Phytochemistry, 1978, Vol 17, pp 391–396 Pergamon Press. Printed in England

STARCH BREAKDOWN IN THE SPADIX OF *ARUM MACULATUM*

PAUL V. BULPIN and TOM AP REES

Botany School, University of Cambridge, Downing Street, Cambridge CB2 3EA, U.K.

(*Received* 12 *August* 1977)

Key Word Index—*Arum maculatum*; Araceae; cuckoo-pint; starch breakdown; enzymes.

Abstract—The aim of this work was to discover the pathway of starch breakdown during thermogenesis in the club of the spadix of *Arum maculatum*. The conventional α-amylase of higher plants could not be demonstrated in extracts of clubs although such extracts did exhibit considerable hydrolytic activity towards starch. This activity had an action pattern characteristic of an endo-amylase, was destroyed by heating to 70°, and was not inhibited by either 7 mM ethylenediaminetetra-acetic acid or 100 mM *N*-ethyl maleimide. Measurements of this hydrolytic activity, and of the maximum catalytic activities of starch phosphorylase, phosphoglucomutase and hexokinase, were made at different stages of club development. These measurements were compared with estimates of the rate of starch breakdown at thermogenesis. This comparison indicates that phosphorolytic cleavage does not play a large role in such starch breakdown, and that this process is mediated, mainly, by the hydrolytic activity, described above, and by hexokinase.

INTRODUCTION

We have no general understanding of the precise routes of starch breakdown in higher plants. The available evidence strongly suggests that the initial step is catalysed by α-amylase (EC 3.2.1.1). This is the only enzyme known to be capable of directly attacking starch grains. Starch phosphorylase (EC 2.4.1.1) and β-amylase (EC 3.2.1.2), both of which can catalyse the breakdown of α-1,4-glucans, are widely distributed in plants, but their roles in starch metabolism in general are not known. Both of these enzymes may contribute to the metabolism of the initial products of α-amylase: there is some evidence that suggests that phosphorylase makes the greater contribution where starch breakdown is intra-cellular, and β-amylase where the breakdown is extra-cellular [1–3]. The aim of the work described in this paper was to discover the pathway of starch breakdown in the club of the spadix of *Arum maculatum* so as to increase our understanding of the relative roles of amylases and phosphorylases in starch metabolism.

The advantage of using the club of *Arum maculatum* for the study of starch breakdown is that at one stage of development the rate of breakdown increases very rapidly to an extremely high value. This sudden consumption of starch is precisely controlled, intra-cellular, and has been measured directly. The club passes through a number of developmental stages that culminate in a brief period of thermogenesis at a specific stage in flowering. This thermogenesis is marked by a rapid rise and subsequent fall in the rate of respiration, and is generally confined to a period of 8 hours or so. Prior to thermogenesis clubs contain 220–300 mg starch/g fr. wt. Almost all of this is consumed during thermogenesis, and estimates of the rate of breakdown gave values of 6–7.5 μmol hexose produced/g fr. wt per minute [4, 5].

Our experimental approach was to estimate the maximum catalytic activities of amylase, starch phosphorylase, hexokinase (EC 2.7.1.1) and phosphoglucomutase (EC 2.7.5.1) in clubs at different stages of development, and then to compare these estimates with the rate of starch breakdown at thermogenesis. We hoped that the enzymes responsible for starch breakdown would be distinguishable by their high activities, and by increases in these activities during club development. Amylase and hexokinase were chosen as indicators of a hydrolytic route of starch consumption, and phosphorylase and phosphoglucomutase as indicators of phosphorolysis.

RESULTS

During the three weeks prior to thermogenesis, the club of the spadix of *Arum maculatum* enlarges steadily by cell expansion as the inflorescence develops. We have designated successive stages of this development as α, β, γ, pre-thermogenesis, and thermogenesis. We have described these stages and how to recognize them [6]. Stages α, β and γ represent the main period of club growth; pre-thermogenesis is the time 6–18 hr before the start of thermogenesis, and thermogenesis is the stage of rapid starch consumption and heat production.

