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FRUCTOSE METABOLISM IN HIGHER PLANTS"

THESIS

submitted for the degree of

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by

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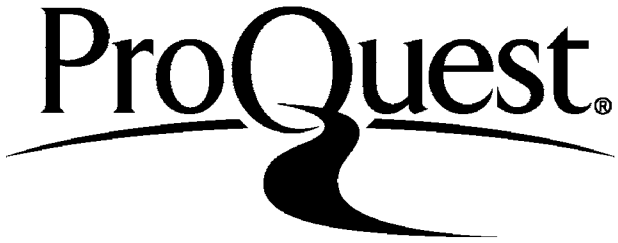

Abstract

Fructose 1-phosphate, an important intermediate in the metabolism of fructose in animals and micro-organisms, has only been tentatively identified as a component of higher plant tissues and nothing was known concerning its formation or metabolism in plants.

In the present study, examination of tissue extracts from a number of different plant species by ion-exchange chromatography did not confirm the presence of endogenous fructose 1-phosphate. However, injection of [U-^{14}C]fructose into developing Jerusalem artichoke tubers resulted in the rapid formation of labelled glucose 6-phosphate, fructose 6-phosphate, fructose-1,6-bisphosphate, and probably glucose 1-phosphate, followed by the appearance of fructose 1-phosphate which was identified by ion-exchange chromatography and hydrolysis. The incorporation of label into fructosans was also noted and examined.

Fructose-1-phosphate was also produced from fructose by tuber callus cultures but not by mature tubers from fructose or by developing tubers from [U-^{14}C]sucrose.

Attempts to detect a kinase in the developing tubers which would specifically transfer phosphate from nucleotide triphosphates or phosphoenolpyruvate to C-1 of fructose failed although ATP:fructose-6-phosphotransferase activity was identified and partially characterised.

Fructose 1-phosphate formation by enzymic isomerization of fructose 6-phosphate or the partial hydrolysis of fructose-1,6-bisphosphate using tuber extracts could not be demonstrated.

The major reaction occurring on incubation of fructose 1-phosphate with artichoke tuber extracts appeared to be dephosphorylation.

CONTENTS PAGE

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"NON SCHOLAE SED VITAE"

ABBREVIATIONS

ADP	Adenosine diphosphate
ADP-Fru	Adenosine diphosphate fructose
ADP-Glc	Adenosine diphosphate glucose
ATP	Adenosine triphosphate
CDP-Glc	Cytidine diphosphate glucose
CIP	Cleavage inhibitory protein
DHAP	Dihydroxyacetone phosphate
DP	Degree of polymerization
E4P	Erythrose 4-phosphate
FFT	ß(2-1')Fructan:ß(2-1')Fructan-1-fructosyltransferase
Fru1P	Fructose 1-phosphate
Fru-1,6-bisP	Fructose 1,6-bis(phosphate)
Fru-2P	Fructose 2-phosphate
Fru-2,6-bisP	Fructose 2,6-bis(phosphate)
Fru6P	Fructose 6-phosphate
G3P	Glyceraldehyde 3-phosphate
GDP	Guanosine diphosphate
Glc1P	Glucose 1-phosphate
Glc6P	Glucose 6-phosphate
NADH	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NAC	2-acetamido-2'-deoxy-D-glucose
OAA	Oxaloacetic acid
PABA	p-Aminobenzoic acid
PEP	Phospho-enol pyruvate

PFK	Phosphofructokinase
PFK-1	Phosphofructokinase-1
PFK-2	Phosphofructokinase-2
2PGA	2-phosphoglyceric acid
3PGA	3-phosphoglyceric acid
P_i	Inorganic phosphate
PP_i	Inorganic pyrophosphate
PVP	Polyvinylpyrollidone
R5P	Ribose 5-phosphate
Ru1,5bisP	Ribulose 1,5-bis(phosphate)
Ru5P	Ribulose 5-phosphate
S1,7bisP	Sedoheptulose 1,7-bis(phosphate)
S6'P	Sucrose 6'-phosphate
S7P	Sedoheptulose 7-phosphate
SPS	Sucrose 6'-phosphate synthetase
SST	Sucrose:Sucrose 1-fructosyltransferase
TEAB	Triethylammonium borate
TDP-Glc	Thymidine diphosphate glucose
TP	Triose phosphate
UDP	Uridine diphosphate
UDP-Fru	Uridine diphosphate fructose
UDP-Gal	Uridine diphosphate galactose
UDP-Glc	Uridine diphosphate glucose
UMP	Uridine monophosphate
UTP	Uridine triphosphate
X5P	Xylulose 5-phosphate

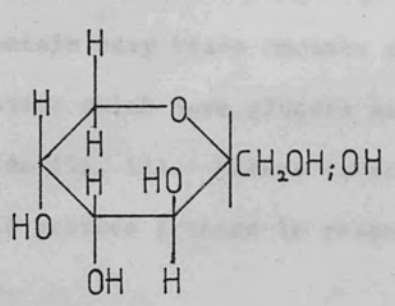
1 INTRODUCTION

1.1 The nature and distribution of D-fructose and its derivatives in higher plants

Fructose and its derivatives are very common components of living organisms and, in the plant kingdom, perform essential roles as intermediates in several important metabolic sequences and as storage materials.

1.1.1 Free fructose

Fructose is the only abundant ketohexose found in nature. In the free form in solution, it exists largely in the pyranoid conformation (I) (1) but when combined it is mainly found in the furanoid form (II) (Fig. 1) (2) Fructose, commonly accompanied by glucose, sucrose and other oligosaccharides, is found in most plant tissues. The tissue concentration of the ketose varies greatly depending on the type of tissue and the stage of development. Amuti and Pollard (3), could not detect free fructose (or glucose) in any of the mature seeds from the 31 plant species they analysed. In the developing pea seed, however, for example, the overall concentration of fructose is said to range from 1.3 mM to 21 mM (4, 5). In spinach leaves the concentration is reported to be 2 mM (6, 7) and in carrot root tissue the ketose can account for 11.7% of the total soluble sugars



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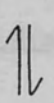
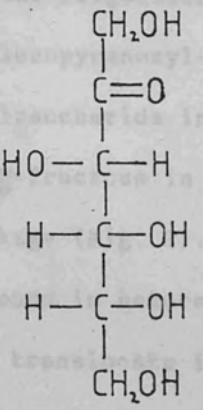
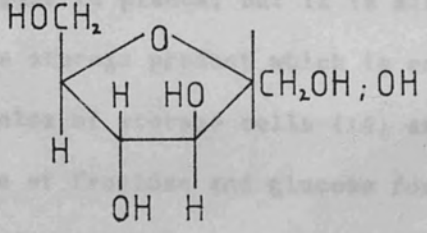






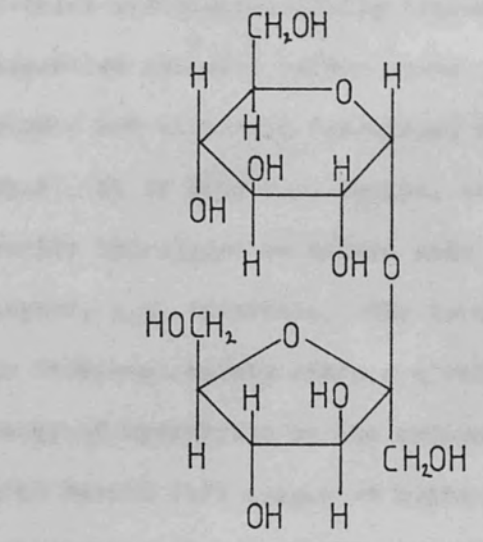
II

Fig 1 The conformers of D-fructose

and 1.05% of the fresh weight (8). Sugar beet roots (9) and leaf petioles and developing tubers from Jerusalem artichoke contain only trace amounts of fructose (10) as do potato tubers which have glucose as the major free monosaccharide (11, 12). Mature tubers from both the latter plants produce frutose in response to decreasing temperatures.

1.1.2 Fructose-containing disaccharides

The most abundant oligosaccharide in plant tissues is sucrose (α-D-glucopyranosyl-ß-D-fructofuranoside), a non-reducing disaccharide in which α-D-glucopyranose is attached to ß-D-fructose in the furanoid form by a 1-2 glycosidic linkage (Fig. 2). The only other non-reducing disaccharide found in nature is trehalose which replaces sucrose as the translocate in some fungi and also occurs in the circulatory system of some insects (12). Sucrose is not only one of the major products of photosynthesis and the main translocatory compound carried from leaves to other organs in plants, but it is also a principal carbohydrate storage product which is compartmentalised in the vacuoles of storage cells (14) and provides a ready source of fructose and glucose for synthetic processes and energy.



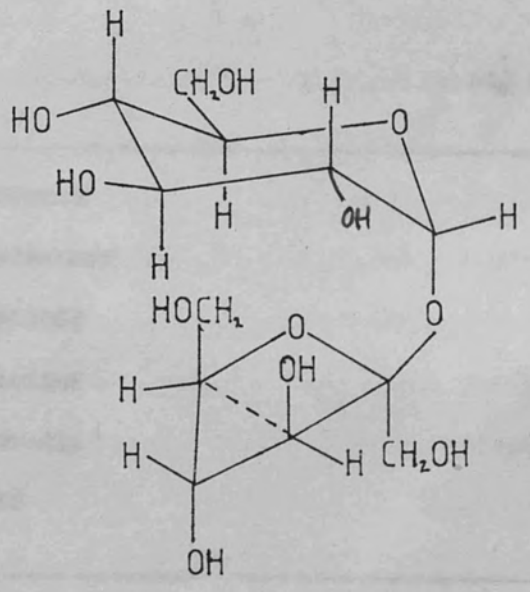


Fig 2 The structure of sucrose showing its stereochemistry

Sucrose is non-reducing because the fructose and glucose moieties are glycosidically linked through their respective anomeric carbon atoms thus blocking the ketonic and aldehydic functions, respectively, of each sugar. It is also very labile, the glycosidic bond being readily hydrolysed by dilute acid or by hydrolytic enzymes, e.g. invertase. The furanose conformation of the fructosyl moiety confers a relatively high free energy of hydrolysis on the molecule (Table 1) (15, 16) which Hassid (17) suggested might account for the distinctive role of sucrose in plant metabolism.

Table 1 ΔG_o^1 hydrolysis values for some oligosaccharides and nucleotide derivatives.

	ΔG_o^1 hyd (Kcal/mol)
SUCROSE	-6.6
TREHALOSE	-5.7
MALTOSE	-3.0
LACTOSE	-3.0
UDP-Glc	-7.0
ATP	-6.9

A question often asked is: why is sucrose so important in plants whereas in mammals, glucose appears to fulfil a very similar role? The replacement of sucrose by glucose in plants could be considered advantageous in terms of less energy being required for the synthesis of the disaccharide glycosidic bond. Arnold (18) also argues that sucrose has little advantage over a glucose equivalent when accounting for the potential number of ATP molecules each sugar can yield. However, in this assessment he forgets that sucrose synthetase releases the energy of the glycosidic linkage yielding fructose and 'energy-rich' UDP-Glc. Arnold suggests that sucrose is a protective derivative of glucose which is relatively unreactive in the cell and thus very suitable for translocation: translocatory glucose would be more susceptible to metabolism.

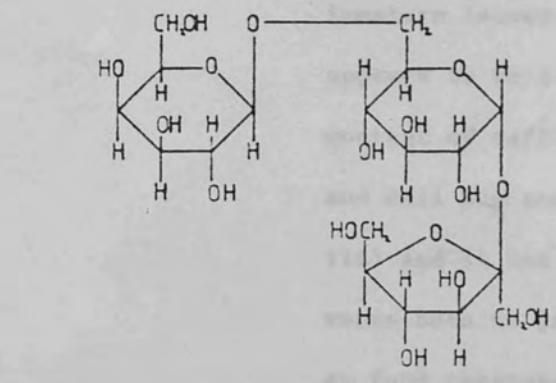
1.1.3 Higher fructose-containing oligo- and poly-saccharides

Apart from sucrose, the two most abundant classes of fructose-containing oligosaccharides in plants are the raffinose series of sugars and the less widely distributed low molecular weight fructosans, both of which are based structurally on sucrose.

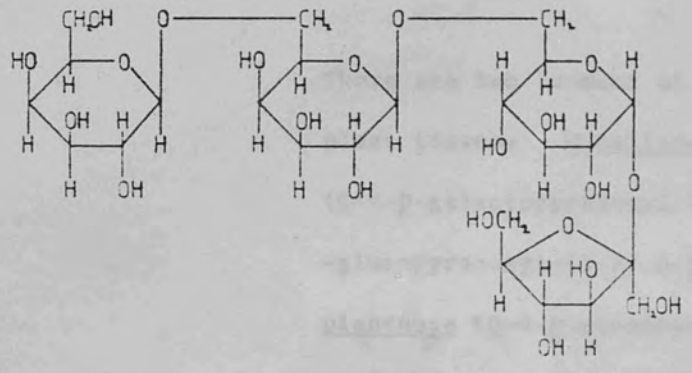
1.1.3.1 Raffinose and homologues and related oligosaccharides

Raffinose (O-α-D-galactopyranosyl-(1-6)-O-α-D-glucopyranosyl-(1-2)-ß-D-fructofuranoside) (Fig. 3) is very widely distributed in the plant kingdom. The successive addition of galactose units to raffinose, by α1-6 linkages, yields the tetrasaccharide stachyose, (which in some seeds is present at higher concentration than raffinose (19)) the pentasaccharide, verbascose, the hexasaccharide, ajugose and longer chain oligosaccharides up to the nonasaccharide (Fig. 3). Raffinose, together with its homologues, is present in seeds from most plant species (19) where it is synthesized as a storage compound during maturation and quickly disappears on germination. These oligosaccharides can also be found in the mature leaves and in the vascular tissue of a more restricted number of plant species (19).

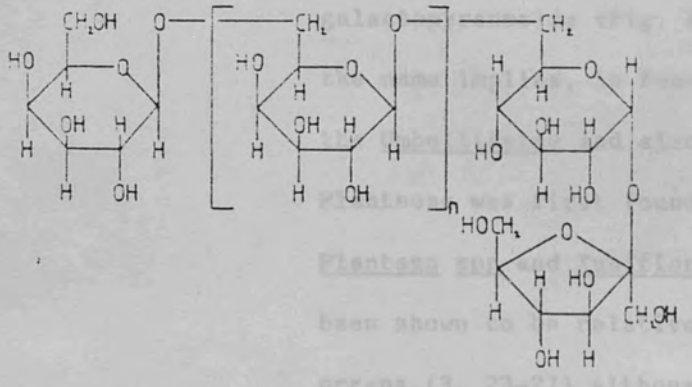
Raffinose and stachyose are important translocatory materials in some plants, such as Cucurbita spp (20), which possess mature leaves which 'export' these oligosaccharides to the



Raffinose



Stachyose



Higher oligomers

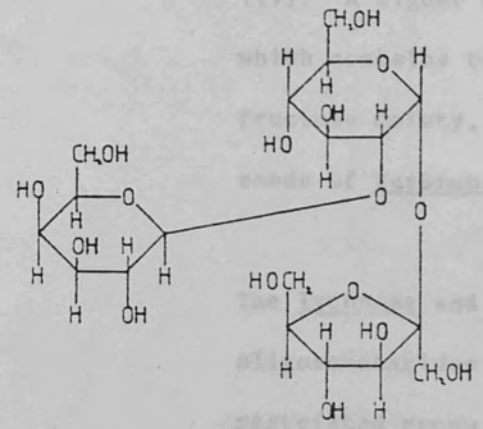
n = 2 Verbascose

n = 3 Ajugose

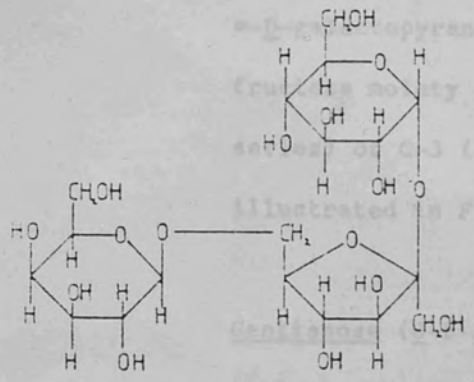
Fig 3 The raffinose series of oligosaccharides

immature leaves. In many perennial plants there appears to be a correlation between an increased content of raffinose and stachyose in bark, buds and cell sap and the approach of the cold season (16) and it has been proposed that these sugars serve both to promote cold hardiness and to act as food reserves.

There are two isomers of raffinose also found in plant tissues: Umbelliferose (O-α-D-galactopyranosyl-(1-2)-O-α-D--glucopyranosyl-(1-2)-ß-D-fructofuranoside) and planteose (O-α-D-glucopyranosyl-(1-2)--O-ß-D-fructofuranosyl-(6-1)-α-D-galactopyranoside (Fig. 4). Umbelliferose, as the name implies, is found in a large number of the Umbelliferae and also the Arallaceae (21). Planteose was first found in the seeds of Plantago spp and Tubiflorae spp (22) but has now been shown to be relatively widespread in these organs (3, 23-27) although less common than raffinose. Planteose has not been found in the vegetative parts of plants (22). Another isomer of raffinose, the so called Lolium trisaccharide (O-α-D-galactopyranosyl-(1-3)-O-α-D-glucopyranosyl-(1-2)-ß-D-fructofuronoside), is found in addition to raffinose and stachyose in caryopses of species of Lolium and Festuca



Umbelliferose



Planteose

Fig 4 The isomers of raffinose

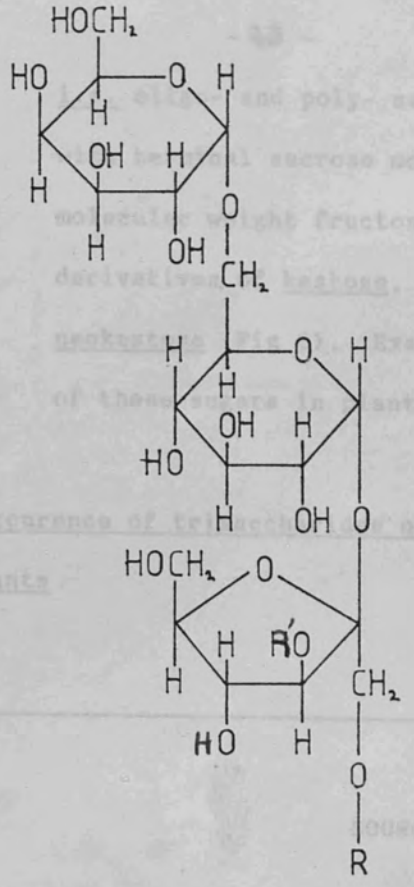
(19). A higher homologue of planteose, sesamose, which contains two galactosyl residues at the fructose moiety, has been isolated from the seeds of Sesamum indicum (27).

The lychnose and isolychnose series of oligosaccharides appear to be a relatively restricted group of compounds, which are found in the roots of Lychnis dioica and other species of the Caryophyllaceae (28). These sugars are derivatives of raffinose with additional α-D-galactopyranosyl residues attached to the fructose moiety of raffinose at C-1 (lychnose series) or C-3 (isolychnose series) as illustrated in Fig 5.

Gentianose (O-B-D-glucopyranosyl-(1-6)-O -α-D-glucopyranosyl-(1-2)-D-fructofuranoside) is a trisaccharide which appears to be confined to species of Gentiana where it is said to serve as a storage carbohydrate (29).

1.1.3.2 Fructosans

Amongst the higher plants most members of the families Campanulaceae and Compositae and the sub-class Monocotyledonae accumulate fructosans,



Lychnose R = Gal (n=1 to 4)
R'= H

Isolychnose R = H
R'= Gal (n=1 to 4)

Gal =

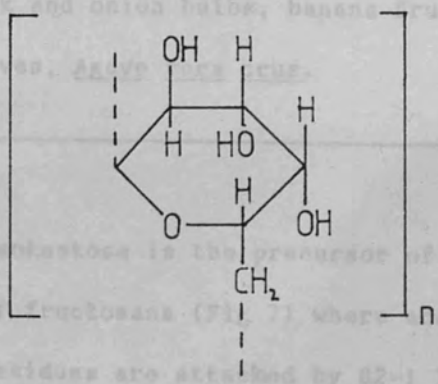


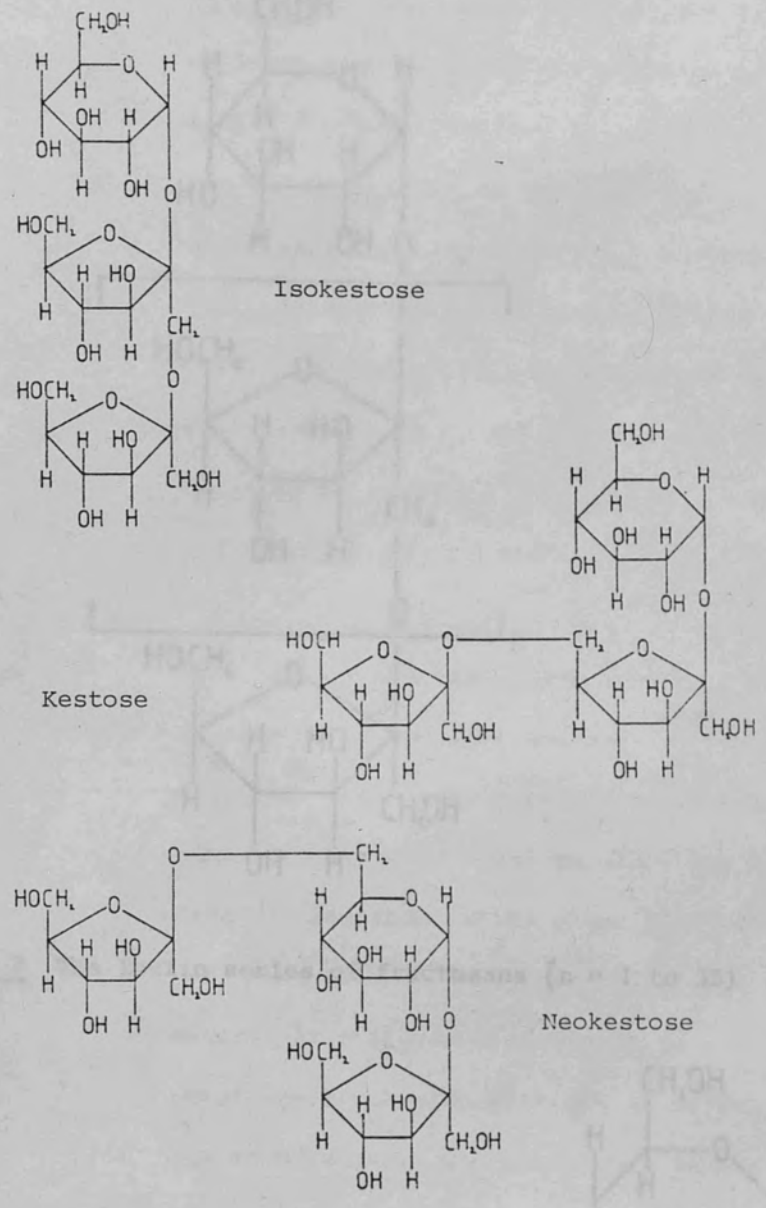
Fig 5 The lychnose and isolychnose series of oligosaccharides

i.e. oligo- and poly- saccharides of fructose with terminal sucrose moieties. The higher molecular weight fructosans may be regarded as derivatives of kestose, isokestose, and neokestose (Fig 6). Examples of the occurrence of these sugars in plants are given in Table 2.

Table 2 The occurence of trisaccharides of the fructosan series in plants

TRISACCHARIDE	SOURCES
ISOKESTOSE	Leek and onion bulbs, Jerusalem artichoke tubers, and the leaves of wheat, barley, rye, Italian ryegrass and oatgrass
KESTOSE	The leaves of Italian ryegrass, oatgrass, perennial ryegrass, wheat, rye and barley.
NEOKESTOSE	Leek and onion bulbs, banana fruit, wheat and oat leaves, Agave vera cruz.

Isokestose is the precursor of the inulin-type of fructosans (Fig 7) where additional fructose residues are attached by ß2-1 linkages to the terminal fructose residue of the trisaccharide.



Isokestose

Kestose

Neokestose

Fig 6 Fructosan trisaccharides

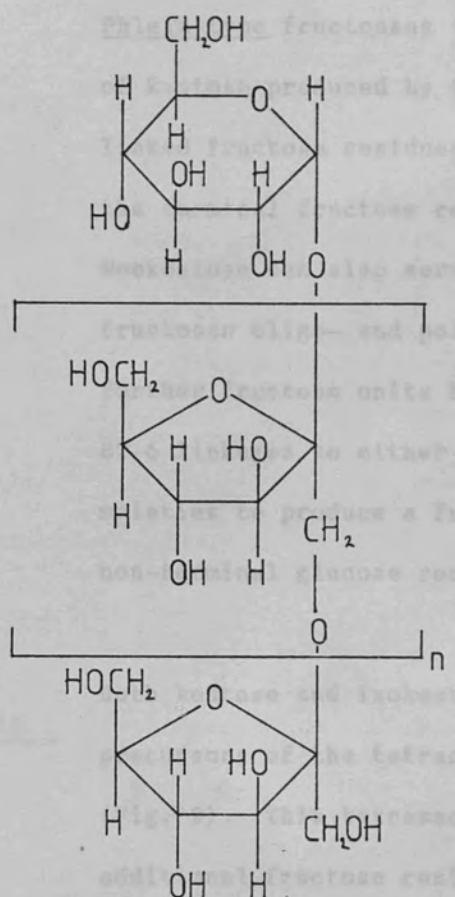


Fig 7 The inulin series of fructosans (n = 1 to 35)

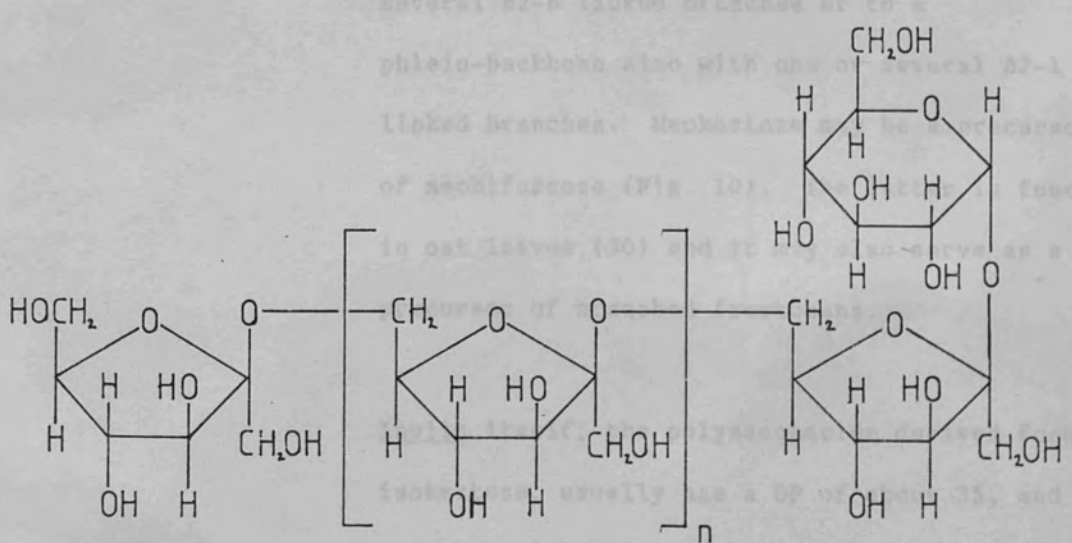


Fig 8 The phlein series of fructosans

Phlein-type fructosans (Fig. 8) are derivatrives of kestose produced by the addition of ß2-6 linked fructose residues to the C-6 position of the terminal fructose residue of kestose. Neokestose can also serve as a precursor of fructosan oligo- and poly-saccharides with further fructose units bonded by either ß2-1 or ß2-6 linkages to either of the terminal fructose moieties to produce a fructosan with a non-terminal glucose residue.

Both kestose and isokestose can be considered as precursors of the tetrasaccharide, bifurcose (Fig. 9). This tetrasaccharide with an additional fructose residue at either of the two terminal fructose groups gives rise to a structure with an inulin-backbone with one or several ß2-6 linked branches or to a phlein-backbone also with one or several ß2-1 linked branches. Neokestose may be a precursor of neobifurcose (Fig. 10). The latter is found in oat leaves (30) and it may also serve as a precursor of branched fructosans.

Inulin itself, the polysaccharide derived from isokestose, usually has a DP of about 35, and together with related oligosaccharides, forms the main reserve carbohydrate in the tubers of

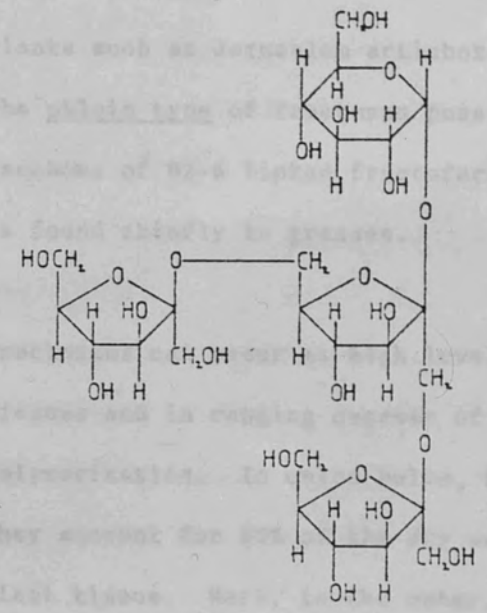


Fig 9 Bifurcose

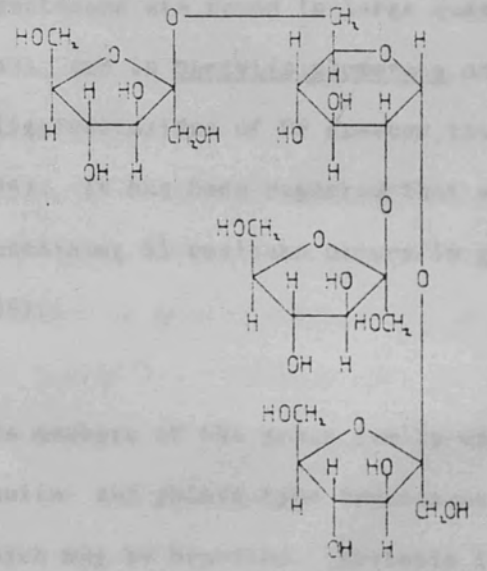


Fig 10 Neobifurcose

plants such as Jerusalem artichoke and Dahlia. The phlein type of fructosan possessing a backbone of ß2-6 linked fructofuranosyl residues is found chiefly in grasses.

Fructosans can occur at high levels in some tissues and in ranging degrees of polymerization. In onion bulbs, for example, they account for 65% of the dry weight of the plant tissue. Here, in the outer, older leaves fructose and the lower oligofructosides predominate, but these low DP molecules decrease from the outer to the inner, younger leaves where oligofructosides with higher (2-11 units) accumulate (31, 32). High molecular weight fructosans are found in large quantities in leek (33), and in Dactylis glomerata only oligofructosides of DP greater than 10 are found (34). It has been reported that a fructosan containing 51 residues occurs in garlic bulbs (35).

The members of the grass family can contain both inulin- and phlein-type fructosans either of which may be branched. Kritesin is an inulin of DP 17-22, found in barley leaves together with hordecin, a phlein of DP11 (36, 37). Couch grass contains inulin and phlein fructosans both of

which are highly branched (36). Mixtures of phlein and inulin fructosans together with fructosans based on neokestose occur in members of the Liliales (Agavaceae, Iridaceae, Amaryllidaceae and Liliaceae) (19, 38) and these neokestose polymers have been studied extensively in Agave vera cruz (39). Neokestose but not its higher homologues, is found in birch sap (40) and banana fruit (41).

Fructosans may occur in plant tissues together with starch or alone. For example in onion bulbs starch is entirely absent (42) whereas in the bulbs of iris and comfrey, the two polysaccharides co-exist (43, 44). In Jerusalem artichoke the leaves contain only starch and the tubers only inulin (42). Taniguchi et al., (45) has shown that ADP-Glc is not present in extracts of Jerusalem artichoke tubers which is consistent with the absence of starch. In cereals, eg wheat, the leaves contain only fructosans and the seeds both starch and fructosans (42).

Apart from providing an energy reserve of carbohydrate, the function of fructosans in plants is somewhat obscure. Many plants which contain these polymers are species which endure

a cold or dry period during their life cycles and it is possible that the osmotic activity of the fructosans may increase the resistance of the plant to freezing or dessication (42, 46).

1.1.4 Nucleotide Fructoses

Since the isolation of UDP-Glc by Leloir and co-workers (47) the number of known, naturally-occurring nucleotide sugars has increased steadily. In essence these compounds consist of a monosaccharide glycosidically linked to the terminal phosphate of a nucleoside-5'-diphosphate with guanine, adenine, cytidine, thymine or uracil present as the nucleoside base. Nucleotide sugars play a fundamental role both in the interconversion of monosaccharides and in the biosynthesis of oligo- and poly-saccharides and hence, are especially important in plant cell wall formation and the formation of carbohydrate reserves (48, 49).

Relatively little is known about the detailed structure and biochemistry of nucleotide derivatives of fructose but uridine diphosphosphate fructose (UDP-Fru) (Fig. 11) has been isolated from a variety of plant species and tissues (see Table 3).

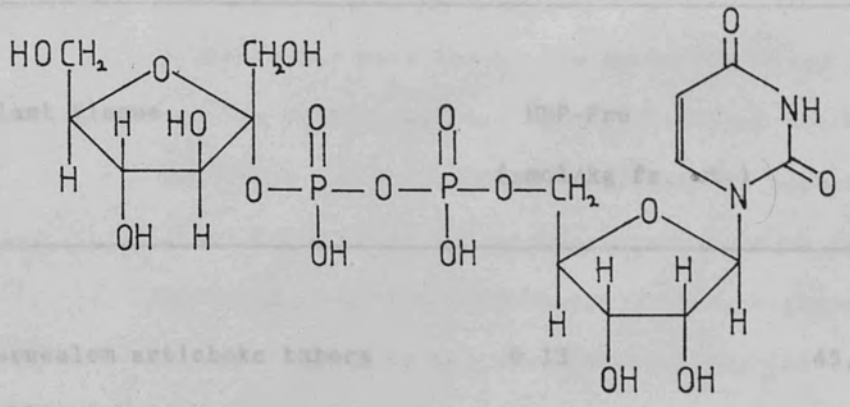


Fig 11 Uridine diphosphate fructose

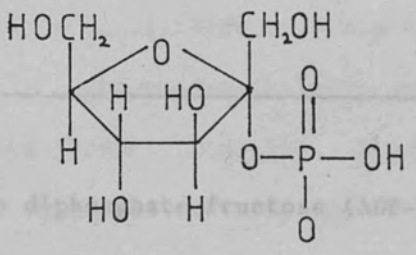


Fig 12 Fructofuranose 2-phosphate

Table 3 The occurence of UDP-Fru in plant tissues

Plant tissue	UDP-Fru (μmol/kg fr. wt.)	Ref
Jerusalem artichoke tubers	0.13	45, 50
Dahlia tubers	-	51, 50
European larch : cambial tissue	0.73	52
: xylem tissue	2.92	52
Parsley leaves	(0.35% fr. wt.)	53, 54
Strawberry leaves	265.00	55, 56
Germinating pea seeds	-	57
Leaf nodes of Balsam	-	57

Adenosine diphosphate fructose (ADP-Fru) is said to occur in European larch tissues (52). A further derivative, guanosine diphosphate fructose, has been detected in the mould, Eremothecium ashbyii (58).

