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BIOSYNTHESIS OF GALACTOSYLSUCROSE DERIVATIVES

A thesis submitted by

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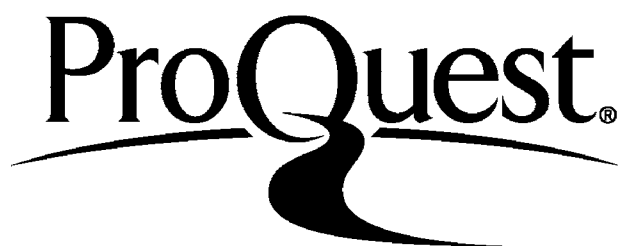
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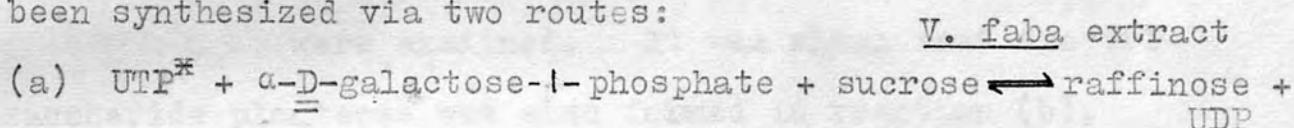
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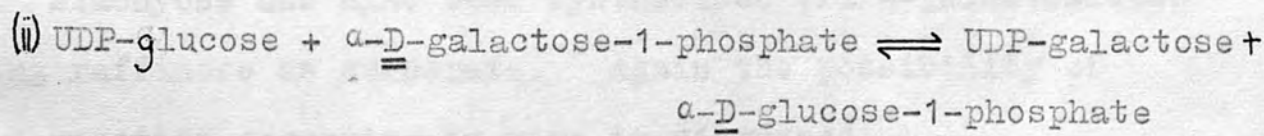
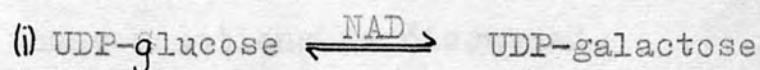
ABSTRACT

The biosynthesis of the "raffinose family" of oligosaccharides in Vicia faba seeds has been investigated in vitro. Preliminary experiments have shown that the oligosaccharides are synthesised as the seeds mature and reach a maximum concentration at the dormant stage.

Raffinose (the lowest member of the series), has been synthesized via two routes:



The following reactions which result in the formation of UDP-galactose were also catalysed by V. faba extracts



#	UTP	Uridine triphosphate
	ATP	Adenosine triphosphate
	UDP	Uridine diphosphate
	UDP-Glucose	Uridinediphospho-D-glucose
	UDP-galactose	Uridinediphospho-D-galactose.
	ADP-galactose	Adenosinediphosphogalactose
	NAD	Nicotinamide adenine dinucleotide.

ADP-galactose was considered as a possible galactose donor, but attempts to synthesize this compound from ATP and α -D-galactose-1-phosphate were unsuccessful. The combined evidence strongly suggests that UDP-galactose is the galactose donor in (a), and that such a reaction could occur in vivo.

(b) Melibiose + sucrose $\xrightarrow[\text{V. faba}]{\alpha\text{-galactosidase}}$ raffinose + glucose

In connection with this, the properties of V. faba α -galactosidase were examined. It was shown that the tri-saccharide planteose was also formed in reaction (b), but in much smaller quantities than the isomeric raffinose.

The biosynthesis of raffinose in vivo by either or both of these reactions is discussed.

Stachyose has also been synthesized via α -galactosidase using raffinose as substrate. Again the possibility of this reaction occurring in vivo is discussed.

Another enzyme found to occur in dormant V. faba seeds is alkaline β -fructofuranosidase. The properties of this enzyme were examined and compared with other β -fructofuranosidases known to occur in plant tissues. The enzyme was shown to hydrolyse β -D-fructofuranosides and to transfer a fructosyl residue to sucrose forming 1^F - β -fructosylsucrose.

The possibility of this enzyme and α -galactosidase being involved in the breakdown of raffinose oligosaccharides in vivo is considered.

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INTRODUCTION

The structure and occurrence of galactosylated derivatives in nature has been extensively studied, but little is known about their metabolism or biosynthesis.

The "raffinose family" of oligosaccharides (Fig. 1) is the most widely occurring of the galactosylated derivatives. The biosynthesis of these compounds is considered to be a metabolic pathway. The lower members of the series (i.e.,



Fig. 1.

raffinose and stachyose ($n = 1, 2$ respectively), have been found in many plant organs (i.e., roots, seeds, shoots and stem tubes). The higher members of the series have been found only in seeds and roots.

The structure of raffinose was originally established by hydrolysis with β -D-fructofuranosylase (β -D-fructofuranosyl fructosylase) which gave D-fructose and

INTRODUCTION

The structure and occurrence of galactosylsucrose derivatives in nature has been extensively studied, but little is known about their metabolism or biosynthesis.

The "raffinose family" of oligosaccharides (Fig.I) is the most widely occurring of the galactosylsucrose derivatives^{1,2,3,4} and may be of considerable metabolic importance. The lower members of the series i.e.

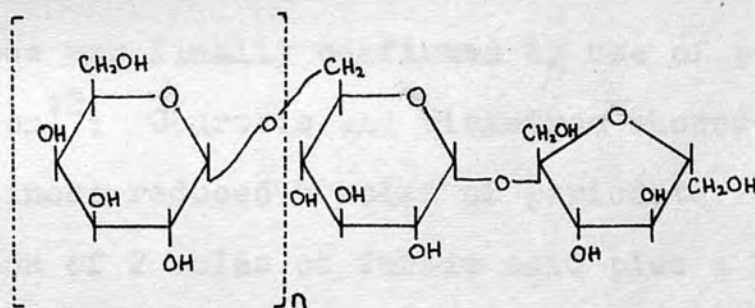


Fig.I.

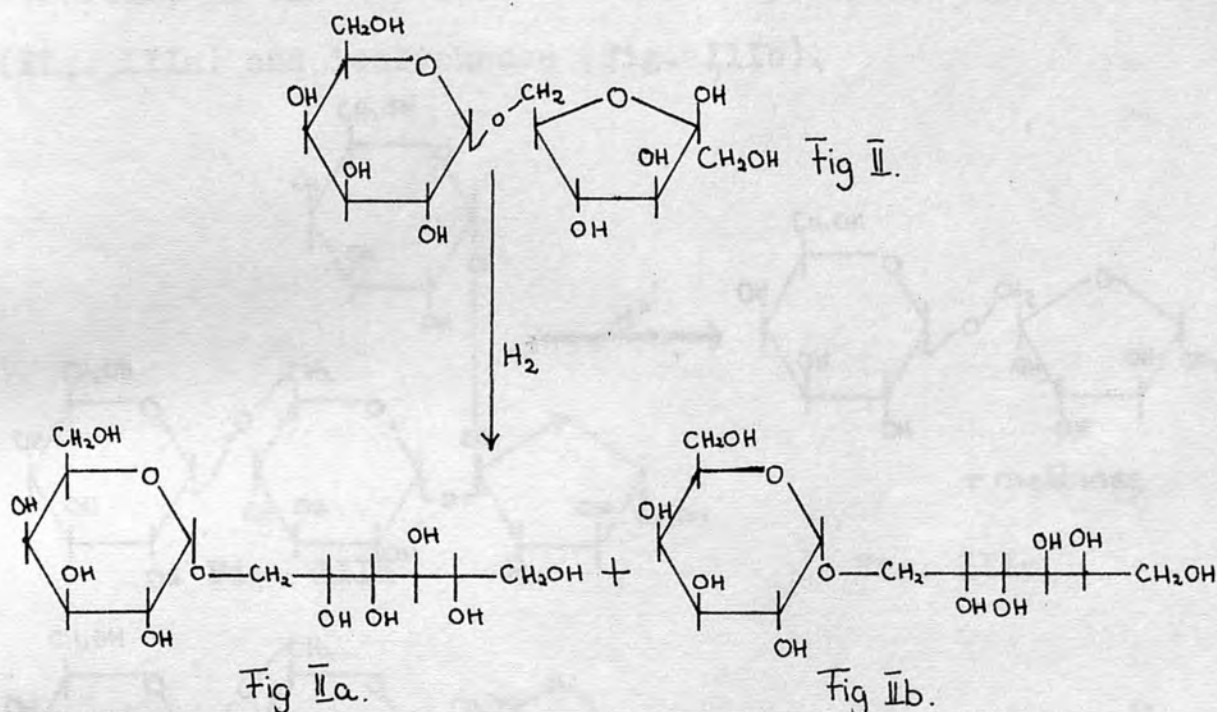
raffinose and stachyose ($n = 1, 2$ respectively), have been found in many plant organs e.g. roots, seeds, shoots and sieve tubes^{3,5,6,7} but higher members of the series have been found only in seeds and roots.

The structure of raffinose was originally elucidated by hydrolysis with β -D-fructofuranosidase (β -D-fructofuranoside fructohydrolase) which gave D-fructose and

melibiose⁸, and almond emulsion α -D-galactosidase (α -D-galactoside galactohydrolase) which hydrolysed the trisaccharide to D-galactose and sucrose⁹. Methylation analysis by Haworth and his associates, which yielded 2,3,4,6-tetra-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-glucose and 1,3,4,6-tetra-O-methyl-D-fructose^{10,11} suggested that there was a (1,6) linkage between the D-galactose and D-glucose units, and that glucose was bonded to fructose as in sucrose. The structure of raffinose was finally confirmed by use of periodate oxidation¹²; Courtois and Wickstrom showed that 1 mole of raffinose reduced 5 moles of periodate with the formation of 2 moles of formic acid plus a hexa-aldehyde. No formaldehyde was produced.