Briggs [7] has emphasized the difficulties involved in the determination of the maximum catalytic activities of the enzymes that metabolize strach. None the less he has devised a specific assay for α-amylase. In this, the tissue must be extracted near pH 6 with a buffer that contains calcium, the extract must then be heated to 70° at pH 6 for 20 min, and finally be assayed by its ability to catalyse a decline in starch–iodine-colour. Repeated application of this procedure to clubs at stages α, β, γ, and thermogenesis failed to reveal any activity of α-amylase. The following features of the procedure were then varied: the pH of the extraction medium, from pH 6 to 7.5; the pH at which the assay was carried out, from pH 5 to 7; and the concentration of starch used in the assay, from 1–3% (w/v). None of these variations produced detectable activity. When we applied Briggs' method to the corm of *Arum maculatum*, we readily obtained appreciable activities of α-amylase (134 starch–iodine-colour units per g fr. wt). We took advantage of this to see if the lack of activity in extracts of clubs was due to the formation or

release of inhibitors during the preparation of the extracts. We took a club at thermogenesis and cut it vertically into two samples. We also cut two samples from a single corm. All four samples had similar weights. Next we prepared and assayed for α-amylase three extracts, one from a half of the club, one from one of the samples of corm, and one from a mixture of the other half of the club and the other sample of corm. The activity recovered from the mixed sample was 107% of the value predicted from the measurements made on the separate samples of club and corm.

Our inability to detect α-amylase activity in extracts of the clubs led us to examine them for any ability to hydrolyse starch. We made the extracts in phosphate–citrate buffer, dialysed them overnight, and then incubated samples with soluble starch. We measured the amounts of reducing sugar liberated and have expressed them as μmol equivalents of maltose. Extracts from clubs at all stages from α to thermogenesis showed considerable activity, henceforth called amylolytic activity. We showed that this activity had a broad optimum between pH 6 and 6.6, was highest with 3% (w/v) starch, and was not increased by removal from the extract of the material that sedimented after centrifuging at 100000 *g* for 30 min. Chromatography of the reaction products revealed a range of oligosaccharides, maltose, and glucose. Subsequent studies of this activity were made under the above optimum conditions, *viz*: the dialysed crude homogenates were incubated at pH 6.25 with 3% (w/v) starch.

Simple fractionation procedures did not resolve this amylolytic activity into different components. The crude extract of a thermogenic spadix was centrifuged at 34000 *g* for 30 min and the supernatant then treated with a saturated solution of $(NH_4)_2SO_4$; the activity was recovered in the fraction that precipitated between 30 and 50% saturation. When this fraction was dissolved in extraction buffer and applied to a column (45 × 2.5 cm) of Sephadex G-200, the activity was eluted as a single peak. In order to investigate whether the amyloytic activity was due to an exo- or an endo-amylase, we determined the action pattern in crude extracts of thermogenic clubs. We did this by the method of Tung and Nordin [8] in which it is necessary to determine, at different times during the incubation, the ratio of total carbohydrate liberated to the number of reducing groups liberated. The results (Fig. 1) show that at the start of the reaction the ratios are quite high but they decline rapidly as the reaction proceeds. In a further attempt to compare the amylolytic activity of the clubs with known α- and β-amylases we determined the effects of 7 mM EDTA, an inhibitor of α-amylase [9], and of 100 mM *N*-ethyl maleimide, an inhibitor of β-amylase [10], on both the activity and the action pattern. Neither treatment, when applied to extracts of thermogenic clubs, affected either the amount of amylolytic activity or its action pattern. Dialysis of crude extracts of thermogenic clubs against 7 mM EDTA for 24 hr also failed to inhibit the activity. Following our determination of the action pattern of the amylolytic activity *in vitro*, we examined the situation *in vivo*. We did this by analysing the neutral fraction of the water-soluble constituents of thermogenic clubs. Paper chromatography of this fraction revealed the presence of glucose, sucrose and a range of oligosaccharides.