No unequivocal structures for nucleotide fructoses have been published. Examination of these compounds has, on the whole, been superficial mainly because of the small amounts that are extractable from natural sources. Taniguchi and his associates (50, 59, 60) have

investigated the structure of UDP-Fru isolated from Jerusalem artichoke tubers. Using chemical methods they synthesized two forms of UDP-Fru; UDP(I)Fru where fructose was attached to the nucleoside-5'-diphosphate via the primary hydroxyl group at C-1 of the ketose, and UDP(2)Fru where the linkage was via the anomeric carbon atom, C-2. The fructose was reported to be in the furanoid form in UDP(2)Fru (59) although the ring size was not stated in the case of UDP(1)Fru (60). By comparing naturally-occurring UDP-Fru with these two synthetic forms Taniguchi argued that the naturally-occurring UDP-Fru was probably UDP(2)Fru with a furanose unit in α-configuration (Fig 11). Incubation of the naturally-occurring UDP-Fru with snake venon pyrophosphatase yielded UMP and a fructose phosphate which was distinguishable from Fru1P, Fru6P and Fru-1,6-bisP, and was tentatively identified as fructose 2-phosphate (Fru2P) (Fig. 12). Naturally-occurring UDP-Fru is also non-reducing and so again this would support the idea that the pyrophosphate group is linked to the anomeric carbon atom of fructose. In dilute hydrochloric acid (0.01 M) at 100°C the order of stability of the naturally-occurring and synthetic nucleotides is:

UDP(1)Fru >UDP(2)Fru (ß configuration) >Naturally-occurring UDP-Fru

Posternak (61) has reported that ß-Glc1P is three times more acid labile than ∝-Glc1P and Mendicino (62) has shown that B-L-apiose 1-phosphate is more acid labile than ∝-L-apiose 1-phosphate. The acid hydrolysis studies would, therefore, suggest that fructose in the naturally-occurring nucleotide has the ∝-configuration. One apparent anomaly in the hydrolysis data is that naturally-occurring UDP-Fru is more stable to acid than fructofuranose 2-phosphate (59, 60) (Fig 11). Normally the O-glycosidic linkage of a nucleotide sugar is hydrolysed at a greater rate than the related sugar phosphate (63). The complete structure for naturally-occurring UDP-Fru, therefore, remains in doubt.

The function of nucleotide fructoses in plants is at present unknown. It has been proposed that UDP-Fru is involved in the biosynthesis of fructosans where it may serve as the fructosyl donor (51). Chaiban (64) has suggested that UDP-Fru in mung bean seedlings might serve as the fructosyl donor in the formation of fructolipids. The pathway for the biosynthesis of UDP-Fru and ADP-Fru has not been established. In germinating pea seeds UDP-Fru accumulation accompanies an increase in free fructose (57).

Feingold (65) reports that Avigad failed to produce radiolabelled UDP-Fru when extracts of ryegrass or dahlia or Jerusalem artichoke tubers were incubated with [fructosyl-^{14}C]sucrose and UDP.

Chaiban (64) tentatively identified UDP-Fru formed *in vitro* from UTP and Fru1P by a crude enzyme preparation from mung bean shoots. The isomerization of Fru1P to Fru2P was considered as a possible first step in this reaction with Fru2P then reacting with UTP, catalysed by a pyrophosphorylase thus:

UTP + Fru2P → UDP-Fru + PPi

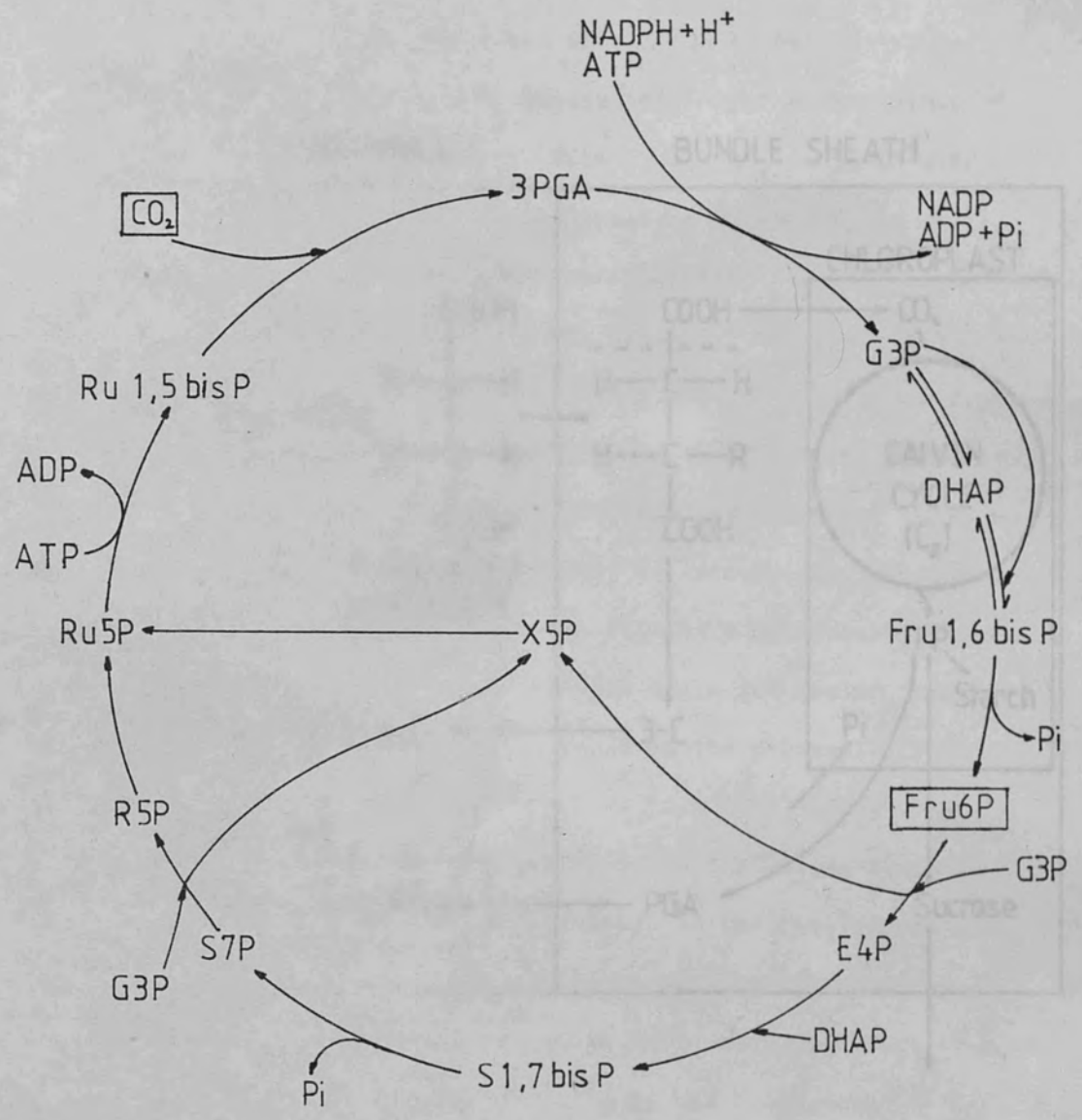
This would be analogous to the UDP-Glc pyrophosphorylase--catalysed formation of UDP-Glc:

UTP + Glc1P → UDP-Glc + PPi

1.2 The origin and metabolism of fructose in higher plants

Fructose originates from the Calvin cycle, the reductive pentose phosphate pathway, in which CO_2 in the chloroplast is fixed to form carbohydrates (Fig. 13). Here the enzyme ribulose bisphosphate carboxydismutase catalyses the initial carboxylation of ribulose-1,5-bisphosphate and its subsequent breakdown to yield two molecules of 3-phosphoglycerate (3PGA).

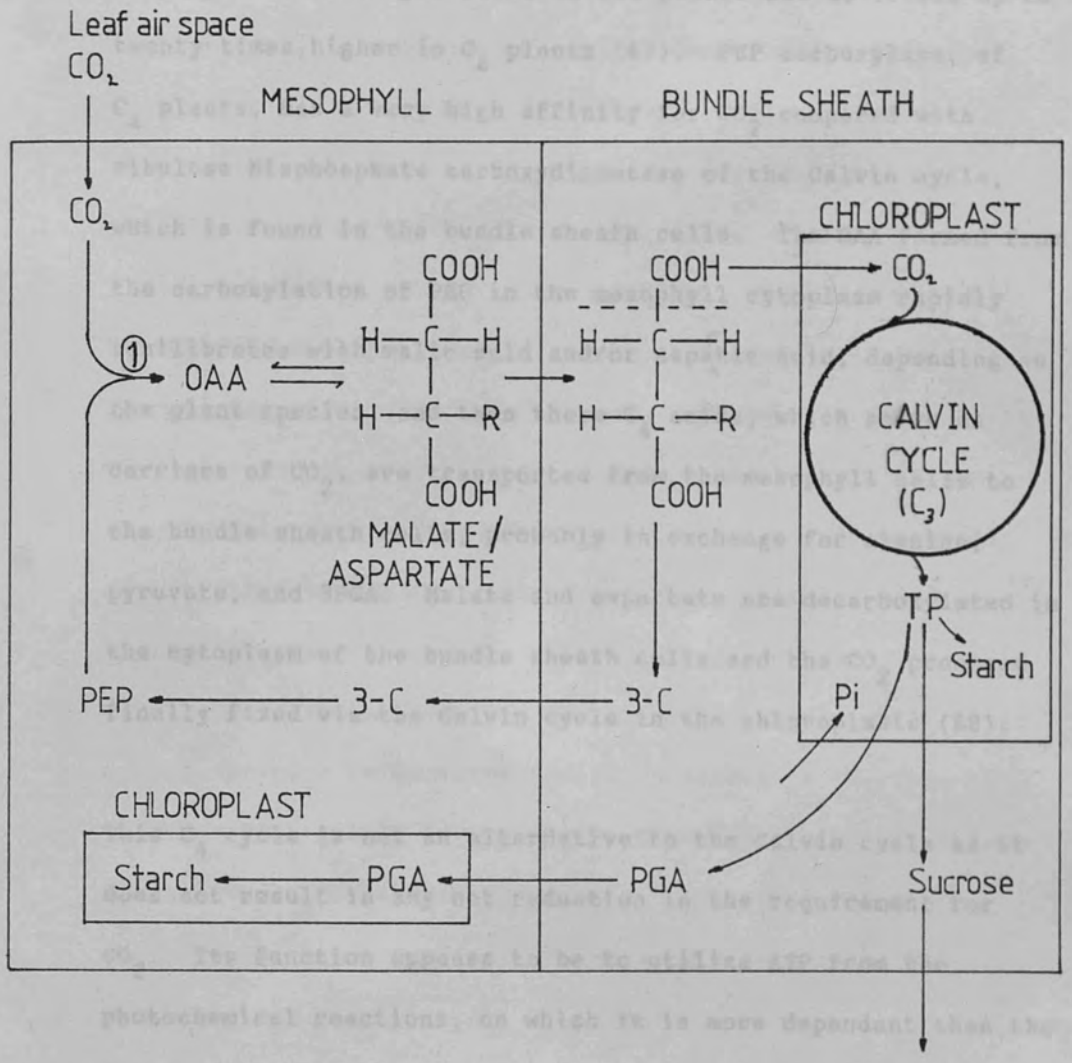
In some plants (C_4 plants), such as the tropical grasses, CO_2 is assimulated by a more complex process. The initial fixation occurs via an alternative cycle in the leaf mesophyll cells whereby PEP is carboxylated to form oxaloacetate (OAA) (Fig 14) (65, 66). This reaction is catalysed by PEP



Overall Reaction

$$6CO_2 + 18ATP + 12NADPH + 12H^+ \rightarrow Fru6P + 17P_i + 18ADP + 12NADP$$

Fig 13 The Calvin cycle



① = PEP carboxylase

R = -OH (Malate) or $-NH_2$ (Aspartate)

Fig 14 Scheme for C_4 pathway of CO_2 assimilation

carboxylase, an enzyme found in all plants but at levels up to twenty times higher in C_4 plants (67). PEP carboxylase, of C_4 plants, has a very high affinity for CO_2 compared with ribulose bisphosphate carboxydismutase of the Calvin cycle, which is found in the bundle sheath cells. The OAA formed from the carboxylation of PEP in the mesophyll cytoplasm rapidly equilibrates with malic acid and/or aspartic acid, depending on the plant species, and then these C_4 acids, which serve as carriers of CO_2, are transported from the mesophyll cells to the bundle sheath cells, probably in exchange for alanine, pyruvate, and 3PGA. Malate and aspartate are decarboxylated in the cytoplasm of the bundle sheath cells and the CO_2 produced finally fixed via the Calvin cycle in the chloroplasts (68).

This C_4 cycle is not an alternative to the Calvin cycle as it does not result in any net reduction in the requirement for CO_2. Its function appears to be to utilize ATP from the photochemical reactions, on which it is more dependant than the Calvin cycle, to produce PEP, and by the subsequent carboxylation of PEP bring carbon into the chloroplast where the Calvin cycle operates. Thus, C_4 plants maintain a higher level of CO_2 than C_3 plants at the local environment of ribulose bisphosphate carboxydismutase which has a low affinity for CO_2. The result is to diminish the oxygenase activity of this enzyme which can convert ribulose-1,5-bisphosphate to glycolate and so lower photorespiration, a wasteful process. Also the C_4 pathway can recapture any CO_2 that is produced by photorespiration. As well as reduced photorespiration C_4

plants have higher rates of photosynthesis, albeit at higher light intensities and higher ambient temperatures than the Calvin (or C_3) plants (68-70). Overall, C_4 plants, are considered to be more efficient at CO_2 fixation than C_3 plants.

C_4 metabolism also occurs in plants belonging to the Crassulaceae, e.g. pineaple (71, 72). These 'CAM plants' are characterized by a diurnal variation in the malic acid and starch contents of their photosynthetic cells. Atmospheric CO_2 is fixed by PEP carboxylase at night and malic acid accumulates in the vacuole. During the day CO_2 is released from malic acid and re-fixed via the Calvin cycle while light energy, trapped by photosynthesis, is stored in the form of starch. This energy is release by glycolysis at night in order to fuel the C_4 metabolic processes. Malic acid may be replaced by citric or isocitric acid in some plants (72).

The 3PGA produced by C_3 and C_4 plants is transported out of the chloroplast in exchange for inorganic phosphate (P_i) (73). A phosphate translocator protein has been identified in chloroplasts of spinach leaves (74) and etiolated leaves of Avena sativa (73) and is thought to mediate this process. 3PGA is exported as the divalent rather than the trivalent anion and thus there is a pH requirement which may be a means of control. Mg^{2+} inhibits 3PGA tansport, and PP_i and citrate inhibit the carrier protein of spinach chloroplasts (74). The transport of 3PGA needs to be carefully controlled in order to permit maximum

export rates without depletion of P_i and 3PGA required for regeneration of ribulose 1,5 bisphosphate which in turn is required for CO_2 fixation in the Calvin cycle. The net flux of 3PGA and Pi will depend on their concentrations on either side of the chloroplast membrane. In the case of C_4 plants, PEP is also thought to be exported out of the chloroplast to the cytosol for carboxylation to OAA, in exchange for P_i again via a carrier protein (73).

In the cytoplasm a reversal of the glycolytic pathway yields fructose-1,6-bis(phosphate) (Fru-1,6-bisP) from 3PGA, and the action of fructose bisphosphatase on Fru-1,6-bisP produces fructose 6-phosphate (Fru6P).

Fructose 6-phosphate can also originate from fats and proteins via gluconeogenesis.

Free fructose in the plant cell may have multiple origins including the hydrolysis of fructose phosphates by phosphatases, but more likely by the action of ß-fructofuranosidases on fructosyl derviatives and from the action of sucrose synthetase on sucrose (see Introduction P55, and REF. 13, 19, 75).

1.3 The metabolism of fructose

Free fructose in the plant cell must be phosphorylated to Fru6P before it can be utilized for general metabolism. Hexose kinases are responsible for this phosphorylation and several of these,

with different specificities, have been isolated from a variety of tissues from many plants (5, 36, 42, 76-87).

Saltman (80) first demonstrated hexose kinase activity in both particulate and soluble fractions from a number of different plant species. Later other workers showed that the hexose kinase activity in plants was similar to that in mammalian tissues i.e. that multiple forms of the enzymes existed with different specificities towards glucose and fructose. Medina and Sols (83) noted the presence of soluble fructokinase (ATP: D-Fructose-6-phospho-transferase EC 2.7.1.4.) in immature pea seeds which phosphorylated glucose at only 8% of the rate of fructose. A particulate hexose kinase, which phosphorylated glucose, fructose and 2-deoxyglucose and which was competitively inhibited by 2-acetamido-2'-deoxy-D-glucose (NAG) was also present in these seeds.

Baldus (6) resolved hexose kinase activity in spinach leaves into three forms, two soluble and one particulate. One of the soluble activities and the particulate activity (the latter was thought to be bound to mitochondria) showed high affinities for glucose and were presumed to be specific glucokinases. However, at saturating levels of hexose substrate, the activities with fructose were twice those with glucose. The second soluble activity in spinach was shown to be a specific fructokinase which was much more active with fructose than with glucose at all concentrations, although the K_m values for these

substrates were 71 μM and 210 μM, respectively. The activity of this enzyme was stimulated by monovalent cations such as K+ and NH_4+.

Turner et al., (81), using mature pea seeds have separated hexose kinase activity into four fractions. Fraction I contained a glucokinase with Km values of 0.07 mM, 0.5 mM and 30 mM for glucose, mannose and fructose, respectively. Glucose 6-phosphate, a product of the reaction with glucose, was not inhibitory, although ADP was (81). Fraction II from peas exhibited non-specific hexose kinase activity and fractions III and IV both contained fructokinases (5, 87). The latter two fractions had similar high affinities for fructose but the catalytic activity of fructokinase IV was 38 times more with fructose than with glucose whereas fructokinase III was only 11 times more active. High fructose concentrations (>0.25 mM) inhibited both III and IV at pH 8 (the optimum for both enzymes) and at pH 6.6 in the case of fructokinase III but not fructokinase IV. Excess magnesium-ATP complex inhibited fructokinase III but not fructokinase IV. Fructokinase IV was inactive in the absence of K+ ions whereas fructokinase III did not show an absolute requirement for this cation.

Fructokinase activity in pea seeds has also been examined by Frankart and Pontis (77). They described an enzyme which was very specific for fructose and had no activity with glucose. It also showed an absolute requirement for K+ ions and had a low affinity for fructose (Km = 20 mM). The enzyme was unusual in

that its pH optimum was 4.5 unlike most other plant, animal and bacterial fructokinases which have optima at alkaline pH values. A similar enzyme has also been tentatively identified in seeds of clover, dandelion and tomato (77).

With all fructokinases and hexose kinases that have been examined from plant sources, the product of the reaction with fructose is Fru6P. Fructose 1-phosphate (Fru1P) has never been detected as a product although it has been tentatively identified in some plant tissues (see Results and Discussion 2.1) and it is the primary fructose metabolite in mammalian liver (88) and in many species of bacteria (89).

Fructose 6-phosphate is a key intermediate in many processes in plants (Fig. 15). It is, for example: (1), a substrate for sucrose synthesis; (2), a precursor of starch via Glc6P, Glc1P and ADP-Glc; (3), a source of pentoses and NADPH and aromatic compounds via the pentose phosphate pathway, and (4), a glycolytic intermediate yielding C_2 and C_3 intermediates and ATP.

In view of the recent discovery of fructose-2, 6-bis(phosphate) (Fig 16), a new fructose metabolite in plants (90), further comment on glycolysis is warranted.

The degradation of Fru6P via the glycolytic pathway commences with a phosphofructokinase (PFK)-catalysed phosphorylation and the formation of Fru-1,6-bisP. PFK from plants appears not to

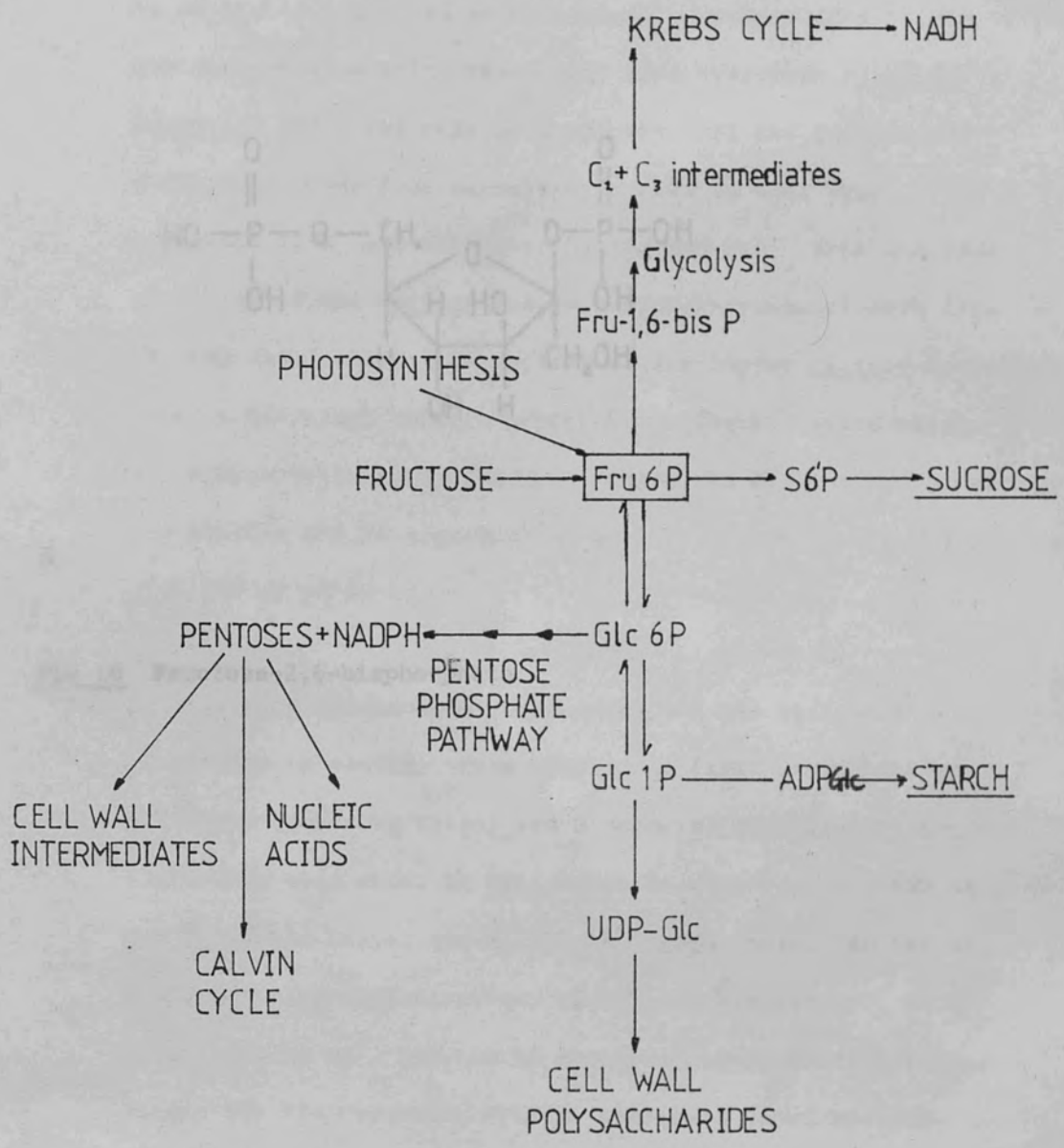


Fig 15 Fructose 6-phosphate, a key intermediate in plant metabolism

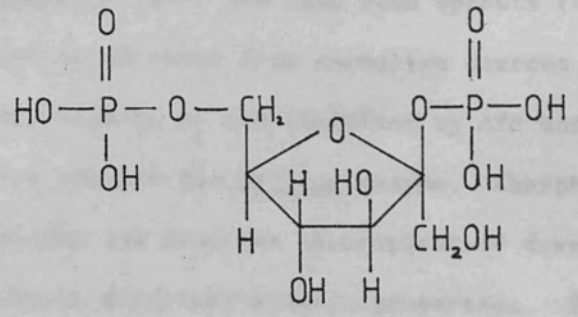


Fig 16 Fructose-2,6-bisphosphate

be as highly regulated as the mammalian enzyme (PFK-1) but in the case of developing castor oil seed endosperm (Ricinus communis) (91), and mung bean sprouts (92) the kinases are similar to those from mammalian sources in that they are activated by P_i and inhibited by ATP and PEP. 3PGA and 2PGA also inhibit the Ricinus enzyme. Phosphofructo-kinases from the cytosol and from the chloroplast of developing Ricinus endosperm exhibit different kinetic properties. The cytosolic enzyme shows hyperbolic kinetics with respect to Fru6P at pH 7 whereas the plastid PFK is sigmoidal at pH 7 but exhibits hyperbolic kinetics at pH 8.

An inorganic pyrophosphate-dependant PFK has also been identified in plants. This enzyme was first isolated from pineapple leaves by Carnal and Black (71) although it had previously been shown to be present in some bacteria (93-96). The pineapple enzyme has an absolute requirement for PPi and Fru6P but high concentrations of PPi are inhibitory. It is stimulated by Mg^{2+} but has no absolute requirements for these ions. The PPi-dependant PFK has also been shown to occur in mung bean shoots (97) and in this case Km values of 2 mM for Fru6P and 0.1 mM for PPi have been reported. Fru-1,6-bisP and glucose-1,6-bis-(phosphate) have been shown to activate this mung bean PFK but it is unaffected by PEP at concentrations up to 1mM. The enzyme was also been reported in seedlings of germinating castor bean (98) where it is confined to the cytosol. In the castor bean, the activity of PPi-dependant PFK is greater than that of the ATP-dependant PFK.

Recently fructose-2,6-bis(phosphate) (Fru-2,6-bisP) was isolated from mung bean shoots (92) and slices of Jerusalem artichoke tubers (99) although it had been previously detected in rat liver (100-102) and in yeast (103, 104). It has also been shown that Fru-2,6-bisP is confined to the cytosol (105). In the liver this phosphoric ester is produced from Fru6P by an ATP-dependant phosphorylation catalysed by phosphofructokinase-2 (PFK-2). The origin of Fru-2,6-bisP in plants is so far unknown although Fru6P is likely to be the precursor.

In the liver, Fru-2,6-bisP is a potent activator of the ATP-dependent PFK-1 but is has no effect on the enzyme isolated from mung bean shoots (91, 92). However, Fru-2,6-bisP does activate the PPi-dependant-PFK from mung bean shoots (92) and Jerusalem artichoke tubers (99). Sabularse and Anderson (92) found that the activity from mung bean shoots was increased 500-fold by very low concentrations (< 1μM) of the ester. This was achieved by a decrease in Km (Fru6P) from 20 μM to 0.3 μM and also an increase in the Vmax. Although Fru-2,6-bisP has no effect on the ATP-dependant PFK-1 from mung bean shoots, Miernyk and Dennis (91) showed that the ester can stimulate the ATP-dependant PFK-1 from the chloroplast of Ricinus endosperm at pH 7 but not pH 8. The stimulation involved a shift in Fru6P substrate kinetics from sigmoidal to near hyperbolic. However, a later study by Cseke (106) demonstrated that the chloroplastic ATP-linked PFK-1 from Ricinus was unaffected by physiological levels of Fru-2,6-bisP. The cytosolic PFK-1 from Ricinus was unaffected by Fru-2,6-bisP

at either pH value. No interaction was observed between Fru-2,6-bisP and Pi, another activator of PFK-1, and Fru-2,6bisP did not alleviate the inhibitory effect of PEP, ATP, 3PGA or 2PGA on PFK-1 from the castor oil plant. From the results reported by Miernyk and Dennis (91) the lack of any stimulatory effect on mung bean PFK-1 by Fru-2,6-bisP may be explained in a number of ways: (1) all plant tissues appear to have multiple forms of glycolytic enzymes and the proportions of cytosolic and chloroplast forms may vary considerably. Only the chloroplast PFK-1 of Ricinus was stimulated by Fru-2,6-bisP and it is possible that the cytosolic enzyme was predominant in the tissues at the particular develpmental stage of the mung bean shoots that were examined; (2) the mung bean PFK-1 was assayed at pH 7-8 at which both forms of PFK-1 from Ricinus were unaffected by Fru-2,6-bisP; (3) mung bean PFK-1 like the cytosolic PFK-1 from Ricinus is unaffected by Fru-2,6-bisP under any conditions.

1.4 The biosynthesis and metabolism of sucrose

Details of the biosynthesis and metabolism of sucrose in plant tissues have been well documented and it is now generally believed that four main enzymes are involved: sucrose phosphate synthetase (SPS) and sucrose-6'-phosphatase for the synthesis of the disaccharide; and sucrose synthetase and invertase for its degradation.

1.4.1 Synthesis

The biosynthesis of sucrose mainly involves the concerted action of sucrose phosphate synthetase and sucrose-6'-phosphatase. Sucrose synthetase, despite recent evidence suggesting implication in synthesis in cucumber fruit penduncles (107), is now considered to be cheifly involved in the breakdown of sucrose (see 1.4.2.1).

Sucrose synthesis is thought to occur in the cytoplasm of photosynthetic cells. Early reports had suggested that sucrose synthesis occurred in chloroplasts and the presence of sucrose phosphate synthetase in these organelles from a number of different plant species was recorded (108). However when Heldt and Sauer (109) demonstrated that the inner membranes of chloroplasts were impermeable to sucrose, further investigations were initiated which resulted in improved methods for separating chloroplasts and cytoplasmic fractions. Using these methods Bird et al., (110) re-examined the subcellular localization of sucrose phosphate synthetase in pea shoots and the leaf tissues of wheat, spinach and field beans and came to the conclusion that the enzyme, and hence sucrose synthesis, was mainly localised in the cytoplasm.

1.4.1.1 Sucrose phosphate synthetase

Leloir and Cardini (111) first discovered sucrose phosphate synthetase (EC 2.4.1.14) in wheat germ. It catalyses the following reaction:

UDP-Glc + Fru6P ⇌ S6'P + UDP

The enzyme is found in photosynthetic tissues as well as in non-photosynthetic tissues such as sweet potato root (112), rice seed scutellum, (113) castor bean endosperm (114) and broad bean cotyledons (114). Sucrose synthesis has also been demonstrated in wheat and barley seeds (115) and in rice scutellum the de novo synthesis of sucrose phosphate synthetase has been observed (113). The enzyme activity may be present at low levels in immature tissues, such as young potato tubers and then increases to high levels during maturation (114, 116).

Although extensive work has been carried out on purified preparations of sucrose phosphate synthetase, the structural nature of the enzyme is not fully understood. The molecular weight of the rice scutellum enzyme is reported to be 4.5×10^5 daltons (113) and 3.6×10^5 daltons has been quoted for the molecular weight

of the wheat germ enzyme (117). Multiple forms of sucrose phosphate synthetase have been detected (118, 119).

Sucrose phosphate synthetase, from a variety of sources, exhibits absolute specificity towards UDP-Glc and Fru6P (120-123). There is no activity with ADP-Glc. The equilibrium constant for the reaction is 3250 at pH 7.5 in the case of the wheat germ enzyme. i.e. favouring S6'P synthesis (62). The optimum pH for the reaction is 6.5. The Km for UDP-Glc lies between 1.3 mM, for the spinach leaf enzyme (124) and 25 mM for the rice seed synthetase (113): for the second substrate, Fru6P, Km values range between 0.6 mM for the ~~from~~ sweet potato root enzyme (112) and 5.9 mM for the rice seed synthetase (113). Although both Fru6P and UDP-Glc show sigmoidal kinetics with wheat germ sucrose phosphate synthetase, this is not true for enzymes from all other sources (108). UDP has been found to be a competitive inhibitor with respect to UDP-Glc and a non-competitive inhibitor with respect to Fru6P using synthetases from various plants (62, 113, 125, 126). Fru-1,6-bisP and P_i both inhibit spinach leaf sucrose phosphate synthetase (125, 127) although the competitive inhibition by Pi

with respect to UDP-Glc could be releived by Fru6P (123). Although sucrose doses not inhibit spinach leaf sucrose phosphate synthetase it is an inhibitor of the wheat germ enzyme (118): turanose, which has a similar structure, and oligosaccarides containing sucrose residues e.g. raffinose, are not inhibitory (118). This sucrose inhibition is non-competitive, with respect to Fru6P, and Hill equation data suggest that the enzyme exhibits negative co-operativity towards sucrose binding. S6'P, a reaction product, does not inhibit the wheat germ enzyme but is a competitive inhibitor, with resepct to UDP-Glc, of the spinach leaf enzyme. Although 50% inhibition of wheat germ synthetase by sucrose can be achieved at physiological concentrations (50 mM), higher concentrations do not give complete inhibition. Salerno and Pontis (118) explained this in terms of two forms of sucrose phosphate synthetase, one susceptible to sucrose inhibition and one not, and speculated that in vivo regulation of the activity could take place via interconversion of the enzyme forms. De Fekete (128) has also reported a possible regulatory function for citrate with the purified enzyme from Vicia faba cotyledons. When citrate was removed activity fell to zero. Magnesium ions activate sucrose

phosphate synthetase from wheat germ, although there would appear to be no absolute requirement for Mg^{2+}. Evidence has been presented for two binding sites on the enzyme for this metal ion (119) and activation by Mg^{2+} is decreased by sucrose (118). The ability of Mg^{2+} to activate the wheat germ enzyme appears to depend on the quarternary structure: the enzyme form with MW 400,000 can be 100% activated by Mg^{2+} whereas the higher MW form (10^6) is unaffected (119). In contrast, the enzyme from barley leaf is inhibited by Mg^{2+} (126). Amir and Preiss (124) showed that purified sucrose phosphate synthetase from spinach leaf was unaffected by Mg^{2+} although the cation did relieve the inhibitory action of UDP and Harbron et al., (125) suggest that, in general, the apparent stimulatory effect of Mg^{2+} may be due to its ability to chelate certain inhibitors.

1.4.1.2 Sucrose 6' phosphatase

Sucrose 6' phosphatase (EC 3.1.3.22) catalyses the irreversible hydrolysis of S6'P to sucrose thus:

$$S6'P + H_2O \rightarrow \text{Sucrose} + Pi$$

Sucrose 6' phosphatase has been detected concomitantly with sucrose phosphate synthetase in several plant tissues (108, 129) and, hence, is probably also a cytoplasmic enzyme. Little work has been carried out on this phosphatase although the enzymes from sugar cane stalks and carrot roots have been shown to be very specific for the hydrolysis of S6'P (Km(S6'P) = 45-170 μM) (123, 130, 131). Sucrose and Pi inhibit the enzyme from both sources and Mg^{2+} is required for the activity of the sugar cane enzyme (130, 131). The irreversible nature of the reaction coupled with the exergonic reactions catalysed by sucrose phosphate synthetase results in a coupled system which strongly favours the synthesis of sucrose. The formation of Pi in the reaction, catalysed by sucrose-6'-phosphatase, fructose-1,6-bis-phosphatase and pyrophosphorylase (coupled with inorganic pyrophosphatase), are important in photosynthetic tissues as the export of photosynthetic triose phosphate from the chloroplasts is balanced by the import of Pi by a specific phosphate translocator in the chloroplast envelope (74, 125). The rate of sucrose synthesis via S6'P therefore, directly affects the availability of those phosphates for the formation of Fru6P, one of the substrates for sucrose synthesis.

1.4.2 Degradation

The cleavage of sucrose to its component monosaccharides can be acheived by the action of either sucrose synthetase or invertase. Sucrose synthetase is particularly important in tissues where the conversion of sucrose to starch takes place although in other tissues, particulary in storage organs, the hydrolytic breakdown of sucrose by invertase appears to be a more common mechanism (75).

1.4.2.1 Sucrose Synthetase

Sucrose synthetase (EC 2.4.1.13) catalyses the reversible reaction:

UDP-Glc + Fru ⇔ Sucrose + UDP

The enzyme was originally discovered by Leloir and Cardini (132) in wheat germ and since that time it has been shown to occur in tissues from many plant species (123). Delmer and Albersheim (121) examined the enzyme distribution in mung bean and found the activities to be high in non-photosynthetic tissue and low in extracts of photosynthetic tissues and, hence, concluded that sucrose synthetase was involved in sucrose cleavage. Other workers found that sucrose

synthetase activity was high in developing seed tissues such as maize and rice endosperms and immature broad bean cotyledons (129) where de novo sucrose synthesis is not important, but in germinating seeds of maize and rice where sucrose synthesis from storage carbohydrate is essential, the enzyme activity was relatively low (129).

Molecular weights of between 2.8×10^5 daltons for the bamboo shoot synthetase (133) and 4.4×10^5 daltons for the enzyme from rice seed (134) have been reported. The sucrose synthetases in rice grains (135), mung beans (136) and maize kernels (137) are tetramers of identical sub-units. Sulphydryl groups are essential for activity.

Unlike sucrose phosphate synthetase, sucrose synthetase is not specific for UDP-Glc: the enzymes from mung bean seedlings (138) and aspen callus (139), for example, can utilize a number of nucleotide sugars, including ADP-Glc, GDP-Glc, CDP-Glc and TDP-Glc for the synthetic reaction and the equivalent nucleoside diphosphates for the cleavage reaction. The Km values for UDP-Glc and ADP-Glc with the mung bean enzyme are 0.21 mM and 1.8 mM respectively

(138) and the Km for fructose using aspen callus synthetase, is 5 mM (139). The sucrose synthetase of the latex of the rubber tree where SPS is absent has a K_m(UDP-Glc) of 0.56 mM and Km(Fru) of 3.85 mM (140). In the sucrose cleavage reaction the Km values for UDP and ADP are both 0.19 mM using the mung bean enzyme and values of 0.8 mM and 3.3 mM, respectively, have been reported with the rice seed sucrose synthetase. The Km for sucrose can vary from 10 mM - 40 mM depending on the enzyme source (123). The Km for sucrose is high at 130 mM for the potato tuber enzyme (116) although a value of 17 mM has been given for the mung bean enzyme (136). Murata (112) has reported that rice grain and potato sucrose synthetases exhibit sigmondal kinetics for both sucrose and UDP. Equilibrium constants for the synthesis reaction catalysed by sucrose synthetase from various sources vary between 1.3 and 6.7 and are dependant on pH (123). It has also been shown that the maximum rate of sucrose synthesis is observed at pH 7.5 to 8.0, whereas the optimum for sucrose cleavage is between pH 6.5 and 7.0 (129).

