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STARCH DEPLETION IN GERMINATING WHEAT, WRINKLED-SEEDED PEAS AND SENESCING TOBACCO LEAVES

I. R. ABBOTT and N. K. MATHESON
Department of Agricultural Chemistry, University of Sydney, N.S.W. 2006, Australia

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Abstract—The residual starches of germinating wheat and barley grains show similar structural changes. Germinating wheat grains produce malto-oligosaccharides and dextrins. The starch of wrinkled-seeded peas showed some structural changes during germination, but the starch from senescing tobacco leaves showed none. Neither peas nor tobacco produced malto-oligosaccharides or dextrins at any stage. Wrinkled-seeded peas showed some differences to smooth-seeded peas in enzyme content, and starch was probably degraded by phosphorylase initially with α-amylase acting after 3 days. In senescing tobacco leaves the only significant enzyme activities were α-amylase and maltase. Wheat closely resembled barley in showing amylolytic breakdown.

INTRODUCTION

STARCH depletion in plants can involve debranching enzymes in conjunction with α- or β-amylases or phosphorylase or a combination of these enzymes.[1,2] Information on the *in vivo* breakdown can be obtained from the oligosaccharides produced, the changes in structure of the residual starches, the production and structure of soluble malto-dextrins and from changes in levels of the starch catabolizing enzymes. The enzymes that can be assayed without extensive prior fractionation are α- and β-amylases, phosphorylase and maltase, although others have been detected after fractionation. The significance of the assay is limited by the ease of extraction and the possibility of localization.

A number of plant tissues, mostly germinating cereal seeds have been examined. In particular, the process of malting in barley seed has been studied. After 4–5 days, the apparent amylose content of starch from isolated granules was found to have increased, the chain length of the amylopectin decreased, the intrinsic viscosity of the amylose decreased slightly and its β-amylolysis limit rose.[3,4] These results were considered to be consistent with a limited preferential β-amylolysis of the amylopectin and a very limited α-amylolysis of the amylose. The presence of glucose, maltose and maltotriose in the ethanolic extract indicated amylolytic breakdown.[3,5] α-Amylase, which is under the influence of endogenous gibberellins, increased in activity from an undetectable value at the onset of germination to a maximum at 4 days and declined to about 1/7 of maximal activity at 7 days.[6] Maltase was also present. Starch hydrolysis in wheat has been attributed to amylases.[7,8] In germinating rice, a marked increase in the production of glucose and malto-oligosaccharides, particularly

[1] D. J. MANNERS, *Advan. Carbohyd. Chem.* **17**, 371 (1962).
[2] C. T. GREENWOOD, *Advan. Carbohyd. Chem.* **23**, 281 (1968).
[3] G. O. ASPINALL, E. L. HIRST and W. MCARTHUR, *J. Chem. Soc.* 3075 (1955).
[4] C. T. GREENWOOD and H. A. THOMSON, *J. Inst. Brew.* **65**, 346 (1959).
[5] G. HARRIS and I. C. MACWILLIAM, *J. Inst. Brew.* **66**, 147 (1960).
[6] D. E. BRIGGS, *Phytochem.* **7**, 513 (1968).
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[8] J. A. JOHNSON, *Cereal Sci. Today* **10**, 315 (1965).

maltotriose was paralleled by an increase in α-amylase activity[9] and maltase activity[10] suggesting an amylolytic breakdown of starch. Maltase and α- and β-amylase have been found in germinating oat seeds.[11] In maize,[12] the α-amylolytic activity increased from a negligible amount at 0–3 days to a maximum 10 days after germination. Phosphorylase was also present.[13]

In smooth-seeded peas with a 45% starch content, which had an amylose content of 35%, there was no accumulation of malto-dextrins or free sugars in the cotyledons during germination.[14] Changes in the chemical structure of starch in granules were ascribed to scission of inner chain α(1–4) linkages by α-amylase in the first 5 days and to the combined actions of α-amylase and phosphorylase, but mainly α-amylase, from 5 to 11 days. The activities of α-amylase and phosphorylase increased on germination[14,15] but no evidence for a phosphatase that hydrolysed D-glucose 1- or 6-phosphates was found, although other phosphatase activities increased.[16]

Granules of starch from potatoes isolated before and after sprouting showed no changes in chemical structure.[17] Examination of some samples from senescing tobacco leaves indicated that this starch may have shown a similar lack of structural change.[18]

In this paper, a comparison has been made of three plant tissues in which starch has been undergoing depletion, germinating wheat seeds (endosperm and bran), wrinkled-seeded pea cotyledons from germinating seeds, and senescing tobacco leaves. The levels of α- and β-amylases, phosphorylase and maltase have been compared, a search for soluble starch breakdown products made and the structure of the residual starch in granules examined.

RESULTS AND DISCUSSION

Quantitative changes in the amounts of residual starch in wrinkled-seeded peas as shown in Fig. 1 involved only slight depletion for up to almost 250 hr after imbibition. Smooth-seeded peas with a much higher initial starch content also behaved in this way.[14] The period covered in the diagram for tobacco leaves is about 10–14 days.

Changes in water-soluble sugars (Fig. 2) showed differences in the different systems. Wheat was characterized by a rapid increase in both total and reducing sugars. In peas, the reducing sugar remained low but total sugars increased and then decreased. In tobacco leaves, both were constant until they increased at the later stages of senescence.

