SUCROSE STARCH INTERCONVERSIONS IN THE POTATO TUBER

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in

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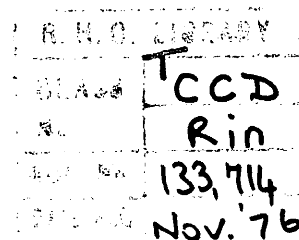
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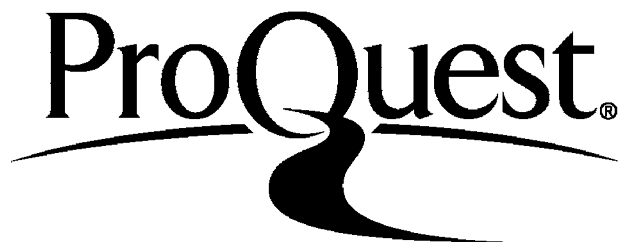

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ABSTRACT

The storage carbohydrate in the potato tuber is starch and it is biosynthesised from translocated sucrose and deposited as starch grains within tuber amyloplasts.

The biosynthetic pathway from sucrose to starch in the potato tuber has been investigated. Young developing tubers have been injected with both [U-^{14}C]sucrose and [U-^{14}C]fructose and the metabolites arising from these labelled compounds have been examined: differences in the distribution of label between the various metabolites were recorded. These results support the theory that sucrose synthetase, and not invertase, is involved in the initial reaction leading to the conversion of sucrose to starch and that ADP-glucose is synthesised preferentially from fructose via hexose phosphate intermediates.

The activities and properties of some of the enzymes believed to be involved in the synthesis of starch from sucrose have been examined. These include sucrose synthetase, ADP-glucose- and UDP-glucose-pyrophosphorylase. The latter enzyme has been shown to occur in multiple forms in the tuber. Starch synthetase activities, associated with starch grains, have also been studied and in particular, the inhibitory effect of nucleotides on starch synthesis. Activation of α-glucan synthesis from ADP-glucose was demonstrated in the presence of both pyrophosphate and inorganic phosphate and the reverse was shown to be true in the case of glucan synthesis from UDP-glucose.

Preliminary experiments were carried out in an attempt to synthesise starch from [^{14}C]fructose and [^{14}C]sucrose using coupled enzyme systems derived from the potato tuber.

The regulation of the metabolic pathway from sucrose to starch which is believed to operate in the potato tuber is discussed.

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ABBREVIATIONS

The abbreviations and symbols used in this thesis are as suggested by the Biochemical Journal (270), except for the following:-

BSA	bovine serum albumin
CL	mean chain length
pCMB	para chloromercuribenzoate
DIECA	diethyl dithiocarbamate
DP	degree of polymerization
E.C.	enzyme commission
EMP	Embden-Meyerhof-Parnas
F1-P	fructose1-phosphate
F1,6-diP	fructose 1,6-diphosphate
F6-P	fructose 6-phosphate
Fru	fructose
G^{o}	free energy of hydrolysis
G1-P	glucose 1-phosphate
G6-P	glucose 6-phosphate
Glu	glucose
HMP	hexose monophosphate
IM	isomaltose
M	maltose
MW	molecular weight
NDP	nucleoside diphosphate
NTP	nucleoside triphosphate
PA	pyruvic acid
PEP	phosphoenolpyruvate
PGA	phosphoglyceric acid
PPO	2,5-diphenyloxazole
S	sucrose
[S]	substrate concentration
⊕Ve	anode
⊖Ve	cathode
Vmax	maximum velocity

I N T R O D U C T I O N

Storage carbohydrates found in plant tissues can either be low molecular weight, water soluble compounds or high molecular weight polymers which are either water soluble or relatively insoluble. The major representatives of these classes of carbohydrates are, undoubtedly, sucrose, amylopectin and amylose, respectively. In some tissues, e.g. sugar cane stems, the storage carbohydrate is virtually all sucrose, whereas in most seeds or tubers amylose and amylopectin predominate.

The tuber of the potato (Solanum tuberosum) is a well known example of a starch-storing organ and the formation of this glucan is intimately connected with sucrose metabolism. This thesis is a report of an investigation of the synthesis of starch from sucrose in the developing potato tuber and the possible regulation of this process.

I. The occurrence and importance of nucleoside diphosphate sugars in plant carbohydrate metabolism.

The importance of nucleoside diphosphate sugars in carbohydrate metabolism is well established; they play a fundamental role in the interconversion and polymerization of monosaccharides (1).

Leloir and his group (2,3) were the first to discover UDP-glucose (Fig. 1a) and since that time many other nucleoside diphosphate sugars, including ADP-glucose (Fig. 1b), with different bases and different sugar moieties have been isolated from both plant and animal tissues (1,4-6).

Nucleoside diphosphate sugars have a high negative free energy of hydrolysis (ΔG^o). Therefore from a thermodynamic view point, they are superior monosaccharide donors for glycoside formation in comparison to other monosaccharide donors such as sugar phosphates (6).

The role of sugar nucleotides in the biosynthesis of various classes of oligo- and poly-saccharides appears to be quite well defined. For example, in plants UDP-glucose is involved in sucrose (7) and cellulose biosynthesis (8), ADP-glucose in chloroplastic starch synthesis (9), and UDP-galacturonic acid in the biosynthesis of pectins (10).

One reaction for the formation of nucleoside diphosphate sugars in plants involves the transfer of a nucleotidyl group from a nucleoside triphosphate to a glycosyl phosphate with the simultaneous release of PPi (see also sucrose synthetase, p. 22). The reaction is

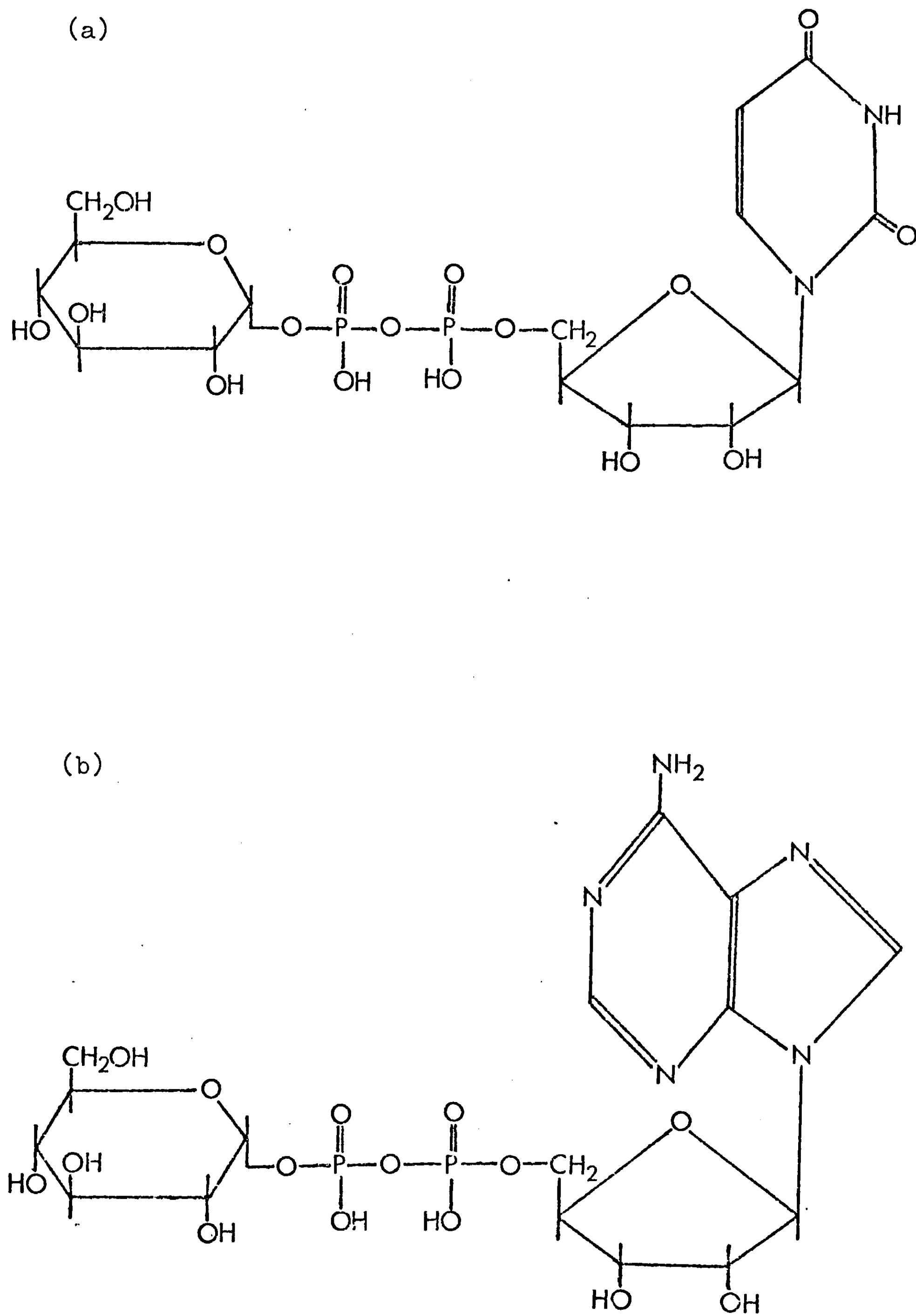


Fig. 1. (a), UDP-glucose; (b), ADP-glucose.

freely reversible and catalysed by nucleoside diphosphate sugar pyrophosphorylases (see pp. 12 and 15).

A. Hexose metabolism.

Glucose 1-phosphate, the hexose donor for the synthesis of both ADP-glucose and UDP-glucose by the pyrophosphorylase reaction, can be formed from either free glucose via glucose 6-phosphate, or from free fructose via the intermediates fructose 6-phosphate and glucose 6-phosphate (11-13). The initial reaction in both cases is a phosphorylation of the free hexose moiety (Fig. 2).

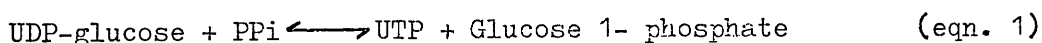
Both fructose and glucose have been identified as common plant constituents (14-16, 175). De Fekete (11) has demonstrated that in developing cotyledons of Vicia faba there is sufficient hexokinase, phosphoglucose isomerase and phosphoglucomutase to account for the conversion of all the fructose (present as sucrose) into glucose 1-phosphate. Isherwood (17) also proposes this mechanism for the conversion of the fructose moiety of sucrose to glucose 1-phosphate in the potato tuber after hydrolysis of sucrose by invertase.

Fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate have all been identified as constituents of many plant tissues, including the potato tuber (18,19). The compounds may be derived from the corresponding free hexoses and also from the degradation of starch and other hexose derivatives.

The formation of nucleoside diphosphate sugars from fructose and glucose is, of course, only one fate of these hexoses. Glucose 6-phosphate may be catabolized by the EMP or the pentose phosphate pathway (hexose monophosphate shunt). Fructose 1-phosphate has been only tentatively identified in extracts from potato tubers and some other plants (20-22). The origin of this ester is not known: one would assume that it is derived from fructose via a specific ketohexokinase (as in animals (23)) or perhaps by the hydrolysis of fructose 1,6-diphosphate.

B. UDP-glucose pyrophosphorylase.

The pyrophosphorolysis of UDP-glucose (eqn. 1) was first reported by Munch-Peterson et al. (24) in 1953 using a Zwischen ferment preparation from yeast.



UDP-glucose + PPi $\rightleftharpoons$ UTP + Glucose 1- phosphate (eqn. 1)

The enzyme, UDP-glucose pyrophosphorylase (E.C.2.7.7.9), was later shown to occur in sugar beet homogenates (25) and since then has

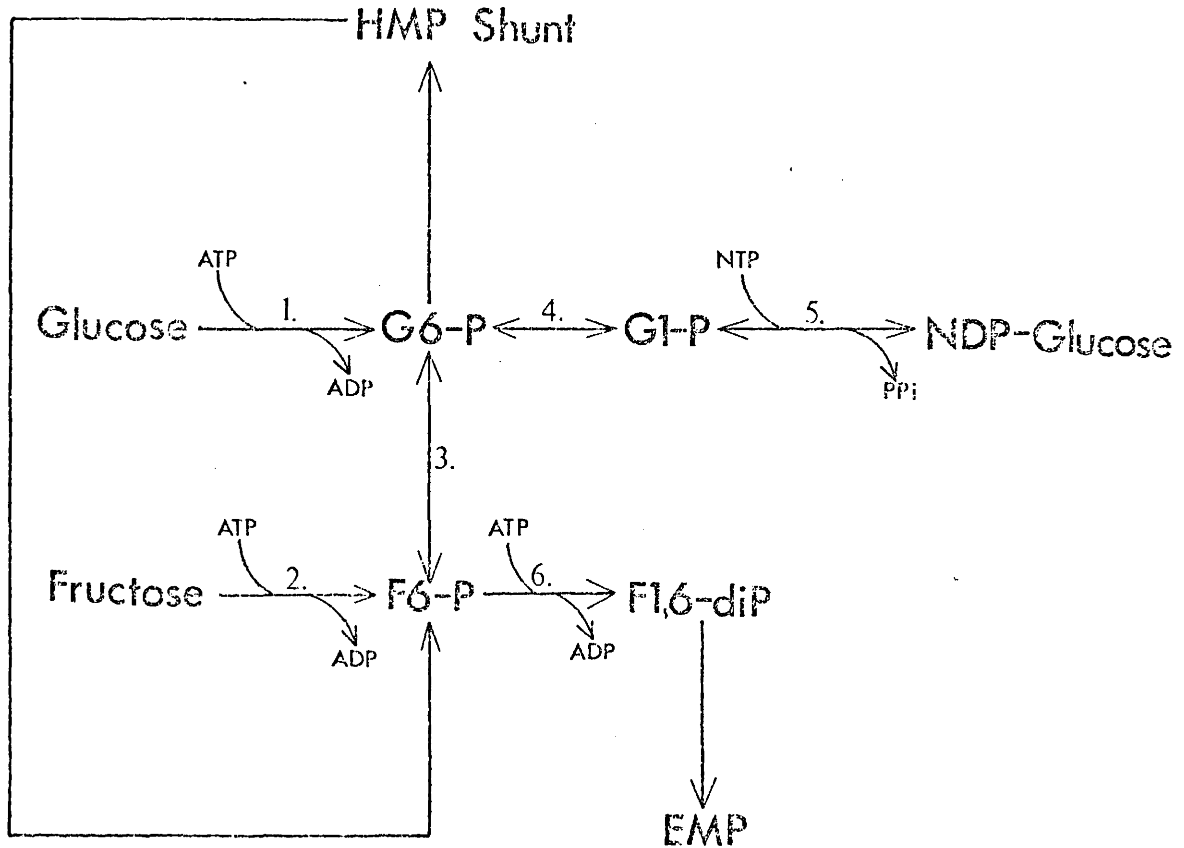


Fig. 2. Schematic representation of glucose and fructose metabolism. Numbers refer to the enzyme that catalyses each particular reaction:

1. and 2., Hexokinase
3. Phosphoglucose isomerase
4. Phosphoglucomutase
5. Nucleoside diphosphate glucose pyrophosphorylase
6. Phosphofructokinase

been identified in many other plant species (26-28) including the potato (27,30). Hopper and Dickinson (29) have commented on the widespread occurrence of the enzyme in the plant kingdom and its abundance in rapidly growing plant cells and also in dividing cells. This presumably reflects the central role of UDP-glucose in plant carbohydrate metabolism.

The enzyme was first purified from seedlings of Phaseolus vulgaris (28); it has a pH optimumof 8.0 and the Km values for UDP-glucose and PPi are 1.1×10^{-4}M and 2.3×10^{-4}M, respectively. The enzyme has an absolute specificity for UDP-glucose. The UDP-glucose pyrophosphorylase prepared from tubers and leaves of the potato by Yuji and Mitsuo (30) showed a pH optimumof 9.5. The tuber enzyme had a higher specific activity than that derived from the leaves. Bird et al. (31) claim that the enzyme is exclusively chloroplastic in Nicotiana tabacum, these results have been confirmed by Nomura et al. using bean leaves (32).

Turner and Turner (33) were the first to suggest that UDP-glucose might have a major role in plant tissues where pronounced changes in sucrose and starch levels occurred. For example, there is a high level of UDP-glucose pyrophosphorylase in developing pea and wheat seeds during the period of rapid transformation of sucrose into starch (34-36). In extracts of germinating peas there is also a high level of the enzyme which can be related to the mobilization of starch and its conversion to sucrose which occurs during germination (33,37,38). Similar views on the role of UDP-glucose pyrophosphorylase in sucrose metabolism are held by several other authors (11,12,39-43).

Delmer and Albersheim (39) using Phaseolus aureus seedlings have shown that UDP-glucose pyrophosphorylase is more active in etiolated seedlings than in photosynthetic tissues. The decrease in pyrophosphorylase activity when etiolated seedlings are placed in the light parallels that of sucrose synthetase (see p. 22) and they conclude that sucrose synthetase and UDP-glucose pyrophosphorylase play a major role in the metabolism of sucrose. Several workers (11,12,40-42) have suggested that during the conversion of sucrose to starch in cereals and potato tubers, UDP-glucose is an intermediary in the formation of glucose 1-phosphate from translocated sucrose.

It is thought that most of the sucrose in plant tissues is formed from UDP-glucose and fructose 6-phosphate (see p. 19), therefore

UDP-glucose pyrophosphorylase also provides the substrate for the synthesis of the disaccharide. Tovey and Roberts (43) have shown that UDP-glucose pyrophosphorylase activity of wheat endosperm tissue increases during germination. They suggest that here UDP-glucose is an intermediate in the mobilization of starch.

Therefore, there is evidence that UDP-glucose pyrophosphorylase catalyses the pyrophosphorolysis of UDP-glucose in tissues synthesising starch from sucrose and that it is also involved in the synthesis of this nucleoside diphosphate sugar during the process of germination.

The rates of reaction catalysed by UDP-glucose pyrophosphorylase may be controlled by various factors. Gustafson and Gander (44) for example, using a highly purified preparation from Sorghum vulgare, have shown that the direction of the reaction is dependent on the Mg^{2+} concentration. Free Mg^{2+} ions were found to activate UDP-glucose synthesis by forming a complex with the enzyme: this is an additional role to the formation of the 'real' pyrophosphorylase substrate, $MgUTP^{2-}$. In contrast, Mg^{2+} had no effect on the rate of pyrophosphorolysis of UDP-glucose beyond its role in the formation of $MgPPi^{2-}$. The physiological significance of these facts may be that the direction of the reaction in vivo is controlled by the Mg^{2+} concentration.

UDP-glucose pyrophosphorylase activity has also been shown to be subject to regulation by nucleoside diphosphate sugars (29,45). Hopper and Dickinson (29) have demonstrated feedback inhibition of the enzyme from Lilium longiflorum pollen by precursors of cell wall polysaccharides (e.g. UDP-glucuronic acid). Gander (45) has shown an activation of the sorghum enzyme by ADP-glucose at low PPi concentrations. These regulatory mechanisms are of interest when the central role of UDP-glucose in the formation of starch and cell wall polysaccharides is considered, since they probably control the differential rates of synthesis of these two types of polysaccharide.

C. ADP-glucose pyrophosphorylase.

ADP-glucose (Fig. 1b) also occurs naturally in plants: it has been found, for example, in Chlorella pyrenoidosa (46), sweet corn, rice and potato starch grains (47-50). ADP-glucose pyrophosphorylase activity (E.C.2.7.7.b) has been detected in the leaves of a variety of plant species (51) including the potato (30).

The enzyme catalyses the reaction:-

ATP + glucose 1-phosphate $\longleftrightarrow$ ADP-glucose + PPi (eqn. 2)

and shows an absolute specificity for its substrates.

Preiss et al. (52) and Kennedy and Isherwood (53) have shown that the enzyme is present in the potato tuber. Earlier reports (30,54) that the enzyme was absent from the potato tuber were probably due to the presence of contaminating phenolic compounds in the soluble protein preparations. ADP-glucose pyrophosphorylase is very sensitive to inhibition by phenolics (7) but the enzyme has been identified in starch-storing tissues from other plant species (36,43,52,55,56).

Ghosh and Preiss (52,57,58) have provided strong evidence for the involvement of the pyrophosphorylase in the regulation of starch synthesis in spinach chloroplasts. They purified the enzyme and studied the effects of photosynthetic intermediates on its activity. 3-PGA, fructose 6-phosphate, fructose 1,6-diphosphate, PEP, 2,3-diphosphoglycerate and ribose 5-phosphate all activated the enzyme. 3-PGA was the most effective activator. Inhibition of the enzyme by Pi and ADP was demonstrated. Kinetic studies with the spinach enzyme suggest that it is allosteric, with 3-PGA, PEP and fructose 1,6-diphosphate sharing a common binding site which is remote from the active site. The activation of the enzyme by these products presumably results in an increased level of ADP-glucose and thus an increase in the synthesis of starch in the chloroplasts during photosynthesis. In the dark, photosynthesis ceases and the concentrations of ADP and Pi rise and the concentrations of 3-PGA and fructose 6-phosphate fall, hence ADP-glucose pyrophosphorylase becomes less active and starch synthesis ceases.

Delmer and Albersheim (39) have shown that in etiolated mung bean seedlings the ADP-glucose pyrophosphorylase activity is low and on exposure of the plants to light the enzyme activity increases. This presumably reflects the role of the enzyme in starch synthesis during photosynthesis. ADP-glucose pyrophosphorylases isolated from different plant sources by Sanwal et al. (51) also show allosteric properties in the presence of photosynthetic intermediates and they suggest a general role for the enzyme in the control of starch biosynthesis in chloroplasts.

The enzyme isolated from potato tubers by Preiss et al. (52) was activated by 3-PGA and to a lesser extent by PEP, fructose 6-phosphate, 2-PGA and fructose 1,6-diphosphate. Kennedy and Isherwood (53) have also

demonstrated activation of the enzyme by 3-PGA and PEP but reported that fructose 6-phosphate had a negligible effect. The exact physiological significance of this activation by the products of photosynthesis in a non-photosynthetic organ is not obvious. Tsai _et al._ (12,59) do not believe that ADP-glucose pyrophosphorylase plays a major part in the regulation of starch synthesis in non-photosynthetic tissues, since in developing maize endosperm, the enzyme requires 2mM 3-PGA for half-maximal activation whereas the chloroplast enzyme shows half-maximal activation in the presence of 0.4mM 3-PGA.

The role of ADP-glucose pyrophosphorylase in the regulation of starch synthesis in non-photosynthetic tissues may be in doubt but the importance of the enzyme in the conversion of sucrose to starch is well known. Tsai and Nelson (12) were unable to show the presence of the enzyme in germinating maize endosperm and Tovey and Roberts (43) showed that during the first 4 days of wheat germination the enzyme activity decreased. Moore and Turner (36) have shown that in developing wheat germ there is a rapid synthesis of starch and a high level of ADP-glucose pyrophosphorylase but in mature grains, where the conversion of sucrose to starch has ceased, the enzyme activity is low.

Several workers have commented on the correlation between the levels of ADP-glucose pyrophosphorylase activity and the starch contents of tissues (56,60-62). Tsai and Nelson (63) have shown that in the maize mutant _shrunken_-2(sh-2), the starch content is greatly reduced (25% of normal) and that there is a higher sucrose content than in other phenotypes. The maize mutant _brittle_-2 also accumulates sucrose and both mutants lack ADP-glucose pyrophosphorylase activity.

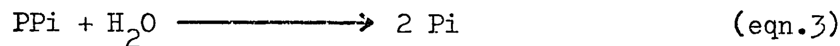
Amir and Cherry (55) have demonstrated that the ADP-glucose pyrophosphorylase of sweet corn is competitively inhibited by PPi and non-competitively inhibited by Pi. They suggest that PPi could control the sugar nucleotide level in the cell by inhibiting the enzyme. Injection of PPi into sweet corn at the time of harvest will stop the post-harvest conversion of sucrose to starch (64); the maintenance of the sucrose level is attributed to the end product inhibition by PPi of ADP-glucose synthesis.

Dickinson and Preiss (65), using a purified ADP-glucose pyrophosphorylase preparation from maize, showed a considerable activation by Mg^{2+} ions when the ratio of Mg^{2+} to ATP exceeded unity: i.e. activation

is caused by free Mg^{2+} (66). The physiological importance of this Mg^{2+} effect is not clear but they (65,66) suggest that a change in the Mg^{2+} concentration in the endosperm cells could affect the rate of starch synthesis.

D. Inorganic pyrophosphatase.

Inorganic pyrophosphatase (pyrophosphate phosphohydrolase, E.C.3.6.1.1) is abundant in plant tissues where it catalyses the reaction:-



$$PPi + H_2O \longrightarrow 2\ Pi \qquad \text{(eqn.3)}$$

The forward reaction is strongly favoured (67) and Mg^{2+} is required (68). The probable substrate for the enzyme is the complex ion, $MgP_2O_7^{2-}$.

PPi is produced in many enzymic reactions and although most are exergonic, the free energy changes are small and hence, they are freely reversible. However, by coupling such reactions with inorganic pyrophosphatase and removing the PPi product, the overall equilibrium can be shifted. An example is the removal of PPi from the reaction catalysed by pyruvate, inorganic phosphate dikinase, a chloroplastic enzyme (69). Bucke (70) has shown that the sugar cane chloroplast pyrophosphatase is Mg^{2+} dependent, and requires a Mg^{2+}:PPi ratio of 4:1. The enzyme also shows a change in pH optima when the Mg^{2+} concentration changes. Bucke has suggested that the fluxes of stromal Mg^{2+} levels in the chloroplast could result in an inactivation of inorganic pyrophosphatase in the dark and an activation in the light. This would result in a low PPi level in photosynthesising plants which in turn would increase the activity of pyruvate, inorganic phosphate dikinase thus bringing about an increase in the regeneration of PEP needed for CO_2 fixation.

A similar situation may arise in the case of the nucleoside diphosphate hexose pyrophosphorylase-catalysed reactions. The reactions are normally freely reversible but if pyrophosphatase is present, PPi would be removed and the equilibrium would favour sugar nucleotide synthesis. Conversely during periods of low pyrophosphatase activity the PPi concentration would remain high and the synthesis of glucose 1-phosphate from the nucleoside diphosphate sugars would be favoured.

II. Sucrose metabolism.

Sucrose is a disaccharide composed of α-D-glucopyranose and β-D-fructofuranose joined by a 1⟶2 link involving the anomeric carbons of both hexose units (Fig. 3). The conformation of the fructosyl group confers a relatively high free energy of hydrolysis (ΔG^{o}-6.6kcal/mol) on the molecule (71).

Sucrose is ubiquitous in green plants, it is presumed to be the first unsubstituted carbohydrate produced by photosynthesis and the major derivative used by the plant for the translocation of carbon from photosynthetic tissues to the rest of the organism (71,72). Arnold (73) has suggested that sucrose is a comparatively unreactive molecule which is most suitable for translocation. The rate of sucrose translocation through the sieve elements of the potato stem has been calculated to be 50cm/h and Badenhuizen and Dutton (74) have demonstrated, using whole potato plants, that the disaccharide is translocated down the stem and along the stolon and enters the tuber spreading evenly through the parenchyma. The conversion of sucrose to starch takes place in the stroma of the tuber amyloplast and the starch grains are built up by a gradual process of apposition (75).

The fact that sucrose is a precursor of starch in the potato tuber is well known (17,74,76,77). Burton (78) has suggested that the high level of sucrose associated with immature tubers is due to the rate of sucrose translocation from the haulm exceeding its rate of conversion to starch.

There are at least four enzymes which are believed to be directly involved with sucrose metabolism in the potato tuber, as well as many other plant tissues. Sucrose phosphate synthetase and sucrose phosphatase are thought to be involved in the synthesis of the disaccharide whereas sucrose synthetase and invertase are believed to degrade it.

A. Sucrose phosphate synthetase.

Since the discovery of sucrose phosphate synthetase (UDP-glucose : D-fructose 6-phosphate 2-glucosyl-transferase [E.C.2.4.1.14] by Leloir and Cardini (79) in 1955, much work has been done to study the physiological role of the enzyme in sucrose metabolism. Present evidence strongly indicates that sucrose phosphate synthetase and the corresponding phosphatase (see p. 22) are the major enzymes involved

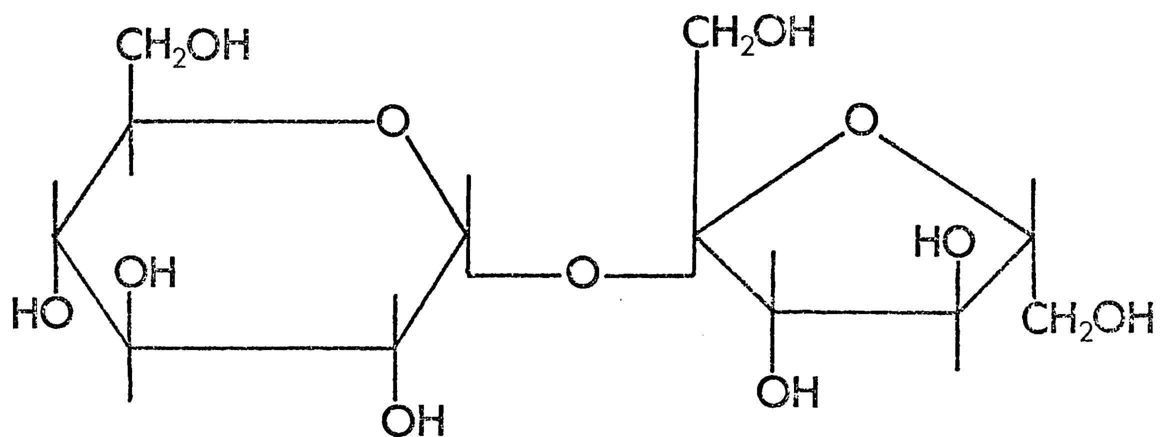
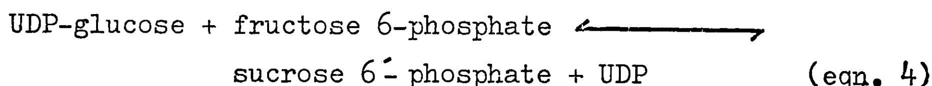


Fig. 3. Sucrose.

in the biosynthesis of sucrose in photosynthetic tissue (39,80-82) and germinating seeds (83).

The enzyme catalyses the reaction:-



UDP-glucose + fructose 6-phosphate ⇌ sucrose 6'-phosphate + UDP (eqn. 4)

The equilibrium constant for the reaction is 3250 (38°C, pH 7.5) and the enzyme is specific for UDP-glucose and fructose 6-phosphate (80).

Sucrose phosphate synthetase was first found in potato tubers by Schwimmer and Rorem (84) and since then several workers have investigated the properties of the enzyme (42,72,85). Pressey (42,72) has found low levels of the enzyme in young tubers, but the activity gradually increases during tuber maturation and also cold storage. In developing tubers sucrose synthetase activity is much higher than that of sucrose phosphate synthetase, but in stored tubers the situation is reversed. Pressey (72) believes that sucrose phosphate synthetase and sucrose phosphatase are both responsible for the formation of sucrose during the cold storage of potatoes.

Slabnik *et al*. (85) using a partially purified sucrose phosphate synthetase from potato tubers has shown that the enzyme is specific for UDP-glucose. The pH optimum is between 6.2 and 7.5 and the Km values for UDP-glucose and fructose 6-phosphate are 7.4mM and 5.5mM, respectively.

Hawker (83) has demonstrated the presence of high levels of sucrose phosphate synthetase and sucrose phosphatase in maize scutella, castor bean endosperm and broad bean cotyledons during seed germination. However, during the development of these tissues the levels of these enzymes are low.

Sucrose phosphate synthetase from both wheat germ and *Vicia faba* cotyledons has been shown to have allosteric properties (86,87). Preiss and Greenberg (86) showed that the wheat germ enzyme is regulated by the concentration of substrates, UDP-glucose and fructose 6-phosphate, and also by the Mg^{2+} concentration.

De Fekete (87) has purified the sucrose phosphate synthetase from *Vicia faba* cotyledons. The enzyme preparation from this source contains an activator which can be removed by freezing and thawing. In the absence of this material the enzyme has little activity but it

can be stimulated by citrate, some dicarboxylic acids and protamine. The saturation curves for these effectors are sigmoidal. Nucleotides and Pi stimulate the enzyme in the absence of the activator but inhibit the activator-bound enzyme. The nature and physiological significance of the naturally occurring activator is not known.

B. Sucrose phosphatase.

This enzyme (sucrose 6′-phosphate phosphohydrolase) catalyses the irreversible hydrolysis of sucrose 6′-phosphate to sucrose. This means that the pathway for the synthesis of sucrose via sucrose 6′-phosphate is not reversible unlike the synthesis involving sucrose synthetase (see p. 22).

The presence of the phosphatase in the potato tuber was inferred from a study of the synthesis of sucrose from fructose 6-phosphate and UDP-glucose. It was believed that sucrose 6′-phosphate was the intermediary which was hydrolysed immediately by the phosphatase (11,72,84,85). However, in these experiments it is possible that fructose 6-phosphate was hydrolysed to give fructose which in turn reacted with UDP-glucose in the presence of contaminating sucrose synthetase to give sucrose.

Sucrose 6′-phosphatase from sugar cane has been examined by Hawker and Hatch (81,88) who have found that the enzyme requires Mg^{2+} for activity. The enzyme is inhibited by Ca^{2+}, Mn^{2+}, Pi, PPi and sucrose. They suggest that inhibition by sucrose may be a controlling factor in the rate of the synthesis of the disaccharide.

C. Sucrose synthetase.

Sucrose synthetase or UDP-glucose : D-fructose 2-glucosyl transferase [E.C.2.4.1.13] catalyses the reaction:-

UDP-glucose +fructose ⇌ sucrose + UDP (eqn. 5)

The reaction is freely reversible and in the case of highly purified preparations from sugar beet and artichoke tubers equilibrium constants, in favour of sucrose synthesis, of 1.3 (pH 7.2) and 1.4-1.8 (pH 7.6), have been reported (89,90). Despite this it is believed that *in vivo* the reaction proceeds mainly in the direction of sucrose degradation and nucleoside diphosphate glucose synthesis (40,41,90-94).

The enzyme was first detected in the potato tuber by Schwimmer and Rorem (84). Pressey (42,72) showed that sucrose synthetase activity was high in young potato tubers but decreased

markedly during maturation: after harvest the activity fell rapidly and remained low during storage. Pressey believes that sucrose synthetase is associated with tuber development and that the high enzyme activity of maturing tubers is evidence that the enzyme plays a role in the conversion of sucrose to starch.

Synthesis and degradation of sucrose by the potato enzyme was studied and the pH optima were 8.8 and 6.6, respectively, for the two reactions. Pressey also claims that under physiological conditions the potato synthetase catalyses sucrose cleavage rather than synthesis. He found that cleavage of sucrose was activated by ethane-1-ol-2-thiol and inhibited by Mn^{2+}, whereas sucrose synthesis was slightly activated by ethane-1-ol-2-thiol and by Mn^{2+}: in the presence of both reagents the synthetase activity was increased two fold. However, Pressey was unable to resolve the synthetase and degradative activities of the enzyme and he concluded that they were functions of a single protein.

Murata (95), working with a purified sucrose synthetase preparation from sweet potato root, has also found marked differences between the synthetic and degradative activities of the enzyme. The substrate saturation curves were hyperbolic for sucrose synthesis and the reaction was competitively inhibited by UDP but for the cleavage reaction both substrate saturation curves were sigmoidal and typical of an allosteric enzyme. At low sucrose concentrations ($<$, 10mM) the rate of cleavage by the enzyme was relatively low but the activity increased rapidly between 10 and 15mM sucrose. This data gives further support to the view that the major role of sucrose synthetase is in the synthesis of nucleoside diphosphate sugars.

The enzyme has been identified in leaves of the potato plant by Arai and Fujisaka (30) and in chloroplast preparations from several plant sources (96-98), but generally the enzyme is considered to be mainly associated with non-photosynthetic tissues (39,99). Delmer and Albersheim (39) using _Phaseolus aureus_ have shown that the enzyme activity is high in etiolated seedlings but decreases rapidly on exposure to light. These workers have demonstrated that the activity profiles for both sucrose synthetase and UDP-glucose pyrophosphorylase, during this inactivation by light, are similar and they suggest a physiological role for both enzymes in the breakdown of translocatory sucrose. This is basically a similar hypothesis to that proposed by many others (11,34,40,41,83,100) for the conversion of sucrose to starch

in non-photosynthetic and starch-storing tissues.

Slabnik et al. (85) using a sucrose synthetase preparation from potato tubers, have shown that UDP, and to a lesser extent ADP, can both act as substrates for the cleavage reaction. The substrate specificity of the enzyme from various sources, including the potato tuber, have been examined (42,90,93), Table 1.

TABLE 1. Substrate specificity of sucrose synthetases from various sources (42,90,93).

Substrate	% Activity relative to UDP		
	Potato tuber	Sugar beet root	Mung bean seedlings (etiolated)
UDP	100	100	100
dTDP	31	52	5.8
ADP	12	16	28.6
CDP	-	12	3.4
GDP	-	6	2.8

It is apparent that the preferred substrate in each case is UDP but none of the enzymes display absolute specificity for this nucleoside diphosphate.

Pressey (42), using a sucrose synthetase preparation from potato tubers has shown that the Km values for sucrose, UDP and ADP to be 130mM, 1.7mM and 1.3mM, respectively. This data can be compared to Km values of 17mM for sucrose and 0.19mM for both ADP and UDP using the Phaseolus aureus enzyme (93).

The specificity of the reaction in the synthetic direction has also been examined. The enzyme from mung beans is not specific for UDP-glucose but can also utilize other nucleoside diphosphate glucose derivatives and the relative Vmax values agree closely with those found by Delmer (93) for sucrose cleavage.