Sucrose degradation by purified sucrose synthetase from mung bean is activated by PPi, NADP and gibberellic acid (141). Mg^{2+} has also been shown to inhibit the cleavage reaction of the enzymes from mung bean (116) and rubber tree latex (140). It activates synthesis of sucrose in the case of the synthetase from wheat germ (142) and rubber tree latex (140).

Pontis and Salerno (143) have recently isolated three protein factors from wheat seeds that inhibit sucrose synthetase cleavage activity: the synthesis reaction is not affected. One of the cleavage inhibitory proteins (CIP), CIP III, has been further purified and shown to exert its effect by changing the affinity of the enzyme for UDP but not sucrose. The hyperbolic kinetics normally exhibited by the enzyme in response to increasing concentrations of UDP change to sigmoidal on addition of this inhibitor. These CIP's may well be involved in the regulation of sucrose synthetase activities *in vivo*.

1.4.2.2 Invertase

Invertases are a group of ß-D-fructofuranoside fructohydrolases (EC 3.2.1.26), which are widely distributed in the plant kingdom and catalyse

the hydrolysis of terminal ß-D-linked fructofuranosyl residues, e.g. from sucrose or related oligosacchardies, to yield free fructose.

Two distinct invertases are found in plants, an acid enzyme having an optimum pH of 4.5-5.3, and an alkaline invertase which exhibits maximum activity between pH 7.5 and 8.0 (144). The enzymes have been shown to occur together in seedlings, and in fruit tissues from a number of different plant species. Although some acid invertase activity is present in the cellular free space (perhaps bound to cell walls or cell membranes where it is thought to play an important role in the mechanism of sucrose mobilization (144)), recent studies with isolated vacuoles from a number of the plant tissues have shown that the major part of acid invertase activity is localized, in particulate form, in these organelles (145). Alkaline invertase is believed to be a soluble, cytoplasmic enzyme.

Invertase activity is high in developing tissues such as root apex, cotyledons and fruits but in mature tissues of storage organs, where sucrose is found as a reserve, the levels of invertase are usually found to be low (145). Invertase is

not present in Jerusalem artichoke tubers in late summer when synthesis of inulin is maximal but appears during sprouting (146, 147). Tissues damage caused by, for example, by cold-shock, mechanical stress and senescence may result in elevated invertase activities (145).

The molecular weights reported for acid invertase range from 2.8×10^4 daltons (sugar beet) (148) to 2.2×10^5 daltons (banana) (149). The enzyme from grape is reported to be a glycoprotein containing 25% carbohydrate (150) and that from radish hypocotyls, 7.7% carbohydrate (151) although the structure of the glycan chains which are supposedly present, is unknown. Acid invertases from a number of plant tissues have also been shown to have high affinity for concanavalin A which is further evidence of their glycoprotein nature (145). It is thought that the difference in molecular weight of the enzymes from various sources may be due to variations in the glycan chain (145). A molecular weight of 6.9×10^5 daltons has been reported for the alkaline invertase from citrus fruit (152): alkaline invertases are probably structurally distinct from the acid invertases (145).

The Km for sucrose with acid enzymes from various plant sources is between 2 and 13 mM, and values of between 9 and 25 mM have been recorded for alkaline invertase (145). Acid invertases hydrolyse raffinose at 10 to 50% of the rate for sucrose: in the case of the alkaline invertases the rate for raffinose is less than 7% that of sucrose (145). The sugar cane acid enzyme is inhibited competitively by free fructose and non-competively by glucose (153). Acid invertase from the leaf sheath is inhibited by excess sucrose unlike the enzyme from other parts of the sugar cane plant (153). Slight inhibition by glucose and Glc6P has been shown to occur with alkaline invertase from potato tubers (154).

A protein factor has been isolated from beet and sweet potato roots (155) and maize endosperm (156) which inhibits acid invertase. This small protein binds irreversibly to the potato enzyme (155) although low pH, high Mg^{2+} concentrations or treatment of the complex with concanavalin A can partially dissociate the enzyme-protein inhibitor complex. The non-competitive binding by the inhibitor, which occurs optimally at pH 4.5, is inhibited by sucrose (2 mM) although sucrose will not dissociate the complex once it

is formed. Free glucose or fructose cannot prevent complexing. Studies on the levels of invertase activity and the concentrations of this enzyme inhibitor in potato tuber support the assumption that the enzyme inhibitor regulates sucrose degradation in vivo (155).

The arrangement by which both sucrose and acid invertase exist together in the same plant compartment, the vacuole, without total hydrolysis of sucrose is not clear. The activity of acid invertase has been found to be highest where the demand for hexose from incoming or stored sucrose is greatest and to be inversely proportional to sucrose concentration in the tissue. In storage roots of beetroot (157) and perhaps in other mature tissues, levels of invertase are usually very low. Sampietro et al., (153) who measured the tissue levels of glucose and fructose (both inhibitors of the enzyme) and sucrose in sugar cane, have suggested that acid invertase from sugar cane leaf sheaths may be modulated by the hexose pool size. Glucose has also been shown to suppress invertase synthesis in sugar cane. As enzyme inhibition by fructose is competitive, high sucrose concentration is required to, either

partially or completely, overcome the effect on acid invertase by fructose. Invertase activity in vivo may also be regulated by hormones as auxins and gibberellins have been reported to induce invertase levels in sugar cane (158) and sweet potato (159). The appearance of the invertase inhibitor protein (see above) could also contribute to the lowering of enzyme activity during sucrose assumulation. Other factors which could affect invertase activity include pH and ionic strength, which may act by altering binding to cell walls and membranes.

1.4.3 Sucrose metabolism : Conclusions

In general, all four enzymes, sucrose phosphate synthetase, sucrose 6' phosphatase, sucrose synthetase and invertase, are detectable concomitantly in many plant tissues, and so it is difficult to unequivocally assign synthesis to the sucrose phosphate synthetase/sucrose 6' phosphatase system, and degradation to sucrose synthetase and invertase. Obviously each of these enzymes is regulated in such a way that, for instance, sucrose synthesis occurs without futile degradation by, for example, invertase. Walker et al., (125) concluded that sucrose synthesis is controlled by sucrose phosphate synthetase and fructose-1,6-bisphosphatase and that the rate of sucrose synthesis is largely dependent upon the

supply of triose phosphates and ATP from chloroplasts. Both SPS and fructose-1,6-bis-phosphatase are very sensitive to inhibition by AMP, UDP and Fru6P. These compounds could inhibit fructose bisphosphatase hence increasing Fru-1,6-bisP levels which in turn inhibit sucrose phosphate synthetase: UDP, Pi and Fru-1,6-bisP inhibit sucrose phosphate synthetase resulting in an increase in Fru6P which could then inhibit fructose-1,6-bisphosphatase. The fact that the maximum activities of both sucrose phosphate synthetase and fructose-1,6-bisphosphatase are much lower than the other enzymes of the pathway also lends support to this theory of their importance in the control of sucrose synthesis. There is also some evidence to suggest that the modifications of the levels of the four enzymes involved in sucrose metabolism occur through the action of hormones. Moreover, the existence of isoenzymes of sucrose synthetase and sucrose phosphate synthetase, which have differential affinities for activators and inhibitors, provides other possible processes by which the activities and hence sucrose metabolism, may be regulated.

1.5 The biosynthesis and metabolism of fructose-containing oligosaccharides

As sucrose is a structural unit in all of these plant oligosaccharides it is not unexpected to find that the disaccharide is a key intermediate in their biosynthesis.

Initial studies on the biosynthesis of raffinose suggested that UDP-Gal was the galactose donor (160-162) and sucrose the acceptor, however more recent work indicates that the raffinose series of oligosaccharides are synthesized from the intermediate, galactinol (O-α-D-galactopyranosyl-(1-1)-L-myo--inositol) (163) a compound with a realtively high ΔG_ohyd (ca -5.5 kcal), which has been found to accompany the raffinose family of oligosaccharides in tissues from many higher plant species (163). In addition, studies with leaves photosynthesizing in the presence of $^{14}CO_2$ show kinetic relationships between galactinol and raffinose which suggest that the latter is a product of the former (38, 163, 164) and a similar relationship is apparent in developing Phaseolus vulgaris seeds (165). Galactinol synthesis from UDP-Gal and myo-inositol has been demonstrated with ~~the~~ enzyme preparations from Pisium sativum (166) and Phaseolus vulgaris (167).

Myoinositol + UDP-Gal → Galactinol + UDP

The raffinose series of oligosaccharides can then be synthesized by the following sequential reactions catalysed by separate α-D-galactosyltransferases: (19, 38, 168).

1) Galactinol + sucrose $\rightleftharpoons_1$ Raffinose + myo-inositol

2) Galactinol + raffinose $\rightleftharpoons_2$ Stachyose + myo-inositol

3) Galactinol + stachyose $\rightleftharpoons_3$ Verbascose + myo-inositol

4) Galactinol + verbascose $\rightleftharpoons_4$ Ajugose + myo-inositol

1-Galactinol:Sucrose-6-galactosyltransferase (GST)

2-Galactinol:Raffinose-6-galactosyltransferase (GRT_1)

3-Galactinol:Stachyose-6-galactosyltransferase (GRT_2)

4-Galactinol:Verbascose-6-galactosyltransferase (GRT_3)

The first reaction catalysed by GST leads to the formation of raffinose. The enzyme from Vicia faba seeds (169) is highly specific for sucrose (Km 1mM) as galactose acceptor and galactinol (km 7 mM) is the best donor: UDP-Gal cannot be utilized and little hydrolysis of galactinol occurs in the reaction. GST has also been isolated and characterized from wheat germ seeds (169) and leaves of Cerastium arvense (170). In Vicia faba seeds these is sufficient enzyme activity to account for the observed rate of total galactosyl sucrose synthesis in maturing seeds (169).

GRT_1 catalyses further polymerization of raffinose to stachyose and is readily reversible. GRT_1 from Phaseolus vulgaris (167, 169, 171) seeds is highly specific for raffinose as acceptor. The enzyme from the leaves of Cucurbita pepo is also specific for raffinose (Km 4.6 mM) although, as in the case of GST from Vicia faba, the Km is high (ca 7 mM) for the donor, galactinol (172).

In Phaseolus vulgaris seeds, GRT_2 activity is very low which can be correlated with the trace amounts of verbascose that are found in these seeds: the main oligosaccharide is stachyose (167). However, in Vicia faba seeds, where GRT_2 activity is

present, verbascose is the major oligosaccharide (165). The enzyme can utilize both stachyose and raffinose as acceptors and Tanner (165) believes that it is responsible for both stachyose and verbascose synthesis in Vicia faba.

Umbelliferose also appears to be synthesized from sucrose but here UDP-Gal is the galactosyl donor and galactinol will not replace UDP-Gal (21). The mechanisms for the synthesis of planteose and the lychnose and isolychnose series of sugars are at present unknown.

The catabolism of galactosylsucrose derivatives would generally appear to involve α-galactosidases, enzymes which have been detected in most plant tissues and which are generally thought to be important for the mobilization of oligosaccharide reserves in germinating seeds (173-174). In vitro, invertase can cleave oligosaccharides with terminal β-D-fructofuranoside residues but there is little evidence that this enzyme plays an important role in vivo except in the case of sucrose hydrolysis (see p56).

1.6 The biosynthesis and metabolism of fructosans

Fructose polymers in higher plants have a limited distribution, nevertheless where they do occur they can reach very high levels: as much as 80% of the dry weight of some tissues. However, limited studies have been carried out on the biosynthesis and metabolism of these oligo- and poly-saccharides.

1.6.1 Biosynthesis

At the present time there are two hypotheses to explain the biosynthesis of fructosans in higher plants. The first is based on the transfer of fructose from one sucrose molecule to the fructosyl moiety of another with the process continuing so as to build up a chain of fructose residues on the original sucrose acceptor molecule. There is a significant amount of experimental evidence to support this concept (42). The second hypothesis maintains that a nucleotide fructose derivative is involved in the transfructosylation reaction (51).

1.6.1.1 Synthesis with sucrose as donor

Edelman and co-workers (42, 175-178) have studied the occurence and biosynthesis of fructosans in a number of higher plants and in particular, the Jerusalem artichoke, Helianthus tuberosus (42).

It is clear, that they can be synthesized from translocated sucrose. Edelman and Popov (177) illuminated leaves of Jerusalem artichoke plants in the presence of $^{14}CO_2$ and then analysed the outer part of a selected tuber (Table 4).

Table 4 Incorporation of label from $^{14}CO_2$ into carbohydrates of Jerusalem artichoke tuber

	% incorporation	Specific Activity (CPM/mg)
Glucose	6	5.42
Fructose	0	0
Sucrose	59	36.65
GF_2	19	15.87
GF_3	7	4.88
$>GF_3$	6	0.77
Inulin	4	0.35

GF_2 = fructosylsucrose (probably isokestose)

GF_3 = fructosyl-(fructosylsucrose)

The majority of the radioactivity was incorporated into sucrose and trisaccharide, GF_2 (presumably largely isokestose), and there were successively decreasing amounts incorporated into the higher fructosans upto inulin.

The biosynthetic mechanism that Edelman proposed (42) bears a superficial resemblance to that first described for the synthesis of bacterial levan which involves the enzyme levansucrase (179-181). Dedonder (10, 182) first reported the occurence of an inulosucrase in Jerusalem artichoke tubers which transferred fructosyl residues from sucrose to the growing fructosan chain:

$$\underset{\text{sucrose}}{G - Fru} + \underset{\text{fructosan}}{G(Fru)_n} \rightarrow G + G(Fru)_{n+1}$$

However Edelman (175, 183) later showed that two quite distinct enzymes were involved in a concerted action. The first of these enzymes is sucrose: sucrose-1-fructosyltransferase (SST) which synthesizes the trisaccharide isokestose, from sucrose (175, 183) thus:

$$\underset{\text{sucrose}}{G - Fru} + \underset{\text{sucrose}}{G - Fru} \rightarrow \underset{\text{Iso-kestose}}{G - Fru - Fru} + G$$

SST has been found in a number of tissues including sugar beet tubers, Jerusalem artichoke tubers and onion bulbs (42, 175, 178, 183-186). It exhibits high specificity for sucrose and the reaction is essentially irreversible, showing

little activity with iso-kestose as donor or with glucose as acceptor. As free fructose is not produced, SST is not an invertase. The enzyme is unable to promote polymerisation above the trisaccharide level (186). SST levels in Jerusalem artichoke tubers are dependant on the sucrose concentration in the tissue. This can be demonstrated by detaching a tuber from a plant where upon SST activity decreases to nil within a few days while the activities of hydrolytic enzymes (B-D-fructofuranosidases) increase (42). Daughter buds produced on storing an artichoke tuber contain SST but the parent tuber does not (42).

The second enzyme, which is responsible for further chain elongation, is B(2-1') Fructan; B(2-1') fructan-1-fructosyltransferase (FFT) which transfers single B-D-fructofuranosyl residues from fructosylsucroses to the C-1 position of fructose residues in sucrose or fructosylsucroses thus:

$$\underset{\text{donor}}{G\text{-}Fru\text{-}(Fru)_n} + \underset{\text{acceptor}}{G\text{-}Fru\text{-}(Fru)_m} \rightleftharpoons G\text{-}Fru(Fru)_{n-1} + G\text{-}Fru\text{-}(Fru)_{m+1}$$

The donor in this reaction can be any fructose polymer where n = 1 to 34 (i.e. not sucrose).

For the acceptor 'm' may range from 0 to 34. In the final equilibrium state of this series of reactions catalysed by FFT the products are a range of fructosans with sucrose as the smallest molecule, where the weight and not the molar ratio of fructose per oligomer is unity (42). This equilibrium can also be reached with 'mid-range' oligofructosides as initial substrates. When isokestose is incubated with a crude artichoke preparation possessing both FFT and SST activities a 'self-transfer' reaction occurs which can lead to a so-called 'clutch' mechanism. That is, the sucrose liberated by FFT more readily accepts fructosyl residues yielding isokestose via the SST-catalysed reaction than does isokestose (yielding tetrasaccharide) in the FFT reaction, hence, further polymerization is inhibited (42). This can be demonstrated by incubating isokestose and [U-^{14}C]sucrose with FFT, which leads to the rapid equilibriation of radioactivty (*) in the sucrosyl residues thus:

G - Fru - Fru + G* - Fru* = G - Fru + G* - Fru* - Fru

Isokestose [U-^{14}C]sucrose

It has been suggested that these disproprotionation reactions may be the main function of artichoke FFT and that this enzyme does not normally take part in fructosan biosynthesis. However, polymers of high DP (>20) when incubated with isokestose and FFT are converted to higher polymers instead of disproportionating to yield lower oligomers, the reaction which takes place in the absence of trisaccharide. This is due to the high affinity of fructosans with DP > 20 for fructose transferred from the trisaccharide (180). Onion bulb FFT, on the otherhand, will not utilize these high DP fructosans as acceptors, although this may be linked to the fact that onion only contains oligomers up to DP 12. In onion bulbs (186) and some other tissues, FFT would appear to be responsible for the conversion of isokestose to neokestose. FFT, like SST, is not an invertase.

There is insufficient evidence to suggest that FFT is responsible for the production of the complete range of fructosans found in tissues, such as Jerusalem artichoke, particularly those of intermediate DP. It is possible that the role of FFT is to keep the ratio of various oligofructosides to unity (on a mass basis) by

disproportionation reactions. Edelman's hypothesis (42)also does not account for the synthesis of phlein-type and branched fructosans. Furthermore, although there is no direct evidence that nucleotide fructoses play a role in the biosynthesis of fructosans it is logical, on the basis of present knowledge, to suspect that they are involved. The two fructose transfer mechanisms may even function together for the biosynthesis of fructosans.

1.6.1.2 Synthesis with nucleotide fructose as donor

Since the isolation of nucleotide fructoses from plants, a second hypothesis has been proposed to explain the biosynthesis of fructosans (45, 50-55, 54, 56). Nucleotide sugars are known to play a fundamental role in the biosynthesis of many oligo- and poly-sacchardies (38) and it has been suggested (50) that the mechanism for the synthesis of inulin and other fructosans is similar to that for other reserve polysaccharides, such as starch and glycogen.

i.e. NDP-Fru + Sucrose → NDP + G-Fru-Fru

NDP-Fru + G-Fru(Fru)$_n$ → NDP + G-Fru-(Fru)$_{n+1}$ etc

As inulin possesses ß-D-fructofuranosyl residues, then in comparison with the biosynthesis of other oligo- and poly-saccharides containing ß-D-glycosyl residues, synthesis should occur from a nucleotide fructose with the ketose residue in the α-D-configuration. In addition, all nucleotide derivatives of D-monosaccharides in plants appear to have the α-D-configuration and naturally-occurring UDP-Fru may well be identical in this respect (see Introduction p34).

However, although nucleotide fructoses have been isolated from plants which are known to synthesize fructosans (45, 50, 51) no one has so far been able to find an enzyme system which will synthesize inulin and related oligosaccharides *in vitro* from such derivatives. Umemura *et al.*, (60) incubated radiolabelled UDP(1)Fru (see p 33) with sucrose and a crude enzyme preparation from Jerusalem artichoke tubers, in an attempt to synthesize inulin but they were unsuccessful. Structural studies (see p33) make it unlikely that naturally-occurring UDP-Fru is this isomer, however, as does the fact that all other nucleotide sugars are O-glycosides.

1.6.2 Metabolism

Edelman et al., (42, 187) have described two enzymes from Jerusalem artichoke tubers with similar properties which degrade fructosans. These β-(2 →1')-fructan-1-fructanhydrolases, A and B, can be distinguished by their mobilities on DEAE-cellulose columns and their relative activities with inulin and oligofructosides as substrates. Although the two enzymes are similar to FFT, in that they break only ß2-1 linkages between terminal and adjacent fructose residues, neither A nor B possesses any transfructosylase activity. Their sole activities are thus:

$$G\text{-}Fru\text{-}(Fru)_n + H_2O \rightarrow G\text{-}Fru\text{-}(Fru)_{n-1} + Fru$$

Furthermore, the enzymes are not invertases as they do not hydrolyse sucrose, although sucrose (but not fructose) does inhibit their activites. Edelman and his colleagues explain this in terms of the conformation of the fructose residue involved: free fructose exists largely in the pyranoid form, whereas it is in the furanoid conformation in sucrose and the fructosans. As expected, therefore, 2-O-ß-D-methyl fructofuranoside does produce some inhibition of both hydrolases. Two hydrolases isolated from the roots of dandelion, Taraxacum officinale, which showed similar characteristics to these from Jerusalem artichoke, were not inhibitied by sucrose (188, 189).

Smith (190) has shown the presence of a ß-fructofuranosidase, in fescue grass (Festuca arundinocea) which is specific for the cleavage of ß2,6 linkages and which did not hydrolyse inulin. Again this enzyme is not an invertase (which was also found in the same tissue) as it did not catalyse the hydrolysis of sucrose.

2 RESULTS AND DISCUSSION

2.1 The occurence of fructose 1-phosphate in higher plants

The occurence of fructose 1-phosphate (Fru1P) in both animals and microorganisms is well established. However, the evidence for the presence of Fru1P in plants is not quite so convincing.

Schwimmer et al. (191, 192), tentatively identified a sugar phosphate from an acidic extract of potato as Fru1P by a combination of paper chromatographic and electrophoretic techniques. The studies of Cole and Ross (193) indicated that labelled Fru1P was produced in corn roots growing in a radiolabelled phosphate medium although they did not comment on the significance of the discovery. Somewhat stronger evidence for the existence of the ester in plants is recorded by Graham and Ap Rees (194) who, using anion-exchange chromatography and paper chromatography, observed a fructose monophosphate, which was not Fru6P, in aqueous ethanolic extracts of carrot and white

turnip. The compound was cleaved by rat liver aldolase (yielding DHAP) and identified as Fru1P. It was claimed that the carrot and white turnip contained 19.5 and 21.1 nmol/g fresh weight, respectively, of the phosphate. The detection of sorbitol 1-phosphate in apricot leaves may be an indication of the natural occurence of Fru1P as this was suggested as a possible precursor of the hexitol derivative (195). In addition plant aldolases (e.g. from jack bean (196) and spinach leaf (197)) can utilize both Fru-1,6-bisP and Fru1P as substrates and this has also been interpreted as possible evidence for the occurence of Fru1P in plants. Indirect evidence of this kind should, of course, be treated with extreme caution.

The identification of Fru1P in plant extracts is difficult. At the commencement of this study it was clear that the simple techniques of paper chromatography and electrophoresis which work so well for the resolution of carbohydrate mixtures were less effective for the separation of Fru1P from other commonly-occurring phosphoric esters such as Glc1P, Glc6P and Fru6P. One of the initial aims of this work, therefore, was to search for and to develop a method whereby Fru1P could be routinely and clearly separated from other sugar phosphates.

A literature survey revealed that several chromatographic methods for the separation of sugar phosphates had been published and a number of these were tested. A summary of the results is given in Tables 5 and 6.

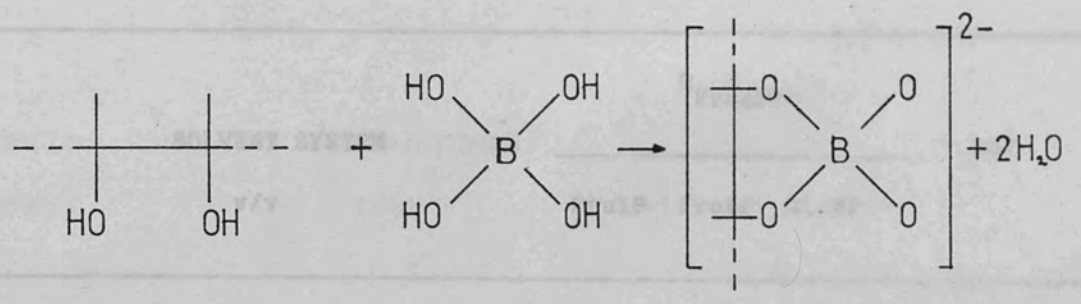
Only one procedure, that was examined, gave a reasonable separation of Fru1P, Fru6P and Glc6P. This was thin-layer chromatography on cellulose using an ethanol/ammonium acetate solvent system. However, in view of the fact that relatively large amounts of plant extracts had to be applied to the thin-layer plate in order to detect small quantities of sugar phosphates, the method was considered to be impracticable for the routine analysis of plant extracts.

High voltage paper electrophoresis under both acid and alkaline conditions was also examined (Table 7). None of these systems however, gave an adequate resolution of a standard mixture of Fru1P, Fru6P and Glc6P.

Gas liquid chromatography (GLC) of trimethylsilyl derivatives of sugar phosphates was attempted (205). However, reproducibility of the method was poor, it gave complex multiple peaks for each sugar phosphate and the GLC method was, therefore, rejected.

Ion-exchange chromatography has been commonly used for the resolution and analysis of sugar phosphate mixtures. However, separations within the series of hexose monophosphates ~~is~~ are complicated by the fact that nearly all have identical dissociation constants and hence, have the same affinities for the ion-exchange material. The use of tetraborate to improve the separation of sugar phosphates by ion-exchange chromatography was introduced by Khym and Cohn (206). Tetraborate ions form negatively charged complexes in alkaline

solution with cis-hydroxyl groups as shown in Fig. 17. The degree to which a given sugar or sugar phosphate will react with tetraborate ions is dependent on its conformation and the configuration of adjacent hydroxyl groups. There is a greater degree of complexing with furanoid forms possessing cis-hydroxyl groups than with pyranoids as the former give structures in which the adjacent hydroxyls are in the optimum position for reacting with tetraborate. Borate complex formation also affects the dissociation constants of the phosphate groups which are doubly ionized under the alkaline conditions required for reaction with tetraborate. The tetraborate, therefore, exaggerates the relatively minor differences in structure which exist amongst the hexose monophosphates and allows separation on anion-exchangers.



DIOL (D) TETRABORATE (B) BD complex

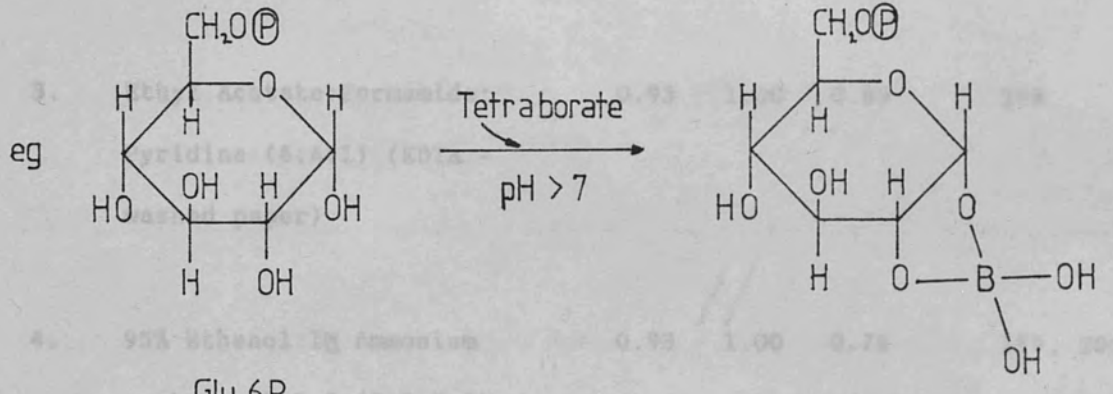


Fig 17 Complexing of carbohydrate with alkaline solutions of tetraborate

Table 5 Paper chromatography of sugar phosphates

	SOLVENT SYSTEM v/v	R_{Fru6P}*			Ref
		Fru1P	Fru6P	Glc6P	
1.	Ethyl Acetate:Formamide: Pyridine (4:4:1)	0.89	1.00	0.84	198
2.	Ethyl Acetate:Formamide: Pyridine (6:4:1)	0.93	1.00	0.89	198
3.	Ethyl Acetate:Formamide: Pyridine (6:4:1) (EDTA - washed paper)	0.93	1.00	0.89	198
4.	95% Ethanol:1M Ammonium acetate pH 3.9 (7.5:3.0)	0.93	1.00	0.76	199, 200
5.	95% Ethanol:0.1M Ammonium borate pH 10 (2:1)	0.92	1.00	0.94	201
6.	2-Methoxyethanol:Methylethyl--ketone:3N NH_4OH (7:2:3)	0.94	1.00	0.76	202

* Distance travelled relative to Fru6P.

Table 6 Thin Layer Chromatography of sugar phosphates

SOLID PHASE	SOLVENT SYSTEM (v/v)	R_{Fru6P}*			Ref
		Fru1P	Fru6P	Glc6P	
1. Silica	95% Ethanol:1M Ammonium acetate pH 3.9 (7.5:3.0)	0.83	1.00	0.84	199, 200
2. Cellulose	95% Ethanol:1M Ammonium acetate pH 3.9 (7.5:3.0)	0.86	1.00	0.73	199, 200
3. Cellulose	Propanol:Water:Ethyl Acetate (7:2:1)	0.86	1.00	0.73	203

* Distance travelled relative to Fru6P.

Table 7 High Voltage Paper Electrophoresis

	ELECTROLYTE SOLUTION	R_{Picric}			Ref
		Fru1P	Fru6P	Glc6P	
1.	0.02M Ammonium Formate, pH 1	0.57	0.54	0.51	204
2.	0.02M Ammonium Formate, pH 1 (EDTA washed)	0.62	0.63	0.65	204
3.	0.05M Ammonium Formate, pH 1	0.96	0.88	0.90	204
4.	0.05M Ammonium Formate, pH 3.5	0.85	0.82	0.88	204
5.	0.3 M Sodium Tetraborate, pH 9.8	1.28	1.64	1.59	192

** Distance travelled relative to picric acid.

In the present study, a modification of the method used by Rintoul (131) based on that of Khym and Cohn (206) was initially used to separate phosphoric esters as their borate complexes. The modification consisted of gradient elution of the complexes from the column with increasing concentration of ammonium chloride instead of the original step-wise elution. Although this gave a separation of Fru1P from Fru6P and Glc6P, the recovery of the separated sugar phosphates for further analysis was made difficult by the presence of the salts in the eluate.

Lefebvre et al (207) claimed to have overcome this particular problem by using the triethylammonium salt of boric acid both as the complexing reagent and for elution. Although this procedure gives higher concentrations of borate in the eluate than the method of Khym and Cohn (206), triethylamine can be easily removed by freeze-drying and, in theory, borate ions can be removed by distillation with methanol. A modified version of the Lefebvre method was examined and eventually good separations of Fru1P from Fru6P and Glc6P and other sugar phosphates were obtained. A shorter but wider column than that recommended by Lefebvre, was used and also a slower flow rate was found to be advantageous. A typical separation profile of sugar phosphate standards is shown in Fig. 18. This anion-exchange chromatographic method was, therefore, chosen for the routine analysis of mixtues of sugar phosphates derived from plant extracts (see Materials and Methods 4.1.4 b)). However, although the method gave satisfactory separations, the long elution times required were a serious disadvantage which had to be accepted together with the small number of samples that could be examined at one time. The alkaline environment of the eluting system also meant that eluted sugars and sugar phosphates were susceptible to alkaline degradation before recovery. This was kept to a minimum by operating the chromatographic columns in a cold room at 4°C and by freeze-drying the pooled fractions as rapidly as possible. A further important problem encountered was the difficulty of removing borate by distillation with methanol: even after repeated distillation (up to four times) it was suspected that borate was still present (see p. 100).

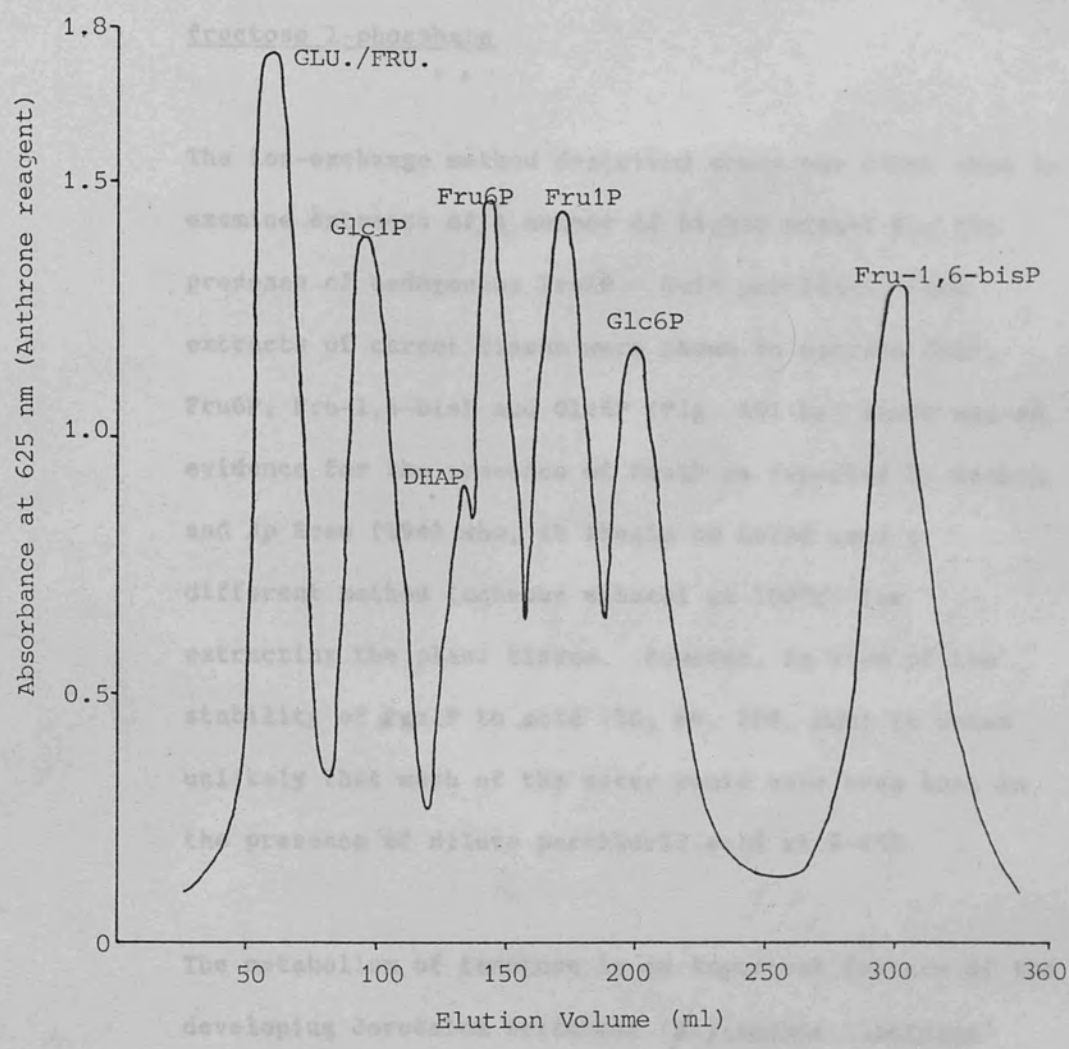


Fig 18 Anion-exchange chromatography of a standard mixture of monosaccharides and monosaccharide phosphates (12.5 µmol each) (see Materials and Methods 4.1.4)

2.1.1 Examination of plant tissues for endogenous fructose 1-phosphate

The ion-exchange method described above was first used to examine extracts of a number of higher plants for the presence of endogenous Fru1P. Cold perchloric acid extracts of carrot tissue were shown to contain DHAP, Fru6P, Fru-1,6-bisP and Glc6P (Fig. 19) but there was no evidence for the presence of Fru1P as reported by Graham and Ap Rees (194) who, it should be noted used a different method (aqueous ethanol at 100°C) for extracting the plant tissue. However, in view of the stability of Fru1P to acid (56, 60, 208, 209) it seems unlikely that much of the ester would have been lost in the presence of dilute perchloric acid at 0-4°C.