Some measurements of chemical structural changes in residual starches (Table 1) showed that wheat resembled barley,[3,4] with an initial increase in apparent amylose content, β-amylolysis limit and a decrease in amylopectin chain length. When barley (var. Cape) was grown for 192 hr the properties of the residual starch returned towards those of ungerminated starch in the same way as wheat. Pea starches showed some variability but the reproducibility of measurement was reduced, probably due to associated protein. Smooth-seeded peas

[9] T. MURATA, T. AKAZAWA and S. FUKUCKI, *Plant Physiol.* **43,** 1899 (1968).
[10] T. NOMURA, Y. KONO and T. AKAZAWA, *Plant Physiol.* **44,** 765 (1969).
[11] G. M. SIMPSON, *Can. J. Bot.* **40,** 1659 (1962).
[12] L. S. DURE, *Plant Physiol.* **35,** 925 (1960).
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[14] B. O. JULIANO and J. F. VARNER, *Plant Physiol.* **44,** 886 (1969).
[15] R. R. SWAIN and E. E. DEKKER, *Biochem. Biophys Acta* **122,** 75 (1966).
[16] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84,** 71 (1959).
[17] W. BANKS and C. T. GREENWOOD, *Biochem. J.* **73,** 237 (1959).
[18] N. K. MATHESON and J. M. WHEATLEY, *Austral. J. Biol. Sci.* **15,** 445 (1962).

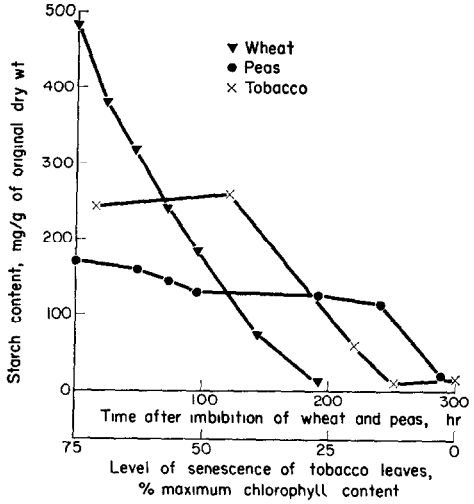

FIG. 1.

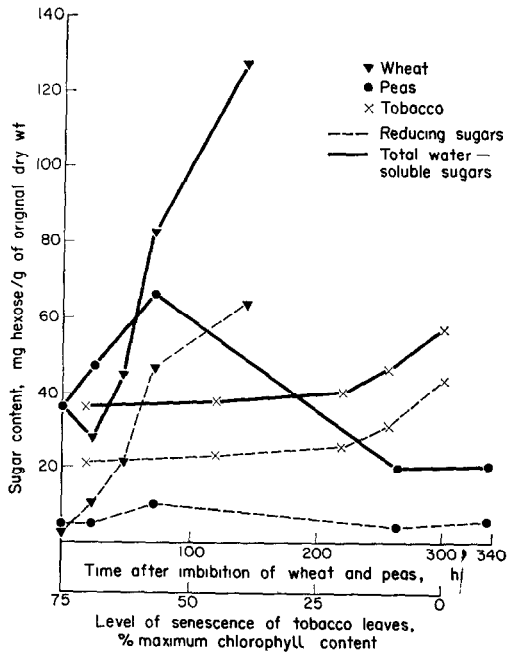

FIG. 2.

FIG. 1. STARCH DEPLETION IN GERMINATING WHEAT AND PEAS AND SENESCING TOBACCO LEAVES.

FIG. 2. TOTAL WATER-SOLUBLE SUGAR AND REDUCING SUGAR CONTENTS OF GERMINATING WHEAT AND PEAS AND SENESCING TOBACCO LEAVES.

were also found to have some differences in chemical properties.[14] Tobacco starches retained the same values over the whole period of depletion.

Gel chromatography on 2% agarose, which excluded polysaccharides above a molecular weight of 2×10^6 gave the elution patterns shown in Fig. 3 for wheat, 4 for peas and 5 for tobacco. Three samples were taken; before and during breakdown and when little starch remained. Two fractions were found. One was excluded and had, like amylopectin, an iodine absorption maximum at 560 nm and the other was retarded with a maximum at 600 nm or above, like amylose. In wheat, the amount of excluded fraction, estimated from peak area, was similar at 0 and 48 hr and rose at 144 hr. The retarded fraction increased markedly from 0 to 48 hr with a further increase to 144 hr. The K_{av} (elution volume — void volume/total volume — void volume) decreased between 0 and 48 hr and 144 hr. In pea starches where the large area of retarded fraction reflected the high amylose content of wrinkle-seeded peas, there were variations in amounts but no regularity of pattern and no significant differences in K_{av} values. In tobacco any differences were at the level of reproducibility.

