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PLANT GLYCOLIPIDS

A Thesis submitted by

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a candidate for the Degree of

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in

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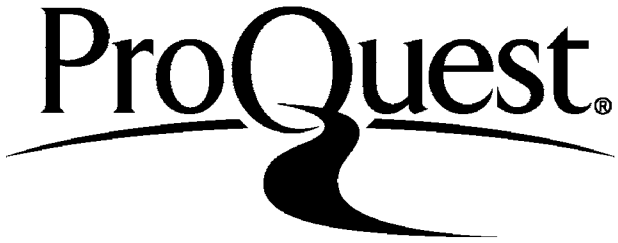

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ABSTRACT

The complex nature of the glycolipids from mung bean has been examined. The lipid-bound monosaccharides are similar to the monosaccharides constituents of polysaccharides which occur commonly in the plant kingdom. The glycosyl groups in the lipids appear to be acid labile and are bound as glycosylglycerides and as non-saponifiable glycolipids.

Glycolipids in mung bean do not seem to be associated with a definite particulate fraction. Glycosylglycerides with relatively short sugar chains have been identified; glucose and fructose are the two main monosaccharide constituents of these lipids. A fructolipid containing a 2,3-linked furanose ring system has been isolated. Arabinose, most of the galactose, mannose and rhamnose all appear to be associated with a non-saponifiable lipid fraction.

A preliminary investigation of the incorporation of label from sucrose-^{14}C into the glycosyl residues of mung bean lipids showed that they became rapidly labelled and there was a tendency for the monosaccharides bound to non-saponifiable lipids to have the highest specific activities. Glucose fructose and mannose may be metabolically more active than galactose, arabinose and rhamnose. The latter sugars may be components of a 'structural lipid.'

As a preliminary to examining the pathway for the formation of the fructolipid present in mung bean shoots, the enzyme synthesis of UDP-fructose, a likely precursor, was studied. The results suggest that fructose-2-phosphate is the probable substrate for a UDP-fructose pyrophosphorylase and that tissue extracts contain a 'phosphofructomutase' which can convert fructose-1-phosphate to fructose-2-phosphate. The nature of the linkage between UDP and fructose has not been established beyond doubt, however.

CONTENTS

ABBREVIATIONS

c.p.m.	counts per minute
r.p.m.	revolution per minute
TLC	thin-layer chromatography
GLC	gas-liquid chromatography
TMS	trimethylsilyl ether
TTC	2,3,5-triphenyltetrazolium chloride
MGDG	monogalactosyldiglyceride
DGDG	digalactosyldiglyceride
SQDG	sulphoquinovosyldiglyceride
PGL	phytoglycolipid
Gal	galactose
Glu	glucose
Man	mannose
Fru	fructose
Ara	arabinose
Xyl	xylose
Rib	ribose
Rha	rhamnose
Gly	glycerol
G-1-P	D-glucose-1-phosphate
G-6-P	D-glucose-6-phosphate
Fru-2-P or F-2-P	D-fructose-2-phosphate
F-2-P(fur) or F-2-P$_f$	D-fructofuranose-2-phosphate
F-2-P(pyr)	D-fructopyranose-2-phosphate
F-1-P	D-fructose-1-phosphate
F-6-P	D-fructose-6-phosphate
PPi	pyrophosphate
ATP	adenosine triphosphate
ADP	adenosine diphosphate
UTP	uridine triphosphate

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UDP	uridine diphosphate
UMP	uridine monophosphate
GTP	guanosine triphosphate
GDP	guanosine diphosphate
CTP	cytidine triphosphate
UDPG	UDP-D-glucose

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

Plant lipids can be subdivided into two groups, neutral compounds, for example hydrocarbons, waxes, sterols, ester sterols, simple glycerides and long-chain alcohols, and polar derivatives such as phospholipids and glycolipids.

The term glycolipid is very vague and it has been used mainly to refer to the glycerolglycolipids (1,2). Taken in its broadest sense the term glycolipid should mean a compound where a lipid moiety is linked in some way to a glycosidic moiety. Thus, glycerolglycolipids (glycosyldiglycerides), sphingolipids and sterol glycosides should all be classified as glycolipids. Phospholipids should be considered as a class on their own although there might be a tendency to consider inositol phospholipids as glycolipids because of the similarity in structure between inositol and the hexoses. This discussion, however, will be concerned only with glycolipids as defined above.

Glycerolglycolipids which include the sulpholipids are characteristic of photosynthetic tissues (3) but they also occur in dormant seeds (4). In animal tissues, however, their presence is less common but small amounts of glycerolglycolipids have been found in mammalian brain and bovine spinal cord but their role is not clear (5,6). Glycerolglycolipids are believed to play a role in photosynthetic reactions but this fact has not been proved conclusively (7,8,9).

Sphingolipids which constitute the main lipid fraction of animal tissues, particularly the brain, have also been found in a wide range of plants. The structures of the two classes of sphingolipids appear to be quite different however; for example, two of the most typical groups of sphingolipids in animal tissues, namely sphingomyelins and gangliosides, have not been detected in plant tissues (10).

Most of the work on plant glycolipids has been carried out during the past decade. The studies have been mainly concerned with isolation, characterization, and structural determinations of a variety of these compounds. The metabolism of the group has only been examined superficially.

Regarding the function of glycolipids in plant tissues, two main

theories have been advanced, one that they play a role in photosynthesis (7,8,9,11) and the other that they are involved in the biosynthesis of cell-wall polysaccharides (12-15). These theories will be discussed later under individual compound headings.

A number of general reviews have been published on the subject of plant glycolipids (16-19). A very comprehensive review has been written by Reinisova and Michalec (10).

Methods of Isolation

A number of methods for the isolation of plant glycolipids have been elaborated in recent years. These methods are mainly based on different chromatographic techniques.

The methods used for the isolation of animal glycolipids were difficult to apply at first to compounds from plant tissues because the latter contained large amounts of interfering pigments such as chlorophyll and its degradation products. However, purification of plant glycolipids has been effected by a combination of the several techniques which were available for animal lipids.

Extraction of Lipids

Lipids are generally extracted from plant tissue with mixtures of chloroform and methanol as in the case of animal tissues. Lipids can also be extracted with ethanol, isopropanol, chloroform, benzene or hexane or any mixture of these solvents (10).

Solvent fractionation is generally inadequate for the isolation of pure components of naturally occurring lipid mixtures, but they can be of great help in preliminary separations of large quantities of material. Solvent fractionation is generally most effective when applied to simple mixtures which have already been partially fractionated by other means (20).

Counter-current distribution

Varying degrees of separation of complex mixtures of polar plant lipids have been accomplished by this method but individual lipid classes have rarely been separated. The main contribution of counter-current

distribution seems to be in the preliminary separation of the lipids into simpler mixtures rather than in the isolation of individual lipid classes free of contaminants (20).

Glycerolglycolipids were separated from less polar compounds in a n-heptane-90% methanol system (4,21,22). Allen et al (19,20) carried out a preliminary fractionation of lipids from spinach with carbon tetrachloride : methanol : water, 62:35:4 (v/v) and 1-butanol saturated with water : 95% methanol : hexane, 2:3:1 (v/v) systems (20). This method was found to be a convenient way to remove chlorophyll and to effect a preliminary separation of lipids according to their hydrophilic character. The presence of chlorophyll has always been a major problem in the isolation of lipids.

Column chromatography

Chromatography on silicic acid columns is one of the most widely used techniques for fractionating plant lipids. A crude fraction of glycosyl diglycerides from wheat flour (4,23) was obtained by this technique but it was contaminated with cerebrosides and neutral lipid. Zill and Harmon (24) tried to separate the lipids of photosynthetic tissues (spinach leaves) by this method but chlorophyll and its degradation products contaminated the fractions. Repetition of this column process brought a certain improvement in the purification of monodigalactosyldiglycerides and cerebroside fractions from bean leaves (25,26).

Fractionation on Florisil and DEAE cellulose is a usual technique in isolating animal lipids (27,28) and it has also been proved to be useful when applied to some plant materials (22): the method gave relatively pure pigment-free lipid fractions.

A good separation of glycerolglycolipids, sulpholipids and glycerolphospholipids was obtained by a combination of DEAE-cellulose and silicic acid column chromatography (19,20). Nichols et al (29,30) fractionated the lipids of blue green algae and plant bulbs by introducing some modifications to the existing methods of lipid fractionation. Phospholipids and sulpholipids of plants have been separated on Dowex -

2 x 8 (acetate form) columns (31) and column chromatography on Sephadex LH-20 proved to be successful for the separation of lipids from spinach leaves (32) which, prior to column fractionation were separated from most of the phospholipids and non-polar compounds by counter-current distribution and acetone precipitation. The residual phospho-lipids contaminants were separated neatly on the column and monogalacto-syldiglyceride and digalactosyldiglyceride were very well separated from one another.

Thin-layer chromatography

By this method one can only separate small amounts of lipids but a high level of purity is commonly obtained.

A number of solvent systems have been tested and two in particular (chloroform : methanol : water, 65:25:4 (v/v) and diisobutyl ketone : acetic acid : water, 40:25:5 (v/v) (33) are capable of good resolution. Two dimensional TLC on silica gel G using these two solvent systems gave very good qualitative results (34,35) with lipids from different plants such as Narcissus bulb (30). Nichols (36) and Pelick (37) tested the separating ability of various solvent systems on silica gel and Adsorbosil-1.

Paper chromatography

Silicic acid impregnated papers developed with diisobutyl ketone : acetic acid : water, 40:25:5 (v/v) (38, 39,25,26) have been used extensively. O'Brien and Benson (22) separated galactosylglycerides and sulpholipids on silicic acid impregnated paper in chloroform : methanol, 9:1 (v/v) and after a preliminary column chromatographic fractionation and Beiss (40) described a series of other solvent systems which are useful for the separation of lipids on silica gel impregnated papers (Schleicher and Schull N.289). Mumma and Benson (41) separated the lipids from wheat leaves on aminoethylcellulose paper (Whatman AE30). Improved separation of the faster moving compounds was achieved at 4°.

Analysis of deacylation products (sugars and glycerol) of glyco-lipids has been carried out on untreated chromatography papers. Acid and

alkali hydrolysis products of MGDG and DGDG were, for example, fractionated with phenol : water, ethylacetate : pyridine : water, butanol : pyridine : water and other normal solvent systems used for carbohydrates (3,26). Several methods of separation by TLC and paper chromatography are summarized in Table 1.

Various staining techniques have been developed to locate the lipids (42), such as Rhodamin 6G (34,43,44,45), ninhydrin (44,45) and periodate-schiff reagent (27) which are used on paper chromatograms. Charring agents such as sulphuric, perchloric and phosphoric acids, 20% aqueous ammonium bisulphate and I_2 vapour (42) are widely used on thin layer chromatograms.

Abbreviations used in Table 1

PI	: phosphatidyl inositol
PCH	: phosphatidyl choline
PG	: phosphatidyl glycerol
PE	: phosphatidyl ethanolamine
PS	: phosphatidyl serine
PA	: phosphoric acid
Glyc-b	:glycolipid b
Glyc-c	: glycolipid c
SQDG	: sulphoquinovosyldiglyceride
DGDG	: digalactosyldiglyceride
MGDG	: monogalactosyldiglyceride

Table I - Chromatographic system useful for separating plant lipids (10)

System	Chloroform: methanol:water 65:25:4 (v/v) (34)	Diisobutylketone: acetic acid:water 80:50:60 (v/v) (34)	Chloroform: methanol 9:1 (v/v) (22)	Diisobutylketone: acetic acid:water 40:25:5 (v/v) (41)	Phenol:water 100:40 (v/v) (38)	Diisobutylketone acetic acid:water 40:25:5 (v/v) (26)
Paper	-	-	Whatman No.3 silica gel impregnated	Whatman AE	Whatman No.1	Whatman No.3 silica acid impregnated
Adsorbent	Silica gel	Silica gel	-	-	-	-
R_f 0.0						
0.1		• PI • PCH			• PI	
0.2	• PI	• SQDG • DGDG			• SQDG	
0.3	• PCH	• PG		• PI • SQDG		• SQDG + PI
0.4	• SQDG	• PE	• SQDG	• PG	• PG • DGDG	• DGDG
0.5	• PG	• MGDG	• DGDG	• DGDG • PE		• PG + PS + Glyc-b • PE
0.6	• DGDG, PE			• PCH	• MGDG + PE	• Di-PG + Glyc-c
0.7	• MGDG			• MGDG		• MGDG
0.8						• PA
0.9	• neutral lipids		• MGDG			
1.0		• neutral lipids		• neutral lipids		

Sphingolipids

Sphingolipids were first discovered as components of nerve tissue, but they were later found to be distributed throughout a wide variety of animal tissues. Studies on the metabolism of these lipids were undertaken only recently (46).

Sphingolipids have also been found in lower and higher plants but in this case studies were mainly concerned with the isolation and the determination of structure of the compounds; their function and metabolism have not been studied in great detail.

Sphingolipids can be divided into four major groups according to their chemical structure:

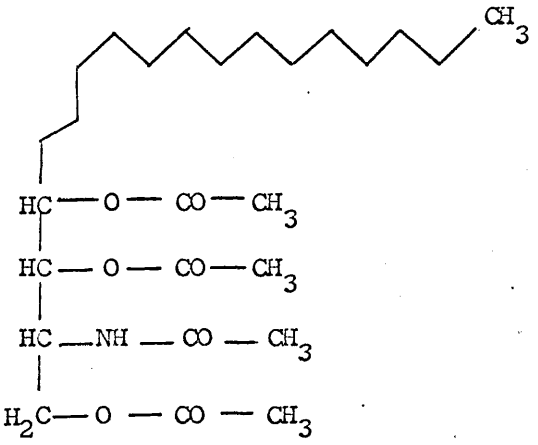
1) derivatives of sphingosine and phytosphingosine bases
2) cerebrin-ceramides
3) cerebrosides (ceramide monohexosides)
4) phytoglycolipids (mycosphingoglycolipids)

Derivatives of sphingosine and phytosphingosine bases

Derivatives of sphingosine and phytosphingosine bases in a free form have been isolated from several microorganisms. Stodola *et al* (47-50) found that the yeast *Hansenula ciferri* produced extra-cellular acetylated sphingosine bases. The compounds were isolated by counter-current distribution followed by crystallization from petroleum ether. Two main components were identified, tetraacetylphytosphingosine (I) and a small amount of triacetyldihydrosphingosine (II).

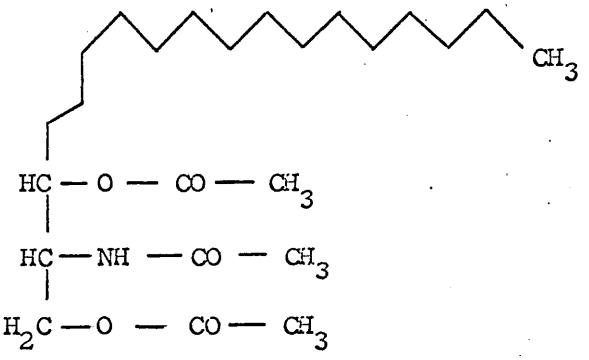
Green *et al* (51) studied the biosynthesis of these compounds and produced evidence which indicated that the pathway to phytosphingosine and other sphingolipids in whole yeast cells, was similar to the pathway to sphingosine and dihydrosphingosine in animal systems where it was found that the base was formed by the condensation of serine or a derivative of serine, with some derivative of palmitic acid or palmitaldehyde.

DL - [3-^{14}C] serine and [9, 10-^{3}H]-palmitic acid were added to the yeast culture medium and it was shown that the hydroxymethyl group of serine was incorporated into carbon-1 of the sphingolipid bases and



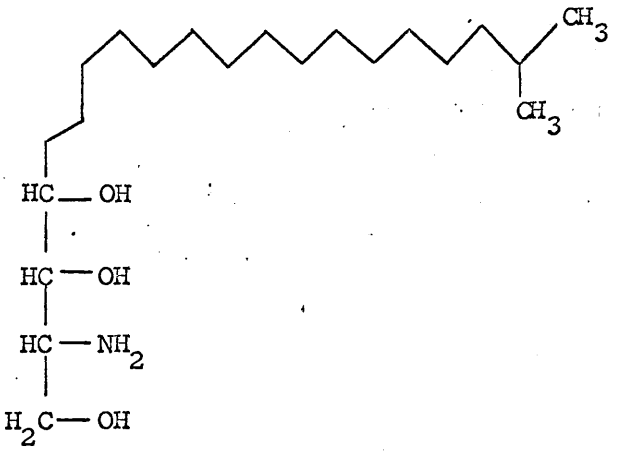
I

tetraacetylphytosphingosine



II

triacetyldihydrosphingosine



III

19-methyl-C_{20}-phytosphingosine

that palmitic acid provided the framework for much of the aliphatic chain.

Hexadec-2-enoic acid was as readily incorporated as palmitic acid and it was assumed that this was because the unsaturated acid was rapidly reduced to the saturated acid.

The low incorporation of 2-hydroxypalmitic acid compared to palmitic was difficult to interpret. The basic pathway leading to all of the sphingolipid bases seemed to be identical but the origin of the extra hydroxyl group in phytosphingosine remains unanswered.

About 50% of the total sphingolipid bases in these yeast experiments were extracted with petroleum ether from the medium and most of the remainder was extracted from the cells with a chloroform; methanol mixture. The composition of sphingolipids in both cases was practically the same.

The sphingosine bases were oxidized with periodate-permanganate reagent and their structures determined on the assumption that sphingosine was oxidized to tetradecanoic acid, phytosphingosine to pentadecanoic acid, and dihydrosphingosine to hexadecanoic acid. The resulting fatty acid mixture consisted of 90.6% pentadecanoic, 6.0% hexadecanoic, 2.4% tetradecanoic acids. C_{18}-phytosphingosine and C_{18}-dihydrosphingosine were thus identified as components of H.ciferri, but there was no definite evidence for sphingosine because C_{16}-dihydrosphingosine would also give $C_{14:0}$ acid upon oxidative degradation. Karlsson (52) reported that the sphingosine type bases in H.ciferri consisted of C_{18}-phytosphingosine (89%) C_{18}-dihydrosphingosine (7%) and a total of 3.5% of C_{20}-, C_{17}-, C_{19}-phytosphingosine, C_{16}-, C_{17}-, C_{19}-, and C_{20}-dihydrosphingosine.' The high base content of yeast might be due to a failure of the organism to incorporate them into complex sphingolipids. Instead the bases are then detoxified by acetylation and excreted.

Cerebrin-ceramide

The first reports of the existence of this type of compound in

lower and higher fungii appeared in the early part of this century in papers by Zellner (53) and later by Rosenthal (54). Twenty years later the problem received more attention when Ruppol (55,56) and Reindel (57) analyzed the sphingolipids of bread yeast and found that they were essentially amides of α-hydroxyacid and sphingosine bases. Many other authors (58-62) looked at the sphingolipid composition in similar organisms, such as Aspergillus sydowi (58) and Torulopsis utilis (62) and showed that the most commonly occurring bases were C_{18} and C_{20} - phytosphingosine or C_{18}-dihydrosphingosine and that the fatty acids were mainly α-hydroxy-C_{26} and α-hydroxy-C_{24} acids.

Wagner and Zofcsik (63) determined the sphingolipid composition of Candida utilis and Saccharomyces cerevisiae. The mixture of sphingolipids, obtained by extraction of lyophilized material from both organisms, was fractionated on a silicic acid column. A cerebrin fraction was isolated which, after hydrolysis gave C_{18}-phytosphingosine (29.1%) C_{20}-phytosphingosine (68.0%), C_{18}-dihydrosphingosine (6.00%) and cerebronic and α-hydroxycerotic acids.

Carter et al (64) separated the lipids of Crithidia fasciculata into polar and neutral fractions by silicic acid chromatography. Eighteen per cent of the polar lipids were stable to treatment by mild alkali and, when examined by silica gel thin-layer chromatography, were shown to consist of two sphingolipid subfractions of different polarity.

These two subfractions could also be isolated, without prior alkaline treatment, by silicic acid column chromatography. The less polar component (10% by weight of the total polar lipids) contained over 90% stearic acid while the more polar component (8% of total polar lipids) contained over 95% α-hydroxystearic acid.

Following hydrolysis, both of the sphingolipid subfractions yielded a long-chain base fraction which was unresolved by silica gel TLC but corresponded to phytosphingosine and gave positive ninhydrin and periodate tests.

The trimethylsilyl derivatives of the bases were prepared and examined by GLC. C_{20}-phytosphingosine was identified together with a new base

19-methyl-C_{20}-phytosphingosine (III).

In the less polar ceramide fraction the ratio of branched-chain base to C_{20}-phytosphingosine was 2.5:1 whereas in the more polar fraction it was 6.0:1.

The structure of the new base (III) was confirmed by mass-spectroscopy and infrared spectroscopy. The intact ceramides from Crithidia were treated with periodate and the resulting aldehydes reduced to alcohols which were then examined by GLC. The presence of 16-methyl-heptadecanol was noted and this confirmed the branched-chain structure of the new base (III).

Cerebrosides (ceramide monohexosides)

Wagner and Zofcsik (65) isolated a cerebroside fraction from Candida utilis by silicic acid column chromatography. The lipid was hydrolyzed with acid and the liberated sugar component identified as galactose. Only one fatty acid component (α-hydroxystearic acid) was detected by GLC analysis. The bases released by acid hydrolysis were examined by TLC on silica gel and detected with ninhydrin. The spots corresponded to C_{16}- and C_{18}-dihydrosphingosine and C_{16}- C_{18}-sphingosine. Oxidation of bases with chromic acid yielded adipic, lauric, myristic and palmitic acids.

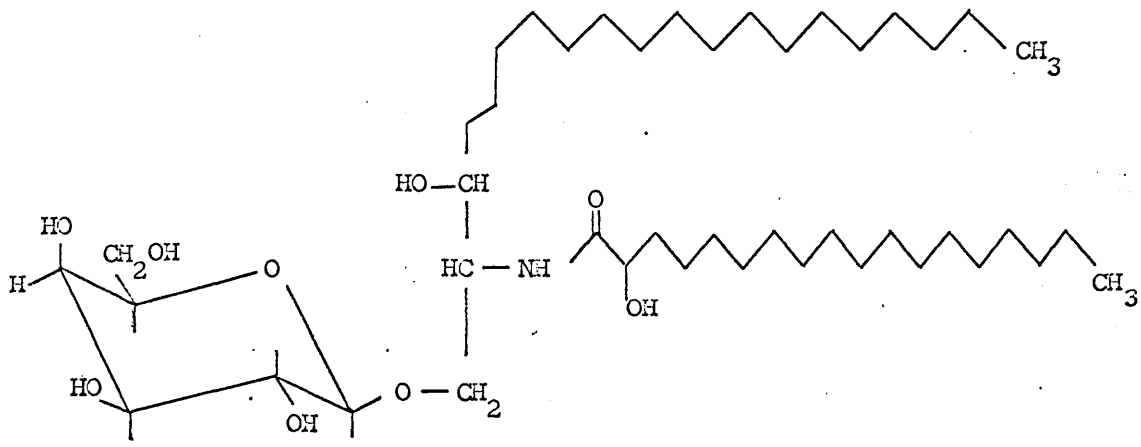
The formation of myristic acid indicated the presence of C_{16}-dihydrosphingosine and C_{18}-sphingosine, and palmitic acid the presence of C_{16}-dihydrosphingosine and C_{18}-sphingosine. Adipic and lauric acids were probably produced by the oxidation of C_{20}-dehydrosphingosine. From these hydrolysis and oxidation studies the structure of the cerebroside was deduced (see structure IV).

A cerebroside fraction similar to that from Candida was identified in wheat flour by Carter et al (66,67).

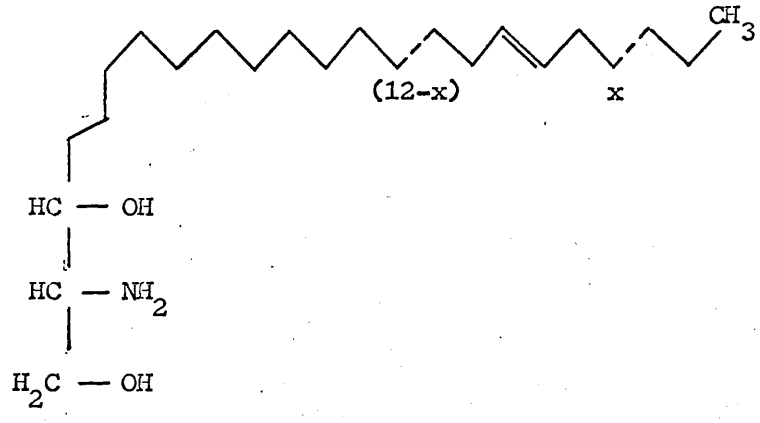
The only monosaccharide detectable in the cerebroside fraction was glucose and four bases were identified:

1) $CH_3(CH_2)_{13}-\underset{OH}{CH}-\underset{OH}{CH}-\underset{NH_2}{CH}-CH_2OH$

phytosphingosine



IV



V

new base isomer of sphingosine

2) $CH_3(CH_2)_8CH{=}CH{-}(CH_2)_3{-}\underset{\displaystyle CH}{\overset{\displaystyle |}{CH}}{-}\underset{\displaystyle CH}{\overset{\displaystyle |}{CH}}{-}\underset{\displaystyle NH_2}{\overset{\displaystyle |}{CH}}{-}CH_2OH$

dehydrophytosphingosine

3) $CH_3(CH_2)_{14}{-}\underset{\displaystyle CH}{\overset{\displaystyle |}{CH}}{-}\underset{\displaystyle NH_2}{\overset{\displaystyle |}{CH}}{-}CH_2OH$

dihydrosphingosine

4) new base isomer of sphingosine (V)

α-Hydroxystearic acid was the major fatty acid component of the fraction.

A cerebroside fraction was also isolated from Aspergillus niger (68). This contained C_{18}-sphingosine and C_{18}-dihydrosphingosine, galactose and a mixture of fatty acids containing more than 80% of 2-hydroxyoctadecenoic acid. A cerebroside fraction, probably a trimannoside, was also detected in wheat flour (4). In runner bean leaves Sastry and Kates (26,69) identified a glucocerebroside fraction which contained a mixture of C_{16}- C_{20}-α-hydroxyacids and a series of long-chain bases the main component being C_{18}-dehydrophytosphingosine (64%) together with smaller amounts of C_{18}-phytosphingosine (2.5%) C_{18}-dihydrosphingosine (8.0%), C_{18}-sphingosine isomer (2.1%) and an unidentified base (19.3%).

The fatty acid and long-chain base composition of runner bean leaves differed considerably from that of wheat flour glucocerebrosides.

Phytosphingoglycolipid (mycosphingoglycolipid)

Chromatographically homogeneous fractions were obtained from the sphingolipids of Candida utilis and Saccharomyces cerevisiae by elution with chloroform from a silicic acid column (70). On acid hydrolysis both yielded mannose, inositol, phosphoric acid and calcium ions. C_{18}- and C_{20}-phytosphingosine and C_{18}-dihydrosphingosine components were characterized by TLC and periodate oxidation to the corresponding aldehydes. The bases were qualitatively the same in both microorganisms but differed quantitatively (Table II). Differences in the fatty acid composition were more marked (Table III). The nature of the linkages involving mannose and inositol were not determined.