The metabolism of fructose is an important feature of the developing Jerusalem artichoke (Helianthus tuberosus) tuber (see Introduction 1.1.3.2) and, hence, it was decided to examine this tissue for the presence of Fru1P. Cold aqueous ethanolic extracts were shown to contain various sugar phosphates (Fig. 20 a, b): in undiluted column fractions the anthrone reagent clearly revealed Fru-1,6-bisP but the remaining material was unresolved (Fig. 20 a). However, when the latter fractions were diluted, anthrone positive peaks corresponding to the Fru6P and Glc6P radioactive markers were found (Fig. 20 a). Further examination of the

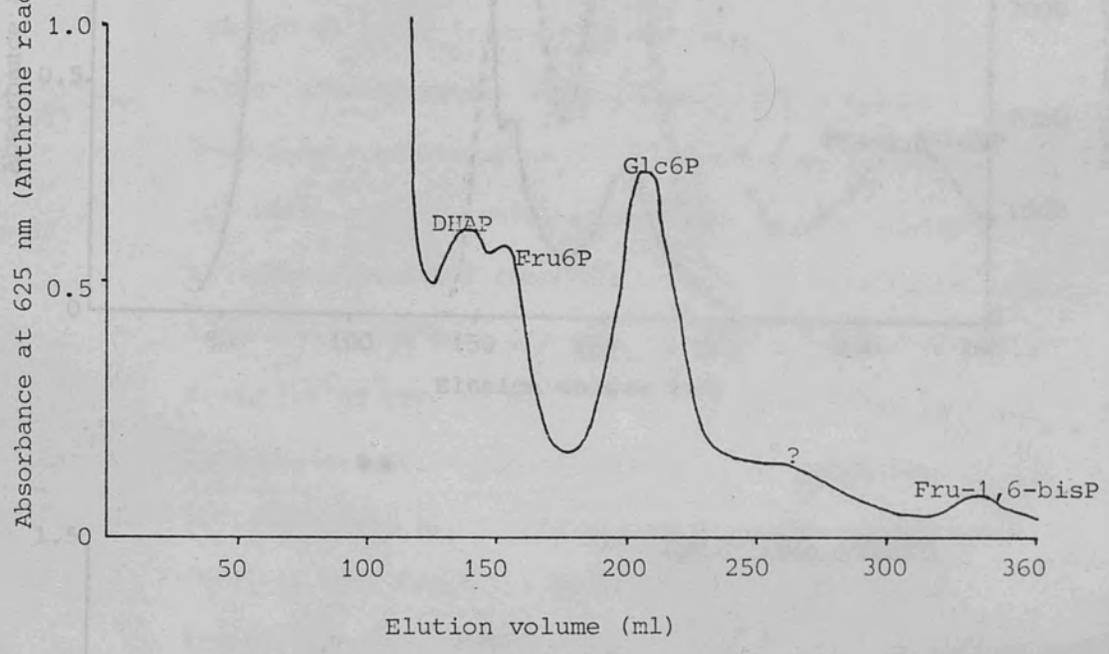


Fig 19 Anion-exchange fractionation of sugar phosphates in a perchloric acid extract of carrot root (see Materials and Methods 4.2.3 i) and 4.2.4 ii))

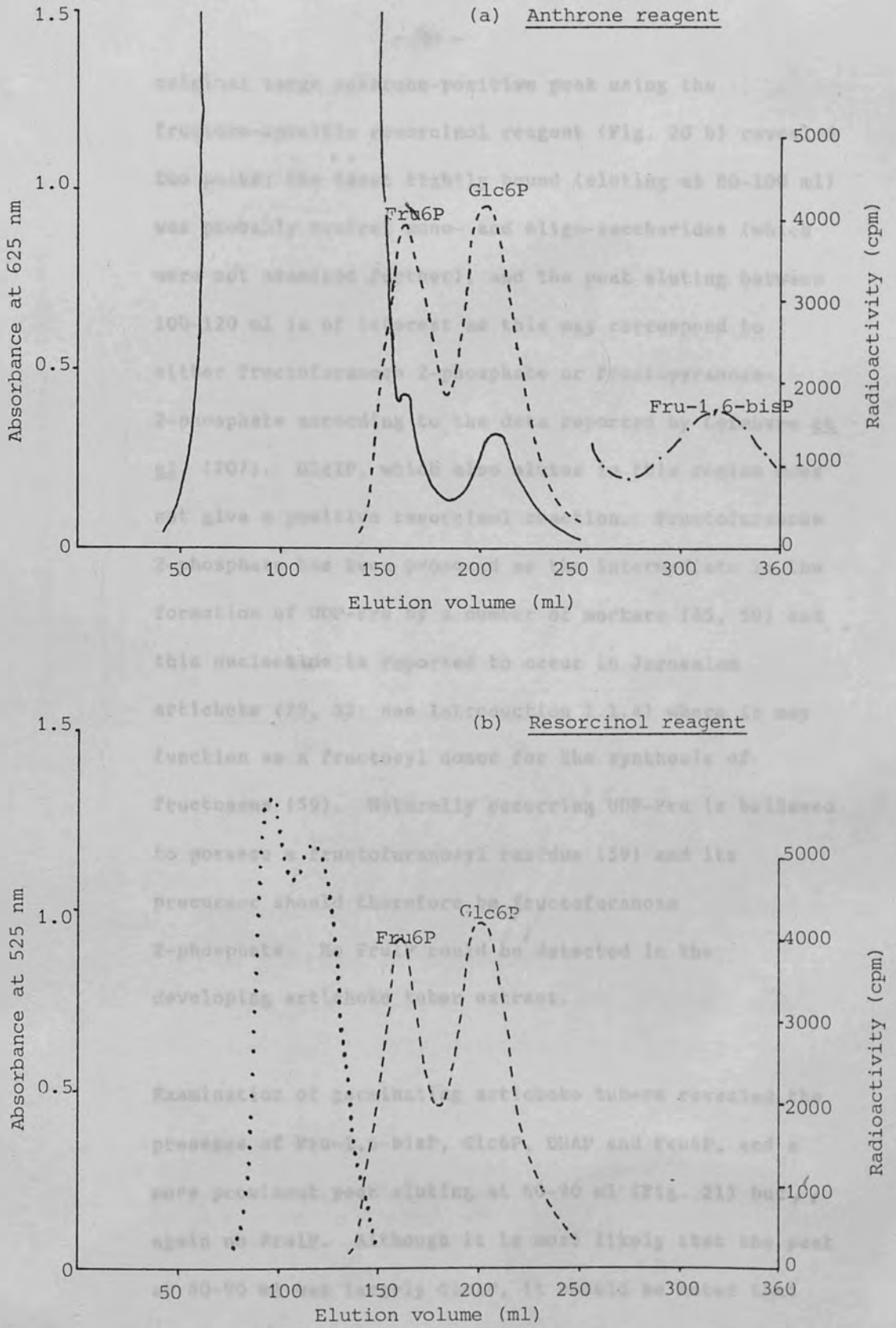


Fig 20 Anion-exchange fractionation of an 80% aqueous ethanolic extract of developing Jerusalem artichoke tuber containing [^{14}C] labelled Fru6P and Glc6P as markers.

(a) Anthrone reagent: -·-·- undiluted fractions; —— diluted (1:10)
(b) Resorcinol reagent: ..... diluted (1:10) fractions. fractions
In (a) and (b) ----- represent [^{14}C] labelled markers (Fru6P & Glc6P)
(Materials and Methods 4.2.3 ii), and 4.2.4 ii),)

original large anthrone-positive peak using the fructose-specific resorcinol reagent (Fig. 20 b) revealed two peaks; the least tightly bound (eluting at 80-100 ml) was probably neutral mono- and oligo-saccharides (which were not examined further), and the peak eluting between 100-120 ml is of interest as this may correspond to either fructofuranose 2-phosphate or fructopyranose-2-phosphate according to the data reported by Lefebvre et al. (207). Glc1P, which also elutes in this region does not give a positive resorcinol reaction. Fructofuranose 2-phosphate has been proposed as the intermediate in the formation of UDP-Fru by a number of workers (45, 50) and this nucleotide is reported to occur in Jerusalem artichoke (29, 32; see Introduction 1.1.4) where it may function as a fructosyl donor for the synthesis of fructosans (59). Naturally occurring UDP-Fru is believed to possess a fructofuranosyl residue (59) and its precursor should therefore be fructofuranose 2-phosphate. No Fru1P could be detected in the developing artichoke tuber extract.

Examination of germinating artichoke tubers revealed the presence of Fru-1,6-bisP, Glc6P, DHAP and Fru6P, and a more prominent peak eluting at 60-90 ml (Fig. 21) but again no Fru1P. Although it is most likely that the peak at 60-90 ml was largely Glc1P, it should be noted that fructose 2-phosphate could also have been present. The peak eluting between Glc6P and Fru-1,6-bisP remains unidentified.

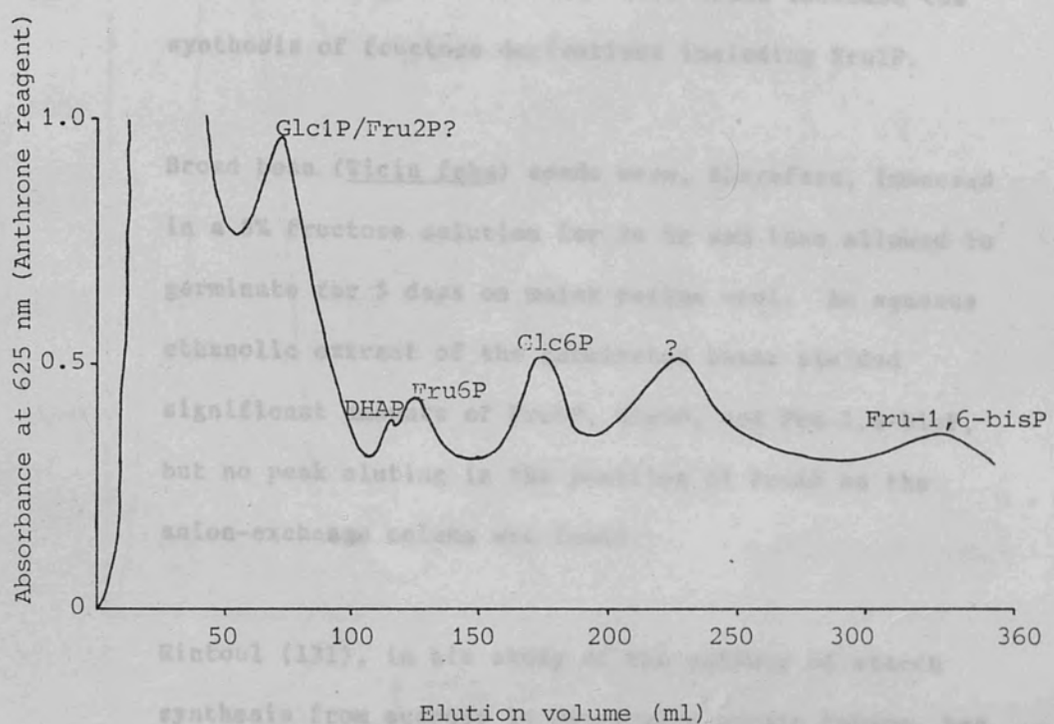


Fig 21 Anion-exchange fractionation of an 80% aqueous ethanolic extract of germinating Jerusalem artichoke tuber (see Materials and Methods 4.2.3 ii) and 4.2.4 ii))

2.1.2 Metabolism of exogenous fructose by plant tissues

It was thought that by challenging a plant with large amounts of exogenous fructose this would increase the synthesis of fructose derivatives including Fru1P.

Broad bean (Vicia faba) seeds were, therefore, immersed in a 5% fructose solution for 24 hr and then allowed to germinate for 5 days on moist cotton wool. An aqueous ethanolic extract of the germinated beans yielded significant amounts of Fru6P, Glc6P, and Fru-1,6-bisP, but no peak eluting in the position of Fru1P on the anion-exchange column was found.

Rintoul (131), in his study of the pathway of starch synthesis from sucrose in developing potato tubers, had found that the injection of [U-^{14}C]tracer into intact tubers, using a hypodermic syringe, was a satisfactory and convenient method for introducing label. This technique was, therefore, used for the introduction of [U-^{14}C]fructose into storage organs in a further attempt to detect Fru1P formation. Following injection of developing potato tubers with labelled fructose and a period of incubation, the sugar phosphates were extracted and analysed as before. The elution profile after 4 hr incubation at room temperature (20-25°C) is shown in Fig. 22. Over this and shorter periods of incubation no

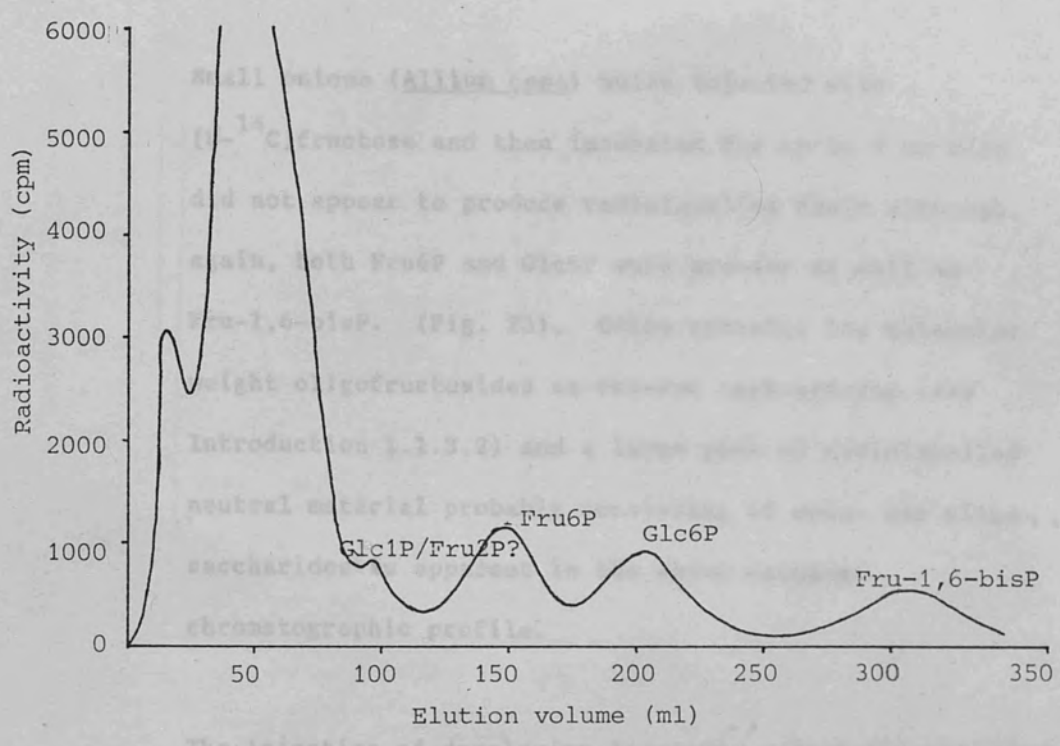


Fig 22 Anion-exchange fractionation of an 80% aqueous ethanolic extract of developing potato tuber injected with [U-^{14}C]fructose and incubated for 4 hr (see Materials and Methods 4.2.1., 4.2.3 ii) and 4.2.4 ii))

radiolabelled peak corresponding to Frul P was detected although the commonly occurring sugar phosphates were present in the extracts.

Small onions (Allium cepa) bulbs injected with [U-^{14}C]fructose and then incubated for up to 4 hr also did not appear to produce radiolabelled Frul P although, again, both Fru6P and Glc6P were present as well as Fru-1,6-bisP. (Fig. 23). Onion contains low molecular weight oligofructosides as reserve carbohydrate (see Introduction 1.1.3.2) and a large peak of radiolabelled neutral material probably consisting of mono- and oligo-saccharides is apparent in the anion-exchange chromatographic profile.

The injection of developing Jerusalem artichoke tubers with [U-^{14}C]fructose followed by an 8 hr incubation period at room temperature (20-25°C) yielded Fru-1,6-bisP and a single, labelled sugar monophosphate peak corresponding to Frul P (Fig. 24). (Mature, germinating tubers treated similarly produced no detectable Frul P.) The fractions containing the acidic material were pooled, frozen and freeze-dried. Borate was removed by repeated distillation with methanol and a sample was then mixed with unlabelled Frul P and re-applied to the anion-exchange column. The Frul P 'marker' was located by the use of resorcinol/HCl reagent and there was exact coincidence on the elution profile between the marker and

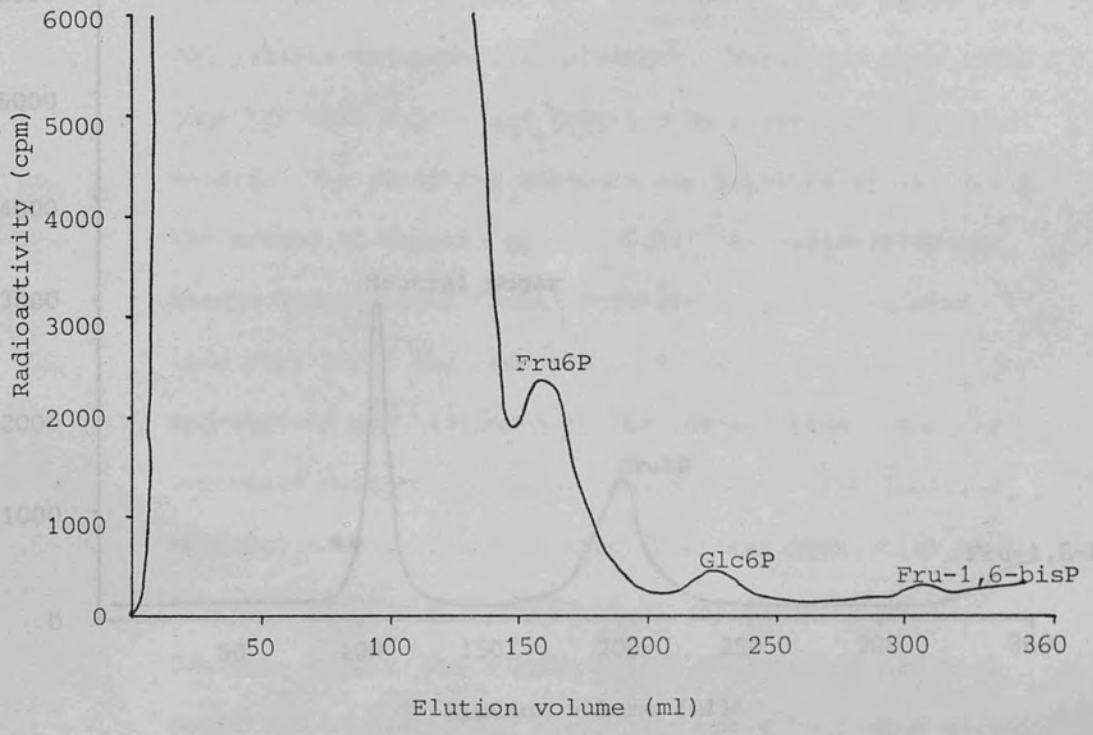


Fig 23 Anion-exchange fractionation of an 80% aqueous ethanolic extract of an onion bulb injected with [U-^{14}C]fructose and incubated for 4 hr (see Materials and Methods 4.2.1., 4.2.3 ii) and 4.2.4 ii))

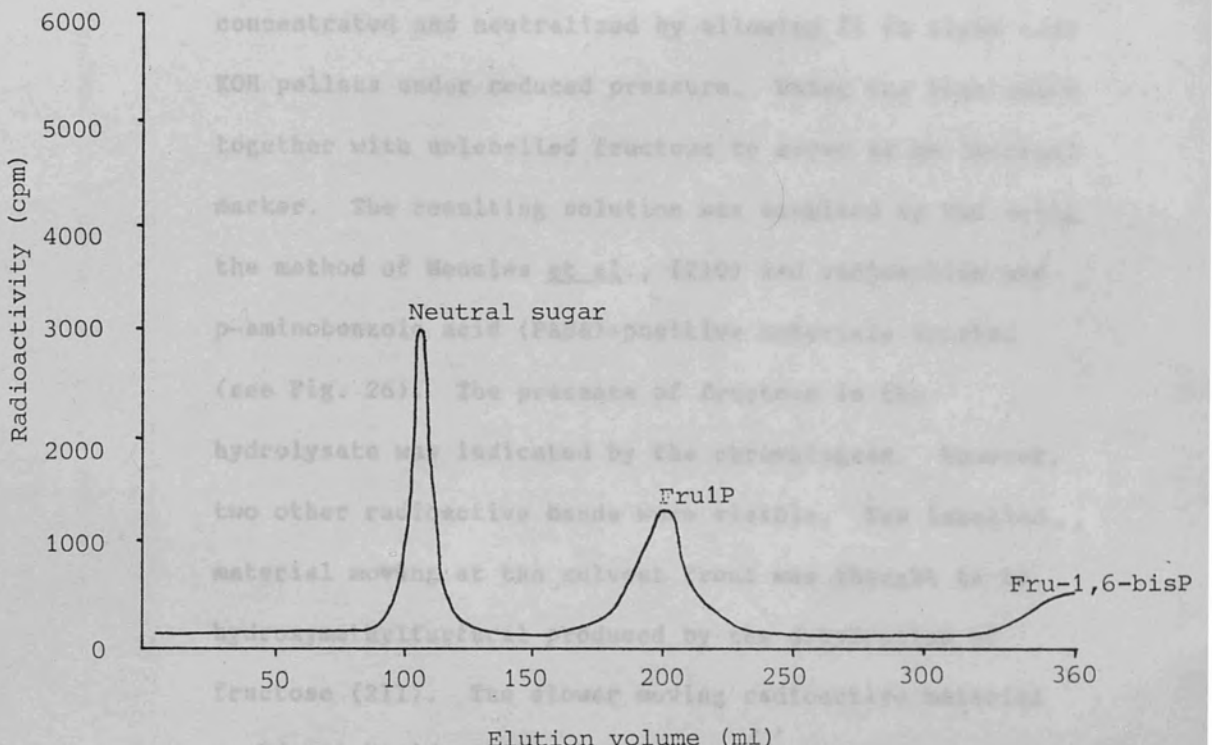


Fig 24 Anion-exchange fractionation of an 80% aqueous ethanolic extract of a developing Jerusalem artichoke tuber injected with [U-^{14}C]fructose and incubated for 8 hr. (see Materials and Methods 4.2.1., 4.2.3 ii) and 4.2.4 ii))

the radioactive compound (Fig. 25). Acid hydrolysis of the putative Fru1P was carried out by reacting with 2M HCl for 30 min at 100°C. The hydrolysate was concentrated and neutralized by allowing it to stand over KOH pellets under reduced pressure. Water was then added together with unlabelled fructose to serve as an internal marker. The resulting solution was examined by TLC using the method of Menzies et al., (210) and radioactive and p-aminobenzoic acid (PABA)-positive materials located (see Fig. 26). The presence of fructose in the hydrolysate was indicated by the chromatogram. However, two other radioactive bands were visible. The labelled material moving at the solvent front was thought to be hydroxymethylfurfural produced by the dehydration of fructose (211). The slower moving radioactive material could not be identified but it was noted that when fructose was incubated with 0.8M triethylamonium borate for 30 min at room temperature then freeze-dried and the borate removed with methanol, a compound with similar chromatographic properties was produced. This may have been an alkaline degradation product of fructose, conceivably of the saccharinic acid type (c.f. the low mobility of sugar acids in the TLC solvent used (210)), or possibly a stable borate complex resulting from the incomplete removal of this ion.

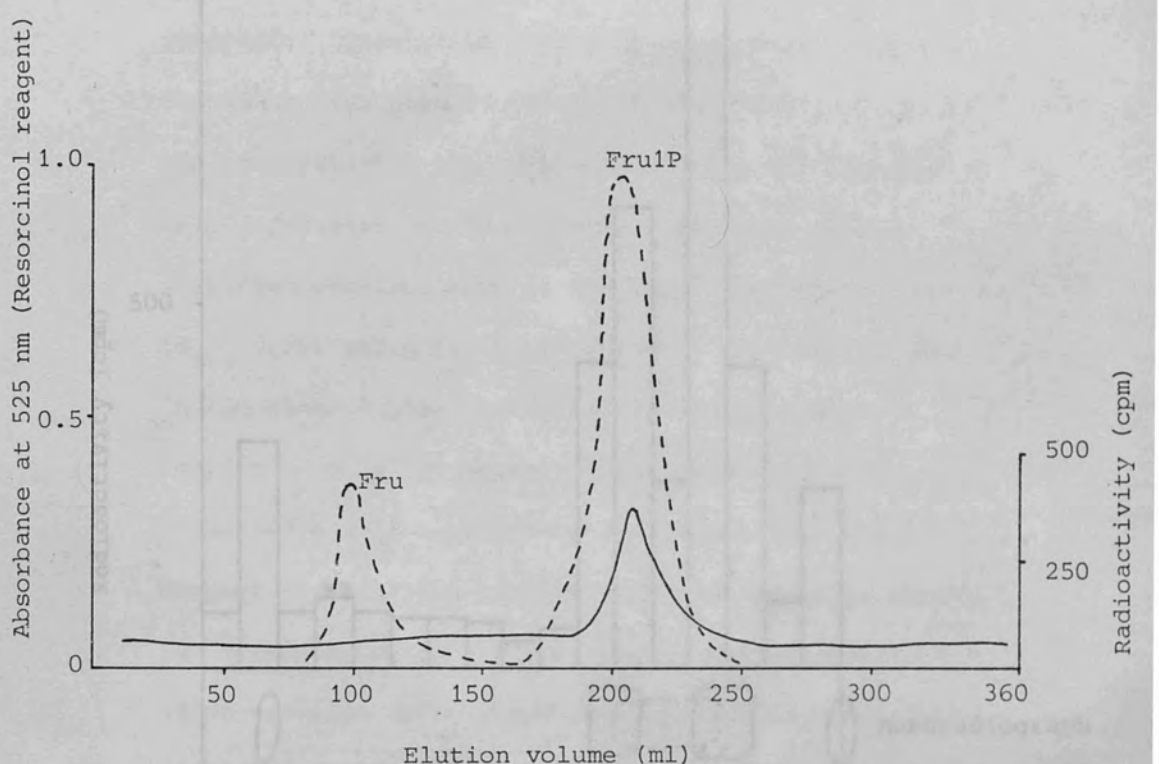


Fig 25 Co-chromatography of Fru1P and the labelled acidic compound (elution volume 170-220 ml) obtained by anion-exchange chromatography of an extract of a developing Jerusalem artichoke tuber injected with [U-^{14}C]fructose and incubated for 8 hr (see Fig. 24). Standard fructose and Fru1P were detected with the resorcinol reagent (-------). Radioactivity (———). (See Materials and Methods 4.2.5 i))

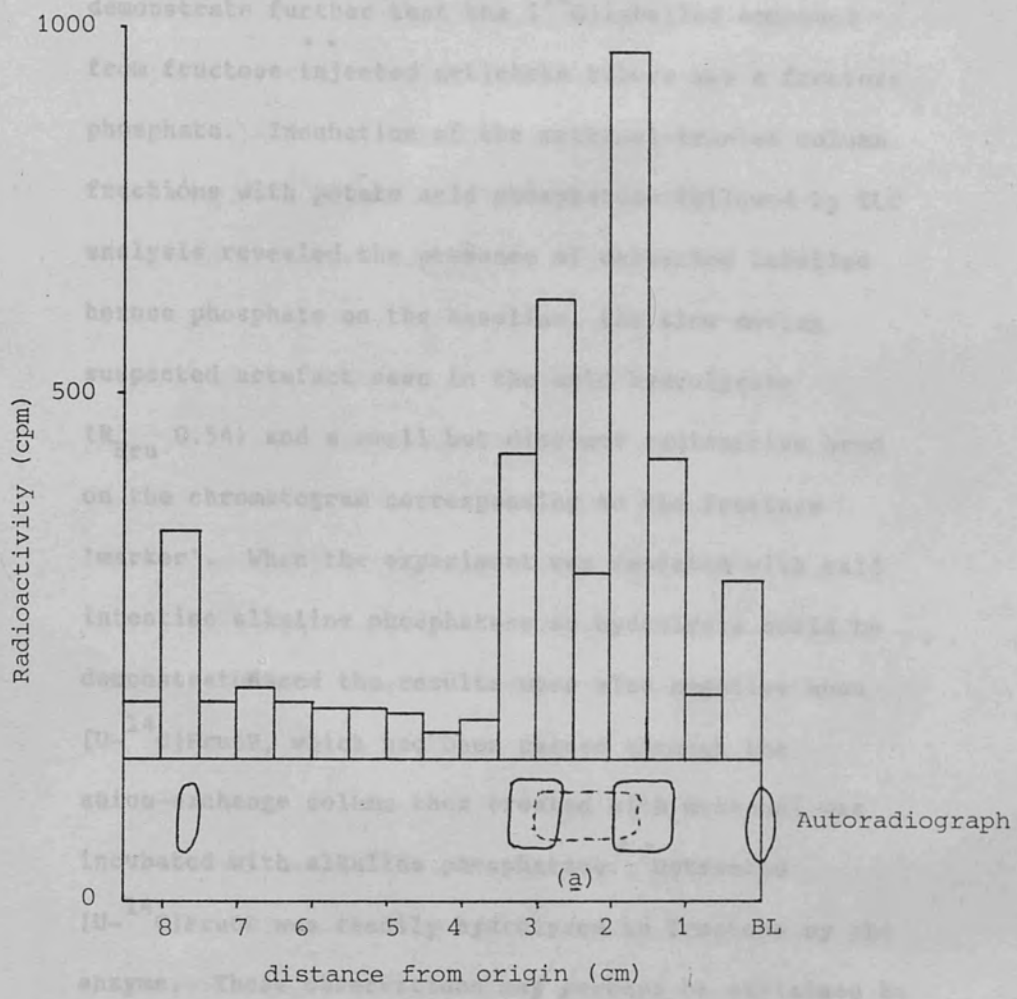


Fig 26 Thin-layer chromatography of an HCl hydrolysate of putative Fru1P isolated by anion-exchange chromatography of an extract of developing Jerusalem artichoke tuber injected with [U-^{14}C]fructose and incubated for 8 hr (see Fig 24) ((a) 'Internal' fructose marker detected with PABA (- - -)) (see Materials and Methods 4.2.5 ii).)

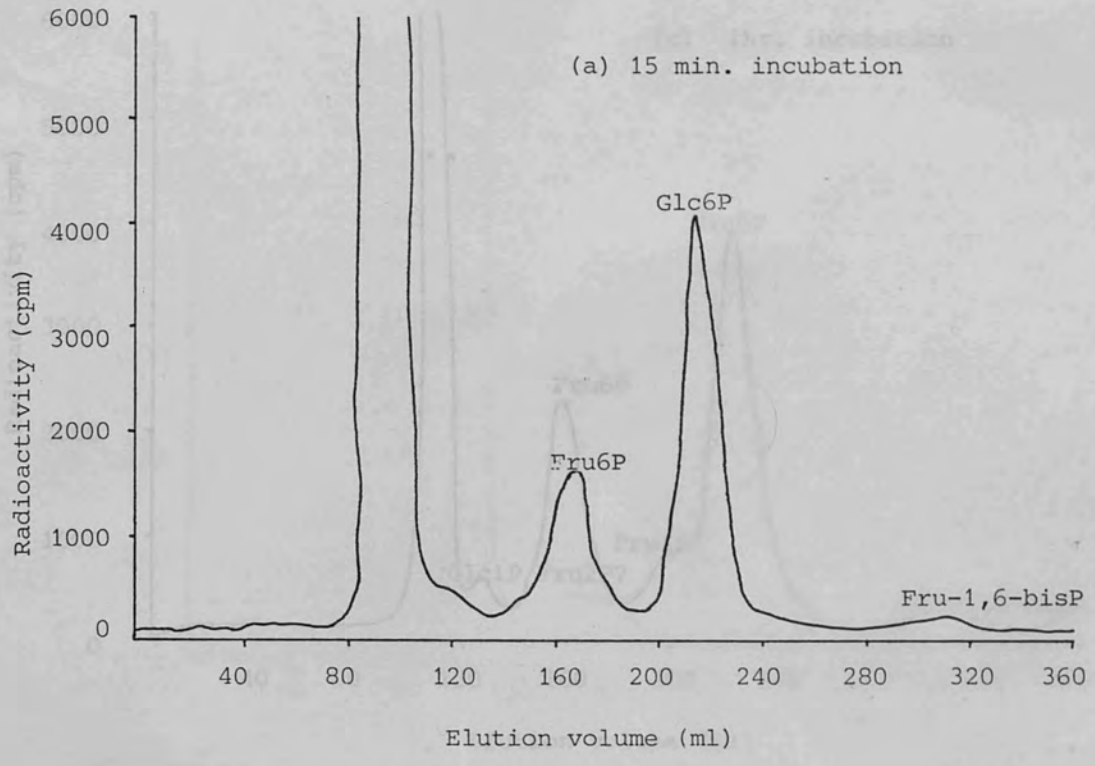
Enzymic hydrolysis was also used in an attempt to demonstrate further that the [^{14}C]labelled compound from fructose injected artichoke tubers was a fructose phosphate. Incubation of the methanol-treated column fractions with potato acid phosphatase followed by TLC analysis revealed the presence of unreacted labelled hexose phosphate on the baseline, the slow moving suspected artefact seen in the acid hydrolysate (R_{Fru} 0.54) and a small but distinct radioactive band on the chromatogram corresponding to the fructose 'marker'. When the experiment was repeated with calf intestine alkaline phosphatase no hydrolysis could be demonstrated and the results were also negative when [U-^{14}C]Fru6P, which had been passed through the anion-exchange column then treated with methanol was incubated with alkaline phosphatase. Untreated [U-^{14}C]Fru6P was readily hydrolysed to fructose by the enzyme. These observations may perhaps be explained by borate contamination of the sample; borate is reported to be an inhibitor of alkaline phosphatase (212).

Further confirmation of the structure of the sugar phosphate was sought by incubating the artichoke product with rat liver aldolase. It had been hoped that this would cleave the ester yielding glyceraldehyde and DHAP and that the latter would be detectable by anion-exchange chromatography. No DHAP was detected, however, and it is again conceivable that borate inhibtion of the enzyme occurred.

On the basis of the behaviour on anion exchange columns (in comparison to standard compounds), and the release of fructose by acid and acid phosphatase hydrolyses, it was concluded that the labelled material produced by injecting [U-^{14}C]fructose into developing Jerusalem artichoke tubers was Fru1P.

Developing Jerusalem artichoke tubers were next injected with [U-^{14}C]fructose and incubated for various periods of time from 5 min up to 8 hr. Similar studies were carried out on three separate occasions (A, B, and C) using different batches of freshly harvested tubers. In Fig. 27a-f elution profiles from anion-exchange columns for one study, A, are given. The percentage incorporation of radioactivity into the various (acidic and neutral) metabolites at various times for all three studies is shown in Figs. 28-31. (The data from which these Figures are derived are given in Appendices I-III).