In wheat, the increase in K_{av} of the retarded peak indicated an increase in molecular size. As this was accompanied by an increase in the proportion of this fraction, it could have been due to the addition of partly degraded amylopectin, possibly combined with a limited degradation of amylose, or to a preferential degradation of amylopectin to soluble products with a limited preferential utilization of lower molecular weight molecules in the amylose fraction. Evidence that the latter is more probable is indicated by the increase in apparent

TABLE 1. PROPERTIES OF STARCH GRANULES ISOLATED DURING DEPLETION IN GERMINATING WHEAT AND PEA SEEDS AND SENESCING TOBACCO LEAVES

Plant and stage of depletion	Apparent amylose content (%)	Chain length of amylopectin (glucose units)	β-Amylolysis limit of amylopectin (%)	Intrinsic viscosity (g/dl).
Wheat (hr of germination)				
0	18	31	56	n.d.
24	20	27	64	n d.
48	20	n.d.*	60	n.d.
72	20	26	60	n.d.
96	20	n.d.	58	n.d.
144	24	30	56	n.d.
192	17	n.d.	53	n.d.
Peas (hr of germination)				
0	61	21	80	n.d.
50	58	21	77	n.d.
90	58	n.d.	81	n.d.
200	61	23	79	n.d.
250	62	22	77	n.d.
300	60	n.d.	77	n.d.
Tobacco (Proportion of original chlorophyll)				
71%	25	n.d.	67	1·53
45%	25	n.d.	69	1·56
20%	25	n.d.	69	1·61
12%	25	n.d.	69	1·58
0%	25	n.d.	68	1·62

* n.d.—not determined.

amylose content (Table 1) and from gel chromatography of the products of *in vitro* amylase action on starch (Fig. 6). The wavelength of maximum absorption of the glucan-iodine complexes of all the eluates was 560 nm. On α-amylolysis all excluded fraction rapidly disappeared leaving a single peak of low molecular weight material. On β-amylolysis the retarded (amylose) fraction disappeared but an excluded fraction remained. Neither enzyme produced a fraction with K_{av} values of the increasing peak in wheat starch. Also, the low wavelength of absorption maximum of the iodine complexes of these degradation products would probably reduce the wavelength of maximum absorption of this peak, when added in the quantities indicated by the increase in area.

The water-soluble extracts at various stages of starch depletion were fractionated by gel chromatography on a column of cross-linked dextran with an exclusion limit of 100,000 (for globular protein). The elution patterns of glycan for two extraction times of wheat and peas are shown in Fig. 7. Both wheat and peas contained a fully retarded fraction, that increased with time in wheat, but which, in peas decreased after an initial increase (compare Fig. 2). Wheat differed from peas in having an excluded glycan fraction of relatively high molecular weight, that increased up to 48 hr and then decreased. A large scale separation of the higher molecular weight water-soluble glycans was effected by ultra-filtration using a membrane with an exclusion limit of 20,000 Daltons. Significant amounts of α(1–4) (1–6) glucan were

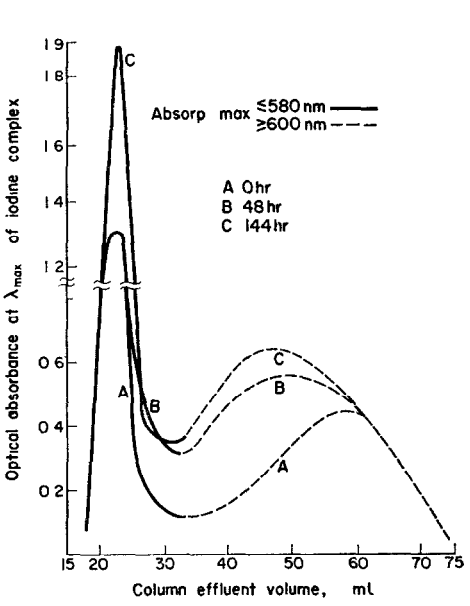

FIG. 3.

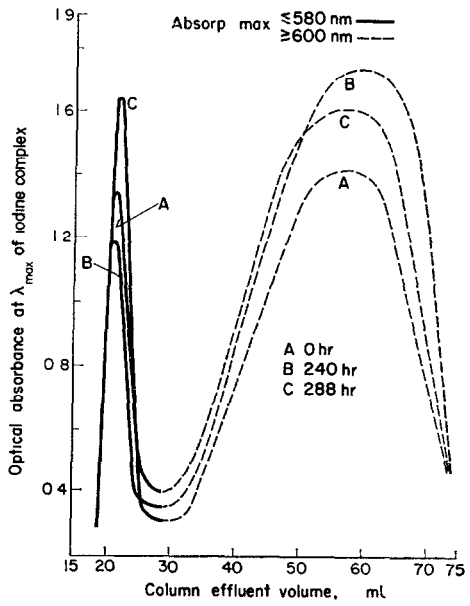

FIG. 4.

FIG. 3. GEL CHROMATOGRAPHY ON 2% AGAROSE OF STARCHES FROM GERMINATING WHEAT.

FIG. 4. GEL CHROMATOGRAPHY ON 2% AGAROSE OF STARCHES FROM GERMINATING PEAS.

only obtained from wheat extracts (Table 2). These were of high molecular weight, as TLC on silica-kieselguhr with *n*-propanol, nitromethane, water (5:2:3) as solvent[19] indicated no oligosaccharides below a DP of at least 25 and on chromatography on 10% agarose gel, the bulk of the material was eluted at or near the void volume. Amyloglucosidase hydrolysis indicated that 5·8% was α(1–4) (1–6) glucan and acidic hydrolysis that about 1/6 of the reducing sugar was glucose. Xylose and arabinose were also present. β-Amylase gave 49%

TABLE 2. RETARDED FRACTIONS FROM MEMBRANE ULTRA-FILTRATION OF WATER SOLUBLE EXTRACTS FROM GERMINATING WHEAT, PEAS AND SENESCING TOBACCO LEAVES

Sample	Wt. of fraction retarded (mg/g original dry wt.)	% α-(1–4) (1–6) glucan in fraction	Wt. of α-(1–4) (1–6) glucan (mg/g original dry wt.)
Wheat, 48 hr	147	5·8	8·5
Wheat, 144 hr	103	0·4	0·4
Peas, 48 hr	2·1	0·1	0·1
Peas, 288 hr	0·6	1·2	0·1
Tobacco, 40–20% chlorophyll	5·6	0·3	0·1

[19] C. N. HUBER, H. D. SCOBELL, H. TAI and E. E. FISCHER, *Analyt. Chem.* **40**, 207 (1968).