Planteose (α -D-glucopyranosyl 1 \rightarrow 2 β -D-fructofuranosyl 6 \leftarrow 1 α -D-galactopyranose), is a non-reducing isomer of raffinose which has an R_f value very similar to the latter on paper chromatograms. Planteose may be distinguished from raffinose by the action of β -fructofuranosidase which hydrolyses the latter but not the former. Planteose also reacts with α -galactosidase to give galactose and sucrose, but unlike raffinose partial acid hydrolysis yields a reducing ketose disaccharide,

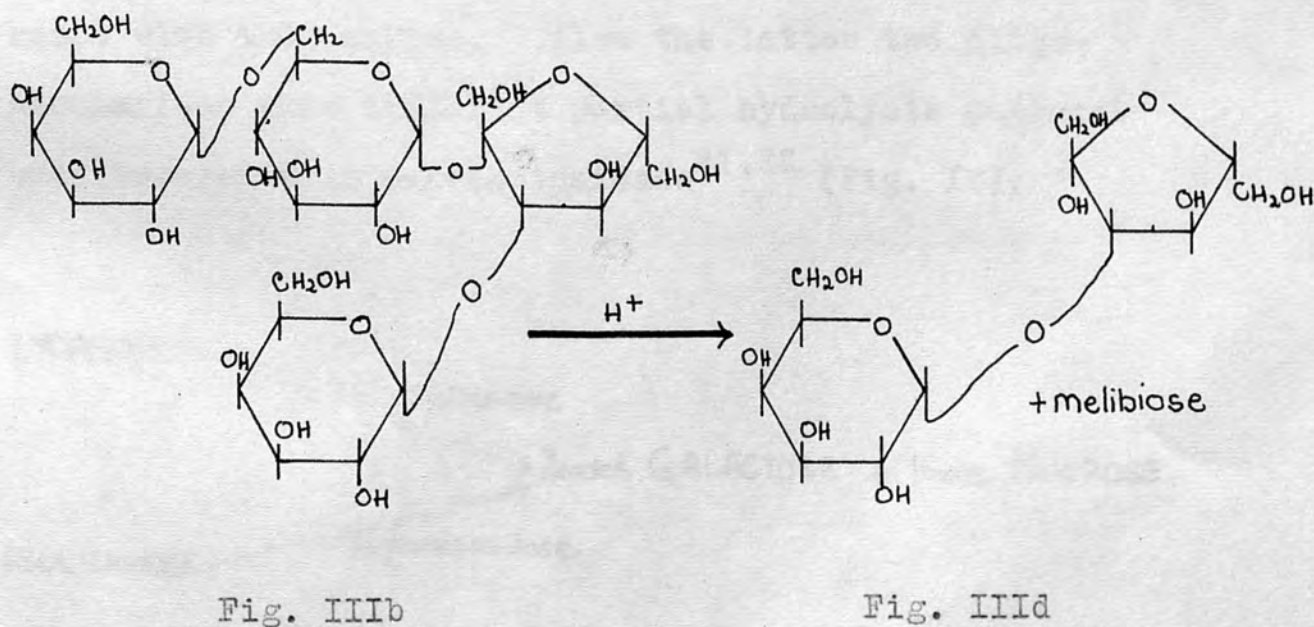
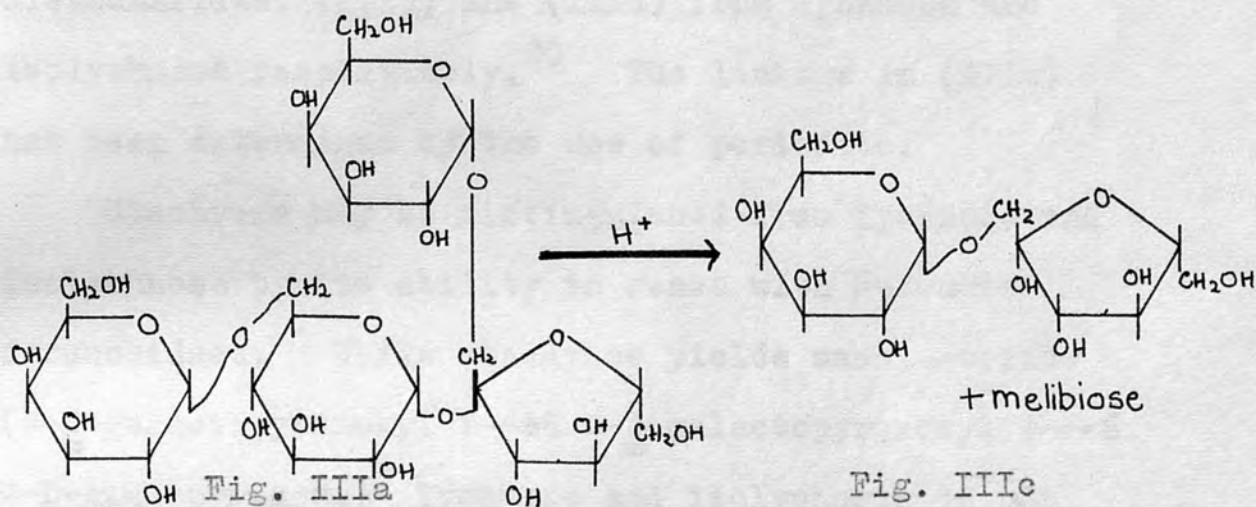
planteobiose (Fig. II). This may be characterized by its reduction to a mixture of melibiose (Fig. IIa) and epimelibiose¹³ (Fig. IIb).



The next member of the raffinose series, stachyose, has long been an object of study in the oligosaccharide field. It was first isolated by Tanret in 1902 from Stachys tuberosa¹⁴ and has since been shown to occur in 65 different plant species. Its structure has been elucidated in a way similar to raffinose. Methylation by Onuki¹⁵ proved the presence of (1,6) linkages between the two galactose units and the galactose and glucose units. Periodate oxidation showed that 7 moles of periodate were reduced by 1 mole of stachyose giving

3 moles of formic acid and an octa-aldehyde. No formaldehyde was produced.^{16,17,18.}

Periodate oxidation has been used to prove the structure of the two isomers of stachyose, lychnose (Fig. IIIa) and isolychnose (Fig. IIIb).



Incubation of these two tetrasaccharides with $0.4N-H_2SO_4$ at 37° for 2 days brings about selective scission of the fructose-glucose linkage giving in both cases melibiose and galactosylfructose disaccharides, (IIIc) and (IIId) from lychnose and isolychnose respectively.²⁰ The linkage in (IIIc) has been determined by the use of periodate.

Stachyose may be distinguished from lychnose and isolychnose by its ability to react with β -fructofuranosidase. While stachyose yields manninotriose (α -D-galactopyranosyl 1 \rightarrow 6 α -D-galactopyranosyl 1 \rightarrow 6 α -D-glucopyranose)⁸ lychnose and isolychnose do not react with this enzyme. Also the latter two oligosaccharides show different partial hydrolysis patterns when treated with α -galactosidase^{21,22} (Fig. IV).