In the three studies the changes in incorporation of label with time into the hexose phosphates were very similar although in studies B and C maximum incorporation into Glc6P and Fru6P was somewhat later (ca 90 min) than in A. These differences may be due to one or a combination of reasons. For example, the tubers obviously vary in physiological state depending on the exact age of the tuber and the condition of the parent plant. Although tubers were used throughout the growing



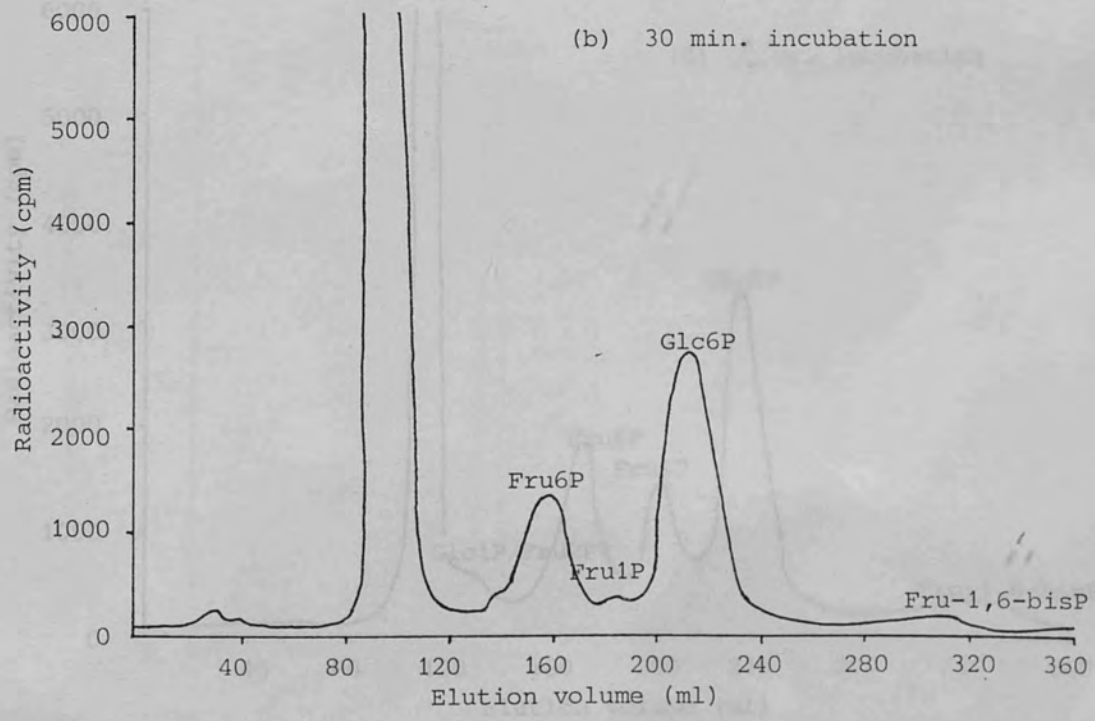


Fig 27 Anion-exchange fructionation of 80% aqueous ethanolic extracts of developing Jerusalem artichoke tubers injected with [U-^{14}C]-fructose and incubated for: (a), 15 min. and (b), 30 min (STUDY A) (see Materials and Methods 4.2.1., 4.2.3 ii) and 4.2.4 ii))

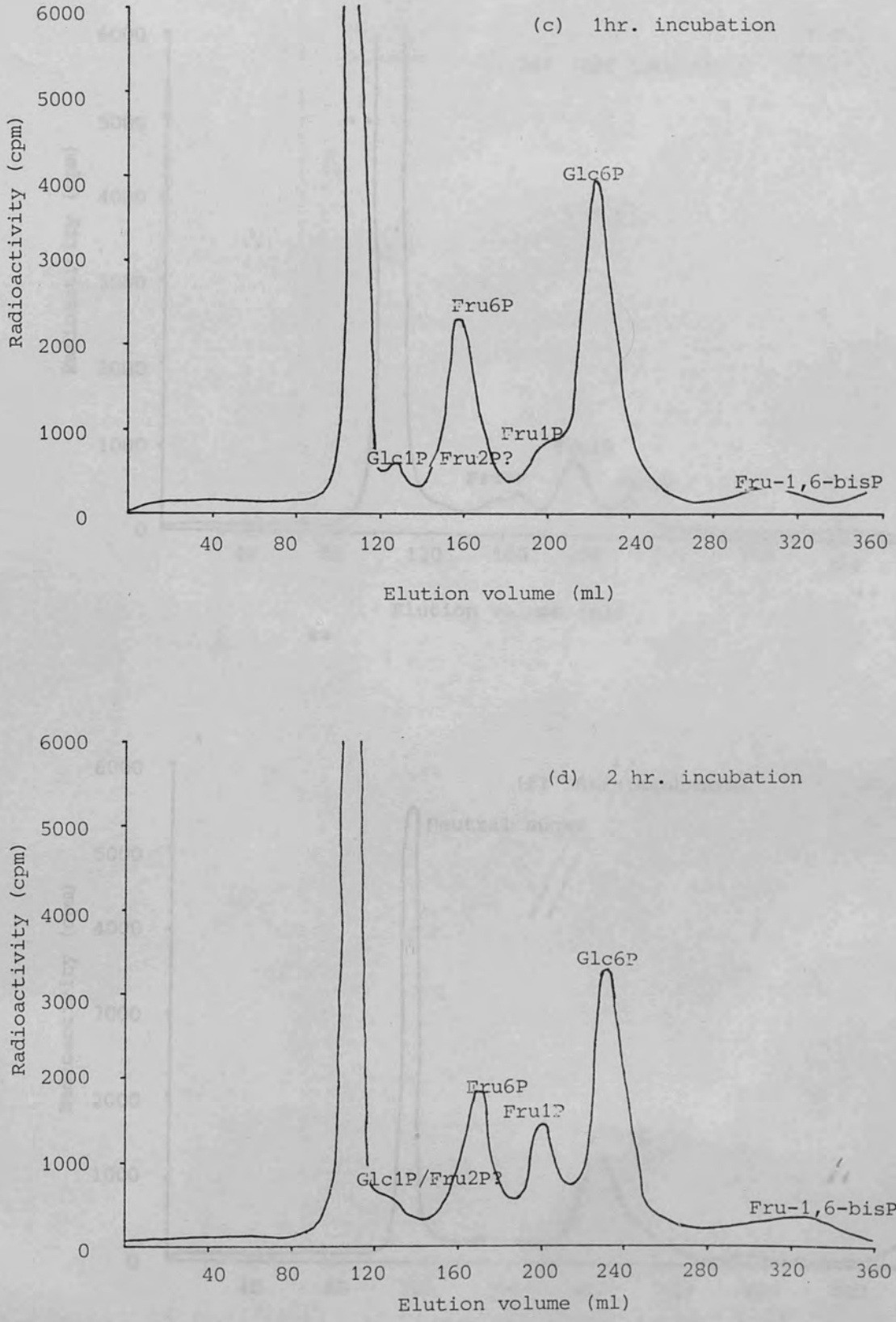
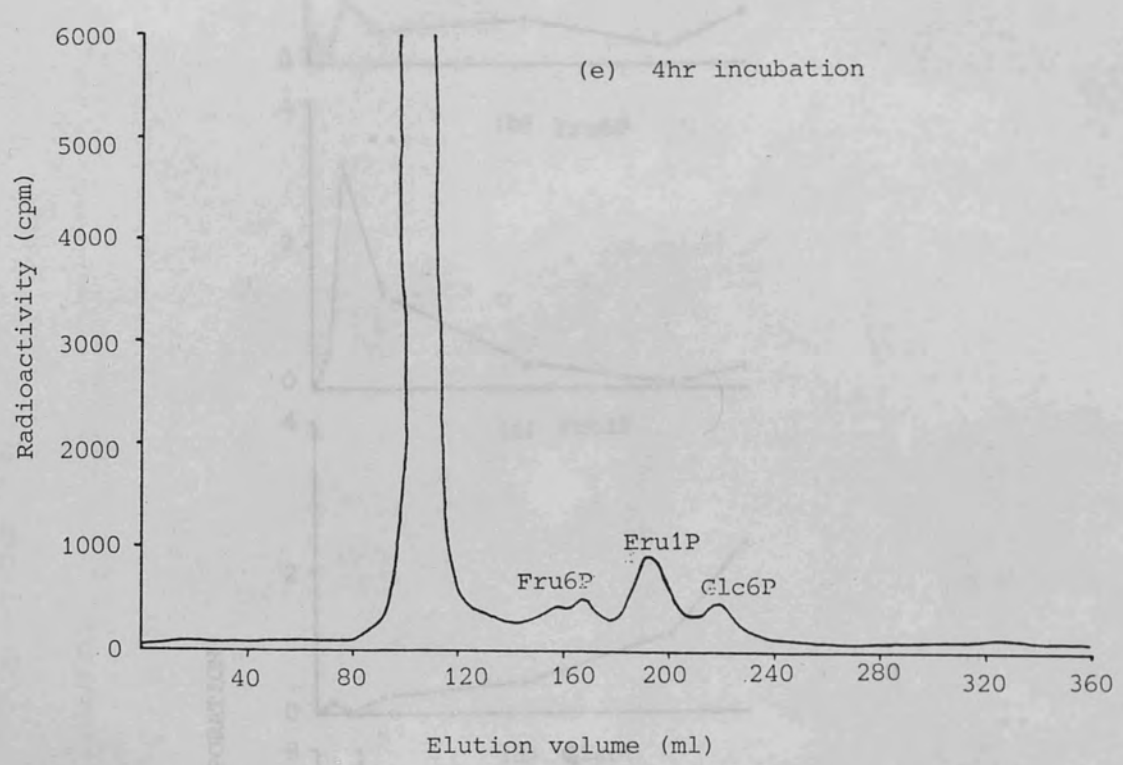


Fig 27 Anion-exchange fractionation of 80% aqueous ethanolic extracts of developing Jerusalem artichoke tubers injected with [U-^{14}C]-fructose and incubated for: (c) 1hr and (d) 2 hr. (STUDY A) (see Materials and Methods 4.2.1, 4.2.3 ii) and 4.2.4 ii))



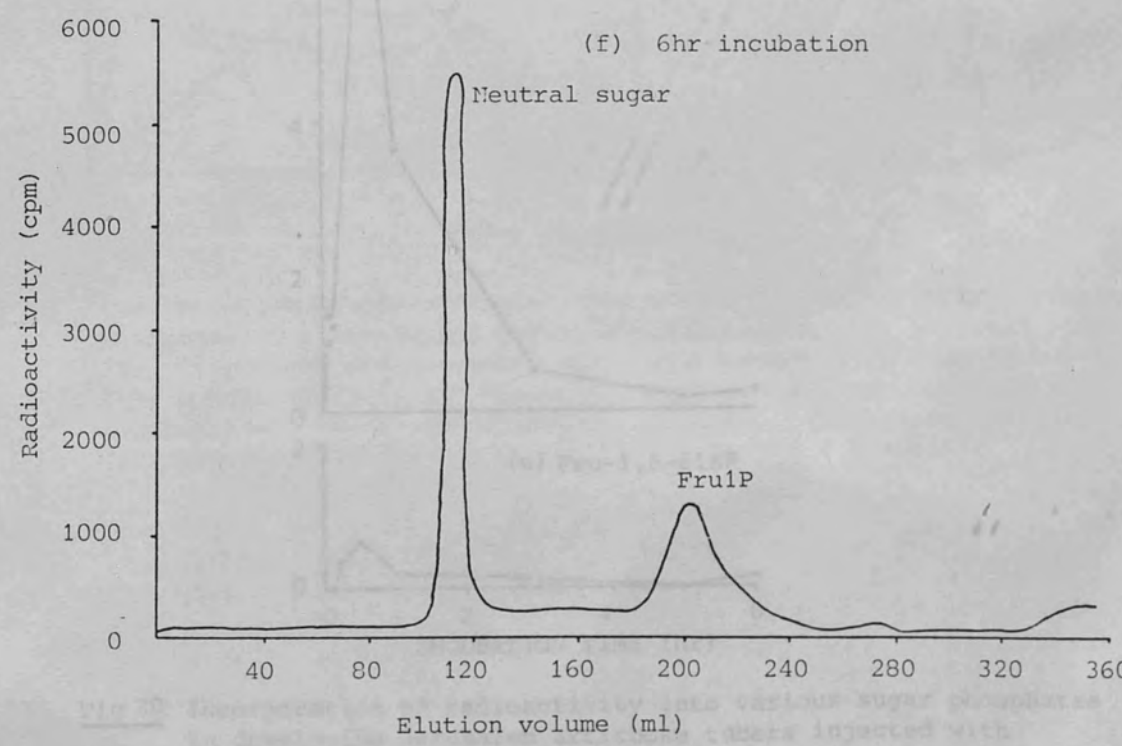


Fig 27 Anion-exchange fractionation of 80% aqueous ethanolic extracts of developing Jerusalem artichoke tubers injected with [U-^{14}C] fructose and incubated for (e) 4hr and (f) 6hr (STUDY A) (see Materials and Methods 4.2.1, 4.2.3 ii) and 4.2.4 ii)).

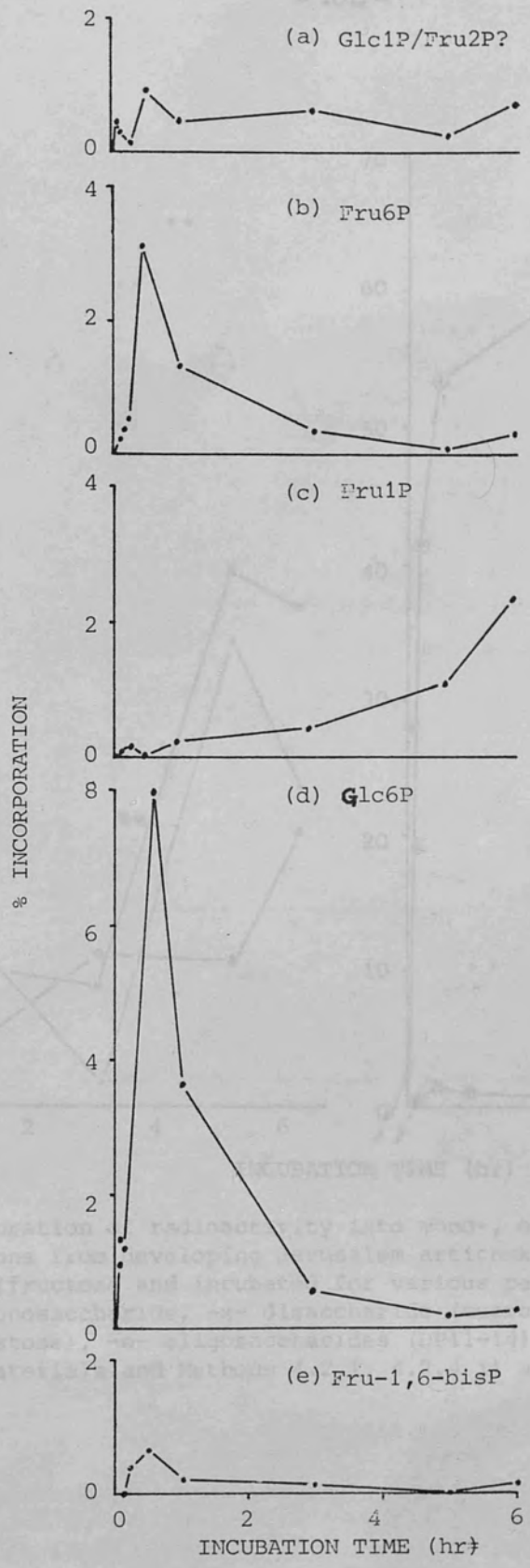


Fig 28 Incorporation of radioactivity into various sugar phosphates in developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose and incubated for various periods of time (Study A) (see Materials and Methods 4.2.1 and 4.2.4 ii))

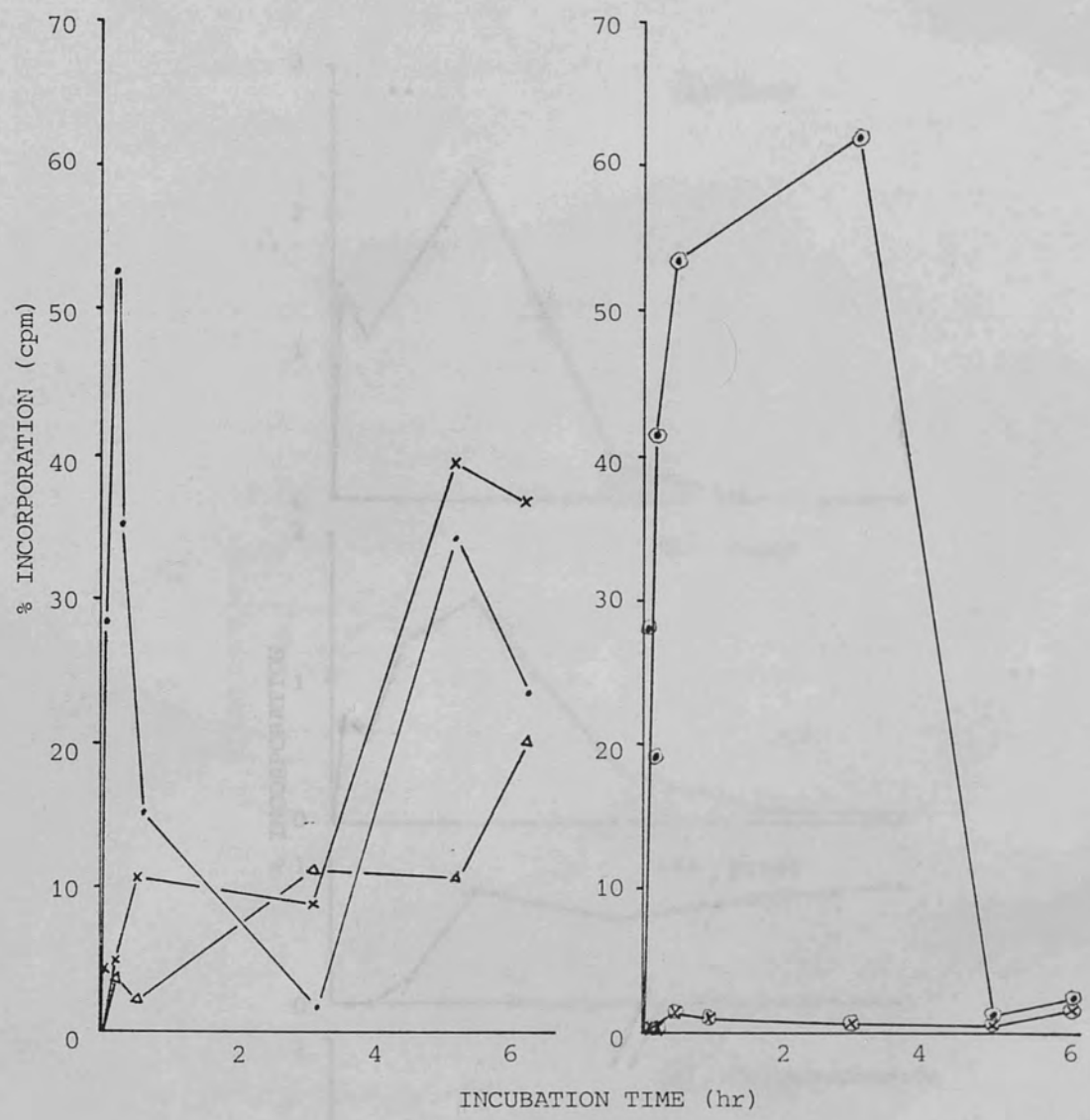


Fig 29 Incorporation of radioactivity into mono-, oligo- and poly-saccharide fractions from developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose and incubated for various periods of time (Study A) (-·- monosaccharide, -x- disaccharide (sucrose), -Δ- trisaccharide (isokestose), -ʘ- oligosaccharides (DP11-14), -⊗- polysaccharide) (see Materials and Methods 4.2.1, 4.2.4 i) and 4.2.4 iii))

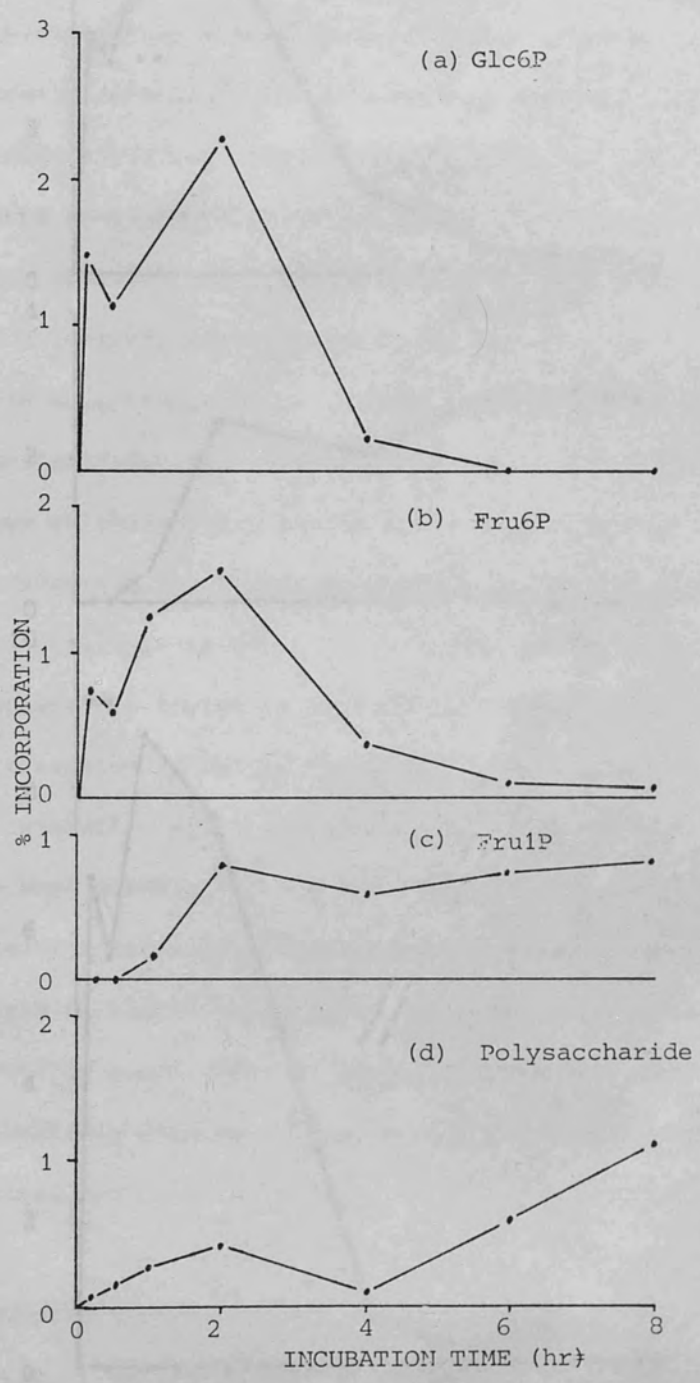


Fig 30 Incorporation of radioactivity into various sugar phosphates and polysaccharide in developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose and incubated for various periods of time (Study B) (see Materials and Methods 4.2.1, 4.2.4 ii) and 4.2.4 iii))

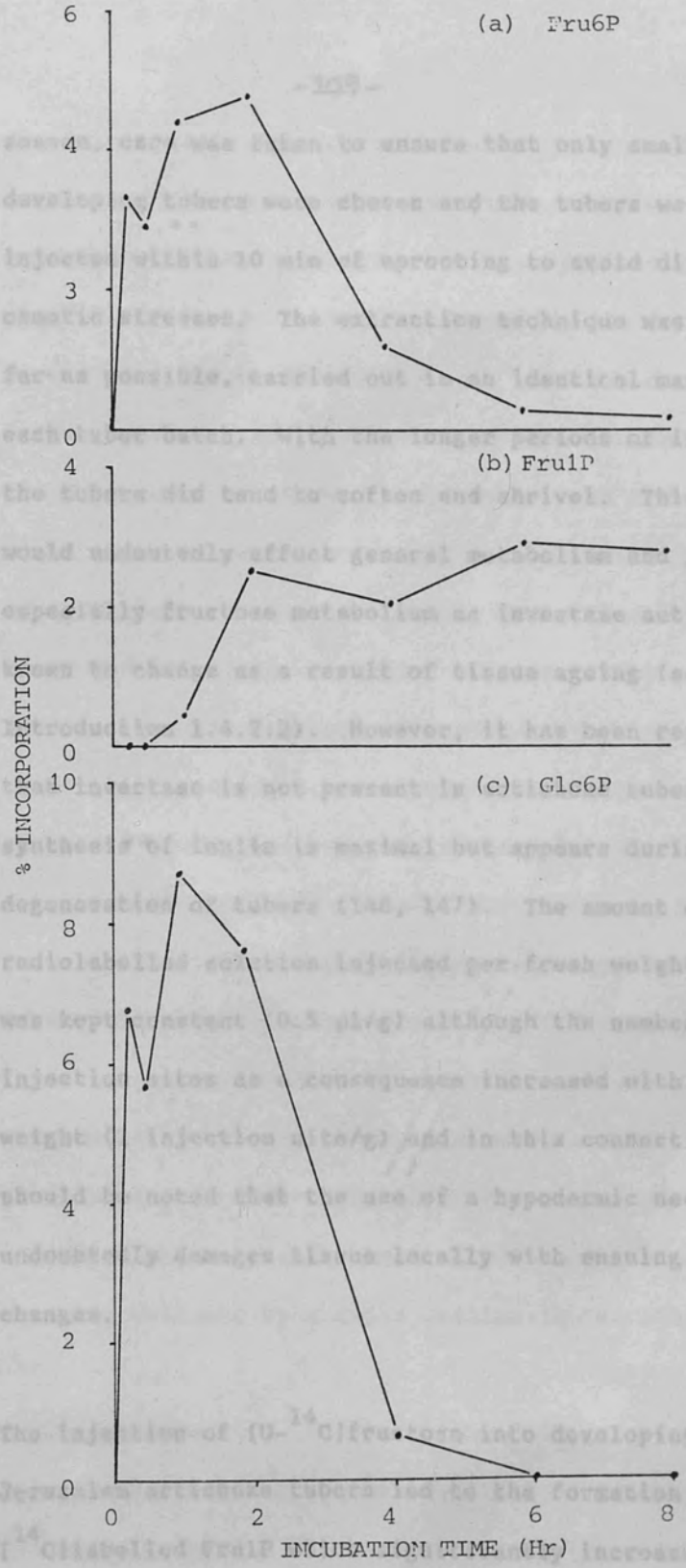


Fig 31 Incorporation of radioactivity into various sugar phosphates in developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose and incubated for various periods of time (Study C) (see Materials and Methods 4.2.1 and 4.2.4 ii))

season, care was taken to ensure that only small developing tubers were chosen and the tubers were injected within 10 min of uprooting to avoid different osmotic stresses. The extraction technique was also, as far as possible, carried out in an identical manner with each tuber batch. With the longer periods of incubation the tubers did tend to soften and shrivel. This 'ageing' would undoutedly affect general metabolism and perhaps, especially fructose metabolism as invertase activity is known to change as a result of tissue ageing (see Introduction 1.4.2.2). However, it has been reported that invertase is not present in artichoke tubers when synthesis of inulin is maximal but appears during degeneration of tubers (146, 147). The amount of radiolabelled solution injected per fresh weight of tuber was kept constant (0.5 µl/g) although the number of injection sites as a consequence increased with tuber weight (1 injection site/g) and in this connection it should be noted that the use of a hypodermic needle undoubtedly damages tissue locally with ensuing metabolic changes.

The injection of [U-^{14}C]fructose into developing Jerusalem artichoke tubers led to the formation of [^{14}C]labelled FrulP which significantly increased with time of incubation in Study A (Figs. 27-29 and Appendix I). There appeared to be an initial lag period of about 60 min followed by a very slow uptake of label

and then a rapid incorporation after 3hr into Fru1P. The lag period may be due to a precurser requirement and/or may represent the time taken for the fructose to reach the site of Fru1P synthesis. Regarding the former hypothesis, it can be clearly seen that the rapid formation of Fru-6P, Glc6P, Fru1,6-bisP and, possibly, Glc1P preceded the formation of Fru1P. In the case of Glc1P there is some doubt regarding the exact composition of the column fraction as other phosphoric esters such as Fru2P and G3P elute at similar volumes. Although this fraction was not further analysed to determine its exact composition, the incorporation of label into this hexose phosphate would have been of interest in relation to the formation of Fru1P and nucleotide fructoses. The three, possibly four, commonly-occurring hexose phosphates presumably arose by phosphorylation of fructose by fructokinase followed by the combined actions of phosphohexoisomerase, phosphoglucomutase and phosphofructokinase. Maximum incorporation of label into all of these phosphates occurred after 30 min. and this was then followed by a rapid decline in the labelling.

In the complementary studies, B and C (Figs. 30 and 31 and Appendices II and III), the labelling of Fru1P again showed a lag period of about 30 min during which there was a rapid labelling of Glc6P and Fru6P. However, after approximately 2 hr no further increase of label in the Fru1P pool was apparent.

Taking the three studies together, the patterns of incorporation of label from fructose could mean that one of the commonly occurring hexose phosphates is either directly, or indirectly, a precursor of Fru1P although no known pathways from these esters to Fru1P are known. Further possibilties for Fru1P synthesis include the existence of unidentified Fru1P precursors such as Fru2P (which, as stated earlier,is difficult to separate from Glc1P) and the direct phosphorylation of fructose to Fru1P by a specific fructokinase as occurs in animal tissues. (Further consideration of the possible precursor(s) of Fru1P is given in the Introduction 1.3).

Finally, it should be noted that there was no indication in any of the studies that Fru1P, once formed, was metabolized further. This is in direct contrast to the other hexose phosphates produced in the feeding experiments. In comparison with the liver system, the breakdown of Fru1P by aldolase would be expected with the formation of DHAP and glyceraldehyde. Aldolase activity is reported to be present in plant tissues (213-217) and these enzymes are, presumably, generally capable of reacting with Fru1P (196) as in the case of Jack bean (127) and spinach leaf (197) aldolases. If this is true in the case of the Jerusalem artichoke tuber then perhaps the apparent failure of the tissue to metabolize Fru1P is a function of compartmentalization of the ester away from

the aldolase site; or that Frul P, relative to Fru-1,6-bisP, is a poor substrate (higher Km) for the enzyme.

The incorporation of label from [^{14}C]fructose into the individual hexose phosphates never exceeded 10% in any of the three studies so the possible fate of the remaining label was investigated in Study A by examining the degree of incorporation of ^{14}C into the mono-, oligo-, and poly- saccharide fractions.

The mono- (glucose and fructose) and oligo- saccharides (three fractions: sucrose, isokestose and fructosans with DP11-14) obtained by extracting the tissues with aqueous ethanol, were separated by paper chromatography and the radioactivity determined by counting the appropriate paper strips in a scintillation counter. Fructosans of DP >14 were not analysed as they remained on the baselines of the chromatograms along with other immobile compounds. Fructosans of DP4-11 co-chromatographed with sugar phosphates and again were not analysed due to this interference. The polysaccharide fractions were obtained by alcohol precipitation of the aqueous extracts of the residual tuber tissue remaining after aqueous ethanol extraction.

Ten minutes after the injection of [U-^{14}C]fructose, a high level of radioactivity (55% incorporation, Fig 29) was detected in the monosaccharide fraction from the tubers. This fell to zero after 3 hr then appeared to peak again at 5 hr. A high proportion of the label (approx 80%) appeared in the fructose-containing oligo- and poly-saccharides after 3 hr but it is difficult to interpret in any detail the complex changing patterns of labelling, probably reflecting anabolism and catabolism, that were observed in the various fractions during the 6 hr. incubation period. Some changes may have been artifacts produced by tissue damage (injections) or by general deterioration of the excised tubers during the incubation period. It is presumed that the incorporation of label from fructose into the various fructosan fractions was initiated by sucrose synthesis (42) which either occurred via Fru6P and sucrose phosphate synthetase or directly from fructose via sucrose synthetase. The latter enzyme is abundant in Jerusalem artichoke tubers (218) where its main function is probably the catabolism of sucrose during sprouting. Sucrose phosphate synthetase has not been reported in this particular storage tissue, but it does occur in others such as developing cotyledons and roots (see Introduction 1.4.1.1).

In Study A (Fig. 29) a 10% incorporation of ^{14}C into sucrose was observed after 30 min incubation, then no further increase occurred for approximately 3 hr and this latter period corresponded with the incorporation of label (max 12%) into the isokestose fraction (Fig. 29) presumably produced by the action of SST on sucrose (see Introduction 1.6.1.1). The labelling of the fructosan fraction, DP11-14, (Fig. 29) showed a striking increase over the first 30 min of incuabtion and then continued to rise slowly up to 3 hr; these oligosaccharides were supposedly synthesized from isokestose by FFT (Introduction p71).

Following an incubation time of 3 hr. there was further labelling of sucrose (which reached 40% incorporation after 5 hr). Also, after a lag period (between 3 and 5 hr), there was further incorporation of label into isokestose (maximum 20% after 6 hr). In contrast to the fructose and di- and tri-saccharide fractions, the higher fructosan fraction lost all of its label after 5 hr and this, at least in part, could have accounted for the second phase increase in the radioactivity of fructose, sucrose and isokestose which may have arisen directly from the degradation of the fructosan fraction by the action of hydrolases associated with the vacuole (see Introduction 1.6.2) or by the stress induced (ageing, injection damage) elevation of invertase activity.

Very little label from fructose (maximum 1.5%; Fig. 29) appeared in the polysaccharide fraction at any time. Incorporation appeared to peak at 30 min. then fall, to be followed by further incorporation after 5 hr. (A two-phase incorporation of ^{14}C into the polysaccharides fraction was also observed in Study B (Fig. 30) and possibly where tuber callus tissue was incubated with [U-^{14}C]fructose (see p.122 and Fig. 34). The two phases of incorporation into the polysaccharide fraction (Figs. 29 and 30) could be accounted for by the formation of different polysacchardies at different incubation times. Hence, the peak of activity at 30 min. may represent, in the main, inulin synthesis which is followed by rapid degradation to lower DP materials. The increase in incorporation of label over the final hour of incubation could be the result of the passage of fructose carbon into other polysaccharides, possibly cell wall materials.

2.1.3 Metabolism of exogenous sucrose by Jerusalem artichoke tubers

As sucrose is considered to be the major source of carbon entering the tuber from the leaves in vivo, it was decided to compare the metabolism of this disaccharide with that of fructose in artichokes.

Developing tubers were, therefore, injected as previously described for fructose, with [U-^{14}C]sucrose and after various periods of incubation labelled sugar phosphates and neutral carbohydrates were isolated by anion-exchange chromatography and paper chromatography or alcohol precipitation (polysaccharide), respectively and the percentage incorporations of label determined (Fig. 32 and Appendix IV).

In comparison with the studies with fructose (Fig. 28, 30, 31) little ^{14}C appeared in the sugar phosphates over a 4 hr incubation period (Fig. 32(c)). Significant but small amounts of label were only detected in Fru6P and Glc6P (Fru6P < Glc6P), the former becoming labelled before the latter, and, as in the case of the fructose injections, the radioactivity in both fractions rapidly declined with increasing incubation time. The order of appearance of label in Fru6P and Glc6P are consistent with an initial sucrose synthetase-catalysed conversion of sucrose to UDP-Glc and fructose, with the latter being phosphorylated by fructokinase. However, labelled monosaccharides, which may have included glucose, were quickly released after sucrose injection (see below), and hence direct phosphorylation of glucose and fructose may well have contributed to the hexose phosphate labelling patterns.

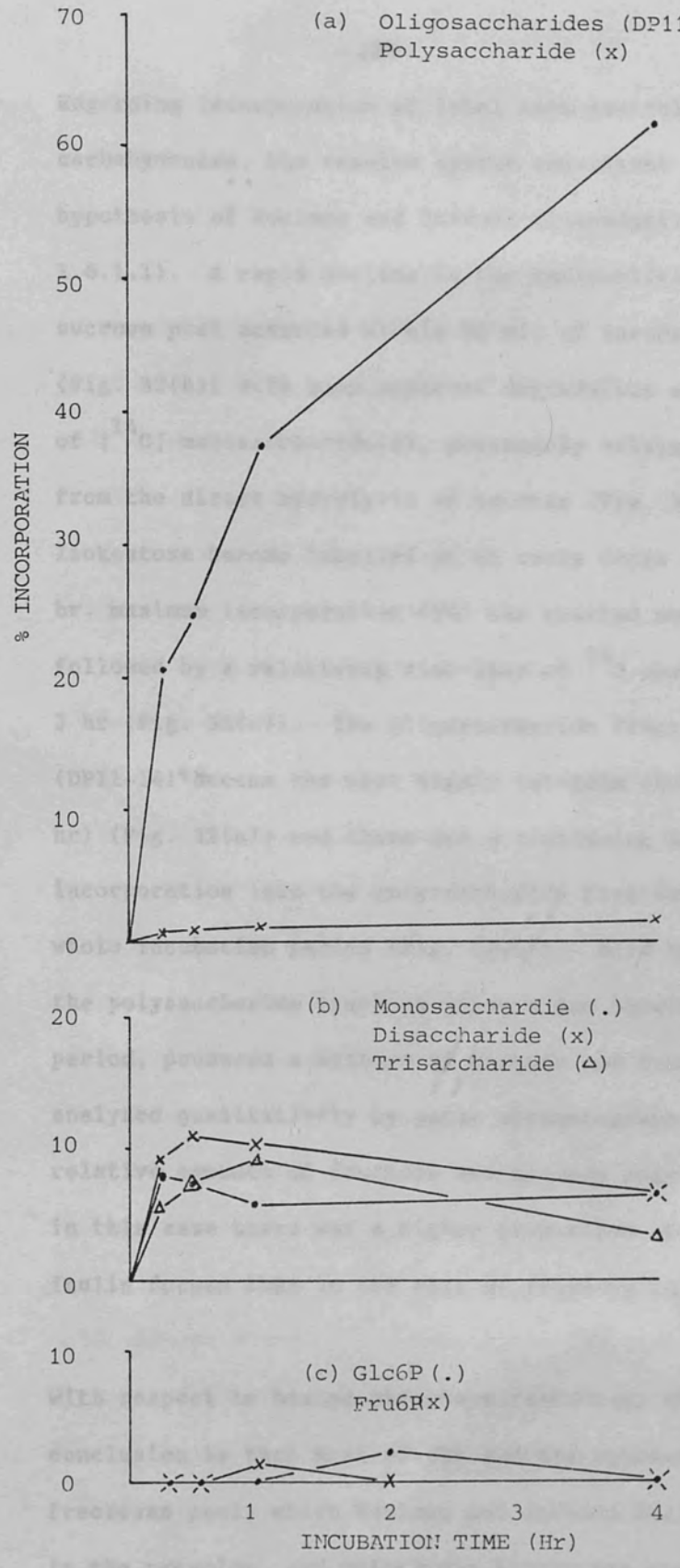


Fig 32 Incorporation of radioactivity into sugar phosphates mono-, oligo- and poly-saccharides in developing Jerusalem artichoke tubers injected with [U-^{14}C]sucrose and incubated for various periods of time (see Materials and Methods 4.3 and 4.2.4).