The molecular weights of sucrose synthetase from various sources has been studied. Values of between 2.9×10^5 and 5.4×10^5 have been estimated for the enzyme from potato (42), mung bean (93), rice grains (101) and sweet potato (95). In addition, the enzyme from mung beans and rice grains has been shown to be composed of four inactive subunits.

Grimes *et al*. (102) believe that the mung bean enzyme is associated with a lipid and that the lipoprotein complex has a molecular weight of 1 x 10^6. The physiological characteristics of the complex suggest that the enzyme may be membrane bound.

Delmer has purified the enzyme from *Phaseolus aureus* to homogeneity (94) and has shown the PPi, NADP, iodoacetate and gibberellic acid all activate sucrose degradation but inhibit sucrose synthesis. Conversely, fructose 1-phosphate and Mg^{2+} inhibit sucrose degradation but activate sucrose synthesis. PPi and fructose 1-phosphate are only effective when accompanied by Mg^{2+}. All the effectors (except gibberellic acid) were tested at relatively high concentrations so the physiological significance of some of the effects may be questioned. However, it is evident that mung bean sucrose synthetase is subject to a number of complex regulatory factors. Pressey (42,72), using a potato tuber sucrose synthetase, has also shown that Mg^{2+} inhibits sucrose degradation but activates the synthesis of the disaccharide.

De Fekete and Cardini (40) have shown the enzyme from sweet corn has a much higher affinity for UDP than ADP and that UDP-glucose is a potent inhibitor of ADP-glucose synthesis, whereas ADP-glucose has only a small effect on the synthesis of UDP-glucose. Since the level of UDP-glucose in sweet corn is between five and ten times higher than that of ADP-glucose, it is unlikely that ADP-glucose synthesis by the enzyme is important. Murata and collaborators (41) have reached similar conclusions in their work on rice grain preparations. They also believe that sucrose breakdown proceeds directly through the reversal of the sucrose synthetase reaction. The inhibition by UDP-glucose, of ADP-glucose synthesis via the sucrose synthetase reaction, has led Grimes (102) to suggest that UDP-glucose may control the synthesis of the other nucleoside diphosphate sugars.

D. Invertase.

Invertase or β-fructofuranoside fructohydrolase [E.C.3.2.1.26] hydrolyses sucrose yielding fructose and glucose.

Pressey (103) has found that freshly harvested potato tubers contain low levels of both 'acid' and 'neutral' invertases and high levels of an invertase inhibitor. Total invertase activity increases sharply when the potatoes are placed in cold storage and in some potato varieties, but not all, there is a reduction in the invertase inhibitor.

Invertase is not generally believed to be involved in the degradatión of sucrose during its conversion to starch, since the invertase activity of starch-storing tissues is usually low (104).

Tsai *et al*. (12) believe that invertase may be involved in the cleavage of sucrose during the first 12 days of the development of the *Zea mays* endosperm and that the sucrose synthetase-catalysed reaction comes into operation later in the maturation process.

Isherwood (17) has proposed a scheme for sucrose-starch interconversions in the potato tuber which involves invertase. He believes that the enzyme catalyses the initial reaction of the process, cleaving sucrose into its component hexose units which are in turn converted to starch via hexose phosphates and nucleoside diphosphate-glucose derivatives. It must be emphasised that this scheme is based solely on observations from cold stored potato tubers and not developing tubers.

III. Starch metabolism.

Sucrose is presumed to be the translocatory sugar of most plants including the potato and the conversion of this disaccharide to starch takes place in the stroma of the tuber amyloplast (75). The plastid is enveloped by a double membrane (105) and consists of a complicated aqueous mixture of enzymes, carbohydrates, proteins, fatty substances, salts and the starch granule. All these substances are known to have an influence on the formation of starch molecules and on their association which produces a paracrystalline grain with a characteristic form and composition which is often species specific (75,106). The plastid also contains DNA and RNA and in the case of the chloroplast, which is derived from the same proplastid as the amyloplast, it has been shown that the starch-synthesising enzymes are autonomically produced proteins (107).

Starch granules appear to be composed of layers which partially or completely encircle the hilum, and Badenhuizen (74) has demonstrated that the grain is built up by a gradual process of apposition. These apparent layers arise from discontinuities in the refractive index of the deposited material and Meyer (108) attributes this ordered radial arrangement of molecules to the presence of spherulites which consist of aggregates of needle shaped crystals held together by secondary valence forces.

Storage starch consists of mixtures of amylose and amylopectin (109), which are easily separated and distinguished by a variety of physical, chemical and enzymatic techniques (110). The proportion of amylose and amylopectin is variable and is controlled by genetic factors (111). The amylose/amylopectin ratio is characteristic of the botanical source (Table 2).

TABLE 2. Percentage of amylose in whole granular starches (112).

Starch source	% Amylose
Algal (Floridean)	<1
Oat (*Avina sativa*)	27
Potato (*Solanum tuberosum*)	23
Apple (*Malus* sp.)	19

Amylose (Fig. 4a) is a heterogeneous mixture of single chain polymers (DP $\sim 10^3$) consisting of D-glucosyl units linked α-(1⟶4). Most chains possess a limited degree of α-(1⟶6)-branching (111).

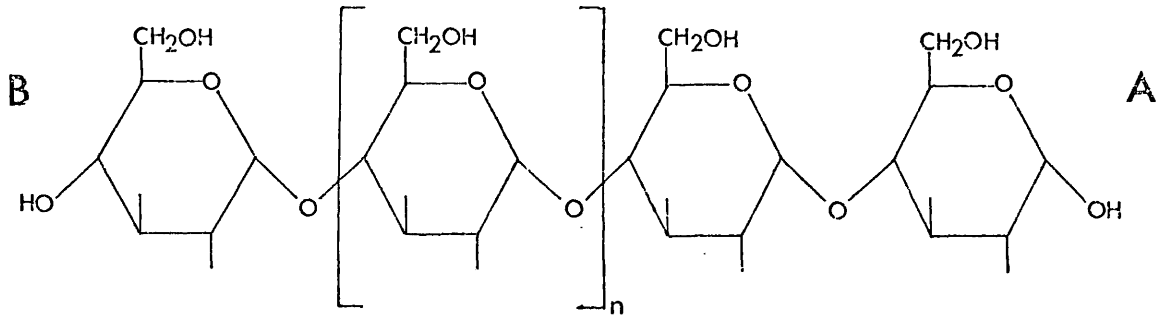
Amylopectin (Fig. 4b) is a highly branched D-glucose polymer, possessing mainly α-(1⟶4)-linkages but with α-(1⟶6)-linkages at the branch points. The side chains consist of 20-25 glucosyl units. The exact architecture of the molecule is still unclear but the structure proposed by Gunja-Smith *et al.* (113) best fits the available analytical data (Fig. 5).

The distinction between amylose and amylopectin is not absolute; 5-10% of the granule is composed of material intermediate in character (114,115).

Phytoglycogen is a glycogen-type polysaccharide found in sweet corn (116). It resembles the characteristic glycogen of animals with respect to $\overline{CL}$ and β-amylolysis limit, but its iodine binding power is 3-4 times greater. This may be related to the size distribution of the individual chains which are longer than those present in animal glycogen (117).

A peculiarity of potato tuber starch is the association of ester phosphate groups with the amylopectin fraction (118). Approximately 1 glucosyl residue in 400 is esterified, this being mainly at

(a) Amylose



(b) Amylopectin

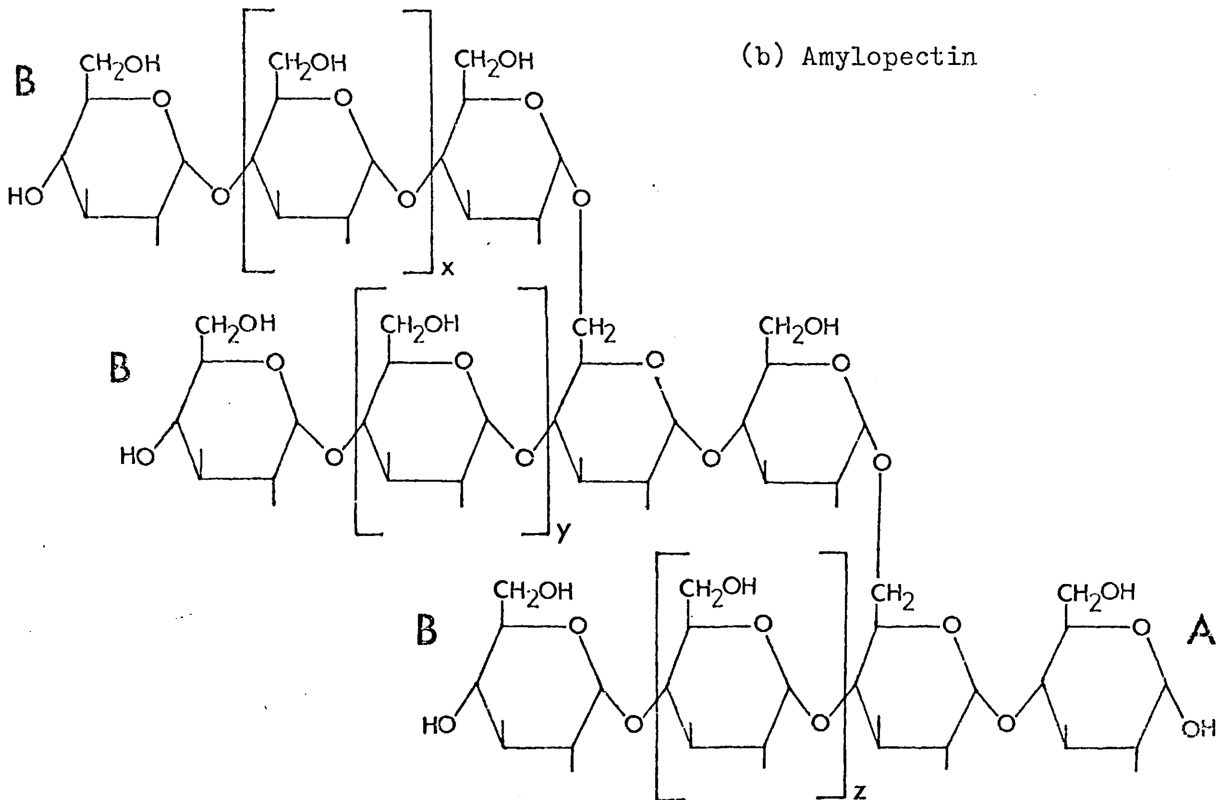


Fig. 4. Structure of (a), Amylose; (b), Amylopectin.
A, Reducing end; B, Non-reducing end;
$n = \sim 10^3$; x, y and z = 17-22

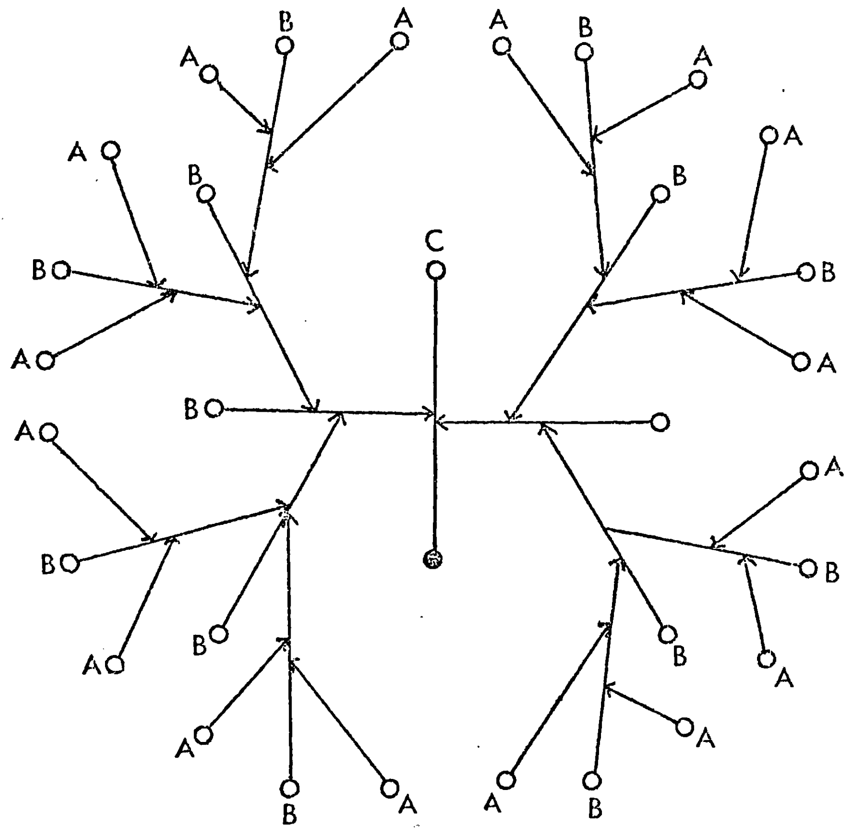


Fig. 5. Schematic representation of amylopectin, after Gunja-Smith *et al*. (113)

o—— , Non-reducing end

●—— , Reducing end

←— , α-(1⟶6)-linkage

—— , Chains of α-(1⟶4)-linked glucose units

A , Chains linked to the molecule only by their potential reducing group.

B , Chains linked to the molecule by their potential reducing group and also carrying one or more A chains.

C , Chains carrying a reducing group.

carbons 3 and 6 (119). Esterification occurs throughout the development of the tuber (120). Somatus and Schwimmer suggest that this starch grain-bound phosphate may act as an important phosphate reserve, which is mobilized during sprouting of the tuber.

Although in higher plants the amylose/amylopectin ratio is generally characteristic of the species (Table 2), in the potato the ratio and molecular properties of the two polymers change during tuber maturation, (Table 3). As the tuber develops the amylose percentage increases together with its DP. Associated with these changes in the amylose fraction is a corresponding increase in the molecular weight of the amylopectin with a concomitant decrease in $\overline{CL}$. As the tubers continue to grow their starch content increases and the starch granules increase in size.

TABLE 3. Changes in the content and properties of amylose and amylopectin in potato tubers at different stages of tuber maturity (121, 122).

Tuber size (cm)	Starch content (% wet weight)	Average grain diameter (μ)	Amylose		Amylopectin	
			%	DP	$\overline{CL}$	Molecular Weight (x 10^6)
2-3	6.4	20	13.9	-	-	14
4-5	9.2	25	16.4	1600	26	24
5-6	11.0	28	17.2	-	-	30
6-7	13.4	32	18.5	3100	24	35
8-9	17.5	43	19.0	3600	23	35
10-11	18.0	45	19.8	4000	22	76
15	18.5	48	20.0	3900	22	120

The role of the enzymes involved in starch metabolism has always been subject to speculation and hypothesis. For many years it was assumed that polysaccharides were formed by the reversal of hydrolysis, but with the discovery of starch phosphorylase a new controversy began.

The reaction catalysed by this enzyme is reversible and thus it seemed probable that the enzyme was involved in both the formation and breakdown of starch. However, in 1957 Leloir and Cardini (123) discovered hepatic glycogen synthetase which transfers glucose from

UDP-glucose to glycogen. This led to the discovery of a similar enzyme in plants which catalyses the transfer of glucose to amylose and amylopectin (124,125).

In the past decade plant biochemists have become increasingly confident that the starch synthetases are responsible for the synthesis of starch in vivo. Originally, phosphorylase and amylases were thought to be involved in starch degradation. However, the importance of phosphorylase in starch biosynthesis has recently become apparent (126-129) and some doubt in the accepted role of starch synthetase has been expressed (75,130).

A. Starch synthetase.

Leloir et al. (124, 125) demonstrated the presence of enzymes in Phaseolus vulgaris and potato tubers which could effect chain elongation of starch and certain oligosaccharides using UDP-glucose as a glucose donor (eqn. 6). The enzyme, UDP-glucose : α-(1→4)-glucan α-4 glucosyl transferase [E.C.2.4.1.21], was subsequently shown to occur in tissues of many other plants.

$$(G)_x + \text{Glucose 1-phosphate} \rightleftharpoons G\text{-}\alpha\text{-}(1\rightarrow 4)\text{-}(G)_x + Pi \qquad \text{(eqn. 6)}$$

where $(G)_x$ represents an α-(1→4)-glucan containing X number of glucose residues.

The enzyme isolated by Leloir and his collaborators was found to be bound to the starch grains and they were able to demonstrate the irreversible incorporation of radioactivity from UDP-[U-^{14}C]glucose into the grains. Degradation of the product by β-amylase gave [^{14}C]maltose, indicating that the transferred glucose residues were attached by α-(1→4)-linkages. Incorporation of radioactivity into both the amylose and amylopectin fractions occurred. The smallest primer for the reaction is maltose: with maltooligosaccharides of higher DP the transfer is more efficient.

The chemical synthesis of ADP-glucose led to the discovery that this nucleoside diphosphate glucose was a better substrate for starch synthesis than UDP-glucose (4,131-133). The enzyme in this case is referred to as ADP-glucose: starch transglucosylase (ADP-glucose: α-(1→4)-glucan α-4 glycosyl transferase [E.C.2.4.1.b]). It is not clear whether the enzyme utilizing UDP-glucose is a different protein from that using ADP-glucose.

The isolation and identification of ADP-glucose from corn (134),

rice (47,133) and potato tubers (18), as well as the presence of ADP-glucose pyrophosphorylase in these starch-storing tissues (30,47, 51,52), supports the theory that this sugar nucleotide is important in starch synthesis. Tsai and Nelson (63) have shown that the starch content of the maize mutant shrunken-2 is only 25% of the level formed in normal maize. The mutant lacks ADP-glucose pyrophosphorylase activity and Tsai concludes that the bulk of the starch synthesised in the normal maize endosperm is derived from ADP-glucose.

A starch grain-bound starch synthetase preparation from the potato tuber has been extensively studied (124,135-140). Frydman (135, 138) showed that the preparation was similar to that obtained from mung beans (131). It utilized ADP-glucose as a substrate at a much faster rate than UDP-glucose and the transglucosylation from the latter was strongly inhibited by ADP-glucose. Frydman suggests that ADP-glucose is the preferred precursor of starch in the tuber. This is of interest, since both ADP-glucose and UDP-glucose have been identified in the potato tuber (18), although only ADP-glucose appears to be associated with the starch grain (141-143).

Leloir (4) suggests that both nucleoside diphosphate glucose derivatives may participate in starch synthesis for although ADP-glucose is a better substrate than UDP-glucose for starch synthesis in vitro, the former nucleotide is generally present in much smaller amounts than UDP-glucose in plant tissues. A different situation is found in photosynthetic tissues because chloroplastic starch synthetases utilize ADP-glucose almost exclusively although UDP-glucose is available (132,137, 144-146). Arai and Fujisaka (137) have made a survey of several plant tissues and have confirmed that starch synthetases involved in the biosynthesis of assimilatory starch are specific for ADP-glucose whereas the starch synthetases from starch-storage tissues show a dual specificity.

The close connection between starch metabolism and K^+ is well known (147,148). Potato plants are sometimes referred to as Kalipflanza because of the high K^+ level in the tuber. Decreased starch production by the tubers in potassium deficient soils has been observed (149). This has led several workers to examine the effects of K^+ on starch synthetases from several starch-storing tissues, including the potato (101,137,139,140,148). All workers have demonstrated activation of the ADP-glucose starch synthetase reaction by K^+. Unlike the maize seed enzyme, which has an absolute requirement for K^+, potato tuber starch

synthetase activity is detectable in the absence of K^+. Nitsos and Evans believe that this may be due to the presence of residual K^+ in the potatostarch grain. Nitsos (140) and Murata (139) are of the opinion that K^+ may be involved in the association of amylose and the enzyme.

Starch synthetase forms an integral part of the starch granule and cannot be removed even after the meshes of the paracrystalline network have been widened (75). It is believed that the enzyme is adsorbed on the amylose and becomes occluded inside the starch granule during the biosynthetic process. The properties of the potato tuber enzyme were found to be modified by mechanical disruption (138). Mechanical grinding of the potato starch grains in glycine buffer (0.2M, pH 8.4) results in the loss of UDP-glucose: starch glucosyl transferase activity, whereas the activity with ADP-glucose is increased. Badenhuizen (150) was also able to increase the ADP-glucose: starch glucosyl transferase activity by grinding potato starch granules. This is of particular interest, since the properties of soluble forms of starch synthetases, which are specific for ADP-glucose, suggest that both soluble and bound synthetases may be different forms of the same enzyme (138,151). Tanaka and Akazawa (152) however, were unable to alter the nucleoside diphosphate glucose specificity of rice grain soluble starch synthetase by forming an enzyme-amylose complex. This complex was specific for ADP-glucose and was inactive with UDP-glucose.

Soluble starch synthetases have been isolated from a variety of plant sources (151-159) including the potato tuber (151,160). The soluble potato enzyme (160) was found to have similar properties to those of other soluble starch synthetases isolated from chloroplasts (151,155,156) and endosperm tissue (158,159). ADP-glucose, and to a lesser extent deoxy-ADP-glucose, are the most efficient glucose donors; UDP-glucose is not utilized by the soluble enzyme. The smallest effective primer for the reaction is maltose; isomaltose and other isomaltooligosaccharides cannot act as primers. The best primers for the reaction are amylopectin and glycogen, starch grains are poor primers.

The pH optima for the soluble potato synthetase is 8.5. The Km for ADP-glucose was found to be 0.15mM and 0.3mM using amylopectin and phytoglycogen, respectively as glucose acceptors. Glucose 6-phosphate and glucose at concentrations of 5×10^{-3}M and isomaltose, adenine,

Ca^{2+} and Mg^{2+} at 1×10^{-3}M have no effect on the enzyme activity. ADP(2×10^{-3}M), AMP(2×10^{-3}M) and ATP(4×10^{-3}M) all inhibit the enzyme (25%, 20% and 20%, respectively. The enzyme was completely inhibited by p-CMB(1×10^{-4}M), which suggests that sulphydryl groups are important for enzyme activity. A second soluble enzyme has also been identified in the potato tuber by Frydman and Cardini (151). In this case ADP-glucose is the most efficient glucose donor for the enzyme and starch grains are the only effective primers. It has been suggested (151) that this enzyme may be a soluble form of the starch grain-bound enzyme.

Recently, multiple forms of soluble starch synthetases have been obtained from several plant tissues (152,161-167). Hawker et al. (162), using a potato tuber preparation, have characterized a soluble starch synthetase that is able to catalyse the transfer of glucose from ADP-glucose to several different acceptors but can also synthesise an α-(1→4)-glucan in the absence of added primer. Prior treatment of the enzyme with glucoamylase, in an attempt to remove endogenous primer, did not appreciably decrease glucan synthesis in the absence of added primer.

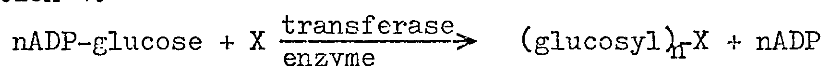
Ozbun et al. (161,164), using a spinach leaf starch synthetase preparation, showed by DEAE-cellulose column fractionation, that there were four fractions with enzymic activity. Two of the fractions (I and II) utilized amylose, amylopectin and glycogen as substrates with equal efficiencies. A third fraction (III) synthesised an α-(1→4)-glucan in the absence of added primer and the activity was stimulated 1000-fold by high salt concentrations (e.g. 0.5M sodium citrate) and BSA (0.5g/l). The fourth component (IV) from the spinach enzyme preparation has similar properties to fractions I and II.

Hawker et al. (167) have shown that the primer-independent α-glucan synthetase preparation (III) from spinach can achieve a de novo synthesis of an α-(1→4)-glucan with a limited degree of α-(1→6)-branching. This fraction III has been further resolved into separate starch synthetase and branching enzyme (p. 40) activities. When the two enzymes are recombined α-glucan synthesis is stimulated in comparison to the rate of synthesis by the starch synthetase (III) alone. This intimate association of starch synthetase III and branching enzyme is thought to reflect the existence of an in vivo complex for de novo amylopectin synthesis. The authors believe that amylose chains are

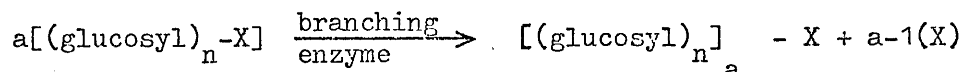
synthesised by another α-(1⟶4)-glucan synthesising enzyme, possibly phosphorylase or another starch synthetase. Schiefer *et al*. (163), from their studies on the multiple forms of starch synthetase of maize mutants, have also proposed a similar mechanism for the synthesis of amylopectin.

Fox and co-workers (168) have advanced a scheme for amylose and amylopectin synthesis involving starch synthetase and branching enzyme. Reaction 1. is catalysed by starch synthetase and forms a linear glucose chain attached to an acceptor molecule X, which may form part of the enzyme. Branching enzyme then catalyses the transfer of these linear chains to form a branched product still attached to X, (Reaction 2). The stimulation of the unprimed reaction (Reaction 1.) by the branching enzyme, which was observed for example by Hawker *et al*. (167), may be explained by the release of X for further reaction and also by the formation of new non-reducing sites which can act as acceptors for the transfer of glucose from ADP-glucose.

Reaction 1.



Reaction 2.



In a recent paper, Lavintman *et al*. (169) report that a similar situation may exist in the potato. These workers have demonstrated the *de novo* synthesis of α-(1⟶4)-glucan from UDP-glucose by a soluble enzyme from the plastids. Here a protein serves as an acceptor for the oligosaccharide chains, and the resulting glucoprotein can in turn act as an acceptor for the synthesis of larger glucan chains from UDP-glucose, ADP-glucose and glucose 1-phosphate *in vitro*.

Marshall (62) is of the opinion that, in general in plants, ADP-glucose: starch glucosyl transferase, together with the branching enzyme, is responsible for the formation of amylopectin. He cites evidence from several workers (158,159,170) that in waxy maize seeds only ADP-glucose:starch glucosyl transferase activity can be detected, which corresponds to the fact that the starch in this seed consists entirely of amylopectin. Schiefer *et al*. (163) have demonstrated the presence of multiple forms of starch synthetase in two maize mutants, sweet corn (SU_1) and amylomaize (ae), using the technique of disc gel

electrophoresis. The isozymic pattern of the two mutants was found to differ. These two varieties produce characteristic forms of starch. The SU_1 mutant synthesizes amylose and amylopectin in the normal ratio together with phytoglycogen, whereas the ae mutant produces a starch composed almost entirely of amylose. These different starches are presumably related to the different enzyme patterns of the mutants.

However, Badenhuizen (75) is critical of the view that starch synthesis occurs through chain elongation by starch synthetase. He proposes that the strong affinity of the enzymic protein for amylose may prevent its conversion to amylopectin by branching enzyme (130). Several workers have pointed out that there is a correlation between the amylose content and starch grain-bound starch synthetase levels in seeds and potato tubers (125,130,171-174).

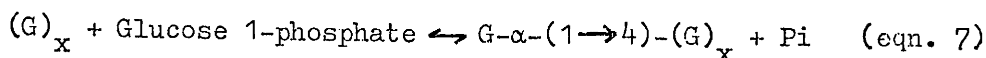
Salema and Badenhuizen (107) have found that Beta vulgaris chloroplasts lose ADP-glucose:starch glucosyl transferase and starch phosphorylase activities when the leaves are kept in the dark. The enzyme activities are restored in the light. They conclude that chloroplasts possess a mechanism for the control of protein synthesis since the process of light re-activation can be reversibly inhibited by chloramphenical and actinomycin D. Chandorkar and Badenhuizen (172) have demonstrated that during a period of darkness the ADP-glucose: starch glucosyl transferase activity of spinach chloroplasts decreases as the assimilatory starch is broken down. It is possible that the presence of starch stabilizes the enzyme so that when the starch is broken down the protein is also degraded. This may explain the rapid loss of activity when the leaf is placed in the dark.

In some germinating seeds the situation is similar to that in chloroplasts. For example, when degradation of wrinkled pea starch occurs there is a concomitant loss of starch synthetase activity (75). However, in sprouting potato tubers the starch synthetase activity actually increases during exocorrosion of the starch grain (75).

B. Starch phosphorylase.

Cori and his collaborators (179) were the first to demonstrate the synthesis of the α-(1$\rightarrow$4)-glucan, glycogen, from glucose 1-phosphate by phosphorylase derived from muscle. Shortly afterwards Hanes (221,222) demonstrated the presence of starch phosphorylase (α-(1$\rightarrow$4)-glucan: orthophosphate glucosyl transferase [E.C.2.4.1.1.]) in pea seeds and potato tubers.

The phosphorylase reaction (eqn. 7) is readily reversible. The glucan can be either a branched or linear chain. In the direction



$$(G)_x + \text{Glucose 1-phosphate} \leftrightarrow G\text{-}\alpha\text{-}(1\rightarrow4)\text{-}(G)_x + Pi \quad \text{(eqn. 7)}$$

$(G)_x$ represents a α-(1→4)-glucan containing x number of glucose residues

of glucan synthesis, glucosyl units are added to the non-reducing end of the starch primer. Similarly, in the degradative reaction, glycosyl units are removed from the non-reducing end of the polymer (182). The phospholytic action on amylopectin yields β-limit dextrins which are branched glucans where the A and B chains have been shortened so that an average of 4 glucose units proceed each branch point. Linear α-(1→4)-glucan chains cannot be shortened to less than 4 glucose units by phosphorylase.

For the past decade there has been controversy over the exact role of phosphorylase in the metabolism of starch. In animals, it is generally accepted that the enzyme is involved in glycogen mobilization, but in plants the enzyme is thought to have both a synthetic and a degradative role (126,127,182-192).

The equilibrium constant for the reaction is dependent on the Pi/glucose 1-phosphate ratio. In developing pea seeds (187) and the bark of the black locust tree (188) where starch accumulation is occurring, it has been observed that the Pi/glucose 1-phosphate ratio does not favour starch synthesis. The observation by Thorpe and Mercer (193) that gibberellic acid-treated tobacco callus tissue contains lower quantities of starch and higher phosphorylase levels than untreated tissue, also suggests a degradative role for the enzyme. The equilibrium constant for the reaction is also pH-dependent (181,185,186). In developing rice seeds there is a parallel increase in phosphorylase activity and in starch formation. However, during this process the pH of the kernel increases and thus the phosphorolysis of starch would be favoured.

The primer requirements for starch synthesis by potato starch phosphorylase are well established (189), the smallest primer being maltotriose; maltotetrose and higher maltooligosaccharides are much more efficient. Amylopectin is a superior donor to amylose because of the greater number of non-reducing groups per molecule (190). Synthesis of

starch takes place by a multichain reaction in which all the primer molecules are increased in length at about the same rate (189).

Phosphorylases, able to catalyse the de novo synthesis of α-(1→4)-glucans in the absence of added primer have been reported for the potato (126-128) and other plant species (191,192,194). This primer-independent reaction is characterized by a lag phase which proceeds the synthesis of the polysaccharide.

Potato starch phosphorylase appears to be comparable in many respects to rabbit muscle glycogen phosphorylase (195). The molecular weight has been estimated to be between 180,000 and 220,000 (196-200). Normally the protein exists as a dimer, containing one molecule of pyridoxal phosphate per monomer unit (195,197-199, 201). The potato enzyme also resembles muscle phosphorylase in its amino acid composition (202), Stokes-radii (196), circular dichroism profile (203), and reactivity towards glyoxal (204). The important difference between the two enzymes is that muscle phosphorylase is an allosteric enzyme whereas potato phosphorylase shows no allosteric properties. However, Sing and Sanwal (205) have described an allosteric α-glucan phosphorylase in banana fruits where starch synthesis is inhibited by ATP and tyrosine. The substrate saturation curve for glucose 1-phosphate in the presence of tyrosine is hyperbolic and freezing and thawing of the enzyme preparation led to a desensitization with respect to inhibition by tyrosine.

Polysaccharide synthesis by primer-independent phosphorylase prepared from potato tubers (126-128) has been shown to be 40% inhibited by both cAMP(4mM) and ATP(20mM). Magnesium ions (80mM) produced a 60% inhibition of the enzyme. Tsai and Nelson (191,206) using a primer-independent phosphorylase from maize endosperm, have demonstrated that ATP and ADP both inhibited the enzyme. De Fekete (207) has reported that ADP-glucose and to a lesser extent UDP-glucose inhibit glucan synthesis by phosphorylase prepared from Vicia faba cotyledons. Burr and Nelson (194) have commented that since the enzyme activity appears to be regulated by ADP-glucose and UDP-glucose, both of which are the immediate substrates for the soluble and bound starch synthetases, then the reactions of the latter enzymes and phosphorylase must be separated in time or in space in order to allow starch formation to occur in vivo.

The discovery of different potato phosphorylase isozyme patterns

in the developing tuber, where starch synthesis is occurring, and the sprouting organ, where starch mobilization is taking place (129, 196,208,209), lends support to the current view that this enzyme is involved in both the anabolism and catabolism of starch _in vivo_. Gerbrandy (129) reported the presence of nine phosphorylase isozymes during starch synthesis in the potato tuber but only two during the period of starch breakdown. He has also shown that there is a relationship between starch metabolism and the occurrence of phosphorylase isozymes in _Vicia faba_ and in _Phaseolus vulgaris_. Gerbrandy and Verleur (196) have further demonstrated that some of the potato isozymes are related in that dimers, tetramers and octomers of a single subunit occur. These workers have also shown that during the breakdown of starch by the potato tuber enzyme, most of the phosphorylase activity is associated with the tetrameric isozyme; they suggest that this isozyme may be concerned with the mobilization of starch. During the period of starch synthesis however, the dimeric isozyme is more active than during starch breakdown and this may indicate that the dimeric form of the enzyme is concerned with starch synthesis.

Gerbrandy and his collaborators suggest that the interconversion of these multiple enzyme forms may represent a fine mechanism for the control of starch metabolism in the potato tuber. For example, in a recent paper Gerbrandy (200) has made a detailed study of the dimeric phosphorylase and shown that, unlike the tetrameric and octomeric forms, it is adsorbed by glycogen and this process is enhanced at low temperatures. Gerbrandy has related this effect to starch degradation and the sweetening of tubers held at low temperatures.

De Fekete (210) has demonstrated a similar adsorption-desorption effect using a _Vicia faba_ phosphorylase which is associated with amyloplasts. The enzyme is adsorbed by the amyloplasts in the presence of EDTA and NaCl at pH values between 6.3 and 8.0 but sucrose and other sugars inhibit this process. She suggests (_cf._ Gerbrandy (200)) that adsorption and desorption of the enzyme onto and off of the amyloplast could be of importance in the regulation of starch grain biosynthesis.

Badenhuizen firmly believes that phosphorylase plays an important role in both synthesis and degradation of starch. He has shown (211-213) that the relative activities of phosphorylase and branching enzyme (see p. 40) (P/Q ratio), parallel the amylose content

of starch, and he also believes that phosphorylase and starch synthetase both play a part in amylose synthesis, though their function may be very different (75).

The opposing view (60) is that phosphorylase plays a predominantly degradative role but that it may be involved in the initial stages of starch formation. That is, primer-independent isozymes are involved in the *de novo* synthesis of an α-(1→4)-glucan primer and then further glucose units are added from ADP-glucose by starch synthetase. Baun *et al.* (214) have reached similar conclusions in their work on developing rice grains and they believe that the high phosphorylase activity detected during the initial stages of starch accumulation, reflects the role of the enzyme in the formation of the primers which are required as substrates by starch synthetases.

C. Q-enzyme.

Q-enzyme (α-(1→4)-glucan:α-(1→4)-glucan 6-glucosyl transferase [E.C.2.4.1.18]) was first identified in the potato tuber (215) and was subsequently isolated from this source in a crystalline form by Gilbert *et al.* (216,217). The enzyme acts by breaking an α-(1→4)-linkage in a glucan chain and then attaches the fragments released to another α-(1→4)-glucan by an α-(1→6)-linkage. Intra-chain transfer is also possible but less likely (218).

Q-enzyme differs from branching enzyme in that the former will branch amylose or amylopectin but the latter is only active with amylose (219,220). The polysaccharide product of Q-enzyme action resembles amylopectin in chain length, solubility, iodine staining and β-amylolysis limit (221). Barker *et al.* (222) have synthesised an amylopectin-type molecule from glucose 1-phosphate using a coupled enzyme system containing potato phosphorylase and Q-enzyme. When the ratio of Q-enzyme to phosphorylase was increased a more highly branched amylopectin was formed. Recently, Drummond and co-workers (223) have shown that the amylopectin type polysaccharide, synthesised either by potato Q-enzyme acting directly on amylose or by the combined action of Q-enzyme and phosphorylase on glucose 1-phosphate, is not identical to natural amylopectin. This was achieved by treating synthetic and natural amylopectins with pullulanase and examining the size and distribution of the linear glucan chains released. The two types of

amylopectins yielded different products. Doi (224) has coupled a purified spinach ADP-glucose:starch glucosyl transferase with potato Q-enzyme and also produced a branched polysaccharide similar to natural amylopectin with respect to iodine colour, β-amylolysis limit and average chain length. This latter coupled enzyme system is of particular interest, since Marshall's (62) suggestion that ADP-glucose:starch glucosyl transferase and branching enzyme are involved in amylopectin synthesis in vivo is supported by the isolation of such an enzyme complex from spinach chloroplasts (161,162,167,168).

D. R-Enzyme.

The phospholytic degradation of starch has been discussed and the fact that α-(1→6)-branch points limit the action of phosphorylase. In vivo for the complete metabolism of starch, these branch points must be hydrolysed. This can be achieved by R-enzyme (debranching enzyme; amylopectin 6-glucohydrolase [E.C.3.2.1.9]). The enzyme was first discovered by Hobson et al. (225) in broad beans and potatoes. The enzyme debranches amylopectin and β-limit dextrins liberating α-(1→4)-linked glucans. Potato R-enzyme cannot debranch glycogen (226) but has been shown to hydrolyse pullulan (227).