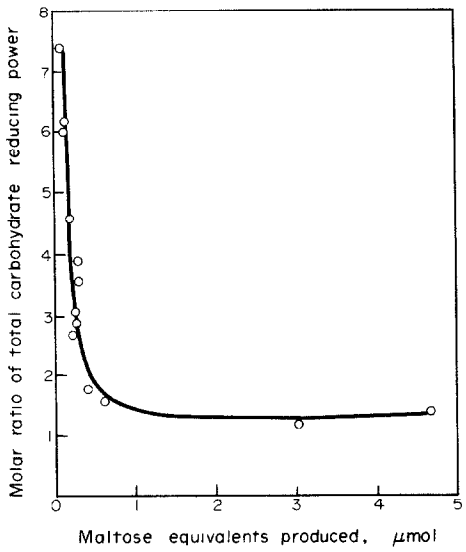

Fig. 1. Action pattern of amylolytic activity in extracts of thermogenic clubs of *Arum maculatum*. Extracts were incubated with soluble starch for varying times. Unmetabolized starch was removed by passage through Sephadex G-50, and then both the total carbohydrate and the number of reducing groups, liberated by enzyme action, were measured.

We investigated whether the amylolytic activity of the

Table 1 Amylolytic activity during the development of the club of the spadix of *Arum maculatum*

Stage of development*	Activity* (μmol equivalents maltose/min per club)				
	α I	β II	γ III	Pre-thermogenesis IV	Thermogenesis V
	0.47 ± 0.08 [12]	3.40 ± 0.44 [5]	10.83 ± 1.64 [5]	13.23 ± 1.20 [5]	11.55 ± 2.12 [5]

		Fisher's *P* values†							
I vs II	I vs III	I vs IV	I vs V	II vs III	II vs IV	II vs V	III vs IV	III vs V	IV vs V
<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.01	N.S.	N.S.	N.S.

*Values are means ± S.E. The number of clubs assayed is shown in parenthesis.

†Fisher's *P* values are given for comparison of the activities of clubs at different stages of development. Values of 0.05 or less are considered significant. Values greater than 0.05 are given as N.S (not significant).

clubs changed during development. The assays were carried out under the optimum conditions described above. The results are expressed per club because the number of cells in a club changes much less than either its weight or protein content during development [6]. The results (Table 1) show that the activity increased from α to β stage and from β to γ stage but not thereafter. The overall increase was about 20-fold. We checked to see if the difference in activity between young and thermogenic clubs was due to the formation or liberation of inhibitors during the extraction of the young clubs. An α stage club and a thermogenic club were both split longitudinally into halves. Then three samples of tissue were prepared, extracted, and assayed: one sample was of a half-club at α stage, one of a half-club at thermogenesis, and one was a mixture of half the α stage club and half the thermogenic club. The activity recovered in the extract of the mixture was 129% of the value predicted from the measurements made on the other two samples.

