as described in the Experimental. Only one peak of activity was obtained which showed both primed and unprimed activity (Fig. 1).

[7] DOWNTON, W. J. S. and HAWKER, J. S. (1973) *Phytochemistry* **12**, 1551.
[8] HAWKER, J. S., OZBUN, J. L. and PREISS, J. (1972) *Phytochemistry* **11**, 1287.
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ADPG starch synthetase from maize leaves and kernels

Preliminary experiments showed that with the extraction medium containing PVP (see Experimental), about 60% of the ADPG starch synthetase from maize leaves was in the supernatant fraction after centrifugation. This fraction was concentrated by $(NH_4)_2SO_4$ precipitation and applied to a DEAE-cellulose column.

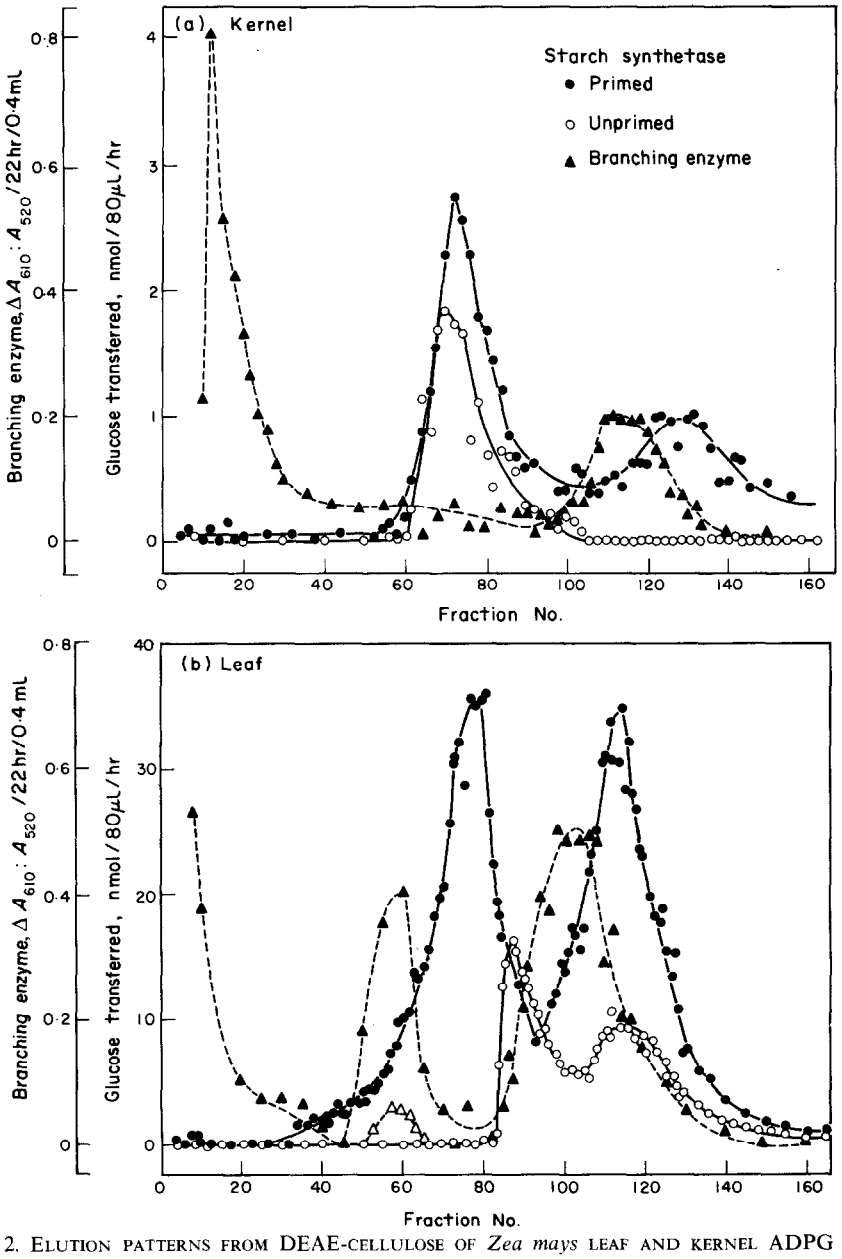

FIG. 2. ELUTION PATTERNS FROM DEAE-CELLULOSE OF *Zea mays* LEAF AND KERNEL ADPG STARCH SYNTHETASE AND BRANCHING ENZYME.