The relatively low affinity of R-enzyme for amylopectin (227, 228) suggests the function of the enzyme in vivo is to hydrolyse the limit dextrins formed by the action of β-amylase or phosphorylase on amylopectin (62).

E. D-Enzyme.

R-enzyme liberates some α-(1→4)-glucan chains (maltodextrins) which are too short for further degradation by phosphorylase. Longer chains can be synthesized from the maltodextrins by disproportionation reactions involving D-enzyme. This enzyme (α-(1→4)-glucan:α-(1→4)-glucan 4-glycosyl transferase [E.C.2.4.1.25]) catalyses the transfer of maltosyl or larger units from maltodextrins to other maltodextrin acceptor molecules (e.g. eqn. 8). Further transfer reactions lead to

2 maltotriose units ⇌ maltopentaose + glucose (eqn. 8)

an equilibrium situation where the products are a mixture of malto-saccharides and glucose. If the equilibrium is disturbed by the continual removal of glucose, larger chains of maltodextrins are produced (218,229).

D-enzyme was first found in potatoes (230) and it is present at

a level which suggests that it is of importance in vivo. Roles in both the synthesis (231) and the degradation (232) of starch have been postulated.

F. Amylolytic enzymes.

In vivo, plants appear to have both a phosphorolytic and hydrolytic capacity for starch degradation. Hence amylolytic enzymes which are capable of acting on amylopectin and amylose by breaking α-(1→4)-glucosidic linkages are widespread in the plant kingdom. Two main types of amylase, α and β, are known.

1. α-Amylase.

α-Amylase(α-(1→4)-glucan 4-glucohydrolase [E.C.3.2.1.1]) occurs widely in plants (233). The enzyme has been shown to occur in multiple forms in a variety of different plants (234) including the potato (208).

α-Amylases contain Ca^{2+}, which does not participate directly in the formation of the enzyme-substrate complex, but holds the enzyme molecule in the correct conformation for activity and maximum stability (111). The enzyme hydrolyses the α-(1→4)-bonds in amylose and this is characterized by a rapid loss in viscosity and iodine staining value together with an increase in reducing power. The final products are mainly maltose and maltotriose. The action on amylopectin is comparable, again there is a rapid fall in the iodine staining value of the product and an increase in reducing power. Cleavage of the α-(1→4)-glucosidic linkages again occurs but the α-(1→6)-branch points are unaffected.

The action of the enzyme is essentially random but this is only true of the reaction in the early stages and certain linkages nearer chain ends are not readily hydrolysed. The enzyme exhibits a multiple attack mechanism (235,236) but the degree of multiple attack depends on the source of the enzyme and the pH of the incubation mixture (237).

The action of the enzyme on granular starches is slow and the potato starch granule is the most resistent to attack (238,239). This is somewhat surprising since the function of the enzyme in vivo is probably the degradation of starch. Varner and Chandra (240) have shown that the de nova synthesis of cereal α-amylase increases markedly during germination and Emilson and Lindblosn (241) have

demonstrated the importance of the enzyme in starch breakdown in sprouting potato tubers. De Fekete and Vieweg (184,242), have shown that maltose inhibits α-amylase. They believe that the disaccharide may play an important role in the regulation of the enzyme during starch degradation by a feedback control mechanism.

2. β-Amylase.

The occurrence of β-amylase (α-(1→4)-glucan maltohydrolase [E.C.3.2.1.2]) is restricted to higher plants. The enzyme has been shown to have a multiple attack mechanism, acting in a stepwise fashion starting at the non-reducing end of the polysaccharide and hydrolysing alternate α-(1→4)-glucosidic linkages. Since hydrolysis takes place with inversion of configuration, β-maltose is liberated. This is the major product of the enzyme on linear α-(1→4)-glucans. The enzyme is unable to by-pass α-(1→6)-glucosidic linkages in amylopectin and the degradation of this branched polysaccharide is incomplete and results in the formation of large β-limit dextrins.

The biological function of β-amylase is believed to be the mobilization of chloroplast starch. The enzyme level increases rapidly in chloroplasts which have been subject to long periods of illumination and the increase corresponds to a rapid disintegration of assimilatory starch (243). Native starch grains are completely resistent to attack by β-amylase and Duncan (244) suggests that the function of the enzyme *in vivo* is complementary to α-amylase, it being involved in the break-down of oligosaccharides produced by the action of the α-amylase. Alexander (245) believes that the high β-amylase levels in the tissues of *Saccharum* species are responsible for the lack of starch in commercial sugar cane.

IV. The aim of the investigation.

The potato (*Solanum tuberosum*) is extensively cultivated in temperate regions of the world. The cultivated plants are normally 0.6m high and have small star shaped white flowers. Tuber development occurs on underground branches or stolons during the growth period of the plant. The mature tuber, which is present after the haulm dies, enters a period of dormancy which usually lasts for not less than three months and then, at the end of this period, providing conditions are favourable, sprouting occurs and a new potato plant begins to grow.

The potato is a major source of dietary starch in the western world and it could, perhaps, be argued that the predominent biochemical reactions occurring in the tuber tissue are directed towards the formation and degradation of starch; the former during tuber development and the latter during sprouting. Industry enquired whether it would be possible to increase the rate of sucrose formation at the expense of starch in potatoes and for this reason and because of fundamental interests, a study of the mechanism and control of sucrose-starch equilibrium in the potato was undertaken.

The pathway by which sucrose is converted to starch in plant tissues has been subject to speculation and hypothesis by biochemists for many years. This thesis describes attempts to unravel this process in the developing potato tuber.

The first part of the investigation was to try to identify the intermediate compounds directly involved in the conversion of sucrose to starch: radioactive sugars were used as tracers for this purpose. Following the results of these feeding experiments, some of the enzymes likely to be involved in the formation of starch from translocated sucrose were investigated and a metabolic sequence by which the whole process might occur was proposed.

The final part of the work was an attempt to reconstruct the proposed pathway, using potato tuber enzyme preparations, in vitro.

RESULTS AND DISCUSSION

V. The metabolism of [U-^{14}C]sucrose and [U-^{14}C]fructose by developing potato tubers

Tracer techniques have been used to obtain information concerning the conversion of sucrose to starch *in vivo* in both wheat and rice grains (158,176,177). The present study has extended these techniques to the potato tuber; radioactive sucrose and fructose were used as tracers. It was hoped that such studies would allow the identification of the metabolites involved in the conversion of sucrose to starch and, hence, the major pathway for starch synthesis in the developing tuber.

Initial experiments, using potato tuber tissue slices incubated with solutions of [U-^{14}C]sucrose, using a method similar to that described by Murata *et al*. for rice grains (136,176), were unsuccessful. The slices respired rapidly and relatively large volumes of solutions were needed to bathe the tissues. Injection of the [^{14}C]tracer solutions into intact tubers with a hypodermic syringe (64,177,178) was later found tobe the most successful and convenient method for introducing label into the tissue carbohydrates.

A. Metabolism of [U-^{14}C]sucrose

Injection of developing tubers with [U-^{14}C]sucrose followed by a 30 min incubation period yielded a [^{14}C]glucan which was presumed to be starch. The polymer was characterised as a labelled α-(1→4)-glucan by α- and β- amylolysis and acid hydrolysis (Fig. 6). The incorporation of label from [U-^{14}C]sucrose into the starch proceeded after an initial lag period which was presumably related to penetration of the cells by the substrates (Fig. 7).

Developing tubers were next injected with varying amounts of [U-^{14}C]sucrose and then incubated for 15 min. Radioactive ADP-glucose, UDP-glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, glucose, fructose and sucrose were extracted with perchloric acid solution and the [^{14}C] metabolites isolated and characterised by a combination of ion-exchange, chromatographic and electrophoretic techniques (see Materials and Methods, sections X.C.1.-3.).

A typical elution profile from a Dowex-1 anion-exchange column for ADP-glucose and UDP-glucose is shown in Fig. 8 and for the hexose phosphates in Fig. 9. The radioactivity associated with the isolated metabolites is shown in Table 4. The data indicates that the

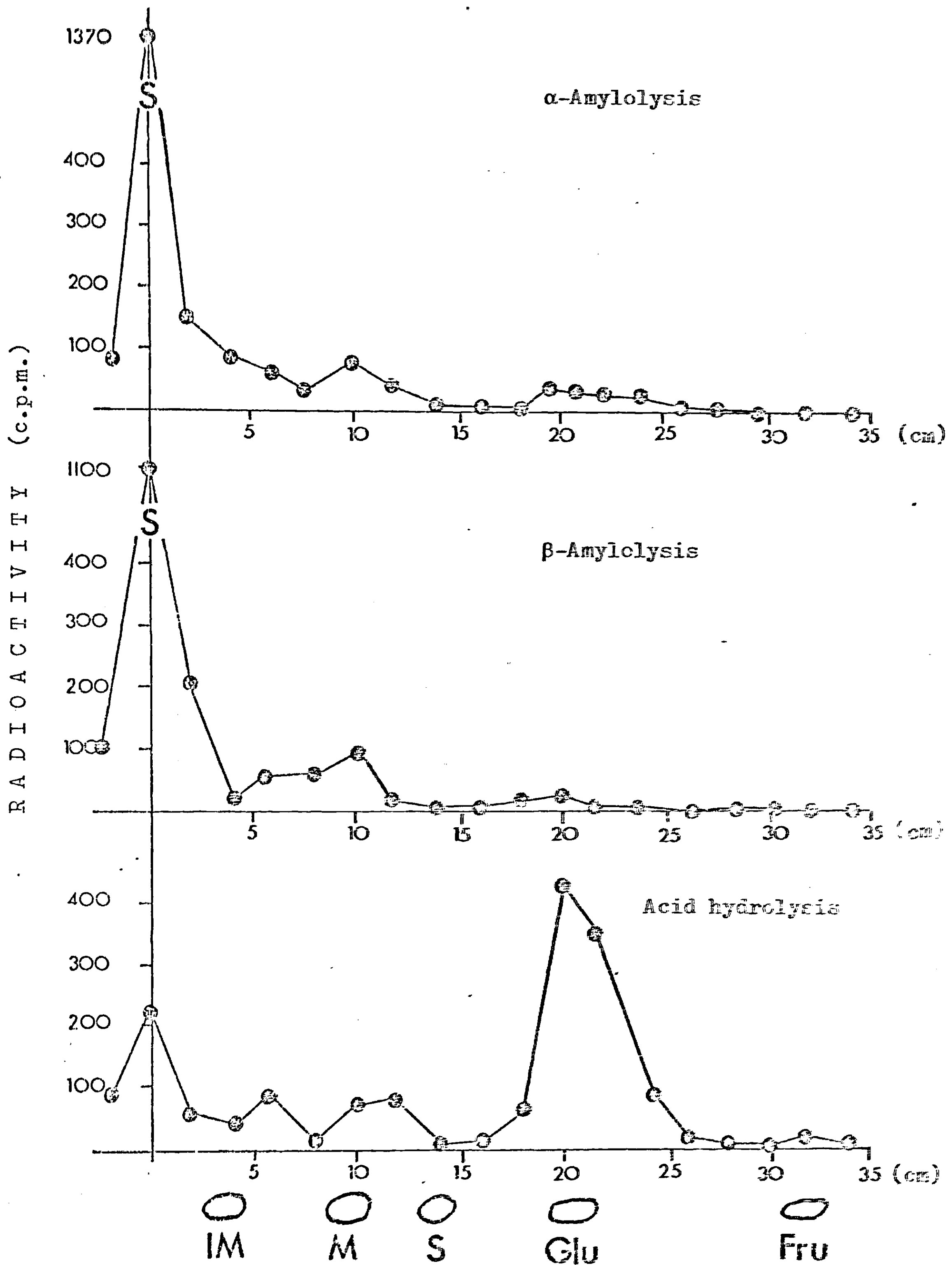


Fig. 6. Radioactive hydrolytic products derived from [^{14}C]starch. The starch was produced by injection of [U-^{14}C]sucrose into developing tubers. For experimental details see Materials and Methods, section X.D.2.

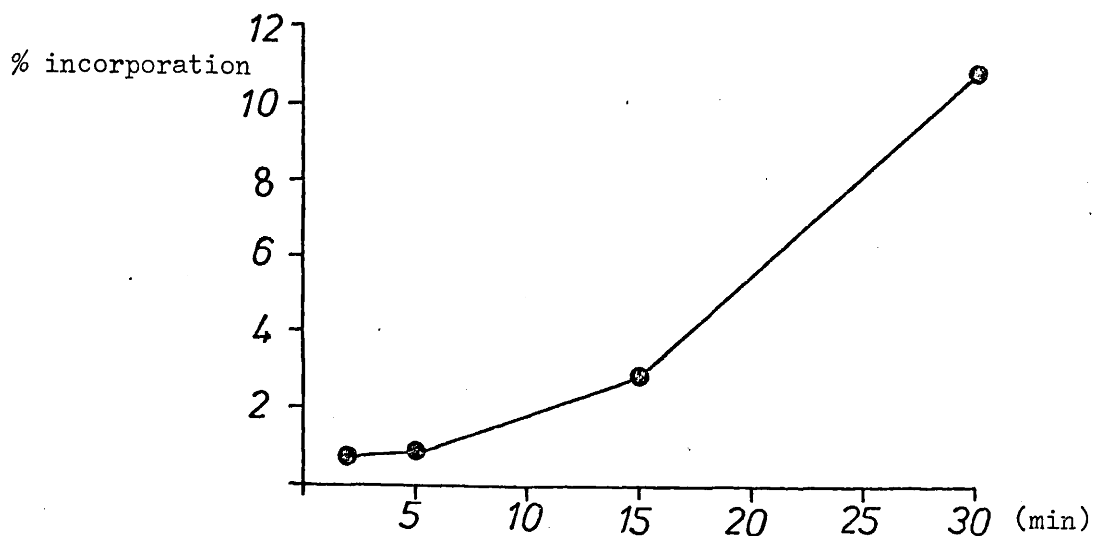


Fig. 7. Time course for the incorporation of [U-^{14}C]sucrose into starch. Injection and incubation of the tubers and extraction of the starch was as described in Materials and Methods, sections X.A., X.B. and X.C.4.

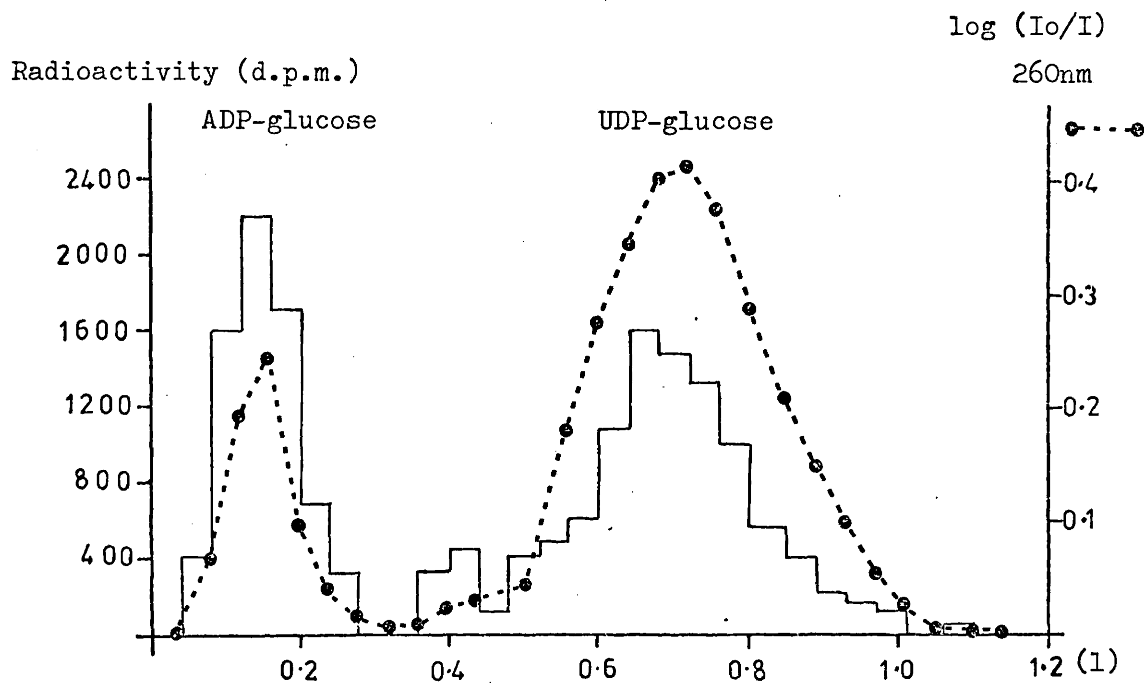


Fig. 8. Elution profile of the sugar nucleotide fraction (derived from [U-^{14}C]sucrose) from a Dowex-1(Cl′)anion-exchange column (experiment E, TABLE 4). See Materials and Methods, section X.C.3. for experimental details.

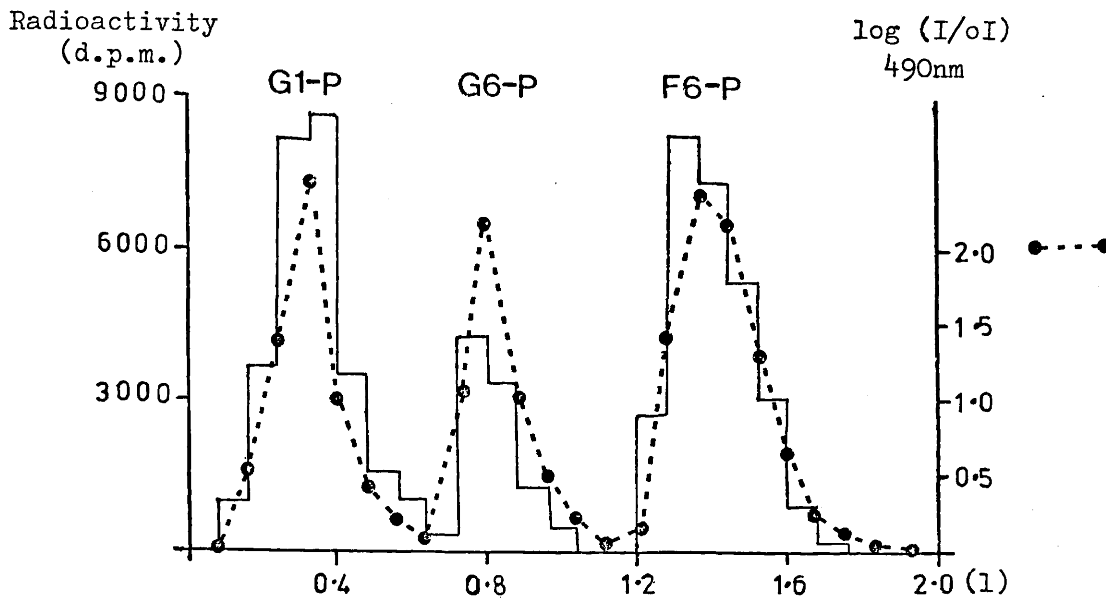


Fig. 9. Elution profile of the sugar phosphate fractions (derived from [U-^{14}C]sucrose) from a Dowex-1(Cl$^-$)anion-exchange column (experiment H, TABLE 4). See Materials and Methods, section X.C.2. for experimental details.

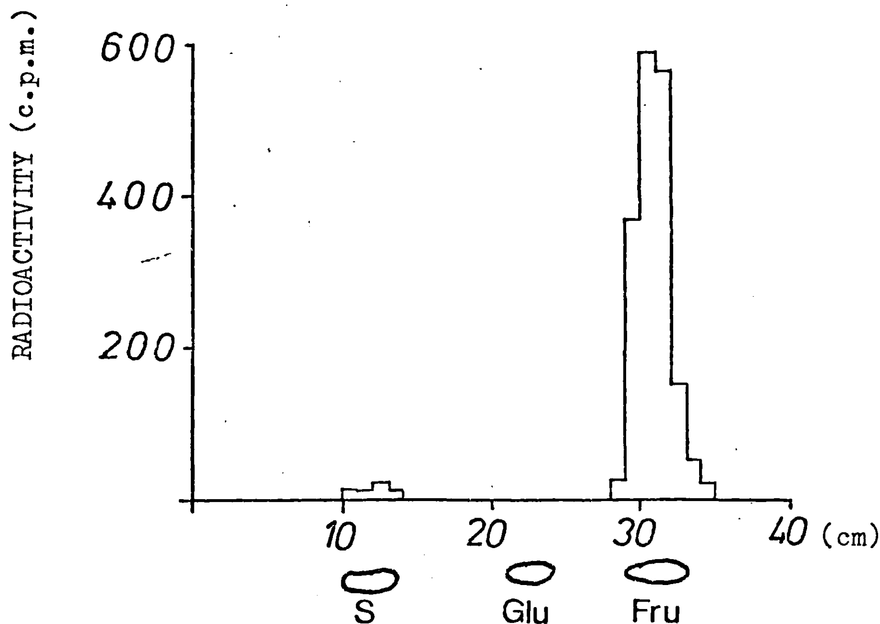


Fig. 10. Paper chromatographic analysis of the products of invertase hydrolysis of [^{14}C]sucrose obtained by the reaction of [U-^{14}C] fructose and UDP-glucose in the presence of potato tuber sucrose synthetase. (Hydrolysis of [^{14}C]sucrose obtained from ADP-glucose and [U-^{14}C]fructose yielded an essentially similar chromatographic pattern). See Materials and Methods, section XI.C.1. for experimental details.

Experiment	µCi[U-^{14}C] sucrose injected	Conversion of [U-^{14}C] sucrose to starch (%)	ADP-glucose (d.p.m.)	UDP-glucose (d.p.m.)	Total NDP-glucose (d.p.m.)	d.p.m. UDPG / d.p.m. ADPG	F6-P (d.p.m.)	G6-P (d.p.m.)	G1-P (d.p.m.)	Total hexose phosphates (d.p.m.)	d.p.m. Hexose phosphates / d.p.m. NDPG	Fructose (d.p.m.)	Glucose (d.p.m.)	d.p.m. Fructose / d.p.m. Glucose
A	1.8	2.9	9,122	17,278	26,400	1.9	-	-	-	-		-	-	
B	2.0	3.0	4,388	7,627	12,015	1.7	-	-	-	-		-	-	
C	2.7	3.0	6,486	11,586	18,072	1.8	-	-	-	29,241	1.6	19,054	1,714	11.1
D	2.8	3.0	4,872	7,998	12,870	1.6	-	-	-	24,232	1.9	20,222	1,800	11.2
E	3.5	2.5	6,327	11,515	17,842	1.8	-	-	-	-		-	-	
F	4.8	2.6	12,088	22,777	34,865	1.9	-	-	-	61,955	1.8	-	-	
G	5.2	2.6	16,666	31,111	47,777	1.9	-	-	-	-		-	-	
H	8.1	3.1	12,627	23,486	36,113	1.9	28,074	10,696	25,000	63,770	1.8	-	-	
I	8.6	2.4	13,823	28,098	41,921	2.0	43,133	9,762	41,591	94,485	2.3	84,582	13,380	6.3
J	8.6	3.5	14,942	29,585	44,527	2.0	37,028	9,346	34,038	80,412	1.8	90,237	11,428	8.0
Average*		2.9±0.1				1.9±0.04					1.9±0.1			9.2±2.5

TABLE 4. Distribution of ^{14}C in carbohydrate metabolites after incubation (15 min, abient temperature) of developing potato tubers with [U-^{14}C]sucrose (specific activity 600µCi/µmol). (-) indicates that no quantitative measurements of radioactivity were made. Extraction, fractionation and analysis of the [^{14}C]metabolites is described in Materials and Methods, sections X.A.-C.

*Values are means ± the standard error for the sample.

percentage incorporation of label from [U-^{14}C]sucrose into starch is independent of the amount of radioactive sucrose injected. The total incorporation into the nucleoside diphosphate glucose fraction shows variation but generally increases as the level of [U-^{14}C]sucrose injected increases whereas the UDP-glucose/ADP-glucose radioactivity ratio remains constant. There are no significant variations in the hexose phosphate/nucleoside diphosphate glucose radioactivity ratio. Significantly more label appears to be present in the free fructose fractions than in the glucose fractions.

When dormant tubers were injected with [U-^{14}C]sucrose no incorporation of radioactivity into starch was observed. The level of radioactive ADP-glucose, UDP-glucose, glucose 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, glucose and fructose from these tissues was also insignificant. The difference in the levels of these [^{14}C]metabolites in developing and dormant tubers presumably reflects the loss of starch-synthesising ability following the cessation of tuber growth.

B. Metabolism of [U-^{14}C]fructose

The injection of developing potato tubers with [U-^{14}C]fructose, followed by incubation for periods of 15 and 30 min, resulted in 13.7 and 23.4% incorporation of label into starch fractions, respectively. The [^{14}C]starch produced was again characterized as an α-(1→4)-glucan by α- and β- amylolysis and acid hydrolysis (see Materials and Methods, section X.D.2.).

The more efficient incorporation of label into starch from [U-^{14}C]fructose, in comparison with incorporation from [U-^{14}C]sucrose, is probably due to a relatively large endogenous sucrose 'pool' in the tuber. Burton (78) has demonstrated that in immature potato tubers the sucrose content is at least five times greater than fructose.

Radioactive ADP-glucose, UDP-glucose, glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, glucose, sucrose and fructose were all identified in perchloric acid extracts of tubers which had been incubated with [U-^{14}C]fructose. These metabolites were characterized as described in sections X.C.1.-3. of Materials and Methods. The radioactivity associated with the individual [^{14}C]metabolites is shown in Table 5.

As with [U-^{14}C]sucrose, the percentage incorporation of label

Experiment	µCi[U-^{14}C] fructose injected	Conversion of [U-^{14}C] fructose to starch (%)	ADP-glucose (d.p.m.)	UDP-glucose (d.p.m.)	Total NDP-glucose (d.p.m.)	d.p.m. UDPG / d.p.m. ADPG	Total hexose phosphates (d.p.m.)	d.p.m. Hexose phosphates / d.p.m. NDPG	Sucrose (d.p.m.)	Glucose (d.p.m.)	d.p.m. Sucrose / d.p.m. Glucose
15 min incubations											
K	1.5	13.5	22,788	30,377	53,165	1.3	307,524	5.8	10,753	11,111	1.0
L	2.0	13.0	56,398	67,677	124,675	1.2	813,858	6.5	137,400	14,350	9.6
M	2.5	14.0	20,799	24,947	45,746	1.2	227,120	5.0	109,688	16,911	6.5
N	3.2	11.0	52,495	66,144	118,639	1.3	-	-	-	-	
AVERAGE*		12.9±0.5				1.3±0.03		5.8±0.3			5.7±2.5
30 min incubations											
O	2.0	24.8	40,864	53,613	94,477	1.3	229,065	2.4	393,555	16,811	23.4
P	3.2	22.0	72,484	83,888	156,377	1.2	-	-	-	-	
AVERAGE*		23.4±1.4				1.3±0.05		2.4±0.0			23.4±0.0

TABLE 5. Distribution of ^{14}C in carbohydrate metabolites after 15 min and 30 min incubation of developing potato tubers with [U-^{14}C]fructose (specific activity 100µCi/µmol). (-) indicates that no quantitative measurements of radioactivity were made. Extraction, fractionation and analysis of the various [^{14}C]metabolites is described in Materials and Methods, sections X.A.-C.

*Values are means ± the standard error for the sample.

from [U-^{14}C]fructose into starch is independent of the amount of radioactive fructose injected. The total incorporation into nucleoside diphosphate glucose and hexose phosphate fractions is variable, although the UDP-glucose/ADP-glucose and the hexose phosphate/nucleoside diphosphate glucose radioactivity ratios remain essentially constant. The average hexose phosphate/nucleoside diphosphate glucose ratios, observed after 15 and 30 min incubation, may suggest a substrate-product relationship between these [^{14}C]metabolites.

No information is available on the 'pool' sizes of the various carbohydrate metabolites, other than fructose, in developing tubers. However, attempts can be made to compare and explain the total incorporation of label from [U-^{14}C]sucrose and [U-^{14}C]fructose into the various intermediates for starch synthesis, bearing in mind problems of interpretation such as compartmentalization which might result from feeding exogenous substrates. For example, the hexose phosphate/nucleoside diphosphate glucose ratio following incubation with [U-^{14}C]sucrose is 1.9 (Table 4), whereas that for a similar incubation with [U-^{14}C]fructose (Table 5) is 5.7. This suggests that sucrose is more directly associated with nucleoside diphosphate glucose formation than fructose. This is consistent with the hypothesis that the first step in the conversion of sucrose to starch involves sucrose synthetase and not invertase (see Introduction p.22 and p.25).

The introduction of labelled sucrose into the potato tuber in these experiments using a hypodermic needle undoubtedly damages the tissues and presumably allows the disaccharide to mix with some released invertase. Therefore, a proportion of the sucrose is probably metabolized via its free components, glucose and fructose.

The UDP-glucose/ADP-glucose ratio following incubation with [U-^{14}C]sucrose is also 1.9 (Table 4), whereas in the case of incubations with [U-^{14}C]fructose it is 1.3 (Table 5). This suggests that ADP-glucose is being formed preferentially from fructose. This hypothesis is supported by other experiments which are described later (see Results and Discussion, section V.C.p.55).

The [^{14}C]sucrose produced after incubation of developing potato tubers with [U-^{14}C]fructose (Table 5) was treated with invertase and the products separated by paper chromatography. In all cases the radioactivity in the fructose fraction was greater than in the glucose (Table 6). The original sucrose was therefore probably synthesised

via the sucrose synthetase reaction as the activity of the related enzyme, sucrose phosphate synthetase, is extremely low in developing tubers (42,72).

Experiment	Radioactivity (d.p.m.)		% recovery from [^{14}]sucrose
	Fructose	Glucose	
K	7,500	1,000	88
M	1,855	711	79
O	4,027	1,555	89

TABLE 6. Distribution of radioactivity in fructose and glucose produced by the invertase hydrolysis of [^{14}C]sucrose obtained after a [U-^{14}C]fructose injection into tubers (Table 5). For experimental details of the invertase hydrolysis see Materials and Methods, section XI.C.1.

Jaarma and Rydstrom (178) have also carried out experiments which suggest that the sucrose synthetase reaction occurs in potato tubers _in vivo_. They injected tubers with UDP-[U-^{14}C]glucose and showed that [^{14}C]sucrose was produced. In general, however, sucrose synthetase is thought to be more important _in vivo_ for the degradation rather than the synthesis of sucrose (see Introduction, p.22).

Dormant tubers injected with [U-^{14}C]fructose were unable to synthesise radioactive starch, although traces of [^{14}C]labelled ADP-glucose, UDP-glucose, glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate were identified in perchloric acid extracts of the tissues. This observation is not unexpected in view of the results obtained when dormant tubers were injected with [U-^{14}C]sucrose (see Results and Discussion V.A.p.51).

C. Metabolism of [U-^{14}C]sucrose in the presence of high concentrations of fructose

Developing potato tubers were injected with [U-^{14}C]sucrose dissolved on 0.1M fructose solution and then left at ambient temperature

for 15 min. If the fructosyl moiety of sucrose formed ADP-glucose preferentially, then it was thought that the UDP-glucose/ADP-glucose ratio would be increased in the presence of high levels of unlabelled fructose. The results of these experiments are shown in Table 7.

The average UDP-glucose/ADP-glucose ratio of 3.3 obtained when unlabelled fructose and [U-^{14}C]sucrose were injected together, in comparison to 1.9 for [U-^{14}C]sucrose alone (see Table 4), is further evidence to suggest that the fructosyl moiety of sucrose is converted preferentially to ADP-glucose.

From Table 7 it can be seen that the incorporation of label from [U-^{14}C]sucrose into starch, after 15 min, is only 1.7% in the presence of fructose compared to 2.9% in the absence of fructose (see Table 4). This relatively low incorporation presumably reflects the dilution of the labelled fructose component from [U-^{14}C]sucrose by the unlabelled fructose. Unexpectedly, the total activity of the free glucose obtained after incubating tubers with [U-^{14}C]sucrose alone is less than that obtained by using [U-^{14}C]sucrose with 'cold' fructose (Tables 4 and 6). The significance of this difference is difficult to explain. It may mean that the high level of unlabelled fructose which is introduced with the labelled sucrose into the tuber inhibits the cleavage of sucrose by sucrose synthetase. In this case invertase, particularly that released from the damaged tissues (249), plays the major role with the formation of significant amounts of glucose and fructose. These would then both be phosphorylated by hexokinase but concentration differences resulting from the introduction of 'cold' fructose could allow the fructose to be removed more rapidly than the glucose which would be reflected in the total radioactivities of the two monosaccharide fractions after incubation.

A probable sequence of events based on the literature (see Introduction) and encompassing all the observed results obtained with potato in the present study is shown in Fig.11.

The possible reason for the heavy labelling of the hexose phosphate fractions following [U-^{14}C]fructose injection (Table 5), in comparison with [U-^{14}C]sucrose (Table 4), is apparent in this scheme which shows a more direct conversion of the fructose moiety of sucrose, than the glucose moiety, to hexose phosphates. The greater radioactivity associated with fructose, compared to glucose, following injection of [U-^{14}C]sucrose, together with the observation that more label from

Experiment	µCi[U-^{14}C] sucrose injected	Conversion of [U-^{14}C] sucrose to starch (%)	ADP-glucose (d.p.m.)	UDP-glucose (d.p.m.)	Total NDP-glucose (d.p.m.)	d.p.m.UDPG / d.p.m.ADPG	Total hexose phosphates (d.p.m.)	d.p.m.Hexose phosphates / d.p.m. NDPG	Fructose (d.p.m.)	Glucose (d.p.m.)	d.p.m. Fructose / d.p.m. Glucose
Q	1.5	1.7	1,123	3,572	4,695	3.2	10,846	2.3	30,866	45,822	0.7
R	3.6	1.5	3,173	10,344	13,517	3.3	-	-	-	-	
S	6.7	1.9	4,100	13,867	17,967	3.4	44,444	2.5	11,299	15,397	0.7
Average*		1.7±0.01				3.3±0.8		2.4±0.1			0.7±0.0

TABLE 7. Distribution of [^{14}C] carbohydrate metabolites in developing potato tubers 15 min after injection of [U-^{14}C]sucrose (specific activity, 600µCi/µmol) in the presence of unlabelled fructose (0.1M). (-) indicates that no quantitative measurements of radioactivity were recorded. For experimental details see Table 4.

*Values are means ± the standard error of the sample.

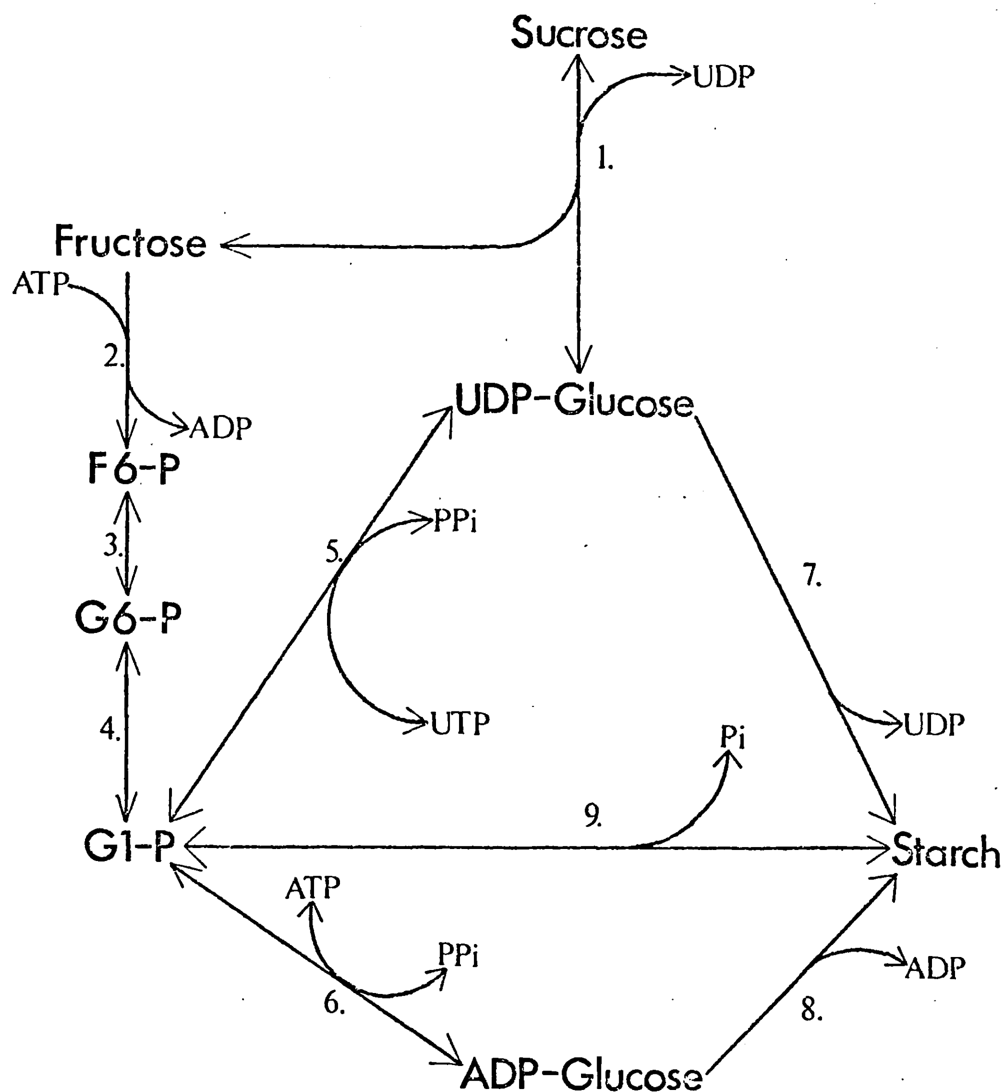


Fig. 11. Proposed *in vivo* pathway for the formation of starch from sucrose in the developing potato tuber. Numbers refer to the enzymes that catalyse each particular reaction: 1., Sucrose synthetase; 2., Hexokinase; 3., Phosphoglucose isomerase; 4., Phosphoglucomutase; 5., UDP-glucose pyrophosphorylase; 6., ADP-glucose pyrophosphorylase; 7., UDP-glucose:starch transglucosylase; 8., ADP-glucose:starch transglucosylase; 9., Starch phosphorylase.

fructose appears to enter ADP-glucose than from sucrose, is perhaps a further indication that the first enzyme in the reaction sequence is sucrose synthetase rather than invertase. However, if in these experiments invertase does play a major role in sucrose metabolism and produces significant amounts of glucose and fructose, then the difference in the radioactivities of the isolated glucose and fructose fractions could result from different rates of phosphorylation of these two hexoses. In this case one would expect to obtain the same ADP-glucose/UDP-glucose ratio from both fructose and sucrose provided both sugars feed into the same pools leading to the nucleoside diphosphate sugars.