Table II - Base composition of mycosphingoglycolipid isolated from Candida utilis and Saccharomycds cerevisiae (70)

Microorganisms	% C_{18}-dihydro-sphingosine	% C_{18}-phyto-sphingosine	% C_{20}-phytosphin-gosine
Candida utilis	7.36	17.63	75.01
Saccharomyces cerevisiae	6.25	37.3	56.26

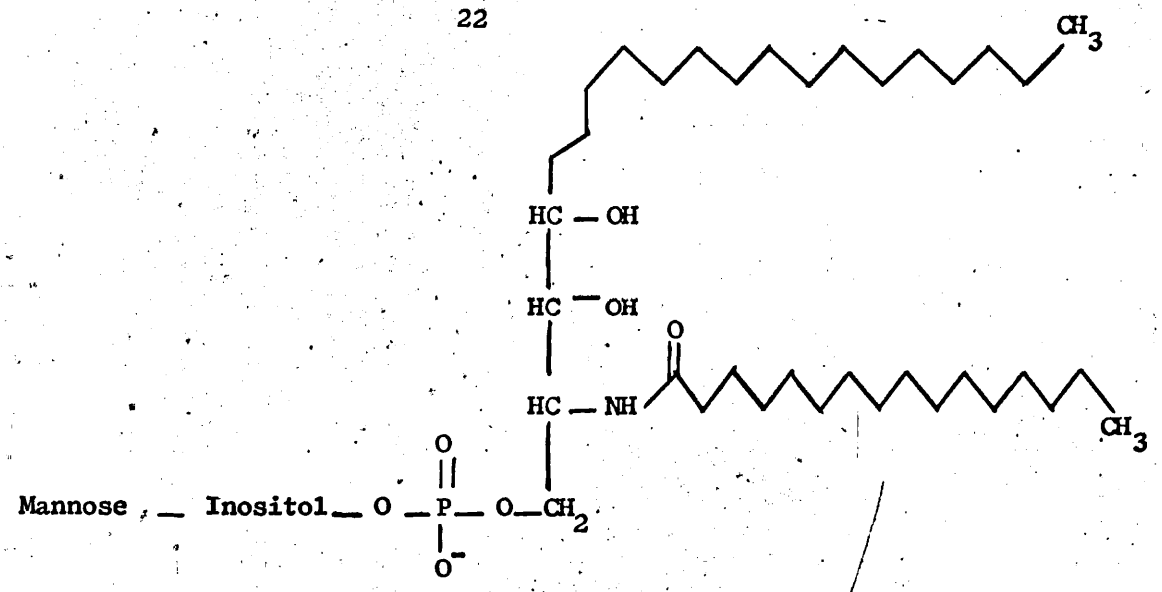
Table III - Fatty acid composition of mycosphingoglycolipid isolated from Candida utilis and Saccharomyces cerevisiae (70)

Fatty acids*		Candida utilis	Saccharomyces cerevisiae
Arachidonic acid	20:0	< 0.1	< 0.1
Behenic acid	22:0	6.99	11.4
Lignoceric acid	24:0	8.45	3.94
Cerotic acid	26:0	< 0.1	34.1
α-hydroxybehenic acid	22:0(OH)	1.27	14.45
α-hydroxylignoceric acid	24:0(OH)	56.40	36.20
α-hydroxycerotic acid	26:0(OH)	23.19	-
C_{30}-fatty acid (uncharacterized)		3.91	-

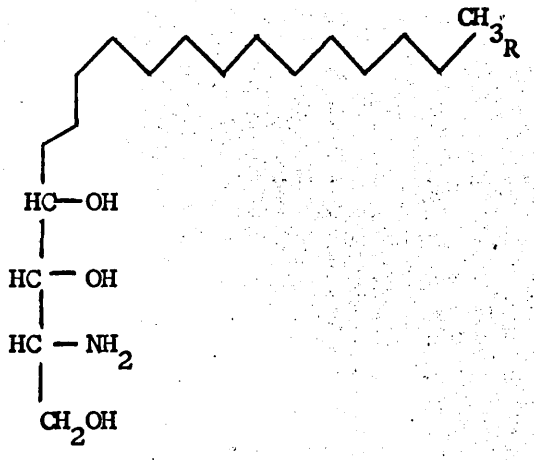
The structures of yeast mycoglycolipids (VI) were similar to that of the phytoglycolipid isolated by Carter et al (71,72) from a variety of plant seeds.

The first detailed reports on the presence of sphingoglycolipids in higher plants were published in 1953. Van Handel (73) isolated, from soybean phospholipids, an acidic fraction which was stable to treatment with mild

*Expressed as % total fatty acids



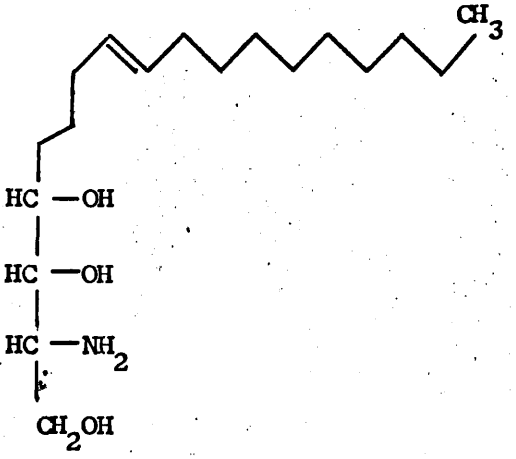
VI



VII ; VIII

R=C_{14}[C_{18}-phytosphingosine: VII]

R=C_{16}[C_{20}-phytosphingosine: VIII]



IX

C_{18}-dehydrophytosphingosine

alkali and had a nitrogen/phosphorus ratio of 2.

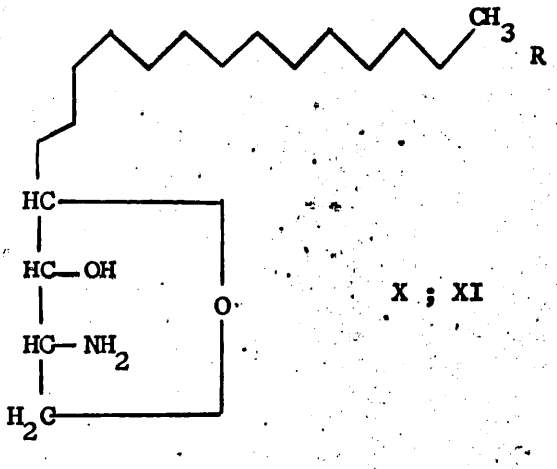
Carter et al (74) separated a crude inositol lipid from corn and soybean phosphatides and after purification by counter-current distribution obtained a mixture of sphingolipids. Hydrolysis of the sphingolipids produced phytosphingosine. The main base in the corn lipid was found to be D-1,3,4-trihydroxy-2-amino octadecane (C_{18}-phytosphingosine) (74,75) (VII). The unsaturated base dehydrophytosphingosine (trans-Δ^8-phytosphingosine (IX)) was found in small amounts in the corn derivative but occurred as a major component of the sphingolipid of soybean and of flax seed (75). C_{20}-Phytosphingosine (D-ribo-1,3,4-trihydroxy-2-amino eicosan) was also detected in soybean, peanut and yeast phosphatide fractions (61,76) (VIII).

Wagner and Wolff (77) also characterized the bases present in the sphingolipids of soybean and peanut by TLC. In agreement with other authors (72,78) they reported the presence of C_{18} and C_{20}-phytosphingosine, C_{18}-dihydrosphingosine, C_{18}-dehydrophytosphingosine, C_{18} and C_{20}-anhydrophytosphingosine (X, XI). Two unidentified bases were also observed in soybean sphingolipids.

The sphingolipids, containing inositol, which had been isolated from soybean, corn, wheat, peanut, sunflower seed, cotton seed and flax were found to possess similar chemical compositions (Carter et al (72)) and were termed phytoglycolipids (PGL). These lipids were soluble in pyridine, dimethyl-sulphoxide and in mixtures of chloroform and methanol, but were insoluble in benzene, ethyl ether and other non-polar solvents.

At one time it was suggested that PGL was an artifact (79) arising from the action of mild alkali (which is used to separate sphingolipids from hydrolysable lipids), on the lipid fraction, but Carter et al (80) proved that PGL was a genuine component of the lipid fraction. A flax inositol lipid mixture consisting of a phosphatidyl inositol and a "phytoglycolipid precursor" was isolated without the use of alkali by counter-current distribution.

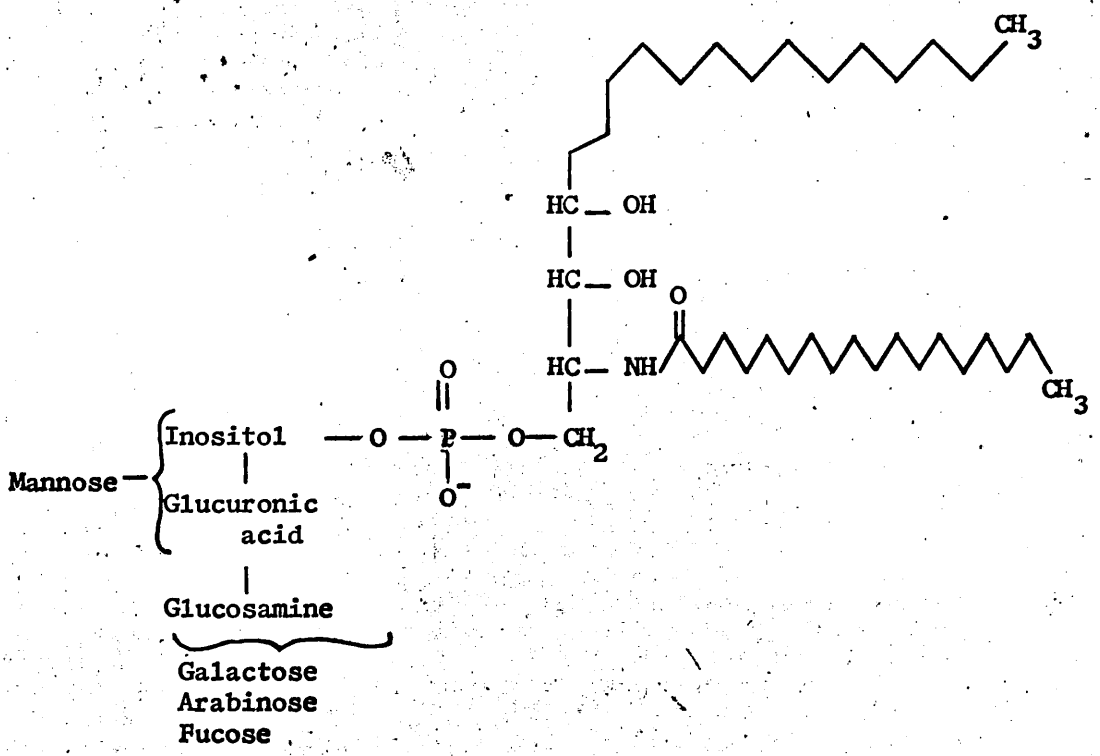
The "PGL-precursor" contained calcium and magnesium and the analytical data obtained suggested that it might represent a molecule of PGL bound,



X ; XI

R=C_{14}[C_{18}-anhydrophytosphingosine: X]

R=C_{16}[C_{20}-anhydrophytosphingosine: XI]



XII

as a mixed Ca-Mg salt, to a molecule of phosphatidyl inositol. The complex was stable when subjected to various chromatographic procedures.

On the basis of degradation studies the following general structure was suggested for PGL (XII) (71,72,81).

PGL (XII) contains a cerebronyl phytosphingosine phosphate and an oligosaccharide moiety. A basic trisaccharide unit, glucosaminido-glucuronido-inositol, was found to be common to PGL from all sources studied. In addition Carter et al (71) showed that other monosaccharides could be present, for example, mannose bonded to inositol or glucuronic acid and galactose, arabinose, and fucose linked through glucosamine. The aldohexoses, arabinose and fucose contents showed species variation.

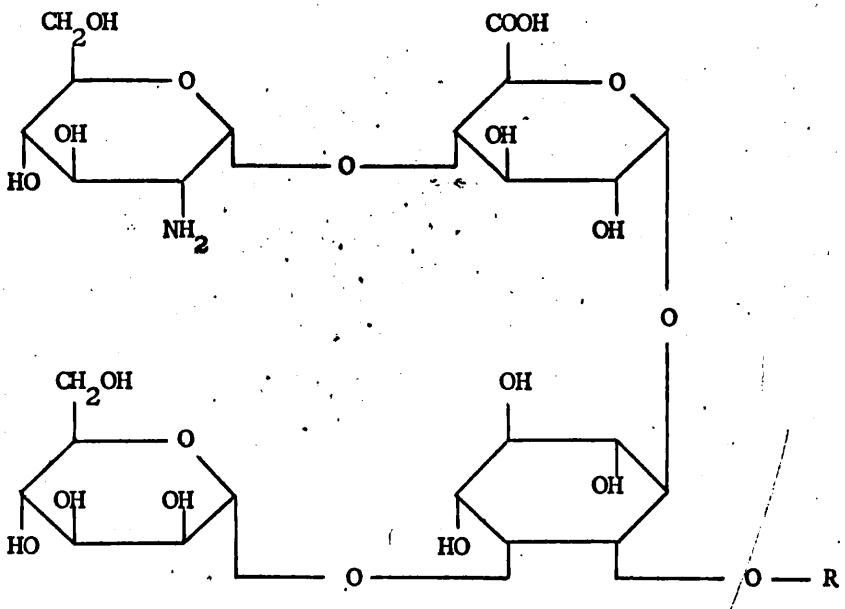
PGL of maize and soybean, for example, does not contain fucose and PGL of flax seed (which contains fucose) yields less mannose and more galactose on hydrolysis than the equivalent preparation from corn PGL. A PGL from groundnut differed from the PGL of soybean and maize in that it had low mannose and high arabinose contents (73). The base compositions of PGL from different plants show large quantitative differences (Table IV).

Table IV - Base composition of phytoglycolipids isolated from different sources (10)

Source	Dehydrophytosphingosine %	Phytosphingosine %
Flax seed	85	15
Soybean	80	20
Peanut	50	50
Maize	10	90

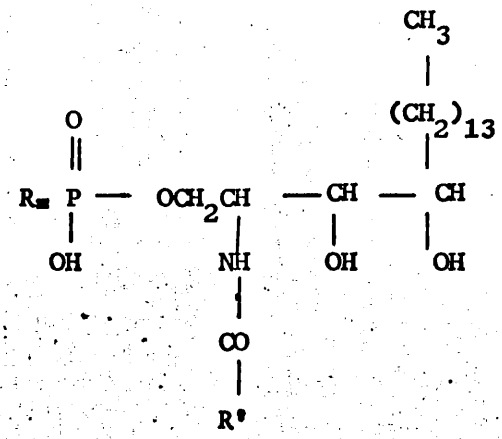
Recently the complete structure of a tetrasaccharide obtained by partial hydrolysis of the sphingolipids from a variety of plant seeds (corn for example) was reported by Carter et al (83) (XIII).

Another type of PGL has been isolated from flax seed and this contains inositol, galactose, arabinose and fucose in the molar ratio of 1:10-11:3:2.



XIII

The 6-O-[2-amino-2-deoxy-D-glucosyl-(1—> 4)-D-glucuronosyl]-2-O-mannosyl-inositol unit present in PGL from a variety of plant seeds (eg : corn, soybean, wheat)



This PGL is devoid of glucosamine and glucuronic acid (80).

Recently PGL-type compounds have been characterized from a wide range of plants. An inositol-free phytosphingolipid was detected in the alga Scenedesmus obliquus (84) which was similar to the phytosphingolipids from peanut and soybean and gave, on hydrolysis, C_{18}-, C_{20}-phytosphingosine, C_{18}-sphingosine and C_{18}-dihydrosphingosine. It contained galactose, mannose, rhamnose, glucosamine, and an unidentified sugar. The fatty acids present were lauric, myristic, palmitic, stearic, oleic, linoleic, arachidic, behenic, lignoceric and two unidentified acids. The difference between algal PGL and that of peanut and soybean was the absence in the former of inositol, glucuronic acid, hydroxy fatty acids and the lower phosphorus content (84).

PGL has also been isolated from plant leaves (Phaseolus vulgaris) (85). In this case the PGL was found to be almost identical to that isolated from flax seed. It consisted of a ceramide attached through a phosphate diester linkage to an oligosaccharide consisting of the basic trisaccharide unit (inositol, hexuronic acid, hexosamine) to which were attached mannose, galactose and arabinose. The major fatty acids were 2-hydroxy C_{22}, C_{24} and C_{26} saturated acids. The major long-chain bases were dehydrophytosphingosine (53%) and phytosphingosine (32%).

A new ceramide-phosphate-polysaccharide was isolated from plant seed phosphatides (corn, soybean, flax, safflower). The basic structural unit consisted of a ceramide-phosphate-inositol-hexuronic acid moiety to which other sugars (galactose, mannose, arabinose) were attached. Flax ceramide-phosphate-polysaccharide contained fucose in addition to other sugars (86).

PGL seems to be a very widely distributed lipid but its function in plant organisms is still not clear. Preliminary studies have been made concerning the biosynthesis of PGL and its function by analyzing the changes in concentration of PGL in the seeds of corn at various stages from one week after pollination to maturity. PGL did not appear to be used as a reserve storage material (87).

Sterol Glycosides

Examples of this type of compounds are not very common in plants and where they have been reported they have not been studied extensively.

A sterol containing glycolipid has been extracted from wheat endosperm (88) and sitosteryl-β'-D-glycoside has been isolated from wheat flour (41) together with phytosteryl-6-O-acyl-β-D-glucopyranoside (89). Nichols (36) identified glycoside sterols in leaves of lettuce and cabbage.

It was also reported that bamboo shoots contained a glycolipid mixture from which was isolated four fractions all containing sterols together with glucose and N-acetylgalactosamine in different proportions. The fatty acids present were palmitic, oleic, linoleic, linolenic and arachidonic acids and two of the fractions contained glycerol (90).

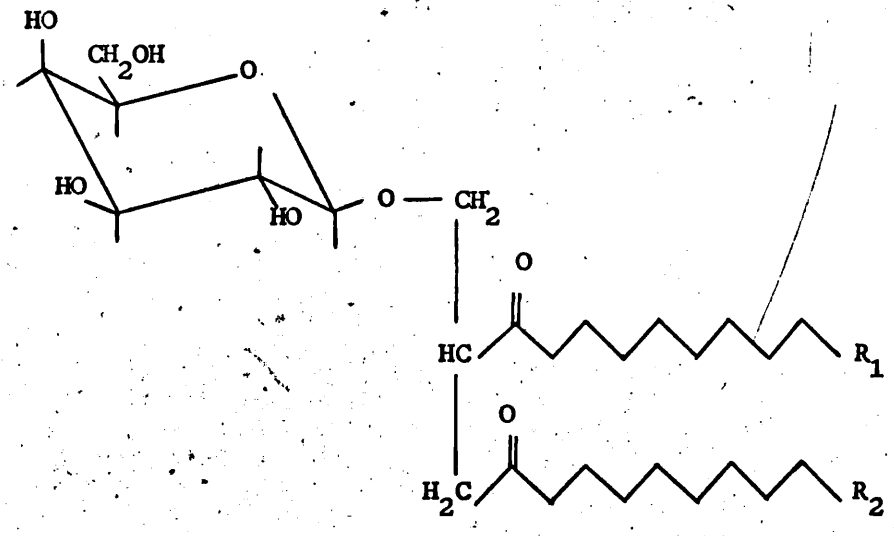
Glycosylglycerides

Galactosyldiglycerides

MGDG and DGDG fractions were first isolated by Carter et al (21) from a benzene extract of bleached wheat flour. A detailed chemical analysis of the deacylated products was carried out, the configuration of glycerol was determined (4,23) and the following structures were deduced (XIV, XV).

In comparison with bleached flour, unbleached wheat flour was found to contain mainly digalactosylglyceride (91) and the authors postulated that chlorine in the bleaching process might have degraded the digalactosylglycerides to monogalactosylglycerides. Galactosylglycerides were also found in wheat gluten (92,93).

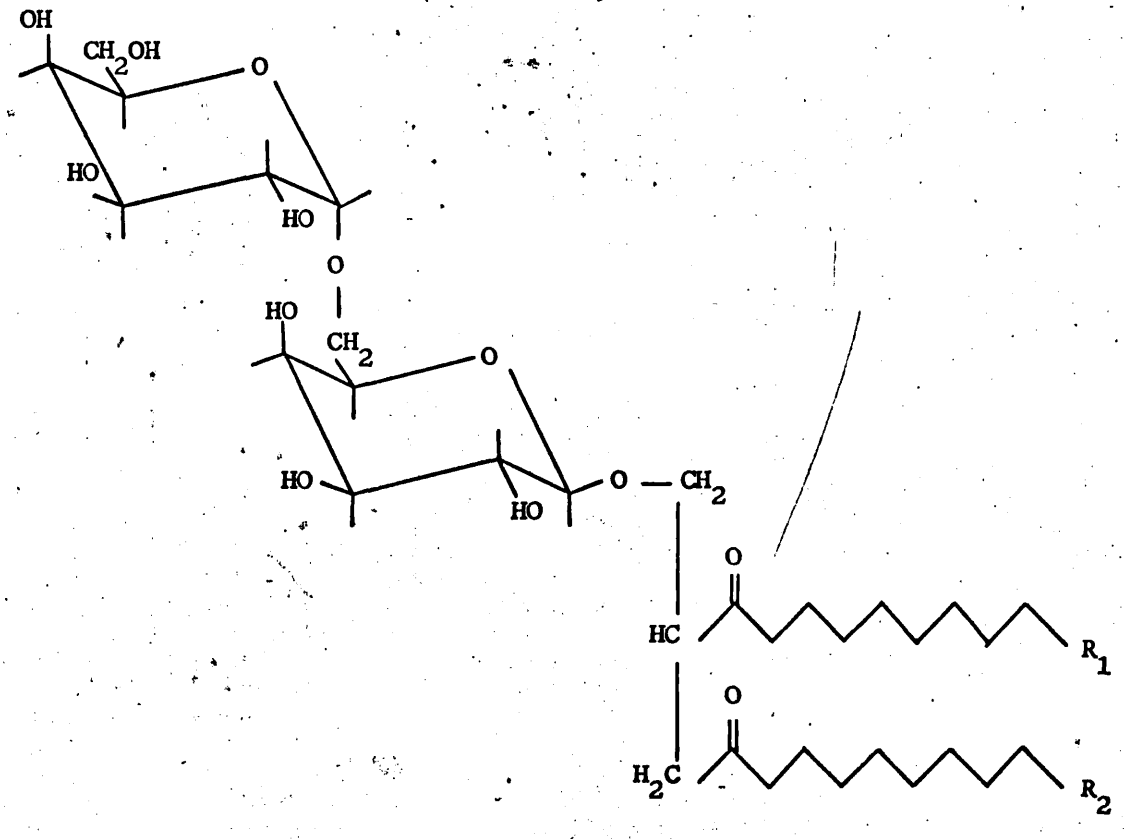
Chlorella pyrenoidosa was observed to contain MGDG and DGDG (94-96) which had similar structures to the compounds present in wheat flour. Quantitative analysis of the lipids in this alga showed that the glycerol-glycolipids concentration was more than twice that of the phosphatides and that the two together formed the major part of the total lipid fraction (38). O'Brien and Benson (22) reported the existence of the



XIV

MGDG

2,3-diacyl-1-β-D-galactopyranosyl-D-glycerol (R_1 and R_2 = fatty acids)



XV

DGDG

2,3-diacyl-1-[α-D-galactopyranosyl-(1⟶ 6)-β-D-galactopyranosyl]-D-glycerol (R_1 and R_2 = fatty acids)

unsaturated acids linolenic and oleic in the galactosylglyceride fractions isolated from Chlorella. MGDG represented 4.2% of the total extractable lipid and DGDG 7.2%.

The lipids of two blue green algae Anacystis nidulans and Anabaena variabilis were fractionated and identified by a combination of DEAE cellulose column chromatography and TLC (97). Both algae resembled Chlorella and the photosynthetic tissues of higher plants in that they contained the four major fatty acid-containing lipids found in chloroplasts, namely, MGDG and DGDG, sulpholipids (eg sulphoquinovosyldiglyceride (SQDG) see p. 36) and phosphatidyl glycerols.

Hirayama (29) compared the glycolipids of the blue green alga Anacystis nidulans with those of spinach leaves; these results are shown in Tables V and Va. Whole cells of Anacystis contained 11% lipids and chloroplast lamellae fragments 20%, both based on dry weight measurements. Spinach showed a much higher lipid content (whole leaves 15%, lamellae 50%).

In contrast to higher plants the galactolipids of blue green algae did not contain major proportions of polyenoic acids (97). DGDG of Anabaena variabilis was an exception in that its fatty acid composition corresponded to that of higher plants.

Wood et al (7) studied the lipid composition of 5 strains of photosynthetic bacteria Rhodopseudomonas spheroides, R.capsulatas, R.palustris, R.gelatinosa and Rhodospirillum rubrum. R.spheroides was the only strain found to contain sulphoquinovosyl diglyceride. No MGDG and DGDG were found in any of the bacteria. The only lipid common to all five organisms to green algae and to plant chloroplasts was phosphatidyl glycerol.

Kates (39,98,99) separated into 10 components the lipids from runner bean leaves by chromatography on silicic acid impregnated paper; these included one sulpholipid and four glycolipids (a,b,c,d). The identification of the individual fractions was based on chromatographic behaviour and reaction to different sprays and on the analysis of acid

Table V (29) - Lipid composition of Anacystis and Spinach
(values : in moles/10 moles of sulpholipids)

Lipids	Anacystis nidulans		spinach leaves	
	whole cells	lamellae	Chloroplasts (38)	lamellae
MGDG	32	30	80	37
DGDG	12	9	33	19
SQDG	10	10	10	10

Table Va (29) - Lipid composition of Anacystis and spinach leaves
(values given as % of total lipid extracts)

Lipids	Anacystis nidulans		Spinach lamellae
	whole cells	lamellae	
MGDG	30	23	24
DGDG	12	10	16
SQDG	11	10	7

and alkali hydrolysis products. However, complete separation of the glycolipids was not achieved.

Improved isolation techniques on silicic acid columns (25,26) permitted the identification of glycolipids a and d as 2,3,-di-O-linolenyl-1-O-β-D-galactopyranosyl-D-glycerol and 2,3-di-O-linolenyl-1-O-β-(6-O-α-D-galactopyranosyl-D-galactopyranosyl)-D-glycerol, respectively,and they accounted for 16.6% and 4%, respectively, of the total bean leaf lipids.

MGDG and DGDG were isolated from the acetone soluble lipid fraction of red clover by Weenink (100,2); they were present in the ratio 2.7:1. The acetone insoluble fraction also contained 25% of galactolipids (101).

Nichols (36) analyzed the lipid content of chloroplast and whole leaves of lettuce and cabbage by thin-layer chromatography with different solvent systems. This was preceded by preliminary fractionation of the lipid mixture on silicic acid column to remove most of the neutral lipids and pigments. The author obtained a very good separation of MGDG and DGDG from phospholipids and sterol glycosides.

Dark green outer leaves of cabbage were observed to contain larger amounts of glycolipids than the light green inner leaves, a fact which might indicate an interdependence between chlorophyll and glycolipid contents. Glycerolglycolipids constituted the major lipid component of spinach, beet and some other leaf chloroplasts (19,20,24,102,103). The glycosylglyceride composition of a number of plant leaves is shown in Table VI (104).

Analysis of MGDG and DGDG in beet showed that these compounds accumulated mainly in chloroplasts and that cell cytoplasm contained negligible amounts (Table VII) (3).

The total lipid content of spinach chloroplasts corresponded to 50% of their dry weight (20,105) but the composition was simple and consisted mainly of glycerol glycolipids. The lipid composition of spinach leaf chloroplast lamellae is shown in Table VIII (105).

Table VI (104) - Glycosylglyceride composition of plant tissues

	µMoles of lipid per g freshweight of leaf		
	MGDG	DGDG	SQDG
Mesotaenium caldoriorum	10.00	5.50	1.30
Marchantia berteroana	1.07	0.66	0.19
Moss	2.68	1.50	0.48
Blechnum fluviatile	5.60	2.30	0.38
Ginkgo biloba	4.70	2.80	0.30
Pinus radiata	2.80	1.95	0.52
Rose (Rosa cv)	5.60	4.60	0.54
Rowan (Sorbus aucupania)	10.20	7.16	0.46
White clover (Trifolium repens)	8.60	5.20	0.76
Lucerne (Medicago sativa)	8.60	5.20	1.72
Poplar (Populus italica)	4.95	3.80	0.62
Camellia japonica	3.10	3.10	0.23
Squash (Cucurbita pepo)	4.10	2.70	0.30
Tomato (Solanum lycopersicum)	5.08	2.46	0.31
Lettuce (Lactuca sativa)	0.68	0.68	0.03
Xanthium orientale	6.10	5.90	0.62
Cocksfoot (Dactylis glomerata)	8.00	5.10	0.62
Perennial ryegrass (Lolium perenne)	5.10	3.95	0.95
Paspalum dilatatum	6.00	3.60	0.62
Maize (Zea mays)	3.10	2.30	0.35
Range	0.68-10.20	0.68-7.16	0.03-1.72
Parsnip root (Pastinaca sativa)	0.17	0.34	Trace

Table VII (3) - Glycolipids in beet leaves. (Amounts per unit volume of fresh leaves expressed as (moles/l) x 10^4)

Material	SQDG	DGDG	MGDG
Whole leaf	9	13	24
Chloroplasts (20% of leaf volume)	3	13.5	24
Cytoplasm (by difference)	6	0	0

Table VIII (105) - Representative distribution of lipids, proteins and inorganic ions in spinach chloroplast lamellae expressed in mole/mole of manganese ion.