The difficulties of measuring the maximum catalytic activity of starch phosphorylase caused us to assay each extract for this enzyme in three different ways. These involved, respectively, the measurement of the incorporation of ^{14}C into amylopectin from [U-^{14}C]-glucose-1-phosphate, the release of inorganic phosphate from glucose-1-phosphate, and the formation of glucose-1-phosphate, The latter was measured by following NADP reduction in the presence of added phosphoglucomutase and glucose-6-phosphate dehydrogenase. Each assay was studied in detail in an attempt to obtain optimum conditions. For the assay involving the incorporation of ^{14}C into amylopectin, and that depending on the release of phosphate, we optimized: the pH of the assay medium, and the concentrations of glucose-1-phosphate and amylopectin. We did this at α stage and at thermogenesis. For the other assay we compared glycylglycine, imidazole and MES as buffers at α stage. Then we optimized the pH, and the concentrations of each component of the reaction mixture for extracts from clubs at α stage and at thermogenesis. We checked for the presence of inhibitors in the extracts by the type of experiment described under our estimation of the amylolytic activity. The activities recovered in the mixed sample of α stage and thermogenic clubs were 90, 113 and 139% of the predicted values for the assays involving incorporation of ^{14}C, release of inorganic phosphate, and reduction of NADP, respectively. Our estimates of phosphorylase (Table 2) show that the values obtained varied somewhat according to the assay used. None the less the estimates by the different methods change similarly during club development. The essential points about these results are two. First, they indicate that the activity of phosphorylase rises between α and β stages but not thereafter. Second, the highest value obtained was only 5.3 μmol/min per club.

We also optimized our assays of phosphoglucomutase and hexokinase. For each we varied the pH and the concentration of every component of the assay mixtures. We did this at α stage and at thermogenesis. For the assay of hexokinase the extracts were centrifuged very briefly so as not to lose any activity that was associated with particulate material. The activities of phosphoglucomutase and hexokinase in a mixed sample of α stage and thermogenic clubs were 99 and 123%, respectively, of the values predicted from measurements made on separate samples of clubs. During club development the activity of phosphoglucomutase rose from α to β stage but no further change in activity was detected (Table 3). The total increase was 5-fold. In contrast the activity of hexokinase increased up to thermogenesis and the overall increase was 25-fold (Table 3).

DISCUSSION

Our results provide firm evidence that starch breakdown during thermogenesis by the club of *Arum maculatum* is not mediated by the type of α-amylase that is usually found in the tissues of higher plants. Although we used Briggs' specific assay for α-amylase, we detected no activity despite variation in the methods of extraction and assay. It is most unlikely that the absence of activity was due to losses during extraction. We have shown previously that it is possible to demonstrate very high activities of quite fastidious enzymes in extracts of *Arum* clubs [6]. Further, such extracts in no way interfered with the recovery of α-amylase from the corm of *Arum*. Thus it is necessary to qualify the view that all starchy tissues of higher plants contain the conventional α-amylase.

The club of *Arum maculatum* is not devoid of the ability of hydrolyse starch. At all stages of development examined we found an appreciable ability to liberate

Table 2. Starch phosphorylase activity during the development of the club of the spadix of *Arum maculatum*

Assay	Stage of developments:	Activity* (μmol/min per club) α I	β II	Pre-thermogenesis III	Thermogenesis IV
[^{14}C]-Glc-1-P incorporated		0.82 ± 0.18	2.55 ± 0.28	4.52 ± 1.10	2.91 ± 0.46
Glc-1-P formed		0.33 ± 0.07	0.94 ± 0.12	1.42 ± 0.33	1.38 ± 0.39
Pi released		0.99 ± 0.24	3.77 ± 0.51	5.27 ± 1.34	5.29 ± 1.21

	Fisher's *P* values†					
	I vs II	I vs III	I vs IV	II vs III	II vs IV	III vs IV
[^{14}C]-Glc-1-P incorporated	<0.001	<0.01	<0.01	N.S.	N.S.	N.S.
Glc-1-P formed	<0.01	<0.01	<0.05	N.S.	N.S.	N.S.
Pi released	<0.001	<0.02	<0.01	N.S.	N.S.	N.S.

*Value are means ± S.E. Seven clubs were assayed at thermogenesis, and six at each of the other stages.
†Fisher's *P* values are given as in Table 1.