The isolation of small quantities of radioactive glucose from tissues fed [U-^{14}C]sucrose may be an indication of invertase activity, perhaps only at the site of injection. Some glucose could arise from the breakdown of its derivatives during the extraction procedures.

D. Distribution of ^{14}C in the amylose and amylopectin fractions synthesised from [U-^{14}C]sucrose and [U-^{14}C]fructose.

The feeding studies discussed on pp. 53 and 55 suggest that ADP-glucose is formed preferentially from fructose and UDP-glucose from the glucose moiety of sucrose. If the theory proposed by Marshall (62) is correct, one would expect label to be incorporated preferentially from fructose into amylopectin and from sucrose into amylose. Hence, developing potato tubers were fed with [U-^{14}C]sucrose and with [U-^{14}C] fructose and the starch isolated and fractionated into amylopectin and amylose in both instances. Measurement of the radioactivity of the two fractions gave the results shown in Table 8.

Injected [U-^{14}C]carbohydrate	Starch (c.p.m./mg)	Amylose (c.p.m./mg)	Amylopectin (c.p.m./mg)	[^{14}C]Amylopectin / [^{14}C]Amylose
Sucrose	0.2	0.3	0.2	0.66
Fructose	4.2	5.0	4.0	0.80

TABLE 8. Distribution of radioactivity in amylose and amylopectin fractions following a 30 min incubation with either [U-^{14}C] fructose or [U-^{14}C]sucrose. Experimental details and characterization of the fractions is described in Materials and Methods, sections X.D.1.-3.

As in previous experiments, the rate of conversion of [U-^{14}C]fructose to starch was much faster than the rate of conversion of [U-^{14}C]sucrose. The difference between the [^{14}C]amylopectin/[^{14}C]amylose ratios obtained by feeding [U-^{14}C]fructose and [U-^{14}C] sucrose were insignificant.

VI. In vitro studies with potato enzymes

Feeding experiments with sucrose and fructose suggested different pathways (see Fig. 11) for the conversion of these sugars to starch in the developing potato tuber. Hence, an examination of some of the enzyme activities associated with the proposed pathways was next carried out with a view to producing an in vitro system for the synthesis of starch from sucrose and fructose.

A. Sucrose synthetase

Sucrose synthetase was extracted from tubers and assayed by a radiochemical method using [U-^{14}C]fructose as a substrate (see Materials and Methods, section XI.B.1. and XI.B.w.(ii)). The radioactive product, obtained by incubating [U-^{14}C]fructose with either ADP-glucose or UDP-glucose as glucose donors, was identified as [^{14}C]sucrose by paper chromatography (see Materials and Methods, section CI.V.3.). The sucrose was further characterised by invertase hydrolysis which yielded [^{14}C]fructose (Fig. 10). The products obtained by incubating the enzyme preparation with [U-^{14}C]sucrose and either ADP or UDP were [^{14}C]fructose and the corresponding radioactive nucleoside diphosphate glucose (see Materials and Methods, section XI.B.3.).

The specific activity of the enzyme was found to be much higher in immature developing tubers than in dormant tubers (Table 9). The protein levels in both types of tuber were essentially similar. These results are in agreement with the observations of Pressey (42,72). The enzyme from both mature and developing tubers showed a greater specificity for uridine than adenine nucleotides (Table 9); this is also in agreement with the results of other workers (7,40,42,85,90).

The evidence that sucrose synthetase catalyses the initial reaction in the conversion of sucrose to starch in higher plants is convincing (see Introduction, p.22 and Results and Discussion, pp. 53, 55 and 58). Since the activity of the tuber enzyme with ADP as substrate is

Substrates	Developing tubers		Dormant tubers	
	Specific activity*/	Activity/g fresh weight	Specific activity*/	Activity/g fresh weight
[U-^{14}C] sucrose + ADP	55	11	0	0
[U-^{14}C] sucrose + UDP	547	109	19	4
[U-^{14}C] fructose + ADP-glucose	26	5	15	3
[U-^{14}C] fructose + UDP-glucose	215	43	23	5

TABLE 9. Sucrose synthetase levels in developing and dormant tubers measured in both the synthetic and cleavage directions using either adenine or uridine nucleotide derivatives.

*The specific activity for the cleavage reaction is expressed in nmol fructose liberated/h/mg protein.

/The specific activity for the synthetic reaction is expressed in nmol of sucrose formed/h/mg protein.

For experimental details see Materials and Methods, sections XI.B.1-2.

inhibited by both UDP and UDP-glucose (40,41,85,95,246), it is perhaps unlikely that ADP-glucose would be synthesised by this reaction _in vivo_ (40,41). The specificity of the enzyme for UDP rather than ADP can be equated with the observations which suggest that sucrose is a better precursor of UDP-glucose than ADP-glucose (see Results and Discussion, sections V.A.-V.C.pp.46-58). The fructose liberated from the sucrose synthetase reaction is presumably phosphorylated and converted to the nucleoside diphosphate sugars via glucose 1-phosphate.

B. Fructose phosphorylation and the formation of ADP-glucose

A crude protein fraction from developing tubers was incubated with [U-^{14}C]fructose and ATP (see Materials and Methods, section XI.D.1.). Radioactive ADP-glucose, glucose 1-phosphate, glucose 6-phosphate and fructose 6-phosphate in the resulting reaction mixture were identified

by paper chromatography (Fig. 12). The identity of ADP-glucose was confirmed by high voltage paper electrophoresis (Fig. 13). Glucose 6-phosphate and fructose 6-phosphate, obtained from paper chromatograms, were further characterised by anion-exchange chromatography on Dowex-1 (Cl^-) resin (Fig. 14). The identification of fructose 6-phosphate, glucose 6-phosphate, glucose 1-phosphate, and ADP-glucose in the reaction mixture is supporting evidence for the proposed pathway, given in Fig. 11, for the conversion of fructose to starch.

The incorporation of radioactivity from [U-^{14}C]fructose into ADP-glucose and the hexose phosphates is very rapid during the first 5 min (Fig. 15) and there is little further incorporation after this time. The initial phosphorylation of fructose is presumably rapid and possibly is inhibited after 5 min incubation, by the accumulation of ADP.

The initial high level of [^{14}C]glucose 1-phosphate and its steady decline, together with the concomitant increase in [^{14}C]ADP-glucose, probably reflects the substrate-product relationship of these metabolites and the relatively slow velocity of the pyrophosphorylase reaction as compared to the other enzymic activities involved in the formation of ADP-glucose.

The experiment was repeated using an enzyme preparation from mature tubers and here the incorporation of label from [U-^{14}C]fructose into [^{14}C]ADP-glucose and [^{14}C]hexose phosphates was less than in the case of the developing tuber preparations (Table 10). The low incorporation of label prevented a detailed time course investigation with mature tuber preparations. The results of all these in vitro experiments can be related to the feeding studies with [U-^{14}C]fructose (see Results and Discussion, section V.B.p. 51), which showed that fructose can be rapidly converted to starch in developing tubers but not in mature tubers.

No previous study of the conversion of fructose into ADP-glucose by potato tubers has been reported, but de Fekete (11) has commented that adequate levels of hexokinase, phosphohexoseisomerase and phosphoglucomutase are present in Vicia faba cotyledons to allow the fructose moiety of sucrose to serve as an important starch precursor. The importance of these enzymes in the formation of starch from sucrose in the potato tuber is indicated by the dramatic fall in the conversion of fructose to glucose 1-phosphate that follows the cessation of both sucrose translocation and tuber growth.

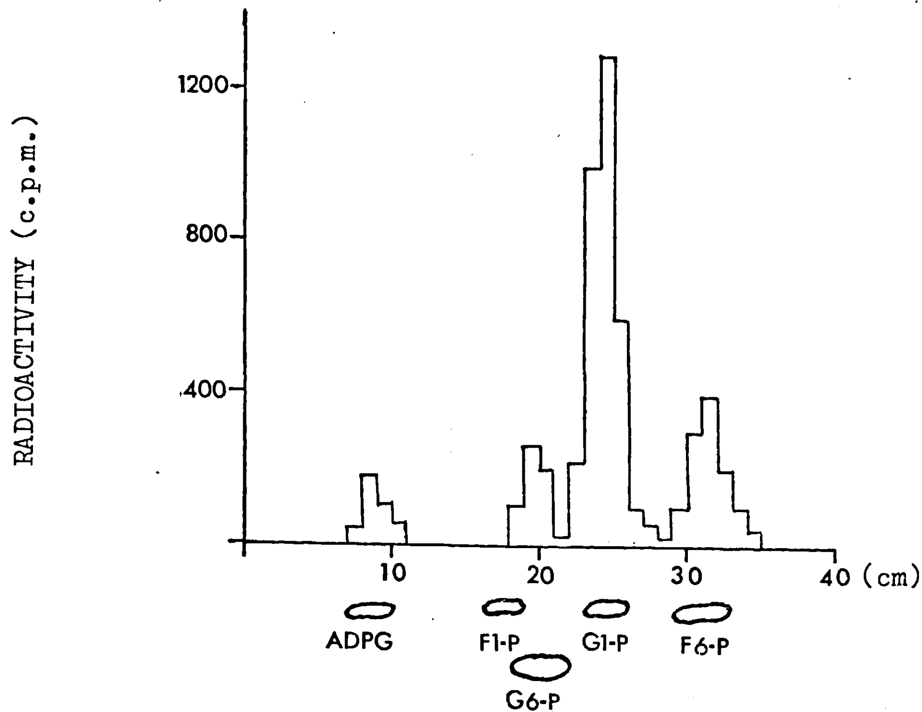


Fig. 12. Paper chromatographic separation of the radioactive products obtained by incubating (5 min) [U-^{14}C]fructose and ATP with a crude enzyme preparation (100µg protein) from developing potato tubers. See Materials and Methods, section XI.D.1. for experimental details.

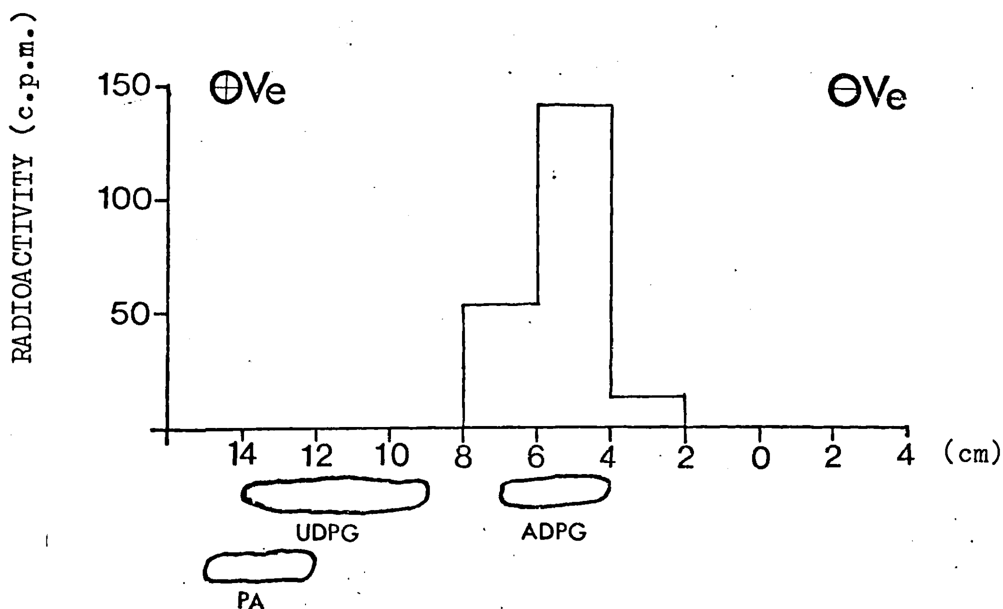
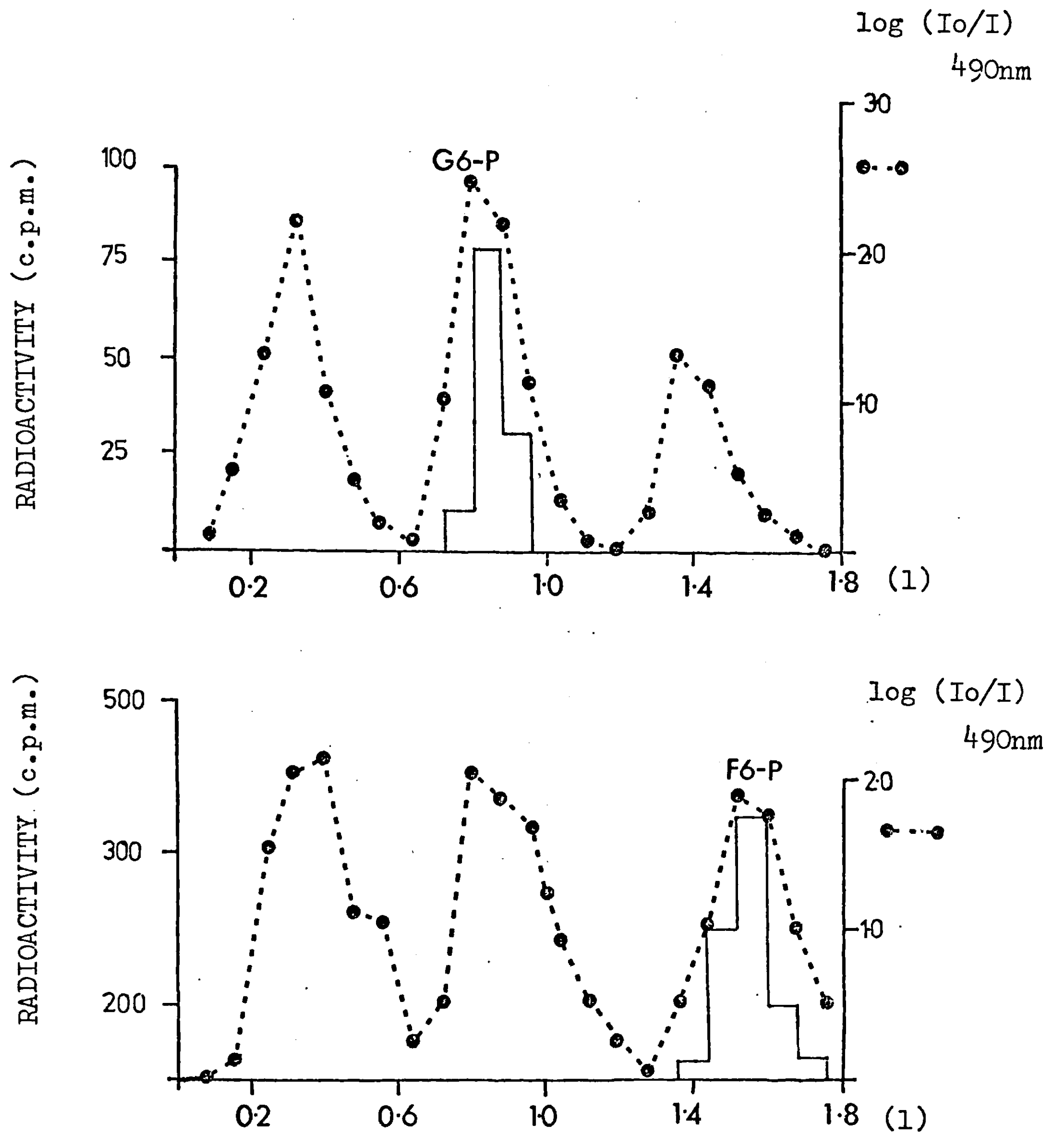


Fig. 13. Paper electrophoresis of ADP-glucose obtained from the reaction described in Fig. 12. See Materials and Methods, section XI. D.3. for experimental details.



<u>Fig. 14.</u> Dowex-1(Cl^-) anion-exchange chromatography of products obtained by incubating [U-^{14}C]fructose and ATP with a crude protein fraction from developing tubers. For experimental details see Materials and Methods, section XI.D.3.

Enzyme source	Radioactive products (d.p.m.)				
	ADP-glucose	Fructose 6-phosphate	Glucose 6-phosphate	Glucose 1-phosphate	Total
Mature tuber	175	416	241	178	1010
Developing tuber	1620	854	777	3030	6481

TABLE 10. Analysis of the radioactive products derived from the incubation (15 min) of [U-^{14}C]fructose and ATP with a crude enzyme preparation from developing (100μg protein) and mature (110μg protein) tubers. Experimental details are described in Materials and Methods, section XI.D.2.

C. <u>Sucrose cleavage and the formation of ADP-glucose</u>

This series of experiments was designed to demonstrate the conversion of fructose, produced by the reversed sucrose synthetase reaction, to hexose phosphates and ADP-glucose, using protein preparations from developing tubers.

In the first instance an attempt was made to demonstrate sucrose cleavage in the presence of UDP and under the conditions of pH(7.5)and Mg^{2+} concentration (10.5mM) required for fructose phosphorylation and subsequent formation of ADP-glucose. The experiment was successful as shown in Fig. 16.

Incubation of the crude enzyme preparation with sucrose specifically labelled in the fructose moiety (α-D-glucopyranosyl-[U-^{14}C]β-D-fructofuranoside), ATP and UDP resulted in the formation of radioactive ADP-glucose, glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate (Fig. 17).

The identification of radioactive ADP-glucose, glucose 6-phosphate and fructose 6-phosphate was made by paper electrophoresis and ion-exchange chromatography (see Materials and Methods, section XI.D.3).

With increasing incubation time (Fig. 17) the pattern of product formation closely resembled that obtained by incubating [U-^{14}C] fructose with ATP (Fig. 15). Hence, the fructose moiety of sucrose

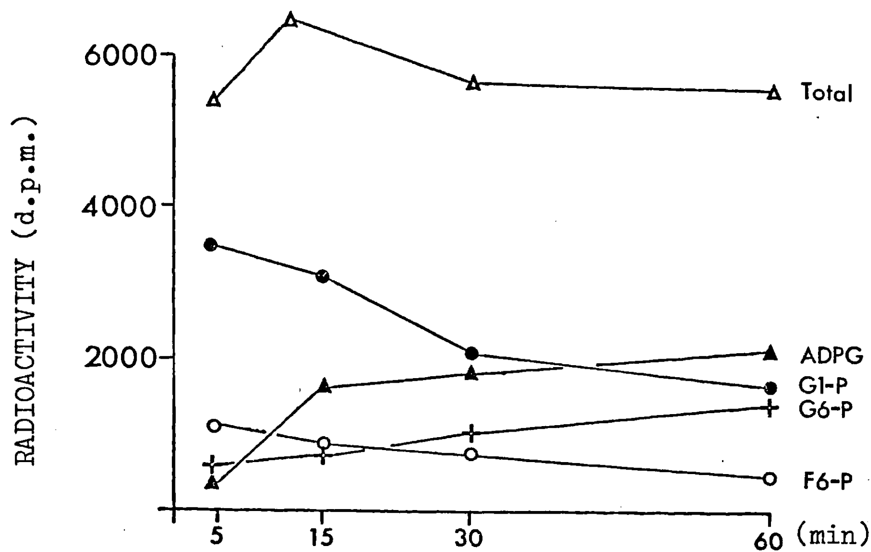


Fig. 15. Time course analysis of the radioactive products from the incubation of [U-^{14}C]fructose and ATP using a crude enzyme preparation (100µg protein) from developing tubers. Experimental details are described in Materials and Methods, section XI.D.1.

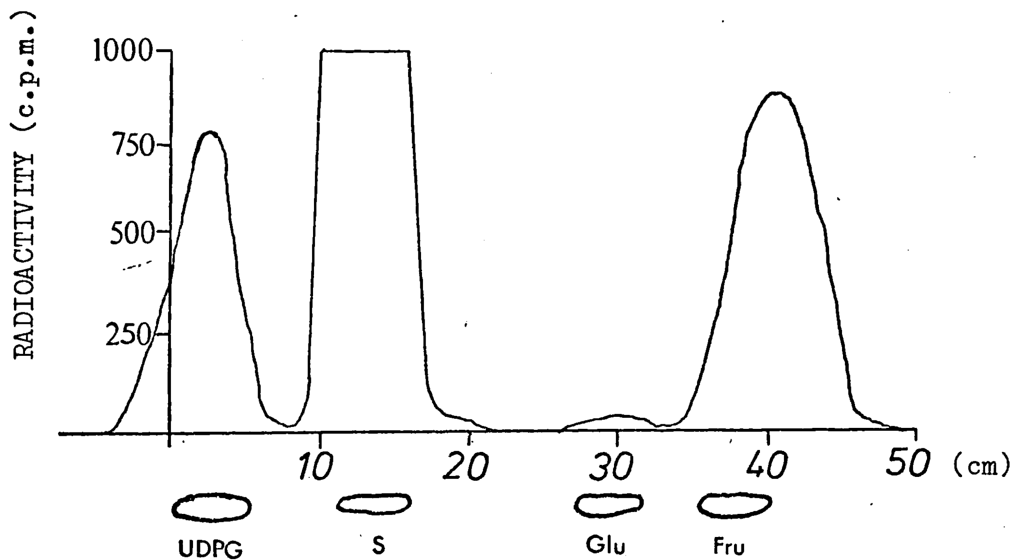


Fig. 16. Paper chromatographic separation (solvent (iii)) of the products obtained by incubating [U-^{14}C]sucrose and UDP with an enzyme preparation from developing tubers at pH 7.5 and a Mg^{2+} concentration of 10.5mM, see Materials and Methods, section XI.E.1.

can be converted to ADP-glucose in the presence of UDP, ATP and a soluble tuber protein preparation. A comparison of Figs. 15 and 17 suggests that sucrose synthetase activity is not rate limiting under the conditions used.

The experiment was repeated using an enzyme preparation from mature tubers. As expected, the formation of [^{14}C]ADP-glucose and [^{14}C]hexose phosphates from the [^{14}C]sucrose was found to be much lower (Table 11), and further kinetic investigations were, therefore, not possible. This can be correlated with the fact that exogenous sucrose was more readily converted to starch by developing tubers than by mature tubers (see Results and Discussion, section V.A.p. 51).

Enzyme source	Radioactive Products (d.p.m.)				
	ADP-glucose	Fructose 6-phosphate	Glucose 6-phosphate	Glucose 1-phosphate	Total
Mature tubers	69	180	218		467
Developing tubers	2061	1326	1205	3444	8036

TABLE 11. Analysis of the radioactive products formed from the incubation of [^{14}C]sucrose, ATP and UDP with crude enzyme preparations from mature (110µg protein) and developing (134µg protein) tubers. Experimental details are described in Materials and Methods, section XI.E.1.

The results of the tracer experiments *in vivo* with tubers (see Results and Discussion, sections V.A.-V.C,pp. 46-58) and the *in vitro* experiments using a soluble protein preparation (see Results and Discussion, section VI.A.-VI.C.pp. 59 - 66) are consistent with the view that sucrose synthetase is the first step in the conversion of sucrose to starch in the tuber and that the liberated fructose moiety is converted to starch via the sequence of reactions shown in Fig. 11.

ADP-glucose and UDP-glucose appear to be intermediates in the conversion of sucrose to starch. Hence some of the properties of potato ADP-glucose and UDP-glucose pyrophosphorylase were next examined.

D. ADP-glucose pyrophosphorylase

The enzymic synthesis of ADP-glucose from glucose 1-phosphate and ATP was demonstrated (Fig. 18) using a crude homogenate of developing potato tubers (see Materials and Methods, section XI.F.1.). The [^{14}C]ADP-glucose formed was eluted from the chromatography paper and further characterized by paper chromatography and paper electrophoresis (Fig. 19).

The effect of glycolytic intermediates on a 60-75% $(NH_4)_2SO_4$ protein fraction from developing potato tubers was examined (Table 12, cf. Preiss *et al.* (52)).

Glycolytic intermediate (2.67mM)	Specific activity (nmol ADP-glucose produced /h/ mg protein
No addition	3.2
Fructose 1,6-diphosphate	5.5
Fructose 6-phosphate	4.9
PEP	4.2

TABLE 12. Effect of glycolytic intermediates on ADP-glucose pyrophosphorylase activity (60-75% $(NH_4)_2SO_4$ fraction). For experimental details see Materials and Methods sections XI.F.1. and XI.F.2.(ii).

Fructose 1,6-diphosphate, fructose 6-phosphate and PEP increased the specific activity of the enzyme, as has been shown with ADP-glucose pyrophosphorylase from other plant sources (52).

An attempt was made to further purify the enzyme from developing potato tubers but this was largely unsuccessful because of its high instability (see Results and Discussion, section IV.D.p. 70). However, several interesting observations, which are worthy of comment, were made.

Starting with a crude tuber extract the specific activity of the ADP-glucose pyrophosphorylase could not be raised by $(NH_4)_2SO_4$ fractionation. When a 40-80% $(NH_4)_2SO_4$ fraction was chromatographed on a DEAE-cellulose column, using a gradient of pH and buffer molarity, two main fractions with ADP-glucose pyrophosphorylase activity were obtained (Fig. 20). Most of the activity was associated with the more acidic fraction (II).

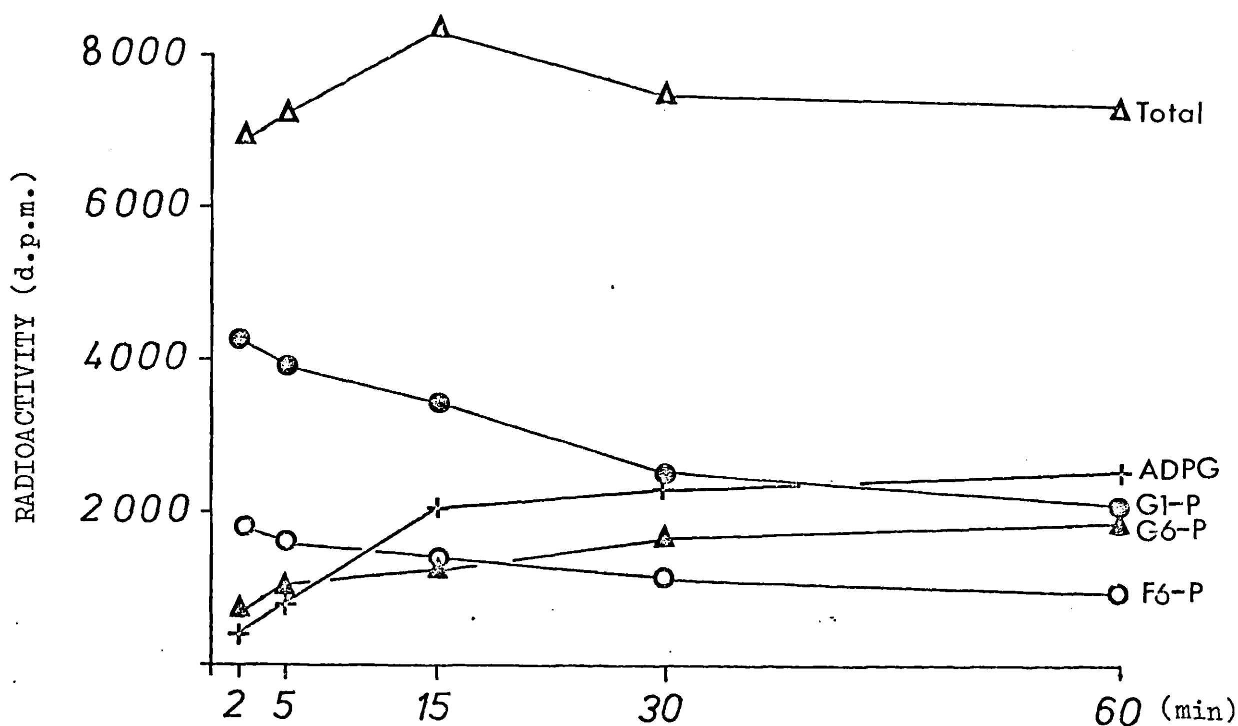


Fig. 17. Time course analysis of the radioactive products formed by incubation of α-D-glucopyranosyl-[U-^{14}C]β-D-fructofuranoside, ATP and UDP with a crude enzyme preparation (134μg protein) from developing potato tubers. Experimental details are described in Materials and Methods, section XI.E.1.

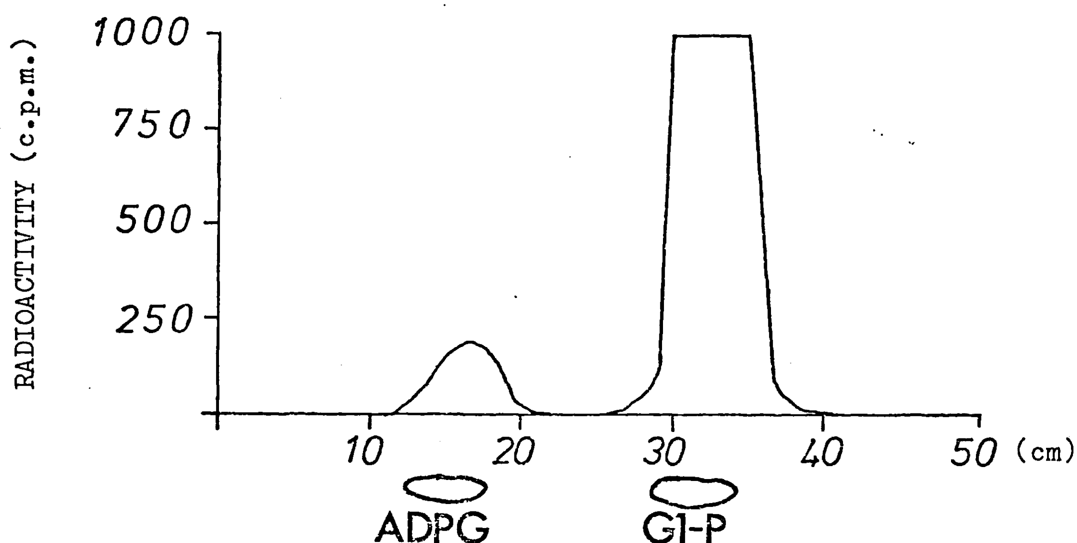


Fig. 18. Paper chromatographic examination of the products obtained by incubating [U-^{14}C]glucose 1-phosphate and ATP with a crude potato tuber homogenate. For experimental details see Materials and Methods, sections X.F.1. and XI.F.2.(ii).

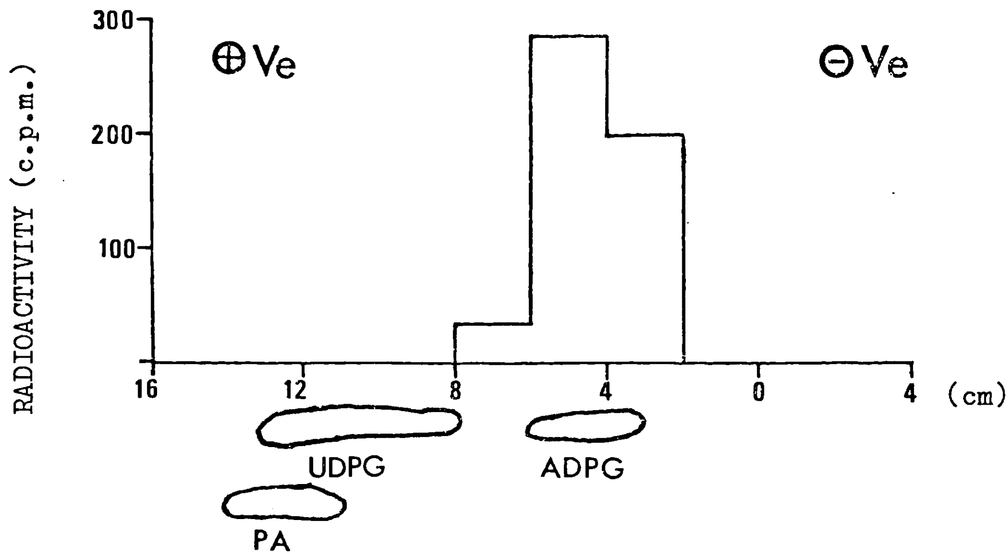


Fig. 19. Identification, by paper electrophoresis, of ADP-glucose produced by the reaction of [U-^{14}C]glucose 1-phosphate and ATP in the presence of a crude potato tuber enzyme preparation. For experimental details see Materials and Methods, section XI.F.3.

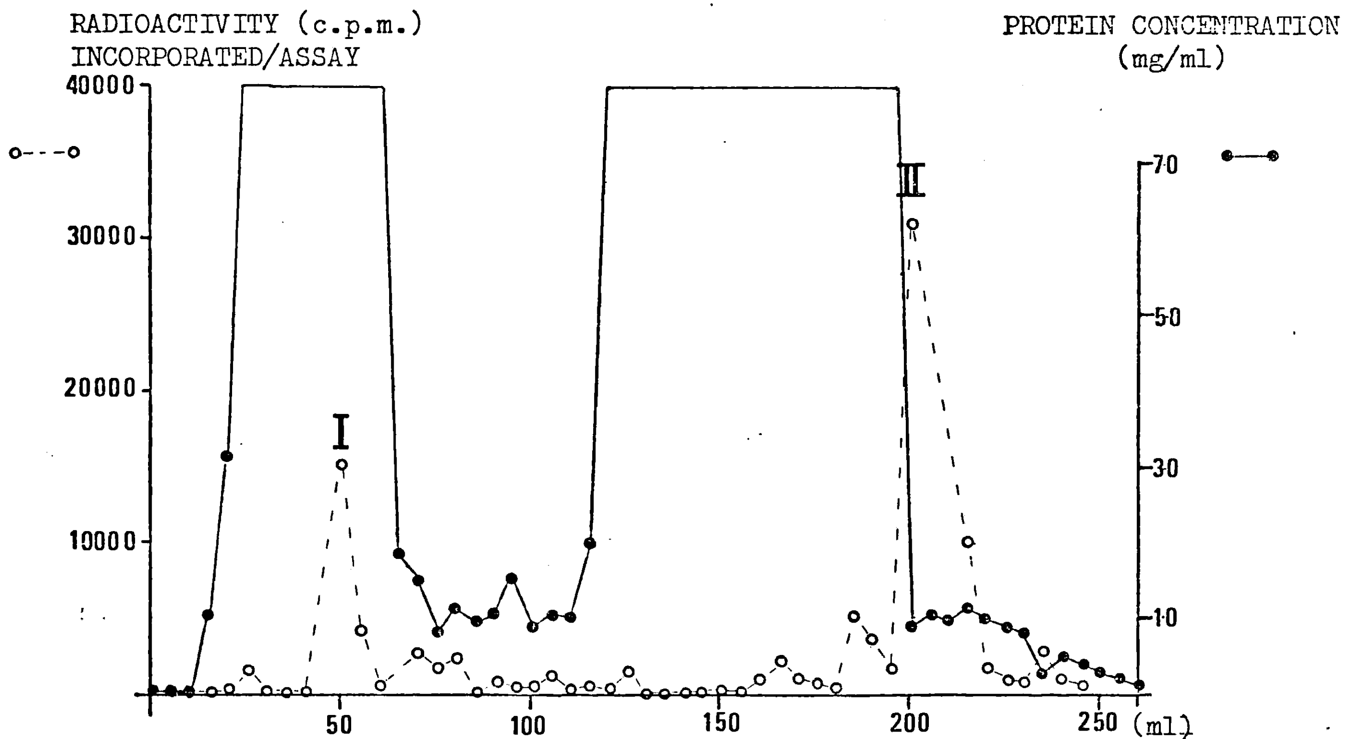


Fig. 20. DEAE-cellulose column chromatography of 40-80% saturated $(NH_4)_2SO_4$ protein fraction from developing potato tubers. The column was eluted with a pH-concentration gradient (10mM tris-succinate, pH 7.5 to 50mM tris-succinate, pH 5.8). See Materials and Methods, section XI.F.1.(i) and XI.F.2.(i).

Storage of fraction II for 3 days at 4°C or at -15°C resulted in a complete loss of activity. Several unsuccessful attempts were made to stabalize the enzyme, for example, by the addition of ATP, KCl or phosphate buffer.

Fraction II (Fig. 20) was examined for specificity with respect to nucleoside triphosphates, soon after elution from the DEAE-cellulose column, using ATP, GTP, UTP, dTTP and CTP. The results (Table 13) showed that the enzyme fraction was most specific for UTP. However, the possibility that the fraction contained several pyrophosphorylases with different stabilities was considered and investigated. Table 14 shows the reaction of fresh and aged samples of the enzyme fraction on UTP and ATP. It was clear that it contains two activities (enzymes?) and that the one yielding ADP-glucose is much more labile than that producing UDP-glucose.