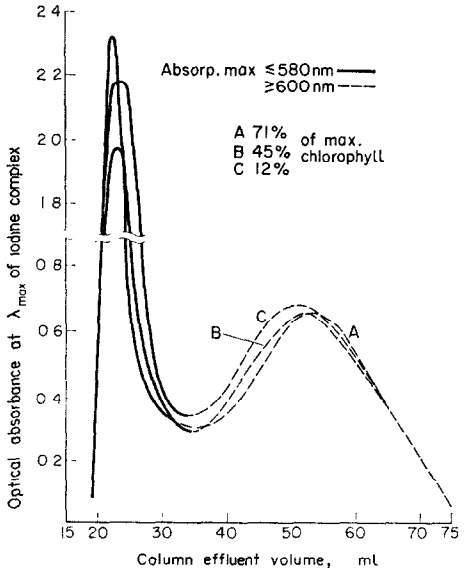

FIG. 5.

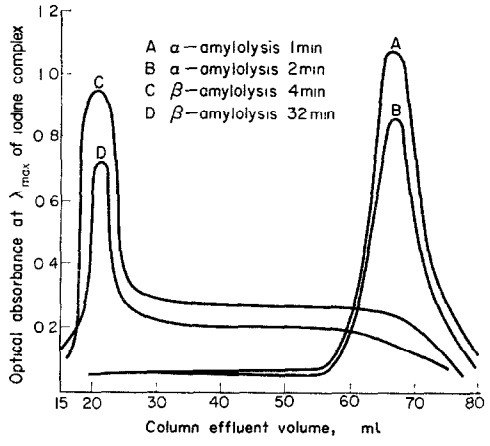

FIG. 6.

FIG. 5. GEL CHROMATOGRAPHY ON 2% AGAROSE OF STARCHES FROM SENESCING TOBACCO LEAVES.

FIG. 6. GEL CHROMATOGRAPHY ON 2% AGAROSE OF PRODUCTS FROM α- OR β-AMYLOLYSIS OF STARCH.

conversion to maltose, indicating a polysaccharide with a shorter chain length than amylopectin. Iodine gave a reddish–brown colour with a maximum at 510 nm. Products of α-amylolysis of extracted wheat starch, amylose and amylopectin gave no colour with iodine and those from β-amylolysis, absorption maxima at 585, 530 and 580 nm respectively.

The fully retarded fractions from chromatography on dextran were fractionated by gel chromatography on polyacrylamide with a fractionation range of 200–2600 for globular proteins. Wheat gave no excluded material. At 24 hr two peaks were present and both increased with time. TLC on cellulose of the retarded fractions and the whole water-soluble extracts showed similar patterns. In wheat, before germination a small amount of material that co-chromatographed with glucose and traces of maltose as well as sucrose, fructose and *myo*-inositol were the main sugars. Glucose and maltose increased with time. Maltotriose appeared at 24 hr and then increased along with smaller quantities of higher malto-oligosaccharides. The retarded fractions from peas gave no excluded fraction and a single peak on polyacrylamide gel. TLC on cellulose showed a trace of glucose, *myo*-inositol, fructose and sucrose. Glucose concentration increased with time. There was a trace of higher oligosaccharide at 0 and 24 hr which then disappeared, but at no time was there any material that co-chromatographed with maltose, maltotriose or higher malto-oligosaccharides.

Water-soluble extracts of tobacco leaves, taken when the chlorophyll contents were 45, 20, 12 and 0% showed glucose, fructose, sucrose and *myo*-inositol on chromatography but no maltose or malto-oligosaccharides.

The levels of activity of α- and β-amylases, phosphorylase and maltase were assayed during starch depletion using similar extraction and assay procedures for each plant and

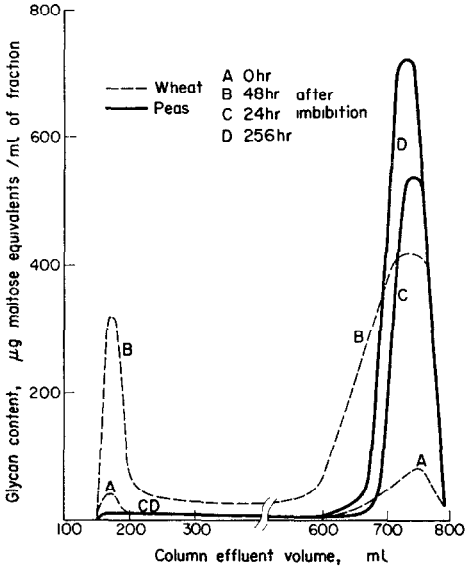

FIG. 7.