Compounds	mole/mole Mn^{2+}
Chlorophyll	115
Carotenoid	24
Quinone	23
Phospholipid	58
MGDG	173
DGDG	72
SQDG	24
Sterols and unidentified compounds	?
proteins	4690N (nitrogen atoms as proteins
Mn^{2+}	1
Fe^{3+}	6
Cu^{2+}	3

Glycosylglyceride containing other sugars

Galactose is not the only sugar component present in plant glycosyldiglycerides. Duncan and Rees (106) isolated glucose-containing lipids, in addition to galactolipids, from starch grains.

An acyl galactosyldiglyceride fraction was isolated from homogenized spinach leaves and this contained glycerol, galactose and fatty acid molecules in the ratio of 1:1:3. The fraction was shown to be a mixture of isomers,the main component being 2,3-di-O-acyl-1-O-(6-O-acyl-β-D-galactopyranosyl)-D-glycerol. The acylated glycolipid fraction was not found, however, when the leaves were boiled in water prior to homogenisation. This fact suggested that the glycolipids were probably formed by an enzymic acyl transfer reaction from di- to monogalactosyl diglyceride during homogenization (107) as, for example, in equation 1. A similar acylated glycolipid fraction was also found in wheat flour (89).

Glycolipids in leaves of Sapium rebiferum Roxb. consisted of monogalactosylglycerol lipids and, diglycosyl and triglycosylglycerol lipids containing galactose, glucose and arabinose (108).

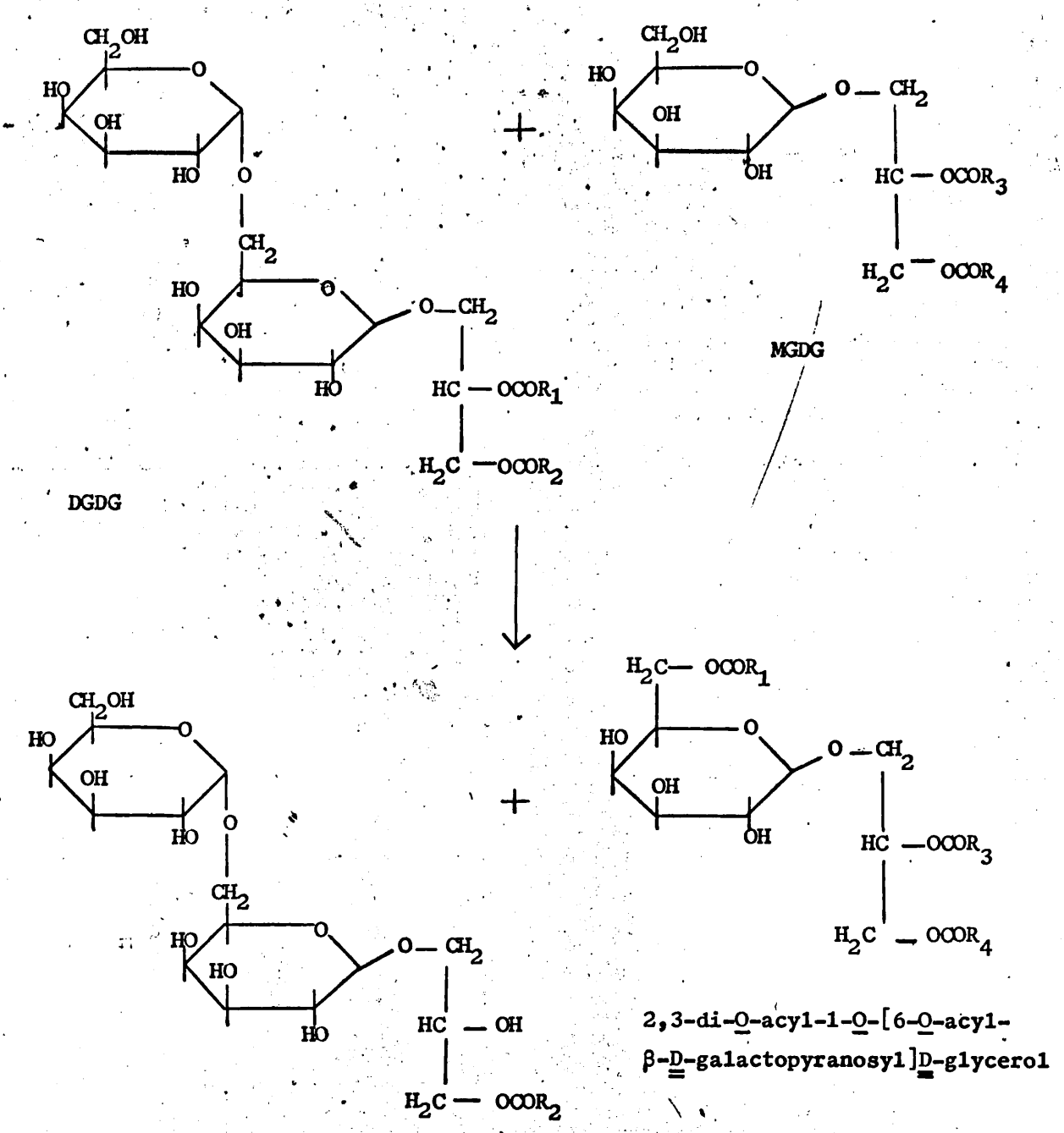
A mannose and erithritol-containing glycolipid fraction was found in Ustilago maydis,a strain of corn smut fungus (109). The general structure XVI was proposed for these compounds.

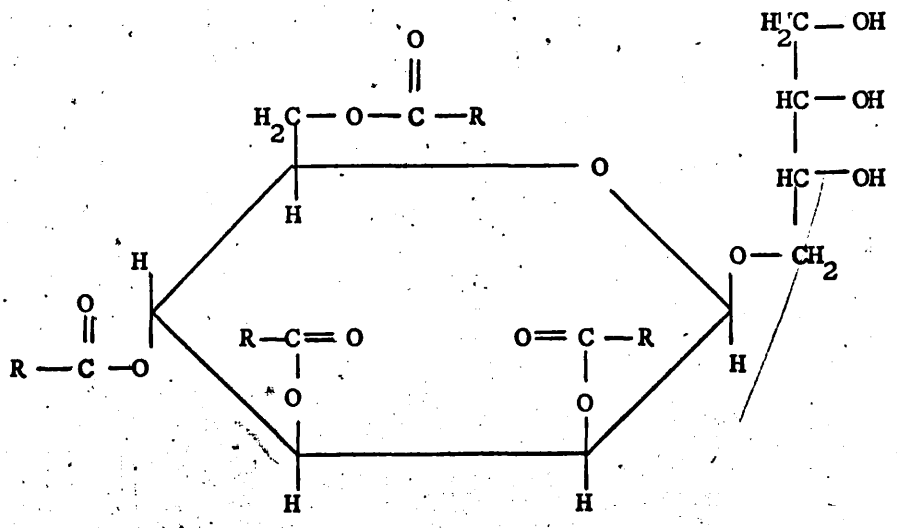
Sulpholipids

A sulphoglycolipid fraction was isolated by Benson et al (110) from the photosynthetic seaweeds, Chlorella, Scenedesmus, from the photosynthetic bacteria Rhodospirillum rubrum, and from higher plant tissues and chloroplasts. The compounds contained a sulphonic acid group ($R-SO_3-H$) which is different from the sulphur-containing lipids of animals (sulphatides); these possess sulphate ester groups ($R-O-SO_3H$). The term sulpholipid was used to denote this difference. The sugar component of the sulpholipids was identified as 6-sulpho-6-deoxy-α-D-glucopyranoside (6-sulpho-α-D-quinovoside) (III). D-glycerol substituted with two acyl groups was also shown to be present (96,112). On the basis of the available data,structure XVII was proposed for the sulpholipids.

Sulpholipids have been found in all photosynthetic tissues so far

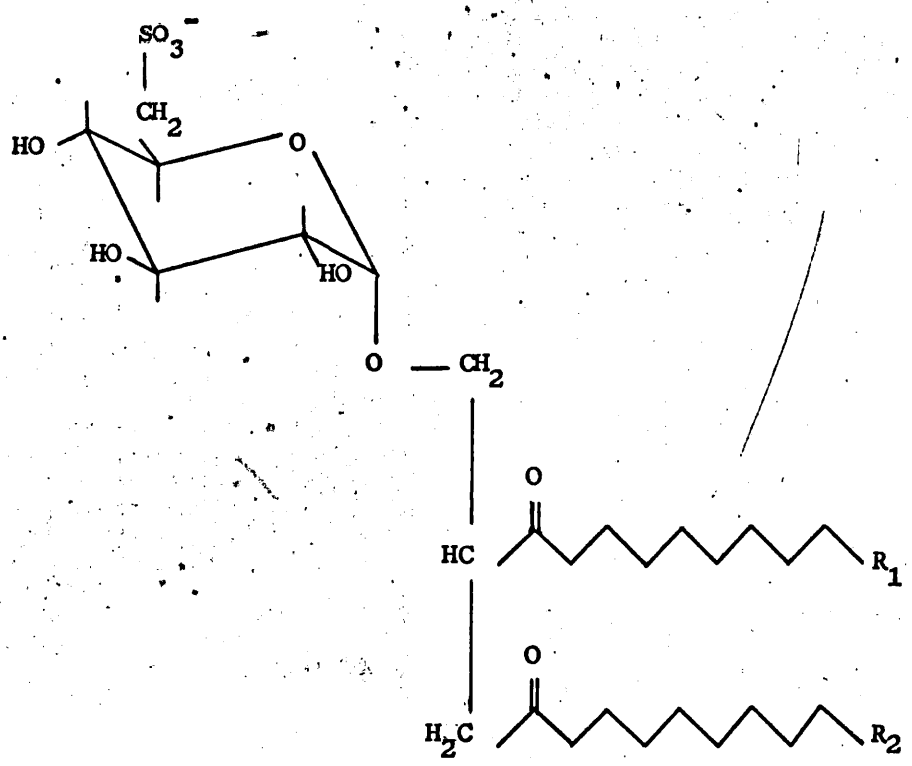
Equation 1


DGDG
MGDG
digalactosylmonoglyceride
2,3-di-O-acyl-1-O-[6-O-acyl-β-D-galactopyranosyl]D-glycerol



XVI

4-O-(2,3,4,6-tetra-O-acyl-β-D-mannopyranosyl)-D-erithritol



XVII

2,3-diacyl-1-(6-sulpho-α-D-quinovopyranosyl)-D-glycerol
(R_1 and R_2 = fatty acids)

investigated (113) eg cabbage and clover (36), narcissus bulbs (30), potato leaves and tubers (34,114) and spinach chloroplasts and whole leaves (19,20,102,105).

The concentration of sulpholipids in a particular tissue always appears to be lower than that of the other glycosylglycerides (104).

O'Brien and Benson (22) isolated sulpholipids from alfalfa leaves and from Chlorella pyrenoidosa cells; they observed that they represented 2.1 and 1.9%, respectively, of the total lipid concentrations in these two plants. Sulpholipids have also been found in blue green algae (29, 91) (Table Va).

Kates (39) isolated a sulpholipid from runner bean leaves and reported that it was different from other plant sulpholipids. This was subsequently disproved by Davies et al (115), however, who identified the same sulpholipid in Euglena gracilis and maize. The latter workers showed that sulpholipids accumulated in chloroplasts but that some were also present in other parts of the cell. Etiolated Euglena cells and maize seedlings, sections of maize stems and cultured cambial cells, all of which contained little or no chlorophyll, also contained sulpholipids but in low concentrations.

Wintermans (3), examining the glycolipids of beet leaves, reached similar conclusions. He identified sulphoglycolipids in the cytoplasm of beet leaf cells (Table VII).

Collier and Kennedy (116,117) fractionated sulpholipid mixtures from several plants by paper chromatography; three components were detected which, according to the authors, probably corresponded to complexes of sulpholipids with chlorophyll.

A sulphoquinovosyl-diacyl glycerol was reported to occur in Antirrhinum leaves (5.2%), Dryopteris filix-mas (4%), Fucus vesiculosus (18.3%), Bactrachospermum moniliforme (14.9%), Oscillatoria chalybia (13.9%) and Rhodopseudomonas spheroides (2.6%) (118).

Kates et al (119) isolated an unusual sulpholipid from Halobacterium cutirubrum which was identified as a derivative of di-O-phytanyl-L-

glycerol (3',7',11',15'-tetramethyl-hexadecyl-L-glycerol). On the basis of analytical and degradative data two possible structures for the glycolipid sulphate ester were suggested:

a) $^{-}O_3S$-O-3-Gal-(1→ 6)-Man-(1→ 2)-Glu-(1→ 1')-2',3'-di-O-phytanyl-L-glycerol

b) $^{-}O_3S$-O-3-Gal-(1→ 2)-Glu-(1→ 6)-Man-(1→ 1')-2',3'-di-O-phytanyl-L-glycerol

Fatty acid composition of plant glycerolglycolipids

GLC studies on the fatty acid composition of plant glycerol-glycolipids have shown that galactolipids of photosynthetic tissue always contain a high concentration of unsaturated long-chain acids, mainly α-linolenic acid.

α-Linolenic acid was shown to be primarily concentrated in the MGDG and DGDG fractions of the blue green alga Anabaena variabilis (97), but Anacystis nidulans resembled photosynthetic bacteria and contained mainly monoenoic acids (ie $C_{16:1}$ and $C_{18:1}$) and palmitic acid (29,97).

O'Brien and Benson (22) have studied the fatty acid composition of galactosyl glycerides in alfalfa leaves and Chlorella pyrenoidosa. The MGDG of alfalfa leaves was shown to be highly unsaturated, containing 95% α-linolenic acid whereas DGDG in the same plant was less unsaturated with 82% α-linolenic acid and a small amount of palmitic acid. In Chlorella the fatty acid compositions of MGDG and DGDG were shown to be similar and to consist mainly of 18:1, 18:3, 16:1 and 16:0 acids.

The galactosyl diglycerides of Chlorella vulgaris (9) contain a higher proportion of polyunsaturated acids than the other lipids present. A higher proportion of these acids was found in MGDG than in DGDG. In fact MGDG contains most of the hexadecanoic and trienoic acids present in Chlorella and in this respect is similar to higher plants (19,25,30). The difference in fatty acid composition of Chlorella pyrenoidosa (22), where $C_{18:1}$ acids predominate, and Chlorella vulgaris (9), which contains

mainly $C_{18:3}$ acids, both growing under the same conditions, has not so far been explained.

Galactolipids isolated from bean and clover leaves (2,26,100) contain a high concentration of α-linolenic acid.

MGDG in spinach contains 67% linolenic acid and a relatively large amount (30%) of hexadecatrienoic acid which is concentrated mainly in the MGDG fraction. DGDG contains only small amounts of hexadecatrienoic acid together with 84% linolenic acid (19).

A different fatty acid composition is found in the glycolipids of corn flour which has been reported to be rich mainly in linoleic, oleic, and palmitic acids. Very little linolenic acid was found (4).

The fatty acid composition of bush bean leaf chloroplasts and proplastids has been examined. It was found that high concentration of chlorophyll corresponded to high concentrations of unsaturated fatty acids in comparison with saturated acids, and also there were more C_{18} acids than C_{16} (120).

The fatty acid composition of MGDG and DGDG fractions from several plants is shown in Table IX.

The substitution pattern of the fatty acids on the glycerol moieties of galactolipids of Artemisia princeps leaves was determined by Noda et al (121). The glycolipids were first hydrolyzed with a pancreatic lipase to the corresponding lysocompounds which were then further hydrolyzed by acid or by β-galactosidase. The fatty acids released were determined by GLC. Saturated and monoenoic acids were found to be attached mainly to the 3-position of glycerol and linolenic to the 2-position.

Plant sulpholipids are more saturated than the corresponding galactosyldiglycerides. The sulpholipids of alfalfa leaves contain almost equal quantities of palmitic and linolenic acids, and with the sulpholipids from Chlorella pyrenoidosa palmitic acid predominates and there is a low (10%) linolenic acid content (122).

The sulpholipids of spinach leaves contain mainly linolenic, linoleic and palmitic acids (19) and the chloroplast lamellae lipids

Table IX (101) - Fatty acid composition of galactolipids of different plant tissue. (contents expressed as %)

Fatty acid	Corn flour (4)		Chlorella pyrenoidosa (22)		Spinach leaves (19)		Alfalfa leaves (22)		Bean leaves (26)	
	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG	MGDG	DGDG
14:0	0.5		trace	trace			trace	trace		
16:0	13.9	41.6	2.7	11.6	trace	6	2.7	14.0	2.3	4.5
16:1	3.4		9.7	9.5			trace	trace	trace	trace
16:3					30	3	trace	6.7		
17:0			1.5	1.2			0.7			
18:0	1.3	4.4	0.3	0.4		1	0.2	3.3	trace	1.0
18:1	17.2	12.1	40.5	36.8	1	4	0.3	0.4	trace	trace
18:2	57.0	29.3	4.5	5.8	1	3	1.7	0.8	2.2	1.3
18:3	2.0		26.8	27.0	67	84	95.0	82.0	95.5	93.2
19:0			12.0	3.3						
18:4			2.6	3.3						
Others	5.1	12.6								

are rich in trienoic acids (more than 90% of 16:3 and 18:3) (20) (Table X).

Lipids were extracted from alfalfa samples collected at intervals over the growing season and these were fractionated to yield a pure sulpholipid fraction. The major fatty acids were palmitic, linolenic and linoleic. Palmitic acid increased as the plant matured whilst the linolenic acid concentration decreased. Thus there was an apparent increase in saturation of the sulpholipids with tissue maturation. If this observation can be generally applied to plants then it will not be possible to assign a characteristic fatty acid composition to a plant lipid on the basis of only one analysis (122).

Table X (101) - Fatty acid composition of sulpholipids from different plant tissues. (contents expressed as %)

Fatty acid	Chlorella pyrenoidosa (22)	Alfalfa leaves (22)	Spinach leaves (19)	Spinach leaves lamellae (20)
14:0	trace	trace		0.2
16:0	67.5	37.4	27.0	38.5
16:1	1.6	1.9		
16:2				0.1
16:3				0.6
18:0	0.4	7.1		0.5
18:1	18.3	2.6	6.0	0.8
18:2	2.0	1.9	39.0	6.5
18:3	9.8	49.0	28.0	52.5

Metabolism of Glycosyldiglycerides

The biosynthesis of glycosylglycerides in Chlorella pyrenoidosa (38) was examined by allowing the organism to photosynthesize for varying periods of time in the presence of $^{14}CO_2$. More than 50% of the total label incorporated into lipids appeared in the galactolipid fraction and the metabolism of these compounds was shown to be independent of phosphatide metabolism. The results showed that MGDG was probably converted to DGDG by galactosylation involving UDP-galactose. The following sequence of reactions for the formation of galactosylglycerides from CO_2 was suggested:

$CO_2 \longrightarrow$ phosphoglyceric acid $\longrightarrow$ UDP-glucose $\rightleftharpoons$ UDP-galactose

UDP-gal + D-2,3-diglyceride $\longrightarrow$ galactosyldiglyceride

Galactosyldiglyceride + UDP-gal $\longrightarrow$ digalactosyldiglyceride

O'Brien and Benson (22) later showed that both MGDG and DGDG had similar fatty acid compositions and hence this supported the theory that MGDG was the precursor of DGDG.

Galactolipid formation in Chlorella vulgaris has been studied by Nichols (10) with the alga growing on purely inorganic media in the light, and on organic media both in the light and in the dark. The proportions of MGDG, DGDG and SQDG increased relative to the total lipid when the organism was grown in the light. Nichols suggested that the metabolism of these three galactolipids was probably similar in both higher plant chloroplasts and Chlorella. Prolonged cultivation of Chlorella in the dark produced etiolated cells but these were not devoid of these chloroplast lipids, therefore it was suggested that photosynthesis was probably not an essential prerequisite for their formation. This behaviour was in contrast to that of Euglena gracilis (a photosynthetic microorganism) which when grown in the dark produced little galactosyldiglycerides (123, 124).

Abraham and Bachhawat (125) studied the metabolism of sulpholipids in Euglena gracilis by examining the incorporation of $^{35}SO_4^{=}$ into the cells. The results indicate that the sulphate uptake and activation is

light-independent whereas the synthesis of the sulpholipid is dependent on light. This may be due to the fact that one of the most important steps in the utilization of sulphate by higher plants and bacterial systems is the reduction of sulphate to sulphite. In microorganisms, sulphate must be activated to PAPS (3'-phosphoadenosine-5'-phosphosulphate) before it can be reduced to sulphite. Although the formation of PAPS by the Euglena enzyme system is light-independent, the impairment of sulpholipid synthesis, when Euglena is grown in the dark, might be due to diminished photosynthetic formation of ATP.

Neufeld and Hall (126) found that isolated spinach chloroplasts catalyzed the transfer of galactose from UDP-D-galactose-^{14}C to an endogenous acceptor, yielding alkali-labile products which were similar although not identical to the normal constitutive galactolipids from plant tissues. The products arising from alkali hydrolysis of the synthetic products were identified as mono-, di-, tri-, and possibly tetra-galactosylglycerols. The synthetic MGDG possessed a β-D-galactosyl residue but the terminal residue of the DGDG was 17% α and the remainder β; DGDG normally possesses only α-linked terminal galactose moieties.

Enzyme preparations from etiolated spinach leaves and non-chlorophyllic tissues were also shown to catalyze the synthesis of galactolipids(130)and a particulate enzyme from mung beans catalyzed the incorporation of glucose from UDP-glucose-^{14}C into two compounds which were soluble in a chloroform-methanol mixture (13).

Sastry and Kates (127) observed that runner bean leaves contained an enzyme system that catalyzed the hydrolysis of mono- and di-galactosyl dilinolenin to galactosylglycerol and linolenic acid. Two enzymes were thought to be involved (125). The preparation was active only towards unsaturated galactolipids. There was no evidence for the formation of "lyso" compounds (galactosyl monoglycerides) although they were thought to be intermediates in the hydrolysis. Both α- and β-galactosidases were

also detected in the leaf enzyme system which catalyzed the hydrolysis of mono- and di-galactosylglycerols to galactose and glycerol. On the basis of these results the following pathway for the breakdown of galactosylglycerides *in vivo* were suggested:

a) Removal of the fatty acid residues

(di) monogalactosyl diglyceride ⟶ (di) monogalactosyl-monoglyceride + fatty acid

(di) monogalactosylmonoglyceride ⟶ (di) monogalactosyl-glycerol + fatty acid

b) Hydrolysis of the galactosyl residues

digalactosylglycerol $\xrightarrow{\alpha\text{-galactosidase}}$ monogalactosylglycerol

monogalactosylglycerol $\xrightarrow{\beta\text{-galactosidase}}$ glycerol + galactose

A galactolipase was found in spinach leaf extracts by Helmsing (128) and from the results of the investigation it was concluded that the hydrolyses of MGDG and DGDG were both catalyzed by one enzyme system; this is in direct contrast to conclusions of Sastry and Kates (125).

Analyses of the glycolipids of *Chorella vulgaris* (9) showed that MGDG contained a higher proportion of polyunsaturated acids than DGDG, including most of the hexadecadienoic and trienoic acids that were present in *Chlorella*. The same observation was made in the case of spinach leaves (19). This difference between fatty acid residues in MGDG and DGDG appears to refute the hypothesis that DGDG is formed by the direct galactosylation of MGDG (22,38) unless there exists a selective deacylation-reacylation enzyme system in the case of these lipids (19). Furthermore, Nichols *et al*, (129) fractionated MGDG from *Chlorella vulgaris* into several components with differing fatty acid compositions. These differences are thought to be due either to the varying specificities of the acyl transferases involved in the synthesis of the diglyceride precursors of these lipids, or to changes in fatty acid structure occuring after the *de novo* synthesis of the MGDG. The authors favoured the second hypothesis.

Yagi and Benson (112) located a specific deacylase (sulpholipase) in *Scenedesmus* extracts which catalyzed the hydrolysis of sulphoquinovo-

syldiglyceride to a lyso-sulpholipid (containing one acyl group). A similar sulpholipase was found in Chlorella, alfalfa leaves and roots, and corn roots.

Function of plant glycolipids

Euglena gracilis can be grown as an autotroph or a heterotroph; the metabolism is obviously changed by different conditions of culture(11).

When the organism is grown in the light galactolipids and sulpholipids accumulate and these decrease when it is transferred to the dark (47,123, 131). At the same time the fatty acid composition changes. In the light, highly unsaturated acids, mainly α-linolenic acid, predominate whereas in cells cultured in the dark the monoenes are prevalent (123,131). It was suggested by Rosenberg et al that galactolipids could be energy reserves in green cells, and that galactolipids containing particular fatty acid components might also perform some function in the assembly of chloroplasts. α-Linolenic acid in Euglena gracilis, as in higher plants, is primarily a chloroplast component (11). It is found universally in photosynthetic organisms but there are different degrees of organisation and morphological complexity of the various photosynthetic apparatuses.

Photosynthetic bacteria with a level of morphological organisation which is identical with that of blue-green algae and equally primitive, are devoid of α-linolenate or any other polyenoic acid and these bacteria are the only photosynthetic organisms which are unable to evolve oxygen during photosynthesis (8).

Anabaena variabilis (8), a blue-green alga, is related morphologically to photosynthetic bacteria but functionally and biochemically resembles higher plants. It produces oxygen during photosynthesis and contains α-linolenic acid. Galactolipids were found to have stimulatory effects on the rate of cytochrome C photoreduction by intact chloroplasts obtained from fresh spinach (132).

These facts suggest that in higher plants and algae α-linolenic acid might be involved in electron transport or in the production of molecular oxygen (8,132).

However, Holton et al (123) did not find α-linolenic acid in the blue-green algae Anacystis nidulans (which performs the Hill reaction). Nichols et al (97) confirmed this result and showed also that the algae

does not contain trans-Δ^3 - hexadecenoic acid which might have, according to the author, a relationship with the Hill reaction (9,134). It is therefore difficult to support the concept of involvement of unsaturated fatty acid in the major pathway of photosynthesis (135), but it seems that "the basic lipid requirement for photosynthesizing cells capable of performing the Hill reaction is the presence of the galactosyl diglycerides, sulpholipid and phosphatidyl glycerol. These lipids are probably required for the maintenance of the highly organised structures present in the chloroplast, and their fatty acid compositions may be important only in providing molecules of a required geometry" (135).

Glycolipids are thought by some authors to play a role in the biosynthesis of cell-wall polysaccharide.

This theory was advanced as an answer to the problem of transport of molecules through cell membranes composed of proteins and lipids (including glycolipids). Cell-walls are arranged outside the membrane and thus two problems arise regarding their formation. If cellulose is synthesized within the cell, then how can such insoluble polymers be transported through the membrane to the growing wall? If instead one assumes that the polysaccharides are formed outside the cell then, how can highly polar precursor molecules such as sugar nucleotides go through lipophilic membranes? (136)

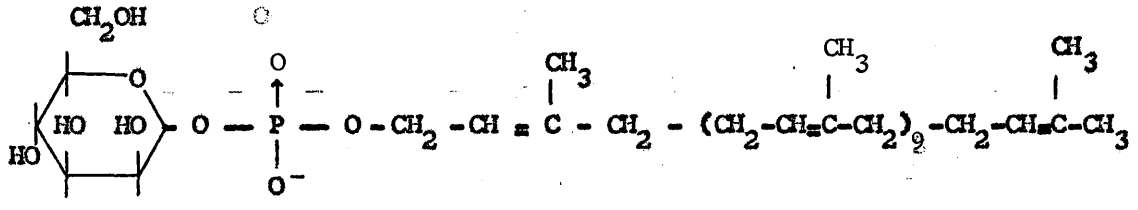
On the other hand it has not been demonstrated that sugar-nucleotides cannot be transported through the cell membrane. Higher plants might also synthesize polysaccharides within the cell-membrane thus completely dispensing with the necessity of transporting precursors. A number of cellular polysaccharides, such as chitin, are synthesized from sugar-nucleotides (136,137).

Many workers in recent years have examined the theory that glycolipids are involved in cell-wall biosynthesis, in both bacteria and higher plants.

A glycolipid was found to be an intermediate in the biosynthesis of cellulose by Acetobacter xylinum. The compound extracted with 80% ethanol

Was converted to cellulose when placed in an aqueous medium. The conversion took place in the absence of bacterial cell walls and the formation was accelerated by a heat-labile extracellular substance presumably an enzyme (14). Furthermore, using the cellulose-synthesizing enzyme of Acetobacter Colvin observed the formation of cellulose from precursors extracted from higher plants with 80% ethanol (15).