Primed ADPG starch synthetase was assayed with rabbit liver glycogen. Reducing sugar formation from amylose (Δ) by leaf fraction 57 was 58 nmol glucose equivalent/20 hr/0·4 ml enzyme.

With potato starch as primer, two peaks of ADPG starch synthetase activity were detected after chromatography of the leaf extract on DEAE-cellulose. Fractions in the first peak were 9 times more active and fractions in the second peak were twice as active when rabbit liver glycogen was used in place of potato starch as primer. Hence rabbit liver glycogen was used in subsequent assays (Fig. 2). In the presence of citrate ions and bovine serum albumin, but in the absence of added primer, two peaks of activity were obtained. However, only the second of these corresponded to one of the peaks obtained when primer was used (Fig. 2b). The first of the unprimed peaks occurred between the two primed peaks. A peak of branching enzyme activity was situated between the two peaks of unprimed activity. A peak of activity centered about fraction 58 giving a change in the ratio A_{610}/A_{520} released reducing sugars from amylose and was therefore probably amylase.

Chromatography of extracts of kernels revealed two peaks of ADPG starch synthetase in the presence of primer, with the first peak also showing activity in the absence of primer (Fig. 2a). Two peaks of branching enzyme were apparent, the second being between the two peaks of ADPG starch synthetase activity as was seen in the leaf chromatogram (Fig. 2).

Stimulation of unprimed ADPG starch synthetase activity by branching enzyme

Branching enzyme from either leaves or kernels did not stimulate ADPG starch synthetase in the presence of rabbit liver glycogen. The rate of the unprimed reaction of ADPG starch synthetase in the first peak of the kernel chromatogram could not be increased by the addition of any other fractions from either leaves or kernels. No unprimed activity was detected in the second peak of ADPG starch synthetase from the kernels even after prolonged incubation.

On the other hand fractions in the first peak of ADPG starch synthetase from leaves (which did not have any unprimed activity) showed considerable unprimed activity on the addition of fractions containing branching enzyme from leaves (L) or from kernels (K) (Table 1). A 14-fold stimulation was obtained by the addition of leaf branching enzyme (101 L) to leaf ADPG starch synthetase (79L) and a 100-fold stimulation by the addition of branching enzyme from kernels (110K or 112K) to the leaf ADPG starch synthetase (75L or 76L). The leaf starch synthetase (75L or 79L) was not stimulated by leaf fractions showing high unprimed activity (87L) or by fractions from the peak releasing reducing sugar from amylose (60L). Fractions from this latter peak (57L) did not inhibit the unprimed reaction (87L). Branching enzyme from kernels (12K) did not increase the rate of the unprimed reaction nor did heat-inactivated enzyme from the second branching enzyme peak from kernels (114K). However, when 114K was incubated with ADPG^{14}C for 2 hr and then heat inactivated, incubation with 75L resulted in some transfer of glucose from ADPG to form a methanol insoluble product. It seems probable that a small amount of polyglucan was produced by the branching enzyme peak (114K) which acted as a primer for the leaf enzyme (75L). When 114K and 75L were incubated together, higher unprimed activity was observed probably due to the concerted action of branching enzyme and starch synthetase producing a branched primer which acted as a favoured acceptor of glucose catalysed by starch synthetase (75L). The product formed was branched since β-amylase resulted in only 68% conversion of product to maltose (Table 2).