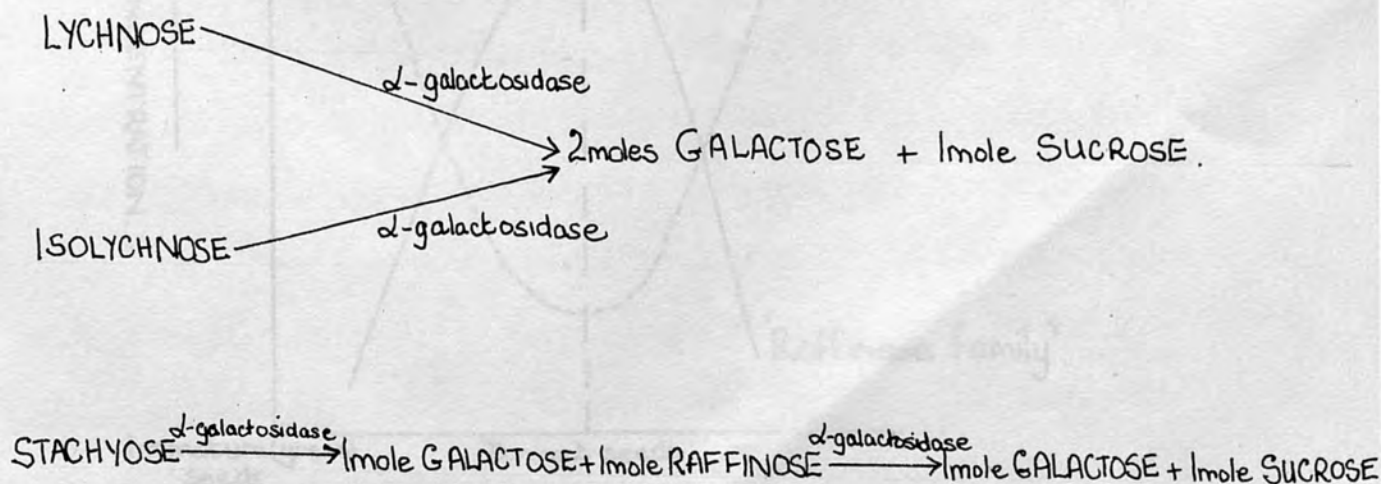
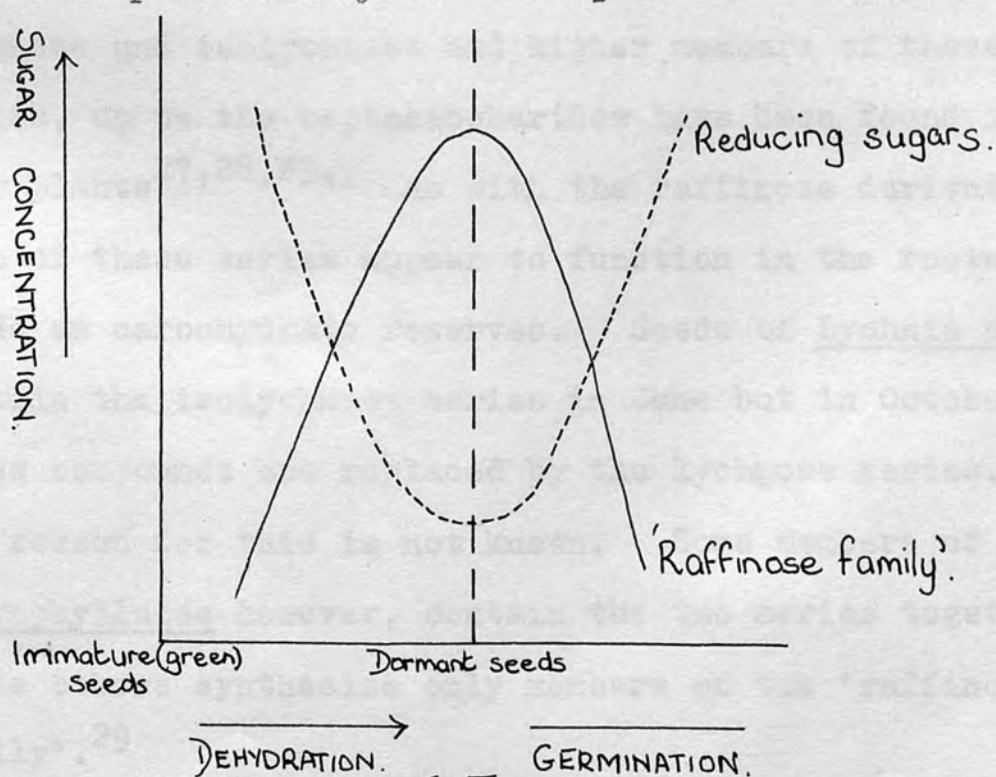


Fig IV

Higher members of the raffinose series ($n = 3-6$ Fig. I) have been found in Verbascum thapsus¹⁹ and all appear to be formed by the addition of one or more ($\alpha 1,6$) linked galactose units to stachyose. The methods used to elucidate these structures are the same as those used for the lower members of the series. Korytnyk and Metzler²³ have shown that the raffinose family of sugars are synthesized in Phaseolus lunatus seeds towards the end of the maturation period and are present in highest quantities when the seeds are dormant. On germination the oligosaccharides rapidly disappear and at the same time, there is an increase in the concentration of sucrose, glucose and fructose, but free galactose is absent or present only in trace quantities at this stage.^{24,25.}



The synthesis of these galactosylsucrose derivatives probably takes place in a stepwise fashion, raffinose being synthesized first, then stachyose, followed by verbascose etc. Conversely, at the onset of germination, the higher members of the series are probably the first to disappear.²⁶ This rapid utilization of the oligosaccharides suggests that they are present as important reserve carbohydrates.

The 'raffinose oligosaccharides' are sometimes found together with other galactosylsucrose derivatives in the same plant. In Plantago major⁹⁵ for example, sucrose and planteose are found in the seeds, while sucrose, raffinose and stachyose occur in the roots. Although no higher homologues based on planteose are known, lychnose and isolychnose and higher members of these series, up to the heptasaccharides have been found in many plants^{27,28,29}. As with the raffinose derivatives, both of these series appear to function in the roots and seeds as carbohydrate reserves. Seeds of Lychnis dioica contain the isolychnose series in June but in October these compounds are replaced by the lychnose series.³⁰ The reason for this is not known. Some members of the Caryophyllaceae however, contain the two series together while others synthesize only members of the 'raffinose family'.²⁹

No experimental evidence has been presented to show how galactosylsucrose derivatives are synthesized in plant tissues, although some of these compounds have been synthesized in vitro using α -galactosidase.

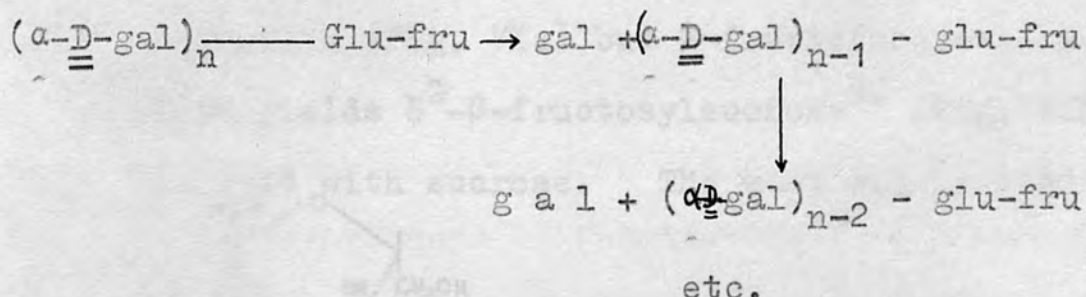
Blagoveschenski claimed to have synthesized raffinose from almond emulsin α -galactosidase, sucrose and galactose.¹⁴ Anagnostopoulos³¹ has utilized a crude α -galactosidase from Coffea arabica to transfer galactose from phenyl- α -D-galactoside to sucrose forming raffinose together with smaller quantities of planteose.

α -Galactosidases from Plantago psyllium, P. ovata and other plants have also been used to synthesize raffinose by a similar method.³² Recently Courtois has resolved a Plantago ovata extract into two separate α -galactosidases, possessing slightly different optimum pH's. While one (pH 4.8) synthesizes raffinose from phenyl- α -D-galactoside and sucrose the other (pH 5.5) synthesizes planteose from the same starting products.³³ Previous work by To Dong and F. Petek, however, showed that α -galactosidase from Coffea arabica could be resolved into two fractions which both synthesized raffinose from phenyl- α -D-galactoside and sucrose.³⁴

Galactomannan has been used as a source of galactose

for the synthesis of raffinose using an enzyme from Trigonellum foenum graecum seeds.³⁵

Sucrose is a probable acceptor molecule for the biosynthesis of galactosylsucrose derivatives in vivo, but it seems unlikely that any simple galactose derivative is involved in this reaction, nor the enzyme α -galactosidase (see p. 65). The role of α -galactosidase as a purely hydrolytic agent in plant tissues is also suspect. In vitro it can be used for the stepwise hydrolysis of the galactosylsucrose derivatives:-



The final products being galactose + sucrose. Whether such reactions bring about the very rapid disappearance of galactosylsucrose derivatives from germinating seeds is unknown. If this is the case then the galactose must be utilized immediately it is liberated, perhaps via a phosphorylated intermediate.

Other known enzymes could theoretically play a role in the breakdown of galactosylsucrose derivatives.

β -fructofuranosidase, for example, would split off fructose from raffinose and leave the disaccharide melibiose. Higher galactosylsucrose derivatives would be split in a similar way to give fructose and higher galactosyl-glucose oligosaccharides. There is no evidence for this however.

β -Fructofuranosidase occurs widely in higher plants^{36,37,38}. In vitro it possesses transferase as well as hydrolytic activities. Incubation of β -fructofuranosidases from sugar beet leaves³⁹ and Aspen soft-xylem tissues⁴⁰ with sucrose, both yield 1F- β -fructosylsucrose (Fig. VIa) but β -fructofuranosidase from banana yields 6G- β -fructosylsucrose³⁶ (Fig. VIb) when incubated with sucrose. The most widely studied

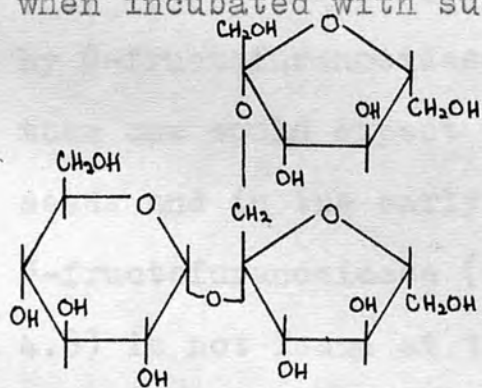


Fig VIa: 1F- β -fructosylsucrose.

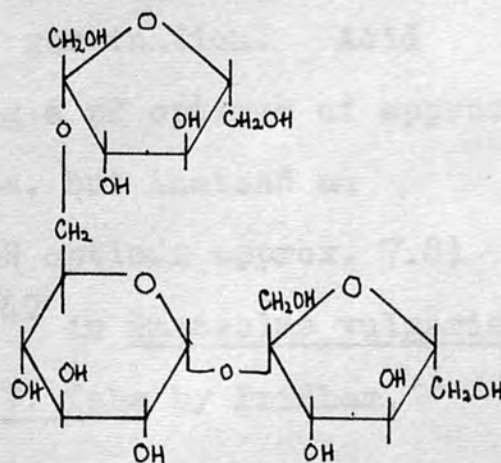


Fig VIb: 6G- β -fructosylsucrose.