Regarding incorporation of label into neutral carbohydrates, the results appear consistent with the hypothesis of Edelman and Jefford (Introduction 1.6.1.1). A rapid decline in the radioactivity of the sucrose pool occurred within 30 min of incubation (Fig. 32(b)) with some apparent degradation and release of [^{14}C]-monosaccharide(s), presumably arising mainly from the direct hydrolysis of sucrose (Fig. 32(b)). Isokestose became labelled at an early stage and after 1 hr. maximum incorporation (9%) was reached and this was followed by a relatively slow loss of ^{14}C over the next 3 hr (Fig. 32(c)). The oligosaccharide fraction (DP11-14) became the most highly labelled (60% after 4 hr) (Fig. 32(a)) and there was a continuing but small incorporation into the polysaccharide fraction over the whole incubation period (Fig. 32(a)). Acid hydrolysis of the polysaccharide fraction after a 4hr incubation period, produced a mixture of glucose and fructose when analysed qualitatively by paper chromatography. The relative amounts of fructose and glucose suggested that in this case there was a higher proportion of labelled inulin formed than in the case of fructose injections.

With respect to hexose phosphate formation, the main conclusion is that most of the sucrose entered the fructosan pool, which Edelman and Jefford (42) suggest is in the vacuoles, and relatively little was converted either directly or indirectly to monosaccharides, and, hence, labelling of hexose phosphate was minimal.

2.1.4 Fructose metabolism by callus cultures of Jerusalem artichoke tubers

A supply of developing Jerusalem artichoke tubers was only available from late June to early October and, furthermore, some variability in metabolism was observed with different tuber samples (e.g. the pattern of Fru1P production in Study A (Fig. 28 & 29) compared with studies B and C (Figs. 30, 31)). At a later stage in the project, therefore, a preliminary attempt was made to develop a tuber cell culture system which could be used, without seasonal tissue supply problems, for studying Fru1P formation. Callus tissue was cultured from discs of developing Jerusalem artichoke tubers using an agar growth medium containing sucrose as the carbon source (for details see Materials and Method 4.1.8). Callus tissue, in the log phase of growth, was injected at several different points with a solution of [U-^{14}C]fructose.

The metabolism of [U-^{14}C]fructose by the tissue (Fig. 33, 34 and Appendix V) was similar to that observed with intact, developing tubers (Figs. 28-31). Incorporation of label into Fru6P, Glc6P, Glc1P (or Fru2P, see p116) and Fru-1,6-bisP was observed and [^{14}C]Fru1P was formed, but only after a lag period of about 3 hr. Evidence for fructosan synthesis via sucrose formation was again evident although the levels of

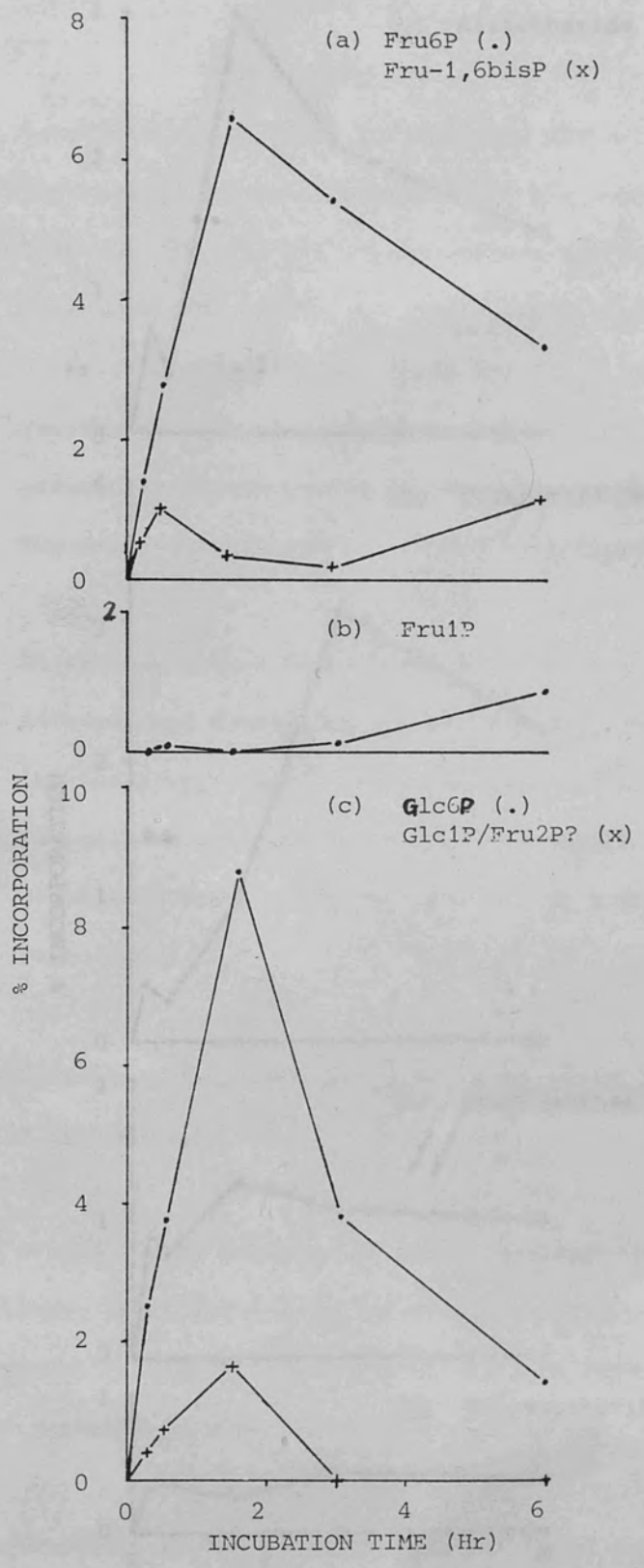


Fig 33 Incorporation of radioactivity into sugar phosphates in callus cultures of Jerusalem artichoke tubers incubated with [U-^{14}C]fructose for various periods of time (see Materials and Methods 4.2.1 and 4.2.4 ii)).

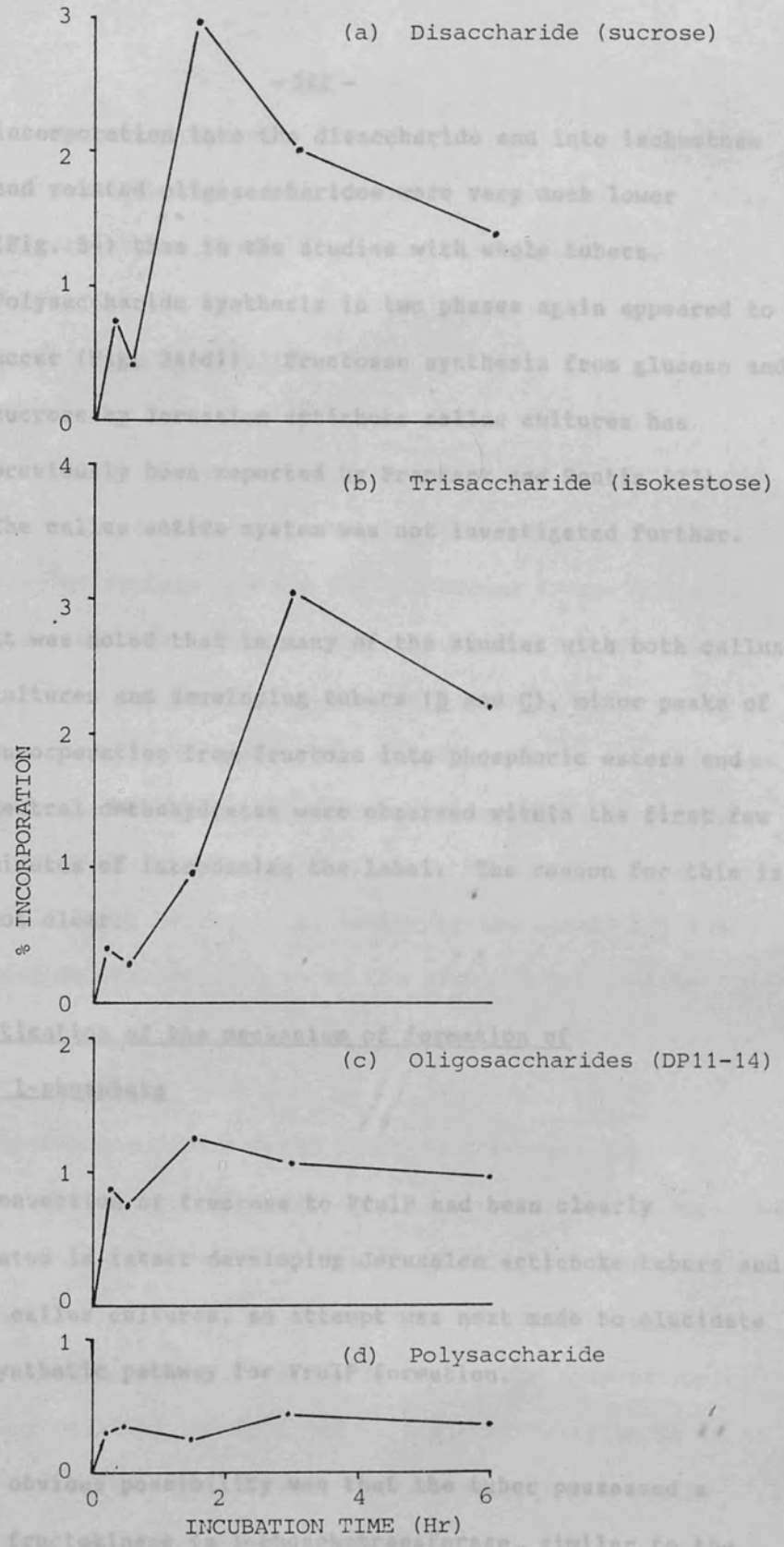


Fig 34 Incorporation of radioactivity into oligo- and polysaccharides in callus cultures of Jerusalem artichoke in callus cultures of Jerusalem artichoke incubated with [U-^{14}C]fructose for various time periods (see Materials and Methods 4.2.1 and 4.2.4 i) and iii)).

incorporation into the disaccharide and into isokestose and related oligosaccharides were very much lower (Fig. 34) than in the studies with whole tubers. Polysaccharide synthesis in two phases again appeared to occur (Fig. 34(d)). Fructosan synthesis from glucose and sucrose by Jerusalem artichoke callus cultures has previously been reported by Frankart and Pontis (77). The callus active system was not investigated further.

It was noted that in many of the studies with both callus cultures and developing tubers (B and C), minor peaks of incorporation from fructose into phosphoric esters and neutral carbohydrates were observed within the first few minutes of introducing the label. The reason for this is not clear.

2.2 An investigation of the mechanism of formation of fructose 1-phosphate

As the conversion of fructose to FrulP had been clearly demonstrated in intact developing Jerusalem artichoke tubers and in tuber callus cultures, an attempt was next made to elucidate the biosynthetic pathway for FrulP formation.

The most obvious possibility was that the tuber possessed a specific fructokinase (a 1-phosphotransferase, similar to the mammalian liver enzyme (232) (see Introduction 1.3)) which

brought about a direct conversion of fructose to Fru1P. This hypothesis was investigated by examining soluble extracts from developing artichokes tubers for hexose kinase activity.

2.2.1 Hexose Kinase activity

Initially, dialysed 0.1M Tris-HCl buffer (pH 8.2) extracts of the tubers were incubated with [U-^{14}C]fructose and ATP and the hexose kinase activity measured by the use of DEAE cellulose discs to 'trap' labelled phosphorylated compounds (219). Other assay methods were investigated (including PC and ion-exchange procedures for the detection of phosphorylated products (220), and the spectrophotometric measurement of ATP utilization by a coupled enzyme system (221)) but the disc method appeared to be the simplest for routine studies.

The tuber extracts were found to possess hexokinase activity with a high affinity for fructose: the apparent Km using this substrate was 22 μM (Fig. 35) and the Vmax 232 pmol.min^{-1}. The optimum pH for the reaction was in the range 7.2-8.5 (Fig. 36). The preparations were unstable and lost 30% of their activity in 24 hr at 4°C and frozen (-18°C) stored tubers lost all hexose kinase activity after 3 months.

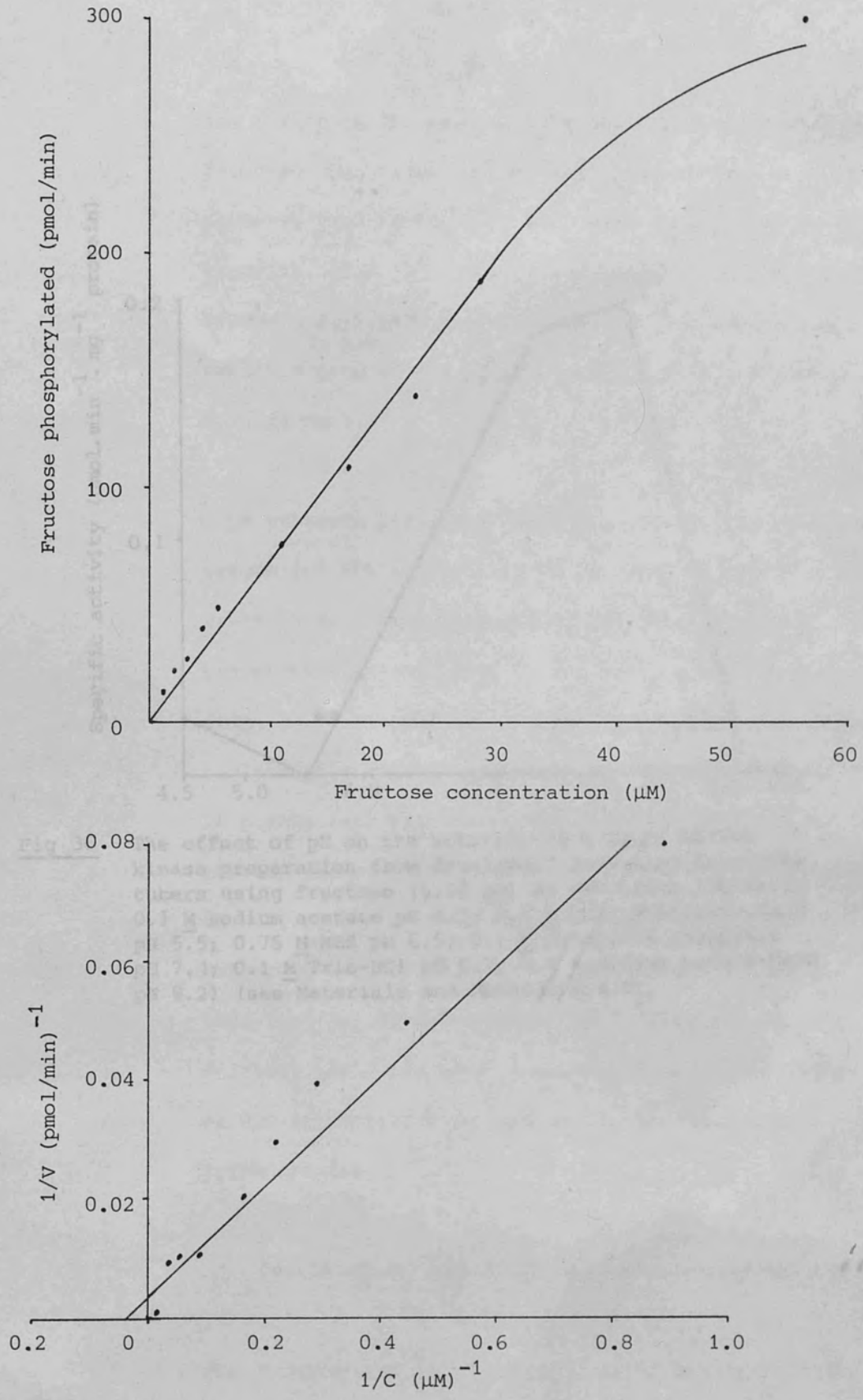


Fig 35 The effect of fructose concentration on a crude hexose kinase activity from Jerusalem artichoke tubers. Incubation mixture contained ATP (3.1 mM), $MgCl_2$ (1.75 mm) NaF (31 mM) and KCl (62 mM) (see Materials and Methods 4.4.1).

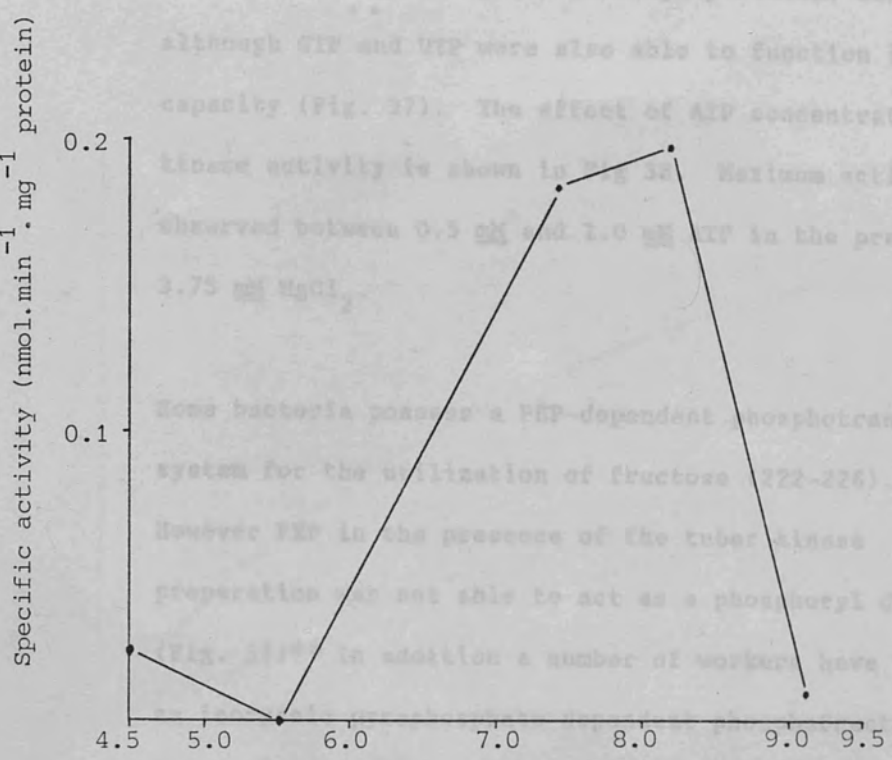


Fig 36 The effect of pH on the activity of a crude hexose kinase preparation from developing Jerusalem artichoke tubers using fructose (5.25 μM) as substrate (Buffers: 0.1 M sodium acetate pH 4.5; 0.1 M sodium citrate-NaOH pH 5.5; 0.75 M MES pH 6.5; 0.1 M potassium phosphate pH 7.4; 0.1 M Tris-HCl pH 8.2; 0.1 M sodium borate-NaOH pH 9.2) (see Materials and Methods 4.4.1).

The preferred phosphoryl donor for the phosphorylation of fructose, catalysed by the crude preparation, was ATP although GTP and UTP were also able to function in this capacity (Fig. 37). The effect of ATP concentration on kinase activity is shown in Fig 38. Maximum activity was observed between 0.5 mM and 1.0 mM ATP in the presence of 3.75 mM $MgCl_2$.

Some bacteria possess a PEP-dependent phosphotransferase system for the utilization of fructose (222-226). However PEP in the presence of the tuber kinase preparation was not able to act as a phosphoryl donor (Fig. 37). In addition a number of workers have reported an inorganic pyrophosphate dependent phosphofructokinase in plants (see Introduction 1.3) but here again, in the present study, there were no indications that PPi could be utilized to phosphorylate fructose (Fig. 37). A number of workers (227-229) have claimed that phytic acid (myo-inositol hexaphosphate) in the presence of plant extracts could act as a phosphoryl donor using either ADP or GDP as acceptors to produce the corresponding triphosphates:

Phytic acid + XDP → inositol-pentaphosphate + XTP

The phosphorylation of fructose using phytic acid as phosphate donor could not be demonstrated in the present study (Fig. 39). However, when phytic acid was added

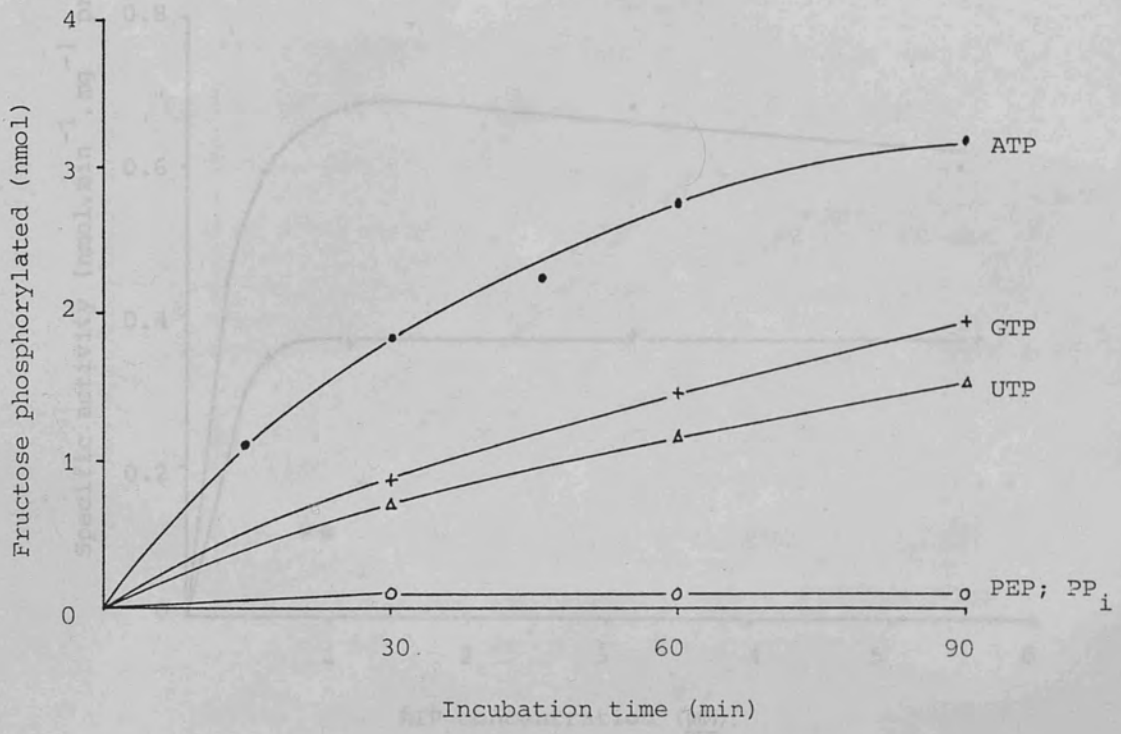


Fig 37 The activity of a crude hexose kinase preparation for developing Jerusalem artichoke tubers with various phosphoryl donors (3.1 mM) using fructose (5.25 µM) as substrate (see Materials and Methods 4.4.1).

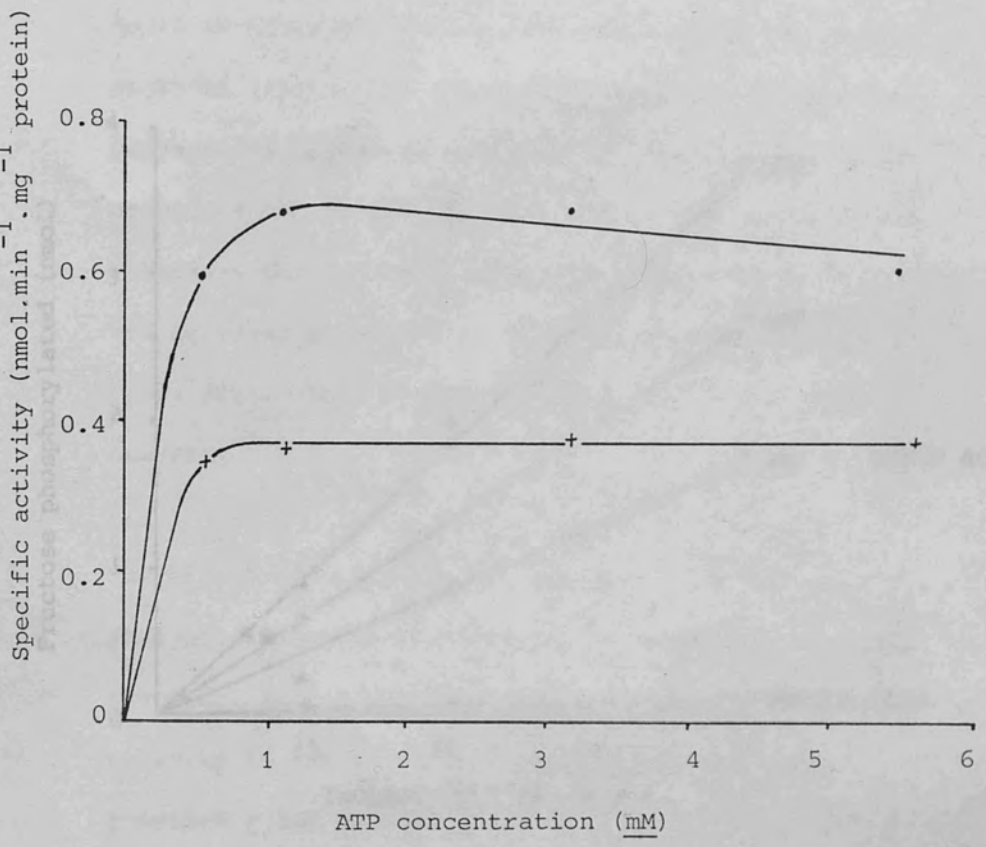


Fig 38 The effect of ATP concentration on the activity of a crude hexose kinase preparation from developing Jerusalem artichoke tubers at two fructose concentrations (5.25 μM (+) and 11.3 μM (.)) (see Materials and Methods 4.4.1).

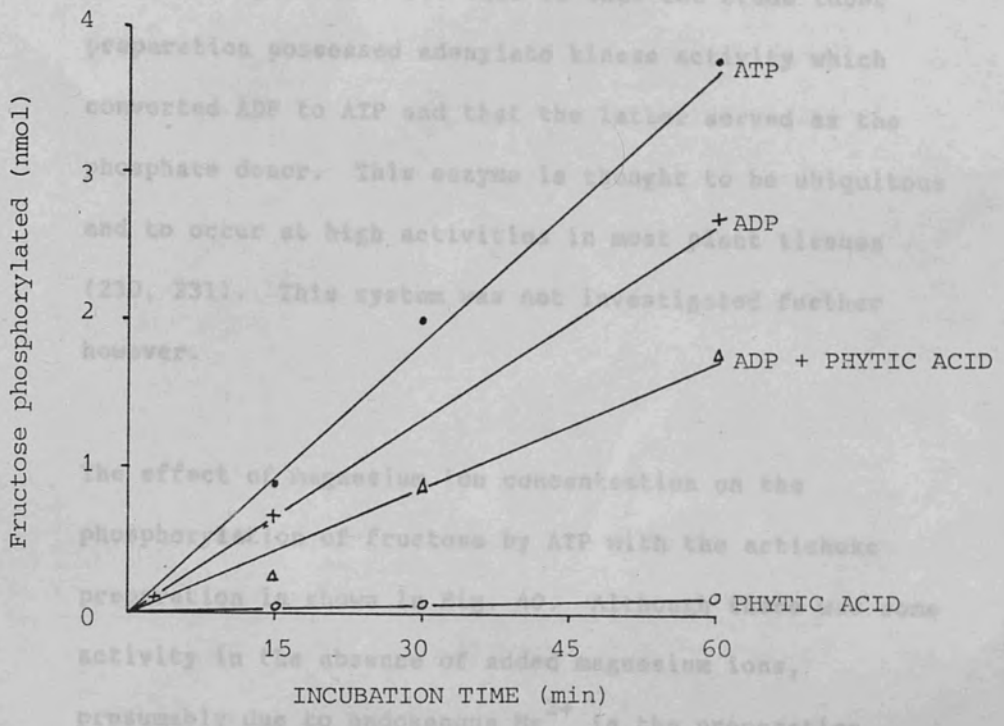


Fig 39 The activity of a crude hexose kinase from developing Jerusalem artichoke tubers using fructose (5.25 μM) as substrate with ADP and phytic acid (3.1 mM alone; 1.55 mM of each when combined) or ATP (3.1 mM) (see Materials and Methods 4.4.1).

together with ADP to the reaction mixture phosphorylation did occur but further work indicated that this reaction could be achieved by using ADP alone (Fig. 39). The probable explanation for this is that the crude tuber preparation possessed adenylate kinase activity which converted ADP to ATP and that the latter served as the phosphate donor. This enzyme is thought to be ubiquitous and to occur at high activities in most plant tissues (230, 231). This system was not investigated further however.

The effect of magnesium ion concentration on the phosphorylation of fructose by ATP with the artichoke preparation is shown in Fig. 40. Although there was some activity in the absence of added magnesium ions, presumably due to endogenous Mg^{2+} in the preparation, the addition of 4mM $MgCl_2$ was required for maximum activity.

There have been various reports of increased activity of fructokinases resulting from the addition of monovalent cations such as potassium, rubidium and ammonium (5, 6, 87). Frankart and Pontis (77) maintained that potassium ions were necessary for the stability of pea fructokinase: these ions are also required for liver ketohexokinase activity (232). The artichoke preparation possessed hexose kinase activity in the absence of added potassium ions but the activity was, stimulated two-fold by 20 mM KCl (Fig 41).

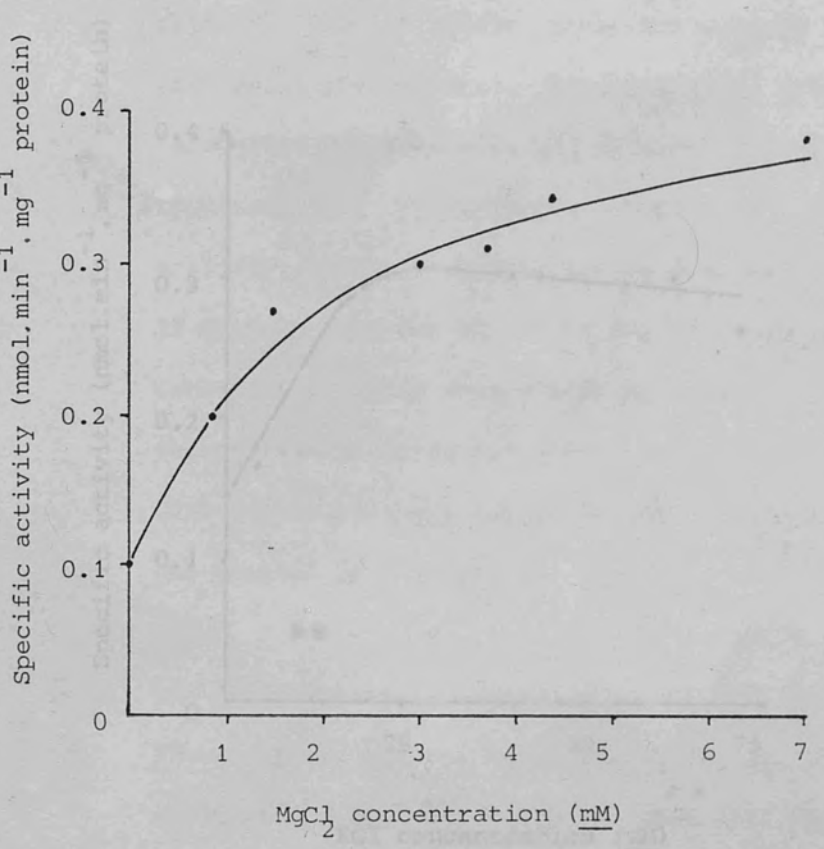


Fig 40 The effect of magnesium ion concentration on the activity of a crude hexose kinase preparation from developing Jerusalem artichoke tubers using fructose (5.25 µM) as substrate (see Materials and Methods 4.4.1).

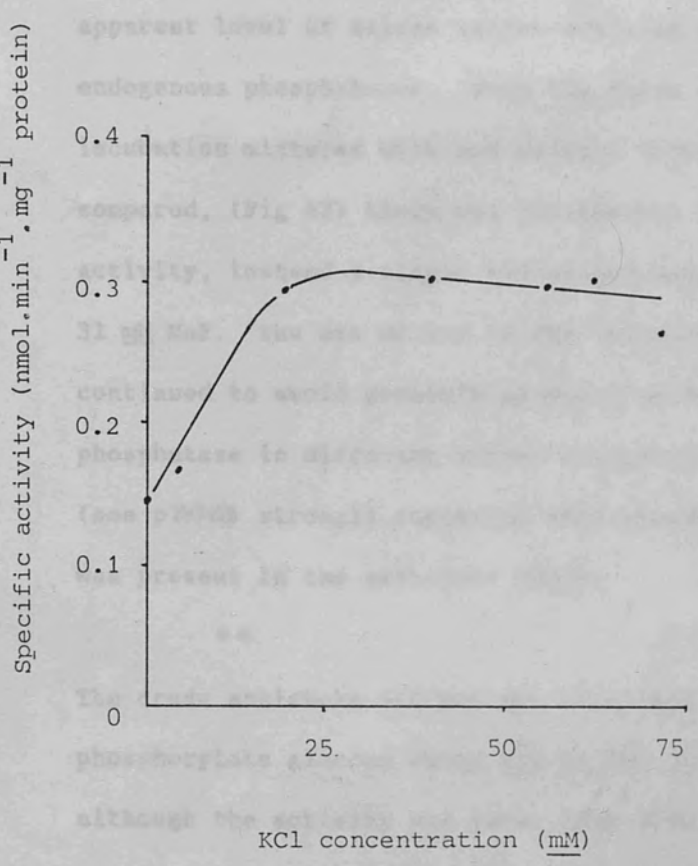


Fig 41 The effect of potassium ion concentration on the activity of a crude hexose kinase preparation from developing Jerusalem artichoke tubers using fructose (5.25 µM) and ATP (3.1 mM) as substrates (see Materials and Methods 4.4.1).

The effect of fluoride ions on the phosphorylation of fructose was also investigated in an attempt to raise the apparent level of hexose kinase activity by inhibiting endogenous phosphatases. When the rates obtained with incubation mixtures with and without added fluoride were compared, (Fig 42) there was no apparent stimulation of activity, instead a slight inhibition was observed with 31 mM NaF. The use of NaF in the reaction mixtures was continued to avoid possible problems caused by phosphatase in different enzyme preparations. Later work (see p147-50) strongly suggested that phosphatase activity was present in the artichoke extract.

The crude artichoke extract was also able to phosphorylate glucose using ATP as the phosphoryl donor, although the activity was lower than with fructose (Fig. 43). This hexokinase (or glucokinase) activity was probably due to an enzyme distinct from fructokinase as NAG, a competitive inhibitor of hexokinase (233), at high concentrations (35 mM) dramatically inhibited the phosphorylation of glucose whereas the activity with fructose as substrate was little affected even at much higher concentrations (up to 96 mM) of NAG (Fig. 43).

The products of fructose phosphorylation under all the various conditions described above were checked using the anion-exchange method (see Materials and Method 4.1.4(b)) but in no case was FrulP detected. In all cases labelled

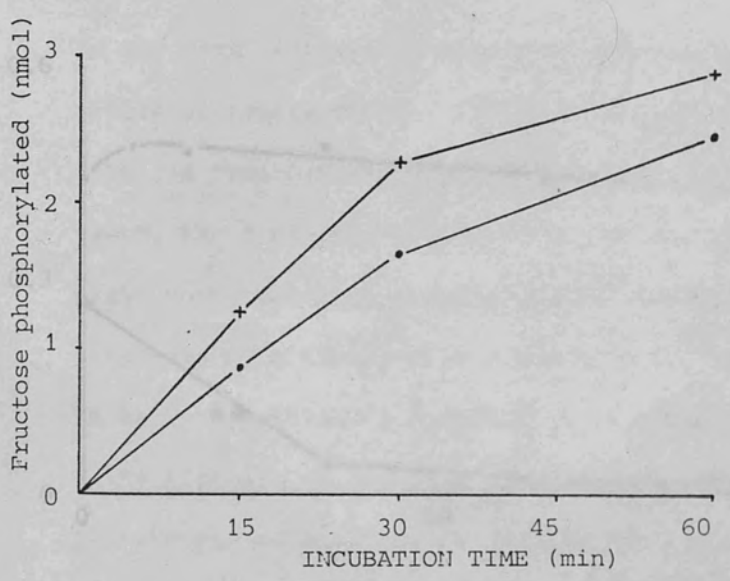


Fig 42 The activity of a crude hexose kinase preparation from developing Jerusalem artichoke tubers using fructose (5.25 μM) as substrate in the presence (.) and absence (+) of fluoride ions (31 mM) (see Materials and Methods 4.4.1).