Nucleoside triphosphate substrate	Pyrophosphorylase specific activity (nmol NDP-glucose formed/h/mg protein)
ATP	0.4
GTP	2.8
UTP	50.6
dTTP	0.0
CTP	1.0

TABLE 13. Nucleoside triphosphate specificity of pyrophosphorylase fraction II (Fig. 20). The assays were performed as described in Materials and Methods, section XI.F.2.(ii). Fraction II was obtained from a DEAE-cellulose column (Fig. 20) and saturated to 80% with $(NH_4)_2SO_4$. The precipitated protein was collected by centrifugation, redissolved in buffer A and dialysed against this same buffer overnight. The dialysed protein solution was used as the enzyme source.

ADP-glucose- and UDP-glucose-pyrophosphorylase activities (d.p.m. of NDP-glucose*)			
Fraction II immediately eluted from column		Fraction II after storage (3 days, 4°C)	
UDP-glucose	ADP-glucose	UDP-glucose	ADP-glucose
26,666	33,333	15,111	111

TABLE 14. Comparison of ADP-glucose and UDP-glucose pyrophosphorylase activities of Fraction II (Fig. 20) immediately on elution from the DEAE-cellulose column and after storage.

*Radioactivity in product after a 30 min incubation of the reaction mixture at 30°C (see Materials and Methods, sections, XI.F.1.(i) and XI.F.2.(i), for experimental details.

A further examination of the effect of fructose 1,6-diphosphate on ADP-glucose pyrophosphorylase, using fraction II (Fig. 20), again showed that the enzyme was strongly activated but the UDP-glucose pyrophosphorylase activity, which was also present in this fraction, was unaffected by the sugar phosphate (Table 15).

Fructose 1,6-diphosphate (mM)	Specific Activity (nmol NDP-glucose formed/h/mg protein)	
	ADP-glucose pyrophosphorylase	UDP-glucose pyrophosphorylase
0.0	0.4	50.6
2.67	1.4	52.8

TABLE 15. Specific activities of ADP-glucose and UDP-glucose pyrophosphorylases in fraction II (Fig. 20) in the presence and absence of fructose 1,6-diphosphate. The enzyme solution was prepared as for Table 13 and assays were performed as described in the Materials and Methods, section XI.F.2.(i).

No ADP-glucose pyrophosphorylase activity could be detected in crude enzyme preparations from dormant tubers. This could be due to absence of the enzyme or the presence of an endogenous inhibitor (54): in either case it presumably relates to the lack of starch synthesis in these tissues.

From the present study, it is apparent that the properties of the ADP-glucose pyrophosphorylase from developing tubers are similar to those of the enzyme from other non-photosynthetic tissues. However, only tentative conclusions may be drawn because of the unstable nature of the potato enzyme.

E. UDP-glucose pyrophosphorylase

UDP-glucose pyrophosphorylase activity in developing potato tubers was determined by incubating a crude tuber homogenate with [U-^{14}C]glucose 1-phosphate and UTP. The UDP-glucose produced was identified by paper chromatography (Fig. 21) and by high voltage paper electrophoresis (Fig. 22).

The UDP-glucose pyrophosphorylase activity in the crude homogenate was resolved into several fractions by the use of $(NH_4)_2SO_4$ (Table 16). Most of the enzyme activity was precipitated between 0 and 50% saturation but a significant proportion (16%) was also precipitated between 50% and 100% saturation. The specific activities of the crude enzyme and the $(NH_4)_2SO_4$ fractions were all increased by the addition of fructose 1,6-diphosphate but the fractions obtained between 50 and 100% saturation were activated to a much greater extent than those obtained with the lower salt concentrations. The activation profile obtained with fructose 1,6-diphosphate suggests that two forms of the enzyme are present in the crude homogenate.

The activation of UDP-glucose pyrophosphorylase, from any source, by fructose 1,6-diphosphate, or other sugar phosphates, has not previously been reported. It may, of course, be due to the interaction of fructose 1,6-diphosphate with another contaminating, regulatory nucleoside diphosphate glucose pyrophosphorylase which has a low specificity with regard to nucleoside triphosphate substrates. This is unlikely, however, as most pyrophosphorylases appear to possess high specificities towards their substrates (54,55).

The two protein fractions which were obtained from a crude tuber homogenate by $(NH_4)_2SO_4$ precipitation (0-50% and 50-100% saturation) were further purified. They were subjected separately to

Fraction	Total activity (units/g fresh weight)	Specific activity (µmol UDP-glucose/h/mg protein)		Activation by fructose 1,6-diphosphate (%)
		+F1,6-di Pi	-F1,6-di Pi	
Crude	-	40.7	20.2	101
$(NH_4)_2SO_4$ 0-30	699	21.0	13.7	53
30-40	883	25.2	12.0	110
40-50	596	13.3	8.2	62
50-60	132	40.1	4.8	735
60-70	106	167.5	7.4	2163
70-100	181	86.1	7.6	1032

TABLE 16. Specific and total activities of UDP-glucose pyrophosphorylase fractions and activation by fructose 1,6-diphosphate (2.67mM). For experimental details see Materials and Methods, sections XI.G.1. and XI.G.2. (XI.F.2.(ii)).

chromatography on a Sephadex G-100 column and the results suggest that multiple forms of the pyrophosphorylase exist (Fig. 23). The 0-50% $(NH_4)_2SO_4$ fraction was partially resolved into two components with molecular weights corresponding to 17,800 and 20,000 (Enzymes I and II, respectively), whereas the 50-100% fraction gave one peak of activity with an estimated molecular weight of 45,700, (Enzyme III).

Enzyme III was chosen for further study, because on gel-filtration it appeared as a single peak of activity and because it was strongly stimulated by fructose 1,6-diphosphate.

The literature (44) suggested that the Mg^{2+} content of the UDP-glucose pyrophosphorylase assay mixture would be critical and that the ion could have a regulatory function. Hence, the effect of Mg^{2+} on the activity of form III, of the enzyme, was first examined.

Enzyme III from a Sephadex G-100 column was concentrated by ultrafiltration and the activity was assayed at different Mg^{2+} concentrations. Maximal activity was observed in the presence of 1.35mM $MgCl_2$, (Fig. 24) and, hence, this level of Mg^{2+} ion was used for all subsequent enzyme assays.

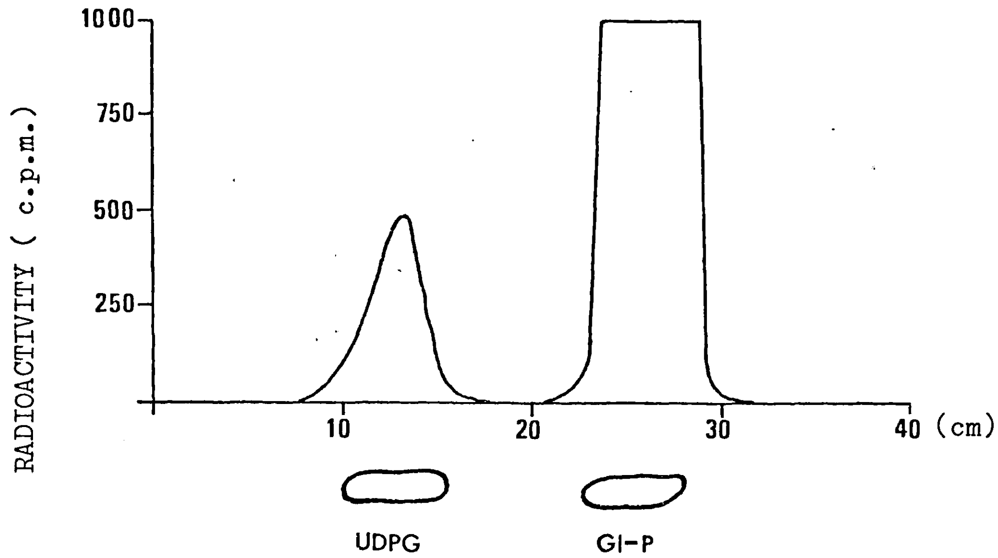


Fig. 21. Paper chromatographic identification of the radioactive product formed after incubating [U-^{14}C]glucose 1-phosphate and UTP with a crude tuber homogenate. For experimental details see Materials and Methods, sections XI.G.1. and XI.G.2. (XI.F.2.(ii)).

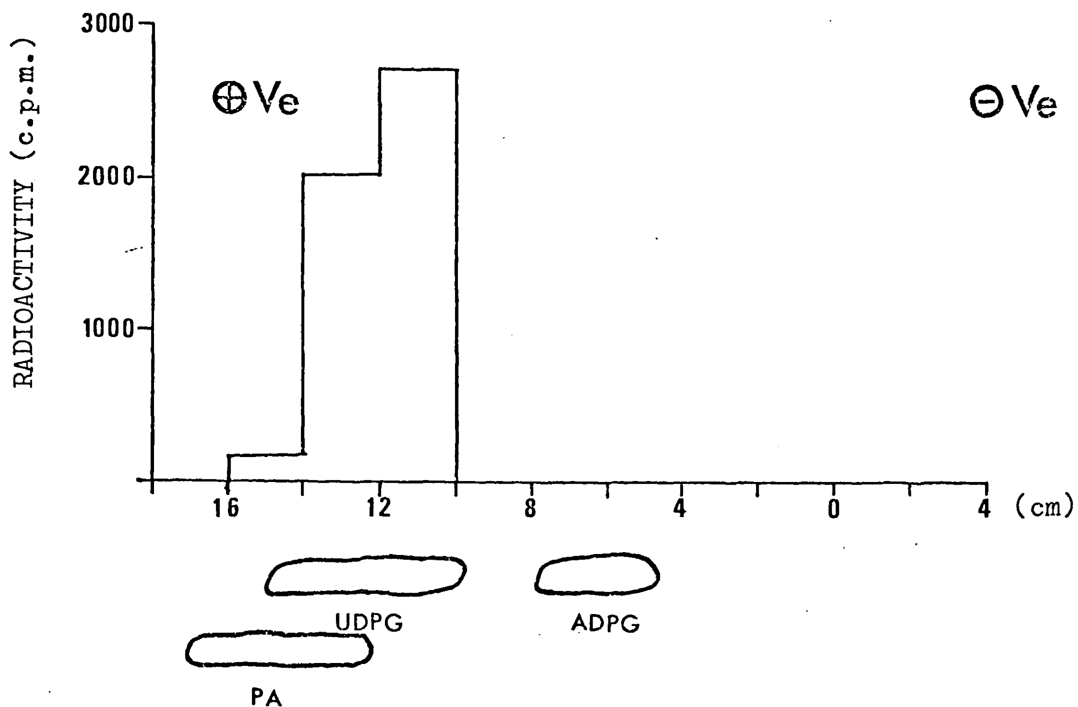


Fig. 22. Paper electrophoretic identification of UDP-glucose obtained as described in Fig. 20. For experimental details see Materials and Methods, section XI.G.3.

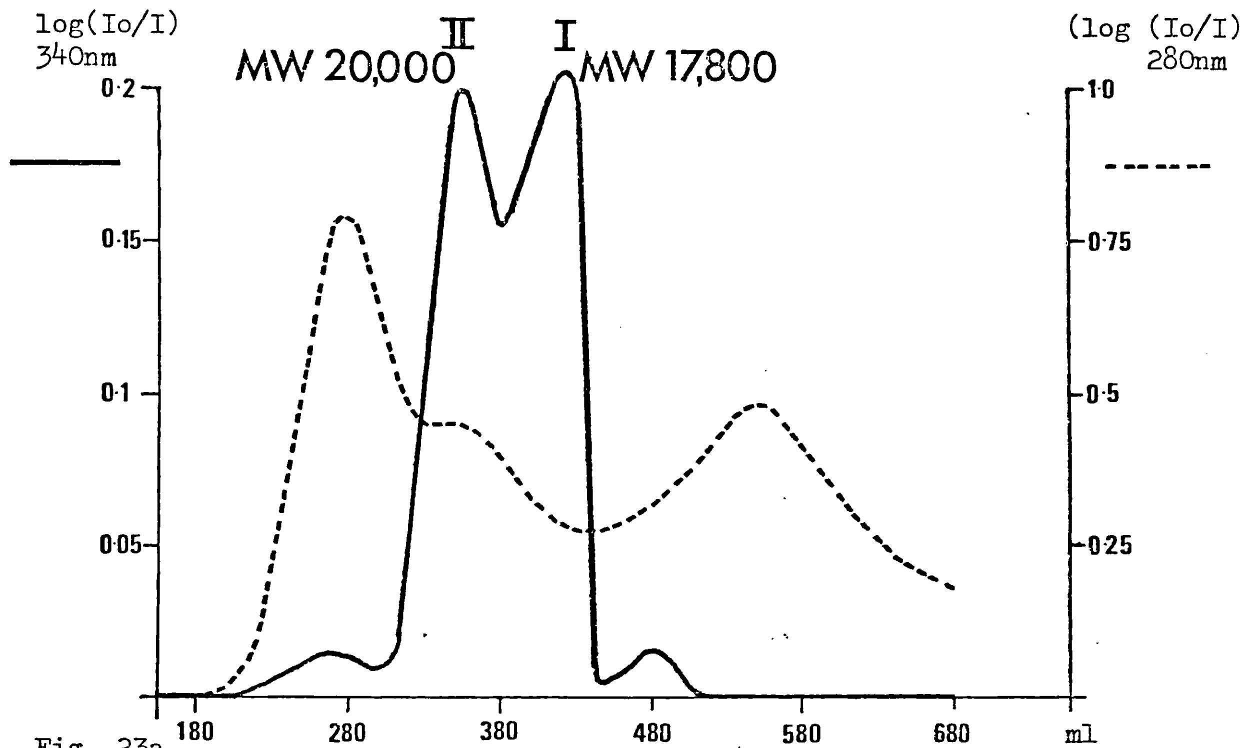


Fig. 23a

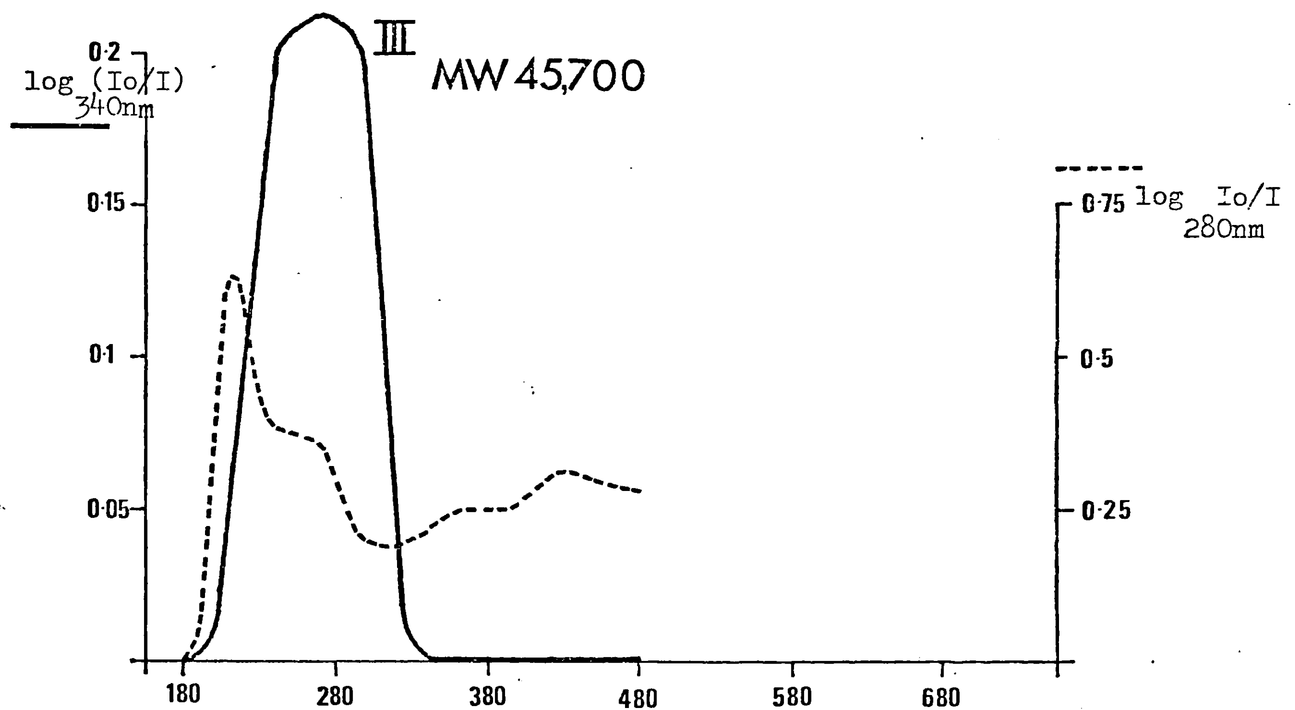


Fig. 23b

Fig. 23. Elution profiles of potato tuber UDP-glucose pyrophosphorylase from a Sephadex G-100 column. Fig. 23a), resolution of a 0-50% $(NH_4)_2SO_4$ fraction from a crude developing tuber homogenate; Fig. 23b), pattern obtained with a 50-100% $(NH_4)_2SO_4$ fraction from the crude homogenate. For experimental details see Materials and Methods, sections XI.G.1. and XI.G.2.

Attempts were next made to improve the purity of enzyme III (Table 17). The $(NH_4)_2SO_4$ fractionation of the crude enzyme and the Sephadex G-100 chromatography stages were repeated as before. The marked decrease in total activity resulting from the $(NH_4)_2SO_4$ step presumably occurred because forms I and II were discarded. Enzyme III, obtained from the Sephadex G-100 column, was concentrated and applied to a DEAE-cellulose column which was eluted with increasing concentrations of sodium chloride (0.0M-0.7M) (Fig. 25). A broad peak of activity came off the column and a 7-fold increase in specific activity was obtained. This fraction was then concentrated and applied to a second DEAE-cellulose column which was eluted with a tris-succinate gradient (10mM, pH 8.0 - 50mM, pH 6.0); four active enzyme fractions resulted (III_A, III_B, III_C and III_D; Fig. 26).

The activities of these forms of the pyrophosphorylase were checked for nucleotide specificity by assaying with UTP, CTP, ATP, GTP and dTTP as substrates (Table 18). The preferred substrate in each case was UTP.

It appears therefore, that the developing potato tuber does possess multiple forms of UDP-glucose pyrophosphorylase, although the possibility that some of the forms are artifacts produced by the purification procedures should not be ruled out.

The initial work on UDP-glucose pyrophosphorylase was carried out using actively growing tubers, but by the time a suitable purification scheme for the enzyme had been developed the tubers were approaching maturity. The activation by fructose 1,6-diphosphate, which had been observed previously with immature tissue enzyme (Table 16), did not occur with crude, $(NH_4)_2SO_4$,(50-100%) or Sephadex (enzyme III) fractions prepared from these more mature tubers; the latter two fractions appeared to be significantly inhibited by the sugar phosphate (Table 19). However, the ADP-glucose pyrophosphorylas of these more mature preparations could still be stimulated by fructose 1,6-diphosphate (Table 19).

An unsuccesful attempt was made to discover why fructose 1,6-diphosphate no longer activated UDP-glucose pyrophosphorylase preparations from mature tubers. However, during this part of the investigation enzyme digests were examined on paper chromatograms which revealed the presence of an unknown radioactive material (X), (Fig. 27).

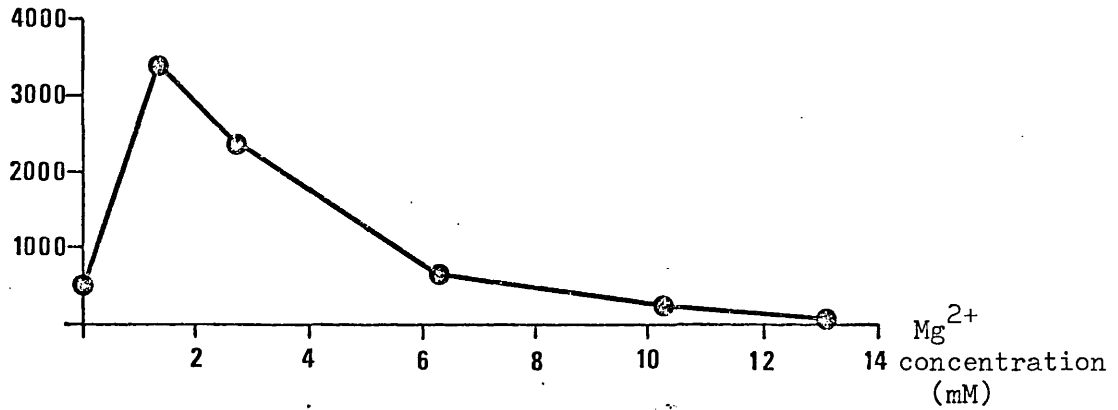


Fig. 24. Effect of $MgCl_2$ on UDP-glucose pyrophosphorylase activity. Enzyme III was prepared from a crude tuber homogenate by $(NH_4)_2SO_4$ fractionation, Sephadex G-100 chromatography and ultrafiltration as described in Materials and Methods, section XI.G.1. Assays were carried out as described in XI.G.2. (XI.E.2.(ii)) except that the $MgCl_2$ concentration was varied.

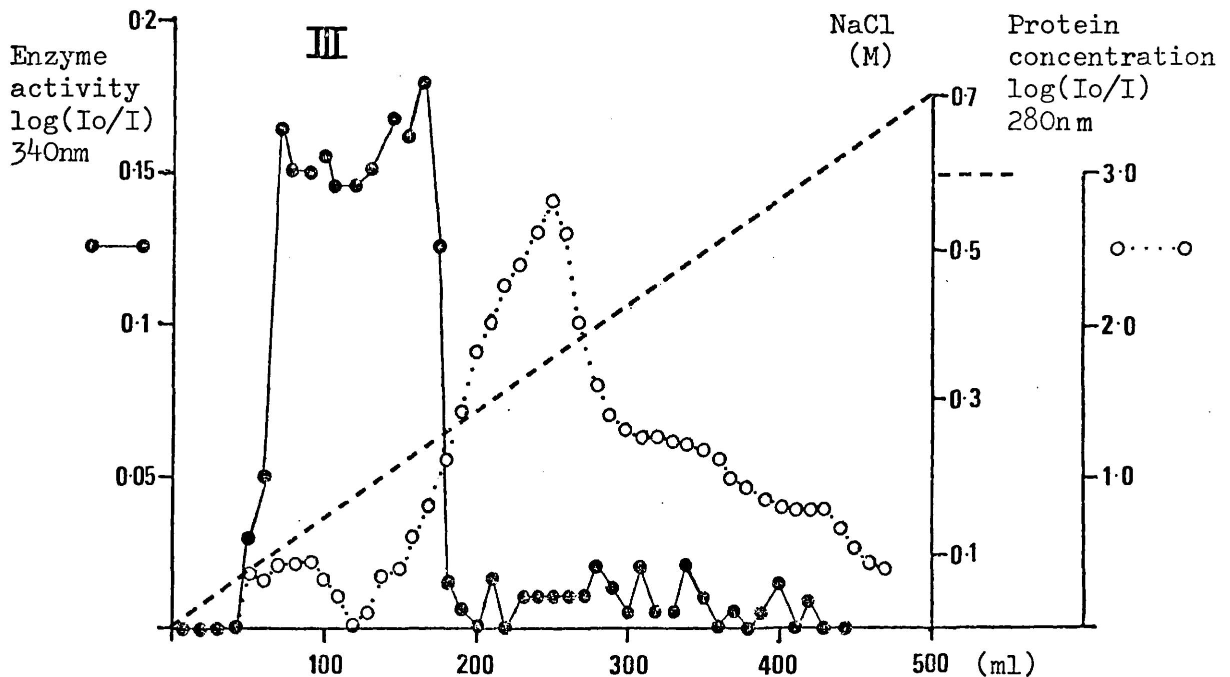


Fig. 25. First DEAE-cellulose fractionation of UDP-pyrophosphorylase III obtained from a Sephadex G-100 column. See Materials and Methods, section XI.G.1.(i) for experimental details.

Fraction	Volume (ml)	Protein concentration (mg/ml)	Specific activity (nmol UDP-glucose/min/mg protein	% Recovery
Crude	305	1.5	97.4	100
50-100% $(NH_4)_2SO_4$	55	7.5	10.6	10
Sephadex chromatography (Enzyme III)	4	10.0	6.9	0.6
1st DEAE-cellulose column	4.8	1.0	40.9	0.4
2nd DEAE-cellulose column				
IIIa	2.2	0.04	774.0	0.4
IIIb	2.2	0.03	989.0	
IIIc	1.2	0.05	365.0	
IIId	2.4	0.11	63.0	

TABLE 17. Purification of UDP-glucose pyrophosphorylase (enzyme III). For experimental details see Materials and Methods, section XI.G.1.(i)-(ii). The enzyme assay was performed as described in section XI.G.2.(XI.F.2.(ii)) except that $MgCl_2$ (1.35mM) was included in the assay mixture.

NTP substrate	Specific activity (nmol NDP-glucose/min/mg protein)			
	IIIa	IIIb	IIIc	IIId
UTP	752	931	357	60
CTP	23	36	11	4
dTTP	14	7	5	6
GTP	6	17	3	2
ATP	-	-	8	-

TABLE 18. Nucleotide specificity of multiple forms of UDP-glucose pyrophosphorylase. Forms IIIa - IIId were prepared as described in section, XI.G.1.(ii) of the Materials and Methods.

Enzyme assays were performed as described in section XI.G.2. (XI.F.2.(i)) of the Materials and Methods except that the $MgCl_2$ concentration was 1.35mM.

Enzyme fraction	Pyrophosphorylase specific activity*					
	ADP-glucose			UDP-glucose		
	Fructose 1,6-diphosphate ABSENT	Fructose 1,6-diphosphate PRESENT (2.7mM)	% Activation	Fructose 1,6-diphosphate ABSENT	Fructose 1,6-diphosphate PRESENT (2.7mM)	% Inhibition
Crude	0.07	0.09	38	68.0	55.0	20
$(NH_4)_2SO_4$ (50-100% saturated)	0.07	0.08	16	7.3	2.5	66
Sephadex (Enzyme III)	0.08	0.26	250	5.9	2.9	51

TABLE 19. Specific activity of ADP-glucose and UDP-glucose pyrophosphorylase in the presence and absence of fructose 1,6-diphosphate. The enzyme was prepared from nearly mature tubers as described in section XI.G.1. of the Materials and Methods.

ADP-glucose pyrophosphorylase was assayed as described in Materials and Methods, section XI.F.2.(i) and UDP-glucose pyrophosphorylase as described in section XI.G.2. (XI.F.2.(i)), except that $MgCl_2$ (1.35mM) was added to the assay medium.

*Specific activity is defined as nmol NDP-glucose formed/min/mg protein.

The chromatographic mobility of X, using 95% ethanol/M-ammonium acetate (pH 3.8) as solvent, was intermediary between the values for neutral sugars and those for the hexose phosphates (Fig. 27). Acid hydrolysis of X yielded radioactive glucose (Fig. 28). The electrophoretic mobility of X using a strongly acidic buffer was similar to that of the hexose phosphates, but treatment with E.coli alkaline phosphatase did not change the electrophoretic mobility of the unknown compound.

X was not produced when crude enzyme preparations were incubated with [U-^{14}C]glucose 1-phosphate in the absence of UTP.

Various fractions of UDP-glucose pyrophosphorylase with different degrees of purity were obtained from mature tubers and the effects of fluoride, phosphate, sugar phosphates and sucrose on the synthesis of both UDP-glucose and X, using these fractions, was examined (Table 20). In general, fluoride ions increased the synthesis of UDP-glucose and decreased the synthesis of X. Fructose 1,6-diphosphate, with most of the enzyme fractions, inhibited the synthesis of UDP-glucose and X, although the variability of the data cannot be explained. Addition of other sugar phosphates, inorganic phosphate or sucrose to the UDP-glucose pyrophosphorylase reaction mixture had little effect on the formation of UDP-glucose or X. The stimulation of UDP-glucose synthesis in the presence of fluoride could result from the inhibition of phosphatases which would prevent hydrolysis of pyrophosphorylase substrates. If X is a glucoside formed from the product of the pyrophosphorylase reaction, i.e. UDP-glucose, and a suitable acceptor in the reaction mixture, then it is difficult to see why fluoride interferes with the transfer of glucose from UDP-glucose.

A recent paper by Storm et al. (247) has revealed that there is an enzyme in germinating seeds of Phaseolus aureus and Pisum sativum which is capable of catalysing the glucosylation of dithiothreitol by UDP-glucose. Dithiothreitol is widely used as an enzyme anti-oxidant and during this study with the potato pyrophosphorylase another thiol, ethane-1-ol-2-thiol, was employed for the same purpose. The possibility that X is a glucoside of ethane-1-ol-1-thiol is, however, considered to be unlikely because of its electrophoretic behaviour which suggested that it was acidic. In addition, when dithiothreitol was substituted for ethane-1-ol-2-thiol in the pyrophosphorylase reaction

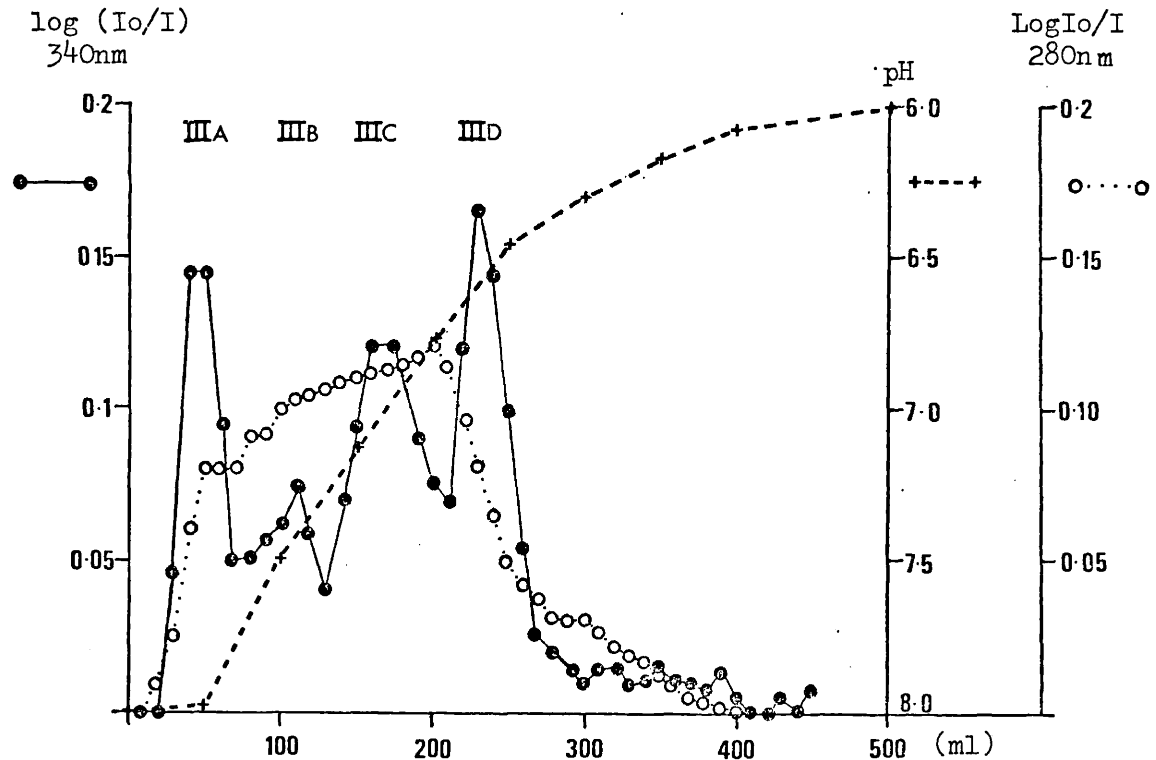


Fig. 26. Second DEAE-cellulose column fractionation of UDP-glucose pyrophosphorylase. See Materials and Methods, section XI. G.1.(ii) for experimental details.

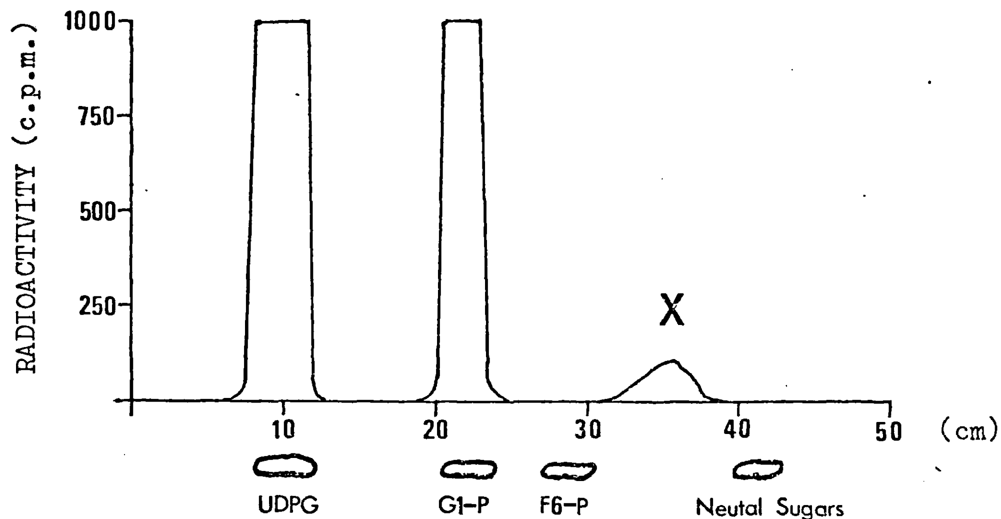


Fig. 27. Paper chromatographic separation of the products formed during the incubation of [U-^{14}C]glucose 1-phosphate and UTP with a crude enzyme preparation from mature tubers (see Materials and Methods, section XI.G.1. The assay was performed as described in Materials and Methods, section XI.G.2. (XI.F.2.(ii)), except that $MgCl_2$ (1.35mM) was added.

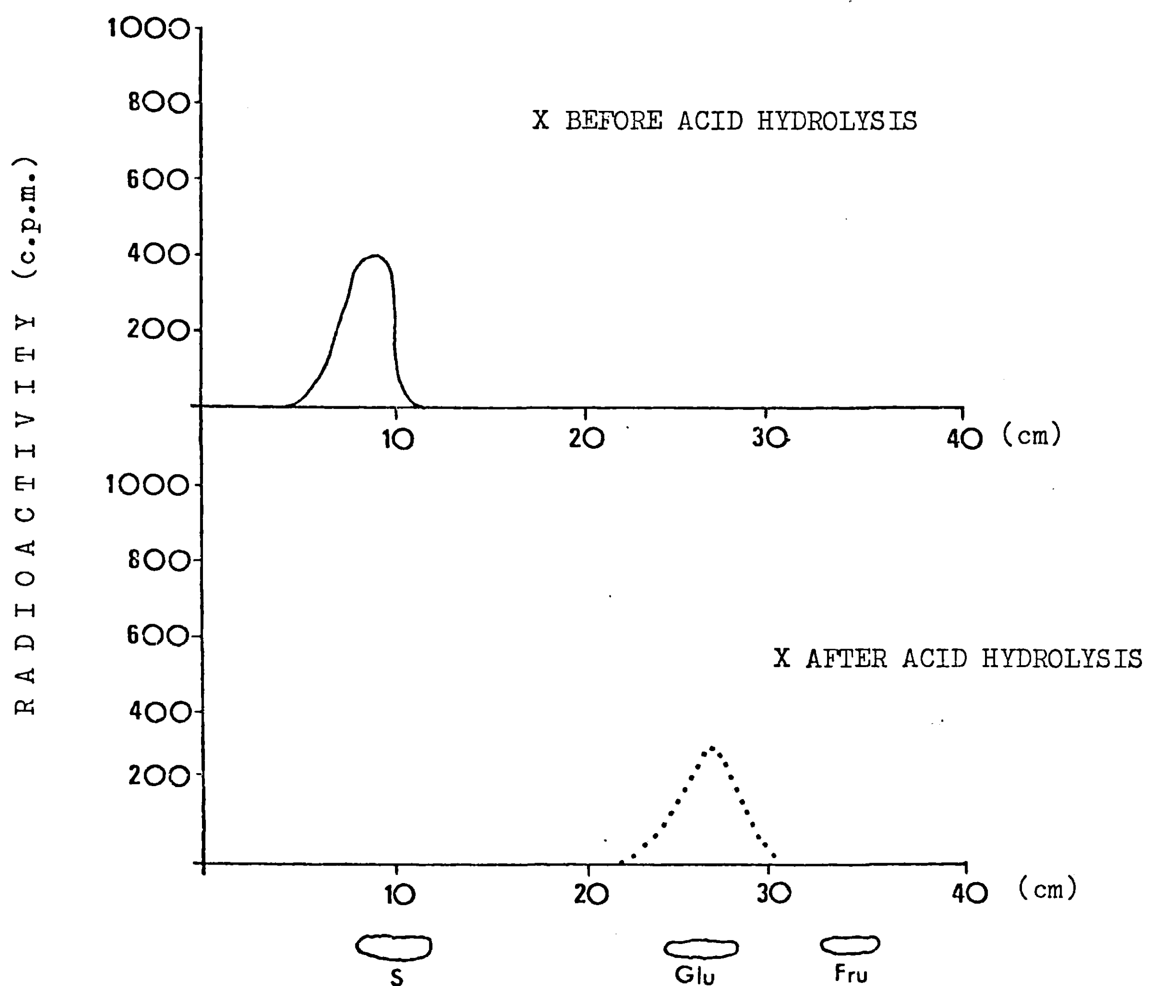


Fig. 28. Paper chromatographic separation (solvent (i)) of the products from the acid hydrolysis of X (1N-HCl at 100°C for 1hr).