Table 3. Activities of phosphoglucomutase and hexokinase during the development of the club of the spadix of *Arum maculatum*

Enzyme	Stage of development	Activity* (μmol substrate consumed/min per club)			
		α I	β II	Pre-thermogenesis III	Thermogenesis IV
Phosphoglucomutase		5.40 ± 0.85	20.96 ± 4.45	28.66 ± 9.10	24.31 ± 5.25
Hexokinase		0.54 ± 0.10	3.32 ± 0.59	7.07 ± 1.81	12.28 ± 1.52

	Fisher's *P* values†					
	I vs II	I vs III	I vs IV	II vs III	II vs IV	III vs IV
Phosphoglucomutase	<0.01	<0.05	<0.01	N.S.	N.S.	N.S.
Hexokinase	<0.001	<0.01	<0.001	N.S.	<0.001	<0.05

*Values are means ± S.E. Seven clubs were assayed at thermogenesis, and six at each of the other stages.
†Fisher's *P* values are given as in Table 1.

reducing groups from starch. The extent to which this activity may be ascribed to a single enzyme cannot be decided until complete purification has been achieved, a process greatly hindred by the short season for which the clubs are available. None the less, these experiments indicate that the activity may represent a single enzyme of the endo-amylase type. The action pattern (Fig. 1) is almost identical to that reported for purified α-amylase [8], but the activity differs from α-amylase in its sensitivity to heat and response to EDTA. We suggest that the amylolytic activity of the clubs has been sufficiently characterized to permit its measurement. This allowed us to investigate its role in starch breakdown.

The presence of abundant α-amylase in the corms and its absence from clubs suggest that the amylolytic activity is peculiarly associated with the rapid breakdown of starch at thermogenesis. This view is sustained by our measurements of enzyme activities. We argue that these measurements reflect the maximum catalytic activities of the clubs. The experiments with mixed samples of tissue do not reveal any substantial inhibition or activation of the enzymes during the preparation of the extracts. Each assay was carefully optimized, and sufficient clubs were assayed to permit statistical analysis. There is some discrepancy between our estimates of phosphorylase. Assay by release of inorganic phosphate is probably an overestimate because phosphatase activity could contribute to such release. The assay involving reduction of NADP may be an underestimate because the pH optima of the coupling enzymes differ from that of phosphorylase itself. Further evidence that our estimates of phosphorylase were not seriously affected by artefacts is provided by the fact that during club development these estimates varied in the same way as those for phosphoglucomutase. Both enzymes increased from α to β stage but showed no further increase. Such co-ordinated behaviour would be expected as one enzyme metabolizes the product of the other.

Examination of our measurements of phosphorylase leads us to conclude that this enzyme does not play a major role in starch breakdown during thermogenesis in the club of *Arum maculatum*. Estimates of the rate of starch breakdown during thermogenesis gave values of 4.6–5.9 μmol hexose produced/min per club. The rate at the peak of thermogenesis must have been somewhat higher [5]. Thus the maximum catalytic activity of phosphorylase at thermogenesis is not high enough to support the observed rate of starch breakdown. There are two further arguments against any major role for phosphorylase in starch breakdown in the club. First, the changes in maximum catalytic activities of phosphorylase and phosphoglucomutase during club development are characteristic of enzymes that are not involved in thermogenesis. The two dehydrogenases of the pentose phosphate pathway also show an increase from α to β stage but not thereafter. Independent evidence showed that this pathway is active in young clubs but makes no detectable contribution at thermogenesis [6]. Second, at thermogenesis the maximum catalytic activity of phosphorylase is about a quarter of that of phosphofructokinase [6]. This would not be expected if phosphorylase were the major source of substrate for phosphofructokinase. In those animal tissues in which glycolysis is dependent upon phosphorolysis of intra-cellular glycogen, the activity of phosphorylase is as great or greater than that of phosphofructokinase [12, 13]. In the leaves of *Kalanchoë daigremontiana*, where starch breakdown appears to be primarily phosphorolytic, the activity of phosphorylase is reported to be four times that of phosphofructokinase [11].