Unprimed starch synthesis by maize kernels

The first peak of starch synthetase of kernels catalysed the synthesis of glucan in the absence of added primer. No branching enzyme could be detected in this peak (72K,

TABLE 1. EFFECT OF BRANCHING ENZYME ON UNPRIMED ADPG STARCH SYNTHETASE ACTIVITY FROM *Zea mays* LEAVES AND KERNELS*

Fraction†	cpm Incorporated into glucan	Fold stimulation by mixing fractions
79L	186	
101L	718	
79L + 101L	12680	14
75L, 76L	120	
12K, 110K, 112K, 114K	0	
75L + 12K	150	1·3
75L + 110K	11500	96
76L + 112K	12250	102
75L + 114K	12100	100
75L + (114K boiled)	120	1
75L + (114K 2hr then boiled)	3750	31
75L + (74K boiled)	120	1
87L	3900	
87L + 79L	3353	0·8
60L	71	
75L	80	
60L + 75L	200	1·3
57L	48	
87L	3900	
57L + 87L	3537	0·9

* Enzyme (40 μl of each fraction) was incubated for 2 hr without primer.
† Fractions used were from DEAE-cellulose chromatography of leaf (L) and kernel (K) extracts shown in Fig. 2.

74K) either with amylose or potato starch (containing about 80% amylopectin) as substrates. Addition of branching enzyme from either leaf or kernels did not stimulate the unprimed activity. Despite the apparent absence of branching enzyme, the unprimed

TABLE 2. β-AMYLASE TREATMENT OF PRODUCT FORMED BY STARCH SYNTHETASE IN THE ABSENCE OF PRIMER

Product treated	% of total ^{14}C incorporated origin	Maltose
75L + 114K	32	68
72K	21	79
74K	22	78

product was not completely hydrolysed to maltose by β-amylase (Table 2) suggesting the presence of α-1,6 links. Treatment of the product with α-amylase resulted in almost 100% conversion to maltose and glucose.

DISCUSSION

From our previous work[6] we had suspected that grapevines might contain multiple forms of ADPG starch synthetase, and by analogy with spinach leaf and maize kernels, that one of these forms would have catalysed an unprimed reaction. Since grape berries contain little starch and low activities of starch synthetase, we further argued that possibly the activity of the unprimed form of the enzyme is very low in berries. However, grape leaves have only one form of ADPG starch synthetase as determined by DEAE-cellulose chromatography (Fig. 1) and this form also catalyses the unprimed reaction. Potato tubers also have only one form of ADPG starch synthetase.[8] The low activity of ADPG starch synthetase[6] in grape berries, the loss of this activity upon concentration by $(NH_4)_2SO_4$, together with a high phenol content currently blocks progress on the study of ADPG starch synthetase in grape berries.

The results with maize kernels (Fig. 2a) show that normal starchy maize has comparable forms of ADPG starch synthetase to those of waxy maize.[2] Two forms are present and with rabbit liver glycogen as primer the ratio of peak heights is similar in both varieties. The first form synthesizes glucan in the absence of primer in each case. No branching enzyme appears to be associated with this first form in NES 1002 (Fig. 2a) and branching enzyme did not stimulate the unprimed reaction. However, the radioactive product formed had branch points (Table 2). Whether there is a branching enzyme present which we cannot detect or whether glucose residues are added at the reducing terminals of the starch molecule in the manner demonstrated for the biosynthesis of the *O*-antigenic side chain of the lipopolysaccharide of *Salmonella anatum*[10] cannot be resolved here. Schiefer *et al.*[3] could not detect branching enzyme in the fast moving bands of ADPG starch synthetase which gave a branched product. They suggested that branching enzyme might act more rapidly on a growing chain than on a pre-existing amylose molecule or that starch synthetase and branching enzyme might exist as a two-enzyme complex.