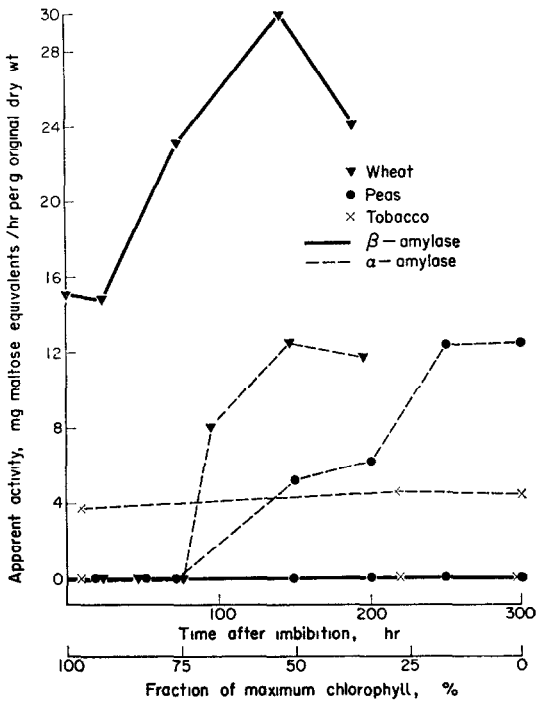

FIG. 8.

FIG. 7. CHROMATOGRAPHY OF WATER-SOLUBLE EXTRACTS FROM GERMINATING WHEAT AND PEAS ON SEPHADEX G-100.

FIG. 8. APPARENT ACTIVITIES OF α- AND β-AMYLASES IN GERMINATING WHEAT AND PEAS AND SENESCING TOBACCO LEAVES.

these are reported on the same activity per original weight basis. To avoid phenolic denaturation of tobacco leaf enzymes, the leaves were ground in liquid nitrogen and extracted with buffer mixed with insoluble polyvinylpyrrolidone and a reducing agent.[20] This procedure was applied to the other tissues and no significant change in activities were found. When tobacco leaves and pea or wheat grains were mixed and extracted, the activities were equal to the sum of the individual values.

The changes in the levels of activity of α- and β-amylases are shown in Fig. 8. Since the ascorbate used as a reducing agent in the extraction medium for tobacco leaves was a possible inhibitor of α-amylase,[2] sodium dithionite was substituted or the enzyme was twice precipitated with ammonium sulphate or dialysed, but no significant differences in readings were found. All three plant organs contained α-amylase and the maximum levels reached in peas and wheat were similar. Tobacco leaves showed a lower and constant activity from the growing to yellowing phase. The time of increase in wheat was later than has been found in barley[6] but similar to rice.[9] Only wheat had any significant β-amylase activity, and the apparent hydrolytic activity was higher than α-amylase, which is the reverse of the situation in germinating maize.[12] From 72 hr onward, wheat appeared to contain a debranching activity that was stable to the low pH used to inactivate α-amylase, as the extract developed

[20] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 243 (1966).

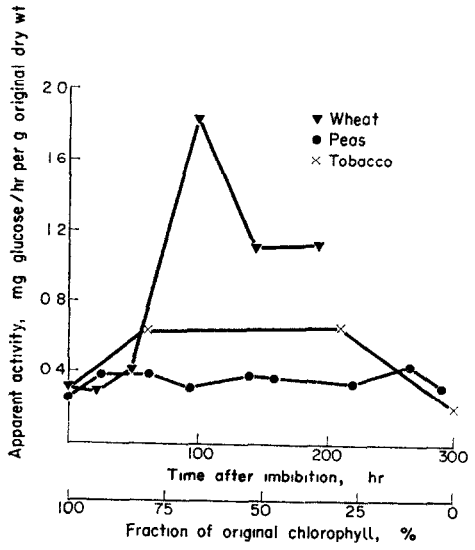

FIG. 9.

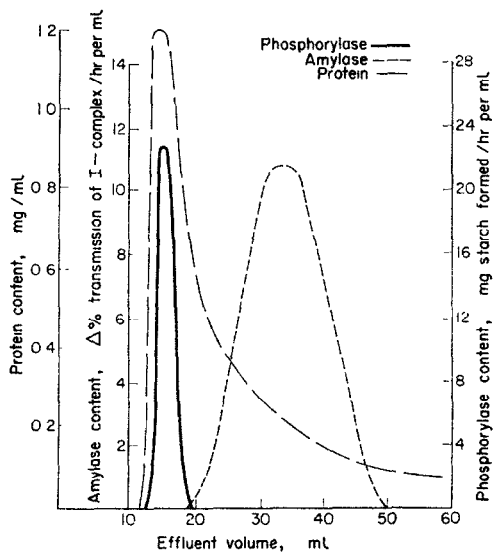

FIG. 10.

FIG. 9. APPARENT MALTASE ACTIVITIES IN GERMINATING WHEAT AND PEAS AND SENESCING TOBACCO LEAVES.

FIG. 10. CHROMATOGRAPHY OF GERMINATING PEA EXTRACT (300 hr) ON BIOGEL P-150.

the ability to hydrolyse a β-limit dextrin. This was probably not due to α-amylase as no measurable α-amylase activity appeared in wheat before 96 hr. Also, in peas no activity towards β-limit dextrin in the β-amylase extracts appeared.

The levels of maltase are shown in Fig. 9. This activity may not be specific for maltose but may have been a general glucosidase or glucose transferase. Wheat showed a rapid increase in activity at the same time as α-amylase, which is consistent with an amylolytic mechanism. Tobacco leaves gave a slight increase at the onset of senescence followed by a decrease and the values in peas remained low and constant.