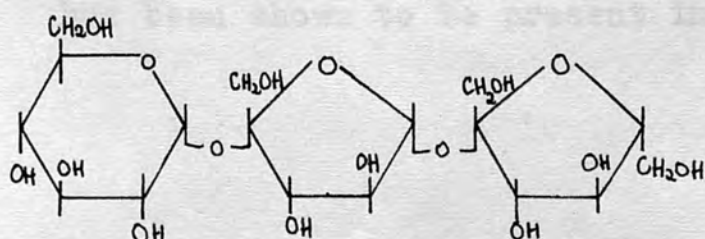


Fig VIc: 6F- β -fructosylsucrose.

β -fructofuranosidase is that of Helianthus tuberosus. This inulin-containing plant, which belongs to the Compositae, contains an abundance of fructose-containing oligosaccharides, the homologous series starting with the trisaccharide 1^F - β -fructosylsucrose. The β -fructofuranosidase isolated from this plant has little or no transferase action on sucrose alone^{37,38} but yields higher oligosaccharides when incubated with sucrose in the presence of 1^R - β -fructosylsucrose. The occurrence of a fructosylraffinose in wheat reported by White and Secor⁴¹ suggests that similar trans-fructosylation reactions involving raffinose may occur in other higher plants.

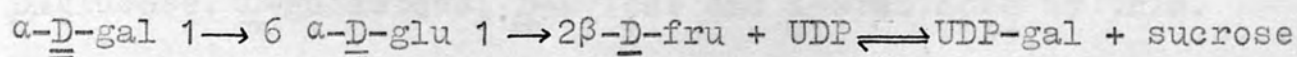
If raffinose does undergo simple hydrolytic action by β -fructofuranosidase in germinating Vicia faba seeds then one would expect to find this enzyme in the dormant seeds and in the early stages of germination. Acid β -fructofuranosidase (i.e. having a pH optimum of approx. 4.8) is not found at these stages, but instead an alkaline β -fructofuranosidase (pH optimum approx. 7.8) first discovered by Greenshields⁴² in Phaseolus vulgaris has been shown to be present in V. faba by Pridham.⁴³

This V. faba enzyme appears to hydrolyse raffinose. No melibiose however is found in the beans at the onset of germination and therefore it seems improbable that hydrolysis by alkaline β -fructofuranosidase occurs in the tissues unless the melibiose formed is utilized very rapidly.

In addition to the action of hydrolases there are two other main pathways which could theoretically be involved in the formation and breakdown of 'raffinose oligosaccharides'.

(a) Phosphorolysis of the gal-glu or the gal-gal bonds with the direct formation of α -D-galactose-1-phosphate. This would be similar to that catalysed by sucrose glucosyltransferase in plant tissues.⁴⁴ By analogy with the latter enzyme however, this reaction would favour breakdown of the galactosylsucrose derivatives.

(b) The involvement of a uridine-type compound e.g.

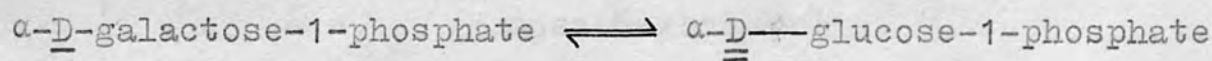


Here again by analogy with UDP-glucose:D-fructose 2-glucosyltransferase this reaction would favour synthesis of the galactosylsucrose derivative.⁴⁵

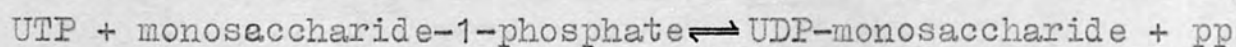
Although in isolation reactions (a) and (b) favour breakdown and synthesis of 'raffinose oligosaccharides' it is

possible that they could be reversed in vivo under the influence of suitable coupled reactions.

Uridine diphosphate derivatives have been shown to act as co-factors in the biosynthesis of many sugars. Uridinediphospho-D-glucose (UDP-glucose), the first compound of this type to be studied, was shown by Leloir et al. ^{46,47,50} to be a necessary intermediate for the reaction:-



It has since been shown that UDP-sugar derivatives can be synthesized from Uridine triphosphate (UTP) and monosaccharide-1-phosphates in the presence of UTP : monosaccharide-1-phosphate uridyltransferases:-



These latter enzymes occur widely in higher plant tissues ^{48,49} and Neufeld et al. ^{45,48} have prepared UDP-derivatives of

D-glucose, D-galactose, D-xylose and L-arabinose by this reaction. Only the $\alpha\text{-}\underline{\underline{D}}$ -anomers of the hexose-1-

phosphates undergo reaction with uridyl transferases,

but since the configuration of the $\beta\text{-}\underline{\underline{L}}$ -arabinose-1-phosphate around C-1 is the same as the α -anomer of the

D-series, it is the β rather than the α -anomer of the

L-arabinose-1-phosphate that reacts in this system.

The above UDP-sugar derivatives have all been isolated from Phaseolus aureus by Ginsburg et al.⁵¹, but UDP-glucose has, so far, been the one most extensively studied.

Extracts from the leaves and roots of sugar beet and from wheat germ have been shown to catalyse the formation of sucrose from UDP-glucose and fructose.^{52,53,54,68} They also catalyse the formation of sucrose phosphate from UDP-glucose and α -D-fructose-6-phosphate.^{52,54} Keys and Skews⁵⁵ have shown that labelled glucose-6-phosphate, fructose-6-phosphate and sucrose are formed when living scuttela of wheat seedlings are fed with ¹⁴C-labelled glucose and fructose. This experiment was also carried out by J. Edelman et al.⁷² who showed that in addition to the above compounds ¹⁴C-cellulose was also formed. The time sequence of the appearance of activity in the products suggested that the formation of sugar phosphates preceded the formation of UDP-glucose and sucrose. These observations agree with the scheme proposed by Burma and Mortimer⁵⁶, who fed sugar beet leaves with radioactive α -D-glucose-1-phosphate and extracted sucrose equally labelled in both halves.

They also suggested that fructose-6-phosphate and α -D-glucose-1-phosphate preceded the formation of sucrose phosphate and sucrose.

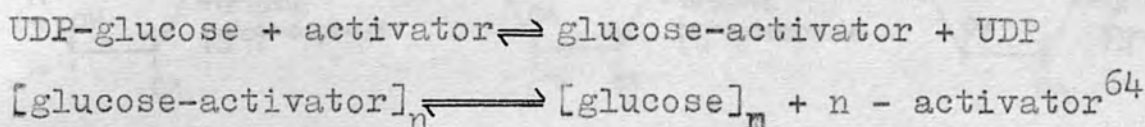
It appears that sucrose phosphate is the precursor of sucrose in plants^{57,58}. Mendicino⁵⁸ has succeeded in separating UDP-glucose-D-fructose-6-phosphate 2-glucosyl-transferase from UDP-glucose-D-fructose 2-glucosyltransferase and partially purifying the former. He showed that the activity of the UDP-glucose-D-fructose-6-phosphate 2-glucosyl transferase was twice that of UDP-glucose-D-fructose 2-glucosyl-transferase.

In a similar way, trehalose phosphate has been synthesized from UDP-glucose and D-glucose-6-phosphate using an enzyme extract from Brewers yeast.^{59,60}

Phenolic glucosides have also been formed from UDP-glucose^{61,62,63} and phenols in the presence of plant extracts. In these latter two reactions, compounds with a β -configuration at the anomeric carbon atom are formed, unlike the formation of sucrose in which the original α -configuration of the glucose in UDP-glucose is retained.

Another field in which UDP-glucose has shown to be of importance is in the synthesis of polysaccharides.

A glucose-containing polysaccharide with a (β 1,3) linkage is synthesized when UDP-glucose is incubated with extracts from Phaseolus aureus, cabbage and parsley. Glucose and various glucosides serve as activators for the reaction which is thought to be of the type:-

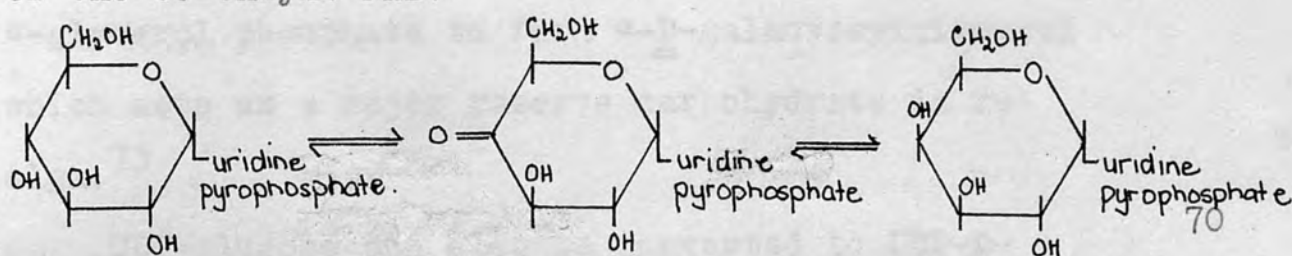


Starch is synthesized when UDP-glucose is incubated with an enzyme preparation from Phaseolus vulgaris⁶⁵, also when the same enzyme preparation is incubated with UDP-glucose and maltose or maltotriose or maltotetraose.⁶⁶ Other UDP-sugar derivatives also give polysaccharides in this way. For example pentosyl groups can be transferred to suitable acceptors. Feingold et al.⁶⁷ have shown that an enzyme preparation from asparagus shoots will transfer D-xylose units from UDP-D-xylose to (α 1,4)-linked D-xylose oligosaccharides of D.P.2-5. This results in the formation of (α 1,4)-linked oligosaccharides, each containing one more xylose unit than the acceptor.