Leaves of maize (*cv* NES 1002) also had two forms of ADPG starch synthetase, the second of which carried out the unprimed reaction. Another peak of unprimed activity did not coincide with either peak. The steep leading edge of this unprimed peak was probably due to the emergence of both branching enzyme and the second peak of starch synthetase at this point. The elution pattern of leaf enzymes differed from the kernels in that only the second of the ADPG starch synthetases from the leaves showed unprimed activity as opposed to the first one in kernels. The second peak in kernels can probably synthesize a small amount of glucan which cannot be detected by normal assays but can be detected as a primer for the leaf enzyme (Table 1). Boiled enzyme from either of the two kernel starch synthetase peaks did not act as primer to the leaf starch synthetase suggesting that primer was not present in the enzyme preparations. Glucoamylase treatment of enzymes from spinach, potato and maize did not affect unprimed activity.[1,2,4,8] However, Schiefer[11] has reported that treatment of sweet corn ADPG starch synthetase with a mixture of glucoamlyase and α-amylase abolished the unprimed reaction suggesting an association of primer with the enzyme.

The unprimed activity of ADPG starch synthetase is probably important in the early stages of starch synthesis in plants whether it reflects *de novo* starch synthesis or the rapid growth of a small glucoamylase-resistant primer. Whatever the mechanism, it

[10] ROBBINS, P. W., BRAY, D., DANKERT, M. and WRIGHT, A. (1967) *Science* **158**, 1536.
[11] SCHIEFER, S. (1973) *Federation Proc.* **32**, 603.

requires the presence of some anions and proteins for optimal activity *in vitro*[4] and it is possible that these conditions simulate the environment *in vivo*. Initially α-1,4-glucan is synthesized with branching enzyme introducing α-1,6 links.[4] Both bacterial enzyme and enzyme from higher plants is stimulated by branching enzyme. The present results show that in some cases the unprimed reaction in higher plants was stimulated by fractions containing branching enzyme. In other cases no stimulation by branching enzyme could be observed despite the apparent lack of branching enzyme in the extracts.

Two main bands of ADPG starch synthetase were demonstrated by disc-gel electrophoresis of kernel extracts from several mutant varieties of maize.[3] An exception was amylomaize (a mutant which contains amylose but little or no amylopectin) which had higher activities of 4 slower moving bands.[3] By the use of a different technique (DEAE-cellulose chromatography) we have shown two forms of the enzyme in the normal (Fig. 2) and waxy maize.[2] In the leaves of the same plant (Fig. 2b), two forms also occur but with different properties from those of the corresponding kernels. The differences between the enzymes in the two organs may account for the different effect of glucan hydrolases on leaf and storage starch grains reported by Bailey and MacRae.[12] Schiefer *et al.*[3] suggested that in maize kernels amylose is synthesized by ADPG starch synthetase *per se* while amylopectin is synthesized by a synthetase—branching enzyme complex and that the ratio of amylose : amylopectin is a function of the ratio of the two forms of the synthetase. By DEAE-cellulose chromatography, the ratio of two forms of ADPG starch synthetase (using rabbit liver glycogen as primer) was about the same in normal (Fig. 2) and waxy maize kernels.[2] Much remains unknown about the interaction of ADPG starch synthetases and branching enzymes in the synthesis and incorporation into starch grains of amylose and amylopectin.

EXPERIMENTAL

Materials. Plants of grapevine (*Vitis vinifera* L. *cv* Cabernet Sauvignon) were grown in a glasshouse[13] or in the field. Maize plants (*Zea mays* L. *cv* NES 1002) were grown in a glasshouse as described previously.[7] Plants were also grown in the field and ears were harvested and frozen 22 days after self pollination. Potato starch, amylose, rabbit liver glycogen, hog pancreas α-amylase and sweet potato β-amylase were purchased from Sigma, St. Louis, Mo., U.S.A.