We suggest that the rapid breakdown of starch at thermogenesis in the club of *Arum* is essentially hydrolytic and is catalysed by amylolytic activity. We also suggest that the products of this hydrolysis enter glycolysis via hexokinase. These suggestions are supported by the following evidence. First, at thermogenesis the amylolytic activity and the activity of hexokinase are well in excess of the observed rates of starch breakdown. Second, the changes in amylolytic activity and in the activity of hexokinase that occur during club development are characteristic of enzymes known to be involved in thermogenesis. The distinguishing feature of such enzymes, exhibited by the glycolytic enzymes [5, 6], is that their maximum catalytic activity shows considerable increases after β stage. Third, the amylolytic activity and the activity of hexokinase increased 25-fold during club development whereas there was only a 5-fold increase in phosphorylase and phosphoglucomutase. Finally, the presence of oligosaccharides in thermogenic clubs would be expected if starch breakdown were due to an enzyme with the action pattern shown in Fig. 1. Thus we suggest that the spadix of *Arum maculatum* provides an example of intra-cellular breakdown of starch that occurs predominantly by a hydrolytic route, and is probably catalysed by an endo-amylase with properties somewhat different from those of the conventional α-amylase.

Comparison of amylolytic activity and hexokinase activity at the different stages of club development with the rate of starch breakdown at thermogenesis shows that the activities at α and β stages are too low to support starch breakdown at thermogenesis. Thus the increase in the activities of these enzymes is a prerequisite for the rapid metabolism of starch at thermogenesis. These increases may therefore be regarded as an example of coarse control of starch br akdown. It is clear that this is not the only form of control operating as the activities of both enzymes at pre-thermogenesis are high enough to support starch breakdown at thermogenesis. Either fine control or compartmentation is likely to be immediately responsible for the rapid acceleration of starch breakdown at thermogenesis. Thus coarse control may be seen as setting the limits within which more immediate mechanisms of control operate.

EXPERIMENTAL

Materials. Apart from the work with corms, all experiments were carried out with the swollen club-shaped portion of the appendix of the spadix of *Arum maculatum* L. Inflorescences at different stages of development were taken from plants growing wild in a range of local sites. The time between collection and the start of the experiments was 1–3 hr. Clubs were not cut off the spadices until the start of the experiments. The stages of development were recognized as described previously [6]. Corms were taken from plants growing wild and were used at once.