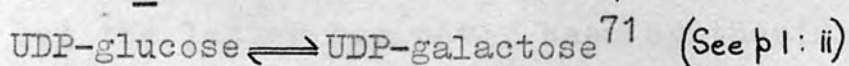
UDP-glucose has also been shown to undergo conversion to UDP-L-rhamnose and UDP-D-galactose in

the presence of Phaseolus aureus leaf extracts^{69,46}.

The formation of the latter nucleotide is believed to proceed by the following reaction in the presence of the co-enzyme NAD:-



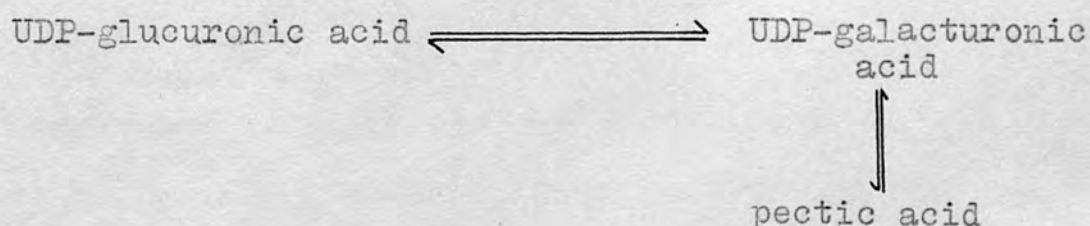
When ¹⁴C-galactose is fed to Canna leaves and wheat seedlings, the glucose moiety of the sucrose formed rapidly becomes labelled and is shown to come directly from the galactose without degradation of the carbon-chain.⁷⁸ This is a further indication that glucose-galactose interconversion can occur in plants, presumably via the corresponding nucleotide derivative. Galactose can also be formed from glucose by the enzyme UDP-glucose: α-D-galactose-1-phosphate|uridyltransferase:-



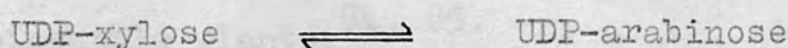
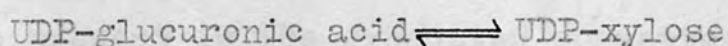
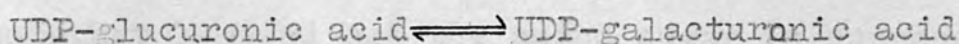
UDP-galactose is thought to be involved in the synthesis of α-D-galactosylglycerol in Iridophycus flaudium which has been shown to contain this nucleotide derivative in addition to UDP-glucose. When ¹⁴C-CO₂ is fed to the

alga, the activity first appears in the ^{14}C -glucose-monophosphate. This is followed rapidly by the formation of labelled UDP-glucose and UDP-galactose. The latter is then presumed to condense with α -glycerol phosphate to form α -D-galactosylglycerol which acts as a major reserve carbohydrate in red alga.⁷³

UDP-glucose can also be converted to UDP-D-glucuronic acid in the presence of NAD. The enzyme (UDP-glucose: NAD oxidoreductase) needed for this reaction which has been purified from pea seedlings⁷⁴ is shown to be specific for UDP-glucose, and has no action on UDP-galactose. UDP-Glucuronic acid and UDP-galacturonic acid occur naturally in Phaseolus aureus seedlings^{75,76}; the former compound also occurs in the red alga Porphyra perforata⁷⁷. This dehydrogenation may be a possible pathway to ascorbic acid but the latter has never been synthesized from UDP-glucose. UDP-glucuronic acid is also thought to be a precursor of pectic acid via the following reactions⁷⁹:-



Phaseolus aureus seedlings have been shown by Feingold et al.⁸⁰ to contain enzymes which catalyse each of the following reactions:-



These workers have succeeded in separating the UDP-D-galacturonic acid-4-epimerase from UDP-L-arabinose-4-epimerase activity thus demonstrating that two separate enzymes are involved. UDP-glucuronic acid and UDP-galacturonic acid can also be formed by reacting the corresponding uronic acid-1-phosphates with UTP in the presence of the appropriate pyrophosphorylases which can be obtained from Phaseolus aureus seedlings^{81,82}. It has been postulated that many of the above nucleotide derivatives are involved in the biosynthesis of hemicelluloses.⁷⁹

Another reaction catalysed by Phaseolus aureus is the formation of UDP-N-acetylglucosamine from UTP and N-acetylglucosamine-1-phosphate.⁴⁴ UDP-N-Acetylglucosamine has been found naturally in Dahlia tubers.⁸³

The latter compound or its derivatives were hitherto unknown in plants; N-acetylglucosamine is known in a polymeric form as chitin, a cell wall constituent of some fungi. Reports of glucosamine in higher plants are somewhat limited but the amino sugar is supposed to be present in the exhausted cossettes of sugar beet and pineapple plants.^{84, 85.}

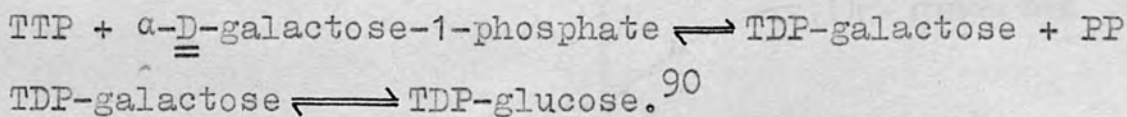
Although uridine-containing compounds have been the group of nucleotides most extensively studied in relation to carbohydrate metabolism, other purine and pyrimidine derivatives have recently been shown to undergo analogous reactions.

Cardini⁸⁶ showed that the synthesis of glucosides and gentiobiosides took place more readily when adenosinediphospho-D-glucose (ADP-glucose) was substituted for UDP-glucose, also the synthesis of starch from ADP-glucose proceeded ten times more rapidly than from UDP-glucose.⁸⁷ Although UDP-glucose was inhibited by ADP-glucose in this latter reaction, ADP-glucose was not similarly inhibited by UDP-glucose. ADP-glucose however is not quite so effective as UDP-glucose in the synthesis of sucrose. In contrast to starch synthesis by these two nucleotide derivatives, sucrose synthesis via UDP-glucose is unaffected by ADP-glucose, but synthesis from ADP-glucose is inhibited by UDP-glucose.⁸⁸

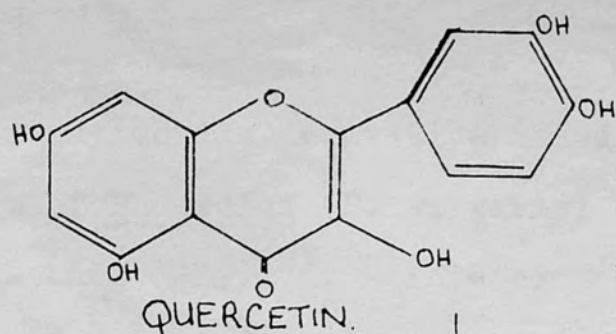
ADP-glucose has been synthesized from ATP and α -D-glucose-1-phosphate in the presence of an enzyme preparation from wheat flour.⁸⁹ Using this enzyme, ADP-xylose has also been synthesized from ATP and α -D-xylose-1-phosphate but the reaction takes place less readily than with the glucose analogue.

No reaction occurs with α -D-fructose-1-phosphate, α -D-galactose-1-phosphate, α -D-mannose-1-phosphate and ATP.

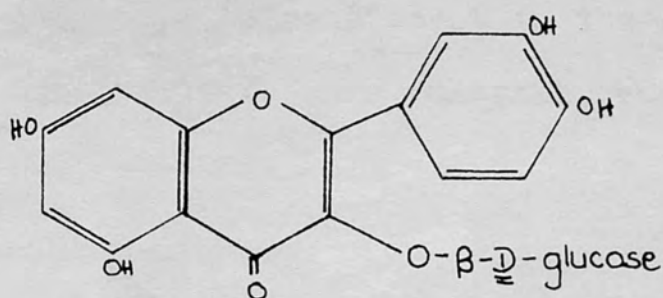
The thymidine compound corresponding to ADP-glucose, (thymidinediphospho-D-glucose: TDP-glucose), has been prepared by reactions which were catalysed by an enzyme preparation from Phaseolus aureus:



Quercetin with TDP-rhamnose and TDP-glucose has been used to synthesize the phenolic glycoside rutin, in the presence of an enzyme preparation from the leaves of the same plant.^{91,92} The reactions were as follows:



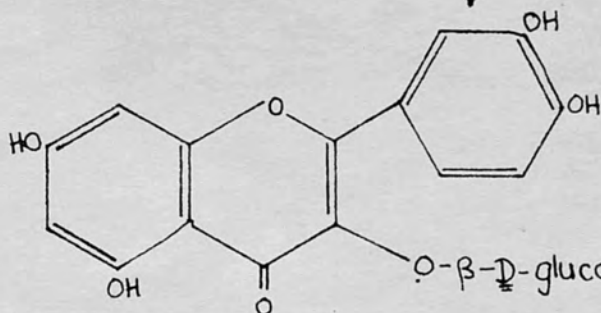
+ TDP-glucose



3-O-β-D-glucopyranosyl-
-3,5,7,3',4'-pentahydroxyflavone.