As amylases interfere with the estimation of phosphorylase, the extracts, after a preliminary ammonium sulphate fractionation, were chromatographed on polyacrylamide gel. The published molecular weights indicated the possibility of their separation. With pea extracts, chromatography on a gel with a molecular weight separation range of 50,000–150,000 gave a complete separation of α-amylase and phosphorylase activities in all extracts (Fig. 10). However, with wheat, amylase appeared in high levels at the void volume and extended across the whole elution pattern. No significant phosphorylase activity could be detected. Using gel with a separation range of 100,000–400,000 still no phosphorylase could be detected. However, it is unlikely that complete separation of phosphorylase and amylase activities was achieved, as a mixture of pea phosphorylase and wheat extract gave the elution pattern in Fig. 11.

The levels of activities in fractionated pea and wheat extracts and unfractionated tobacco extracts are shown in Fig. 12. The tobacco reading plotted against 100% chlorophyll represents a sample from the expanding phase of growth. Tobacco extracts were also assayed by estimation of inorganic phosphate released, using molybdate to suppress phos-

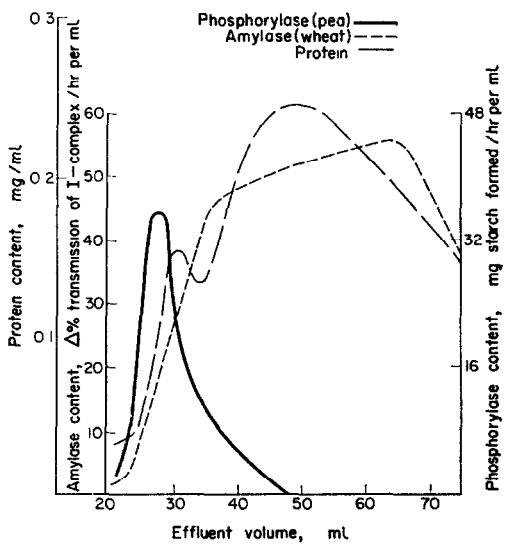

FIG. 11.

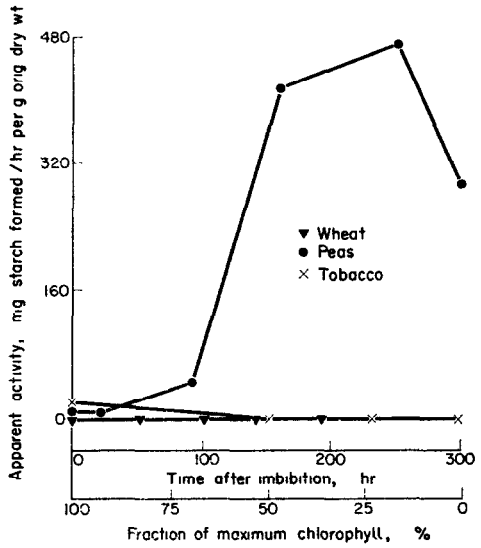

FIG. 12.

FIG. 11. CHROMATOGRAPHY OF WHEAT (0 hr) EXTRACT AND PEA PHOSPHORYLASE ON BIOGEL P-300.
FIG. 12. APPARENT PHOSPHORYLASE ACTIVITIES IN GERMINATING WHEAT AND PEAS AND SENESCING TOBACCO LEAVES.

phatase activity.[21] Expanding leaves produced 1·1 mg inorganic phosphate/hr/g of original dry wt., leaves with 51% and 23% of maximum chlorophyll gave 0·3 mg. For comparison, potato tubers had a value of 12·0 mg and this extract produced 580 mg starch/hr/g original dry wt. when assayed for starch formation.

The inorganic phosphate content of pea cotyledons, expressed as mg/g wet wt., dropped sharply after imbitition (0·55–0·12) and then rose slightly to 0·20 at 250 hr. In wheat the values were constant in the range 0·20–0·30 and in tobacco it was 0·20 in expanding leaves, rose to 0·50 in leaves with 20% chlorophyll and dropped to 0·40 when the leaves were yellow.

In comparing the three plant tissues, only wheat contained significant levels of β-amylase and it probably lacked phosphorylase which was present at least at some time in the other plants. α-Amylase was present in all, but increased with starch depletion only in wheat and peas, and the levels of activity reached in these were similar. Maltase was also detected in all, but increased only in wheat and tobacco. Malto-oligosaccharides and malto-dextrins were characteristic of wheat only. Thus wheat resembled barley[4–6] in which starch depletion involves amylolysis. The malto-dextrins isolated at 24 and 48 hr after imbibition, before significant amounts of α-amylase could be detected, were still susceptible to attack by β-amylase indicating that it may not have been the only active enzyme. The lack of accumulation of β-limit dextrin up to 72 hr, although approximately half of the original starch had been depleted, and the production of malto-oligosaccharides with a DP greater than 2 is in agreement with this. A debranching enzyme or an insoluble α-amylase may have been acting.

Wrinkled-seeded peas appeared to resemble smooth-seeded peas in their pattern, most probably with phosphorylase and α-amylase degrading starch.[14,15] However, there were

[21] D. H. TURNER and J. F. TURNER, *Austral. J. Biol. Sci.* **10**, 302 (1957).

differences. In the latter the peak of phosphorylase and α-amylase activities were well separated in time at 120 and 216 hr,[14] and α-amylase was present at imbibition. Phosphorylase showed a 3-fold increase in activity in one study[14] and 13-fold in another.[15] In the present measurements, the increase in phosphorylase was much greater and coincided with the increase in α-amylase. The maximum level of phosphorylase was similar to values found for potato tubers. There was no measurable α-amylase activity until at least 75 hr after the onset of germination, although a low but definite phosphorylase activity was present even at 0 hr, suggesting that starch is initially attacked by phosphorylase and later by the combined action of phosphorylase and α-amylase. Another possibility is that the α-amylase cannot be extracted initially. These differences may have been the result of the prior separation of α-amylase and phosphorylase by gel chromatography. As with smooth-seeded peas the rate of starch depletion in the early stages was much slower than wheat, suggesting a mo re controlled hydrolysis and utilization.