Glycolipids were shown to be intermediates in the formation of cell-wall components of several microorganisms,for example in the synthesis of glycopeptides in Staphylococcus aureus (138). In addition a lipid linked intermediate (trisaccharide lipid), was found to be involved in the formation of polysaccharide chains of the O-Antigens of different Salmonella (139-141). An alkali-stable, acid-labile lipid formed from GDP-mannose and an endogenous lipid in crude, cell-free preparations of Micrococcus lysodeikticus was shown to be mannosyl-1-phosphoryl-polyisoprenol (MPP).



MPP

This compound could behave as a coenzyme and serve as a mannosyl donor in the enzymic synthesis of mannans, as well as in the formation of more complex cell-wall peptido-glycans (142,143).

It has been repeatedly shown that in plants there exist enzyme systems which catalyze the synthesis of cell-wall polysaccharides from sugar nucleotide derivatives (144-151).

Cellulose has been synthesized with a particulate system from mung bean seedlings using GDP-glucose as glucose donor (145,152). In vitro the rate of formation of cellulose was considerably lower than in vivo. The experiments demonstrated that GDP-glucose was the only effective donor

but there might be a possibility of a more immediate precursor such as glucolipid. None of the data contradicted this hypothesis but it was shown that the addition of different lipid fractions from mung beans did not stimulate cellulose formation (145). However, GDP-glucose is not the only glucose donor in the synthesis of cellulose-like polysaccharides; particulate enzyme preparations from Lupinus albus (147,153) and Avena sativa (154) were reported to catalyze the incorporation of D-glucose-^{14}C from UDP-D-glucose-^{14}C as well as GDP-D-glucose-^{14}C into such polysaccharides. Villemez et al (151) showed that in P.aureus (mung bean) alkali insoluble materials are synthesized from UDP-D-glucose and that the rate of this synthesis is 23 times greater than that from GDP-D-glucose. He reported later (13) the incorporation of D-glucose-^{14}C from UDP-D-glucose-^{14}C into a glucolipid using a particulate enzyme preparation from P.aureus. The properties and the distribution of this enzyme are similar to those of the enzymes involved in cell-wall polysaccharides syntheses, but a function cannot be definitely assigned to this glucolipid.

New evidence was reported recently to support the theory of a glycolipid intermediate in cell-wall polysaccharide synthesis. Incorporation studies of GDP-mannose in cell-wall polysaccharide of P.aureus provide evidence that a lipid soluble material is a direct precursor of higher plant cell-wall polysaccharide. The intermediate is acid labile and has the solubility properties of complex glycolipids. It is also tightly bound to the particulate enzyme system. There is little doubt that the radioactive intermediate incorporate into polysaccharide directly (155). The mannolipid is similar to the intermediate (MPP) in M.lysodeiticus mannan biosynthesis. The lypophilic portion of P.aureus mannolipid is probably a long chain isoprenoid (156).

RESULTS AND DISCUSSION

The evidence which favours the involvement of glycolipids in the biosynthesis of plant polysaccharides has been discussed (see p. 50) and the study described in this thesis was originally undertaken in an attempt to further this theory. The complex nature of the glycolipids from mung beans has been examined together with a preliminary investigation of the incorporation of label from sucrose-^{14}C into the glycosyl residues of the lipids. The occurrence of fructose-containing lipids in bean shoots promoted experiments to find a possible pathway for the biosynthesis of UDP-fructose, a likely precursor of the fructolipids.

Mung bean (Phaseolus aureus) was chosen for this work because of ease of germination and because many facts concerning the carbohydrate content and biochemistry were already known (144,145,146,148,151).

I Qualitative studies on the polysaccharides of mung bean shoots

First experiments were designed merely to determine the nature and variety of the combined monosaccharides (mostly as polysaccharides) present in germinating bean tissues, with a view to possibly relating polysaccharide and glycolipid structures.

Following a preliminary separation of neutral and acidic sugars by anion exchange chromatography, acid hydrolysates of mung bean shoots were examined by paper chromatography, GLC and paper electrophoresis. In addition, a crude fractionation of the various tissue polysaccharides was carried out and the monosaccharide components of each fraction similarly examined. The polysaccharides occurring in 1,000 and 10,000g fractions from the cells were also studied.

The qualitative results (Table XI) obtained from this general survey showed that mung bean shoots contained all of the normal combined monosaccharides which are found in higher plants. Bailey et al (157) have also examined the 1,000 and 20,000g particulate fraction from mung bean shoots and detected some of the bound sugars listed in Table XI.

Table XI - Bound monosaccharide constituents from various Mung bean tissue extracts and fractions

	FRACTIONS							
Monosaccharides	Neutral* sugars	Acidic* sugars	Cold water extract	Hot water extract	1N-HCl extract	1N-NaOH extract	1,000g cell fraction	10,000g cell fraction
Galactose	+		+	+ + +	+ + +	+ + +	+ + +	+ + +
Glucose	+		+ + +	+ + + +	+ + +	+ + + + +	+ + + + +	+ + +
Mannose	+				trace	trace		
Arabinose	+		+	+ +	+ + +	+ + +	+ + +	+ + +
Xylose	+		+	+	+	+	+	+
Rhamnose	+		trace	+	+	+	+	+
Ribose	+			+ +	+		+	+
Glucuronic acid		+						
Galacturonic acid		+						

*Hydrolysate of 80% methanol-extracted shoots

II Structural studies on glycolipids of mung bean seedlings

Isolation of glycolipids and identification of their monosaccharide components. Glycolipids were isolated by extraction of mung bean shoots with 80% aqueous methanol (or ethanol). Similar extractions of 1,000g and 10,000g particulate fractions were also made. Free sugars were absent from all the glycolipid fractions as judged by paper chromatography. The glycolipids were observed to be very labile to acid hydrolysis (eg 0.01N H_2SO_4 at 100°; see Table XII).

Table XII - Release of monosaccharides from total glycolipids with 0.01N - H_2SO_4

Monosaccharide liberated	1hr. hydrolysis	2hr. hydrolysis
Galactose		+ +
Glucose	+ + +	+ + +
Mannose/Arabinose/Fructose	+ + +	+ + + +
Xylose	+	+
Rhamnose	+	+

Hydrolysis of the total shoot glycolipids with 0.01N-H_2SO_4 only released galactose after heating at 100° for 2 hr. Table XIII shows the effect of hydrolysis under more rigorous conditions (N-H_2SO_4 for 2 hr.) which indicates that the total glycolipid and 1,000 and 10,000g fractions have similar compositions. A glycolipid fraction obtained by Bailey et al (157) from a 20,000g particulate preparation was observed to possess a similar monosaccharide composition but the Californian group did not report the presence of mannose or fructose as glycolipid components. It was, therefore, necessary to confirm the presence of the lipid-bound hexoses.

Table XIII - Hydrolysis products resulting from treatment of glycolipid fractions with $\underline{N}$-H_2SO_4 for 2 hr. at 100°

Monosaccharide liberated	1,000g fraction	10,000 fraction	total glycolipid fraction
Galactose	+++	++	+++
Glucose	+++	+++	+++
Mannose/Arabinose/ Fructose	+++	+++	++
Xylose	trace	trace	trace
Rhamnose		+	+

Further isolation of mannose from the hydrolysate of bean shoot glycolipids by preparative paper chromatography followed by paper chromatographic purification yielded an apparently homogeneous hexose with the same mobility as standard mannose in two solvent systems. Further confirmation of structure was obtained by gas-liquid chromatography of the trimethylsilyl derivative which resulted in two peaks (5.2cm, 8.5cm) which corresponded exactly to those given by standard mannose.

Further characterization of fructose was carried out with ketohexose which had been isolated after fractionation of the total glycolipid. This characterization is discussed on page 66.

Results of a quantitative analysis of the monosaccharide components of the total glycolipid fraction from shoots using the Wilson method (158) are shown in Table XIV. Fructose was determined with the triphenyl tetrazolium chloride colourimetric reagent (159,160).

TableXIV - Quantitative determination of the monosaccharide constitutents of total glycolipid fraction*

Monosaccharide	Ratio by weight
Galactose	2.4
Glucose	1.9
Mannose	1.5
Arabinose	1.5
Xylose	Trace
Rhamnose	1

* (hydrolysis with N-H_2SO_4 for 2 hr. at 100°)

Table XV - Monosaccharide constituents of the glycolipids obtained by solvent fractionation

Monosaccharides	Chloroform extract	Acetone extract	80% aqueous Methanol extract
Galactose	trace	+	+
Glucose	trace	+	trace
Mannose	-	-	+
Fructose	-	+	trace
Arabinose	-	-	+
Xylose	-	-	-
Rhamnose	-	+	trace
Glycerol	+	+	trace

Glycerol was also identified in the total glycolipid hydrolysate suggesting that the lipids in mung bean consisted, at least in part, as glycerides with sugar moieties attached through glycosidic linkages to the alcohol groups of glycerol.

Solvent fractionation - In an attempt to fractionate the glycolipids, bean shoots were extracted successively with cold chloroform, acetone and finally boiling 80% aqueous methanol. The glycolipids present in the different extracts were hydrolysed with acid and the resulting monosaccharides identified by paper chromatography (Table XV).

The chloroform fraction contained glycerol but only traces of monosaccharides. This can be explained by the fact that chloroform would tend to extract mainly neutral lipids (e.g. waxes, steroids, hydrocarbons, triglycerides etc.) and only traces of the more polar glycolipids would be removed from the tissues with this solvent. The acetone and 80% aqueous methanol fractions differed in their monosaccharide compositions. Acetone, the less polar solvent, extracted galactose, glucose, fructose, rhamnose and glycerol. It is likely that acetone would only extract glycolipids with a relatively low monosaccharide content in view of the polarity of the solvent. 80% Aqueous methanol, a more polar solvent than acetone, extracted glycolipids containing galactose, arabinose and mannose but only traces of glucose, fructose and glycerol. The absence of glycerol suggested that the glycolipids in this fraction were not glycosylglycerides but were more likely to be of the sphingolipid type (i.e. with the carbohydrate residues linked to the fatty acid through a long chain base of the sphingosine family). The monosaccharides identified in this fraction could be components of a complex glycolipid containing galactose, mannose and arabinose, possibly similar to seed phytoglycolipid (see p. 21) or, alternatively, they could be derived from a series of simpler glycolipids containing a single type of monosaccharide. The first hypothesis seems more likely in view of the many examples of complex glycolipids containing sphingosine bases, galactose, mannose and arabinose, which have been reported to occur in a wide variety of plants (85,86).

Column fractionation - Two glycolipid fractions, soluble in acetone and 80% methanol, respectively, were prepared and fractionated on a silicic acid column using the phenol/H_2SO_4 method to locate peaks. The elution profiles of both fractions (Figures 1,2) revealed a very complex pattern which was difficult to interpret. Many factors could influence the fractionation of the glycolipids such as the nature of the combined sugars and fatty acids. Each of the six peaks of the 80% methanol fraction when examined by thin layer chromatography (on silica gel G; chloroform:methanol:water (90:10:1, v/v) solvent), was fractionated into three groups of components (x(slow), y and z; figure 3). Component x moved from the base line when a more polar solvent (chloroform:methanol:water (60:40:1, v/v)) was used. Peaks 1,2 and 3 from the column contained only traces of x, but in general the only difference between the peaks was the relative proportions of the three components. It is assumed that fractionation on the column with solvents of increasing polarity depended on differing carbohydrate contents, whereas in the case of TLC, using solvents with high concentrations of chloroform, differences in lipid composition determined the fractionation.

The different column fractions obtained from both the 80% methanol- and acetone-soluble glycolipids were hydrolysed and their carbohydrate constituents determined by paper chromatography (Tables XVI, XVII).

Two of the peaks from the acetone-soluble glycolipids contained combined glucose, galactose, fructose and rhamnose, one glucose and galactose and one glucose and fructose. Glycerol was probably present in all of the hydrolysates (see p. 67) but the problem here is that this triol is difficult to detect in small amounts on paper chromatograms.

The column fractions from the aqueous methanol-soluble glycolipids could be divided into two groups : those containing mainly galactose, mannose and arabinose, and those containing only glucose and arabinose. In this case glycerol was more readily detectable in most fractions.

Fig. 1 Silicic acid chromatography of 80% methanol glycolipid fraction from mung bean shoots

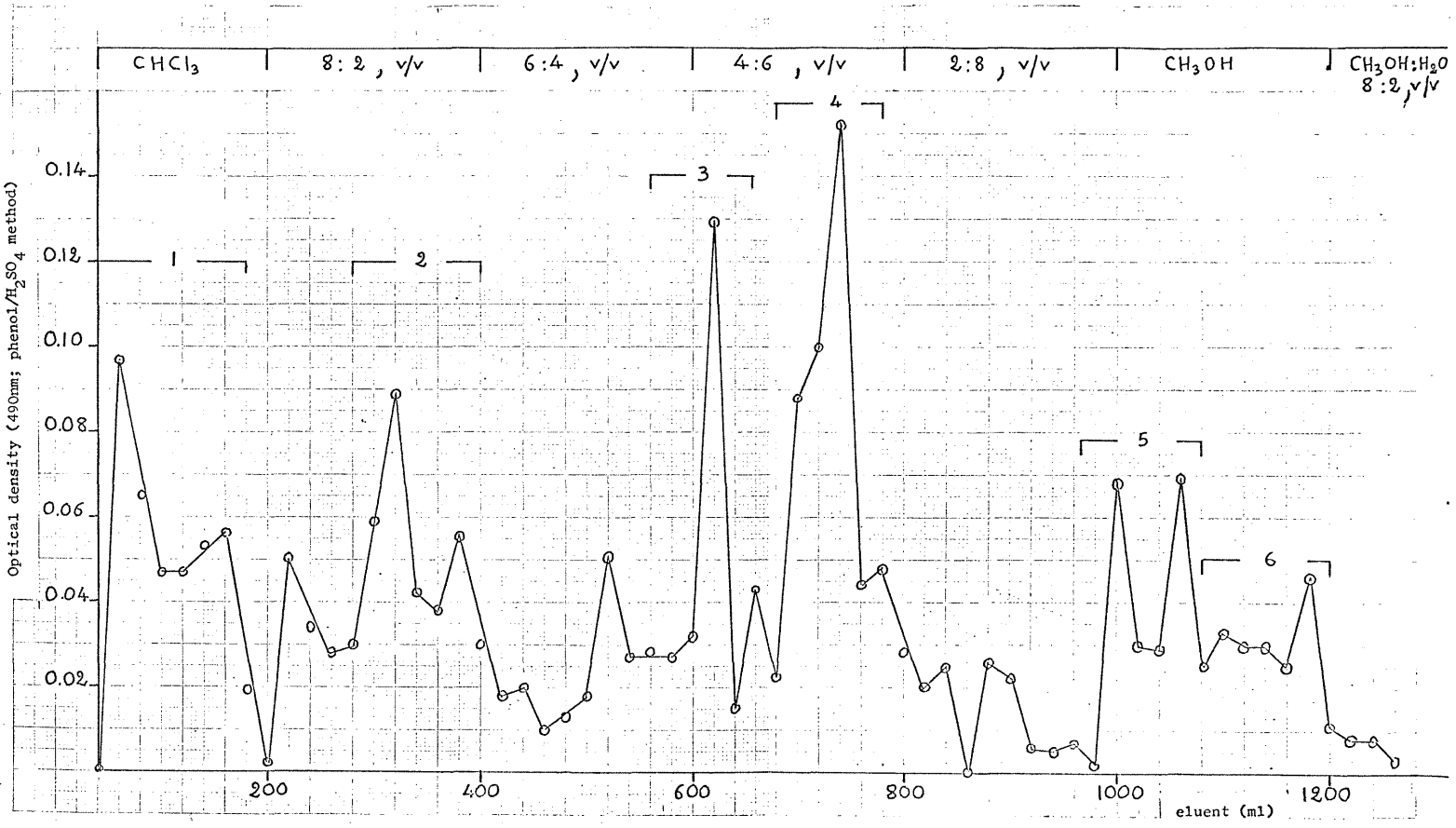
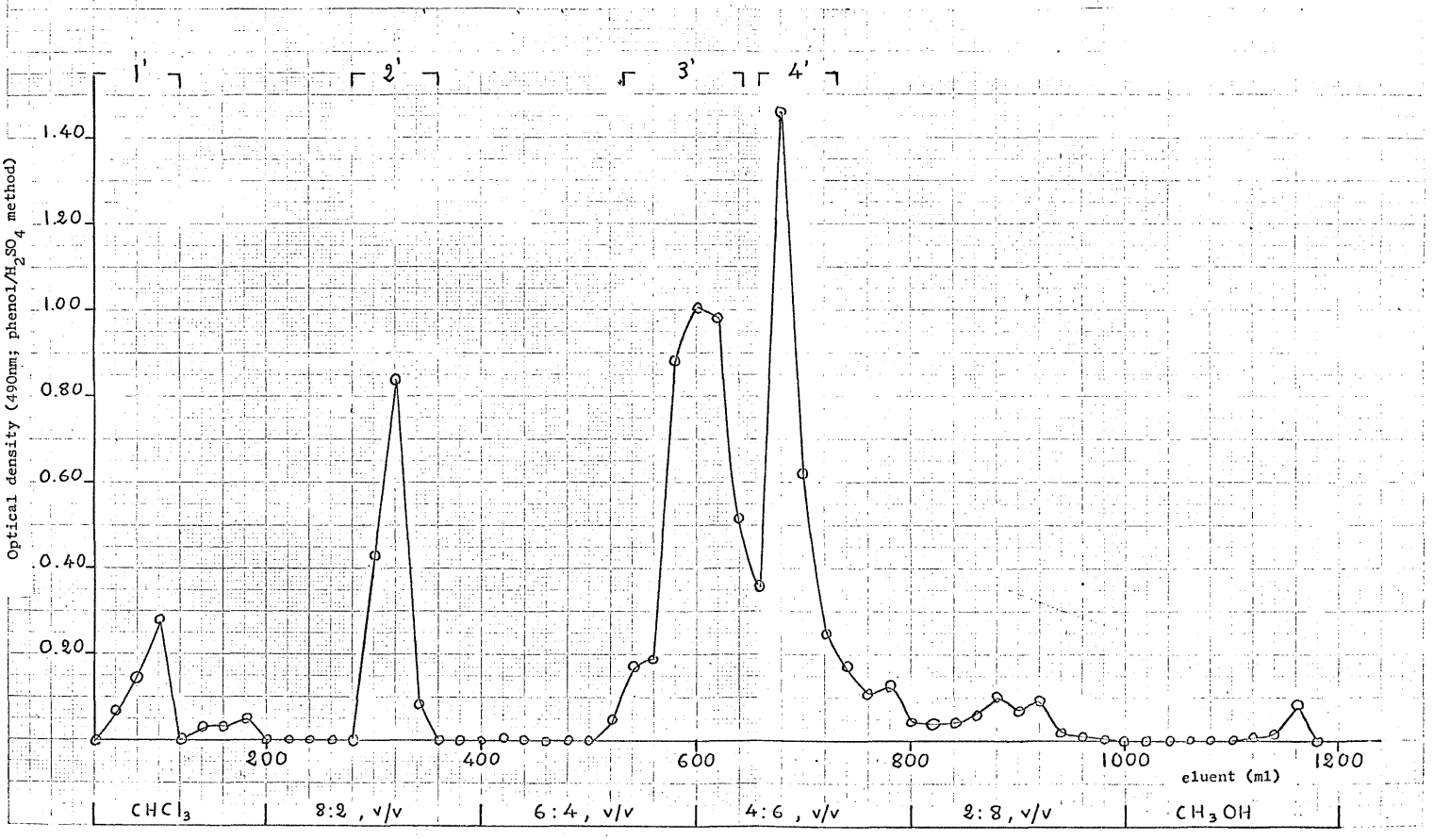


Fig. 2 Silicic acid chromatography of acetone glycolipid fraction from mung bean shoots



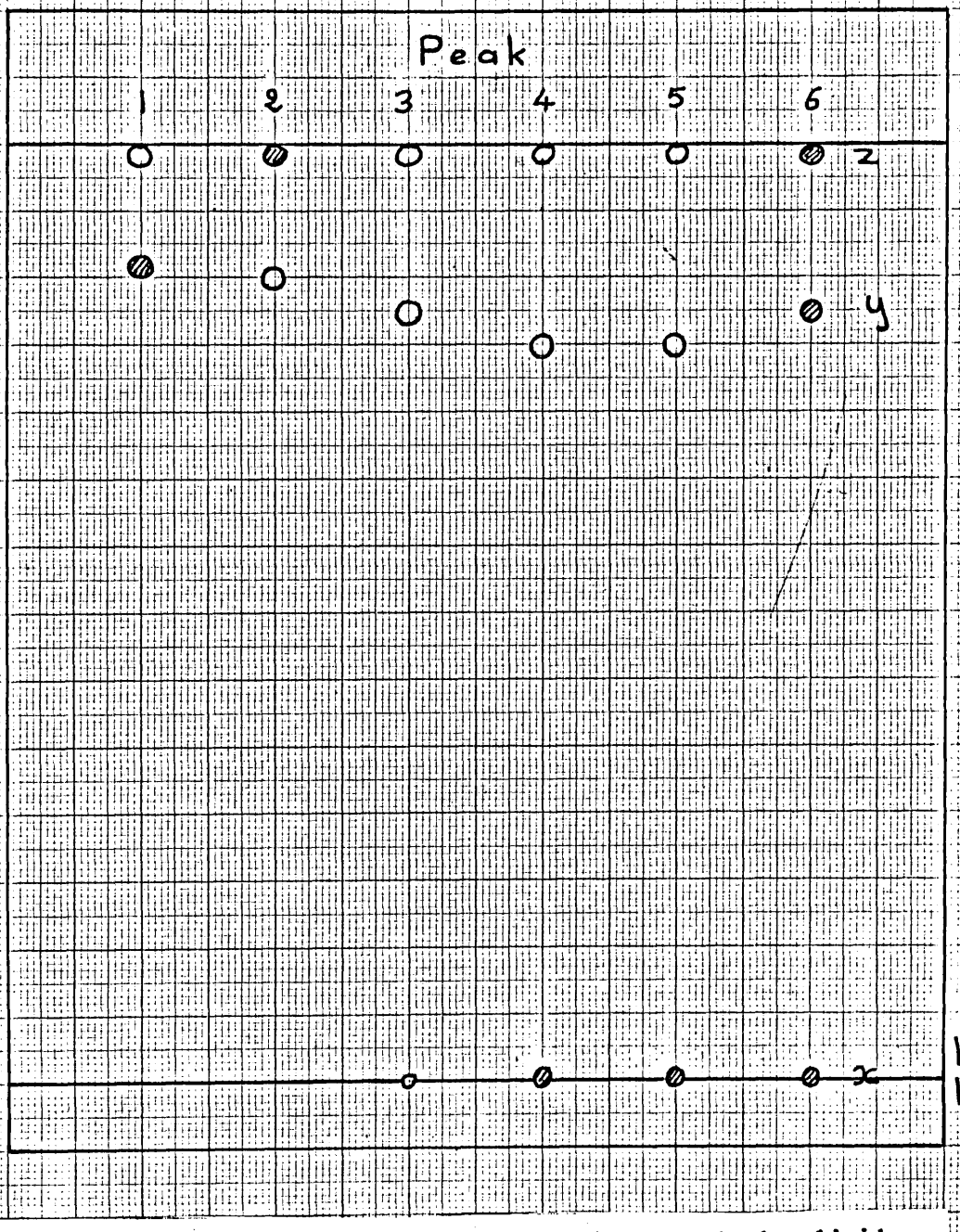


Fig. 3 Thin-layer chromatography of 80% methanol glycolipids

Adsorbent : silica gel G - Solvent system: chloroform : methanol : water (90:10:1, v/v)- Detection : I_2 vapour and vanillin/H_2SO_4 spray.

Table XVI - Monosaccharide constituents of the fractions isolated by column chromatography of the 80% methanol-soluble glycolipids

	FRACTIONS					
Monosaccharides	1	2	3	4	5	6
Galactose	-	-		++	++	+++
Glucose	++	++++	trace	-	-	-
Mannose	-	-	trace	+++	++	+
Arabinose	++	++	trace	++	++	+++
Rhamnose	-	-	-	+	-	-
Glycerol	++++	++	trace	+	+	+

Table XVII - Monosaccharide constituents of the fractions isolated by column chromatography of the acetone-soluble glycolipids

	FRACTIONS			
Monosaccharides	1'	2'	3'	4'
Glucose	++	++	++++	+++
Galactose	-	++	++	+++
Mannose	-	-	-	-
Fructose	++	-	+++	++
Arabinose	-	-	-	-
Rhamnose	-	-	++	++
Glycerol	?	+	?	?

Studies on the deacylation products of glycolipids from mung bean shoots - Schmidt et al (79) have employed mild alkaline saponification to separate sphingolipids from glycerolipids. Saponification hydrolyses ester groups and glycerol derived lipids are converted to salts of fatty acid and water-soluble products (glycerololigosaccharide derivatives). Sphingolipids, namely cerebrosides and phytoglycolipids are stable under these conditions and can be separated readily from the hydrolysis mixture.

This hydrolysis procedure was used with the mung bean total glycolipid fraction. In the deacylation studies the resulting water-soluble fraction was resolved on paper chromatograms into eight main components (I-VIII, VIII being the slowest moving component) (Table XVIII).

Table XVIII - Paper chromatographic fractionation of deacylation products of glycolipids

Fractions	$R_{Glucose}$ values
I	1.79
II	1.54
III	1.25
IV	1.00
V	0.78
VI	0.64
VII	0.46
VIII	0.29

Further purification showed that IV and VI contained other components designated IVa and VIa, respectively. Fractions I and II were found to be acidic and I appeared to contain a phosphate group. The results of a qualitative analysis of the hydrolysates of the deacylation products are shown in Tables XIX and XX*. All the oligosaccharides contained glycerol but the only reducing sugars found in any appreciable amounts were glucose and fructose, together with smaller amounts of galactose. The presence of fructose in III was confirmed by reduction of the hydrolysate with sodium borohydride followed by detection of mannitol and glucitol (as TMS derivatives) by gas-liquid chromatography. Components VI and VIII contained only myoinositol and glycerol, thus could not be considered as glycolipids. Components IVa and I contained unidentified non-reducing components. The latter four components might have come from phospholipids and inositol lipids which would be deacylated under the conditions used. The only fractions which could be considered as glycolipids and which were present in workable quantities were III, IV, VI and VII; these were examined in detail. The amounts of VIa and VII were small in comparison with fractions III and IV. These four fractions were methylated, methanolised and examined by gas-liquid chromatography on two types of column.

Oligosaccharide III which had a paper chromatographic rate of movement greater or equal to that of glucose gave 1,3,4,6-tetra-O- methylfructose and 1,4,6-tri-O-methylfructose. These derivatives were produced in approximately equal amounts and are indicative of a 2,3-linkage which is unusual in higher plant fructose derivatives. The suggested structure for this oligosaccharide is therefore:

*In view of the very small quantities of material which were available for study (the total yield of crude oligosaccharides from 4Kg of plant material was 450mg) D.P. values could only be estimated from chromatographic data. In this connection it should be noted that hexoses and the corresponding mono-hexose-glycerol derivatives have similar R_f values (21,161).

Table XIX - Paper chromatographic analysis of hydrolysates of deacylation products

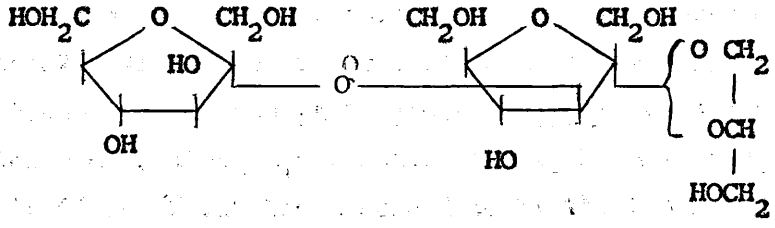
	FRACTIONS								
Monosaccharides	I	II	III	IV	IVa	VIa	VI	VII	VIII
Galactose	-	-	-	-	-	-	-	+	-
Glucose	-	-	-	+	-	+	-	+	-
Mannose	-	-	trace	-	-	-	-	-	-
Fructose	-	-	+	-	-	+	-	-	-
Arabinose	-	-	-	-	-	-	-	-	-
Xylose	-	+	-	-	-	-	-	-	-
Ribose (?)	+ *	-	-	-	-	-	-	-	-
Rhamnose (?)	+ ≠	-	-	-	-	-	-	-	-
Myoinositol	-	-	-	-	-	-	+	-	+
Glycerol	+	+	+	+	+	+	+	+	+
$R_{Glucose}$: 0.5 (solvent B)	-	-	-	-	+	-	-	-	-

* -Co-chromatographed with ribose but gave a different colour with p-anisidine spray reagent.