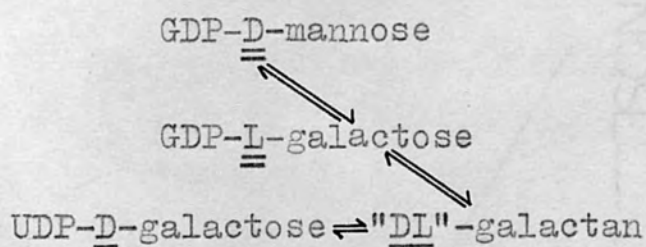
TDP-rhamnose



RUTIN

(3-O-β(-6-O-α-L-rhamnopyranosyl-D-glucopyranosyl)
-3,5,7,3',4'-pentahydroxyflavone)

Another nucleotide derivative found in a different species of Phaseolus (*P. vulgaris*) is guanosine triphosphate (GTP) ⁹³. This compound is one of the main phosphate esters formed in Pisum sativum during orthophosphate uptake.⁹⁴ Guanosinediphospho-D-mannose and guanosinediphospho-L-galactose have been found in Porphyra perforata where, together with UDP-D-galactose (also present in the alga) they bring about the synthesis of a galactan, containing D and L galactose residues.⁷⁷



EXPERIMENTALPaper Chromatography

Paper Chromatography was carried out on Whatman No.1, No.3 and 3 mm papers, using the descending technique with the following solvent systems; all proportions given are by volume unless otherwise stated.

- A Ethyl acetate, acetic acid, water (9:2:2)⁹⁶
- B Butanol, ethanol, water (40:11:19)⁹⁷
- C Ethyl acetate, pyridine, water (2:1:2)⁹⁸
- D Ethyl acetate, pyridine, water (10:4:3)⁹⁹
- E Ethanol, M-ammonium acetate buffer (pH 7.5)¹⁰⁰
- F Isopropanol, acetic acid, water (7:1:2)¹⁰¹
- G Butanol, pyridine, water (6:4:3)¹⁰²

Mobilities of carbohydrates were expressed as R_G or R_{raff} given by

$$R_G = \frac{\text{distance travelled by substance}}{\text{distance travelled by glucose}}$$

and

$$R_{raff} = \frac{\text{distance travelled by substance}}{\text{distance travelled by raffinose}}$$

Mobilities of nucleotides were expressed as R_{AMP} given by

$$R_{AMP} = \frac{\text{distance travelled by substance}}{\text{distance travelled by adenosine|monophosphate}}$$

Paper electrophoresis.

Paper electrophoretic examination of compounds was carried out using Whatman No.3 paper for 90 min. (I) or 30 min. (II) at 57 v/cm. The buffers used were:-

- I 0.2 M - sodium borate (pH 10.0)¹⁰³
II 8.1×10^{-3} M-ammonium molybdate (pH 5.2)¹⁰⁴

The mobilities of the compounds were expressed as

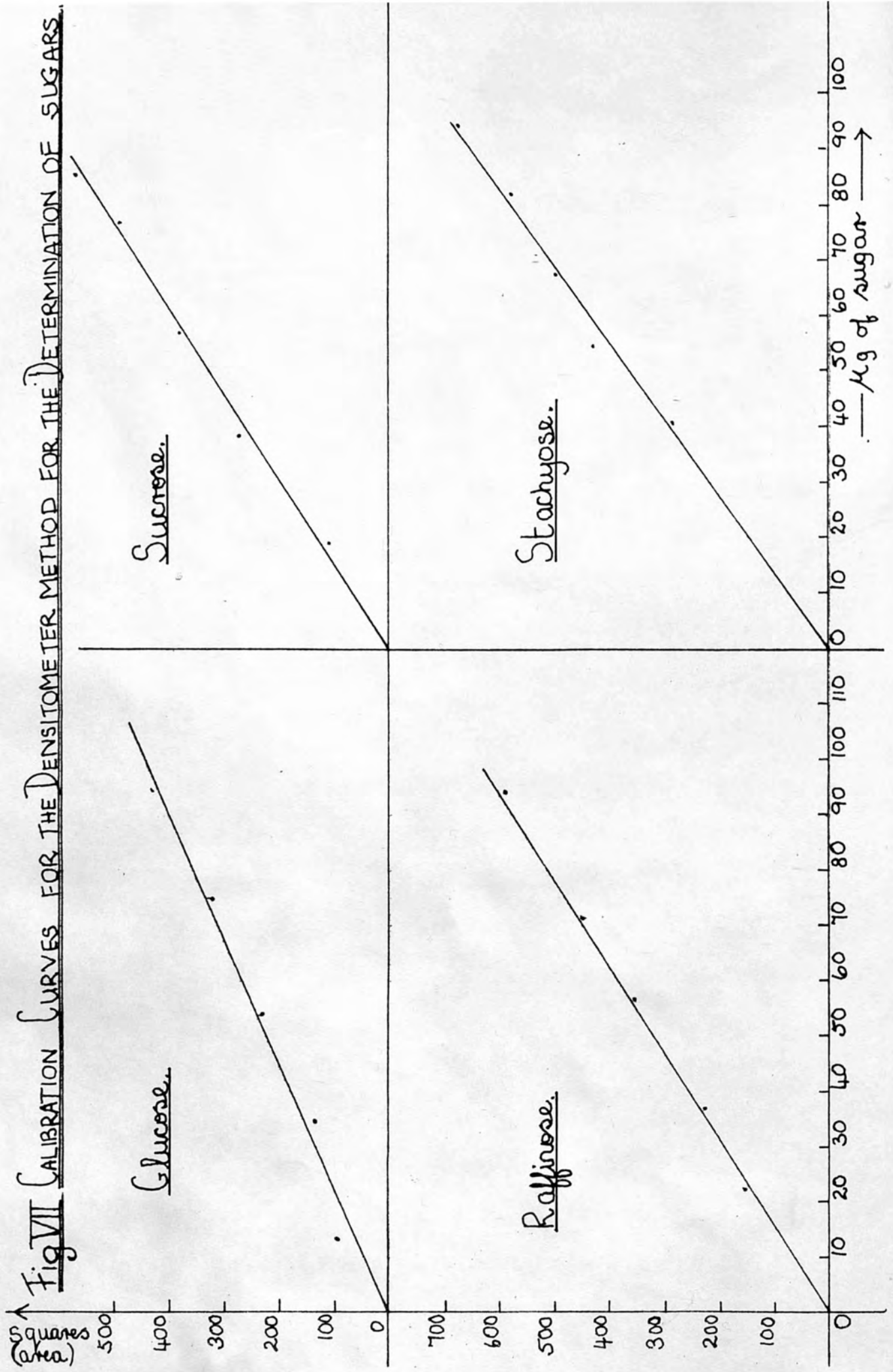
or M_{glucose} or M_{sorbitol} i.e. relative to the movement of glucose or sorbitol using respectively OH-methyl-furfural and glycerol as zero markers.

Spray reagents.

The following spray reagents were used to detect compounds on paper chromatograms and electrophoretograms.

- (a) p-anisidine hydrochloride followed by heating at 120° (reducing sugars and labile non-reducing oligosaccharides).¹⁰⁵
- (b) Urea phosphate followed by heating at 120° (ketoses)¹⁰⁶
- (c) Perchloric acid and ammonium molybdate solution followed by heating at 85° , then spraying with a solution of quinol (phosphates).¹⁰⁷
- (d) Silver nitrate in acetone and ethanolic sodium hydroxide (polyhydroxy compounds).¹⁰⁸

Fig VII Calibration Curves for the Densitometer Method for the Determination of Sugars.



Nucleotides were detected by the use of U.V. light (chromalite) and their U.V. spectra determined in 0.1N NaOH on an S.P.137 spectrophotometer. In all enzyme work, controls were set up using boiled extracts.

1. Determination of carbohydrates.

(a) Reducing sugars.

The Somogyi method^{109,110} was used for the determination of monosaccharides.

(b) Non-reducing sugars.

Oligosaccharides were hydrolysed with N-H₂SO₄

(4 ml for 5 mg oligosaccharide) by heating on a boiling water bath for 1 hr. After neutralization with BaCO₃ and filtration, the solutions were made up to 25 ml and a 5 ml portion treated as for reducing sugars (see (a)).

2. Densitometer method for the determination of sugars

Chromatograms were developed (solvent A) of the unknown quantity of sugar together with 5 different known amounts of the same sugar. The intensity of the coloured spots was determined on an Eel densitometer. Standard curves were prepared by plotting the area under the curve given by the known spots against the amount of sugar present (Fig. VII).

3. Occurrence of galactosylsucrose derivatives in Leguminosae.

Seeds of the following plants were examined:-

Colluta aborescens.

Cytisus scoparius.

Selegia orientalis.

Lupinus polyphyllus.

(a) Seeds (0.4 - 2.0g fresh weight samples) were harvested at the beginning of July and macerated with 75% aq methanol (10 ml). After filtration, the eluate was examined chromatographically (solvent A : spray a).

(b) Another sample of seeds was allowed to dehydrate in the air for 10 days, after which the oligosaccharides were extracted as above. Other samples of matured seeds - Spartium junceum, Pisum sativum, Vicia sativa were also taken and treated as (a) above.