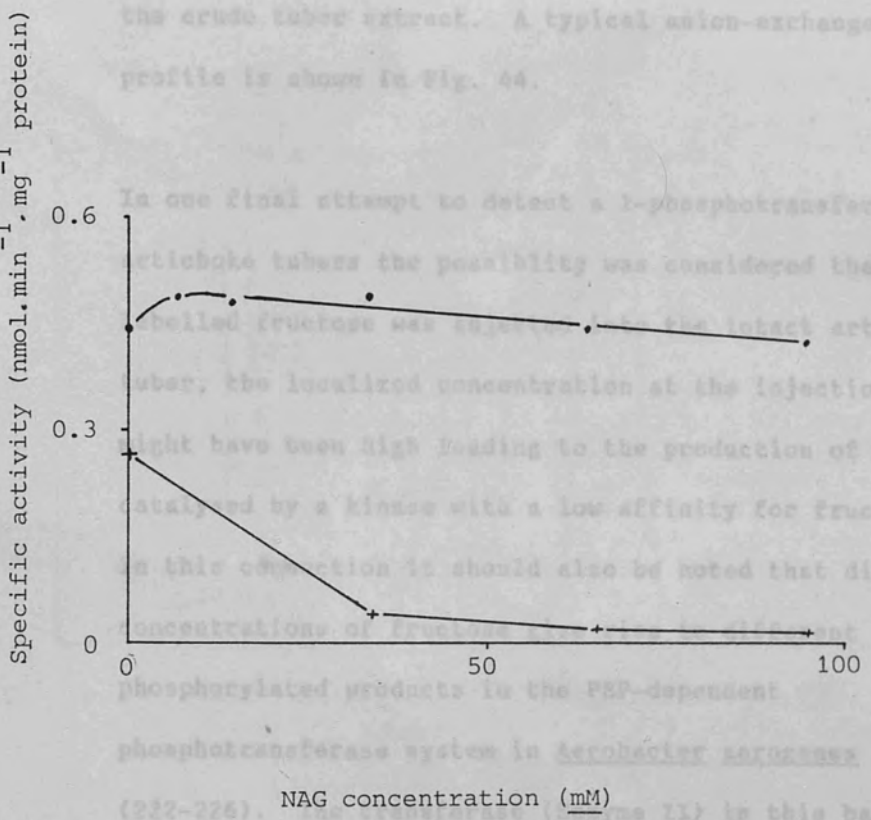


Fig 43 The effect of 2-acetamid-2'deoxy-D-glucose (NAG) on the phosphorylation of glucose (4.3 µM) (+) and fructose (5.25 µM) (.) by ATP using a crude hexose kinase preparation from developing Jerusalem artichoke tubers (see Materials and Methods 4.4.1).

Fru6P was evident together with the expected products of further metabolism (Glc6P, Glc1P (Fru2P?), DHAP etc.) arising from the actions of glycolytic enzymes present in the crude tuber extract. A typical anion-exchange profile is shown in Fig. 44.

In one final attempt to detect a 1-phosphotransferase in artichoke tubers the possiblity was considered that when labelled fructose was injected into the intact artichoke tuber, the localized concentration at the injection site might have been high leading to the production of Fru1P catalysed by a kinase with a low affinity for fructose. In this connection it should also be noted that different concentrations of fructose give rise to different phosphorylated products in the PEP-dependent phosphotransferase system in Aerobacter aerogenes (222-226). The transferase (Enzyme II) in this bacterium produces Fru1P at low fructose concentrations and Fru6P at high concentrations. When the fructose concentration in the crude artichoke kinase reaction was raised to 1 mM, Fru6P again appeared to be the only primary phosphorylated product formed (Fig. 45). With 10 mM substrate, a very small shoulder in the ion exchange elution profile (Fig. 46) corresponding to Fru1P was observed but it was not considered to be significant.

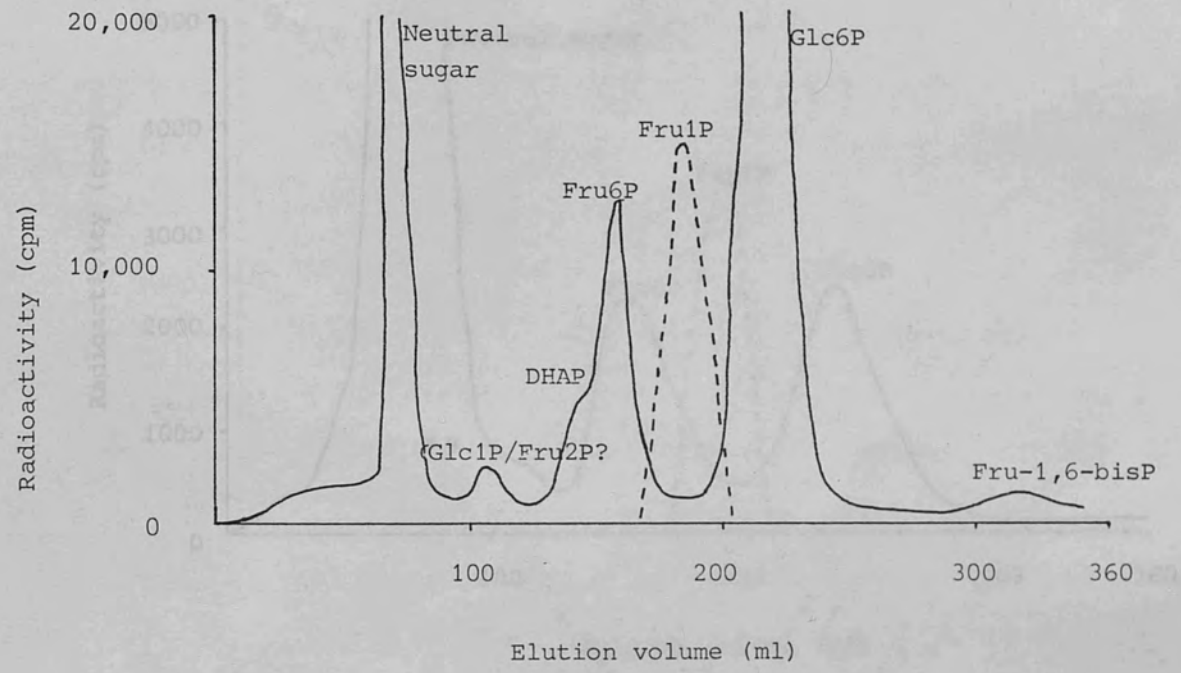


Fig 44 Anion exchange fractionation of a standard incubation mixture containing [U-^{14}C]fructose (5.25 μM) and ATP (3.1 mM) as substrates with a crude hexose kinase preparation from developing Jerusalem artichoke tubers, showing the position of a Fru1P marker (---) (see Materials) and Methods 4.4.1 and 4.1.4).

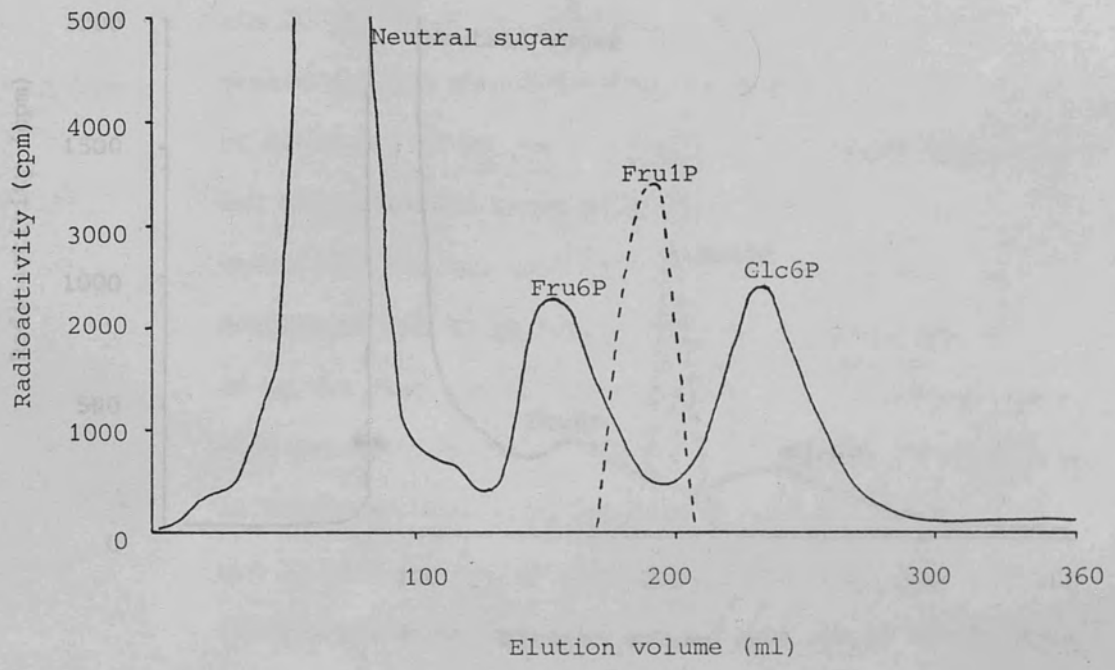


Fig 45 Anion-exchange profile of products arising from the incubation of [U-^{14}C)fructose (1mM), ATP 10 mM) and $MgCl_2$ (15 mM) with a crude hexose kinase preparation from developing Jerusalem artichoke tubers showing the position of a Fru1P marker (---) (see Materials and Methods 4.4.1 and 4.1.4).

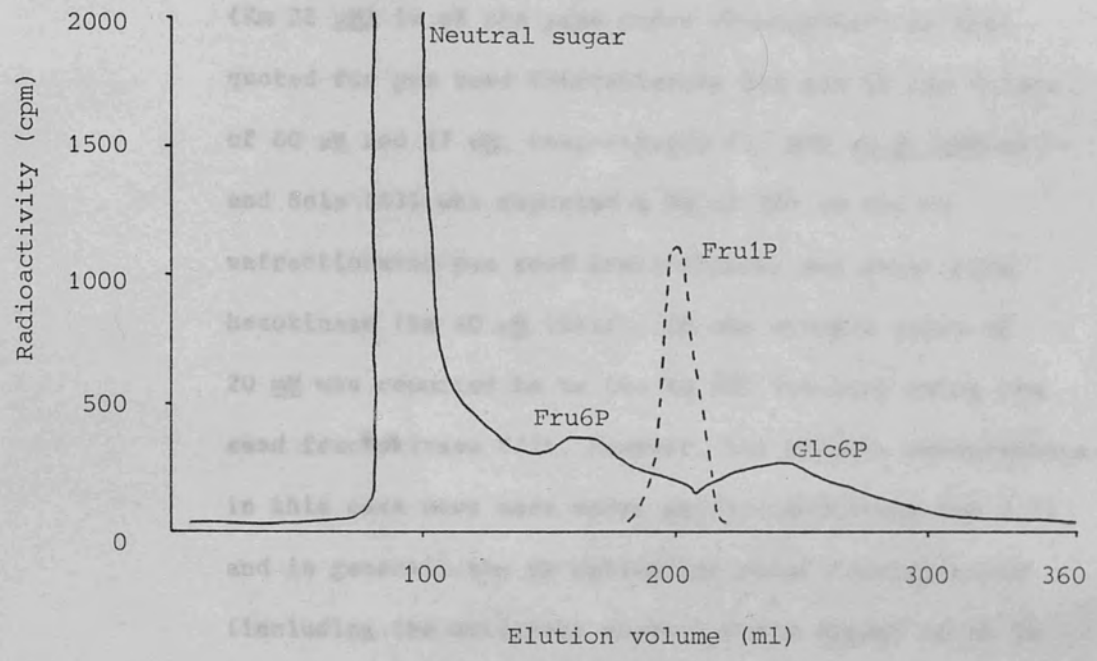


Fig 46 Anion-exchange fractionation of products arising from the incubation of [U-^{14}C]fructose (10 mM), ATP (10 mM) and $MgCl_2$ (15 mM) with a crude hexose kinase preparation from developing Jerusalem artichoke tubers showing the position of a Fru1P marker (---) (see Materials and Methods 4.4.1 and 4.1.4).

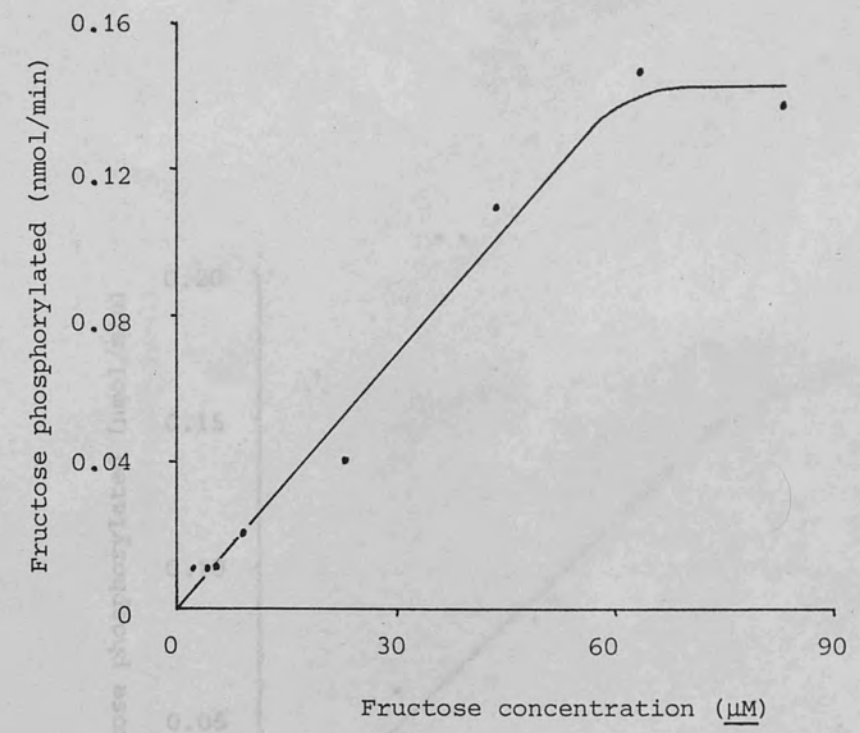
In several ways, the fructokinase activity detected in artichoke tubers appears to be similar to that found in other higher plant tissues. In all past reports no phosphorylated product other than Frul P has been claimed and the affinity of the artichoke enzyme for fructose (Km 22 μM) is of the same order of magnitude as that quoted for pea seed fructokinases III and IV (Km values of 60 μM and 57 μM, respectively (5, 87) (c.f. Medina and Sols (83) who reported a Km of 100 μM for an unfractionated pea seed fructokinase) and wheat germ hexokinase (Km 40 μM (84)). In one study a value of 20 mM was reported to be the Km for fructose using pea seed fructokinase (77), however, the kinetic measurements in this case were made under acidic conditions (pH 4.5) and in general, the pH optima for plant fructokinases (including the artichoke enzyme) would appear to be in the range pH 6.6 to 8.5 (5, 6, 82, 86).

The order of reactivity of the artichoke enzyme with various phosphoryl donors (ATP > GTP > UTP) again is similar to the data quoted for other plant fructokinases (5, 6, 77, 82, 84, 86, 87).

As Frul P formation from fructose had been observed with artichoke tuber callus (see Results and Discussion 2.1.4) the hexose kinase activity of this tissue was also examined.

A crude enzyme extract prepared from callus cultures in the log phase of growth exhibited hexokinase activity with both fructose (Km = 28μM) (Fig. 47) and glucose (10 mM) (Fig. 48) as substrates. As with the tuber extract, separate fructokinase and glucokinase activities could be demonstrated by the use of NAG (Fig. 49) but they were both lower than in the case of the tuber preparation (Fig 43). The rate of reaction with glucose was three times higher than with fructose using the callus preparation (Figs. 47-49) which is opposite to that found with the tuber extract (Fig 43). The effect of varying the concentration of ATP on callus fructokinase activity is shown in Fig. 50: above a concentration of 4 mM the enzyme was inhibited. No reaction was observed when ATP was replaced by UTP or GTP. The enzyme, unlike the tuber preparation, exhibited an absolute requirement for Mg^{2+} (Fig. 51) and, like the tuber preparation was also activated by potassium ions; KCl (62 mM) raised the activity by 88%. Fluoride ions (31 mM) did not have any effect on activity but were used in the incubation mixtures as a precaution against the action of phosphatases on hexose phosphates.

No Frul P was detected, using the anion-exchange chromatographic method, amongst the products of the phosphorylation of fructose by the callus enzyme: various incubation mixtures as described above were examined including those with fructose concentrations ranging from 1.8 μM to 7.5 mM.



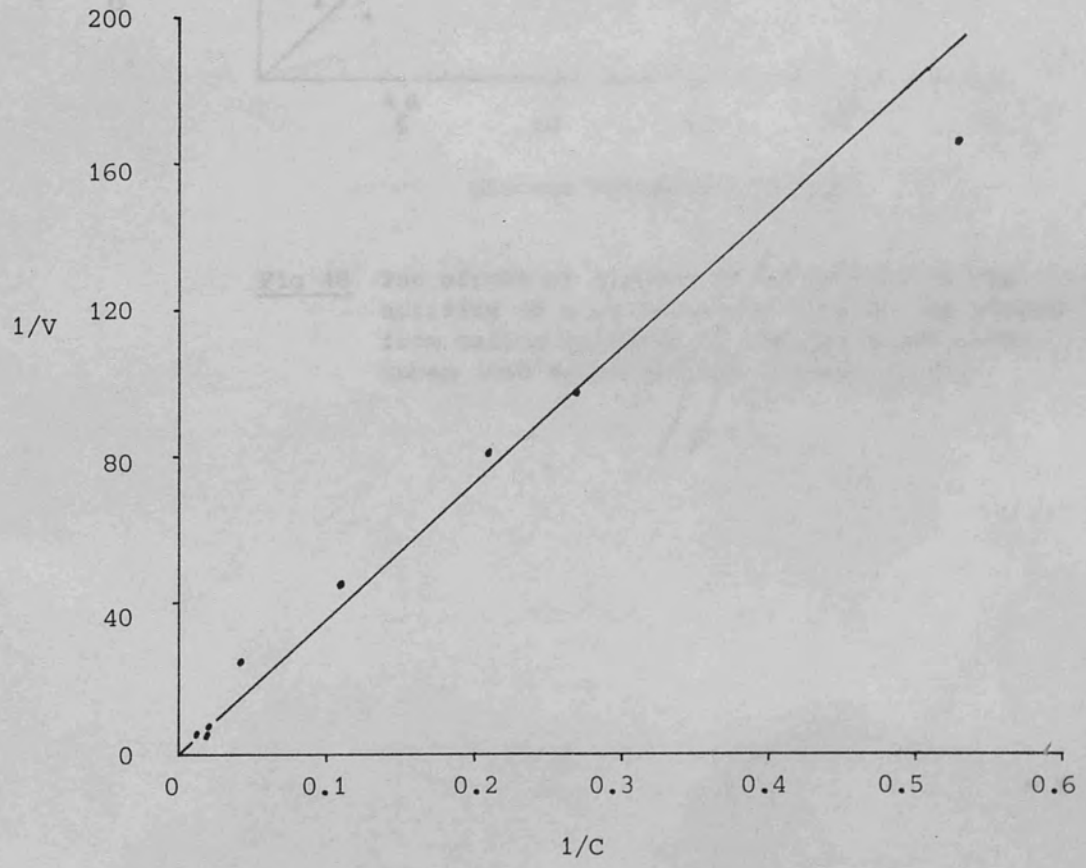


Fig 47 The effect of fructose concentration on the activity of a crude hexose kinase preparation from callus cultures of Jerusalem artichoke tuber (see Materials and Methods 4.4.1).

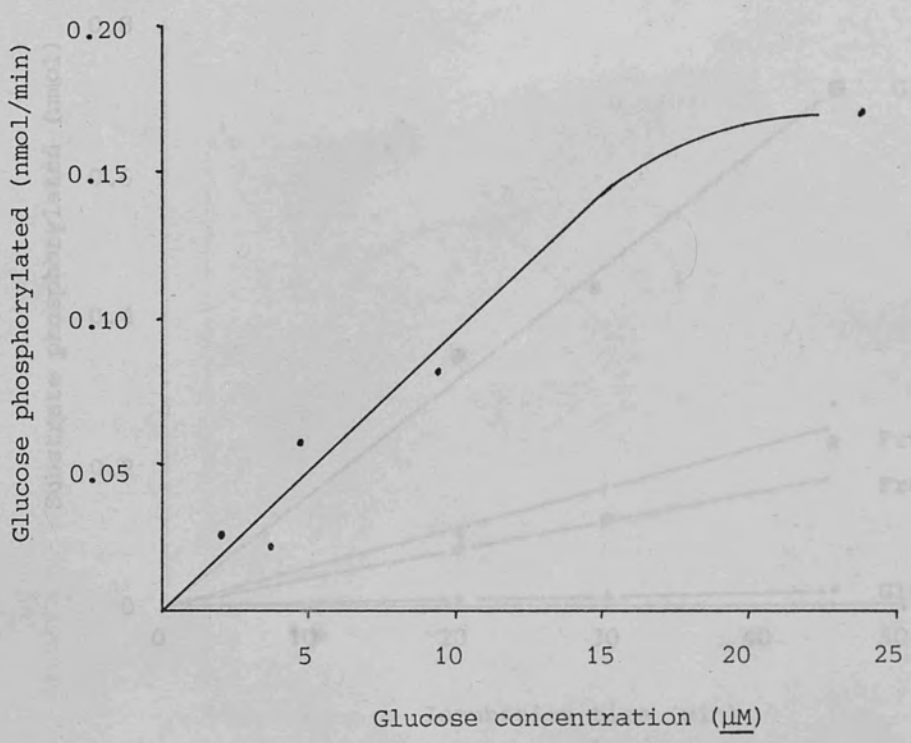


Fig 48 The effect of glucose concentration on the activity of a crude hexose kinase preparation from callus cultures of Jerusalem artichoke tuber (see Materials and Methods 4.4.1)

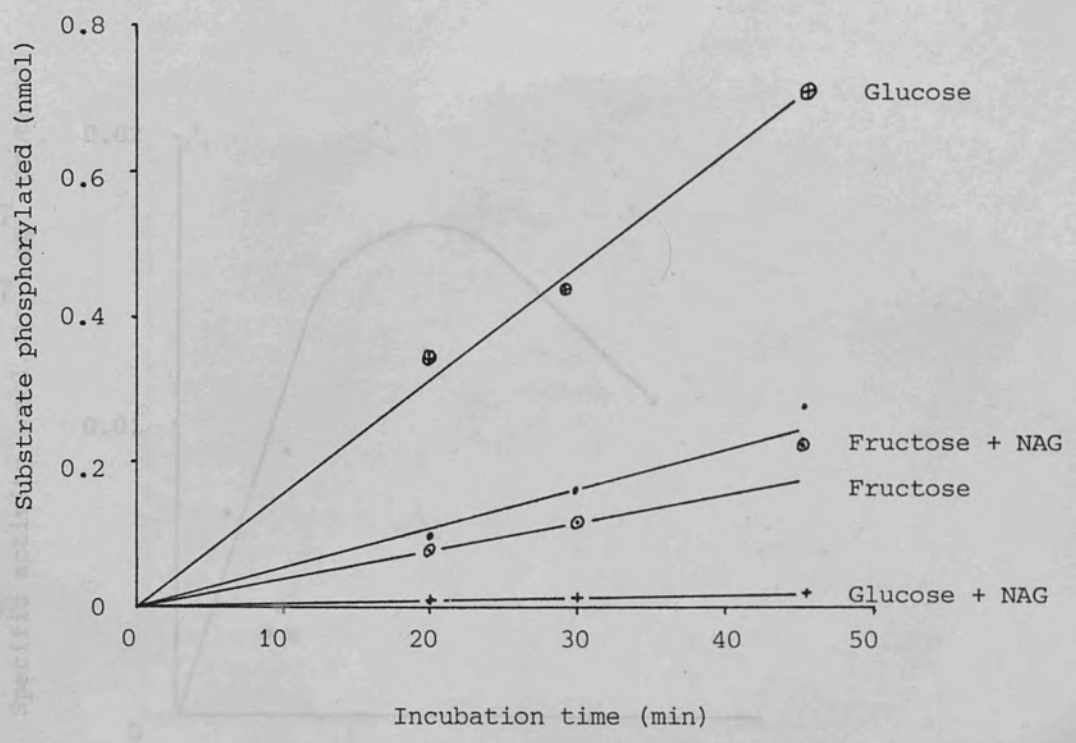


Fig 49 The activity of a crude kinase preparation from callus cultures of Jurusalem artichoke tuber using fructose (5.25 µM) and glucose (4.25 µM) as substrates in the presence (./+) and absence (O/⊕) of 2-acetamido-D-glucosamine (NAG) (96 mM) (see Materials and Methods 4.4.1)

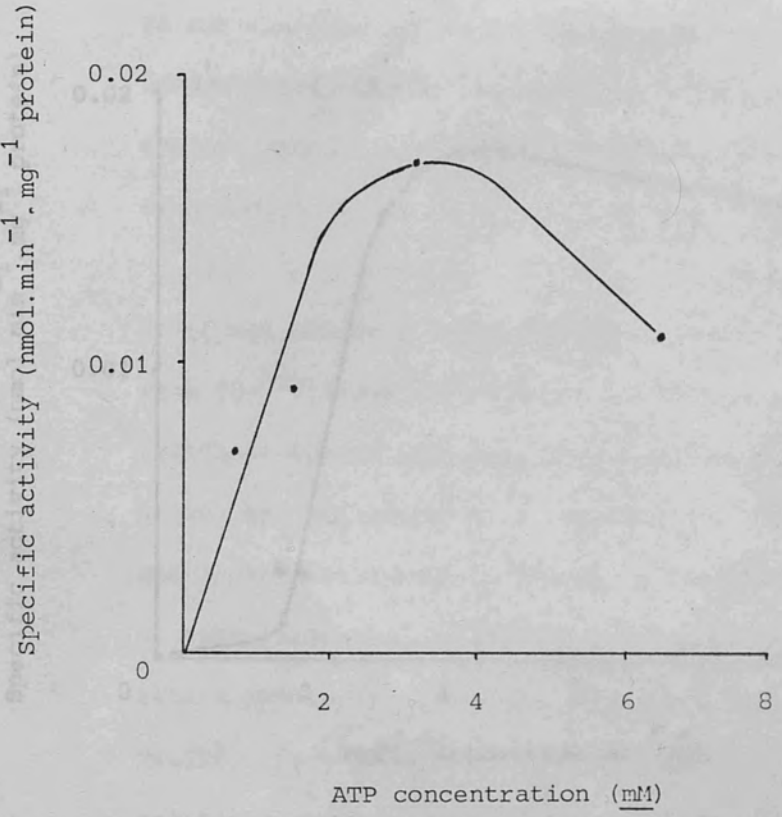


Fig 50 The effect of ATP concentration on the activity of a crude hexose kinase preparation from callus cultures of Jerusalem artichoke tuber using fructose (5.25 μM) as substrate (see Materials and Methods 4.4.1).

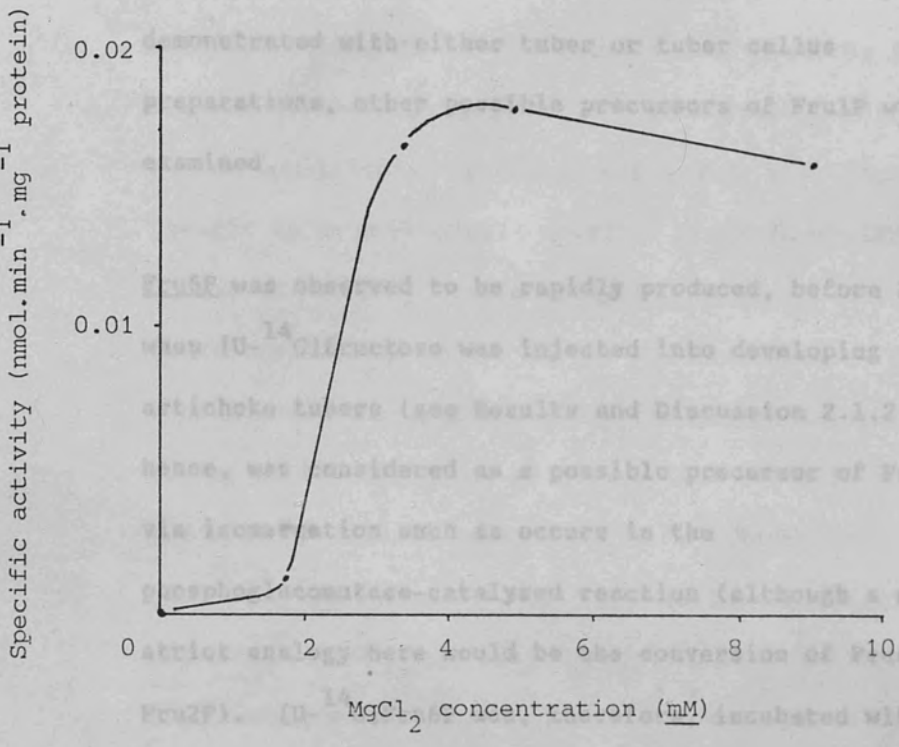


Fig 51 The effect of magnesium ion concentration on the activity of a crude hexose kinase preparation from callus cultures of Jerusalem artichoke tuber using fructose (5.25 µm) and ATP (3.1 mM) as substrates (see Materials and Methods 4.4.1)

2.2.2 Attempted conversion of other phosphorylated hexoses to fructose 1-phosphate in vitro

As the phosphorylation of fructose to Fru1P could not be demonstrated with either tuber or tuber callus preparations, other possible precursors of Fru1P were examined.

Fru6P was observed to be rapidly produced, before Fru1P, when [U-^{14}C]fructose was injected into developing artichoke tubers (see Results and Discussion 2.1.2) and, hence, was considered as a possible precursor of Fru1P via isomerzation such as occurs in the phosphoglucomutase-catalysed reaction (although a more strict analogy here would be the conversion of Fru6P to Fru2P). [U-^{14}C]Fru6P was, therefore, incubated with a soluble protein preparation from developing tubers: unfortunately only frozen tubers were available at this stage of the investigation. The products were examined for phosphate esters by paper chromatography and in some cases by anion-exchange chromatography. However, no Fru1P could be detected under any of the chosen conditions (see Materials and Methods 4.4.2). Fru6P was converted to Glc6P in all cases. Fru-1,6 bisP (10 μM) which was considered to be a possible cofactor in the interchange of phosphate between C-6 and C-1 of fructose, had no apparent effect.

Fructose-1,6-bisphosphatase, a key enzyme in gluconeogenesis in plant and animal tissues specifically removes the 1-phosphate group from Fru-1,6-bisP and, in addition to acid and alkaline phosphatases are widespread in nature (234, 235). The formation of Fru1P by the removal of the 6-phosphate group from Fru-1,6-bisP, either specifically or non-specifically, was, therfore, thought to be a possibile route to Fru1P formation in artichoke tubers. Consequently Fru-1,6-bisP at two concentrations (1 mM and 10 mM) was incubated at 25°C with a crude enzyme preparation from fresh developing tubers at two different pH values, 4.5 and 8.2, and in the presence and absence of added Mg^{2+} ions (see Materials and Methods 4.4.3). After incubation for 45 min the products were anlyased by paper chromatography for phosphorylated derivatives. However, in no case was there any indication that Fru1P had been produced. In one instance the incubation mixture pH 8.2, containing Mg^{2+} was examined by anion-exchange chromatography (Fig. 52) for the presence of Fru1P but none was detected. However Fru6P (presumably arising from the action of fructose-1,6-bisphosphatase) and products of further metabolism were present.

2.3 Metabolism of fructose 1-phosphate

By analogy with mammalian metabolism and the numerous reports of aldolase activity (including Fru1P aldolase activity (196, 197)) in plant tissues (192, 194, 196, 213-215, 234), Fru1P in plants

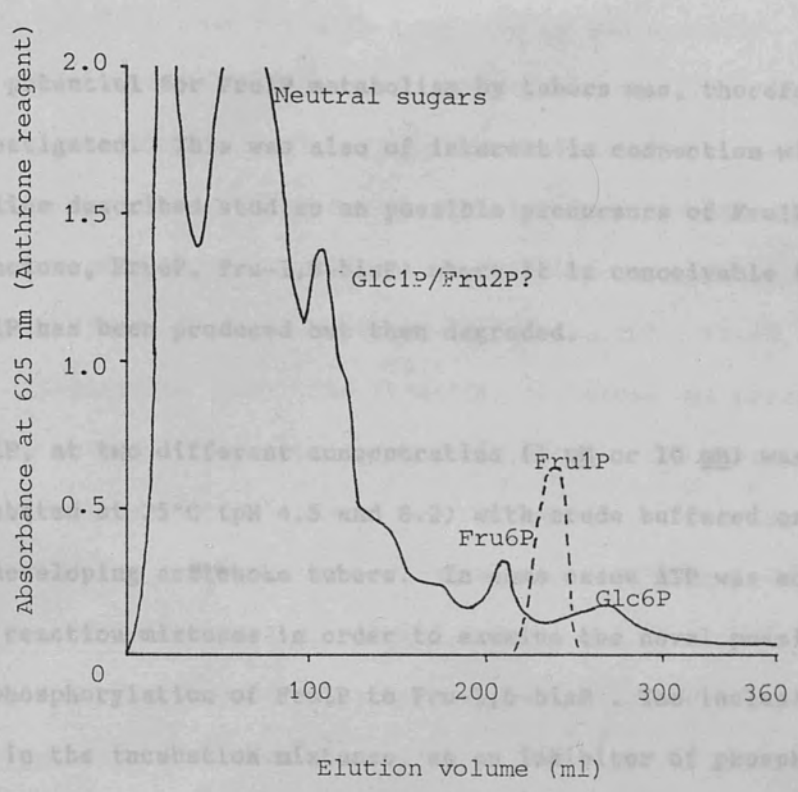


Fig 52 Anion-exchange fractionation of the products of incubation of Fru-1,6-bisP (10 mM) and $MgCl_2$ (10 mM) with a crude enzyme preparation from Jerusalem artichoke tubers at pH 8.2 (see Materials and Methods 4.4.3).

would be expected to be further metabolized although there was no evidence for this in the *in vivo* studies with tubers (see Results and Discussion 2.1.2 and 2.1.4).

The potential for Fru1P metabolism by tubers was, therefore, investigated. This was also of interest in connection with the earlier described studies on possible precursors of Fru1P (Fructose, Fru6P, Fru-1,6-bisP) where it is conceivable that Fru1P has been produced but then degraded.

Fru1P, at two different concentratins (1 mM or 10 mM) was incubated at 25°C (pH 4.5 and 8.2) with crude buffered extracts of developing artichoke tubers. In some cases ATP was added to the reaction mixtures in order to examine the novel possibility of phosphorylation of Fru1P to Fru-1,6-bisP . The inclusion of NaF in the incubation mixtures, as an inhibitor of phosphatase, was also investigated. After 45 min, the products were examined by paper chromatography (see Materials and Methods p.179) for phosphorylated derivatives.

In all cases under acidic conditions hydrolysis of Fru1P to fructose was the only reaction observed (which was not alleviated by the presence of NaF) and as this was also the case using boiled enzyme the hydrolysis must have been due largely to acid-catalysis.

When alkaline conditions were used, again, the main reaction was the hydrolysis of Fru1P to fructose, although to a lesser extent than at pH 4.5 as judged visually from PC. The hydrolysis of Fru1P at pH 8.2 was probably catalysed by non-specific phosphatases as a boiled enzyme control did not yield fructose. The anion-exchange profile (Fig. 53) derived from a reaction mixture containing 10 mM Fru1P, ATP, Mg^{2+} and NaF showed the presence of unreacted Fru1P together with hexose (presumably largely fructose) and also Glc6P, Glc1P (Fru2P?), Fru6P, Fru-1,6-bisP and DHAP. The formation of hexose was probably due to the action of phosphatase on Fru1P despite the presence of fluoride ions. The shoulders present on the neutral sugar peak (Fig. 53) may have been the 5- and 6- membered ring forms of Fru2P (see Lefebvre et al. (207)). It should be remembered that there was also some evidence to suggest that Fru2P was produced in vivo, from [U-^{14}C]fructose (see p. 110). It is most likely that most of the major products were derived from the hydroloysis of Fru1P by phosphatase followed by the action of fructokinase and other glycolytic enzymes and not by the direct conversion of Fru1P to phosphorylated intermediates. In this connection there is also no evidence to suggest that tuber tissue possesses a "phosphfructomutase" (see p 35) which, if it existed, would presumably catalyse the interconversion of Fru1P and Fru6P.