≠ -Co-chromatographed with rhamnose but non-redcing

Table XX - Gas-liquid chromatographic analysis of hydrolysates of deacylation products

	FRACTIONS						
Monosaccharides	I	III	IV	VI	VIa	VII	VIII
Galactose	-	-	-	-	-	+	-
Glucose	-	-	+	-	+	+	-
Mannose	-	trace	-	-	-	-	-
Fructose	-	+	-	-	+	-	-
Myoinositol	-	-	-	+	-	-	+
Glycerol	+ (strong)	+	+	+	+	+	+



Sufficient material was not available for a detailed analysis so the corresponding derivative with two 2,3-linked fructofuranose residues cannot be ruled out. However, the relative amounts of methylated derivatives detected and the chromatographic mobility of the original oligosaccharide make this latter suggestion unlikely.

Oligosaccharide IV gave only 2,3,4,6-tetra-O-methylglucose indicating a glucosyl-glycerol derivative which from chromatographic data would appear to be monoglucosyl-glycerol.

Oligosaccharide VIa a relatively slow moving compound on paper, gave 1,3, 4,6-tetra-O-methylfructose and possibly 2,3,4,6-tetra-O-methylglucose

which would indicate the presence of fructofuranose and glucopyranose end groups. No other information is available at the present time. Oligosaccharide VII gave 2,3,4,6-tetra-O-methylgalactose and possibly a trimethylglucose which could not be fully identified. The compound may therefore be a galactosylglucosylglycerol.

Upon deacylation of the glycolipid fraction, only three monosaccharides were detectable in the water-soluble fraction, namely glucose, fructose and to a lesser extent, galactose. Mannose, arabinose, rhamnose and most of the galactose, which were all clearly identified in the acid hydrolysates of the glycolipid mixture, were not present in the deacylation products. These four monosaccharides must, therefore, constitute the sugar-moieties of sphingolipids which are not hydrolysed under the mild alkaline conditions used in deacylation (79). But it is impossible, at this stage, to know whether rhamnose, arabinose, mannose and glactose form a complex oligosaccharide moiety of a single sphingoglycolipid, as in phytoglycolipids or whether each monosaccharide is present as a relatively simple glycosyl constituent of different sphingoglycolipid as in the case of cerebrosides. The presence of both types of compound in Phaseolus vulgaris (85) could indicate the existence of a similar situation in mung bean (Phaseolus aureus). Glucolipid and fructolipid seemed to be the two main glycosylglycerides in mung bean shoots. The galactosylglyceride, found as a minor component, might have originated from proplastids formed during germination in the dark.

III Studies on the biosynthesis of glycolipids

In view of the complex nature of the glycolipids which were detected in P.aureus tissues it was of interest to examine the rate of synthesis of these compounds in developing shoots.

Preliminary studies on the incorporation of label from sucrose-U-^{14}C into the glycolipids showed that approximately 38% of the total radioactivity of the glycolipids was present in the carbohydrate moieties after feeding with the disaccharide for 24hr. The radioactivities of the individual monosaccharides resulting after hydrolysis of the

glycolipids were calculated (Table XXI), and the most outstanding result was that rhamnose, which was present in small amounts relative to the other main sugars, seemed to contain a high level of radioactivity.

In an attempt to study more completely the pattern of incorporation of sucrose-U-^{14}C into glycolipids, the experiment was repeated and the feeding period varied (figures 4,5,6,7). After 7½ hr. glucose contained a high level of radioactivity as did a compound which co-chromatographed with glucosamine in basic solvent. The identify of this compound could not be confirmed but it should be noted that phytoglycolipids contain glucosamine. The other sugars in the hydrolysate had a relatively low level of radioactivity. After 24hr. feeding the levels of radioactivity in all the sugars had increased and this was particularly noticeable in the case of rhamnose. The unidentified spot which behaved like glucosamine appeared to have lost radioactivity during the extra feeding period.

Table XXI - Radioactivity in the monosaccharide components of glycolipids from mung beans

	Glycolipid hydrolysate	
Monosaccharide	c.p.m. solvent A	c.p.m. solvent B
Galactose	1238	595
Glucose		600
Mannose	608	565
Fructose		
Arabinose		
Xylose	437	278
Ribose	420	377
Rhamnose	875	731
Glycerol	573	

Fig. 4 Incorporation of sucrose-U-^{14}C into the monosaccharide components of glycolipids of mung beans after feeding for 7½ hr. (solvent A)

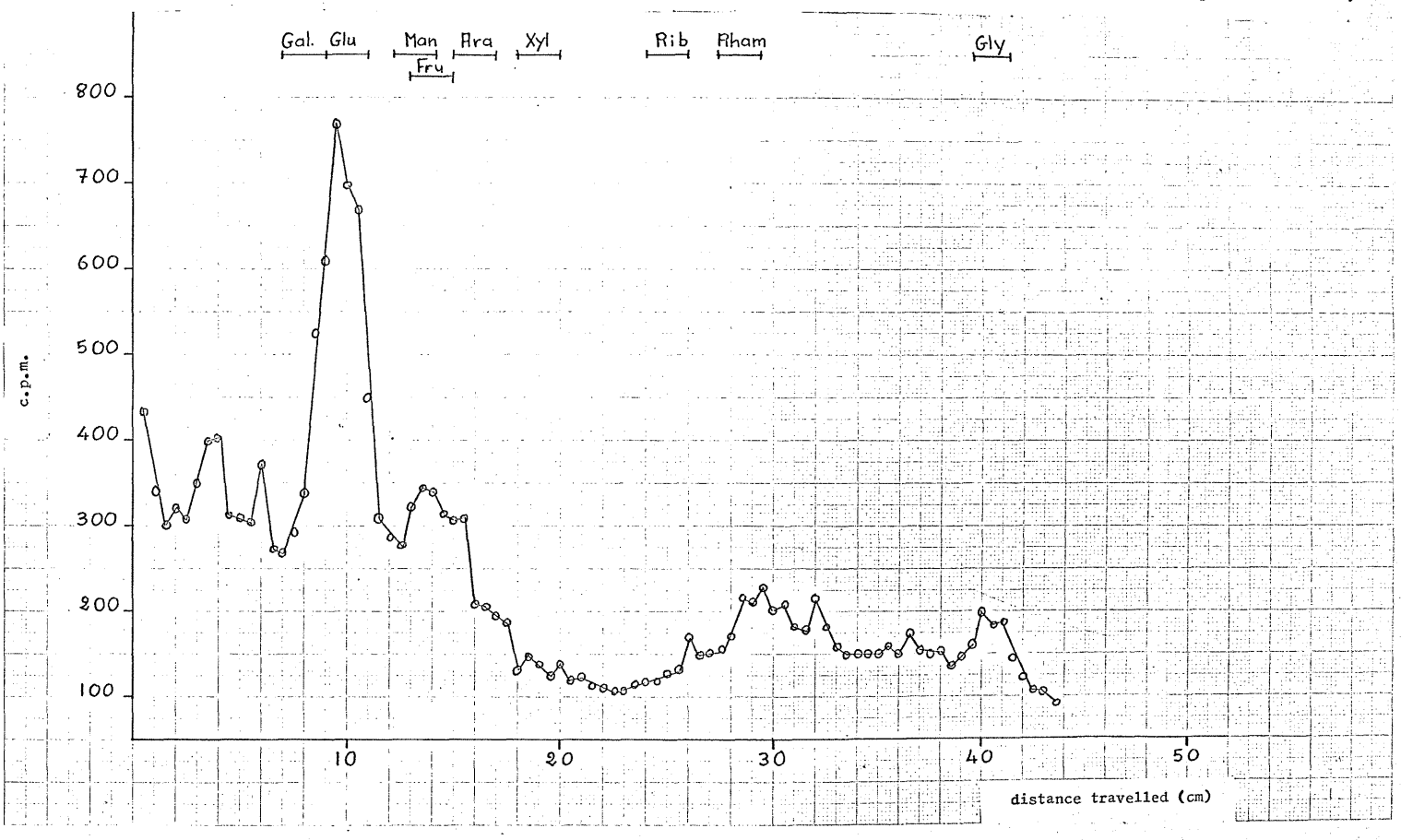
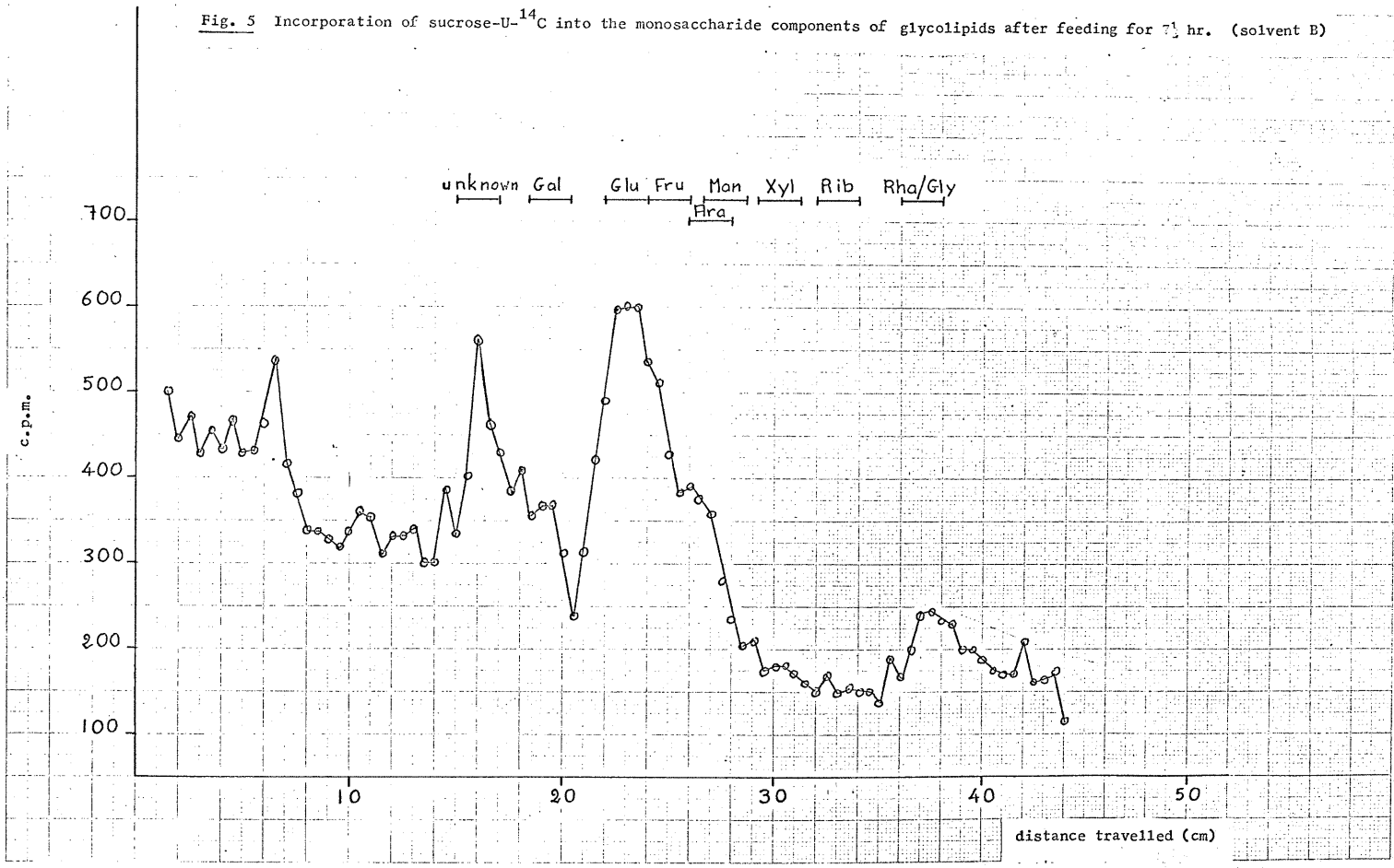


Fig. 5 Incorporation of sucrose-U-^{14}C into the monosaccharide components of glycolipids after feeding for 7½ hr. (solvent B)



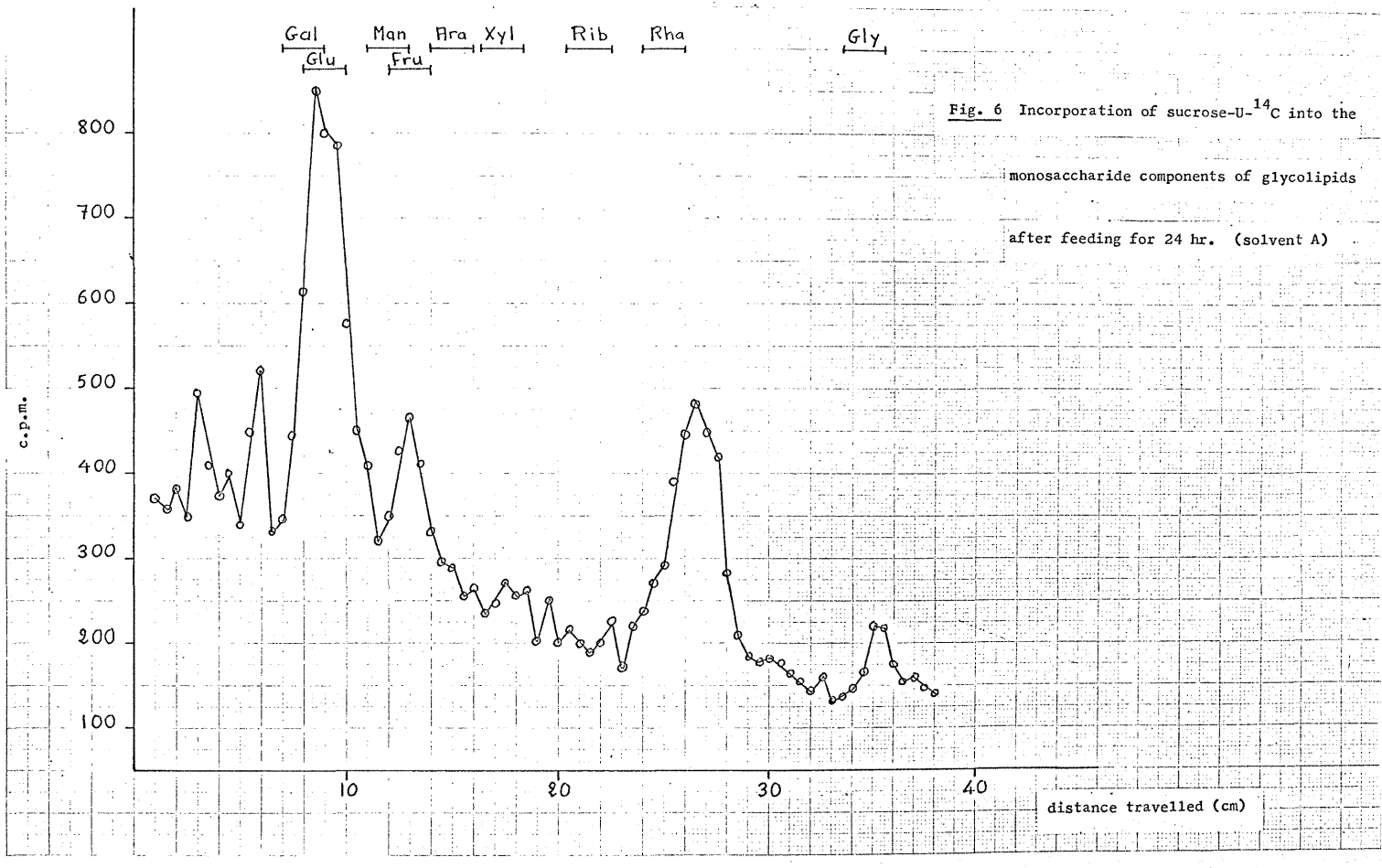
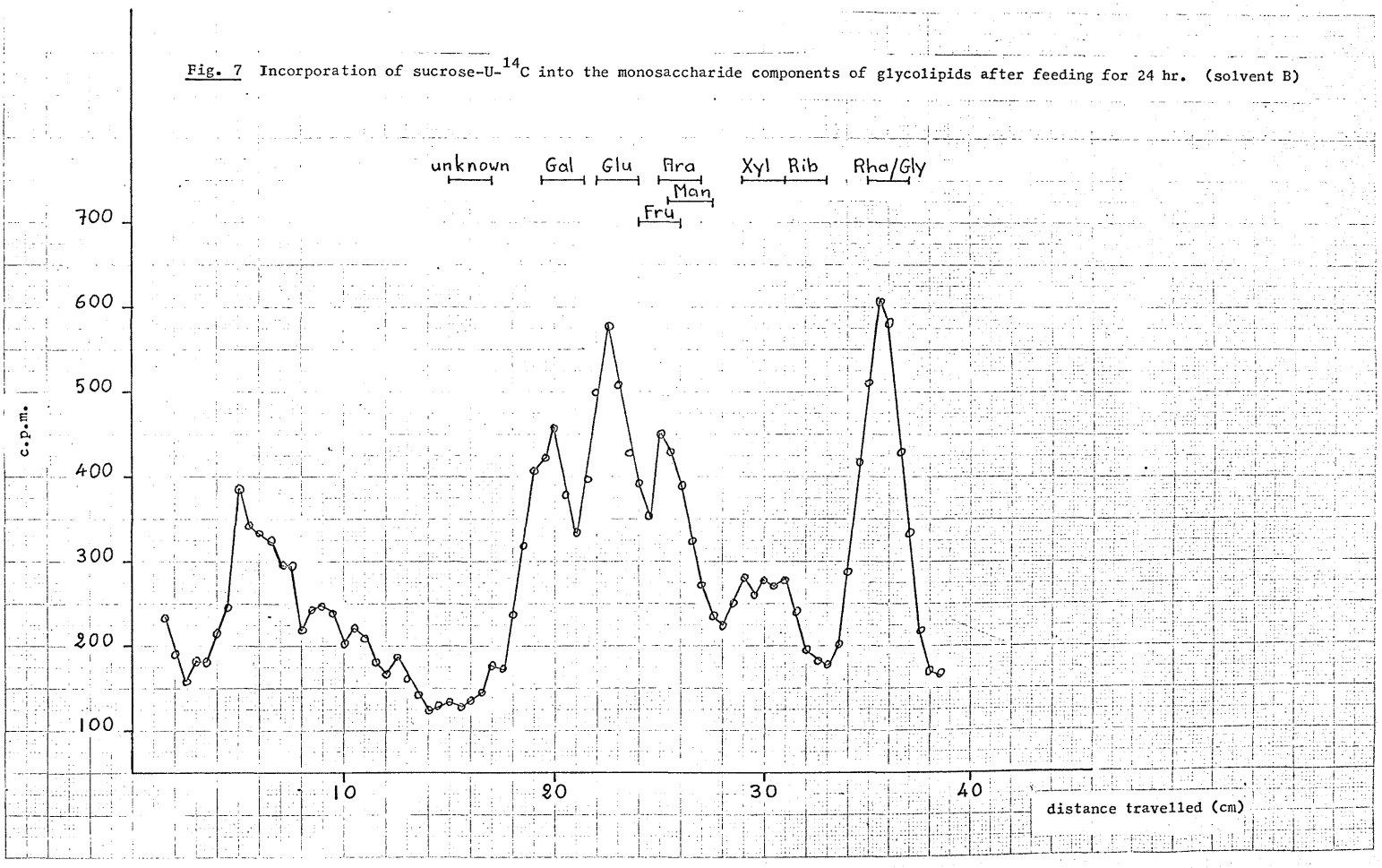


Fig. 6 Incorporation of sucrose-U-^{14}C into the monosaccharide components of glycolipids after feeding for 24 hr. (solvent A)

Fig. 7 Incorporation of sucrose-U-^{14}C into the monosaccharide components of glycolipids after feeding for 24 hr. (solvent B)



The incorporation of sucrose-U-^{14}C into glycolipids after 7½ and 24hr. was then studied in more detail (Table XXII).

Having started with sucrose equally labelled in the glucose and fructose moieties, one would expect approximately equal labelling of glucolipid and fructolipid provided the biosyntheses of both compounds go via comparable routes. However, after the shorter period of incorporation the combined glucose had a specific activity three times that of fructose. It is of course almost impossible to explain this difference as it could depend on marked dissimilarities in biosynthetic routes, and/or rates of individual reactions, utilization of glycolipid substrate for other reactions etc. It is possible, however, that the glucosyl moiety of sucrose can be directly converted to UDP-glucose and, hence, glucolipid by reversed sucrose synthetase reaction (162):

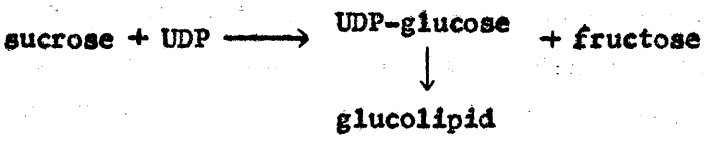


Table XXII - Incorporation of sucrose-U-^{14}C into the monosaccharide components of glycolipids of mung beans

	% Activity		% by Weight		Specific Activity	
Monosaccharides	7½hr.	24hr.	7½hr.	24hr.	7½hr.	24hr.
Glucose	44.4	25.4	29.7	37.3	1.50	0.68
Galactose	14.5	19.4	25.9	25.7	0.56	0.75
Fructose	11.4	6.9	28.4	24.4	0.40	0.28
Mannose	10.1	6.2	5.2	5.5	1.90	1.10
Arabinose	7.4	4.4	6.9	3.5	1.10	1.30
Rhamnose	11.2	19.8	3.9	3.5	2.90	5.70

The fructose liberated by this process would probably have to undergo several reactions via fructose phosphates and, possibly, UDP-fructose before incorporation into fructolipid occurred. The decrease in the specific activities of glucose and fructose (and mannose) after 24hr. is also difficult to explain particularly as this was not the case with combined galactose, arabinose and rhamnose and, in addition, shoots were kept in contact with sucrose-^{14}C throughout the whole incubation period. The decrease, therefore, appears to have some biochemical or physiological significance. It may be that the lipid bound glucose, fructose and mannose are metabolically more active than the other bound sugars, and that the latter (galactose, arabinose and rhamnose) are components of "a structural lipid" which is relatively stable.

It is interesting to note that the monosaccharides with high specific activities namely, galactose, mannose, arabinose and rhamnose, are those which are usually linked to long-chain bases such as sphingosine and its derivatives. In mung bean glycolipids only the fructolipid, the glucolipid and a small amount of the galactolipid were susceptible to mild alkali hydrolysis and hence appear to be glycosylglycerides, whilst most of the galactose, mannose, arabinose and rhamnose were not liberated by this treatment and thus may have been present as sphingolipids. Villemez et al (12) gave evidence that a lipid material in mung bean, having the solubility properties of complex glycolipids acted as an intermediate in the incorporation of GDP-mannose into cell-wall polysaccharide. In view of the high specific activity of the lipid mannose in sucrose-^{14}C fed mung bean shoots, and the decrease in this activity with time, the possibility that this mannose is part of the intermediate detected by Villemez should be considered.

IV Studies on the possible formation of UDP-fructose in mung beans

It has been shown that nucleotide diphosphate sugars are involved in the biosynthesis of glycolipids. For example, UDP-glucose is the glucosyl donor for glucolipid formation in mung beans (13) and UDP-galactose is utilized for galactolipid biosynthesis in chloroplasts (38). By analogy with these reactions, it seemed possible that the metabolically active fructolipid detected in mung beans (see section III) might be synthesised from a nucleotide fructose derivative.

UDP-fructose has been isolated from Jerusalem artichoke (163, 164) and dahlia tubers (165), and from germinating pea seeds and leaf nodes of Impatiens balsamina (166). It has been suggested that the occurrence of UDP-fructose in such a variety of tissues may indicate that the compound is widely distributed in the plant kingdom (166). at present it is not known whether this is a fructofuranose or fructopyranose derivative although Umemura et al (163) suggest that it is the former. There is no known function for the compound. It could conceivably serve as a fructosyl donor for fructosylglyceride synthesis. With the latter possibility in mind, a preliminary study of the mode of biosynthesis of UDP-fructose was undertaken.

Preliminary studies - A common biosynthetic reaction for the formation of nucleotide sugars is the nucleoside diphosphate sugar pyrophosphorylase - catalysed transfer of nucleoside monophosphate to aldose-1-phosphates. For example, both plants and animals can produce UDP-glucose from uridine-5'-triphosphate (UTP) and α-D-glucose-1-phosphate (G-1-P):

$$UTP + G\text{-}1\text{-}P \rightleftharpoons UDP\text{-glucose} + PPi$$

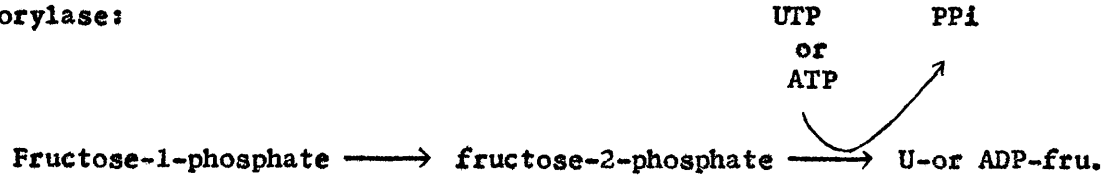
The enzyme required for this reaction is UTP: G-1-P uridylyl transferase (UDP-glucose pyrophosphorylase).

If UDP-fructose is formed in plants by an analogous reaction fructose-2-phosphate would be the expected acceptor for a uridylic acid residue:

$$UTP + Fru\text{-}2\text{-}P \rightleftharpoons UDP\text{-}fru + PPi$$

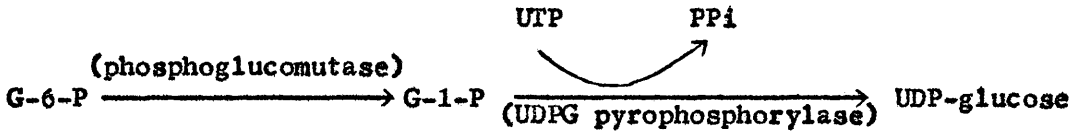
Fructose-2-phosphate has not been reported as a plant cell constituent neither is it obtainable from commercial sources; hence the following initial experiments were carried out.

Fructose-1-phosphate together with UTP or ATP was incubated with an enzyme preparation from mung bean seedlings on the assumption that a nucleotide fructose derivative might be produced by the following reactions involving a phosphofructomutase and a NDP-fructose pyrophosphorylase:



equation 2

A possible reaction between ATP and fructose-1-phosphate was examined in view of the fact that ADP-fructose has been detected in higher plants (167) and this could also act as a donor for fructolipid formation. The reaction summarised in the above equations is, for example, similar to the well known conversion of G-6-P to UDP-glucose (168):



Paper chromatographic analysis of the products obtained by incubating F-1-P with UTP or ATP and a 95% ammonium sulphate fraction from 3 day-old mung bean seedlings, showed uv-absorbing bands, in the case of both triphosphate substrates, with mobilities characteristic of nucleoside diphosphate hexoses; the product from UTP co-chromatographed with UDP-glucose (163). The experiment was repeated on a larger scale and the products isolated by preparative paper chromatography. Compounds derived from both ATP and UTP exhibited u.v. absorbance peaks at 260nm (Figure 8), and when hydrolysed with N HCl for 10 min. at 100° yielded fructose, which was detected by paper chromatography using the ketose specific urea/phosphoric acid spray reagent. A later investigation showed that 12hr. germinated mung bean seedlings were more active in producing nucleotide fructoses.