4. Determination of glucose, sucrose, raffinose and higher oligosaccharides in maturing V. faba seeds.

Ten samples of green beans (each consisting of ten beans), were weighed and allowed to dry out in the dark. At 24-hourly intervals a sample was re-weighed, the seeds cut into small pieces and placed in 75%

aq. methanol (25 ml) (Beans which had been drying out for 5-10 days were hardened with liquid air then ground in a pestle and mortar before maceration). The resulting alcoholic solutions were then combined with the original filtrates, each combined solution concentrated to 1-2 ml and subsequently made up to 5 ml. By spotting these latter solutions quantitatively on Whatman No.1 paper, the amounts of glucose, sucrose, raffinose and higher oligosaccharides present in each sample were determined, using the densitometer method as described in (2).

5. V. Faba enzyme preparations.

The buffers used were:-

M-sodium acetate (pH 4.8)¹¹¹

0.5M-sodium phosphate or McIlvaine (pH 7.0)

0.5M-sodium phosphate (pH 7.8)¹¹²

The testas were removed from all beans before the enzyme preparations were made. During preparation, the temperature was kept below 5°.

Dormant beans (or embryos) were powdered in a vibratory ball-mill followed by maceration with buffer (1.5 ml buffer/g. tissue powder) in an M.S.E. homogenizer (1 min). Centrifugation was carried out

at 0° for 30 min. at 3,000 r.p.m. followed by dialysis of the enzyme overnight against approx. 20 vol. of buffer.

Beans were germinated by immersing in water at room temperature and aerating vigorously for two days.

Extracts were prepared from germinated beans (or embryos) and green beans using the procedure described above.

Enzyme preparations

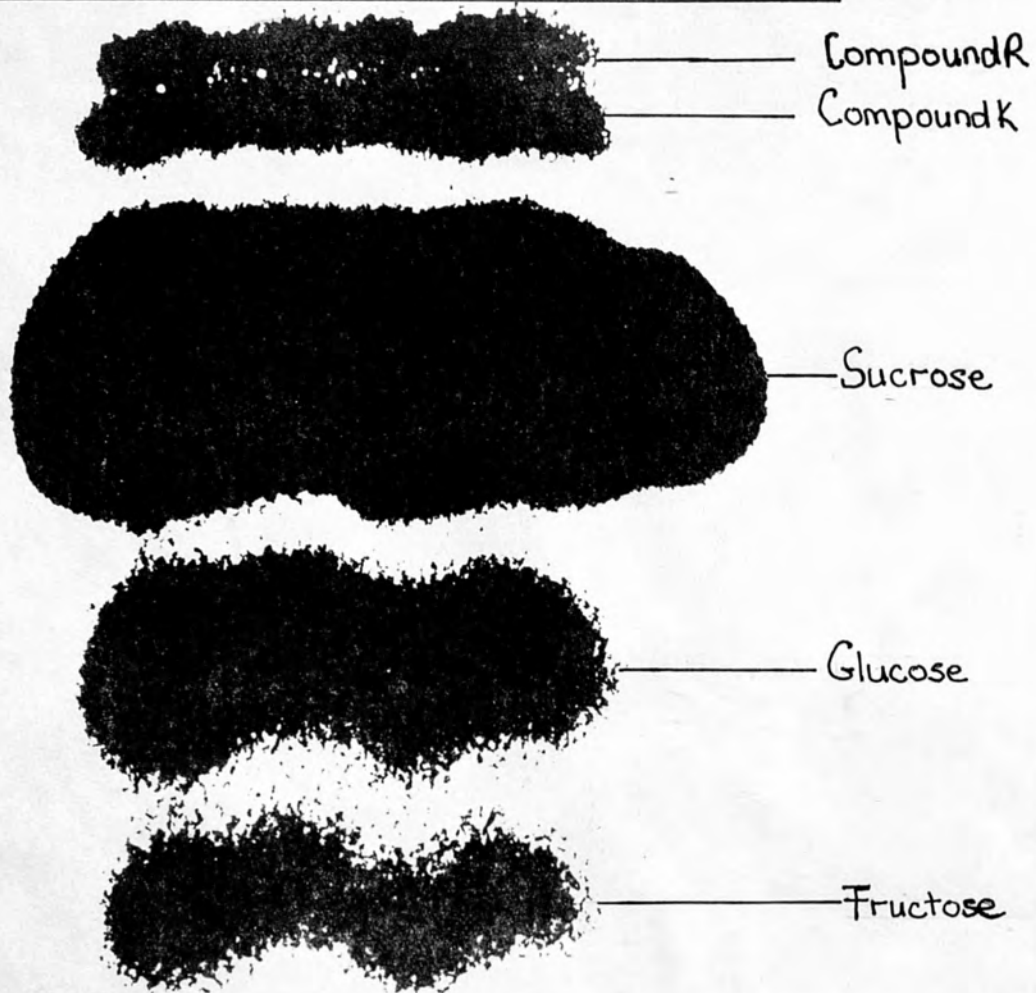
	pH 4.8 (Acetate)	pH 7.0 (Phosphate)	pH 7.0 (McIlvaine)	pH 7.8 (Phosphate)
Whole dormant bean	I	II	IIa	III
Dormant embryo	XII	-	XIII	XI
Whole green bean	IV	-	-	V
Whole germinated bean	VI	-	-	VII
Germinated embryo	X	VIII	VIIIa	IX
Whole steeped bean				XIV

6. Synthesis of raffinose from sucrose using nucleotides

The following reaction mixtures were prepared:-

	<u>A</u>	<u>B</u>	<u>C</u>
Enzyme II (ml. heated for 1 min. in a boiling water bath).	-	0.3	
Enzyme II (ml).	0.3		0.3

Fig VIIIa AUTORADIOGRAPH OF REACTION MIXTURE A

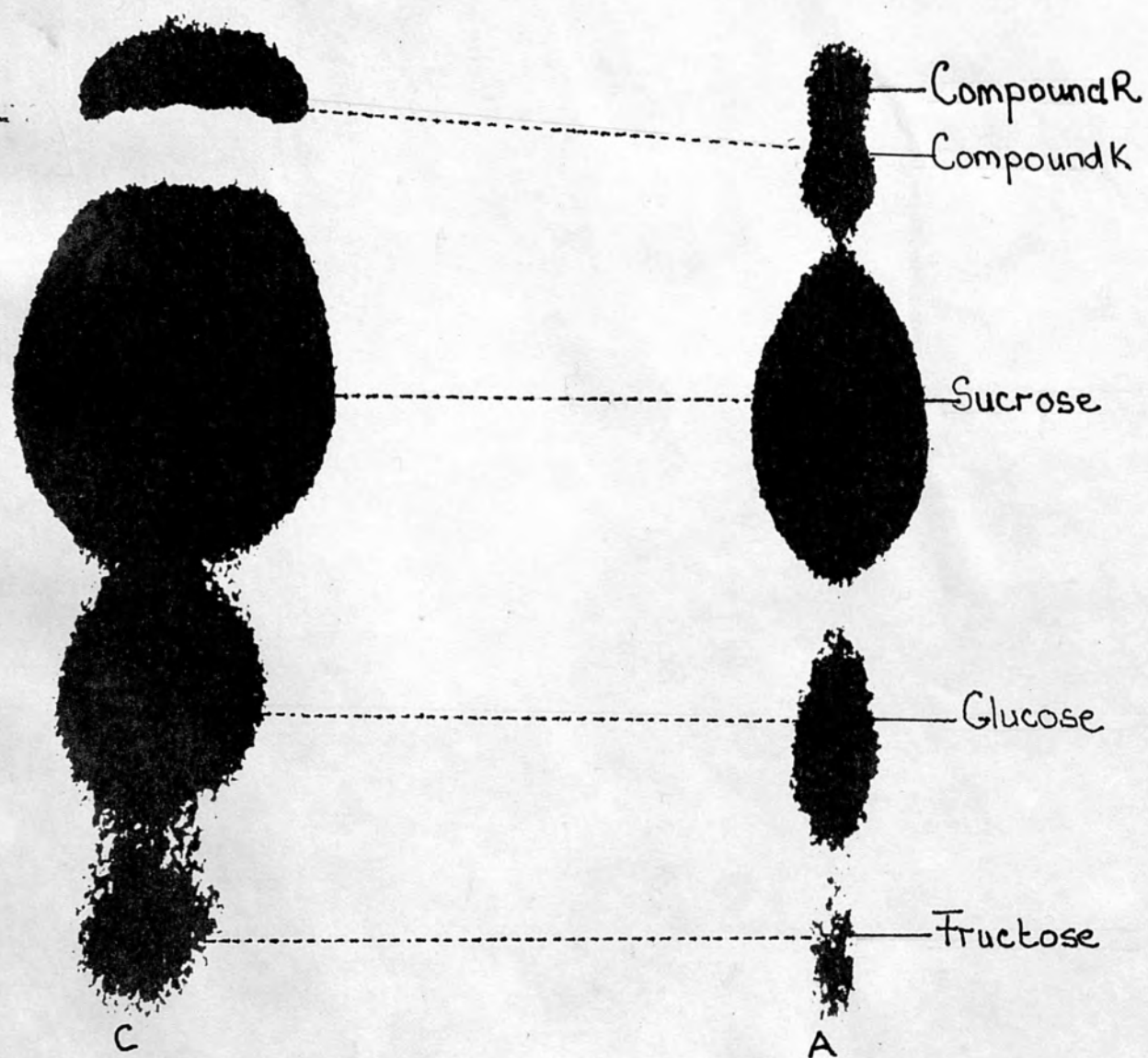


	<u>A</u>	<u>B</u>	<u>C</u>
ATP (μ M)	10	10	-
UTP (μ M)	3	3	-
Galactose-1-phosphate (μ M)	5	5	5
Magnesium chloride (μ M)	10	10	10
^{14}C -sucrose (μ M)	3 (+20 μ C)	3 (+20 μ C)	3 (+10 μ C)
<u>L</u> -Cysteine (μ M)	3	3	3

All three reaction mixtures were incubated at 35° , under toluene. At 30 min. intervals samples were taken from (A) and (B) for paper chromatographic examination. After $2\frac{1}{2}$ hr. ethanol (0.3 ml.) was added to the three reaction mixtures. Small samples of (A) and (C) were spotted on a sheet of Whatman No.1 paper for direct chromatographic comparison and the remaining solutions (A), (B) and (C) each streaked on Whatman No.3 paper. The chromatograms were then developed with solvent A for 24 hr. after which they were removed, dried and autoradiographs prepared (Ilford industrial G X-ray film, 4 days exposure).