The nature of the enzymes effective in starch breakdown in tobacco leaves during senescence is less definite. The rate of breakdown of starch was comparable to that found in peas and wheat as the period of starch depletion (from 45 to 12% of maximum chlorophyll content, Fig. 1) was 150–200 hr. No evidence could be obtained that denaturation, associated with the high phenolic content of leaves, was responsible for the generally low activities. In the lack of structural changes in the residual starches and the absence of malto-oligosaccharides, tobacco leaves resembled sprouting potato tubers where phosphorylase probably degrades the starch,[17] but phosphorylase levels in tobacco were negligible during senescence. If α-amylase degraded starch in tobacco leaves it must have been accompanied by an efficient malto-oligosaccharase activity as no malto-oligosaccharides were detected.

EXPERIMENTAL

Plant material. Wheat (variety Gamut) was grown on filter paper after soaking for 6 hr in distilled water for periods of up to 3 days and in moistened perlite for longer times. Peas (variety Fullpod) were soaked for 18 hr in water and grown in perlite. Filter paper and perlite were initially moistened with water containing 9 ppm of 5,5′-dichlor 2,2′-dihydroxy diphenyl to control fungal infection. All plants were grown in the dark at 25·0°. The roots and shoots were removed before extraction or glucan preparation. With tobacco leaves (variety Hicks) samples of thirty leaves were collected from field grown plants from positions 15–18, counting from the cotyledons, at 10·00 a.m. for the isolation of starch and water soluble products, at 5 different leaf conditions; (a) when fully expanded and still green (71% of maximum chlorophyll content); (b) ripe (45% chlorophyll); (c) overripe (12% chlorophyll); and (e) yellow. Three discs, 3·8 cm in dia. were cut from each leaf to estimate chlorophyll and starch content. The remainder was used for starch isolation. For estimation of enzymic activities and inorganic phosphate contents, plants were grown in perlite. Twelve discs, 3·8 cm in dia. were cut from 4 leaves and immediately dropped into liquid N_2. Another 12 discs 1·3 cm in dia. were dropped into EtOH for estimation of chlorophyll content and dry wt. Samples were taken when the leaves had lost approximately 1/4, 3/4, and all of the maximum chlorophyll content. For inorganic phosphate estimation 8 discs 3·8 cm in dia. were dropped into liquid N_2, and another 8 discs 3·8 cm in dia. were dried with paper tissues and weighed to estimate wet wt.

Starch content. Measured by the method of Pucher *et al.*[22]

Isolation of starch granules and determination of chemical properties. Previously described methods[18,23] for the extraction, isopotential iodine absorption, β-amylolysis limits, chain length of amylopectin, intrinsic viscosities and agarose chromatography[24] were used.

Preparation and fractionation of water-soluble extracts of malto-dextrins and malto-oligosaccharides. Plant material (2500 wheat grains, 200 pea seeds or 1500 cm^2 of tobacco leaves) was macerated in 0·2 M $HgCl_2$ and centrifuged. A portion was deionized on a column of ion retardation resin (Bio-Rad AG11A8) and concentrated by freeze-drying. After dissolving in water a small quantity was examined by TLC on binder

[22] G. W. PUCHER, C. S. LEAVENWORTH, H. B. VICKERY, *Analyt. Chem.* **20**, 850 (1948).
[23] N. K. MATHESON and J. M. WHEATLEY, *Austral. J. Biol. Sci.* **15**, 312 (1962).
[24] N. K. MATHESON, *Phytochem.* in press (1972).

free cellulose (Machery MN 300) using *n*-BuOH–pyridine–benzene–H_2O (5:3:1:3) in 4 ascents as solvent. Sugars were detected by dipping in $AgNO_3$ in acetone followed by ethanolic KOH. The remainder of this portion of the wheat and pea extracts was chromatographed on cross-linked dextran (Pharmacia Sephadex G-100) and the glycan content of fractions estimated with anthrone. Fractions were concentrated by freeze-drying. The retarded fractions were chromatographed on polyacrylamide (Biogel P-2). The excluded fraction from Sephadex chromatography of the wheat extract was then fractionated on 10% agarose (Bio-Rad).

The main fraction from the extract was deionized (Dowex AG1 and AG50W) and filtered through a 3·0 μ membrane filter. The eluate was concentrated by ultra-filtration at 45 lb/in^2 using a Diaflo membrane (UM-20E) with a nominal cut-off in M.W. of 20,000. The concentrated solution was freed of low molecular weight material by passage of another 6 vol. H_2O. After centrifugation (14,000 *g*, 10 min) the supernatant was freeze-dried.