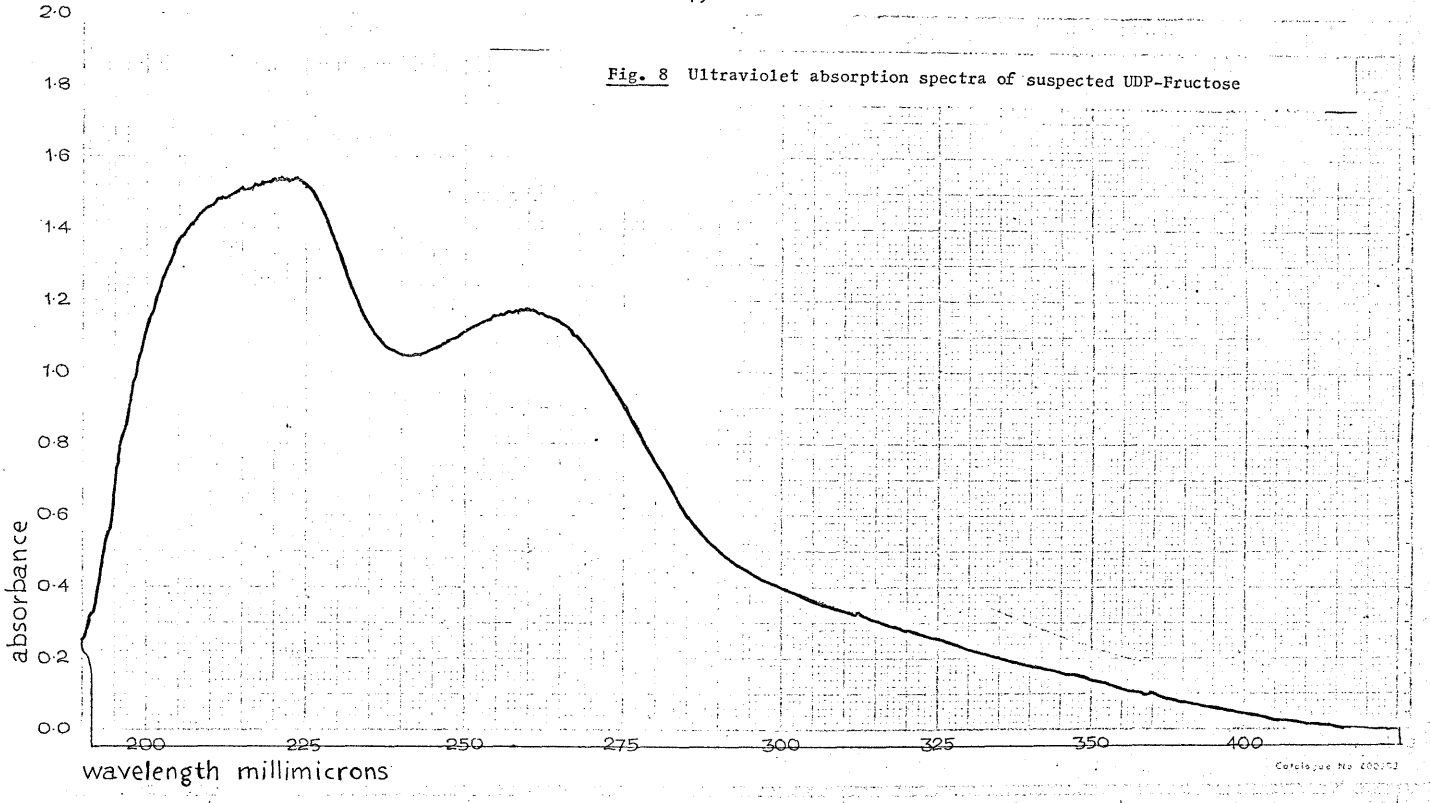
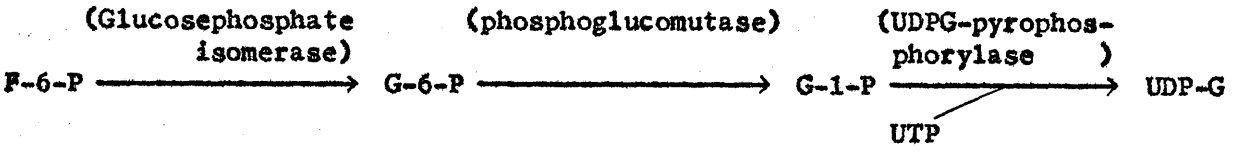


Fig. 8 Ultraviolet absorption spectra of suspected UDP-Fructose

When fructose-(U)-^{14}C-6-phosphate was incubated with UTP and the bean extract the only detectable UDP-sugar derivative on hydrolysis yielded glucose. The reaction occurring in this case was presumably as follows:



These preliminary results, therefore, suggested that the postulated reaction sequence shown in equation 2 had occurred.

Attempted conversion of fructose-1-phosphate to fructose-2-phosphate - In view of the above findings an attempt was made to detect phosphofructomutase activity in seedling extracts. Incubation of extracts with fructose-1-phosphate (both ^{14}C-labelled and unlabelled) produced other compounds which on paper chromatograms and electrophoretograms behaved as sugar phosphates. Yields were too small, however, for any definite conclusions to be drawn.

Attempted conversion of fructose-2-phosphates to nucleoside diphosphate fructoses - As attempts to synthesize, biochemically, fructose-2-phosphate in workable amounts had failed the pyranose and furanose forms of this compound were synthesized chemically by the method of Pontis and Fischer (169,170).

Fructo-furanose and -pyranose-2-phosphates can be separated from each other by paper chromatography and from F-1-P and F-6-P.

No nucleoside diphosphate fructose could be detected, however, when either fructose-2-phosphate was incubated with UTP (or ATP, GTP or CTP), and preparations from germinated mung bean of various ages from 12hr. to 3 days.

Studies on reaction of F-1-P with UTP - As the direct conversion of F-2-P (pyranose and furanose forms) to UDP-fructose could not be demonstrated further attention was paid to the reaction of F-1-P with UTP using a 12hr.

germinated mung bean enzyme preparation. Figure 9 shows the formation of suspected UDP-fructose with tissue using both Tris (pH 7.5) and cacodylate (pH 7.0) buffers. If Mg^{2+} ions were not added to the digests there was no apparent reaction. Further amounts of the UDP-sugar were isolated from paper chromatograms and hydrolysed with 50% aqueous formic acid and the hydrolysis products examined (as TMS derivatives) by GLC; fructose was detected.

Additional investigations were made with fructose-(U)-^{14}C-1-phosphate. This substance was synthesized in the laboratory by the reaction of ATP with Fructose-(U)-^{14}C using a beef liver fructokinase preparation (A detailed study of the optimum conditions for this reaction was carried out; see p.110).

Reactions of labelled F-1-P and UTP with mung bean enzyme preparations were carried out in capillary tubes and the products fractionated on paper chromatograms which were subsequently scanned for radioactivity (Figures 10a, b and c) and accurate determinations of ^{14}C also made by counting the appropriate paper strips with a scintillation counter. Figure 10b shows the formation of suspected UDP-fructose (peak A) after incubation for 60 min. at 35°. In figure 11 it can be seen that inorganic phosphate interferes with the formation of peak A. In addition, a faster moving radioactive component (B) of the digest is also apparent (Figure 10b) and this material appears to increase when the formation of component UDP-fructose (A) is inhibited with Pi (Figure 10c). When B was isolated and re-examined by paper chromatographs it exhibited a similar rate of movement to fructose-2-phosphate.

This observation lends some support to the original idea that the initial reaction in the formation of UDP-fructose is the isomerization of F-1-P to F-2-P. The inhibition of nucleotide fructose formation by phosphate is interesting as ADP-glucose pyrophosphorylases from various plants appear to be allosteric enzymes which are also inhibited by phosphate (171). Figure 12 shows that the rate of nucleotide formation was proportional to F-1-P concentration and it was shown that a two-fold

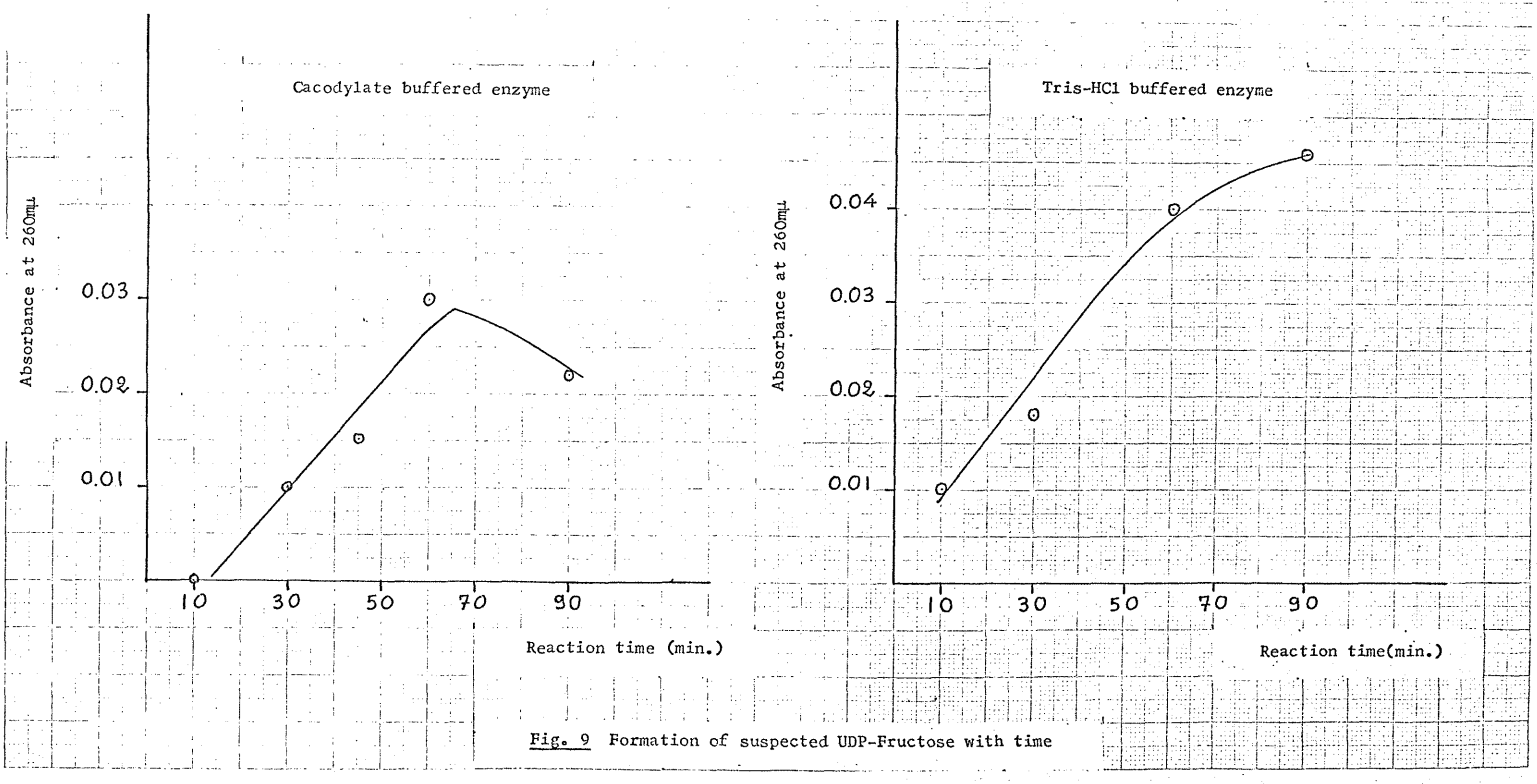


Fig. 9 Formation of suspected UDP-Fructose with time

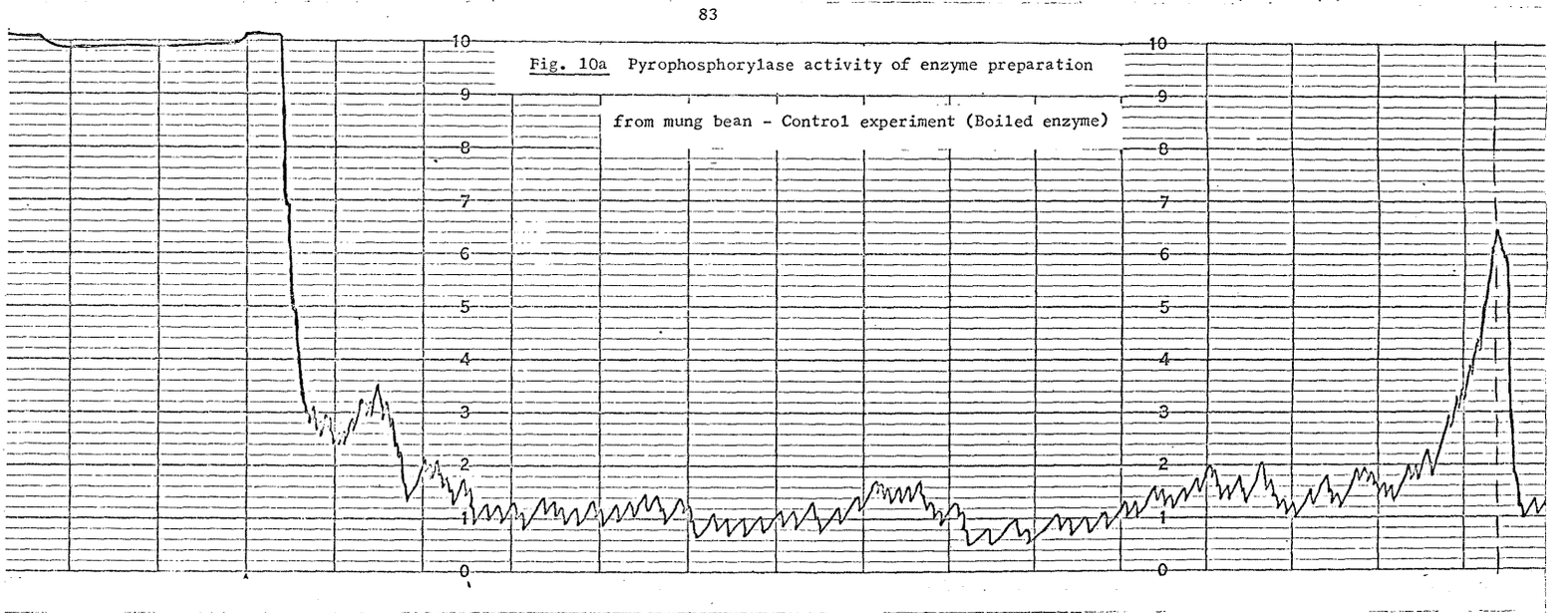


Fig. 10a Pyrophosphorylase activity of enzyme preparation from mung bean - Control experiment (Boiled enzyme)

Fig 10b Pyrophosphorylase activity of enzyme preparation from mung bean -

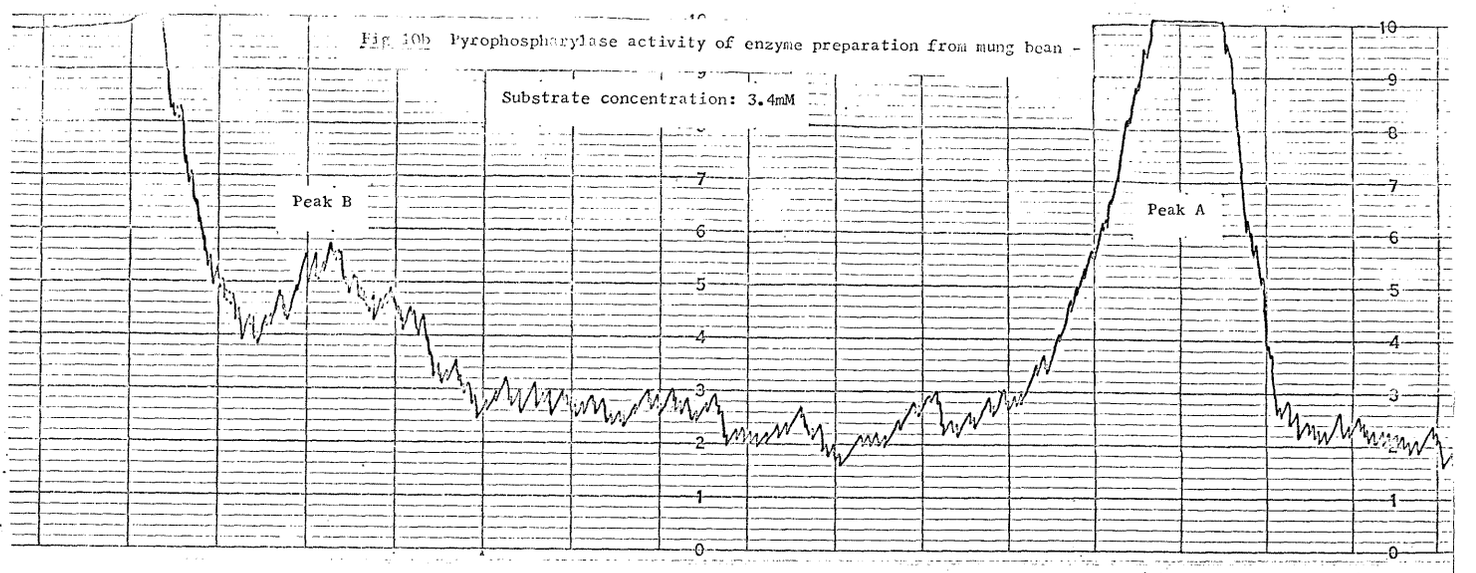
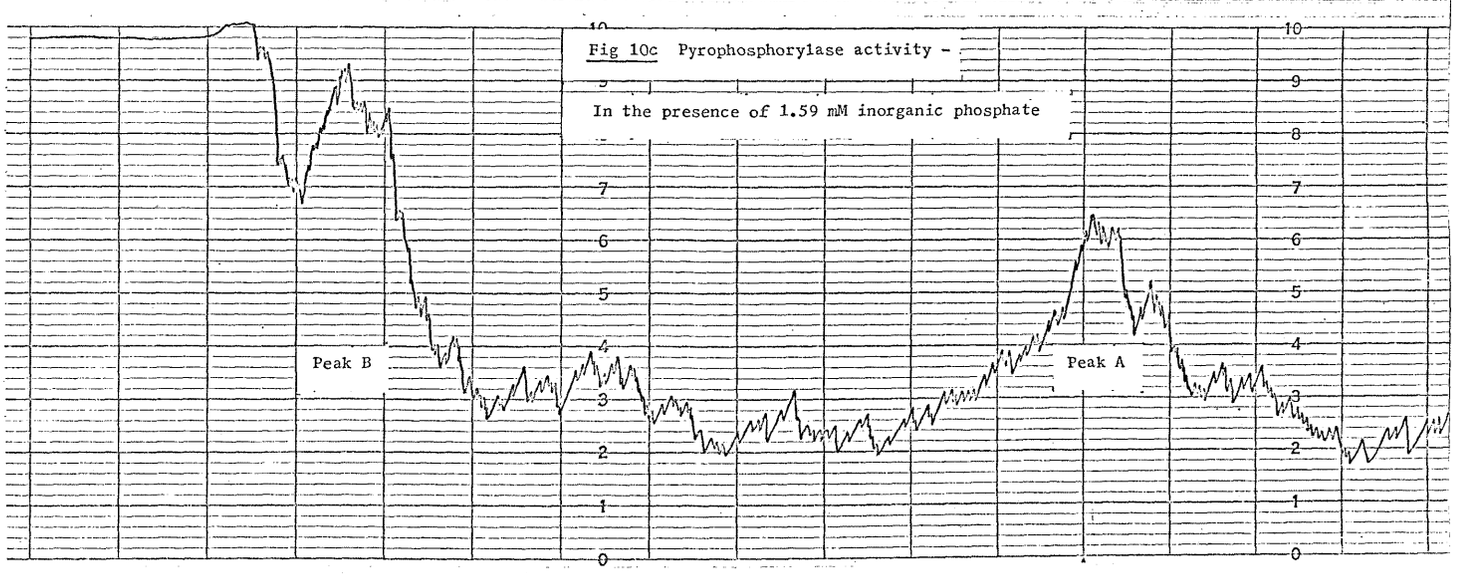


Fig 10c Pyrophosphorylase activity -

In the presence of 1.59 mM inorganic phosphate



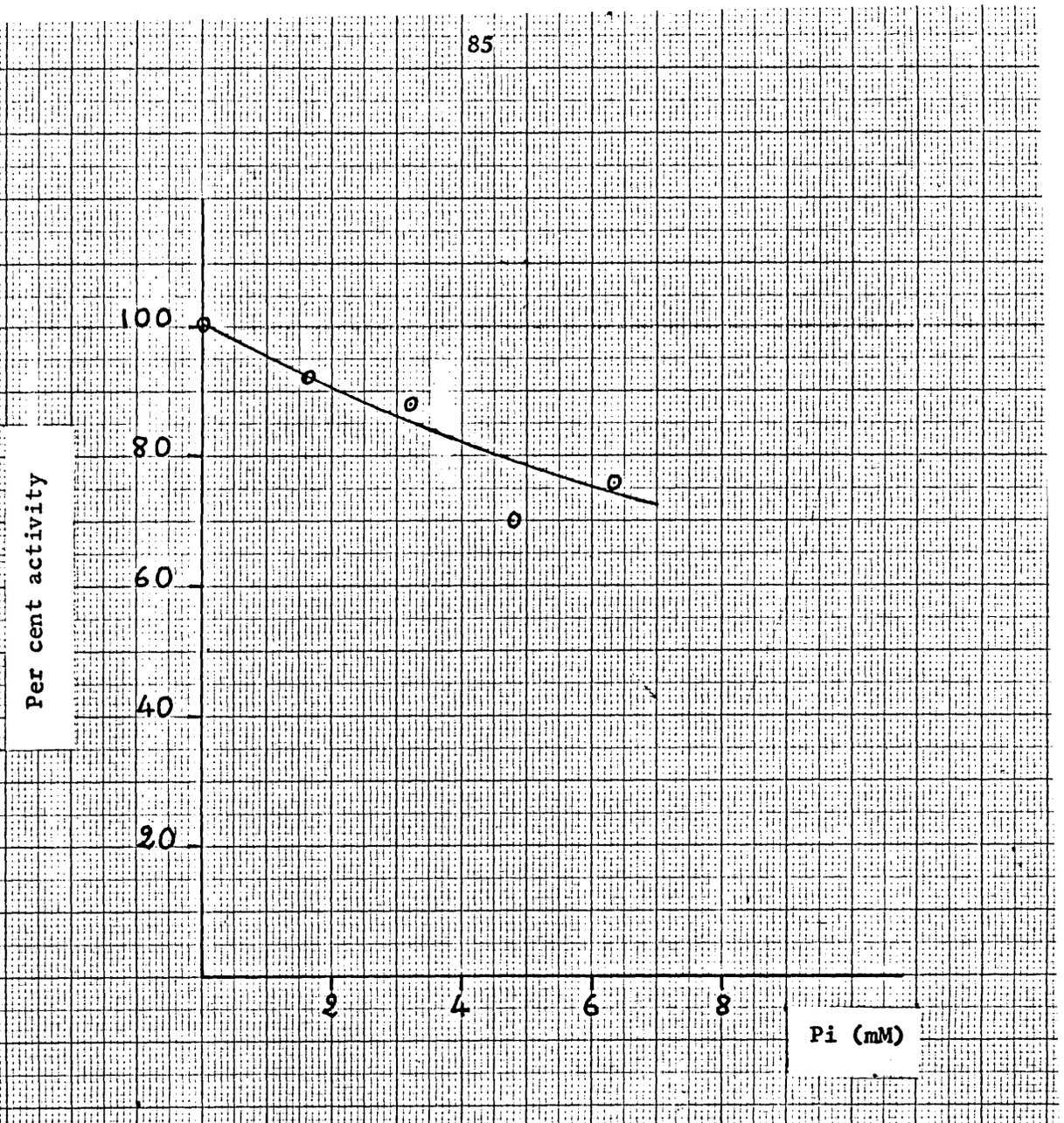


Fig. 11 The inhibition of UDP-fructose synthesis by Pi. One hundred per cent activity pertains to the UDP-fructose synthesis in the absence of inhibitor

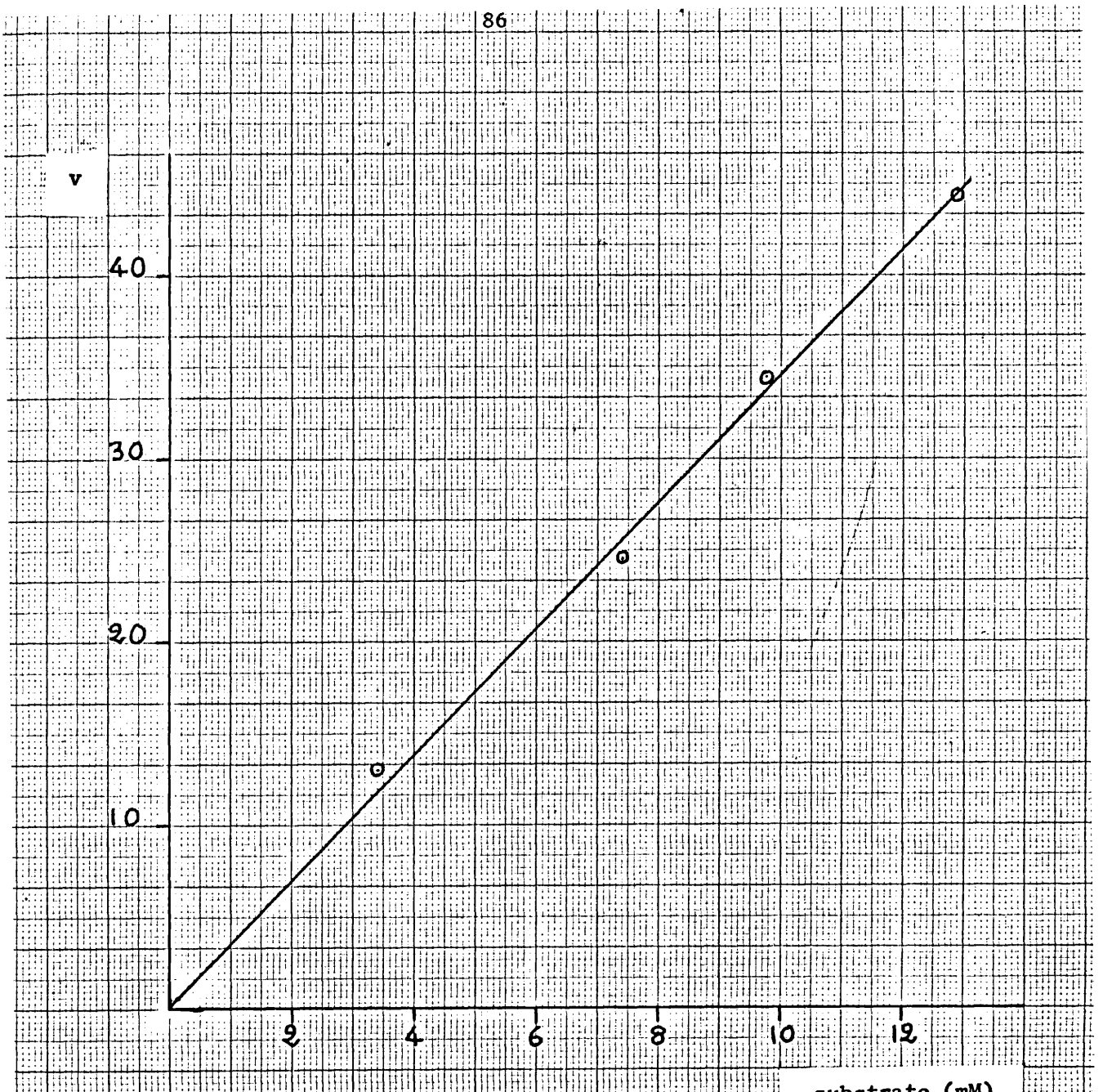


Fig. 12 Effect of substrate concentration on the rate of reaction.

v = millimicromoles of UDP-fructose formed in one hour

increase in the enzyme concentration approximately doubled the yield of product. In the absence of exogenous Mg^{2+} there was a 50% decrease in product formation (Table XXIII)

Table XXIII Effect of enzyme concentration and Mg^{2+} ions on UDP fructose formation

Digest*	UDPfructose (cpm)
Complete (UTP, F-1-P, Mg^{2+}, E)	1670
2 x [E]	2921
-Mg^{2+}	888

* Incubation at 35° for 45 min.

The results of a rate/time study of the enzymic formation of UDP-fructose are given in figure 13. The apparent lag period could be related to a required initial isomerization of the fructose-1-phosphate. Prior incubation of fructose-(U)-^{14}C-1-phosphate with the enzyme preparation before addition of UTP did not appear to affect the rate of UDP-fructose formation. When fructofuranose-2-phosphate was added in varying amounts to the reaction mixture some activation was observed (Figure 14); this was not the case with fructopyranose-2-phosphate. Activation (ie increased cpm) by exogenous fructofuranose-2-phosphate is further evidence that this sugar phosphate is involved in the formation of UDP-fructose and as the pyranose derivative had no effect if further suggests that UMP is transferred to F-2-P_f with the formation of UDP-fructofuranose (cf Umemura et al (163).