Purification of ADPG starch synthetase. All operations were performed at 0–4°. *From grape leaves.* Washed leaves (30 g) were ground with sand in a mortar with 100 ml 0·35 M Tris-acetate buffer pH 8·5 containing 20 mM EDTA, 11 mM sodium diethyldithiocarbamate (DIECA), 15 mM cysteine-HCl and 6% Carbowax 4000. The homogenate was filtered through cheesecloth and centrifuged at 35000 *g* for 15 min. The pellet was suspended in 100 ml 20 mM Tris-acetate buffer pH 8·5 containing 10 mM EDTA and 2 mM dithiothreitol (DTT). After centrifugation at 35000 *g* for 15 min, this supernatant was added to the first supernatant and 10 g of insoluble PVP was added. After further centrifugation sucrose (0·1 g/ml) was added to the supernatant which was dialysed overnight against 2 changes of 50 mM Tris-acetate buffer pH 8·5 containing 5 mM EDTA, 1 mM DTT, 10 mM DIECA and 10% sucrose. PVP (2 g) was added to the dialysate and the suspension was centrifuged at 35000 *g* for 20 min. Part of the supernatant (100 ml, containing 275 mg of protein and 0·4 μmol/min of ADPG starch synthetase activity) was applied to a 25 ml column of DEAE-cellulose which had been equilibrated with dialysis buffer minus DIECA. After passage of 1 column vol. of the same buffer, 1 l. of buffer with increasing KCl concentration (linear gradient 0–0·4 M KCl) was passed through the column and collected in 10 ml fractions. *From maize leaves.* Washed leaves (30 g) were ground with 10 g of washed carborundum 90 (to ensure breakage of bundle sheath cells) in a mortar with 100 ml 50 mM Tris-acetate buffer pH 8·5 containing 10 mM EDTA, 10 mM DTT and 1% insoluble PVP. After passage through 2 layers of Miracloth, the homogenate was centrifuged at 35000 *g* for 15 min. The supernatant was made to 40% saturation with solid $(NH_4)_2SO_4$ and centrifuged at 35000 *g* for 15 min. The precipitate was dissolved in 20 ml 50 mM Tris-acetate buffer pH 8·5 containing 5 mM EDTA, 1 mM DTT and 10% sucrose and dialysed overnight against 2 changes of the same buffer. The dialysate was centrifuged at 35000 *g* for 15 min, the

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supernatant (18 ml, containing 170 mg protein and 0·5 μmol/min ADPG starch synthetase activity) was applied to a 25 ml DEAE-cellulose column and treated as for grape leaves except that 1 l. of buffer (linear gradient 0–0·3 M KCl) was collected in 5 ml fractions. *From maize kernels.* Frozen kernels (30 g) were treated as were maize leaves, except that no carborundum was used and 7 ml enzyme (80 mg protein, 0·3 μmol/min of ADPG starch synthetase activity) was applied to a 20 ml column.

Measurement of enzyme activities. ADPG Starch Synthetase (E.C. 2.4.1.b.) Transfer of glucose-[U-^{14}C] to a primer, or formation of glucan-[-^{14}C] in the absence of primer was determined at 30° as previously described[8] except that GSH and amylopectin were replaced with DTT and either potato starch or rabbit liver glycogen. *Branching enzyme.* (E.C. 2.4.1.18.) Enzyme (0·4 ml of fractions from DEAE-cellulose columns) was incubated with 0·7 ml of solution containing amylose (0·5 mg/ml), 0·5 M Na citrate and BSA (0·5 mg/ml) at 30°. Samples (0·2 ml) were mixed with 2·8 ml of I_2/KI reagent[14] and *A* at 520 nm and 610 nm was measured. Activity was expressed as the decrease in ratio of *A* at 610 nm to *A* at 520 nm. The amount of reducing sugar in the reaction mixtures was also determined.[15] *Amylase treatment of unprimed product.* The washed pellet of starch (mainly carrier starch added at the end of the unprimed assay) dissolved in H_2O was treated with 5 μl of α- or β-amylase for 15 hr at 30°, and then chromatographed on paper using 95% EtOH–M NH_4OAc, pH 7·5 (5 : 2). The radioactive spots were detected with a strip chart detector and counted in a planchet counter.

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[15] NELSON, N. (1944) *J. Biol. Chem.* **153**, 375.