Methods. For the assay of α-amylase, single clubs were homogenized in 4–8 vol. 12.6 mM CaOAc adjusted to pH 6 with 0.1 M HOAc. The homogenate was transfererd to 70° for 20 min, cooled to 4°, and then centrifuged (34000 *g*, 15 min). The supernatant was assayed as described by ref. [14]. Activity is expressed as starch–iodine-colour units as calculated in ref. [15]. For assay of amylolytic activity, single clubs were homogenized in 4–8 vol. 0.134 M Na_2HPO_4–0.034 M citric acid, pH 6.25. The homogenate was dialysed against the same buffer at 0.1 strength at 4° for 24 hr. Samples (1.0 ml) of the dialysis residue were then incubated at 25° for 1 hr with 2 ml 3% (w/v) soluble starch dissolved in 0.134 M Na_2HPO_4–0.034 M citric acid, pH 6.26. The reaction was stopped by heating at 100° for 3 min. The reaction mixture was then centrifuged (3500 *g*, 20 min) and the supernatant was assayed for reducing power, using maltose as a standard, as described by ref. [16]. The action pattern was determined according to ref. [8]. The extract was prepared and incubated as above, except that the time of incubation was varied from 5–60 min. The supernatant obtained after centrifuging the boiled reaction mixture was passed through a column (30 × 0.7 cm) of Sephadex G-50 to separate soluble starch from the reaction products. The latter were assayed for reducing power as above, and for total carbohydrate as described in ref. [17]. For determination of the pH optimum, the reaction mixture was 1.0 ml dialysed extract, 3 ml 1% (w/v) soluble starch and 1 ml of buffer prepared from 0.2 M Na_2HPO_4 and 0.1 M citric acid. Partial purification of the amylolytic activity was achieved as follows. The initial homogenate was centrifuged (34000 *g*, 30 min) and the supernatant was treated with satd $(NH_4)_2SO_4$, pH 6.25, at 4°. The fraction that pptd between 30–50% satn was collected by centrifugation, re-suspended in extraction buffer, and applied to a column (45 × 2.5 cm) of Sephadex G-200 that had been equilibrated with extraction buffer. The column was then eluted with extraction buffer, and fractions of 3 ml were collected and assayed for amylolytic activity, and, by measurement of A_{280}, for protein. For the assay of hexokinase, phosphorylase, and phosphoglucomutase, single clubs were homogenized in 4–8 vol. 40 mM glycylglycine, pH 7.4. For these assays, and for those of amylase, the clubs were ground first with a pestle and mortar and then with an all-glass homogenizer. Examination of the homogenates with a microscope showed that this procedure left extremely few cells unbroken. One of our assays for phosphorylase, and those for hexokinase and phosphglucomutase, depended upon measurement of NADP reduction in the presense of added glucose-6-phosphate dehydrogenase. In crude extracts this type of assay is subject to interference by 6-phosphogluconate dehydrogenase present in the extracts. This problem was solved, as described by ref. [18], either by the addition of excess 6-phosphogluconate dehydrogenase to the assay and then using half the rate of NADP reduction as the measure of activity, or by taking, as our measurement, the difference between the rate of NADP reduction in the presence of added 6-phosphogluconate and the rate in the presence of 6-phosphogluconate plus the substrate of the enzyme to be assayed. For the assay of hexokinase the crude homogenate was centrifuged at 4° (600 *g*, 10 min) and the supernatant was assayed according to ref. [19]. At α stage, β stage and pre-thermogenesis the assay mixture (3 ml) contained: 28 mM glycylglycine (pH 7.7), 0.85 mM NADP, 5.5 mM ATP, 6.7 mM $MgCl_2$, 20 mM glucose, 5 μg (0.7 unit) glucose-6-phosphate dehydrogenase and 2 μg (0.024 unit) 6-phosphogluconate dehydrogenase. At thermogenesis the mixture was the same except that the glycylglycine was 26 mM, and the 6-phosphogluconate dehydrogenase was replaced by 6 mM 6-phosphogluconate. For phosphoglucomutase, the crude extract was centrifuged at 4° (100000 *g*, 30 min) and the supernatant was assayed according to ref. [20]. At α stage, β stage and pre-thermogenesis the assay mixture (3 ml) contained: 31 mM imidazole–HCl (pH 7.4), 0.85 mM NADP, 3.3 mM $MgCl_2$, 0.9 mM EDTA, 8.2 μM glucose-1,6-diphosphate, 3.3 mM glucose-1-phosphate, 5μg (0.7 unit) glucose-6-phosphate dehydrogenase and 2 μg (0.024 unit) 6-phosphogluconate dehydrogenase. The latter component was omitted at thermogenesis as we found no interference from the relatively small amount of 6-phosphogluconate dehydrogenase present in the extracts. For phosphorylase the extract was treated as for phosphoglucomutase and the supernatant was assayed in 3 ways. The first was according to ref. [21] for glycogen phosphorylase. At α stage, β stage and pre-thermogenesis, the reaction mixture (3 ml) contained: 25 mM imidazole–HCl (pH 7), 0.85 mM NADP, 6.7 mM Pi (as a mixture of 0.4 M KH_2PO_4 and 0.4 M Na_2HPO_4 at pH 7), 10 mM $MgCl_2$, 4.1 μM glucose-1,6-diphosphate, 0.25% (w/v) amylopectin, 20 μg (4 units) phosphoglucomutase, 10 μg (1.4 units) glucose-6-phosphate dehydrogenase, and 4 μg (0.048 unit) 6-phosphogluconate dehydrogenase. At thermogenesis the mixture was the same except that 35 μg (0.42 unit) 6-phosphogluconate dehydrogenase was added. The second assay was according to ref. [22]; the reaction mixture (1 ml) contained: 20 mM MES (pH 6.3), 10 mM NAF, 10 mM glucose-1-phosphate and 0.9% (w/v) amylopectin. The third assay was that of ref. [23]; the reaction mixture (0.1 ml) contained. 25 mM MES (pH 6.3), 0.1 μCi [U-^{14}C]-glucose-1-phosphate at 10 mM, and 0.9% (w/v) amylopectin. For the second and third assays the incubation time was 20 min. All enzyme assays were made at 25°. The neutral fraction of the water-soluble components of the clubs was prepared as described in ref. [6] and analysed by PC, using *n*-BuOH–Py–H_2O (2:2:1).