The sugar-nucleotide, that had been produced from fructose-(U)-^{14}C-1-phosphate in the various kinetic studies, was isolated by paper chromatography and subjected to hydrolysis with acetic and oxalic acids,

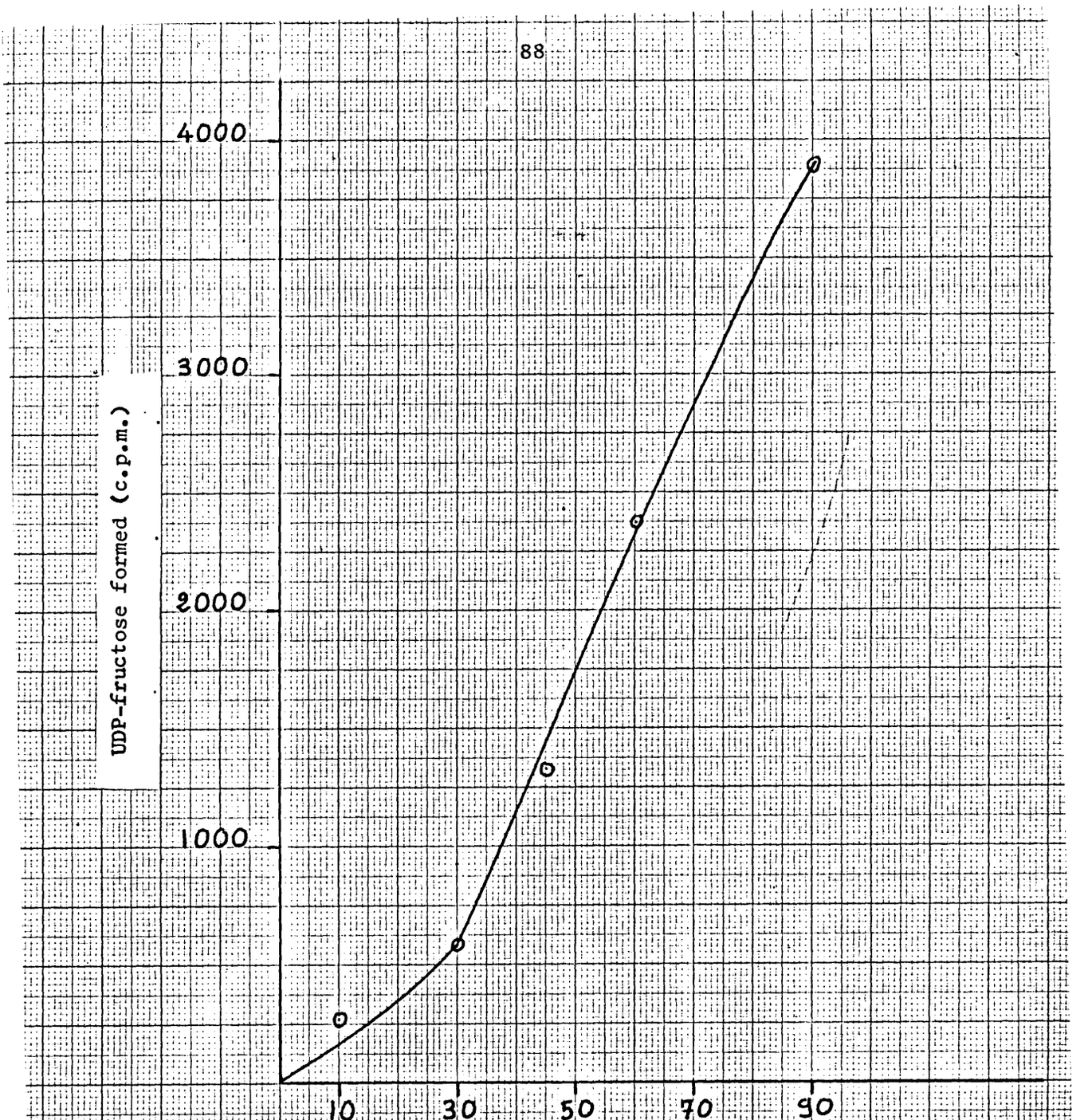


Fig. 13 Effect of time of reaction on the yield of sugar-nucleotide

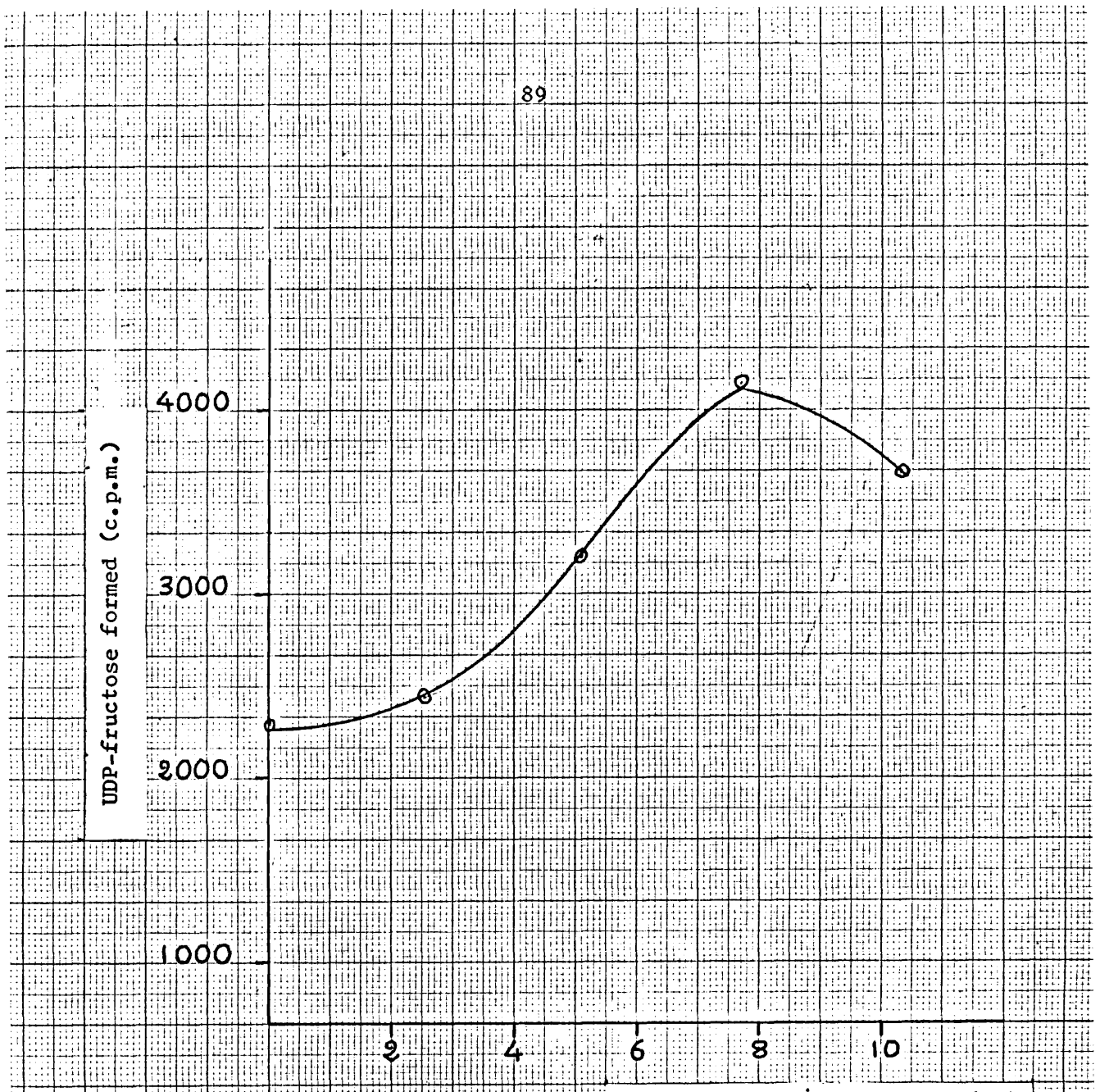


Fig. 14 Effect of concentration of fructofuranose-2-phosphate on rate of reaction

at pH 3.8 (6hr; 100°). Both treatments yielded a slow moving radioactive spot ($R_{fructose} \simeq 0.5$) and when this material was further treated with 2% aqueous oxalic acid (pH 1) for 2 hr. at 100° labelled fructose and a trace of glucose were detected. The partial hydrolysis effected by acetic or oxalic acids probably cleaved the pyrophosphate linkage in the nucleotide liberating fructose phosphate which was subsequently hydrolysed to fructose by the stronger (pH 1) oxalic acid treatment.

Attempts were made to detect a possible linkage between the C-1 primary hydroxyl of fructose and phosphate in the nucleotide fructose derivative by reduction with sodium borohydride followed by hydrolysis. If the 2-hydroxyl of the fructosyl moiety had been free D-glucitol and D-mannitol should have been produced; these hexitols were not detected, however. This type of negative evidence is not entirely satisfactory proof of the absence of C-1-phosphate linkages in the nucleotide but a compound with this type of structure would not be expected to be formed as, up to now, only sugar phosphates with the phosphate group linked to the anomeric carbon atom, have been shown to act as substrates for pyrophosphorylases. Obviously further proof of structure is necessary including a methylation study and an examination of the products produced by an organic pyrophosphatase. The evidence described at present, points to the fact that C-2 of fructose is linked to the terminal phosphate of UDP.

V Conclusion

The lipid-bound monosaccharides found in mung bean shoots are similar to the monosaccharide constituents of polysaccharides which occur commonly in the plant kingdom; these are galactose, glucose, mannose, fructose, arabinose, xylose and rhamnose. The glycosyl groups in the lipids appear to be acid labile and are bound as glycesyl glycerides and as non-saponifiable glycolipids.

Glycolipids in mung beans do not seem to be concentrated in a definite particulate fraction. They have been identified in the 1000,

10,000, and 20,000g (157) fractions from shoots. Column chromatography of the total glycolipids has revealed a complex mixture and glycerol glycolipids with relatively short sugar chains have been identified; glucose and fructose are the two main monosaccharide constituents of these glycolipids. A fructolipid containing 2,3-linked furanose ring system has been isolated. This is the first reported example of a naturally occurring plant fructolipid. Arabinose, most of the galactose, mannose and rhamnose all appear to be associated with a non-saponifiable lipid fraction.

A preliminary investigation of the incorporation of label from sucrose-^{14}C into the glycosyl residues of mung bean lipids showed that they became rapidly labelled and there was a tendency for the monosaccharides bound to non-saponifiable lipids to have the highest specific activities. The decrease, with time, in the specific activities of glucose, fructose and mannose may indicate that these sugars are metabolically more active than the other monosaccharides (galactose, arabinose and rhamnose) which show an increase in specific activity with time. The latter sugars may be components of a "structural lipid". It should be noted that mannose has an initial high specific activity which decreases with time and that Villemez et al (12) have suggested that mannolipids are intermediates in polysaccharide biosynthesis.

In an attempt to study the pathway for the formation of the fructolipid present in mung bean shoots the formation of UDP-fructose, a likely precursor, was studied. The results suggest that F-2-P is the probable substrate for a UDP-fructose pyrophosphorylase and that tissues extracts contain a "phosphofructomutase" which can convert F-1-P to F-2-P. The structure of the nucleotide formed from F-1-P was not studied in great detail. More work is required to establish beyond doubt the nature of the linkage between UDP and fructose. The possible involvement of the C-1 primary hydroxyl of fructose cannot at present be ruled out.

Future work in this general field should be directed towards the further characterisation of the complex oligosaccharide moieties present

in the mung bean lipids and on an understanding of their function. The biosynthetic pathway from the fructose phosphates to fructolipids is also worthy of further attention.

E X P E R I M E N T A L

General Methods

1 - Carbohydrate content

Carbohydrate content was estimated using the method of Dubois et al (172) (phenol/sulphuric acid test).

2 - Acid hydrolysis

a) Formic acid - Carbohydrate (2-10mg) was dissolved in 90% formic acid (1-2ml) in a 25ml round-bottom flask. Solid carbon dioxide was added to saturate the atmosphere with gas and the flask was sealed after the solid CO_2 had evaporated. The flask was heated for 6hr. at 100°C after which it was opened and the solution diluted (x 10) with water and heated unsealed for a further hour. This solution was then reduced to a small volume, treated with methanol (x 2) and finally evaporated to dryness.

b) Sulphuric acid -

(i) Carbohydrate (1-2mg) was dissolved in 2N-sulphuric acid (1ml), the vessel sealed, heated at 100°C for 6 hr. and then cooled. This solution was extracted (x 3) with 5% N,N-di-n-octylmethylamine dissolved in chloroform and the extract discarded. The aqueous layer was washed with chloroform until neutral and then evaporated.

(ii) Carbohydrate (5-10mg) was dissolved in 2N-sulphuric acid (4-5ml) in a hard glass tube. The tube was sealed, and heated at 100°C for 6 hr. then cooled and neutralised with Analar barium carbonate. After filtration the hydrolysate was evaporated to dryness.

3 - Paper chromatography - Analytical paper chromatography was carried out using Whatman No.1 paper and preparative paper chromatography with distilled water-washed (24hr.) Whatman No.1, No.3 and No.17 papers. Whenever No.17 paper was used, a wick of No.3 paper was attached, with the aid of staples, according to the method of Frush (173); compounds were detected by spraying side and centre strips or by the imprint method of Frush (173). All paper chromatography was carried out using descending elution technique.

4 - Solvents for paper chromatography

A : ethylacetate : acetic acid :formic acid : water. (18:3:1:4, v/v) (174)

B : butanol-1 : pyridine : water (6:4:3, v/v) (175)

C : methylcellosolve : methylethylketone : 3N-ammonium hydroxide (7:2:3, v/v) (176)

D : ethanol (95%) : M-ammonium acetate, pH 7.5 (7.5:3, v/v) (177)

E : ethanol (95%) : M-ammonium acetate/acetic acid buffer pH 3.8 (7.5:3, v/v) (177)

F : methylethyl ketone : acetic acid : water saturated with boric acid (9:1:1, v/v) (178)

G : ethylacetate : acetic acid : water (9:2:2, v/v) (179)

H : 1-butanol : ethanol : water (40:11:19, v/v) (180)

I : Benzene : 1-butanol : pyridine : water (1:5:3:3; upper phase) (181)

5 - R_g values

The R_g values quoted refer to the chromatographic mobility of compounds relative to that of glucose.

ie $R_g = \frac{\text{distance moved by component}}{\text{distance moved by glucose}}$

6 - Spray reagents

a) Silver nitrate/sodium hydroxide (182)

b) p-Anisidine hydrochloride (183,184)

c) Ninhydrin (184,185)

d) Ketose sprays

(i) urea phosphate spray (183,184)

(ii) p-Anisidine (1g) in phosphoric acid (183,184)

e) Ammonium molybdate (sugar-phosphates) (186)

f) Vanillin/sulphuric acid (187)

7 - Ionophoresis

Ionophoresis of sugars was carried out using a Shandon High Voltage Electrophoresis apparatus using Whatman No.3 papers as supports. Phosphate (pH 7) and borate (pH 10) buffers were used.

8 - Buffers used for enzyme extractions and assays (188)

a) Enzyme extraction

Tris-HCl buffer : 0.05M tris-HCl, pH 7.5
0.002M cysteine
0.002M EDTA

Cacodylate buffer : 0.05M-sodium cacodylate, pH 7.0
0.002 M-cysteine
0.002 M-EDTA

Tris-succinate buffer : 0.02M tris-succinate, pH 7.2
0.001M EDTA
0.001M cysteine

b) Assays

Glycylglycine buffer 0.5 M, pH 7.5

Tris -HCl, pH 7.5

Cacodylate buffer, pH 7.0

9 - Gas-liquid Chromatography (GLC)

a) - Instrumentation and columns

The instrument used was a Pye Argon Chromatograph fitted with an Argon ionization detector using dry argon as the carrier gas. The gas flow and temperature were varied according to the specific task.

The columns used for analytical procedure were:

(1) Butane-diol-succinate polyester (B.D.S.), 15%

(2) Polyphenyl ether [m-bis-(m-phenoxy-phenoxy)benzene] (PPE), 10%

(3) S E 30, 3%

(4) Apiezon K (ApK), 7.5%

All liquid phases were coated on celite supports which had been previously washed with acid and alkali and coated with dimethydichlorosilane.

b) - Preparation of Trimethylsilyl Ether. (189)

The material for analysis (5-10mg) was dried and to it was added the contents of one Sil-prep capsule [1ml containing trimethylsilyl chloride (1vol), hexamethyl disilazane (3vol.) and pyridine (9vol.)] and the solution

treated at 40° for 45 min. after which the solvent was evaporated and the residue dissolved in ether and analysed by GLC.

10 - Reduction of aldehyde groups to primary alcohols

This was carried out using 2% aqueous potassium or sodium borohydride solution for 24 hr. (190).

Experiments

1 - Separation of neutral and acidic monosaccharide components of the polysaccharide fraction from mung bean shoots.

Shoots (71.5g), grown for 3 days in the dark at 25°, were macerated in an MSE blendor and extracted (3 times) with boiling 80% aqueous methanol. The alcoholic extracts were centrifuged at 2000 rpm and the supernatants discarded. The residue (44g after partial drying) was hydrolysed with $\underline{\underline{N}}$-$H_2SO_4$ (400ml) for 15 hr. at 100°. The hydrolysate was centrifuged and the solution neutralised with barium carbonate and filtered. The filtrate was concentrated under reduced pressure at 30° to approximately 50ml and applied to an anion exchange resin column (IRA-400, acetate form). The neutral fraction was eluted from the column with water and evaporated to dryness under reduced pressure. The acidic fraction was subsequently eluted with $\underline{\underline{N}}$-$H_2SO_4$ and the eluate neutralised with barium carbonate. The acidic sugars were thus obtained as a solution of their barium salts. These were concentrated and passed through Amberlite IR-120(H^+) resin to remove barium. Elution was continued until the eluate gave a negative phenol/sulphuric acid test.

2 - Solvent fractionation of the polysaccharides from mung bean shoots.

Mung bean seeds (200g) were allowed to germinate in the dark for 4 days at 25°. The shoots were macerated and extracted (3 times) with 80% methanol (900ml). The extract (I) was stored at -20°. The residue (I) (obtained by centrifugation of the macerate at 2500 rpm for 15 min.) was then extracted four times (3-4hr/extraction) with cold water (800ml) [Extract II]. The residue (II) was extracted four times (as before) with hot water (800ml at 80°) [Extract III]. This residue (III) was divided in two equal parts (A and B) which were extracted, respectively, with $\underline{\underline{N}}$-$H_2SO_4$ and $\underline{\underline{N}}$-NaOH (1L at room temperature for 4½hr.). All extractions were made in the presence of nitrogen. The acidic (A) and basic (B) extracts were neutralised, respectively, with concentrated NaOH and glacial acetic acid and dialysed (72hr.) against running tap water. All

extracts were freeze-dried. Samples were hydrolysed with $2\underline{N}$-H_2SO_4 for 6hr. at 100° in sealed tubes, and the liberated sugars identified.

3 - Fractionation of the polysaccharides in mung beans by centrifugation.

400g of mung bean seeds were allowed to germinate in the dark for 5 days at 25°. Shoots (1300g) were ground with sand in Tris-HCl buffer (0.1M, 1300ml, pH 7.5) at 4°, and the brei pressed through two layers of muslin. The resulting mixture was centrifuged successively at 1000g, 10,000g, 20,000g, 50,000g and 100,000g. All the fractions were freeze-dried.

4 - Isolation of glycolipids.

In all experiments the whole glycolipid fraction was extracted with 80% aqueous methanol or ethanol (191). Approximately 1L of solvent was used for 100g of shoots (3 days old, grown in the dark at 25°). In a typical experiment 100g of shoots were macerated and then extracted for 5 min. in 500ml. of boiling 80% methanol. The macerate was filtered hot through a Buchner funnel and the residue then re-extracted with aqueous methanol. The filtrates were combined and the solvent removed under reduced pressure. The resulting syrup was suspended in 200ml of water and the mixture centrifuged for 30min. at 25,000g. The residue was again taken up in water and the centrifugation repeated.

Glycolipids were obtained from Extract I in Experiment 2 and from 1000g and 10,000g fractions in Experiment 3. Glycolipids were hydrolysed with $0.01\underline{N}$- and $\underline{N}$-H_2SO_4 for 2 hr. at 100°. The residues from 1000g and 10,000g fractions, after extraction of glycolipids, were hydrolysed with $2\underline{N}$-H_2SO_4 for 6hr. at 100° in sealed tubes.

5 - Qualitative analysis of the fractions obtained by different extraction procedures.

The acid hydrolysates of all fractions were subjected to qualitative analysis using chromatography on Whatman No.1 paper in solvents A,B,G and H. The papers were sprayed with $AgNO_3$/NaOH and p-Anisidine hydrochloride reagents. The acidic sugars were identified by paper

electrophoresis on Whatman No.3 paper in phosphate buffer (pH 7.0) at 2000 volts for 70 min. The neutral monosaccharides were further characterised by gas-liquid chromatography (GLC) of their trimethylsilyl (TMS) derivatives on two columns (BDS; PPE)

6 - Quantitative determination of the monosaccharide components of glycolipids in mung bean shoots.

The Wilson method (158) was used to determine quantitatively the monosaccharide constituents of glycolipids. Standard curves, for each of the identified sugars, were obtained. The sugar mixtures were resolved on paper using solvents A and B.

7 - Identification of mannose.

Glycolipids were extracted from 150g of shoots with 80% ethanol and hydrolysed for 2½ hr. at 100° with $\underline{\underline{N}}$-$H_2SO_4$(50ml). The hydrolysate was neutralised with a solution of 5% N, N-di-n-octylmethylamine in chloroform. The water layer was washed twice with chloroform to remove traces of organic base and then concentrated under reduced pressure. The mixture of monosaccharides was streaked on Whatman No.3 paper which was developed in solvent A for 40hr. The mannose band was identified and the sugar eluted from the paper. After checking its purity by paper chromatography final identification was effected by GLC of a TMS derivative on a PPE column.

8 - Solvent fractionation of the glycolipids.

a) Chloroform extraction - 30g of shoots were extracted twice with chloroform (150ml) for 4 min. in a macerator. The chloroform extracts were washed four times with 150ml of water in order to remove any free monosaccharide. The chloroform was then removed under reduced pressure and the residue suspended in 20ml of $\underline{\underline{N}}$-$H_2SO_4$ and hydrolysed at 100° for 2hr. The hydrolysate was neutralised with a 5% N,N-di-n-octylmethylamine solution and then spotted on papers which were developed with solvents A and B.

b) Acetone extraction - 30g of fresh shoots were extracted twice

with acetone (150ml) for 4 min. in a macerator. The soluble glycolipids were isolated, hydrolysed and their carbohydrate content determined.

c) 80% methanol fractionation - The residue from the acetone extraction was re-extracted twice with boiling 80% aqueous methanol (60ml) and the carbohydrate content of the dissolved glycolipids again determined.

9 - Column fractionation.

150g of mung bean shoots were extracted successively with acetone and 80% methanol. The glycolipids were extracted from each fraction, dissolved in chloroform and passed, separately, through a column consisting of a mixture of silicic acid and hyflo super cel (192). Step-wise elution of the fractions from the column was effected with 200ml of each of the following: chloroform, 20, 40, 60 and 80% methanol in chloroform, methanol, 80% aqueous methanol. 10ml fractions of eluate were collected. The column was finally washed with 200ml of 50% aqueous methanol. A quantitative determination of the carbohydrate content in each fraction was performed, using the phenol-sulphuric acid method on 0.5ml aliquots. The fractions corresponding to the peaks were pooled and the solvent removed.

10 - Qualitative analysis of the peaks isolated by column chromatography.

a) Thin-layer chromatography - Aliquots of the peaks isolated from 80% methanol glycolipid fraction were dissolved in hot methanol and spotted on thin layer plates coated with silica gel G. These were developed with a chloroform:methanol:water solvent (90:10:1, v/v or 60:40:1, v/v) (193). The spots were detected with I_2 vapour and vanillin/sulphuric acid spray.

b) Paper chromatography - Aliquots from all peaks were hydrolysed with $\underline{\underline{N}}$-$H_2SO_4$, neutralised and the monosaccharide constituents determined by paper chromatography in solvents A and B.

11 - Preliminary studies on the deacylation products of glycolipids.

a) Deacylation of the glycolipids - Glycolipids (900mg) from an 80% (v/v) aqueous methylated spirit extract of mung bean shoots (400g) were deacylated with 0.2M-KOH in absolute methanol (100ml) for 3 hr. at room temperature (193). The solution was then treated with IR-120 (H^+form) resin. The resulting acidic solution was made alkaline with concentrated ammonium hydroxide and the excess ammonia removed on a rotary evaporator leaving the solution neutral. This solution was taken to dryness and then dissolved in chloroform. The chloroform solution was extracted with water and the aqueous layer back-extracted with chloroform. The carbohydrate of the water layer, determined by the phenol/sulphuric acid test, was approximately 80mg.

b) Preparative paper chromatographic fractionation of deacylation products - The mixture was fractionated on Whatman No.17 paper in solvent B. The seven fractions obtained were eluted from the paper with water and re-chromatographed in solvent A and B to give finally eight components. These components were again eluted from the paper with water, concentrated and analysed.

c) Qualitative analysis of the components isolated from deacylation products - Samples of all the components were hydrolysed with N-H_2SO_4 and neutralised with N,N-di-n-octylmethylamine in chloroform. The resulting neutral solutions were divided into two parts. One part was reduced with potassium borohydride. TMS derivatives of the resulting sugar alcohols were prepared and examined by GLC on two columns (PPE and SE30). The second part of the hydrolysates was fractionated by paper chromatography in solvents A and B using $AgNO_3$/NaOH and p-anisidine sprays. Specimens of three components were also hydrolysed with 90% formic acid for 6hr. The hydrolysates were examined by paper chromatography in solvents A and B and sprayed with urea-phosphate reagent (specific for ketoses).

c) Methylation of components III and IV - Oligosaccharides (0.5-2.0mg) were shaken with methyl iodide (0.1ml), dimethyl formamide (0.2ml)

and silver oxide (0.2g) at room temperature in the dark for 18hr. (194,195). The mixture was extracted twice with chloroform, filtered, and the combined filtrate was evaporated to dryness under reduced pressure at 30°. The residue was methylated a second time under the conditions stated above, then the methylated products were treated with boiling methanolic-HCl (3.8%) for 4hr. and the resulting methyl glycosides examined by GLC on two columns (B.D.S. and P.P.E.).

12 - Structural studies on the deacylation products of the glycolipids.

a) Isolation and fractionation of the deacylation products - 4Kg of shoots were extracted with 40L of boiling 80% aqueous methylated spirit. The glycolipid was isolated and then dried by successive treatment with methanol, methanol/chloroform mixture and chloroform. The glycolipid pellet (3g) was subjected to mild alkali treatment (see Experiment 11a) and the water-soluble deacylation products were fractionated on Whatman No.3MM papers in solvent A for 24hr. All fractions were eluted from the papers with water and tested for purity in three chromatographic solvents (A,B,I).

b) Qualitative analysis - Aliquots of all the fractions were hydrolysed with 90% formic acid (2ml) in a CO_2 atmosphere at 100° for 6hr. Paper chromatographic analysis (insolvents A and B) of the hydrolysates was then carried out using $AgNO_3$(NaOH and p-anisidine hydrochloride as locating reagents. Aliquots of the hydrolysates were also analysed as TMS derivatives by GLC on two columns (PPE and SE30).

c) Methylation studies on III, IV, VIa and VII - The oligosaccharides were methylated twice as described in experiment 11c. The mixture was then extracted with chloroform, filtered and the filtrate evaporated to dryness. The residue was methylated a third time by dissolving it in methyl iodide and refluxing the solution in the presence of silver oxide for 8hr. (196). The methylated oligosaccharides were methanolysed by refluxing in dry methanol in the presence of IR-120(H^+) previously treated and dried with methanol for 6hr. (197). The mixtures were filtered and the resin washed with dry methanol. The methanolysis products were

examined by GLC on two columns (BDS and PPE).

13 - Preliminary studies on the incorporation of sucrose-(U)-^{14}C into glycolipids.

a) Mung bean shoots (5g; grown for 3 days in the dark at 25°), were placed in a specimen tube with their cut ends in contact with a solution consisting of sucrose-(U)-^{14}C (2μCi, 463 mCi/mM) in water (0.5ml). The shoots were left in the dark at approximately 23° for 24hr. The shoots were macerated in 80% aqueous ethanol and the glycolipids extracted in the usual way. One fifth of the glycolipid solution in chloroform was spotted onto filter paper and its radioactivity content (11,691 cpm) determined with a Beckman CPM-100 scintillation counter. The remaining glycolipids were then hydrolysed with 90% formic acid under an atmosphere of CO_2. Chloroform was added to the resulting syrup and the chloroform solution extracted with water. The water layer was back-extracted with chloroform (one fifth of the chloroform solution, containing fatty acid, gave a count of 7,723 cpm). The monosaccharide fraction was divided equally between two Whatman No.1 papers which were developed separately with solvents A and B. The papers were cut into strips and the radioactivity in each strip was recorded.

b) The experiment was repeated and the monosaccharide fraction obtained by acid hydrolysis of the glycolipid pellet was applied, as a 3cm band, to two paper chromatograms which were developed with solvents A and B, respectively. The radioactive strip was cut into 0.5cm sections which were counted.