α(1–4) (1–6) *Glucan content of the water-soluble dextrins.* Amyloglucosidase (0·2 ml) (Sigma) 0·2% in acetate buffer (0·2 M pH 4·5), acetate buffer and dextrin at a final concentration of 1·5 mg/10 ml in a final vol. of 10 ml was incubated at 37° for 45 min. Glucose produced was estimated by the Nelson–Somogyi method.[25]

Preparation of extracts for enzyme assay and phosphate content and estimation of inorganic phosphate. Tobacco leaves were ground in liquid N_2 and a slurry of high molecular weight insoluble polyvinylpyrrolidone (1·0 g/g fresh wt.) in the required extraction solution which also contained 0·25 M ascorbate was added. Other tissues were macerated directly. The suspension was centrifuged (29,000 *g*, 30 min) and the supernatant used for assay or further purification. To estimate inorganic phosphate, trichloroacetic acid to a final concentration of 3–4% was added to the supernatant from extraction with cold 0·5 M HCl. After filtration the solution was made to volume for estimation of phosphate by a modification of the method of Allen.[26]

Estimation of β-amylase activity. The supernatant from extraction in 0·5% NaCl was adjusted to a pH of 3·3 with HOAc. After 15 min at room temp., the pH was raised to 4·5 with NH_4OH and an equal vol. of satd. $(NH_4)_2SO_4$ added and the solution kept at 4° for 1 hr. After centrifuging (29,000 *g*, 20 min) the precipitate was re-dissolved (1·0 ml for each 0·04 g original dry wt. of plant material) and dialysed against acetate buffer (0·2 M, pH 4·5) at 4° for 24 hr, centrifuged and the supernatant used. An aliquot (0·4 ml) was incubated at 30° with 0·05% starch solution (1·0 ml) in acetate buffer (5·0 ml) (0·2 M pH 4·5). Reducing sugar produced was estimated by the Nelson–Somogyi method. Starch breakdown was also followed by the decrease in the absorption at 625 nm of the starch iodine complex after the addition of 0·002% iodine solution (5·0 ml) to an aliquot (1·0 ml). With tobacco samples 0·2% I_2 was added to the blank until the colour persisted. The same volume was added to the sample followed by 0·002% I_2 solution.

Estimation of α-amylase activity. $CaCl_2$ (0·2 M to a final concentration of 0·02 M) was added to the supernatant from extraction with 0·5% NaCl solution. The pH was adjusted to 7·0 with NaOH and the solution maintained at 70° for 15 min. After cooling, an equal vol. of satd $(NH_4)_2SO_4$ was added and the solution kept at 4° for 1 hr. Precipitated protein was collected by centrifuging (29,000 *g*, 20 min), re-dissolved (1·0 ml for each 0·04 g original dry wt.) and dialysed against acetate buffer (0·2 M, pH 4·5) containing 0·5% $CaCl_2$ at 4° for 24 hr. The supernatant after centrifuging (4·0 ml) was incubated at 30° with 0·5% β-limit dextrin from potato starch[27] (1·0 ml) and acetate buffer (5·0 ml). Reducing sugar produced was estimated in the same way as described for β-amylase.

Estimation of phosphorylase activity. The supernatant from extraction at 4° with citrate buffer (0·05 M, pH 6·0) was brought to 20% saturation with $(NH_4)_2SO_4$. After centrifuging (29,000 *g*, 20 min), the supernatant was brought to 50% saturation with $(NH_4)_2SO_4$. After 30 min at 4° the precipitate was dissolved in citrate buffer (0·05 M, pH 6·0) (1·0 ml for each 5·0 g original dry wt.). Pea phosphorylase was separated from α-amylase by chromatography on polyacrylamide (Biogel P–150, 50–100 mesh). Aliquots (1·0 ml) were applied to a column 1·4 × 50 cm and eluted with citrate buffer. Wheat extracts were chromatographed on Biogel P–150 and P–300. Protein content of fractions was estimated by the Folin–Lowry method. To estimate phosphorylase activity, extract (1·0 ml), 1% maltose soln (0·5 ml) and α-D-glucose 1-phosphate (0·2 M, 0·5 ml) were incubated at 30°. Iodine (0·002%) was added and the optical density at 660 nm was determined. Starch calibration curves were prepared with each estimation. With extracts of tobacco leaves, 0·2% I_2 was added to the blank until the colour persisted. The same vol. was added to the sample followed by 0·002% I_2. The activity of tobacco extracts was also estimated by inorganic phosphate release. Ammonium molybdate to a final concentration of 10^{-3} M was added to inhibit phosphatase.[21] Inorganic phosphate was estimated by a modification of the Allen method.[26]

Estimation of maltase activity. An equal vol. of satd $(NH_4)_2SO_4$ was added to the supernatant from extraction at 4° with maleate buffer (0·2 M, pH 6·0). After 30 min at 4°, precipitated protein was collected by centrifugation (29,000 *g*, 30 min) and re-dissolved in maleate buffer (1·0 ml for 2·0 g original dry wt. of plant material). Extract (0·2 ml) and 0·05% maltose solution (0·2 ml) were incubated at 37°. After heating,

[25] N. Nelson, *J. Biol. Chem.* **153**, 375 (1944)
[26] J. F. Turner, *Biochem. J.* **67**, 450 (1957).
[27] Y. Shain and R. M. Mayer, *Physiol. Plantarum.* **21**, 765 (1968).

denatured protein was removed by centrifuging (29,000 *g*, 15 min). Liberated D-glucose was estimated:[28] digest (0·1 ml) was incubated at 37° with glucose oxidase, peroxidase and *o*-anisidine in Tris buffer (3·4 ml). After 1 hr the colour was read at 420 nm. Glucose calibration curves were prepared with each assay.

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[28] A. DAHLQUIST, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. VIII, p. 584, Academic Press, New York (1966).

Key Word Index—Triticum vulgare; Gramineae; *Pisum sativum*; Leguminosae; *Nicotianum tabacum*; Solanaceae; starch; amylose; germination.