Fraction	Product	Fluoride (40mM)	Radioactivity of product (d.p.m.)						
			Effector absent	Effector (2.66mM)					
				F1,6 diP	F6-P	G6-P	3-PGA	Sucrose	Pi
Crude	UDP-glucose	+	22,867	18,800	23,400	21,575	23,050	23,450	21,075
		-	11,800	11,150			14,420		
	Sucrose	+	1,405	693	1,159	1,179	1,167	1,371	424
		-	6,059	3,583			3,515		
$(NH_4)_2SO_4$ (50-100% saturation)	UDP-glucose	+	12,500	4,260	11,825	11,950	13,850	9,700	11,140
		-	9,680	7,800			6,520		
	Sucrose	+	3,488	309	3,66[illegible]	2,537	506	1,562	1,464
			565	340			0		
Sephadex column	UDP-glucose	+	10,840	6,500	5,850	8,890	9,850	10,000	12,500
		-	2,500	5,300			2,430		
	Sucrose	+	441	467	0	0	343	0	24
		-	413	459			327		
First DEAE-cellulose column	UDP-glucose	+	6,335	8,450			8,400		
		-	3,300	7,650			3,050		
	Sucrose	+					155		
		-	367	200			150		
Second DEAE-cellulose III_A	UDP-glucose	+	48,470	45,000			48,280		
		-	18,950	23,750			22,250		
	Sucrose	+	2,585	2,316					
		-	6,679	5,779			6,173		
III_B	UDP-glucose	+	33,950	25,750			28,600		
		-	15,600	13,500			14,670		
	Sucrose	+	1,669	1,984					
			3,559	5,321			5,219		
III_C	UDP-glucose	+	28,590	29,930			29,320		
		-	14,130	11,820			11,840		
	Sucrose	+	1,680						
		-	3,092	5,417					
III_D	UDP-glucose	+	12,450	10,850			12,550		
		-	10,250	9,250			12,550		
	Sucrose	+							
		-		0					

TABLE 20. Formation of X and UDP-glucose during the UDP-glucose pyrophosphorylase reaction in the presence and absence of various potential effector molecules. The enzyme fractions were prepared as described in Materials and Methods, section XI.G.1.(i)-(ii). Enzyme assays were performed as in section XI.G.2. (XI.F.2.(ii)) of Materials and Methods except $MgCl_2$ (1.35mM) was included.

mixtures, labelled X (or a compound indistinguishable from X on paper chromatograms) was still produced. No further attempts were made to characterize X.

The specific and total activities of UDP-glucose pyrophosphorylase in a crude mature tuber homogenate were shown to be 6.1 nmol UDP-glucose/min/mg protein and 32.9 units/g tissue, respectively. This apparent fall in both activities during maturation (specific and total activities of the enzyme prepared from less mature tubers were 97.4 nmol UDP-glucose/min/mg protein and 225 units/g tissue, respectively and the specific activity of the enzyme prepared from immature developing tubers was 337 nmol UDP-glucose/min/mg protein) may have been due, in whole or in part, to the formation of X and, hence, loss of substrate(s).

VII. Starch grain-bound enzyme studies

Having examined some of the soluble enzymes involved in sucrose metabolism in the potato, attention was next directed towards the enzymes bound to starch grains.

A. Nucleoside diphosphate glucose: starch glucosyl transferase activities.

Acetone-washed starch grains prepared from developing potato tubers were incubated with either ADP-[U-^{14}C]glucose or UDP-[U-^{14}C] glucose. After re-isolation and washing with aqueous alcohol, the grains were hydrolysed with acid, α-amylase and β-amylase, separately. The products of these reactions (Fig. 29) indicated that labelled α-(1→4)-linked glucose residues had been incorporated into the starch.

Starch grains prepared by the acetone-washing technique (see section XI.H.2. of Materials and Methods) were completely devoid of starch phosphorylase, ADP-glucose- and UDP-glucose-pyrophosphorylase activities (see Materials and Methods, sections XI.J.3 and 4.), therefore; the possibility that incorporation of [^{14}C]glucose into starch was occurring via glucose 1-phosphate was considered unlikely. It was also noted that sucrose synthetase and inorganic pyrophosphatase activities were absent in acetone-washed starch grains (see Materials and Methods, sections XI.J.1. and 2.).

The kinetics of the starch grain-bound synthetase activities were examined. The rates of reaction were shown to be linear over a

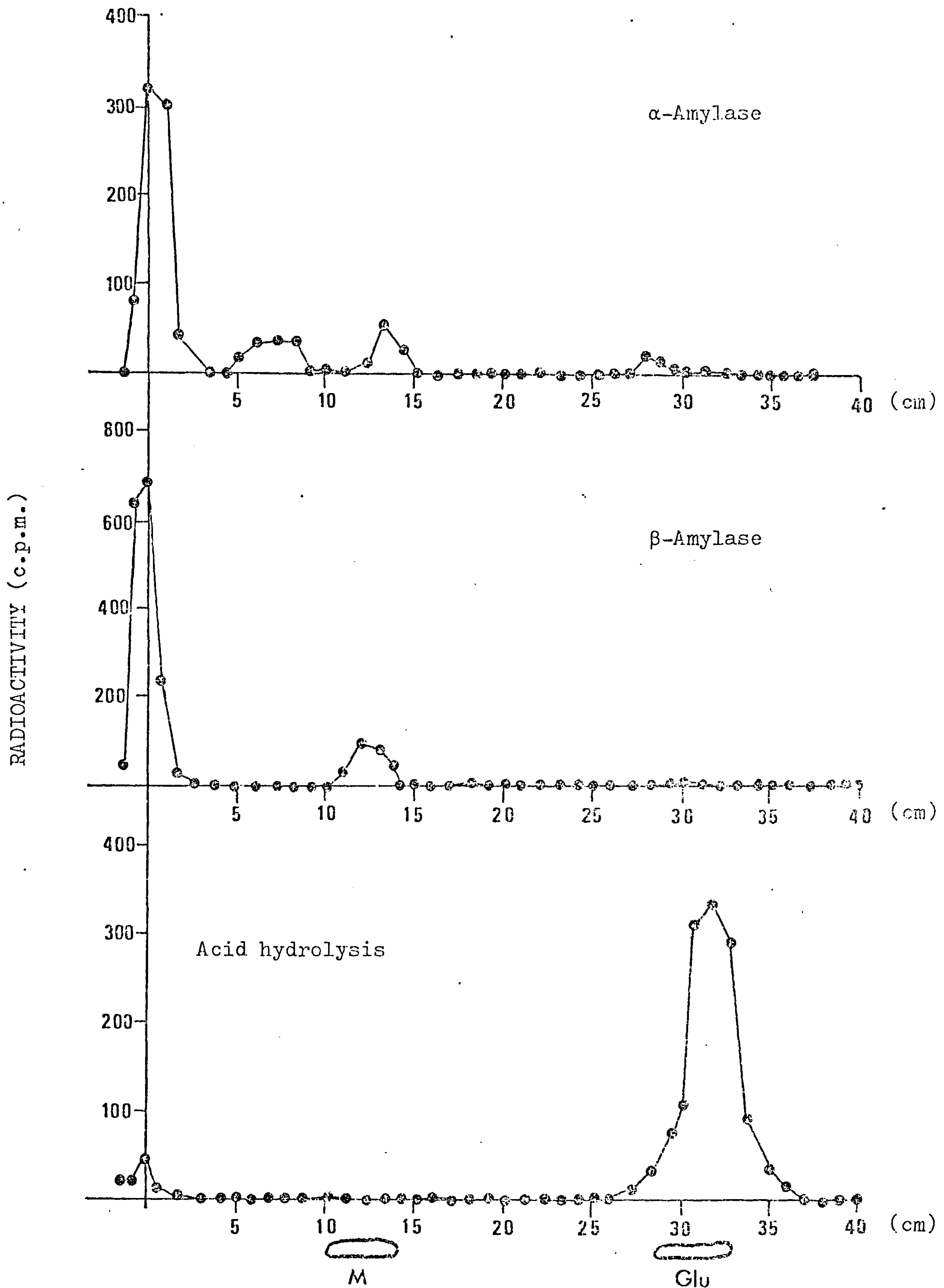


Fig. 29. Paper chromatographic analysis of the [^{14}C]products obtained by the action of α-amylase, β-amylase and acid on [^{14}C]starch synthesised from ADP-[U-^{14}C]glucose. The product pattern for starch synthesised from UDP-glucose was essentially the same. For experimental details see sections XI.I.1. and 2. of Materials and Methods.

period of 15 min with both ADP-glucose and UDP-glucose as substrates (Fig. 30.): the rates of utilization of these nucleoside diphosphate sugar substrates was 3.72 and 0.86 nmol/h/mg starch, respectively. In this connection it should be noted that there is general agreement that ADP-glucose is the preferred nucleotide substrate for starch synthesis in the potato tuber. The incorporation of [^{14}C]glucose into starch from both substrates was proportional to the starch grain concentration under normal assay conditions (Fig. 31).

KCl was observed to activate the formation of starch from both ADP-glucose and to a lesser extent UDP-glucose (Fig. 32). Maximal stimulation of both transglucosylase activities was observed in the presence of 25m M-KCl: the transglucosylation from ADP-glucose (Km 3.3mM) was increased in the presence of K^+ by 82% to 6.6nmol/h/mg starch and from UDP-glucose (Km 6.6mM) by 39% to 1.2nmol/h/mg starch, (Figs. 32 and 33). These results essentially confirm the work of Murata and Akazawa (139).

PPi and Pi, in the presence and absence of KCl, stimulated transglucosylation using ADP-glucose as substrate but with UDP-glucose, inhibition was observed in both cases (Table 21). The Lineweaver-Burk plots which illustrate the nature of the PPi effects on both transglucosylases are shown in Fig. 34. It was noted that the effects produced by PPi were greater than those produced by Pi (Table 21). In order to eliminate the possibility that the PPi was broken down to Pi during the incubation period, the acetone-washed starch grains were tested for inorganic pyrophosphatase activity (see Materials and Methods, section XI.J.2.), which was found to be absent.

The effects of PPi and Pi were essentially the same when starch grains that had been washed from the potato tissue with deionized water and then freeze dried (instead of acetone-washed grains) were used (Table 22).

The activation and inhibition of ADP-glucose and UDP-glucose: starch glucosyl transferase activities by PPi and Pi is of particular interest and tends to confirm that the transferases are separate enzymes, as suggested by Marshall (62), rather than different functions of the same protein. Possible further evidence for the existence of distinct enzymes is seen in Tables 21 and 22. It is evident that if the starch grains are acetone-washed, then there is a 30% loss of ADP-glucose: starch glucosyltransferase activity but a 50% increase in the UDP-

Effector (10mM)	Specific Activity*		Relative Activity (%)	
	ADP-glucose	UDP-glucose	ADP-glucose	UDP-glucose
CONTROL(+KCl)	6.6	1.20	100	100
CONTROL(-KCl)	3.6	0.9	55	70
PPi(+KCl)	8.7	0.7	130	60
PPi(-KCl)	5.0	-	75	-
Pi(+KCl)	7.7	1.0	116	80
Pi(-KCl)	4.2	-	65	-

TABLE 21. Regulation of potato tuber starch synthetase activities by PPi(10mM) and Pi(10mM) in acetone-washed starch grains. The grains were prepared and assayed for enzyme activity as described in Fig. 32. *Specific activity is expressed as nmol glucose incorporated/h/mg starch.

Effector (10mM)	Specific Activity*		Relative Activity (%)	
	ADP-glucose	UDP-glucose	ADP-glucose	UDP-glucose
CONTROL	9.6	0.7	100	100
PPi	12.6	0.0	130	0
Pi	11.0	-	120	-

TABLE 22. Effect of PPi(10mM) and Pi(10mM) on potato tuber starch grain-bound starch synthetase activities. The starch grains were prepared as described in sections XI.H.2. and the enzyme assayed as described in section XI.I.1.(ii) of the Materials and Methods. *Specific activity is defined as nmol glucose incorporated/h/mg starch.

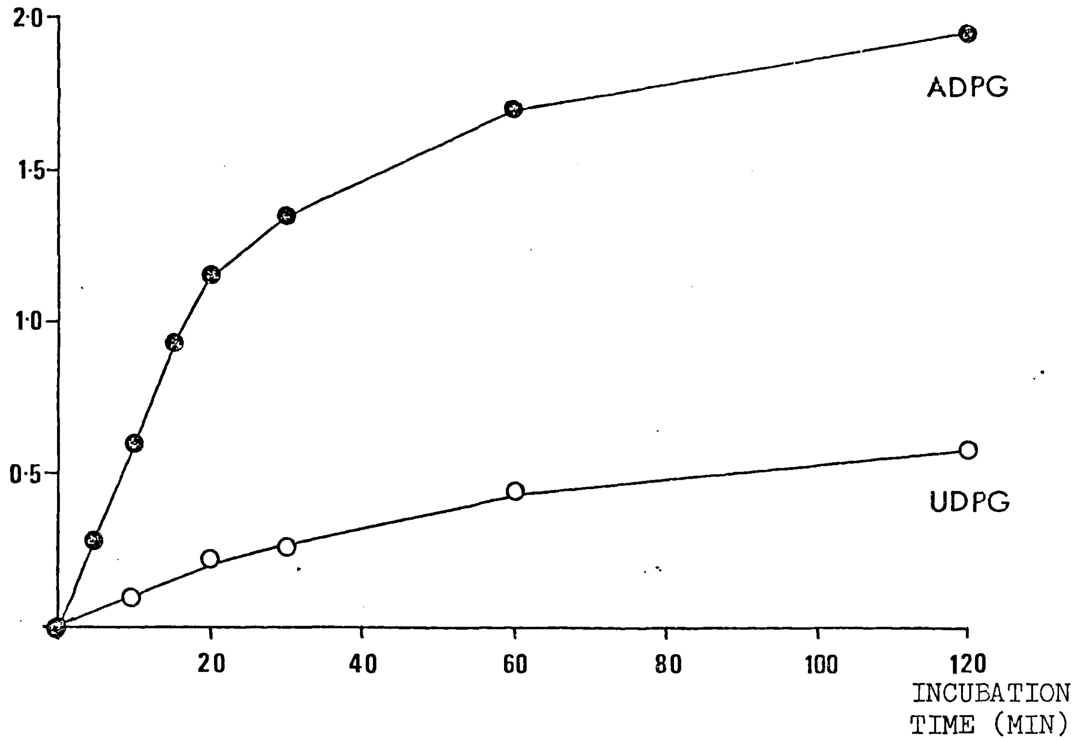


Fig. 30. Time course for the incorporation of glucose from ADP-glucose and UDP-glucose into starch using starch grain-bound starch synthetase. For experimental details see Materials and Methods sections XI.H.1. and XI.I.1.(ii)

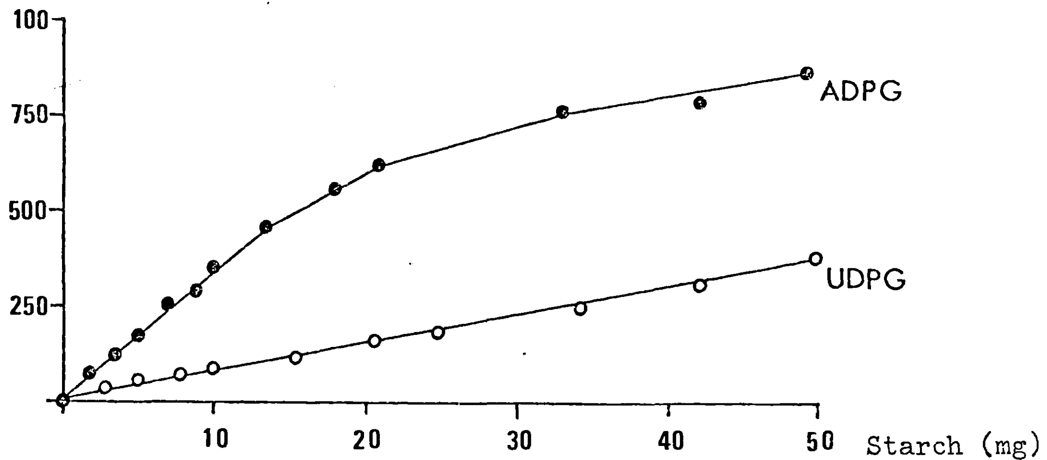


Fig. 31. Effect of increasing starch concentration on the incorporation of ^{14}C from UDP-[U-^{14}C]glucose and ADP-[U-^{14}C]glucose into starch. For experimental details see Fig. 30.

nmol glucose
incorporated/h/mg starch

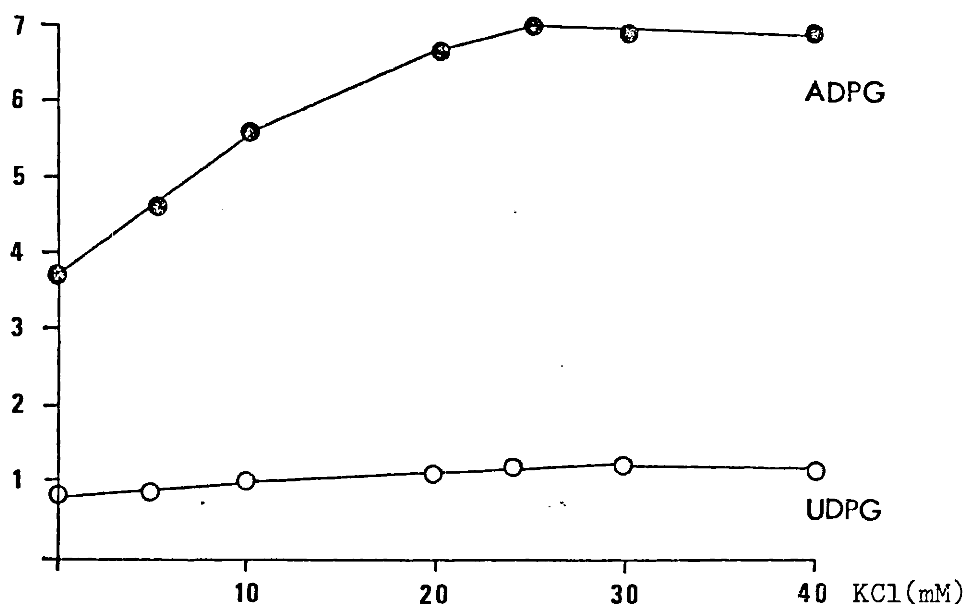


Fig. 32. Effects of KCl on starch grain-bound UDP(ADP)-glucose:starch glucosyl transferase. Experimental details are described in sections XI.H.2., XI.I.2.(i) and (ii) of Materials and Methods.

nmol glucose
incorporated/h/mg starch

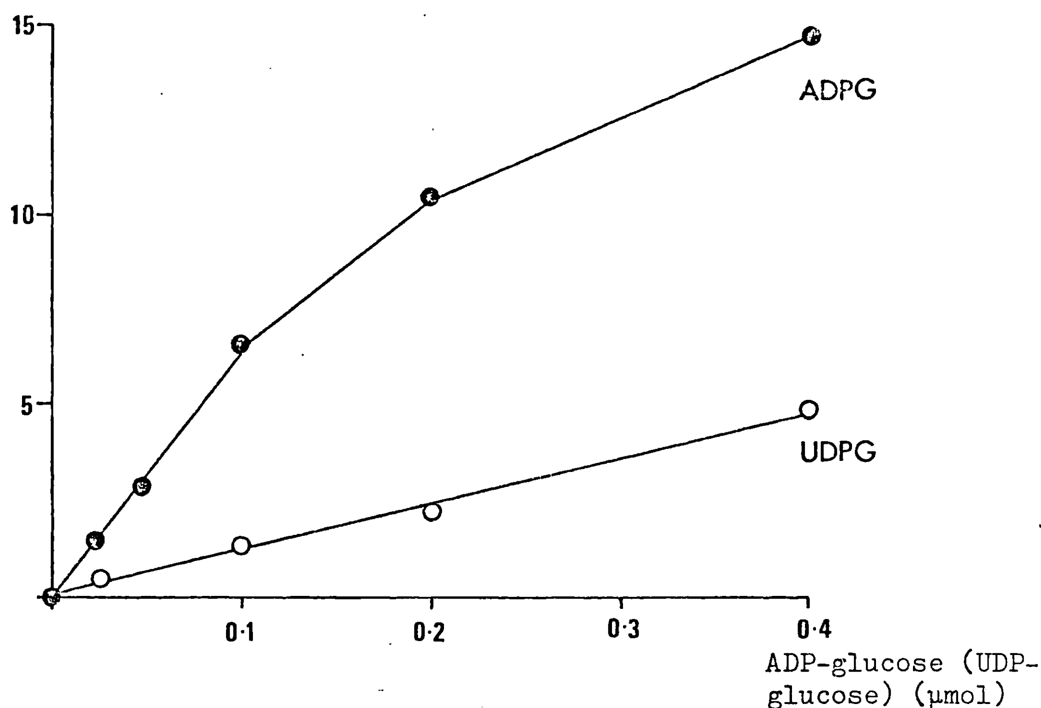
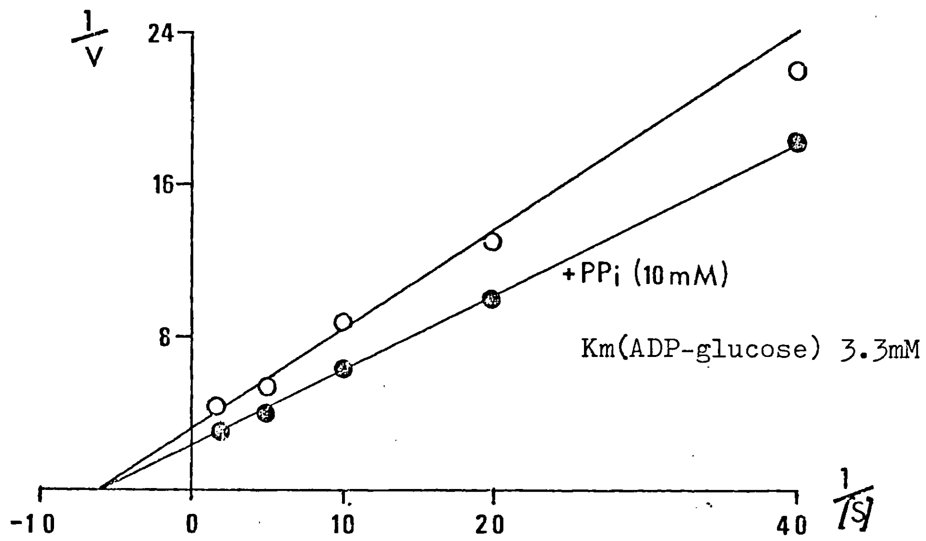


Fig. 33. The effect of substrate concentration on the rate of trans-glucosylation from ADP-glucose and UDP-glucose to starch. Experimental details as in Fig. 30.

ADP-glucose:starch glucosyl transferase



UDP-glucose:starch glucosyl transferase

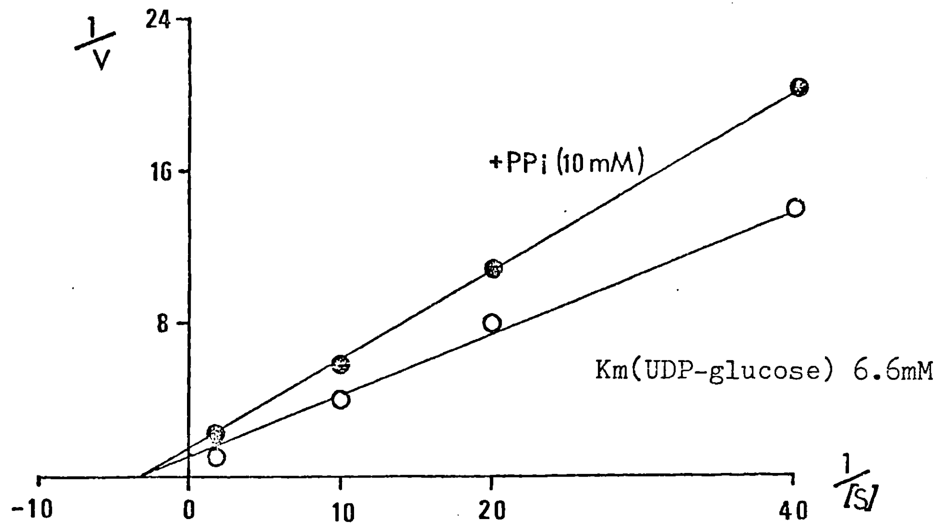


Fig. 34. Lineweaver-Burk double reciprocal plot showing the effect of PPi on UDP-glucose and ADP-glucose starch: glucosyl transferase activities. Experimental details as in Fig. 30.

glucose:starch glucosyl transferase activity. However, these activity differences could conceivably result from the removal of another nucleoside diphosphate glucose-utilizing enzyme by acetone.

The effects of various carbohydrate metabolites on transglucosylation, from both UDP-glucose and ADP-glucose to starch, was also examined. Sucrose, fructose, glucose 1-phosphate and fructose 6-phosphate had no detectable effect on starch synthesis, but characteristic inhibitions were observed in the presence of some nucleotide derivatives, (Table 23). The ADP-glucose:starch glucosyl transferase activity was inhibited by ADP but not AMP or ATP. UDP-glucose:starch glucosyl transferase activity was inhibited by mono-, di- and tri-phosphates of adenosine and also by ADP-glucose. The Ki for the latter inhibitor was 25mM (Fig. 35) and for ATP, 20mM (Fig. 36): both compounds inhibited competitively.

Frydman (135) has also carried out a similar investigation of the effects of various nucleotide derivatives on the potato tuber starch synthetase reaction. However, she reported that ADP-glucose: starch glucosyl transferase was inhibited by ATP and by AMP. Apart from this, her results are in general agreement with those presented in this thesis (Table 23), except that, in all cases, the inhibitor effects she observed were greater.

The evidence obtained from the present study of starch grain-bound starch synthetase (Tables 21, 22 and 23) supports the view that ADP-glucose is the major sugar nucleotide involved in starch synthesis in the potato tuber for the following reasons:

1. Assuming there are two separate enzymes the activity of the starch grain-bound synthetase utilising ADP-glucose is greater than that of the enzyme using UDP-glucose. (Rees and co-workers (49, 50,248)) have also found ADP-glucose associated with potato tuber starch granules, but they failed to detect UDP-glucose).

2. ADP-glucose is a potent inhibitor of UDP-glucose:starch glucosyl transferase.

3. PPi stimulated the synthesis of starch from ADP-glucose but inhibited its synthesis from UDP-glucose. This phenomena, in vivo, is possibly coupled with the reaction of the ADP-glucose pyrophosphorylase

Effector (10mM)	Specific Activity*		Relative Activity (%)	
	ADP-glucose	UDP-glucose	ADP-glucose	UDP-glucose
CONTROL	6.6	1.2	100	100
ATP	6.6	0.6	100	50
ADP	2.1	0.6	30	50
AMP	6.7	0.8	100	70
Cyclic AMP(1mM)	6.6	1.2	100	100
ATP+(cyclic AMP, 1mM)	6.6	0.5	100	40
ADP-glucose	-	0.6	-	50
UTP	6.6	1.1	100	95
UDP	6.6	1.0	100	90
UDP-glucose	6.6	-	100	-

TABLE 23. Effect of nucleotide derivatives on potato tuber starch synthetase activities. For experimental details see Materials and Methods, sections XI.H.2. and XI.I.1.(ii).

*Specific activity is defined as nmol glucose incorporated/h/mg starch.

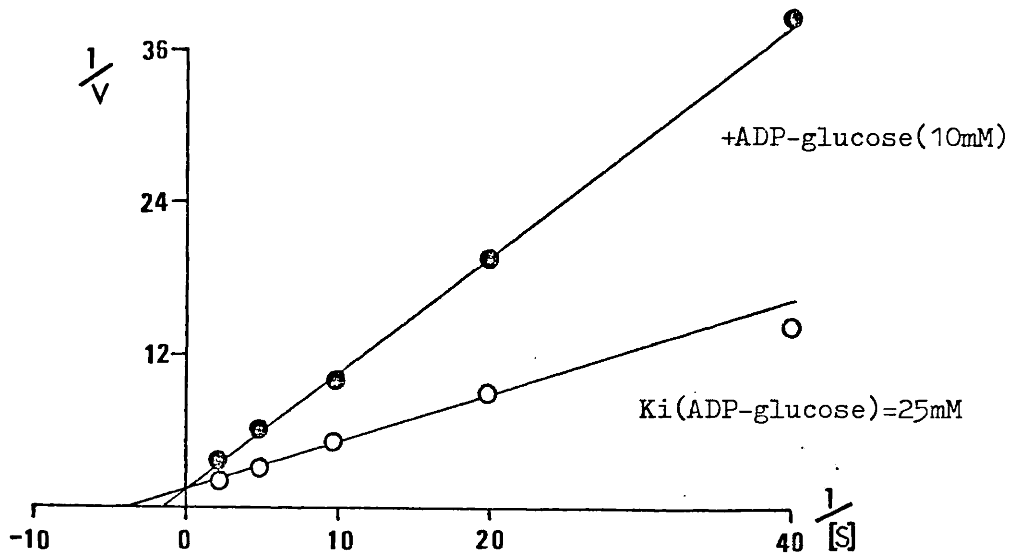


Fig. 35. Lineweaver-Burk double reciprocal plot for the inhibition of UDP-glucose:starch glucosyl transferase by ADP-glucose (10mM). The acetone-washed starch grains were prepared and assayed as described in Table 23.

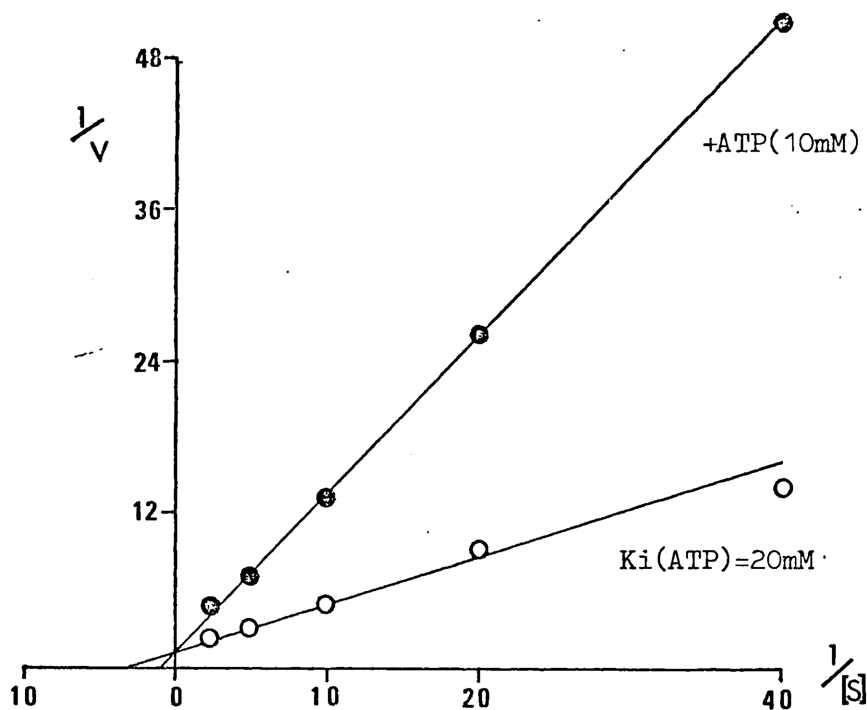


Fig. 36. Lineweaver-Burk double reciprocal plot for the inhibition of UDP-glucose:starch glucosyl transferase by ATP(10mM). The acetone-washed starch grains were prepared and assayed as described in Table 23.

which produces PPi. Thus the product of one reaction is an activator for the next enzyme in the biosynthetic sequence.

4. ADP, a product of starch synthesis, and ATP, a substrate for ADP-glucose pyrophosphorylase, together with the product of this enzyme, ADP-glucose, all inhibit starch formation from UDP-glucose. However, the synthesis of the polysaccharide from ADP-glucose is not affected by UDP, UTP or UDP-glucose.

B. Starch phosphorylase

Acetone-washed starch grains possessed no detectable phosphorylase activity (see Results and Discussion, section VIII.A.p. 85). However, when water-washed starch grains were incubated with [U-^{14}C] glucose 1-phosphate a radioactive α-(1→4)-glucan(starch) was identified as a product of the reaction (Materials and Methods, section XI.J.4.). (Paper chromatographic analysis of the labelled sugars produced by hydrolysis of the glucan with α-amylase and with acid (see Materials and Methods, section X.D.2.) were essentially the same as shown in Fig. 29a) and c)).

When the starch grains were incubated with glucose 1-phosphate and ATP or UTP, nucleoside diphosphate glucose formation could not be detected. This suggests that the incorporation of [^{14}C]glucose into starch was not occurring via a coupled pyrophosphorylase/starch synthetase reaction.

The specific activity of the bound phosphorylase was 23.9 nmol glucose incorporated/h/mg starch. ATP(6mM) was observed to inhibit the phosphorylase completely whereas UTP(6mM) had less effect (72% inhibition) (Table 24).

The inhibition of phosphorylases by ATP and UTP has been well documented (7, 191). Tsai and Nelson (191) detected a phosphorylase isozyme in maize endosperm (phosphorylase II) that appeared only during the period of rapid starch synthesis. The isozyme was markedly inhibited by ATP, GTP, ADP and GDP and to a lesser extent by UTP, CTP, UDP and CDP. Mg^{2+}(10mM) stimulated the activity of the isozyme.

The absence of phosphorylase activity in acetone-washed grains may be due to instability of the enzyme in the solvent or to its ready removal from a surface position, perhaps on the membrane.

The relative specific activities of starch phosphorylase, UDP-glucose:starch glucosyl transferase and ADP-glucose:starch glucosyl

Effector (6mM)	Specific activity (nmole glucose incorporated/ h/mg starch)	Relative Activity %
Control	23.4	100
ATP	0.0	0
UTP	4.6	28

TABLE 24. Effect of ATP and UTP on starch grain-bound starch phosphorylase activity. Extraction and assay procedures were as described in section XI.J.4. of the Materials and Methods.

transferase in water-washed starch grains (23.9:9.6:0.62 nmol glucose incorporated/h/mg starch, respectively) may indicate that phosphorylase plays an important role in starch synthesis in the developing potato tuber (see Gerbrandy and others 191, 196, 206, 208-210). However, this assumes that the pH, glucose 1-phosphate and ATP concentrations are favourable in the environment of the enzyme.

C. Sucrose synthetase

Grimes _et al_ (102) have purified sucrose synthetase from mung beans and shown the enzyme to be associated with a lipoprotein complex with a molecular weight of 1×10^6. The physiological characteristics of the complex suggest that the enzyme may be membrane bound. It was believed that the enzyme could be associated with the amyloplast membrane.

Sucrose synthetase, which was present in the soluble protein fraction from the potato, could not be detected in either acetone-washed, freeze dried or disrupted (sonicated) starch grains.

When starch grains were incubated with [U-^{14}C]sucrose in the presence or absence of UDP the degree of labelling of the grains was high but essentially the same in both cases. No evidence could be obtained to indicate that starch synthesis had occurred and it was supposed that the grains rapidly absorbed the [U-^{14}C]sucrose without further detectable reaction. However, the adsorbed disaccharide could not be removed by washing the starch grains with 75% aqueous methanol.

In view of the association of sucrose synthetase with chloroplasts (7) the lack of enzyme activity in starch grains (amyloplasts) was unexpected.

VIII. The synthesis of starch from sucrose *in vitro*.

Having obtained information on the fate of exogenous sucrose in the potato and the nature of the enzymes likely to be involved in the conversion of sucrose to starch, an attempt was next made to assemble an *in vitro* system capable of synthesising the glucan.

The cleavage of sucrose to UDP-glucose and fructose *in vitro*, together with the phosphorylation of the ketohexose and its conversion to ADP-glucose via glucose 1-phosphate, has already been demonstrated using tuber enzymes (see Results and Discussion, sections VI.B. and VI.C.pp. 60 - 66). As an initial step towards the coupling of this complete system to starch synthesis an attempt was made to incorporate glucose 1-phosphate into starch in the presence of a nucleoside triphosphate.

A. The coupling of nucleoside diphosphate glucose pyrophosphorylase and starch synthetase.

A nucleotide triphosphate dependent transfer of label from [U-^{14}C]glucose 1-phosphate to starch was demonstrated using a system consisting of a partially purified soluble enzyme preparation, from developing tubers, containing both ADP-glucose- and UDP-glucose-pyrophosphorylase activities, together with starch grain-bound starch synthetase (Fig. 37). The enzyme preparations were completely devoid of phosphorylase activity at the pH used since no [^{14}C]starch was produced in the absence of ATP or UTP.

The [^{14}C]glucan product yielded [^{14}C]glucose when hydrolysed with acid and on treatment with α-amylase [^{14}C]glucose and [^{14}C]maltose were formed.

Paper electrophoretic analysis of the incubation mixtures also showed the presence of [^{14}C]ADP-glucose when ATP was a substrate and [^{14}C]UDP-glucose when UTP was present.

The specific activity of the coupled enzyme system (Fig. 37) in the presence of ATP was 2.3 µmol glucose (from glucose 1-phosphate) incorporated/h/mg protein. This reaction was extremely efficient; 78% of the glucose 1-phosphate was converted to starch after 30 min incubation.

In the presence of UTP the specific activity was 0.3µmol glucose (from glucose 1-phosphate) incorporated/h/mg protein. The slower rate of reaction in the presence of UTP can be explained in terms of the low specific activity of UDP-glucose:starch glucosyl transferase, present in the system, in comparison with the corresponding activity utilizing ADP-glucose, in addition, UTP inhibits the UDP-glucose:starch glucosyl transferase.