Acknowledgement—PVB thanks the Science Research Council for a research studentship.

REFERENCES

1. Dunn, G. (1974) *Phytochemistry* **13**, 1341.
2. Manners, D. J. (1974) *Essays in Biochemistry* (Campbell, P. N. and Dickens, F. eds) Vol. 10, p. 37. Academic Press, London.
3. ap Rees, T. (1974) *MTP Int. Rev. Sci. Biochem.* **11**, 89.
4. Lance, C. (1972) *Ann. Sci. Natur. Bot. Biol. Végétale 12 eme Sér.* **13**, 477.
5. ap Rees, T., Wright, B. W. and Fuller, W. A. (1977) *Planta* **134**, 53.
6. ap Rees, T., Fuller, W. A. and Wright B. W. (1976) *Biochim. Biophys. Acta* **437**, 22.
7. Briggs, D. E. (1973) *Biosynthesis and its Control in Plants* (Milborrow, B. V. ed.) p. 219. Academic Press, London.
8. Tung, K. K. and Nordin, J. H. (1969) *Anal. Biochem.* **29**, 84.

9. Fischer, E. H and Stein, E. A. (1960) *The Enzymes* (Boyer, P. D., Lardy, H. and Myrbäck, K. eds) Vol. 4A, p. 313. Academic Press, New York.
10. Spradlin, J. E. and Thoma, J. A. (1970) *J. Biol. Chem.* **245**, 117.
11. Sutton, B. G (1975) *Australian J. Plant Physiol.* **2**, 389.
12. Crabtree, B. and Newsholme, E. A. (1972) *Biochem. J.* **126**, 49.
13. Zammit, V. A. and Newsholme, E. A. (1976) *Biochem. J.* **160**, 447.
14. Briggs, D. E. (1967) *J. Inst. Brewing* **73**, 361.
15. Duffus, J. H. (1969) *J. Inst. Brewing* **75**, 252
16. Hestrin, S., Feingold, D. S. and Schramm, M. (1955) *Methods in Enzymology* (Colowick, S. P. and Kaplan, N. O. eds) Vol. 1, p. 231. Academic Press, New York.
17. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1965) *Anal. Chem.* **28**, 350.
18. Glock, G. E. and McLean, P. (1953) *Biochem. J.* **55**, 400.
19. Fowler, M. W. and Clifton, A. (1974) *European J Biochem.* **45**, 445.
20. Shronk, C. E. and Boxer, G. E. (1964) *Cancer Res.* **24**, 709.
21. Lowry, O. H., Schulz, D. W. and Passonneau, J. V. (1964) *J. Biol Chem.* **239**, 1947.
22. Lee, Y P. (1966) *Methods in Enzymology* (Neufeld, E. F. and Ginsberg, V. eds) Vol. 8, p. 550. Academic Press, New York.
23. Downton, W. J. S and Hawker, J. S. (1973) *Phytochemistry* **12**, 1551