14 - Quantitative studies on the incorporation of sucrose-(U)-^{14}C into glycolipids.

a) 60g of mung bean shoots were left in contact with a solution containing 10μCi sucrose-(U)-^{14}C for 7½hr. and 24hr., respectively. Controls were left in distilled water for the same periods of time. Combined radioactive monosaccharides were again released by acid hydrolysis of the

glycolipid fractions and the hydrolysates applied separately as bands to chromatography papers which were then developed separately in solvent A (20hr.) and solvent B (40hr.). The radioactivities of the sugars were determined as in 13b.

b) Quantitative analysis of the monosaccharide component of glycolipids - Total activities in the monosaccharide components of the glycolipids were calculated. At the same time the monosaccharide components were obtained from the glycolipids that had been extracted from the control shoots which had been left in distilled water. These monosaccharides were fractionated with solvents A and B on Whatman No.1 papers. They were determined quantitatively using the tetrazolium method (159,160). Standard curves were obtained for every determination.

Tetrazolium method (159,160). The paper chromatograms were dipped in a solution of 0.5% 2,3,5-triphenyltetrazolium chloride (TTC) in chloroform and the solvent allowed to evaporate off at room temperature. The papers were then dipped in 2% ethanolic sodium hydroxide and allowed to dry almost completely at room temperature. They were then put in an oven with a water saturated atmosphere at 50-55^{o} for 20 min. Areas of paper containing the coloured spots were immediately cut from the chromatograms and eluted with 4ml of 8% acetic acid in methanol. Absorbance of the resulting solution was measured at 490nm with a Unicam SP.500 spectrophotometer.

15 - Preliminary studies on the formation of UDP-fructose and ADP-fructose.

a) Enzyme preparation - Mung bean seedlings were allowed to germinate for 3 days in the dark at 25^{o}. 100g of shoots were extracted with 200ml of tris-HCl (pH 7.5) buffer, for 2min. in an MSE blendor. The macerate was filtered through two layers of muslin and centrifuged at 10,000g for 20min. One part of the supernatant solution was immediately dialysed overnight against tris-succinate (pH 7.2) buffer in the cold room (crude enzyme). The other part was fully saturated with ammonium sulphate at 0^{o} and then centrifuged at 10,000g for 20min. The centrifugate was suspended in approximately 30ml of tris-succinate (pH 7.2) buffer and dialysed overnight

in the cold room against this buffer (purified enzyme)

b) Enzymic reaction - The following digests were prepared:

UTP (0.1M) or ATP (0.1M)	10µl
$MgCl_2$ (0.1M)	10µl
D-fructose-1-phosphate (0.1M)	10µl
Bovine serum albumin (10mg/ml)	5µl
Glycylglycine buffer (0.5M; pH 7.5)	20µl
Enzyme preparation (crude or purified)	20µl

The reactions were carried out in capillary tubes incubated at 35° for 1hr. Control experiments were carried out for the same time using an enzyme preparation which had been boiled for 10-15 min. The contents of the capillary tubes were streaked on Whatman No.1 papers which were developed with solvent E for 48 hr. The dried chromatograms were examined under ultraviolet light.

The reaction was repeated on a larger scale (10 times) using the purified enzyme preparation. The bands absorbing under ultraviolet light were eluted from the paper and the u.v. spectrum of the solution measured with a Unicam SP-800 spectrophotometer. The compounds formed were also hydrolysed with N-HCl for 10 min. at 100°. The hydrolysates were examined on a paper chromatogram with solvent A.

c) Attempted enzymic conversion of fructose-1-phosphate (F-1-P) to fructose-2-phosphate (F-2-P) - An experiment, in all respects similar to that described in the previous section was carried out but ATP and UTP were excluded. The reaction mixture streaked on to Whatman No.3 papers and electrophoresis effected in borate buffer (pH 10) at 3,500 volts for 1 hr. The reaction was repeated using cacodylate buffer (pH 7.0) in place of glycylglycine and enzyme preparations obtained from mung beans grown for 3 days, 29hr. and 12hr., respectively. The reaction mixtures were studied by paper chromatography using solvent C. The paper electrophoretograms and chromatograms were sprayed with molybdate reagent (see p.95).

16 - Chemical synthesis of D-fructofuranose-2-phosphate [F-2-P(fur)] and D-fructopyranose-2-phosphate [F-2-P(pyr)].

The barium salt of D-fructose-1-phosphate was converted to the pyridinium salt by passage through Dowex 50(H^+form) resin followed by neutralisation with pyridine (169,170).

Pyridinium D-fructose-1-phosphate (2.5mmole), dicyclohexylcarbodiimide (2.5mmole), triethylamine (7.5mmole), and water (4.2ml) were made up to a total volume of 63ml with pyridine and the solution kept at 85° for 25 min.

The solution was then evaporated to dryness under reduced pressure (30°) and the residue dissolved in 23ml of sodium hydroxide and kept at 37° for 8 min. After cooling to 0° the solution was applied to a column (3 x 50cm) of Dowex-1 (200-400mesh; borate form) resin. The column was washed with water and the phosphate esters then eluted with a linear gradient of from 0 to 0.3M ammonium borate, pH 8.8 (total volume, 10L).

Fractions (15ml) were collected at a flow rate of 1.5ml/min. Samples (0.6ml) of every fraction were taken and analysed for phosphate by the method of Bartlett (198,199).

Three small peaks of D-fructofuranose-2-phosphate, D-fructopyranose-2-phosphate and inorganic phosphate appeared first from the column followed by a large peak of D-fructose-1-phosphate (Fig.15).

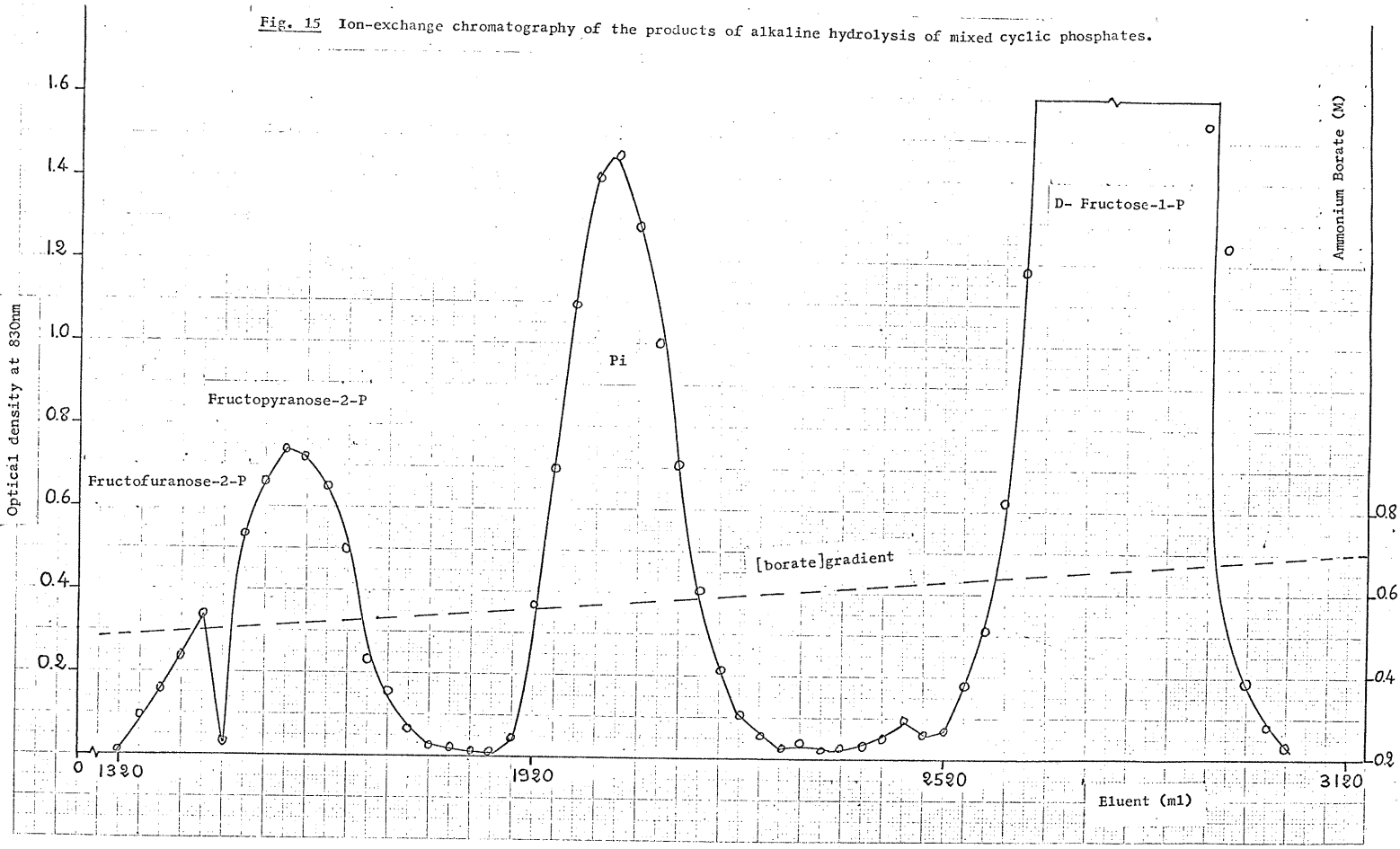
The appropriate fractions were pooled and freeze dried. Methanol was added and the fructose phosphates were freed from ammonium borate by evaporation under reduced pressure at 30°. The resulting solids were neutralised with 0.1N-sodium hydroxide and the fructose-phosphate salts freeze dried.

The phosphates were identified by paper chromatography with solvent C. The solutions of fructose-2-phosphates were stored at -20°.

17 - D-fructofuranose-2-phosphate and D-fructopyranose-2-phosphate as possible substrates for fructose nucleotide formation.

An enzyme preparation was obtained and purified using tris-HCl buffer (pH 7.5) (see experiment 15a)

Fig. 15 Ion-exchange chromatography of the products of alkaline hydrolysis of mixed cyclic phosphates.



a) Effect of time of incubation - The following basic reaction mixtures were prepared:

Tris-HCl buffer (1.0M; pH 7.5):$MgCl_2$(0.1M), 2:1 v/v	20µl
Enzyme preparation	20µl
Bovine serum albumin (10mg/ml)	5µl
ATP(0.1M) or UTP(0.1M)	2µl
F-2-P(fur) (0.08M) or F-2-P(pyr) (0.06M)	5µl

The reactions were carried out in capillary tubes at 35° for 10, 30,60 and 90 min. The mixtures were streaked on Whatman No.1 papers which were developed with solvent D. Papers were scanned for fructose-nucleotide formation under uv light.

b) Effect of different nucleoside triphosphates - Similar digests were prepared using different nucleotides as substrates (UTP,ATP,CTP, and GTP; 0.1M, 10µl). The reaction mixtures were treated as described.

c) Effect of germination time - In this case cacodylate buffer (pH 7.0) was used to extract enzymes from mung beans grown for 3 days and 29hr. respectively. The reactions were carried out in cacodylate buffer. Two nucleotides (UTP and ATP) F-2-P(fur), F-2-P(pyr) and F-1-P were used as substrates.

18 - Attempted direct conversion of D-fructose-1-phosphate to UDP-fructose.

Mung bean seeds were germinated for 12hr. at 25° in the dark. The enzyme fraction was extracted from the whole seedling either with cacodylate buffer (pH 7.0) or with tris-HCl buffer (pH 7.5). The reactions were carried out for 10,30,45,60 and 90 min. In addition, one sample of fructose-1-phosphate was incubated with the enzyme preparation (20µl) in the absence of nucleotide for 30min. and then UTP and more enzyme (20µl) were added; incubation was then continued for another 30min. Paper chromatographic fractionation of reaction mixtures was effected with solvent D and papers were "visualized" with uv light. The uv spectrum of the nucleotides synthesized were examined with a Unicam SP-800 spectrophotometer. Quantitative measurements were made on aqueous solutions at 260nm.

Pooled chromatographic fractions of the suspected UDP-ketose were evaporated to dryness and hydrolysed with 50% aqueous formic acid under an atmosphere of CO_2 at 100° for 30min. TMS derivatives of the hydrolysates were prepared and examined by GLC on an ApK column.

19 - Synthesis of fructose-(U)-^{14}C-1-phosphate.

Fructokinase (ketohexokinase:fructose-1-phosphotransferase) was extracted from beef liver according to the method of Hers (200-202).

a) Fresh beef liver was sliced and homogenised in a Waring blender with ice-cold water (1L).

b) The homogenate, maintained at 0°, was acidified to pH 4.5 with N-HCl and brought back immediately to pH 5.5 with N-NaOH. The bulky precipitate containing agglutinated particles and denatured proteins was centrifuged off (10,000g for 30min.) and the clear supernatant solution adjusted to pH 7.5.

c) The supernatant was treated with ammonium sulphate (60% saturation) and the precipitate centrifuged off at 10,000g for 20min. This was taken up and dialysed against 0.1M-cacodylate buffer, pH 6.5.

The enzyme preparation was only partially purified, however, therefore it was necessary to determine the optimum conditions for obtaining fructose-^{14}C-1-phosphate.

Digests with the following composition were prepared:

Fructose(U)-^{14}C (0.05μCi; 2.8mCi/mM)	10μl
ATP (0.05M)	10μl
Magnesium acetate (0.05M)	10μl
Potassium acetate (4.0M)	30μl

After mixing 0.5M-sodium fluoride (10μl) and enzyme preparation (50μl) were added and the whole taken up in a capillary tube which was then sealed. Control experiments were carried out replacing ATP and magnesium acetate solutions by water.

The activity of the enzyme preparation was first tested in a preliminary experiment (Fig.16), and then the effect of reaction time on

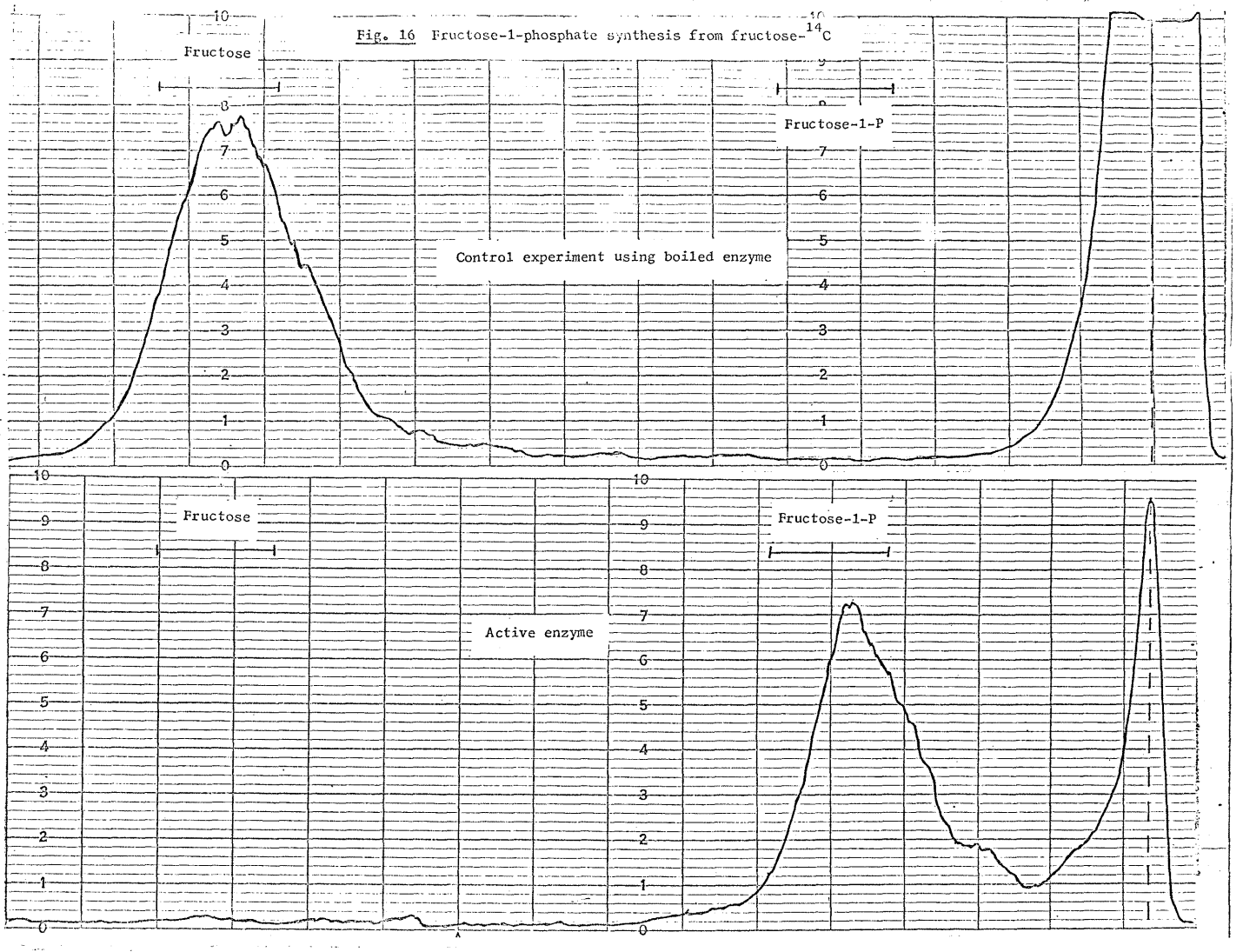


Fig. 16 Fructose-1-phosphate synthesis from fructose-^{14}C

yields was studied. Reaction mixtures were incubated at 35° for 1,5, 10 and 20 min. They were then immediately applied to Whatman No.1 papers which were developed in solvent C. The radioactive strips were examined with a Tracerlab 4II scanner (Fig.17). The total activity of the sugar-phosphate in each reaction was counted with a scintillation counter (Fig.18). Reaction times of 1 and 5 min. produced symmetrical peaks of fructose-1-phosphate when chromatograms were examined with a strip counter. After 15 and 20 min. the yields decreased and more than one peak of radioactive sugar-phosphate may have been present. A reaction time of 5 min. was, therefore, chosen for "large scale" preparations. A solution of fructose-(U)-^{14}C-1-phosphate (15,000cpm/µl) was obtained. When a sample of the product was hydrolysed in N-HCl for 1 hr. at 100° fructose was the only detectable monosaccharide in the hydrolysate.

20 - Attempted conversion of fructose-(U)-^{14}C-1-phosphate to UDP-fructose.

The following basic reaction mixture was used for all kinetic studies:

Fructose-(U)-^{14}C-1-phosphate (15,000cpm/µl)	5µl
Bovine serum albumin (10mg/ml)	5µl
UTP (0.1M)	5µl
Cacodylate buffer (0.1M; pH7.0):$MgCl_2$(0.1M),2:1v/v	20µl
Enzyme preparation (17.5mg protein/ml)	20µl

Reactions were carried out in capillary tubes at 35° and the product (suspected UDP-fructose) fractionated on paper chromatograms using solvent E. Papers were examined with a 4II scanner and accurate determinations of radioactivity made with a scintillation counter.

a) The reaction mixtures were incubated for 10,30,45,60 and 90 min., respectively. A control experiment using boiled enzyme was also incubated for 60 min. Three additional reactions were carried out. In the first the enzyme concentration was doubled and the reaction mixture incubated for 45 min. In the other two, F-1-P was incubated with the enzyme preparation for 30 min. in the absence of nucleotide. Then UTP and a further volume (20µl) of enzyme were added to one digest and UTP alone was added to the other. Both were incubated subsequently for 30 min.

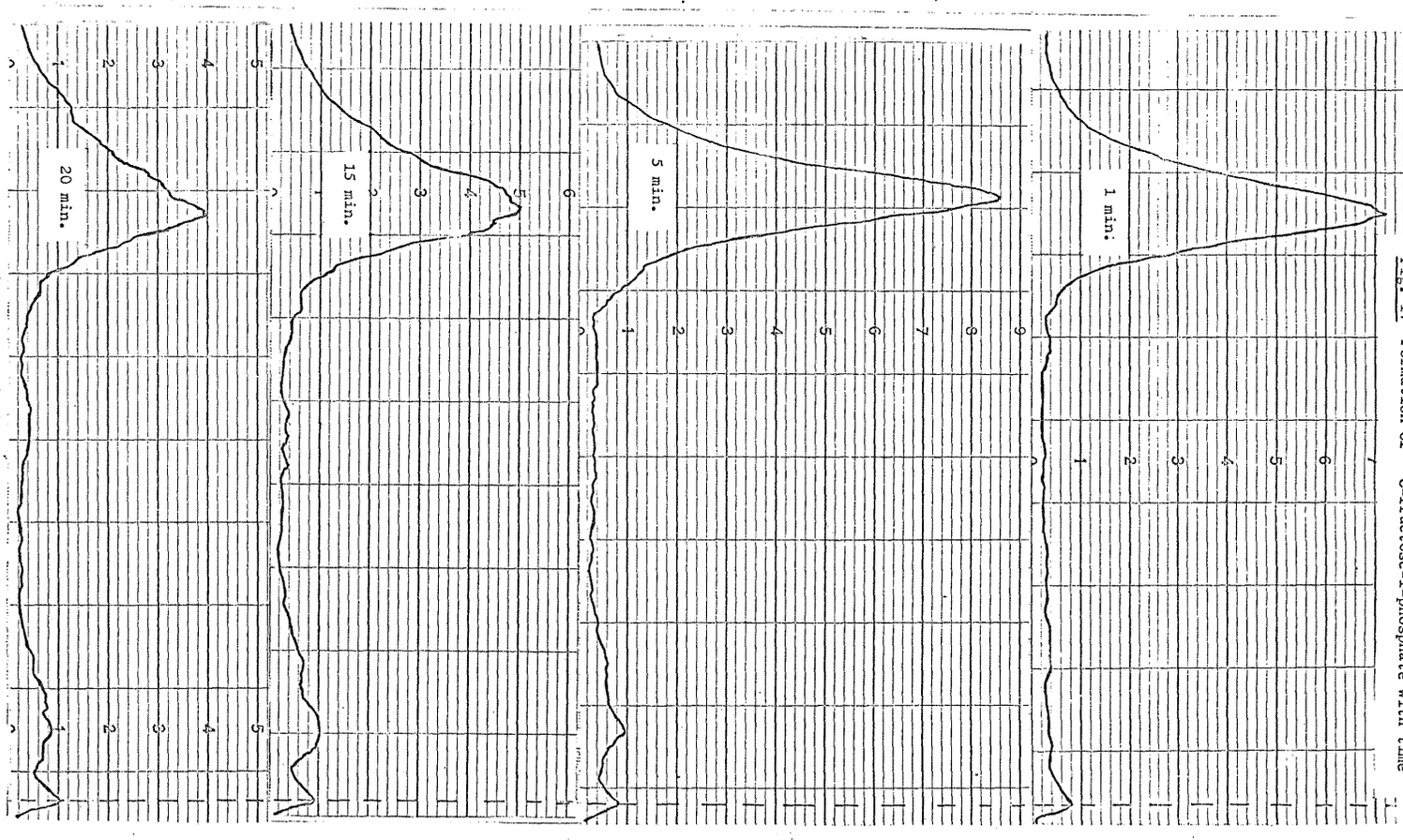


Fig. 17 Formation of ^{14}C-fructose-1-phosphate with time

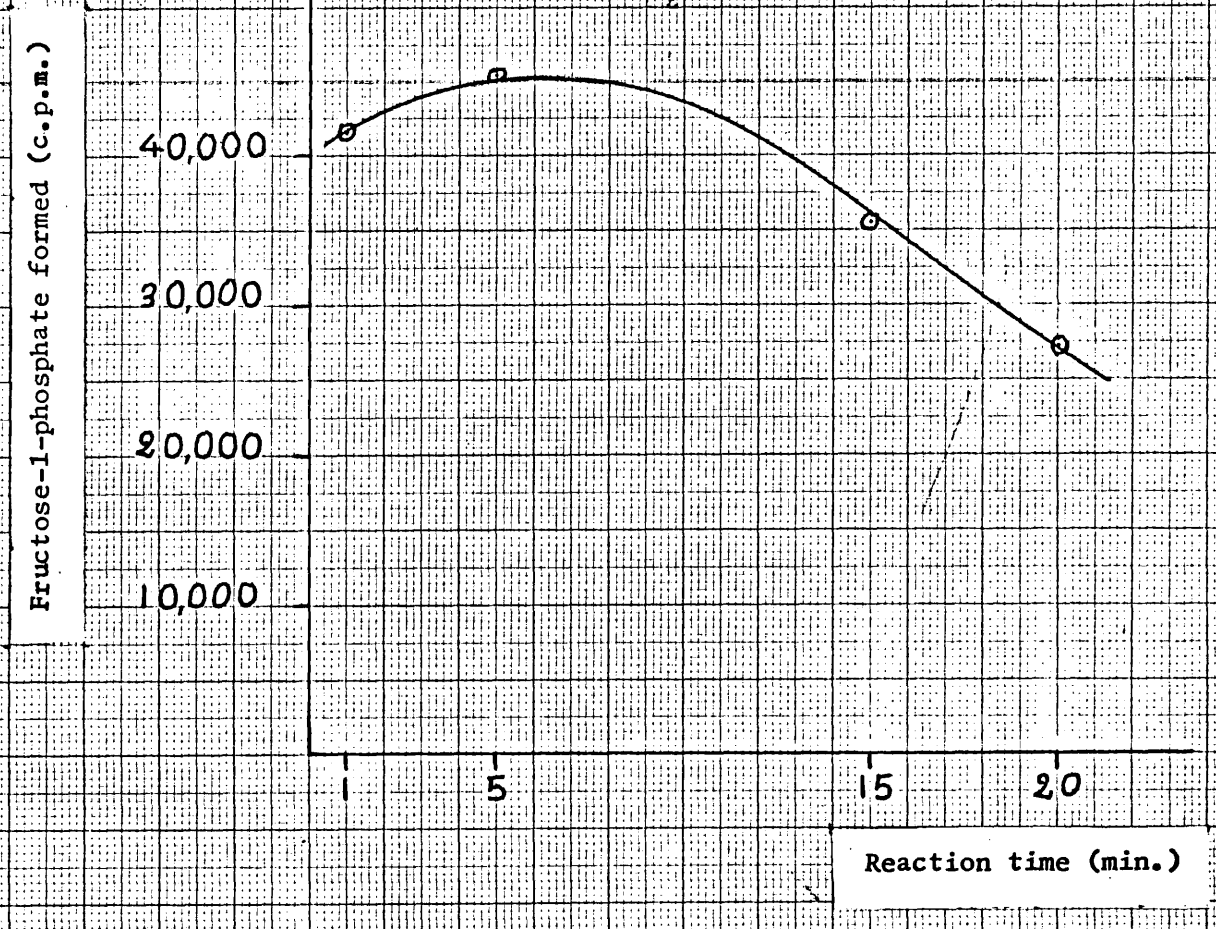


Fig. 18 Formation of ^{14}C-fructose-1-phosphate with time

b) Fructose-nucleotide synthesis was measured in the presence of varying amounts of fructose-1-phosphate (3.40, 7.40, 9.75, 12.9 mM).

c) Fructose-nucleotide synthesis was measured in the presence of varying amounts of fructofuranose-2-phosphate (2.54, 5.08, 7.62, 10.3 mM).

d) Fructose-nucleotide synthesis was measured in the presence of varying amounts of inorganic phosphate (1.59, 3.18, 4.76, 6.35 mM).

21 - Qualitative analysis of the radioactive fructose-nucleotide formed.

The fructose-nucleotide formed in Experiments 20 was eluted from papers and subjected to different acid treatments.

Aliquots were treated with acetic acid and oxalic acid solutions both at pH 3.8 for 6hr. and 2% oxalic acid for 2hr., all at 100°. The products were spotted on Whatman No. 1 papers which were developed with solvent A. A sample of the nucleotide was treated with sodium borohydride for 24hr. and then hydrolysed. The hydrolysate was spotted on paper chromatogram and developed in solvent F.

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