B. The attempted conversion of fructose to starch via ADP-glucose *in vitro*.

The synthesis of [^{14}C]ADP-glucose from [^{14}C]fructose using a soluble enzyme preparation from early developing tubers had been observed (see Results and Discussion, section VI.B. pp. 60 - 64), and therefore, by coupling kinases, isomerases, pyrophosphorylases and starch synthetase reactions it was hoped to be able to effect the conversion of fructose to starch. Hence [U-^{14}C]fructose and ATP were incubated with acetone-washed starch grains and a soluble protein fraction from tubers (see Materials and Methods, section XI.M.). No conversion of [U-^{14}C]fructose to [^{14}C]starch could be demonstrated and no hexose phosphates were detected. The reasons for this was thought to be due to the soluble tuber protein fraction which had been prepared from potatoes in a late stage of development. It was presumed that the preparation lacked hexokinase and/or pyrophosphorylase activities (see Results and Discussion, section VI.D.p.67). Unfortunately no time was available to repeat the experiment using a preparation from younger tuber tissues. Earlier studies with the soluble enzyme preparations (see Results and Discussion, section VI.B. p. 60) suggest that the system would have worked if preparations from early developing tubers could have been used.

C. Coupled sucrose synthetase and starch synthetase reactions.

Attempts to demonstrate the conversion of sucrose to starch were more successful.

Sucrose was incorporated into the glucan by incubating [U-^{14}C] sucrose and either ADP or UDP together with a soluble enzyme preparation from developing potato tubers and acetone-washed starch grains (Fig. 38). Allowances for the absorption of [^{14}C]sucrose by the starch grains were made by using control incubation mixtures composed of starch grains, labelled sucrose and nucleoside diphosphates. Presumably, in these

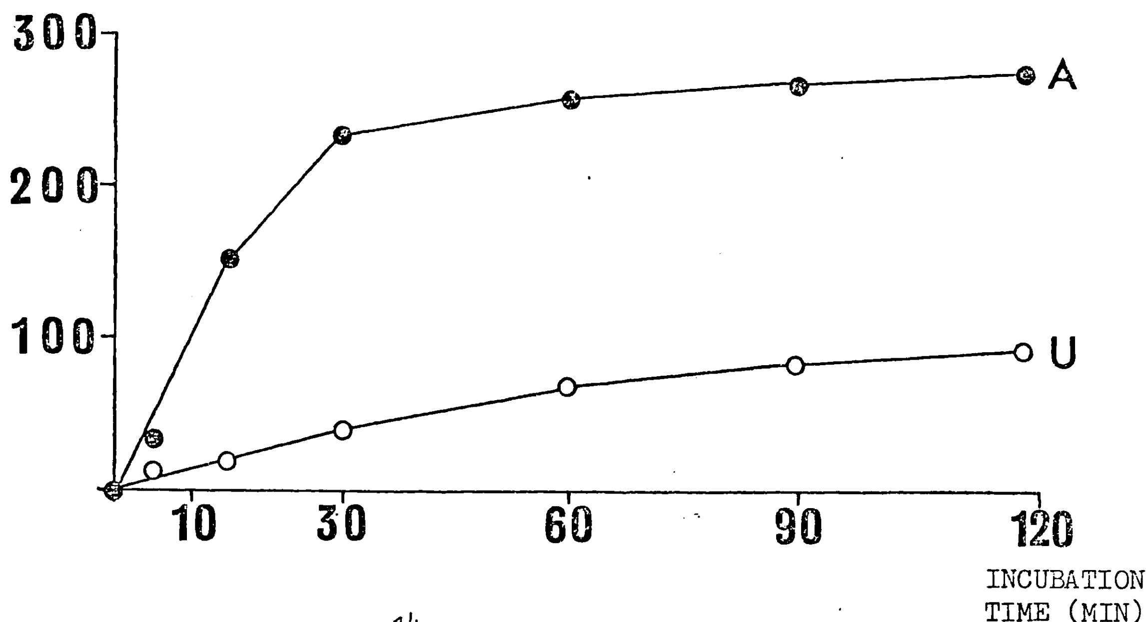


Fig. 37. Conversion of [U-^{14}C]glucose 1-phosphate to starch in the presence of ATP, (A) and UTP, (U). The reaction mixture consisted of acetone-washed starch grains (10mg), [^{14}C]glucose 1-phosphate and ATP or UTP. Experimental details are given in the Materials and Methods, section XI.K.

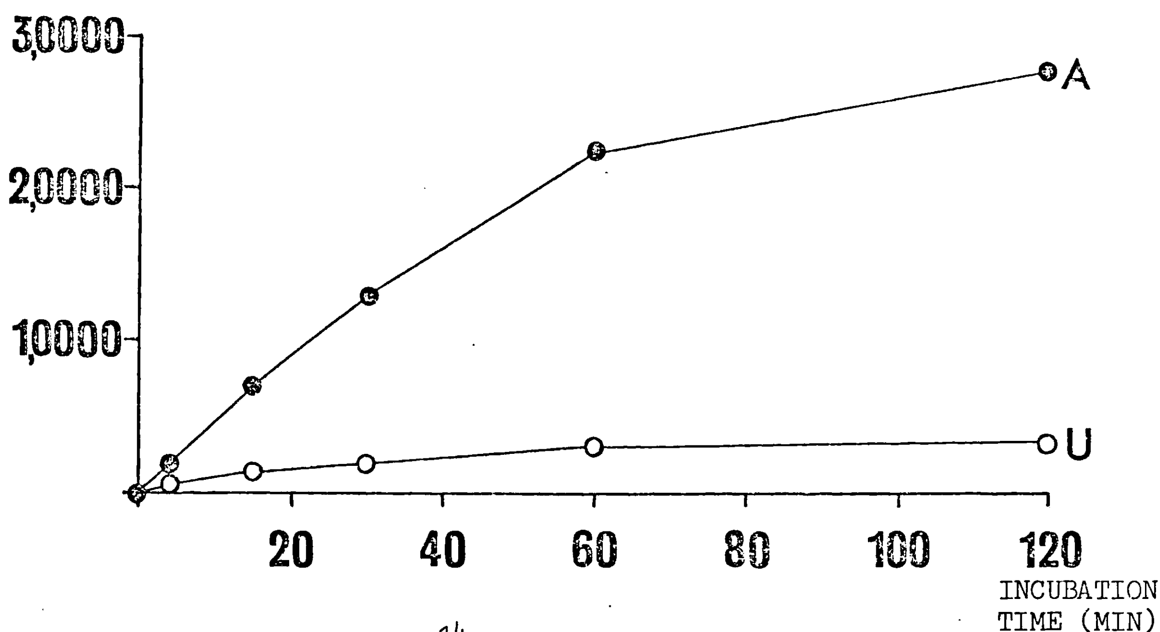


Fig. 38. Incorporation of [^{14}C] into starch following the incubation of a soluble protein fraction and starch grains from developing tubers with [U-^{14}C]sucrose in the presence of either ADP, (A) or UDP, (U). For experimental details see Materials and Methods, section XI.L.

experiments, labelled glucan was derived from the glucosyl moiety of [U-^{14}C]sucrose. The fructose released by the sucrose synthetase reaction would be unlikely to contribute to the labelling of starch because of the absence of ATP.

The greater efficiency (approx. 6 times) of glucose incorporation from sucrose in the presence of ADP compared with UDP, may be explained by the fact that starch grain ADP-glucose:starch glucosyl transferase is more active than the corresponding synthetase utilizing UDP-glucose (Table 24). In other words the formation of nucleoside diphosphate glucoses in these reactions are not rate limiting despite the fact that sucrose synthetase from the tuber converts sucrose to UDP-glucose more efficiently than to ADP-glucose (Table 9).

G E N E R A L   C O N C L U S I O N S

From the evidence presented in this thesis a possible pathway for the conversion of both the glucose and fructose moieties of sucrose to starch is proposed (Fig. 39). It is suggested that the translocated sucrose is cleaved by sucrose synthetase yielding fructose and UDP-glucose which are then both converted to starch, mainly via glucose 1-phosphate.

The results of the investigation (see Results and Discussion, section V.C.p. 54) support the view that sucrose synthetase, rather than invertase, is the initial enzyme involved in the breakdown of sucrose prior to starch formation. The relatively high free energy of hydrolysis of the sucrose molecule is retained in the form of UDP-glucose when the disaccharide is cleaved by sucrose synthetase. This is an obvious thermodynamic advantage in comparison to hydrolysis by invertase.

Studies, in vitro, using potato tuber sucrose synthetase (Results and Discussion, section VI.A.p. 59) and the results of other workers (Table 1), suggest that the reaction catalysed by this enzyme favours the synthesis of NDP-glucose rather than breakdown and that the enzyme shows a greater specificity for uridine diphosphate than diphosphates of other nucleosides. The fructose liberated by the action of sucrose synthetase, in the potato, is probably, in the main, converted, via fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate to ADP-glucose. This pathway is supported by evidence from the feeding experiments with [^{14}C] labelled sugars (Results and Discussion, sections V.B. and C.pp. 51 - 58) and the results of the studies in vitro using soluble preparations of enzymes from developing tubers (Results and Discussion, sections VI.B. and C.pp. 60-66).

The formation of ADP-glucose, from glucose 1-phosphate by the ADP-glucose pyrophosphorylase reaction, is believed to be a key step in the synthesis of assimilatory starch (42,51,52,57,58) and it is stimulated by sugar phosphates. The importance of this reaction in non-photosynthetic starch-synthesising tissues has already been discussed (see Introduction p.15) and the work described in this thesis is consistent with the view that ADP-glucose pyrophosphorylase is involved in the synthesis of starch from sucrose in the potato (Results and Discussion, section VI.D.p. 67).

The regulatory role of ADP-glucose pyrophosphorylase in non-photosynthetic tissues has been questioned (12,59). The results of the

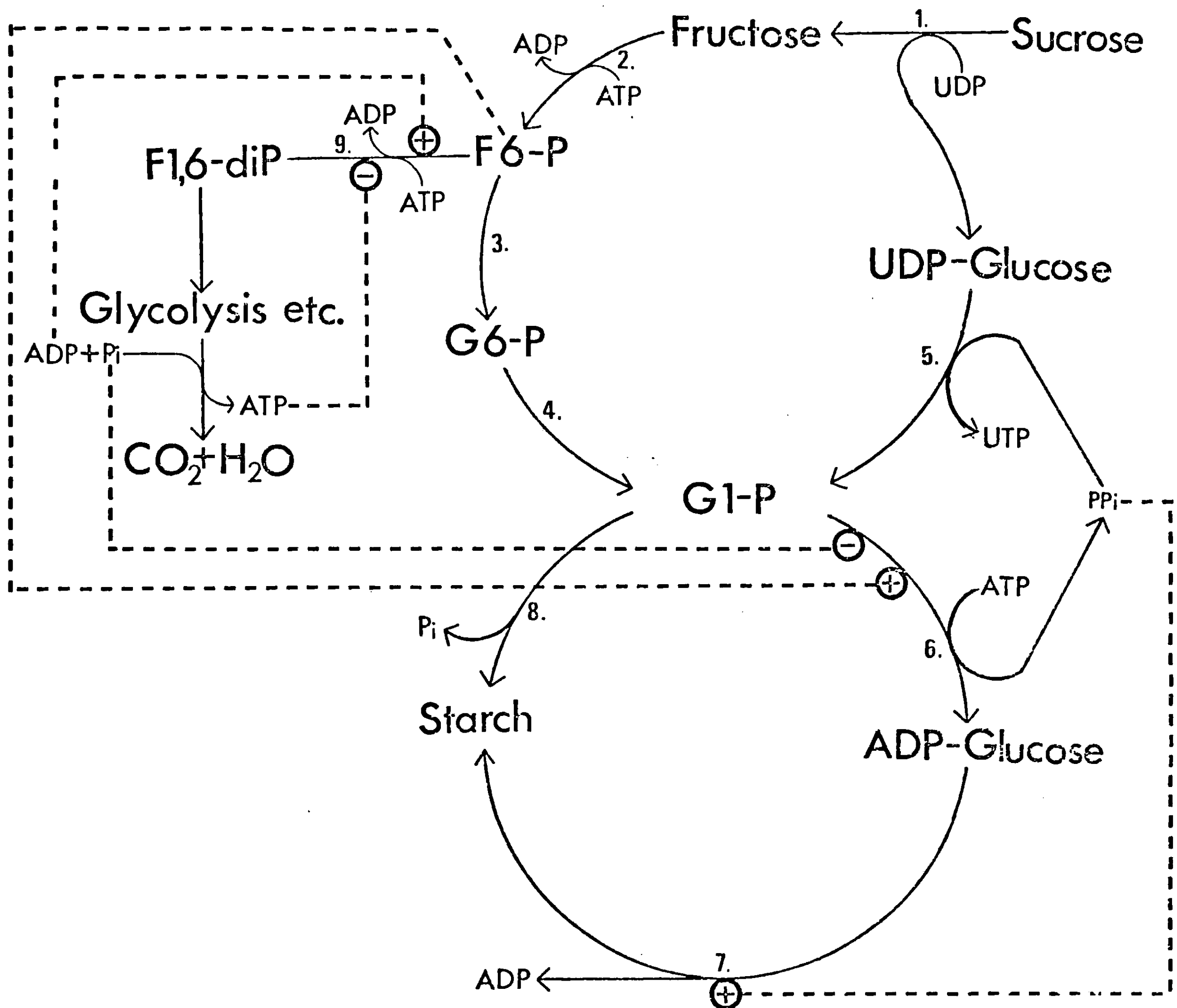


Fig. 39. Proposed pathway for the conversion of sucrose to starch in potato tubers. Numbers refer to the enzyme that catalyses the individual reactions:-

1., Sucrose synthetase; 2., Hexokinase; 3., Phosphoglucose isomerase; 4., Phosphoglucomutase; 5., UDP-glucose pyrophosphorylase; 6., ADP-glucose pyrophosphorylase; 7., ADP-glucose:starch transglucosylase; 8., Starch phosphorylase; 9., Phosphofructokinase.

⊕---------- activating effect

⊖---------- inhibitory effect

present study, are inconsistent with the view that the enzyme in this type of tissue has a key regulatory function in the conversion of sucrose to starch. The enzyme from the potato tuber is only weakly activated by high concentrations of 3-PGA and fructose 6-phosphate, early products of photosynthesis which markedly activate the enzyme from photosynthetic tissues. However, even weak activation of ADP-glucose pyrophosphorylase by fructose 6-phosphate should, perhaps, not be ignored: it may allow the potato pyrophosphorylase to function more efficiently for the synthesis of ADP-glucose and, hence, starch.

Phosphofructokinase, the main regulatory enzyme in the glycolytic sequence, is inhibited by ATP and activated by ADP (269). The antagonistic effects of these adenine nucleotides on the phosphofructokinase and ADP-glucose pyrophosphorylase reactions, together with the effects of fructose 6-phosphate and Pi on the latter reaction, would allow the rate of starch synthesis from both UDP-glucose and fructose 6-phosphate to be governed by the levels of ADP and ATP.

It is proposed (Fig. 39) that of the two nucleoside diphosphate sugars capable of forming starch *in vitro*, ADP-glucose is the major precursor utilized *in vivo* by the potato (see Results and Discussion, section VII.A.p. 85). This proposal is supported by the experiments with coupled enzyme systems (Results and Discussion, sections VI.B. and C. and VIII.A. and B. pp. 60-66 and 97-98). In addition ADP-glucose but not UDP-glucose, appears to be closely associated with the starch grains from potato tuber (49, 50, 243).

It is interesting to note that one of the products of the ADP-glucose pyrophosphorylase reaction, PPi, can activate the next enzyme in the metabolic sequence from glucose 1-phosphate to starch, i.e. ADP-glucose:starch glucosyl transferase. This effect, together with the activation of ADP-glucose pyrophosphorylase by fructose 6-phosphate, would thus ensure the efficient conversion of fructose to starch.

The UDP-glucose produced from sucrose by the sucrose synthetase reaction can be converted to glucose 1-phosphate by UDP-glucose pyrophosphorylase but there is no experimental evidence to confirm that this enzyme functions in this direction *in vivo*. If this UDP-glucose is not utilized directly for starch synthesis and, hence,

increases in concentration, then presumably pyrophosphorylysis of the nucleotide sugar will occur provided PPi is present. However, UDP-glucose would, of course, also be utilized for the synthesis of cell wall polysaccharides in the developing tuber.

If the conversion of UDP-glucose to glucose 1-phosphate does occur in developing tubers then there should exist within the tuber cells a PPi-generating system. This demand may be met by the pyrophosphorylase-catalysed conversion of glucose 1-phosphate to ADP-glucose.

The formation of glucose 1-phosphate from sucrose via UDP-glucose, may be the route whereby the glucose moiety of sucrose is converted to starch.

Glucose 1-phosphate could be converted directly to starch by starch phosphorylase. The evidence that this enzyme has a synthetic as well as a degradative role in starch metabolism is convincing (60, 129, 196, 208, 109). The high levels of phosphorylase reported in this study to be associated with the starch grains in developing tubers (Results and Discussion, section VII.B.p. 95) and the similarity in properties between the potato enzyme and the synthetic phosphorylase II isozyme in wheat (191), supports the view that some forms of starch phosphorylase in the potato are involved in starch synthesis.

The proposed scheme (Fig. 39), for the synthesis of starch from sucrose in developing potato tubers, is also interesting with respect to the nucleotide specificity. In the first place, sucrose synthetase utilizes UDP with greater efficiency than ADP and UDP-glucose and other uridine nucleotides inhibit the synthesis of ADP-glucose:ADP-glucose and other adenosine nucleotides have no effect on the synthesis of UDP-glucose by this enzyme. ADP-glucose is much more readily converted to starch than UDP-glucose. ADP-glucose, ATP, ADP and AMP all inhibit the synthesis of starch from UDP-glucose, unlike UDP-glucose, UTP and UDP which have no effect on starch synthesis from ADP-glucose. This means that cleavage of sucrose and the synthesis of starch could occur in the same environment since the preferred substrate for sucrose synthetase and the product of its action have very little effect on ADP-glucose:starch glucosyl transferase activity and *vice versa.*

Possible control points in the reaction sequence are ADP-glucose pyrophosphorylase and ADP-glucose:starch glucosyl transferase. These enzymes are thought to be subject to fine control mechanisms which regulate the synthesis of starch from glucose 1-phosphate and ADP-glucose. There is evidence to suggest that sucrose synthetase and starch phosphorylase may also be involved in the regulation of the process (see Introduction pp. 22 and 36).

The suggested pathway provides a possible mechanism by which starch synthesis can be regulated; the Pi liberated during the phosphorylase reaction may activate the ADP-glucose:starch glucosyl transferase reaction and thus ensure the synchronized synthesis of starch from both ADP-glucose and glucose 1-phosphate.

Another interesting observation related to control mechanisms is the Mg^{2+} dependency of many of the enzymes involved in this scheme. Both ADP-glucose and UDP-glucose pyrophosphorylase reactions are Mg^{2+}-dependent and the latter enzyme has been shown to be inhibited by high Mg^{2+} concentrations ($<$ 1.35mM) (see Results and Discussions, section VI.E.p. 73). Several workers have commented on the possible involvement of Mg^{2+} in the regulation of pyrophosphorylase activity (44, 65, 66). Gustafson and Gander (44) have suggested that providing sufficient substrates are available, the cell could regulate the direction of catalysis by controlling the intracellular levels of free Mg^{2+}. Burke (70) has shown that pyrophosphatases of spinach chloroplasts are Mg^{2+}-dependent and he has suggested that light-dependent fluxes of stromal Mg^{2+} levels in the chloroplast could regulate the PPi level of the organelle. A similar situation may arise in the case of the pyrophosphorylase reactions. Potato tuber starch grains are formed within plastids (amyloplasts) and Mg^{2+} fluxes may occur in these organelles.

The failure to detect ADP-glucose pyrophosphorylase activity in mature tubers and the drop, both in sucrose synthetase and UDP-glucose pyrophosphorylase activities in the tissue, together with the inability of mature tubers (both *in vivo* and *in vitro*) to metabolize sucrose and fructose, strongly suggests that the regulation of enzyme (or enzyme inhibitor) synthesis are other important factors in the control of starch synthesis.

M A T E R I A L S   A N D   M E T H O D S

Materials: analar grade chemicals were used whenever possible; other chemicals were of the best available purity. Glass distilled, deionized water was used for the preparation of all solutions.
Plants: Solanum tuberosum (variety Ulster Prince) were used throughout the study; they are a late variety, tuberising between July and September. Winter and spring crops were grown in a greenhouse with a 14h day length provided by supplemental flourescent lighting.

IX. General methods

A. Paper chromatography

Whatman No. 1 and No. 3 papers were used and these were developed by the descending elution technique. Whatman No. 3 paper only was used for preparative chromatography: compounds were located by spraying side strips and elution of materials from the paper was effected with deionized water.

1. Chromatography solvents

(i) ethyl acetate/acetic acid/formic acid/water (18:3:1:4, by vol.)(250)

(ii) 95% ethanol/M-ammonium acetate pH 3.8 (7.5:3, by vol.)(251)

(iii) ethyl acetate/pyridine/water (7:2:1, by vol.)(251)

(iv) n-butanol/ethanol/water (52:/33:15, by vol.)(252)

2. Locating reagents

(i) Reducing carbohydrates were located by dipping the chromatograms in a silver nitrate solution in acetone, allowing to dry in air and then dipping in methanolic sodium hydroxide solution (2%). Sodium thiosulphate solution (10%) was used as a fixative (253).

(ii) Sucrose was located by spraying with 2N-HCl/acetone (1:19,v/v) and heating the papers at 100°C for 3-5 min (102)

(iii) Phosphates were detected by the method of Badurski and Axelrod (254).

(iv) Compounds containing purine or pyrimidine bases were visualized under u.v. light.

B. Paper electrophoresis

A Shandon high voltage electrophoresis apparatus (L24) was used with Whatman 3MM paper. The electrophoresis was carried out for 1-2h at 80mA (60V/cm).

1. Buffers

(i) 0.1M-ammonium formate/formic acid, pH 3.8 (255)

(ii) 0.05M-sodium tetraborate, pH 9.6 (102)

2. Locating reagents

Carbohydrates, phosphates and purine and pyrimidine bases were visualized as described in section IX.A.2.

C. Spectroscopic measurements

Absorbance values were measured with Unicam SP 1800 or SP 500 u.v. spectrophotometers.

D. Radioactive measurements

Radioactivity on paper chromatograms and electrophoretograms was located either with a Tracerlab 4π strip counter (13% efficiency for ^{14}C or by autoradiography. Accurate determinations of radioactive compounds on paper were achieved by the use of either a Beckman CPM100 scintillation counter or a Packard TRI-CARB (model 3390) liquid scintillation spectrometer, using a toluene scintillant (5g PPO/1 redistilled toluene). The efficiency of this method was 90-98% for ^{14}C.

[^{14}C]Starch was solubilized and assayed as described in section X.C.4.

E. Protein estimations

The protein content of all enzyme preparations was measured spectrophotometrically by the method of Lowry et al. (256) except that the Folin-Chiocalteau reagent was diluted with an equal volume of deionized water. BSA was used as the standard protein. The protein concentration of column fractions was monitored at 280nm.

F. Buffers used for ion-exchange columns

1.A, tris-succinic acid, pH 7.5 (10mM), containing EDTA (1mM), $MgCl_2$(5mM), reduced glutathione (1mM) and glycerol (2%).

2.B, tris-succinic acid, pH 6.0 (10mM), containing EDTA (1mM), $MgCl_2$(5mM), reduced glutathione (1mM) and glycerol (2%).

3.C, tris-succinic acid, pH 8.0 (10mM), containing EDTA (1mM) and ethane-1-ol-2-thiol (1.4mM).

G. Extraction of enzymes

Enzyme extractions were performed in a cold room at 5°-$8^{\circ}C$; the tubers were washed, peeled, sliced and homogenised with an appropriate

buffer using an MSE Atomix bottom drive macerator for three periods of 30s with 30s intervals.

The oxidation of phenolic compounds during cell disruption and the presence of endogenous polymerised phenolics, may interfere with the assay, localization and purification of enzymes (257). The following methods were therefore used, either singly or in combination, to overcome these effects.

a), an alkaline pH was chosen whenever possible to reduce the hydrogen bonding potential of both condensed and hydrolysed tannins and, hence, combinations with proteins. An alkaline pH has the advantage of reducing polyphenol oxidase activity;

b), ethane-1-ol-2-thiol or cysteine (15-0.66mM) were included in most extraction and assay media to maintain reducing conditions;

c), DIECA(10-20mM) was used in all extraction buffers. This agent forms a stable complex with copper ions and, hence, inhibits the 'browning reaction' catalysed by polyphenol oxidase. EDTA(20-0.4mM) was employed in all solutions as a metal chelating agent;

d), gel-filtration or dialysis were employed whenever possible to remove phenols during enzyme purification.

H. Preparation of nucleotide solutions

All nucleoside phosphates and sugar nucleotide solutions were neutralized with NaOH solution before use.

X. [^{14}C]Sucrose and [^{14}C]fructose feeding experiments

[U-^{14}C]Fructose and [C^{14}]sucrose were injected into developing tubers and the distribution of label in hexose phosphates, sugar nucleotides, free hexoses, sucrose and starch in the tuber was examined after a suitable incubation period (normally 15 min).

A. Injection and incubation

Three actively growing tubers (2-5g) were excised from the plant, the appropriate labelled carbohydrate (2µl) was injected into the tuber which was left at ambient temperature in the dark for the period of incubation. A microsyringe (5µl) was used for the injection, which followed an imaginary line from where the stolon entered the tuber, to the center of the tuber. The [^{14}C]solutions were injected over a 1min period in 0.5µl quantities. At the end of the incubation period the tubers were quickly frozen and stored at -15°C.

Injections and incubations were also carried out using tubers which were still attached by their stolons to the haulm. At the end of the incubation the tubers were again harvested and frozen at -15°C. The two methods of injection produced no detectable differences in the [^{14}C]metabolites pattern.

B. Extraction of [^{14}C]metabolites

The whole tubers were placed in a pre-cooled X-press (X-25AB Biotec, Sweden) and the cell walls ruptured by three passes through the press (12 tons p.s.i.). Subsequent operations were carried out in a cold room (5°-8°C).

A perchloric acid extract of the tuber pellet was prepared by grinding it in a pestle and mortar with 0.46M-perchloric acid (50ml) (c.f. Murata _et al_ (176). The acid insoluble residue was removed by centrifugation (MSE Minor, maximum speed, 15 min) and the residue re-extracted twice with perchloric acid (50ml). The three acid extracts (total volume 150-200ml) were combined.

The acid insoluble residue, consisting of starch grains and cell wall debris, was washed with water (50ml) and centrifuged (MSE minor, maximum 15 min). This process was repeated and finally the residue was washed with acetone (4 x 50ml) and dried to constant weight under reduced pressure at room temperature.

The total perchloric acid extract was carefully neutralized to pH 6.0 with 10N-KOH, with final adjustments to pH being made with 1N-KOH. The precipitated $KClO_4$ was removed by centrifugation after chilling at -15°C (15-20 min), the supernatant solution was concentrated (to approximately 5ml) by rotary evaporation at 30°C. Further precipitation of $KClO_4$ occurred and this was again removed by centrifugation.

Fructose, glucose, fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate (5mg each), ADP-glucose (2mg) and UDP-glucose (4mg) were added to the extract as carrier compounds for subsequent chromatography. The extract was stored at -15°C overnight.

C. Fractionation and characterization of [^{14}C]metabolites

A modification of the method, as described by Mori and Nakamura (18, 259) and Murata _et al_. (18,259) was used.

The neutralized, perchloric acid extract was thawed and the precipitated $KClO_4$ again removed by centrifugation. A column (10 x 15cm)

of Dowex-1 anion-exchange resin (AG1 x 8, chloride form, 200-400 mesh; Calbiochem.) was prewashed overnight with a solution containing sucrose (0.01M), fructose (0.01M) and glucose (0.01M) and finally with water (100ml). The extract was then applied to the column and subsequent elutions were carried out at a flow rate of 90ml/h.

1. Neutral sugars

The Dowex column was eluted with water (300ml) and the resulting fraction, containing the neutral sugars, reduced to 2ml by rotary evaporation.

Initially, this fraction was applied to a second Dowex-1 anion-exchange resin (borate form) column (10 x 1.5cm) and the borate complexes of the sugars were separated as described by Khym and Zill (260). The carbohydrate content of each fraction was determined by the method of Dubois et al. (261) and the radioactivity of each fraction (40ml) was determined by spotting aliquots (4ml) from each of the fractions on to Whatman No. 3 paper (4 x 7cm), air drying and counting by the liquid scintillation method (section IX.D.p.109).

In latter experiments the neutral sugar fraction (2ml), obtained from the original Dowex column, was streaked (50µl or 100µl) onto Whatman No. 3 paper and developed for 48h in solvent (iii) (see section IX.A.1.). The positions of the labelled carbohydrates were located as described under General Methods and the radioactivity assayed by liquid scintillation counting.

Solutions of the individual radioactive carbohydrates, obtained following either the column chromatography or paper chromatography of the original neutral sugar extract, were streaked onto chromatography paper and characterized by high voltage electrophoresis (buffer (ii)) and descending paper chromatography (solvents (i) or (iii)).

2. Hexose phosphates

Following the elution of the original Dowex column with water, to remove the neutral sugars, the column was next eluted with 0.01M-HCl(350ml). The eluate was neutralized by stirring with AG11A8 ion-retardation resin (Calbiochem.) which had previously been treated with a solution containing fructose 6-phosphate, glucose 6-phosphate and glucose 1-phosphate (5mg each). (These phosphates were added to prevent any adsorption of the labelled hexose phosphates by the ion-retardation resin). The neutralized [^{14}C]hexose phosphate solution was

filtered free of the ion-retardation resin and reduced to 2ml by rotary evaporation at 30°C.

The hexose phosphate fraction was applied to another column (10 x 1.5cm) of Dowex-1 anion-exchange resin (chloride form) and successively eluted (flow rate 90ml/h) with solutions containing 0.025M-NH_4Cl, and 0.01M-$Na_2B_4O_7$(400ml); 0.025M-NH_4Cl,0.025N-NH_4OH and 0.001M-$Na_2B_4O_7$(800ml) and finally 0.025M-NH_4Cl,0.025N-NH_4OH and 0.00001M-$Na_2B_4O_7$(480ml) according to the method of Khym and Cohn (262). Fractions (40ml) were collected and the hexose phosphate levels in each assayed by the phenol-sulphuric acid method of Dubois et al. (261). Aliquots (4ml) from each of the fractions were also spotted onto Whatman No. 3 paper (4 x 7cm) and analysed for radioactivity as described for the neutral sugars (see section X.C.1.p.112).

The paper strips containing [^{14}C]glucose 1-phosphate, [^{14}C] glucose 6-phosphate or [^{14}C]fructose 6-phosphate were washed with toluene, dried and then eluted with deionised water. The solutions were streaked on to chromatography paper and subjected to either high voltage electrophoresis using buffer (i) or descending paper chromatography using solvent (ii). The paper chromatograms or electrophoretograms were cut into strips and the radioactivity in each strip measured by liquid scintillation counting.

3. Sugar nucleotides

ADP-glucose was next eluted from the original Dowex-1 resin column with a solution containing 0.01N-HCl and 0.01M-NaCl(400ml). UDP-glucose was then eluted with a solution containing 0.01N-HCl and 0.05M-NaCl(800ml). Fractions (20ml) were collected and the absorbance at 260nm measured. Aliquots (4ml) from each fraction were spotted on to Whatman No. 3 paper (4 x 7cm) and analysed for radioactivity as described for the neutral sugars (see section X.C.1. p.112).

Radioactive ADP-glucose and UDP-glucose were eluted from the paper strips as described for the hexose phosphates and characterized as described in sections XI.F.3. and XI.G.3.; their identities were also confirmed by paper chromatography using solvent (ii).

4. Starch

Aliquots (1-50mg) of the dry, acid insoluble residue (see Materials and Methods, section X.B. p.111) were weighed into scintillation

vials, moistened with distilled water (100µl) and solubilized using NCS tissue solubilizer (Amersham/Searle) (1.0ml). The vials were incubated for 12h at 37°C. Scintillant (5g PPO/1 redistilled toluene) (20ml) was added to each vial and the radioactivity measured by liquid scintillation counting. Quench corrections were applied to all results. The [^{14}C]starch was characterised as described in section X.D.2. of the Materials and Methods.

D. Extraction and fractionation of labelled starch derived from [^{14}C]fructose or [^{14}C]sucrose

The injection and incubation of the tubers was achieved as described in section X.A. of the Materials and Methods except that the tubers were larger (50-100g) and 500g of untreated tubers were also added. The preparation and fractionation of the starch was carried out by the method of Schoch (263).

1. Preparation of starch

The tubers were washed, peeled, sliced and homogenized in an MSE Atomix macerator with ethane-1-ol-2-thiol (60mM) (2 vol.) for 5 min. The slurry was screened with muslin (4 layers) and the starch washed through with a solution of ethane-1-ol-2-thiol (60mM). The pulp was rehomogenized with ethane-1-ol-2-thiol (60mM, 2 vol.) and screened again through muslin (4 layers). The combined starch suspension was passed through muslin (8 layers) into a measuring cylinder (2l) and allowed to settle for 2h. The supernatant solution was decanted off and the top of the starch cake rinsed with a little distilled water to remove any residual fibre. The starch was resuspended in distilled water, screened through musline and allowed to settle for 1h in a measuring cylinder. The process of resuspension, screening and sedimentation was repeated twice.

The whole starch cake was resuspended in ethanol, filtered off with a Büchner funnel, washed with methanol and dried at 40°C overnight to constant weight. Radioactive analysis of the starch was performed as described in section X.C.A. of the Materials and Methods.

2. Characterization of [^{14}C]starch

The radioactive starch was characterized by an examination of the hydrolysis products after treatment with α- and β- amylases and 2N-HCl.

(i) Starch (10mg) was treated with β-amylase (Wallerstein) (625)μg) in sodium acetate buffer, pH 4.9 (1μmol). The reaction mixture (total volume 50μl) was incubated at $37^{\circ}C$ for 16h with continuous shaking.

(ii) Starch (10mg) was treated with salivary α-amylase in sodium acetate buffer, pH 6.5 (1μmol) containing NaCl(0.25μmol). The reaction mixture (total volume 50μl) was incubated at $37^{\circ}C$ for 16h with continuous shaking.

(iii) Starch (10mg) was hydrolysed at $100^{\circ}C$ for 1h with 2N-HCl (200μl). The incubation mixture was neutralized with 10N-KOH (40μl).

In all cases the reaction mixtures were streaked on to chromatography papers which were developed with solvent (i). The chromatograms were cut into strips (1cm) and the distribution of radioactivity assayed byliquid scintillation counting.

3. <u>Fraction of Starch</u>

Starch was fractionated into amylose and amylopectin by the technique of Lansky <u>et al</u>. (264).

XI. <u>General methods in enzymology</u>

A. <u>Microassay technique</u>

Conventional techniques were used for enzyme assays where the total volume of the assay exceeded 150μl. In other cases a microassay technique (cf. Porter and Hoban (265)) was used to cope with the small volumes of liquid.

Solutions of the individual constituents of the enzyme assay were measured and placed separately, as droplets, on to a strip of Parafilm placed on the cover of a petri-dish filled with ice. The solutions were then mixed together thoroughly and taken up in a long capillary tube which was sealed at both ends using a bunsen flame, taking care not to heat the reaction mixture. The incubation was started by placing the sealed tube into a thermostated water bath and the reaction stopped by placing the sealed tubes in a boiling water bath (30s) or in a mixture of acetone and cardice. The reaction mixture was streaked on to paper dried using a hot air blower prior to chromatographic fractionation.

B. Sucrose synthetase in potato tuber

1. Purification

Tubers (300g) were homogenized in 0.1M-tris-HCl buffer, pH 7.5 (200ml), containing EDTA (20mM), cysteine (20mM) and DIECA (20mM). The slurry was strained through muslin (2 layers) and the filtrate centrifuged at 20,000 x g for 15 min.

Solid $(NH_4)_2SO_4$ was added to the supernatant solution to 25% saturation and the precipitate removed by centrifugation. The $(NH_4)_2SO_4$ concentration in the supernatant solution was then increased to 40% saturation. the precipitate again collected by centrifugation and dissolved in a minimum volume of 10mM-tris-HCl buffer, pH 7.0, containing cysteine (1mM) and EDTA (1mM). The resulting protein solution (1ml) was desalted with a Sephadex G-25 column (10 x 1.5cm). and eluted with the same buffer at a flow rate of 42 ml/h. Fractions (2ml) were collected and the protein eluted between fractions 4 and 5, inclusive, used for determination of activity.

2. Enzyme assay

(i) Sucrose cleavage

The incubation mixture (total volume 70µl) contained tris-HCl buffer. pH 6.5 (5.0µmol), ethane-1-ol-2-thiol (70nmol), UDP or ADP (0.25µmol), [U-^{14}C]sucrose (0.05µmol; 174,200 c.p.m.) and enzyme (50µl).

Incubations were carried out (30mins. 30°C) using the micro-assay technique. The reactions were terminated by streaking the mixtures on to chromatograms and air drying. The chromatograms were developed with either solvent (ii) or (iii) and the radioactivity associated with fructose and the nucleoside diphosphate glucose located using a scintillation counting.

(ii) Sucrose synthesis

The incubation mixture (total volume 70µl) contained tris-HCl buffer, pH 7.5 (3.3µmol), cysteine (20nmol), UDP-glucose or ADP-glucose (1.5µmol), [U-^{14}C]fructose (1.0µmol; 67,900 c.p.m.) and enzyme (50µl). The incubations were again carried out at 30°C (30 min) using the micro-assay technique and the products fractionated (solvent (iii) only) and radioactivity determined as described above (see section XI.B.2.(i)).