The question of why Fru1P is apparently not metabolized after it is formed in response to fructose injection into the tuber, therefore, remains unanswered. Aldolase activity is undoubtedly

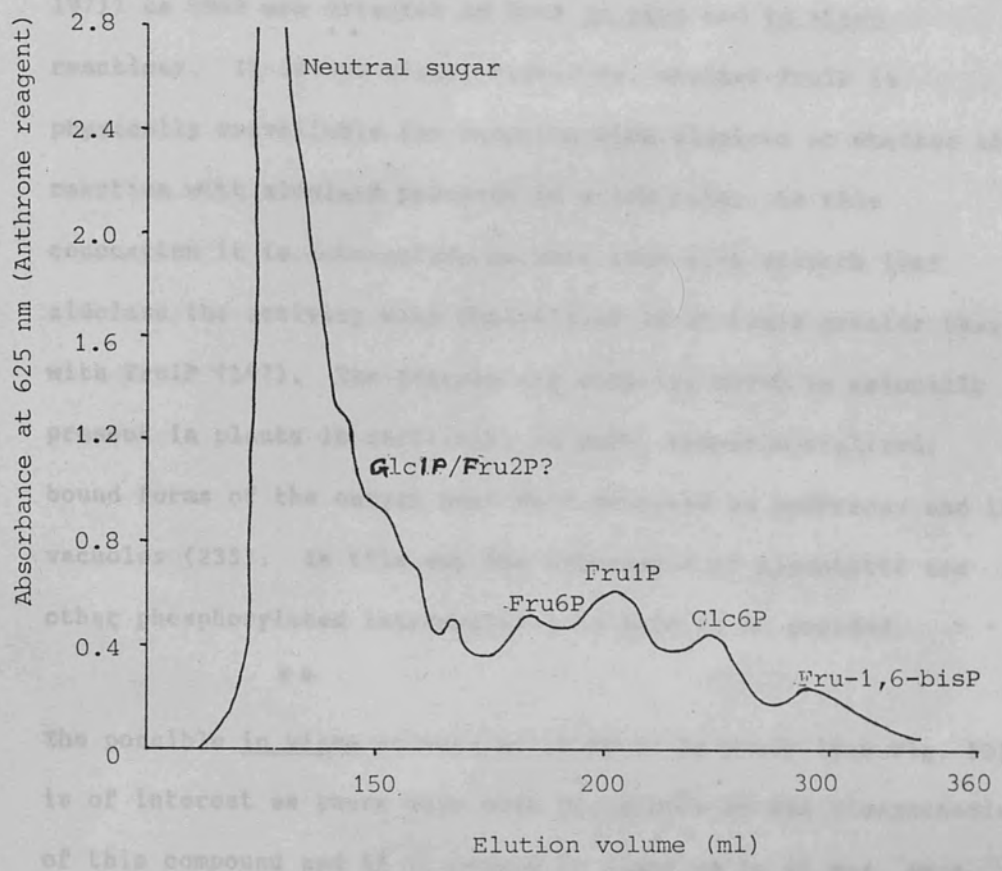


Fig 53 Anion-exchange fractionation of the products of the incubation of Fru1P (10 mM) ATP (25 mM), $MgCl_2$ (30 mM) and NaF (25 mM) with a crude enzyme preparation from developing Jerusalem artichoke tubers at pH 8.2 (see Materials and Methods 4.4.4).

present in the artichoke tissues (cf. other plant tissues (196, 197)) as DHAP was detected in both *in vivo* and *in vitro* reactions. It is not clear, therefore, whether Fru1P is physically unavailable for reaction with aldolase or whether the reaction with aldolase proceeds at a low rate. In this connection it is interesting to note that with spinach leaf aldolase the activity with Fru1,6-bisP is 25 times greater than with Fru1P (197). The phosphatase activity which is generally present in plants is certainly, in part, compartmentalized; bound forms of the enzyme have been detected on membranes and in vacuoles (235). In this way the hydrolysis of glycolytic and other phosphorylated intermediates is said to be avoided.

The possible *in vitro* conversion of Fru1P to Fru2P (see Fig. 53) is of interest as there have been no reports of the biosynthesis of this compound and if it occurs in plant cells it may, as a relatively energy-rich phosphate, have important functions such as serving as a precursor of nucleoside diphosphate fructoses (see Introduction 1.1.4). There are no real indications in any of the present studies of how it may have been produced. The most closely related derivative which occurs naturally is Fru-2,6-bisP which has recently received a great deal of attention as a moderator of glycolysis and gluconeogenesis in animals (235, and Introduction 1.3). This ester is found in plant tissues, including Jerusalem artichoke tubers (99), and it may be synthesized by the ATP-dependent phosphorylation of Fru6P catalysed by a PFK-2-type enzyme, a reaction which occurs in mammalian systems (90). It is unlikely that Fru-2,6-bisP would

have been identified in any of the studies with artichoke tubers as the compound co-elutes with Frul,6-bisP from anion-exchange column (D.R. Davies, personal communcation). If supplies of Fru-2,6-bisP had been available it would have been of interest to examine tuber tissues for the possible presence of a 6-phosphohydrolase which could convert Fru-2,6-bisP to Fru2P. The possibility that Fru2P could arise from FrulP by isomerization (cf. Chaiban (64)) has been discussed (Introduction 1.1.4).

3 CONCLUSIONS

Fructose 1-phosphate has been characterized as a metabolite of fructose which was produced when the ketose was supplied exogenously to developing Jerusalem artichoke tubers or artichoke callus cultures. The time course of formation of Fru1P and other fructose metabolites was followed which led to speculation that Fru1P was being synthesized either directly or indirectly from other sugar phosphates, or by the direct phosphorylation of fructose involving a specific 1-phosphofructokinase. However, investigations of crude enzyme preparations, from both developing tubers and callus cultures, only revealed the presence of a fructokinase which yielded Fru6P as a product. No real evidence could be obtained to suggest that Fru6P or Fru-1,6-bisP was converted to Fru1P. It is conceded, however, that Fru1P may have been formed *in vitro* in the presence of crude enzyme preparations and subsequently broken down, e.g. by aldolase and/or phosphatase activities.

Once formed in developing tubers or callus cultures, Fru1P did not appear to be further metabolized, despite the presence of aldolase activity, and this may have been due to compartmentalization. Some evidence for the conversion of Fru1P to Fru2P was obtained when the former was incubated with an artichoke preparation. There were also some tentative indications that Fru2P was present in extracts of normal, developing artichoke tubers and in developing tubers that had been injected with [U-^{14}C]fructose and then incubated.

Further conclusions on the *in vitro* metabolism of FrulP are difficult to draw as the metabolites formed on incubation of FrulP with an artichoke tuber preparation could be explained by the action of phosphatase and the subsequent metabolism of the product, fructose.

Both the mechanism of formation of FrulP and the apparent inability of intact artichoke tubers to metabolize the phosphate, therefore, remain obscure.

4 MATERIALS AND METHODS

Analar grade chemicals were used whenever possible, and other chemicals were of the best available purity. Glass distilled, deionized water was used for the preparation of all solutions.

Plant Material

Jerusalem artichoke (*Helianthus tuberosus* L.) tubers were supplied by the University of London Botanical Supply Unit, Egham, Surrey. Two stages in tuber development were examined: germinating tubers (10-20 g) were chosen in spring when green shoots had just appeared, and developing tubers (2-8 g) which were excised from freshly uprooted plants and used immediately.

Developing potato tubers (*Solanum tuberosum*) (10-20 g) were freshly picked from plants grown in pots in a greenhouse with a 14 hr day length provided by supplementary fluorescent lighting.

Small onions (*Allium cepa*) obtained locally were used when the shoot ($\triangleq$ 5 cm) had appeared.

Other plant material used was as stated in text.

4.1 General methods

4.1.1 Paper chromatography

Whatman No. 1 papers were used and were developed by the descending elution technique. In the case of EDTA-washed papers, the papers were eluted with 0.1% w/v EDTA solution overnight and then dried before use.

Chromatography solvents:

i) Ethyl acetate:Formamide; Pyridine (6:4:1 v/v) (198)

ii) Ethyl acetate:Formamide: Pyridine (4:4:1 v/v) (198)

iii) 95% Ethanol:1M Ammonium acetate pH 3.9 (75:30 v/v) (199, 200)

iv) 95% Ethanol:0.1 M Ammonium borate pH 10 (2:1 v/v) (201)

v) 2-Methoxyethanol:Methylethylketone:3M NH_4OH (7:2:3 v/v) (202)

vi) Propanol:Water:Ethyl acetate (6:3:1 v/v) (203)

Carbohydrates were located using the PABA reagent (210); compounds containing purine and pyrimidine bases were visualized under u.v. light, and radioactivity as described in Section 4.1.5.

4.1.2 Thin-layer chromatography

Schleicher and Schuell F1440 cellulose or F1500 silica TLC plates were used. Elution was by an ascending method.

Chromatography solvents

i) 95% Ethanol:1M Ammonium acetate pH 3.9 (75:30 v/v) (199, 200)

ii) Propanol:Water:Ethyl acetate (7:2:1 v/v) (203)

iii) Ethyl acetate:Pyridine:Acetic acid:Water (60:30:10:10 v/v) (210)

Carbohydrates and compounds containing bases were located as for paper chromatograms (Section 4.1.1) and radioactivity as described in Section 4.1.5.

4.1.3 Paper electrophoresis

A Shandon high voltage electrophoresis apparatus (192) was used with Whatman No. 1 paper. The electrophoresis was carried out for 2 hr. at 80 mA (30-60 v/cm). Picric acid and caffeine were used as markers.

Electrophoretic buffers:

i) 0.02 M Ammonium Formate pH 1 (204)

ii) 0.05 M Ammonium Formate pH 1 (204)

iii) 0.05 M Ammonium Formate pH 3.5 (204)

iv) 0.3 M Sodium Tetraborate pH 9.8 (192)

Carbohydrates, compounds containing purine and pyrimidine bases, and radioactivity were located as in the case of the paper chromatograms (See Section 4.1.1).

4.1.4 Anion-exchange chromatography

All column operations were carried out in a cold room (4°C).

a) Initially, a modification of the method used by Rintoul (131) based on that of Khym and Cohn (206) was used to separate phosphoric esters as their borate complexes. A sample containing sugar phosphates made to pH 8 with 1 M NaOH was applied to a column (1.5 x 10 cm) of Dowex 1-X8 (200-400) (Cl^-) and eluted (flow rate 90 ml/hr) with a linear gradient (0.025 M NH_4Cl, 0.01 M $Na_2B_4O_7$ (1 l) in the mixing vessel and 0.025 M NH_4OH, 0.025 M NH_4Cl, 0.01 mM $Na_2B_4O_7$ (1 l) in the reservoir).
Fractions (20 ml) were collected and assayed for total carbohydrate using the anthrone-sulphuric

acid method of Scott and Melvin (236), and for fructose by the Resorinol-HCl method of Roe et al (237). Radioactivity was measured as described in Section 4.1.5.

b) For the most part, the anion-exchange method used was a modification of that developed by Lefebvre et al (207). Dowex-1-X4(200-400) (chloride) anion-exchange resin was converted to the borate form by stirring with 0.8 M. triethylammonium borate (TEAB) and then used to make a 1 x 30 cm column. The column was washed with deionized water until the pH of the eluate fell to 7. A sample containing sugar phosphates was adjusted to pH8 using 0.1M TEAB and then applied to the top of the column. [U-^{14}C]Glc6P and [U-^{14}C]Fru6P radioactive markers were added to 'cold' analyses and unlabelled Glc6P and Fru6P (12.5µmol each) to radioactive material prior to application to the column. The column was then eluted with a linear gradient of TEAB (0.1 M → 0.4M; total volume 360 ml) at a flow rate of approx. 0.2 ml/min. Fractions (2 ml) were collected and assayed as appropriate, for total carbohydrate using anthrone reagent$_A$ (236) for ketoses with Roe's reagent$_A$ (237) for trioses using the cysteine-carbazole reagent (238), and for radioactivity as described in section 4.1.5.

Column fractions for further analysis were pooled, frozen and freeze-dried. The residue was then rotary evaporated with methanol (50 ml) up to four times in order to remove the eluting salt (TEAB) as described by Lefebvre et al (207).

4.1.5 Determination of radioactivity

Paper strips from chromatograms and electrophoretograms, and DEAE-cellulose discs from the kinase assay were dried and then placed in toluene scintillant (5 g PPO/l toluene) (10 ml).

Radioactive bands on TLC plates were located by scraping bands (0.5 cm) from the plate and eluting the radioactivity with water (1 ml) by vortex-mixing for 1 min. in a microcentrifuge tube. The chromatographic media was removed by centrifugation and the supernatant counted in scintillation cocktail ((PPO (5 g/l toluene):Triton X100 (2:1 v/v)) (10 ml).

Aliquots (0.2-1.0 ml) of column fractions from anion-exchange chromatography, diluted to 1 ml with water where necessary, were added to the scintillation cocktail (10 ml) (PPO (5 g/l toluene):Triton-X100 (2:1 v/v)).

Polysaccharide was first digested in NCS (Digestion cocktail, Amersham International Ltd.) at 37°C for 18 hr. and, after neutralization with glacial acetic acid, was counted in toluene scintillant containing PPO (5 g/l) (10 ml).

All radioactive determinations using scintillation fluid were made using either a Beckman scintillation counter or the Packard Tri-Carb liquid scintillation spectrometer.

Radioactivity on paper and thin-layer chromatograms and paper electrophoretograms was also determined using autoradiography or by use of a spark chamber apparatus.

4.1.6 Protein estimation

The protein content of all enzyme preparations was measured spectrophotometrically by the method of Lowry et al., (239). BSA was used as the standard protein.

4.1.7 Polysaccharide

Total polysaccharide was determined by the anthrone method of Scott and Melvin (236). Inulin was used as the standard.

4.1.8 Cell culture

Jerusalem artichoke callus cultures were initiated from discs (0.6 cm) of fresh tuber tissue on agar plates of Schenk and Hildebrant medium, which is based on sucrose as the carbon source, at 25°C in the dark, and sub-cultured regularly every six weeks onto fresh agar:medium, according to the method of Dixon & Fuller (240).

4.1.9 Assay for hexose kinase activity

The assay method of Dey (219) was used for the determination of hexose kinase activity in extracts of Jerusalem artichoke tuber and callus cultures.

Aliquots (25 µl) were taken from the various incubation mixtures and spotted onto Whatman DEAE-cellulose discs (2.1 cm dia) previously wetted with water (100 µl). The discs were then placed on a filter and washed with large volumes of distilled water. After drying the discs were counted as described in Section 4.1.5.

Total radioactivity in these aliquots was determined as above but the washing step was left out.

4.1.10 Preparation of crude Jerusalem artichoke enzymes

All operations were carried out at 4°C. The same method was used for both the preparation of crude enzyme from developing Jerusalem artichoke tubers or Jerusalem artichoke callus cultures (used in the log phase of growth).

Jerusalem artichoke tissue was homogenized in 0.1M Tris-HCl pH 8.2 buffer (1.5 vols) containing 2-mercaptoethanol (3 µl/g fresh wt) and PVP (20% w/fresh wt) using a MSE vortex homogenizer. The homogenate was centrifuged (16,000 g, 20 min) and the supernatant dialysed against a large volume (4-5 l) of the buffer for 2 hr. The dialysate was used as the crude enzyme preparation. This crude enzyme preparation was always prepared directly before use as it was found to be unstable, losing 33% of its hexose kinase activity in 24 hr at 4°C. Tubers on freezing (-18°C) were found to contain no hexose kinase activity on thawing.

PVP was used in order to bind to phenolic compounds which might otherwise interfere with the subsequent assay of enzymes. 2-mecaptoethanol was used in order to maintain reducing conditions in the extraction medium, especially with respect to the oxidation of phenols. The sample was dialysed to remove low molecular weight sugars which would competitively inhibit actiity with radiolabelled substrates.

4.2 Metabolism of exogenous and endogenous fructose in vivo

4.2.1 Injection and incubation of [U-^{14}C]fructose into plant tissue

[U-^{14}C]fructose was injected into a number of plant tissues; developing and germinating Jerusalem artichoke tubers, developing potato tubers, small onion bulbs and callus cultures of Jerusalem artichoke tuber; and after different periods of incubation, the distribution of label in neutral sugars, hexose phosphates and/or polysaccharide was examined (Materials and Methods 4.2.4). The same method of injection and incubation was used for each plant tissue.

[U-^{14}C]frutose (241-301 mCi/mmol) was injected (0.5 µCi/g fresh wet) into the appropriate plant tissue (which in except the case of callus tissue, had been washed and sterilzed using 0.1% sodium hypochlorite) using a microsyringe (5 µl) as described by Rintoul (131). The radiolabelled solution was injected in 0.5 µl quantities over a 1 min period at a number of points. The plant tissues were then incubated wrapped in aluminium foil at ambient temperature (20-25°C) , after which time they were frozen in liquid nitrogen and stored at -18°C until analysed.

4.2.2 Imbibition of broad bean seeds with fructose

Broad bean (Vicia faba L.) seeds (variety Bunyards Exhibition) (100 g) were left to imbibe overnight in 5% fructose solution and then transferred to moist cotton wool and allowed to germinate for 5 days. After removal of the testa, the beans were extracted with aqueous ethanol and analysed for hexose phosphates using anion-exchange chromatography.

4.2.3 Extraction of fructose metabolites

Fructose metabolites were extracted from plant tissues using either aqueous ethanol or perchloric acid. All extractions were carried out in a cold room (4°C) using cooled solutions and apparatus.

i) Perchloric acid: The method of Murata et al., (241) as modified by Rintoul (131) was used. Carrot tissue was sliced, into small pieces and then homogenized to a fine slurry in cold 0.46 M perchloric acid (2-3 vols) using a chilled mortar and pestle and a little sand. The extract, and washings (2 x 1 vol) were then centrifuged (2000 g/10 min) and the supernatant decanted from the residue pellet. The supernatant was neutralized with KOH and the precipitated $KClO_4$, after chilling in a

freezer for 20 min, removed by centrifugation. The extract was concentrated (5-10 ml) by rotary evaporation at 30°C and then stored frozen (-18°C) before use. Any further precipitation of $KClO_4$ was removed by centrigation before column chromatography.

ii) Aqueous ethanol: the method of Gonzalez and Pontis (51) was used. Plant tissue was sliced, where necessary, into small pieces and then homogenized to a fine slurry in cold 80% v/v aqueous ethanol (2-3 vol) using a chilled mortar and pestle and a little sand. The extract, and washings (2 x 1 vol) were then centrifuged (2000 g/10 min) and the supernatant rotary evaporated (30°C) to a small volume (5-10 ml) and stored frozen (-18°C) before use. In the cases of plant tissues incubated with radiolabelled hexose, the pellet after centriguation was also frozen before further extraction for polysaccaride (see Section 2.2.3).

4.2.4 Fractionation of fructose metabolites

i) Neutral sugars

An aliquot (25 µl) of the soluble fractions of the plant extract was analysed for mono- and

oligo- saccharides by paper chromatography in solvent vi) and carbohydrate and radioactivity located, as described in Section 4.1.1.

ii) Hexose phosphates

An aliquot (2-4 ml) of the soluble fraction of the plant extract was adjusted to pH 8 with 0.1M TEAB and then analysed using the anion-exchange chromatographic method of Lefebvre et al., (Section 4.1.4).

iii) Polysaccharide

The pellets containing the residue were washed twice with 80% v/v aqueous ethanol (3-5 vol) and, after centrifugation (2,000 g/5 min), hot water (2-3 vol) was added to the residue and the mixture kept at approx. 80°C for 20 min with stirring to aid dissolution. On cooling, polysaccharide was precipitated by the addition of absolute ethanol to 50% v/v, and then removed by centrifugation (2000 g/10 min). Ethanol was removed from the pellet by blowing with nitrogen and then the pellet was re-dissolved in hot water (500 µl) and finally analysed for both radioactivity (see Section 4.1.5) and total polysaccharide (see Section 4.1.7). A sample of

polysaccharide was also hydrolysed using trifluoroacetic acid by the method of Bateman (288) and the monosaccharide components determined by the method of Menzies et al., (210) (see also Section 4.1.2 iii).

4.2.5 Characterization of FrulP from extracts of developing Jerusalem artichoke tubers

Column fractions, relating to peaks corresponding to FrulP from the anion-exchange chromatography of extracts of developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose, were pooled, frozen and freeze-dried. After removal of borate by rotary evaporation with methanol (see p 85) the residue was treated as follows:

i) Anion-exchange chromatography

To a sample of the residue was added a solution of 'cold' FrulP (25 μmol). The mixture was made alkaline by the addition of solid sodium carbonate immediately prior to the analysis by amion-exchange chromatography according to the method of Lefebvre et al (Materials and Methods 4.1.4).

ii) Acid hydrolysis

To a sample of the residue was added hot 2M HCl which was then heated at 100°C for 30 min, after which time the reaction was stopped by freezing in liquid nitrogen. The sample was then freeze-dried. This was then neutralized by standing over KOH pellets under reduced pressure for 24 hr. The resultant solid was dissolved in water (10 µl) containing unlabelled fructose (20 nmol) as an internal marker, and then examined by the TLC method of Menzies et al. (210) (Materials and Methods 4.1.2 iii). The fructose marker was located using the PABA reagent (210) and for radioactivity using both autoradiography and by the analysis of individual bands (Materials and Methods 4.1.5).

iii) Acid phosphatase

A sample of the residue was incubated with potato acid phosphatase (1 unit) (Sigma) in 0.1 M sodium citrate buffer pH 5.6 (1 ml) for 5 min at 37°C. The reaction was stopped by the addition of absolute ethanol (4 vols) and precipitated protein removed by centrifugation. The hydrolysate was freeze-dried, re-dissolved in water (25 µl) and analysed along with

monosaccharide standards by the TLC method of Menzies et al. (210). Carbohydrate and radioactivity were located as for the acid hydrolysate.

iv) Alkaline phosphatase

A sample of the residue was incubated with calf intestine alkaline phosphate (1 unit) (Sigma) in 0.1 M Tris-HCl pH 8.2 buffer (200 µl) for 30 min at 37°C. The reaction was stopped by the addition of absolute ethanol (4 vols) and the precipitated protein removed by centrifugation. A sample of [U-^{14}C]Fru6P similarly isolated from column fractions (anion-exchange chromatography), and an untreated [U-^{14}C]Fru6P sample were incubated in the same manner with alkaline phosphatase. The hydrolysates were then analysed by the TLC method of Menzies et al (210) along with standard hexoses. Carbohydrate and radioactivity were located as for the acid hydrolysate.

v) Rat liver aldolase

FrulP aldolase was extracted from rat liver by the method of Eggleston (221). $(NH_4)_2SO_4$ was removed by dialysis beforehand as it inhibits the enzyme.

A sample of the residue and 'cold' Fru1P (20 µmol) were incubated with rat liver aldolase (equivalent to an activity of 1.26 µmol/min) in 0.05 M Triethanolamine-HCl buffer pH 7.4 (2.5 ml) (containing NaF (50 µmol) (to inhibit phosphatases), for 30 min at 37°C. The sample was then adjusted to pH 8 with 0.1 M TEAB and then analysed by anion-exchange chromatography (along with DHAP (20 µmol) as a standard) as described in Section 4.1.4. Trioses and ketoses, and radioactivity were located as described in Section 4.1.4 and 4.1.5.

4.3 Incubation and extraction of developing Jerusalem artichoke tubers with [U-^{14}C]sucrose

[U-^{14}C]sucrose (382 µCi/µmol) was injected (0.25 µCi/g fresh wt) and incubated as described for that with [U-^{14}C]fructose. After extraction with aqueous ethanol samples were analysed for neutral sugars, hexose phosphates, and polysaccharide as described in Section 4.2.

4.4 Mechanism of FrulP synthesis in developing Jerusalem artichoke tubers

4.4.1 Hexose kinase activity

The crude enzyme preparations from Jerusalem artichoke tuber and callus culture were made as described in Section 4.1.8. Incubations with crude enzyme preparations were carried out at 25°C in small tubes in a shaking water bath. These reaction mixtures were assayed using the DEAE disc assay (Section 4.1.9) and the remainder frozen and later anlaysed for hexose phosphates using the anion-exchange chrmoatographic method (section 4.1.4).

The standard reaction mixtures (total volume 800 µl) contained; crude tuber or callus enzyme preparation (500 µl), [U-^{14}C]fructose (241 mCi/mmol) (4.2 nmol); ATP (solution adjusted to pH 7 with Na_2CO_3 (2.5 µmol), $MgCl_2$ (3 µmol) NaF (25 µmol)' KCl (50 µmol); NaF (25 µmol); and 0.1 M Tris-HCl pH 8.2 buffer (to give 800 µl).

Reactions were normally assayed after 15 min incubation in the case of callus enzyme and after 30 min using the tuber enzyme. In both cases the rates were shown to be linear over a period of 45 min.

In the measurement of Km (Fru), [U-^{14}C]fructose was varied in the standard reaction mixtures to give a final concentration in the range 1-85 μM. The effect of pH on the hexose kinase activity of the crude enzyme prepartion was measured by replacing 400 μl of the enzyme preparation with 400 μl of an appropriate buffer (0.1 M sodium acetate pH 4.5, 0.1M sodium citrate-NaOH pH 5.5; 0.75 M MES pH 6.5; 0.1 M Potassium phosphate pH 7.4; 0.1 M Tris-HCl pH 8.2; 0.1 M sodium borate-NaOH pH 9.2). The acidic digests after incubation were made alkaline by the addition of 0.1M NaOH (800 μl) before being assayed by the DEAE-disc method (Section 4.1.9).

The Km (ATP) was measured by varying ATP concentration in the standard reaction mixture from 0.57 mM - 5.39 mM at two fructose concentrations (5.25 μM and 11.3 μM).

ATP was replaced by the alternative potential phosphoryl donors UTP, GTP, PPi, ADP, Phytic acid (all 2.5 μmol) or also by ADP together with phytic acid (1.25 μmol of each), in the standard reaction mixtures and assayed over a 60 min incubation period.

The effect of magnesium ion concentration on hexose kinase activity was determined by varying the concentration of $MgCl_2$ (0.73 mM - 6.88 mM) in the standard digest.

The effect of potassium ion concentration as hexose kinase activity was measured by varying the concentration of KCl in the standard digest over the range 0-70 mM final concentration.

In order to measure the affect the removal of fluoride ions on hexose kinase activity their place in the standard mixture was taken by Tris-HCl buffer.

Hexose kinase (glucokinase) activity was demonstrated by replacing fructose with [U-^{14}C]glucose (295 mCi/mmol) (3.4 nmol) and compared with fructokinase activity (4.2 nmol fructose) in the presence and absence of NAG (94 mM) over a 60 min period. In the case of callus crude enzume prepartion the effect of varying NAG concentration (0-95 mM) on activity with fructose (4.2 nmol) and glucose (3.4 nmol) was also investigated.

Higher fructose concentrations (1 mM and 10 mM) were also incubated with the crude enzyme preparations using the following digest: [U-^{14}C]fructose (301 mCi/mol) (33.2 nmol); 0.1M fructose (to give 1 µmol and 10 µmol total fructose); ATP (10 µmol); $MgCl_2$ (15 µmol); and 0.1 M Tris-HCl pH 8.2 buffer (to give a final volume of 1 ml). The mixtures after incubation for 20 min at 25°C were frozen and later analysed by anion-exchange chromatography (Section 4.1.4).

4.4.2 In vitro metabolism of Fru6P

[U-^{14}C]Fru6P (268 µCi/µmol, 0.89 nmol) was incubated for 60 min at 37°C in 0.1 M Potassium phosphate (pH 7.5) buffer with a crude enzyme prepartion from frozen developing Jerusalem artichoke tubers (25 µl) (Section 4.1.10) in incubation mixtures (total volume 100 µl) as tabulated in Table 8. The experiment was repeated in 0.1 M sodium acetate (pH 4.5) buffer as per Table 9. Boiled enzyme controls of full compliment reaction mixtures were also incubated.

Table 8 Incubation of Fru6P with artichoke tuber enzyme (pH 7.5)

	A	B	C	D	E	F
[U-^{14}C]Fru6P (0.89 nmol)	+	+	+	+	+	+
ATP (0.5 µmol)	+	+	-	-	-	-
$MgCl_2$ (1 µmol)	+	+	+	+	-	-
Fru-1,6-bisP (1 nmol)	-	+	+	-	+	-
NaF (2.5 µmol)	-	-	+	+	+	+
Enzyme preparation (25 µl)	+	+	+	+	+	+
0.1 M potassium phosphate pH 7.5 to 100 µl final volume						

Table 9 Incubation of Fru6P with artichoke tuber enzyme (pH 4.5)

	G	H	J	K
[U-^{14}C]Fru6P (0.89 nmol)	+	+	+	+
$MgCl_2$ (1 µmol)	+	+	–	–
Fru-1,6-bisP (1 nmol)	+	–	+	–
NaF (2.5 µmol)	+	+	+	+
Enzyme preparation (25 µl)	+	+	+	+
0.1M Sodium acetate buffer (pH 4.5)	to 100 µl final volume			

Aliquots (10 µl) of all samples were analysed by paper chromatography in both solvents (ii) and (iii) and carbohydrate and radioactivity located as described in Section 4.1.1. In addition samples A and B were analysed for phosphate esters using the anion-exchange chromatographic method (Section 4.1.4).

4.4.3 In vitro metabolism of Fru-1,6-bisP

Fru-1,6-bisP (1 µmol and 10 µmol) was incubated at 25°C for 45 min with the crude artichoke tuber enzyme prepartion (900 µl) in the presence and absence of $MgCl_2$ (10 µmol) with 0.1 M Tris-HCl pH 8.2 buffer,

final volume 1 ml. The experiments were repeated except that 0.2 ml aliquots of crude enzyme were used and the reaction mixtures were made to 1 ml with 0.1M sodium citrate (pH 4.5) buffer. Control incubations were also carried out using boiled enzyme.

Aliquots (10 µl) were analysed by paper chromatograhy in solvent (v) (Section 4.1.1). One incubation mixture (Fru-1,6-bisP (10 µmol), $MgCl_2$, pH 8.2) was also analysed by anion-exchange chromatography.

4.4.4 Metabolism of FrulP by crude artichoke enzyme preparation

FrulP (1 mmol and 10 mmol) was incubated with crude artichoke tuber enzyme preparation for 45 min at 25°C at both pH 4.5 (0.1 M sodium citrate buffer) and pH 8.2 (0.1 M Tris-HCl buffer) as tabulated in Table 10. Control experiments using boiled enzyme were also carried out.

Table 10 Incubations of Fru1P with artichoke tuber enzyme

	0.1M sodium citrate pH 4.5				0.1M Tris-HCl pH 8.2			
Fru1P (1 µmol or 10 µmol)	+	+	+	+	+	+	+	+
ATP (25 µmol)	+	+	–	–	+	+	–	–
$MgCl_2$ (30 µmol)	+	+	+	–	+	+	+	–
NaF (25 µmol)	+	–	–	–	+	–	–	–
Enzyme preparation (µl)	200	200	200	200	900	900	900	900
Buffer	to give 1000 µl final volume							

Aliquots (10 µl) were analysed by paper chromatography in solvent v) (Section 4.1.1) and in addition one sample (10 mmol Fru1P, ATP, $MgCl_2$, NaF, pH 8.2) was analysed further by anion-exchange chromatoraphy (Section 4.1.4).

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Appendix I Incorporation of radioactivity into carbohydrate in developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose (Study A) (see Materials and Methods 4.2.1, 4.2.3ii) and 4.2.4)

	% Incorporation of label									
	Method of Isolation									
	Anion exchange chromatography					Paper Chromatography				Ethanol Precipitation
Length of Incubation	Glc1P*	Fru6P	Fru1P	Glc6P	Fru1,6-bisP	MS	DS	TS	OS (DP11-14)	PS
0.08 hr	0.42	0.31	0.01	0.94	0	29.19	5.67	4.22	28.77	0.05
0.17 hr	0.32	0.39	0	1.33	0.01	54.80	2.52	2.19	17.73	0.11
0.25 hr	0.16	0.53	0.06	1.23	0.38	35.34	4.81	3.16	41.80	0.12
0.5 hr	0.95	3.12	0	8.02	0.68	15.33	10.77	1.72	54.15	1.28
1 hr	0.45	1.34	0.13	3.64	0.19	-	-	-	-	0.78
3 hr	0.67	0.37	0.40	0.57	0.17	0	8.26	11.08	63.13	0.39
5 hr	0.27	0	1.09	0.13	0	33.70	40.74	9.96	0	0.47
6 hr	0.77	0.24	2.40	0.20	0.12	13.17	37.44	20.01	0.66	1.51

MS = Mainly Fru, Glu, DS = Mainly sucrose, TS = Mainly isokestose, OS = Oligofructosides

PS = Polysaccharide

* Possibly also contains Fru2P,G3P

Appendix II Incorporation of radioactivity into carbohydrate in developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose (Study B) (see Materials and Methods 4.2.1, 4.2.3 ii), and 4.2.4)

	% Incorporation of Label			
	Method of isolation			
	Anion-exchange Chromatography			Ethanol Precipitation
Length of Incubation	Fru6P	Fru1P	Glc6P	PS
0.25 hr	0.74	0	1.49	0.04
0.5 hr	0.58	0	1.15	0.11
1 hr	1.20	0.12	2.32	0.23
2 hr	1.57	0.82	2.48	0.41
4 hr	0.39	0.57	0.18	0.11
6 hr	0.11	0.75	0	0.59
8 hr	0.06	0.78	0	1.11

PS = Polysaccharide

Appendix III Incorporation of radioactivity into sugar phosphates in developing Jerusalem artichoke tubers injected with [U-^{14}C]fructose and isolated by anion-exchange chromatography (Study C) (see Materials and Methods 4.2.1, 4.2.3ii) and 4.2.4ii))

	% Incorporation of label		
Length of incubation	Fru6P	Fru1P	Glc6P
0.25 hr	3.43	0	6.94
0.5 hr	2.98	0	5.72
1 hr	4.57	0.42	8.82
2 hr	4.90	2.53	7.71
4 hr	1.36	2.00	0.64
6 hr	0.33	2.88	0
8 hr	0.21	2.82	0

Appendix IV Incorporation of radioactivity into carbohydrate in developing Jerusalem artichoke tubers injected with [U-^{14}C]sucrose (see Materials and Methods 4.3 and 4.2.4)

	% Incorporation of label									
	Method of Isolation									
	Anion exchange chromatography					Paper Chromatography				Ethanol Precipitation
Length of Incubation	Glc1P*	Fru6P	Fru1P	Glc6P	Fru1,6-bisP	MS	DS	TS	OS (DP11-14)	PS
0.25 hr	0	0	0	0	0	8.02	9.19	5.32	20.22	0.23
0.5 hr	0	0	0	0	0	7.11	11.01	7.05	24.11	0.44
1 hr	0	0.58	0	0	0	5.99	10.01	9.05	36.78	0.75
2 hr	0.27	0.14	0	1.57	0	-	-	-	-	
4 hr	0	0.05	0	0.08	0	6.51	6.28	3.92	62.22	1.41

MS = Mainly Glu, Fru, DS = Mainly sucrose, TS = Mainly isokestose, OS = Oligofructoside

PS = Polysaccharide

* Possibly also contains Fru2P,G3P

Appendix V Incorporation of radioactivity into carbohydrate in callus cutlures of Jerusalem artichoke tubers injected with [U-^{14}C]fructose (see Materials and Methods 4.2.1, 4.2.3ii) and 4.2.4)

	% Incorporation of label									
	Method of Isolation									
	Anion exchange chromatography					Paper Chromatography				Ethanol Precipitation
Length of Incubation	Glc1P*	Fru6P	Fru1P	Glc6P	Fru1,6-bisP	MS	DS	TS	OS (DP11-14)	PS
0.25 hr	0.41	1.43	0	2.54	0.49	0.67	0.84	0.42	0.92	0.28
0.5 hr	0.70	2.85	0	3.79	1.02	0.33	0.39	0.30	0.76	0.31
1.5 hr	1.64	6.71	0	8.78	0.35	4.37	3.06	0.97	1.74	0.25
3 hr	0	5.55	0.05	3.84	0.20	25.55	1.99	3.07	1.13	0.45
6 hr	0	3.43	0.90	1.53	1.17	39.54	1.40	2.23	1.04	0.40

MS = Mainly Glu, Fru, DS = Mainly sucrose, TS = Mainly isokestose, OS = Oligofructoside

PS = Polysaccharide

* Possibly also contains Fru2P,G3P