3. Identification of products

The [^{14}C]fructose, produced by the sucrose-cleavage reaction

catalysed by sucrose synthetase, was characterized by paper chromatography (solvent (i)) and high voltage electrophoresis (buffer (ii)). [^{14}C]sucrose, produced from the nucleoside diphosphate glucose and [^{14}C]fructose, was identified by the methods described above for [^{14}C]fructose and by hydrolysis with Candida utilis invertase (see Materials and Methods, section XI.C.1.) which yielded glucose and [^{14}C]fructose.

Corroboration of the identities of both [^{14}C] ADP-glucose and [^{14}C]UDP-glucose, produced by the sucrose cleavage reaction, was achieved by high voltage electrophoresis (buffer(i)).

C. Preparation of α-D-glucopyranosyl-[U-^{14}C]β-D-fructofuranoside

Sucrose synthetase was prepared as described in Materials and Methods XI.B.1. The incubation mixture (total volume 200µl) contained glycylglycine buffer, pH 8.0 (15µmol), UDP-glucose (5.0µmol), [U-^{14}C]-fructose (0.1µmol; 30µCi) and enzyme (150µl). The incubation (30°C, 1hr) was carried out in a 1ml test-tube, and the reaction stopped by streaking the mixture on to a Whatman No. 3 chromatography paper. The chromatogram was developed for 48h with solvent (i), the radioactive sucrose located with a 4π scanner and eluted from the paper with distilled water. Further purification was effected by rechromatography twice using solvents (iii) and (iv). The final sample, obtained by elution from the paper with water, was concentrated to dryness and stored at -15°C.

1. Characterization of the synthetic α-D-glucopyranosyl [U-^{14}C]β-D-fructofuranoside.

The [^{14}C]sucrose had the same chromatographic mobility as sucrose in solvents (i), (iii) and (iv). The distribution of the label in the molecule was confirmed by hydrolysis with invertase as follows: the reaction mixture (total volume 30µl), containing sodium acetate buffer, pH 5.0 (1µmol), Candida utilis invertase (1000 units, Sigma grade X) and [^{14}C]sucrose (5,000 c.p.m.), was incubated for 1h at 30°C and the reaction terminated by streaking the mixture on to a chromatogram. The substrate and products were found only to be associated with fructose and the unhydrolysed disaccharide.

D. Metabolism of fructose by potato tuber extracts

1. Enzyme preparation and reaction conditions

Tubers (300g) were homogenized in tris-HCl, pH 7.5 (0.1M, 200ml), containing EDTA (10mM), cysteine (10mM) and DIECA (10mM). The extract was strained through muslin (2 layers) and the cell debris removed by centrifugation (20,000 x g, 15 min). Solid $(NH_4)_2SO_4$ was added to the supernatant solution to give 100% saturation. The precipitate was collected by centrifugation (20,000 x g, 30 min) and redissolved in tris-HCl buffer, pH 7.0 (10mM, 40-50ml), containing EDTA (1mM) and cysteine (1mM). Prior to use this enzyme fraction (1ml) was desalted using a Sephadex G-25 column (10 x 1.5ml) as described previously (see Materials and Methods XI.B.1.).

The reaction mixtures (total volume 90µl) contained glycyl-glycine buffer, pH 7.5 (5.0µmol), ethane-1-ol-2-thiol (15nmol), $MgCl_2$ (1.0µmol), glucose 1,6-diphosphate (25nmol), NaF (10µmol), ATP (0.4µmol), [U-^{14}C]fructose (0.2µmol; 17,000 c.p.m.) and enzyme solution (50µl). The mixtures were incubated at 30°C in polythene test-tubes for varying times up to 120min: reactions were terminated by the addition of absolute ethanol (100µl) and the mixtures then streaked on to chomatograms, which were developed with solvent (60h). The radioactivity associated with ADP-glucose and hexose phosphates was located with a 4π scanner and then measured, after elution, by liquid scintillation counting.

2. Effects of carbohydrate metabolism on fructose metabolism by potato tuber extracts.

Incubation mixtures and subsequent treatment were as described in Materials and Methods XI.D.1., except that ADP-glucose, glucose 1-phosphate, glucose 6-phosphate or fructose 6-phosphate (0.2µmol of each) were included in the reaction mixtures.

3. Characterization of products

[^{14}C]ADP-glucose and the hexose phosphates were identified by paper chromatography (solvent (ii))and by high voltage electrophoresis (buffer (i))

The identity of fructose 6-phosphate and glucose 6-phosphate was also confirmed by column chromatography on a Dowex-1 anion-exchange resin (see Materials and Methods X.C.1.).

E. Enzymic synthesis of ADP-glucose from α-D-glucopyranosyl-[U-^{14}C]β-D-fructofuranoside

1. Enzymic preparation and reaction conditions

The enzyme solution was prepared as described in Materials and Methods, section XI.D.1.

The incubation mixture (total volume 95μl) contained glycylglycine buffer, pH 7.5 (5.0μmol), ethane-1-ol-2-thiol (95nmol), $MgCl_2$ (1.0μmol), glucose 1,6-diphosphate (25nmol), NaF (10μmol), ATP (0.2μmol), UDP (0.25μmol), α-D-glucopyranosyl [U-^{14}C]β-D-fructofuranoside (0.05μmol), 174,260 c.p.m.) and enzyme (50μl). The incubation conditions and analysis of the products were as described previously (see Materials and Methods, section XI.D.1-3.).

F. ADP-glucose pyrophosphorylase

1. Purification

Tubers (250g) were homogenized in tris-HCl buffer, pH 7.5 (0.5M, 200ml), containing EDTA (10mM), ethane-1-ol-2-thiol (10mM), DIECA (10mM), $MgCl_2$ (5mM) and glycerol (2%). The extract was strained through muslin (2 layers) and the cell debris removed by centrifugation (20,000 x g, 15min). This crude preparation had a specific activity of 9.3 nmol ADP-glucose/h/mg protein. The supernatant solution was 40% saturated with solid $(NH_4)_2SO_4$ and the resulting precipitate removed by centrifugation. The $(NH_4)_2SO_4$ concentration was then increased to 80% saturation and the precipitated protein again collected by centrifugation. The pellet was re-dissolved in tris-succinic acid buffer, pH 7.5 (10mM), 20-30ml), containing EDTA (1mM), ethane-1-ol-2-thiol (1mM), $MgCl_2$ (5mM) and glycerol (2%) and the solution dialysed against two changes of this same buffer (2l) overnight. The specific activity of this dialysed protein solution was 7.9nmol ADP-glucose/h/mg protein. This fall in the specific activity of the enzyme, following $(NH_4)_2SO_4$ fractionation, is presumably linked to the unstable nature of the enzyme (see Results and Discussion, section VI.D.p.70).

(i) DEAE-cellulose chromatography

The dialysed solution (10ml) was applied to a DEAE-cellulose 52 (chloride form) anion-exchange column (3 x 9cm, Whatman product), which had been previously equilibriated with buffer A. After elution

with one column volume of buffer A, the adsorbed protein was eluted with a solution of increasing pH and buffer molarity. For this purpose the mixing chamber contained buffer A (250ml) and the reservoir tris-succinic acid buffer, pH 5.8 (50mM, 250ml) containing EDTA (1mM), $MgCl_2$ (5mM), reduced glutathione (1mM) and glycerol (2%). Fractions (5ml) were collected at a flow rate of 30ml/h.

Peak II (see Fig. 20, p.69) was collected and concentrated to 5-8ml using an Amicon micropore ultrafiltration unit (UM-10 membrane). The concentrated solution was dialysed against buffer B.

(ii) Phosphocellulose chromatography

The dialysed protein fraction from the DEAE-cellulose column was next applied to a column of P11 cation-exchange cellulose (H^+ form, 10 x 1.5cm, Whatman product) which had been equilibriated with buffer B. The protein was eluted (30ml/h) with buffer B until the optical density [280nm] of the eluate was less than 0.05 (approximately 1.5 column volumes).

(iii) $(NH_4)_2SO_4$ fractionation

Solid $(NH_4)_2SO_4$ was added to the P11 protein fraction to 80% saturation. The precipitate was recovered by centrifugation, the pellet extracted with glycylglycine buffer, pH 7.5 (10mM, 20ml), 60% saturated with $(NH_4)_2SO_4$ and also containing EDTA (1mM), $MgCl_2$ (5mM) and reduced glutathione (1mM). The undissolved protein was collected by centrifugation and extracted once more with the glycylglycine buffer (20ml) containing EDTA, $MgCl_2$, reduced glutathione and $(NH_4)_2SO_4$, the latter being reduced to 40% saturation. The resulting solution (40-60% $(NH_4)_2SO_4$ fraction) was recovered by centrifugation and dialysed against the glycylglycine, EDTA, $MgCl_2$, reduced glutathione buffer (1l). The dialysed solution, after concentration (5-2ml) with an Amicon Micropore Ultrafiltration unit (UM-10 membrane), was used as the source of ADP-glucose pyrophosphorylase. The specific activity of this preparation was 13.9nmol ADP-glucose formed/h/mg protein.

2. Enzyme assay.

(i) Nucleoside diphosphate glucose synthesis was measured by a modification of the technique of Shen and Preiss (266). The reaction mixture (total volume 75µl) contained glycylglycine buffer, pH 7.2 (20µmol), ethane-1-ol-2-thiol (50 nmol), $MgCl_2$ (1.0µmol), ATP (0.2µmol), NaF (5.0µmol), BSA (50µg), [U-^{14}C]glucose 1-phosphate (0.2µmol; 110,000 c.p.m.)

and enzyme (30μl). The mixture was incubated at 30°C and the reaction terminated after 30 min by boiling for 30 S. (The rate/time curve was linear for the period of incubation under the conditions used). The reaction mixture was then added to glycylglycine buffer, pH 8.0 (30μmol, 50μl) containing E.coli alkaline phosphatase (0.1 unit, Sigma product type IIS) and incubated at 37°C for a further 40 min. The unreacted [U-^{14}C]glucose 1-phosphate was thus hydrolysed to [U-^{14}C]glucose. A sample (100μl) of this phosphatase-treated reaction mixture was adsorbed on to a DEAE-cellulose paper disc (2.3cm diameter, Whatman product DEAE 82) and the disc then washed with distilled water (600ml) in a Buchner funnel (267). After air drying the discs, the [^{14}C]NDP-glucose was determined by liquid scintillation counting.

(ii) The incubation mixture was the same as in assay (i) but the reaction was terminated by streaking onto chromatography paper and drying. The chromatogram was then developed in solvent (ii), and the region containing the [^{14}C]NDP-glucose was assayed for radioactivity by liquid scintillation counting.

3. Characterization of [^{14}C]ADP-glucose

Samples of the paper strips (from XI.F.2.(ii).) were washed with toluene, dried, and then eluted with distilled water. The solution was streaked onto chromatography paper and subjected to high voltage electrophoresis (buffer (i)). UDP-glucose, ADP-glucose and glucose 1-phosphate were used as standards. The electrophoretograms were cut into strips (1cm) and the radioactivity of each strip measured by liquid scintillation counting.

G. UDP-glucose pyrophosphorylase

1. Purification

Developing tubers (200g) were homogenized in tris-succinic acid buffer, pH 8.0 (0.5M, 200ml), containing EDTA (1mM), ethane-1-ol-2-thiol (15mM) and DIECA (10mM). The slurry was strained through muslin (2 layers) and cell debris removed by centrifugation (20,000 x g, 15 min). Solid $(NH_4)_2SO_4$ was added to the supernatant solution to 100% saturation and the precipitate collected by centrifugation (20,000 x g, 30 min). The protein was dissolved in buffer C and dialysed against two changes of this same buffer (2l) overnight. The protein solution (6ml) was passed (40ml/h) through a Sephadex G-100 column (2.5 x 100cm)

which had been equilibriated with buffer C. The active fractions (160ml-240ml) were collected, pooled and concentrated (5-10ml) with the Amicon Micropore Ultrafiltrator (UM-10 membrane).

(i) First DEAE-cellulose chromatography

The concentrated protein solution (2.5ml) was applied to a DEAE-cellulose column (2.5 x 15cm) which had been equilibriated with buffer C and the column was washed (one column volume) with buffer C and the adsorbed protein eluted (30ml/h) with a solution of NaCl of increasing concentration. The gradient was achieved with buffer C (250ml) in the mixing chamber and tris-succinic acid buffer, pH 8.0 (50mM) containing EDTA (1mM), ethane-1-ol-2-thiol (1mM) and NaCl (0.7M) (250ml) in the reservoir. The fractions eluted between 70ml and 220ml were pooled and concentrated (5ml) using an Amicon Ultrafiltrator (UM-10 membrane).

(ii) Second DEAE-cellulose chromatography

The concentrated protein solution (3.5ml) was loaded onto a second DEAE-cellulose column (2.5 x 10cm) that had been equilibriated with buffer C. Unadsorbed protein was eluted with buffer C (2 column volumes) and the enzyme was then washed from the column (30ml/h) with an increasing concentration of tris-succinic acid buffer. (The mixing chamber contained buffer C (250ml) and the reservoir tris-succinic acid buffer, pH 6.0 (50mM, 250ml) containing EDTA (1mM) and ethane-1-ol-2-thiol (1.4mM)). Fractions (5ml) were collected, pooled and then concentrated (2.5 - 5.0ml) using an Amicon Ultrafiltration unit (UM-10 membrane).

Enzyme assay

The enzyme was normally assayed by the methods described in sections XI.F.2.(i) and (ii) except that UTP replaced ATP and the incubation period was usually 15 min.

A spectrophotometric assay was developed for monitoring the enzyme activity of column fractions using the Griffen Bioanalyst (automated chemistry module).

The incubation mixture (total volume 2.9ml) was added to the enzyme (100µl) at three stations. Incubations were at 30°C and all reagents were kept at 0°C. Changes in absorbance at 340nm were followed using an Unicam SP 1800 spectrophotometer fitted with a silica

flow cell (10mm). The enzyme profile of the column eluant was recorded on an Unicam AR25 linear recorder.

All reagents were prepared in tris-succinic acid buffer, pH 8.0, (33mM). Station 1 dispensed 1.0ml of solution containing glucose 1-phosphate (0.6μmol) and NaF (2.0μmol); station 2 dispensed 1ml of a solution containing UDP-glucose dehydrogenase (100 units, Sigma product) and NAD (1.5μmol) and station 3 dispensed 1.3ml of a solution composed of UTP (0.6μmol) and $MgCl_2$ (3.0μmol). Absorbances were measured at 1 min, after the final additions, and at 15 or 30 min. The rate/time curve for the reactions were linear for the incubation period and the conditions used.

In some assays fructose 1,6-diphosphate (0.2μmol) was included in the solution at station 1.

3. Characterizations of UDP-glucose

[^{14}C]UDP-glucose was characterized by the same methods as described for ADP-glucose (see sections IX.F.3.)

H. Preparation of starch grains

1. Freeze dried

Developing tubers (1500g) were washed, peeled and grated. The slices were washed with ethane-1-ol-2-thiol (60mM) and the starch suspension filtered through muslin (2 layers). The grains were collected by centrifugation (MSE Minor centrifuge, maximum speed, 5 min), resuspended in deionized water and centrifuged again. The process of resuspension and centrifugation was repeated a second time. The starch grain slurry was then frozen, using liquid N_2, freeze dried and stored at -15°C.

2. Acetone-washed

The starch grains were prepared using a technique similar to that described by Frydman (135). The grains were extracted as in section XI.H.1., except they were washed only once in deionized water. The starch grain pellet was then washed with acetone (5 vol.) and centrifuged (MSE Minor centrifuge, maximum speed, 2 min). This acetone-washing was repeated four times. The grains were dried under reduced pressure and stored at -15°C.

I. Starch grain-bound nucleoside diphosphate glucose: starch transglucosylase

1. Enzyme assays

(i) The incubation mixture (total volume 50µl), contained acetone-washed or freeze dried starch grains (10mg) and either ADP-[U-^{14}C]glucose (0.1µmol, 20,000 c.p.m.) or UDP-[U-^{14}C]glucose (0.1µmol, 50,000 c.p.m.). Incubations were carried out in test tubes at 30°C for 15 min (the rate/time curve was linear under the conditions used) with continuous shaking. The reaction was terminated with aqueous methanol (75%, 2ml) and the radioactive grains collected by centrifugation (MSE Minor centrifuge, speed 4, 2 min) and washed three times with aqueous methanol (75%, 2ml). The grains were then dissolved in NaOH (1ml, 0.1N) and to an aliquot of this solution (200µl) was added NCS tissue solubilizer (1ml) and the radioactivity assayed as described under General Methods, section IX.D.

(ii) The transglucosylase assays were performed as described in (i) but KCl (1.25µmol) was included in the incubation mixture.

2. Characterization of products

The methanol-washed [^{14}C] starch grains were dried (40°C) and characterized as described in section X.D.2.

J. Analysis of other starch grain-bound enzymes

1. Sucrose synthetase

(i) Preparation

Freeze dried starch grains were prepared as described in section XI.H.1. Grains (1g) were subjected to ultrasonic disruption (MSE Ultrasonic disintegrator, medium power, amplitude 6, 16 microns peak to peak) in tris-HCl buffer, pH 7.0 (10mM, 5ml), containing EDTA (1mM) and cysteine (1mM). The supernatant solution was collected by centrifugation (MSE Minor, maximum speed, 5 min) and used as the enzyme source.

(ii) Enzyme assays

a), The supernatant solution was assayed for sucrose synthetase activity by the methods previously described (see section XI.B.2.).

b), The reaction mixture (total volume 50µl), contained starch grains (10mg), citrate buffer, pH 6.3 (5µmol), EDTA (0.25µmol), ethane-1-ol-2-thiol (1.5nmol), UDP or ADP (0.5µmol) and [U-^{14}C]sucrose (0.5µmol, 867,800 c.p.m.). The incubation and subsequent analysis of the radioactive starch grain product was performed as described previously (see section XI.I. of the Materials and Methods). The radioactive methanol washings

from the grains were pooled and concentrated to 100µl by rotary evaporation and then applied to chromatography paper. The chromatograms were developed using solvent (ii) and the radioactivity associated with either ADP-glucose or UDP-glucose assayed by liquid scintillation counting.

2. Pyrophosphatase

The incubation mixture (total volume 50µl), consisted of freeze-dried or acetone-washed starch grains (10mg), glycylglycine buffer, pH 8.0 (5.0µmol), $MgCl_2$ (1µmol) and PPi (5µmol). The mixture was shaken (30°C) and the reaction terminated after suitable incubation times by the addition of aqueous methanol (75%, 2ml). The methanol was decanted after centrifugation and the grains washed twice in distilled water (2ml). The methanol and water washings were combined and reduced to dryness by rotary evaporation. The extract was dissolved in distilled water (0.6ml) and the Pi was assayed by the Fiske and Subbarow method (268).

3. Nucleoside diphosphate glucose pyrophosphorylase

The incubation mixture (total volume 50µl) consisted of acetone-washed starch grains (10mg), glycylglycine buffer, pH 8.4 (4.0µmol), KCl (1.25µmol), EDTA (0.25µmol), $MgCl_2$(1.0µmol), ATP or UTP (0.3µmol) and [U-^{14}C]glucose 1-phosphate (0.5µmol, 92,000 c.p.m.). The resulting labelled grains were washed and the aqueous methanol washings analysed for either [^{14}C]ADP-glucose or [^{14}C]UDP-glucose as described in section XI.J.1.(ii)b of the Materials and Methods. The assay method described in section XI.I.2. was used to investigate the possible formation of [^{14}C]starch by the combined action of pyrophosphorylases and transglucosylases.

4. Starch phosphorylase

The reaction mixture (total volume 50µl) contained freeze-dried or acetone-washed starch grains (10mg), citrate buffer, pH 6.3 (5.0µmol), EDTA (0.25µmol) and [U-^{14}C]glucose 1-phosphate (0.5µmol, 100,000 c.p.m.). Soluble starch phosphorylase (soluble enzyme preparation, 20µl) was assayed using a similar incubation mixture but with enzymically inactive (acetone-washed) starch grains replacing the active starch grains. Incubations and analysis of the labelled product was carried out as described previously (see section XI.I.).

K. Coupled pyrophosphorylast and transglucosylase reactions

1. Enzyme preparation

The soluble nucleoside diphosphate glucose pyrophosphorylases were prepared as described in section XI.F.1., except that the dialysis step was omitted and the protein solution (1ml) was desalted using a Sephadex G-25 column (1.5 x 10cm) as described in section XI.B.1. The starch grain-bound nucleoside diphosphate glucose:starch transglucosylases were prepared as described in section XI.H.2.

2. Assay

The reaction mixture (total volume 60µl) contained acetone-washed starch grains (10mg), glycylglycine buffer pH 8.0 (5.0µmol), EDTA (0.25µmol), KCl (1.25µmol), NaF (5.0µmol), maltose (3.0µmol), ATP or UTP (0.3µmol), [U-^{14}C]glucose 1-phosphate (0.5µmol, 80,000 c.p.m.) and enzyme (20µl). The mixture was shaken continuously (30°C) and the reaction terminated by the addition of aqueous methanol (75%, 2ml). The [^{14}C]starch grains produced were washed with aqueous methanol and assayed for radioactivity as described in section XI.I.1. of the Materials and Methods. The characterization of the starch was achieved as described previously (see Materials and Methods, section XI.I.2.).

L. Coupled sucrose synthetase and transglucosylase reactions

1. Enzyme preparation

Sucrose synthetase and the transglucosylases were prepared as described previously (see Materials and Methods, sections XI.B.1. and XI.H.2.).

2. Assay

The reaction mixture (total volume 60µl) contained acetone-washed starch (10mg), glycylglycine buffer, pH 7.0 (5.0µmol), ethane-1-ol-2-thiol (1.5nmol), EDTA (0.25γmol), KCl (1.25µmol), NaF (5.0µmol), maltose (3.0µmol) UDP or ADP (0.1µmol), [U-^{14}C]sucrose (0.1µmol, 200,000 c.p.m.) and enzyme (20µl). The reaction mixtures were shaken continuously (30°C) and the reaction terminated by the addition of aqueous methanol (75%, 2ml). The [^{14}C]starch grains produced were washed with methanol and assayed for radioactivity as described previously (see section, XI.I.1.). Characterization of the [^{14}C]starch product was performed as described in section XI.I.2. of the Materials and Methods.

M. Enzymic conversion of [U-^{14}C]fructose to starch

1. Preparation of enzyme fraction

The soluble enzyme fraction was prepared as described in section XI.D.1. of Materials and Methods and the starch grain-bound transglucosylase as described in section XI.I.1.

2. Assay

The incubation mixture total volume (60µl) contained acetone-washed starch grains (10mg), glycylglycine buffer, pH 7.5 (5.0µmol), $MgCl_2$ (1.0µmol, EDTA (0.25µmol), KCl (1.25µmol), NaF (10µmol), glucose 1,6-diphosphate (25µmol), ATP (0.4µmol), [U-^{14}C]fructose (0.2µmol, 200,000 c.p.m.) and enzyme (20µl). The incubation, termination of the reaction and the assay of [^{14}C]starch was performed as described in section XI.I.2.

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Table 1. *Sugars and reserve polysaccharide in susceptible barley as percentage of dry weight after treatment with DDT*

Details are given in the text.

	Time after treatment (day)	*Ethanol-soluble carbohydrate	†Sucrose (%)	Glucose (%)	Fructose (%)	Fructosan (%)
Susceptible barley, DDT-treated	0.5	9.8	8.1	1.0	0.8	—
	2.0	22.5	8.6	7.8	6.1	0.4
	5.0	43.9	9.3	18.3	14.5	—
Susceptible barley, untreated	0.5	7.0	5.1	1.9	—	—
	2.0	10.9	6.2	2.5	2.5	0.6
	5.0	9.8	5.9	2.0	1.8	—

* At day 0 the amount of ethanol-soluble carbohydrate comprised 3.4% of the dry weight.
† In treated barley a small amount of raffinose (tentative identification) was also present.

in dry weight of the treated leaves (Fig. 1). This increase is accompanied by cessation of growth of treated leaves and of leaves which have not contacted DDT, and the weight of the plant as a whole is significantly less than for untreated plants (J. D. Hayes, personal communication). Five days after treatment, ethanol-soluble carbohydrate comprised some 40% of the dry weight of treated leaves, in contrast with untreated controls, or DDT-treated or untreated resistant barley, where it comprised some 10% of the dry weight. Analysis showed that a marked increase in glucose and fructose, together with a small increase in sucrose, accounted for the increase in dry weight (Table 1). Hydrolysis of polysaccharide reserves is not likely to account for the increase since concentrations of fructosans were similarly low in both treated and untreated leaves. These increases explained an earlier puzzling observation in $^{14}CO_2$ fixation studies, that, as well as net fixation of radioactivity in treated susceptible plants being decreased, as noted earlier, there was also a decrease in the specific radioactivity of the ethanol-soluble extract. Thus though net fixation of CO_2 is lowered glucose and fructose, nevertheless, accumulate through some inhibition of utilization of the products of fixation. Accordingly, if susceptible plants are kept in the dark immediately after DDT treatment no increase in carbohydrate concentration in the leaves is evident (Hayes, 1966). In contrast, if susceptible seedlings are treated with DDT and illuminated for some days before being held in darkness the concentration of ethanol-extractable material (principally carbohydrate) increases over the succeeding 20h, although in untreated seedlings the concentration falls.

On the basis of these results we suggest that it is the inhibition of photosynthetic electron flow, and hence ATP formation, that may be the lethal effect of DDT on susceptible barley. This disturbance in energy balance is reflected in a decreased ability to fix CO_2 to form phosphorylated intermediates of the carbon-reduction cycle, and hence glucose and fructose, but more important may be an impairment of their further metabolism and of translocation of products of CO_2 fixation to the rest of the seedling. At this time it is not possible to say whether DDT also affects ATP generation by respiration.

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Control of Starch Synthesis in Potato Tuber (*Solanum tuberosum*)

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Starch-grain-bound enzyme preparations were first used by de Fekete *et al.* (1960) to demonstrate the transfer of glucose from UDP-glucose to starch and a similar reaction with ADP-glucose was reported by Recondo & Leloir (1961). Starch synthases have also been found in soluble forms (Frydman & Cardini, 1964*a*,*b*; 1966), and many laboratories have detected multiple forms of these soluble enzymes (Ozbun *et al.*, 1972; Schiefer *et al.*, 1973).

In the present study starch grains were prepared by the method of Frydman (1963); this technique involves the use of acetone to remove starch phosphorylase from the grains. No starch phosphorylase, sucrose synthase, UDP-glucose pyrophosphorylase, ADP-glucose pyrophosphorylase or inorganic pyrophosphatase could be detected in the acetone-washed grains.

Starch synthase activity was assayed by incubating the grains with either ADP-[U-^{14}C]glucose or UDP-[U-^{14}C]glucose. The standard reaction mixture contained glycine buffer (pH 8.5, 5.0 μmol), EDTA (0.25 μmol), KCl (1.25 μmol), ADP-[U-^{14}C]-glucose (0.1 μmol, 20000 c.p.m.) or UDP-[U-^{14}C]glucose (0.1 μmol, 50000 c.p.m.) and starch grains (10 mg) in a total volume of 50 μl. All incubations were carried out at 30°C with shaking. The reaction was terminated with 95% aqueous methanol (2 ml) and the grains washed three times with 75% aqueous methanol (2 ml). The labelled starch was taken up in 0.1 M-NaOH (1 ml) and 0.5 ml of this solution mixed with NCS tissue solubilizer (1.0 ml; Amersham/Searle, Des Plaines, Ill., U.S.A.) and the radioactivity measured with a scintillation counter.

KCl was found to stimulate both ADP-glucose–starch and UDP-glucose–starch glucosyltransferase (EC 2.4.1.b and EC 2.4.1.21) activities; maximum stimulation was observed in the presence of 25 mM-KCl. The two activities exhibited normal Michaelis–Menten kinetics; the K_m values for ADP-glucose and UDP-glucose were 3.3 and 6.6 mM respectively. The specific activity of the ADP-glucose–starch glucosyltransferase was 0.11 and for UDP-glucose–starch glucosyltransferase 0.02 nmol/min per mg of starch.

PP_i and P_i stimulated transglucosylation using ADP-glucose as substrate but with UDP-glucose, inhibition was observed (Table 1). PP_i was found to have a non-competitive effect on both ADP-glucose–starch and UDP-glucose–starch glucosyltransferase activities. The effects of PP_i and P_i were also essentially the same using starch grains that had been washed from potato tissue with water and then freeze-dried.

The effect of various carbohydrate metabolites on transglucosylation from both UDP-glucose and ADP-glucose was examined. Sucrose, fructose, glucose 1-phosphate and fructose 6-phosphate had no detectable effect on the reactions, but characteristic inhibi-

Table 1. *Regulation of potato tuber glucosyltransferases*

Enzyme assays were performed as described in the text.

		Activation or inhibition of glucosyltransferase activity (% of control)	
Effector (10 mM)	Glucose donor ...	ADP-glucose	UDP-glucose
Control		100	100
Control (−KCl)		55	72
PP_i		132	62
PP_i (−KCl)		75	
P_i		116	81
P_i (−KCl)		64	
ATP		100	46
ADP		32	52
AMP		100	70
Cyclic AMP (1 mM)		100	100
ATP (+cyclic AMP, 1 mM)		100	41
ADP-glucose		—	46 (K_i 25 mM)
UTP		100	95
UDP		100	87
UDP-glucose		100	—

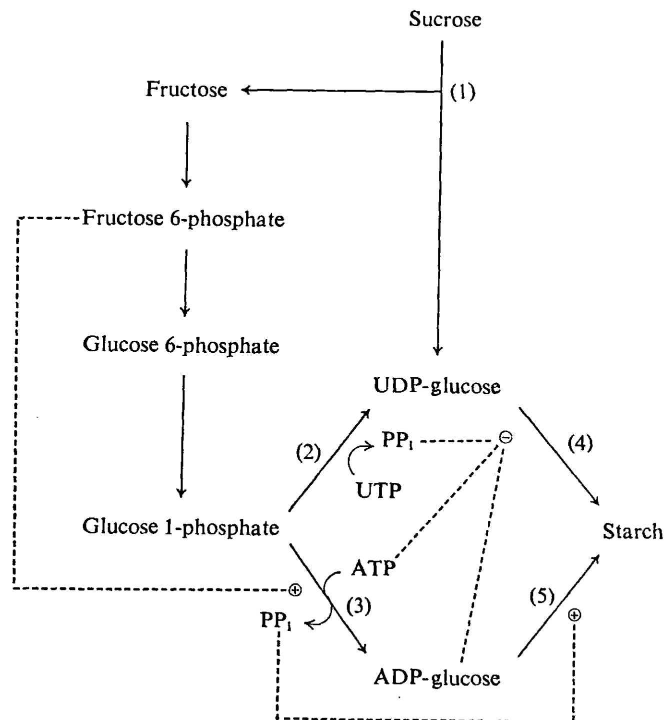

Fig. 1. *Regulation of starch synthesis in developing potato tubers*

(1) Sucrose synthetase; (2) UDP-glucose pyrophosphorylase; (3) ADP-glucose pyrophosphorylase; (4) UDP-glucose–starch glucosyltransferase; (5) ADP-glucose–starch glucosyltransferase.

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tions were observed in the presence of phosphorylated nucleotide derivatives (Table 1). The ADP-glucose–starch glucosyltransferase activity was inhibited only by ADP; this differs from the results of Frydman (1963) who, using a similar enzyme preparation, also obtained inhibition with ATP and AMP. In the case of transglucosylation from UDP-glucose the observations of Frydman were confirmed.

The results of this study suggest various regulating mechanisms which may function during the synthesis of starch from sucrose in the potato tuber. These are summarised in Fig. 1.

Of particular interest is the role of PP_i, which may inhibit starch formation from UDP-glucose but stimulates the synthesis from ADP-glucose. This effect is presumably coupled with the reaction of ADP-glucose pyrophosphorylase which produces PP_i. The latter enzyme is activated by fructose 6-phosphate and other glycolytic intermediates (D. R. Davies, J. B. Pridham & J. Rintoul, unpublished work; Preiss *et al.*, 1967). ADP-glucose, the other product of the ADP-glucose pyrophosphorylase reaction, and ATP, the substrate, also both inhibit the synthesis of starch from UDP-glucose.

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Some Properties of an Endo-1,3-β-Glucanase from Papain

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Enzymes capable of hydrolysing 1,3-*β*-glucosidic linkages are widely distributed in higher plants (Clarke & Stone, 1963). The presence of 1,3-*β*-glucanase activity has been demonstrated in the dried latex of *Carica papaya* (Clarke & Stone, 1963), and in commercial papain (Eschrich, 1959). Eschrich (1959) showed that papain degraded sieve-tube callose (a 1,3-*β*-D-glucan), and suggested that the smallest product was a tri- or higher oligosaccharide. As papain has been used in the extraction of *β*-glucans (Bass *et al.*, 1953) it is of considerable interest to know the nature and specificity of any carbohydrases present. A knowledge of the substrate requirements of any 1,3-*β*-glucanases present is particularly important since it is now apparent that there are two distinct groups of endo-*β*-glucanases capable of hydrolysing 1,3-*β*-glucosidic linkages. One of these groups is highly specific for sequences of adjacent 1,3-*β*-linkages in its substrate (EC 3.2.1.39), whilst the other group (EC 3.2.1.6) will also attack polymers containing both 1,3-*β*- and 1,4-*β*-linkages, e.g. barley *β*-D-glucan and lichenin.

Several samples of commercially available papain were examined for *β*-glucanase activity by both reductometric and viscometric assay methods. Details of these assay procedures are given by Manners & Wilson (1973). It was found that some of the samples contained 1,3-*β*-glucanase activity. These samples showed no significant activity towards lichenin and cellodextrin (reductometric assay), or barley *β*-D-glucan and carboxymethylcellulose (viscometric assay).

The 1,3-*β*-glucanase activity from one sample of papain (supplied by Powell and Scholefield Ltd., Liverpool, U.K.) was purified. The crude powder was dissolved in Tris–HCl buffer (20mM, pH 7.6) and was partially purified by ultrafiltration in Amicon cells, using Diaflo membranes (Amicon Ltd., High Wycombe, Bucks., U.K.). The resulting solution was fractionated on DEAE-cellulose (Fig. 1) followed by Biogel P-60. The final preparation showed a 146-fold increase in specific activity compared with the starting material. It was observed that loss of 1,3-*β*-glucanase activity during purification could be prevented by inhibiting proteolytic activity by the addition of tosyl-L-lysylchloromethane (Whitaker & Perez-Villaseñor, 1968). There was no inhibition of 1,3-*β*-glucanase by this reagent.

The purified enzyme has a pH optimum in the region of pH 4.8–5.0. The K_m value for laminarin was 0.24mM. Enzymic hydrolysis of laminarin yielded laminaribiose and the series of higher laminarisaccharides, together with traces of glucose. The endo-activity pattern of the enzyme was confirmed by the demonstration that it reduced the viscosity of carboxymethylpachyman solutions. Although the enzyme did not attack lichenin it decreased the viscosity of samples of barley *β*-D-glucan which contained long sequences of adjacent 1,3-*β*-linkages (Bathgate *et al.*, 1974). The enzyme thus has a similar specificity to an endo-1,3-*β*-glucanase from malted barley (Manners & Wilson, 1974) in that it requires a number of adjacent 1,3-*β*-linkages in its substrate. It is probable that extraction of barley *β*-D-glucan with papain will result in the degradation of adjacent 1,3-*β*-linkages, if endo-1,3-*β*-glucanase activity is present.

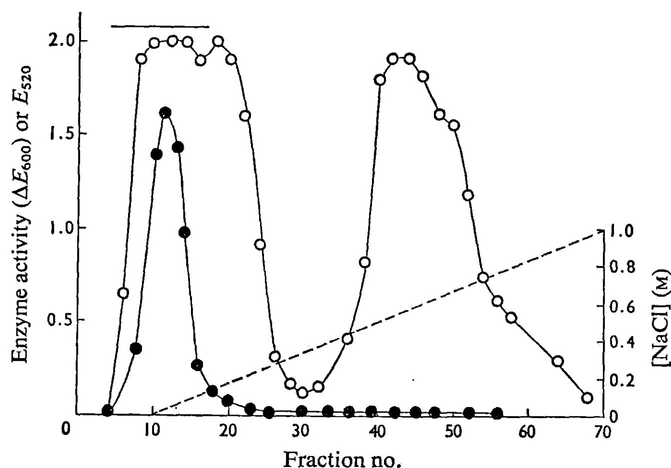

Fig. 1. *DEAE-cellulose chromatography of papain*

Experimental details are given in the text. o, Protein distribution; •, 1,3-β-glucanase activity; ----, NaCl gradient. The fractions under the horizontal bar were pooled, concentrated by ultrafiltration and applied to a column of Biogel P-60.

The effect of potential activators and inhibitors on enzyme activity was tested. The enzyme was not affected by *N*-ethylmaleimide, D-glucono-1,5-lactone or EDTA, but was inhibited by phenylmercurinitrate, Hg^{2+}, Cu^{2+} and *N*-bromosuccinimide. Enzyme activity was increased in the presence of Ca^{2+}, Ba^{2+}, Co^{2+} and bovine serum albumin.

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Control of Glycerokinase Activity by Sex Hormones

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The efficiency of conversion of fructose into plasma triglycerides is sex-dependent in some animals, including man (Macdonald, 1966; Jourdan, 1969; Hill, 1970) and it has been suggested that this pathway may be implicated in cardiovascular disease (Yudkin, 1971; Albrink, 1973).

The formation of triglycerides from fructose can occur by phosphorylation followed by aldolase-catalysed cleavage of the resulting fructose 1-phosphate to dihydroxyacetone phosphate and glyceraldehyde. Glycerol dehydrogenase is available in liver to convert