The autoradiograph of reaction mixture (A Fig. VIIIa) showed the presence of labelled sucrose and four new radioactive substances: Compound R, which co-chromatographed with raffinose; compound K

Fig VIIIb COMPARATIVE AUTORADIOGRAPH OF REACTION MIXTURES A and C

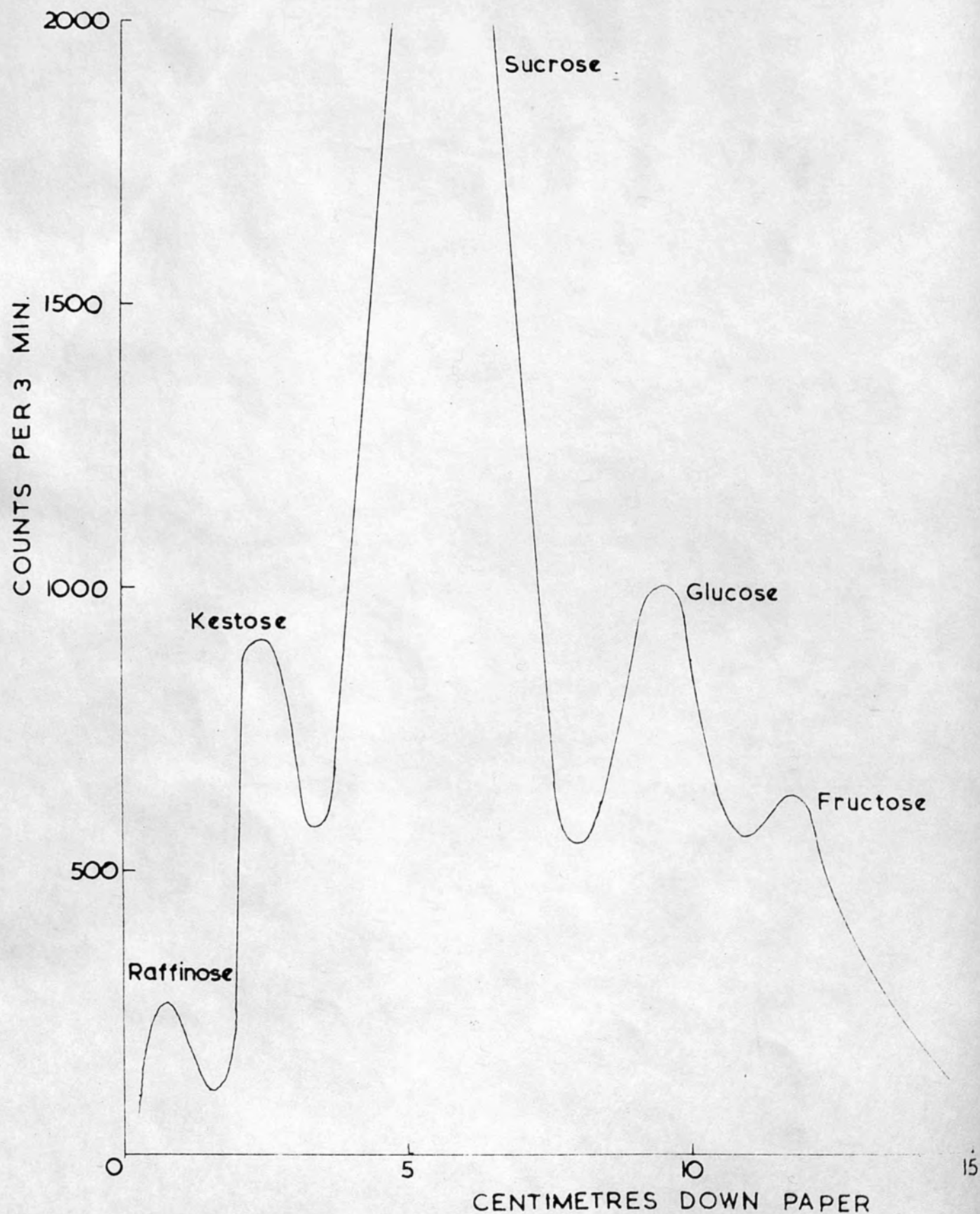


($R_G = 0.23$) and two compounds which co-chromatographed with glucose and fructose. The autoradiograph of (B) did not show the formation of any new labelled compounds. The autoradiograph of (C) however, (Fig. VIIIB) showed that in the absence of the nucleotides three new labelled compounds had been produced: a compound which was chromatographically identical to K, glucose and fructose.

7. Exact location of radioactive bands on the chromatogram of reaction mixture (A).

Strips of paper (1 cm wide) were cut from the edges of the chromatogram and both marked off in 0.5 cm lengths starting from the origin. Each strip in turn was placed between two aluminium plates possessing a 1.0 x 0.5 cm slit. The paper was then drawn through, each 0.5 cm being counted for 3 min. using a Geiger-Muller tube and a plot of the paper length against the counts/3 min. was produced. Both paper strips produced identical plots (Fig. IX) which corresponded with radioactive bands shown in the autoradiograph of the whole chromatogram, compounds R and K could therefore be exactly located.

Fig IX LOCATION OF RADIOACTIVITY IN THE
CHROMATOGRAM OF REACTION MIXTURE A.



8. Isolation of compound R.

Using the autoradiograph, the position of band R was marked on the chromatogram of reaction mixture (A). The band was then cut out and R eluted off the paper with water, into a weighed flask. The water was removed with a rotary evaporator at 40° (Yield of R = 4 mg). Compound R was then diluted with an equal weight of authentic raffinose and the whole dissolved in water (1.5 ml).

9. Paper chromatography and electrophoresis of (R+raffinose).

Chromatograms of (R + raffinose) were developed using solvents A and C and the spots located with spray a. Paper electrophoresis of (R + raffinose) was carried out using buffer I. The paper electrophoretogram was exposed to HCl fumes for 10 min. before being sprayed with a. In each case the strip containing the developed compound was cut out and the point of maximum colour intensity of the spot measured on an Eel densitometer. The strip was tested for radioactivity as in (7).

10. Acid hydrolysis of (R + raffinose).

Solution (R + raffinose: 0.5 ml) was heated with N-H₂SO₄ (3 ml) on a boiling water bath for 3 hr.

The solution was neutralized with IR-4B (OH^- form) resin and examined chromatographically using solvent A (spray a); the colour intensity of the spots was determined as in (9) and any radioactivity in the paper located as in (7).

11. Hydrolysis of (R + raffinose) with yeast β -fructofuranosidase.

B.D.H. invertase concentrate¹¹⁴ (0.1 ml) was diluted with M-acetate buffer (pH 4.8 : 0.9 ml) and 0.1 ml of the resulting solution used.

The solution of (R + raffinose : 0.1 ml) was added to the diluted yeast β -fructofuranosidase (0.1 ml) and the solution made up to 0.5 ml with M-acetate buffer (pH 4.8). After overnight incubation at 35° electrophoresis of the products was carried out using buffer I. After exposing the electrophoretogram to HCl fumes for 10 min. spots were located with spray a. A compound had been formed which co-electrophoresed and co-chromatographed with melibiose.

12. Reduction of melibiose from (11).

The solution from (11), (after β -fructofuranosidase hydrolysis) was evaporated to dryness and the melibiose (0.4 mg) mixed with potassium borohydride (0.02 mg) in water (1 ml). Biodeminrolit (CO_3^- form)

was added to the mixture and an electrophoretogram of the products developed using buffer II. Colour intensity of the developed compound was determined as in (9) after spraying with c and the radioactivity in the paper was located as in (7).

13. Hydrolysis of melibiotol

Melibiotol (0.25 mg) was dissolved in N-H₂SO₄ (0.5 ml) and treated for 3 hr. on a boiling water bath. After neutralization with IR. 4B resin an electrophoretogram of the solution was developed using buffer I. The spots were located with spray d, their colour intensity was determined as in (9) and radioactivity in the paper was located as in (7).

14. Chromatography of glucose and fructose produced in reaction mixture A.

Glucose and fructose were located on the chromatogram of reaction mixture A as in (8) and eluted from the paper with water. Chromatograms of the solutions were developed with solvents A and C, glucose was detected with spray a, fructose with spray b. The colour intensity of the spots was determined as in (9) and the radioactivity in the paper located as in (7).

15. Synthesis of UDP-galactose, ADP-galactose, ADP-glucose.

The following reaction mixtures were prepared (all quantities are in μ moles):-

UDP-glucose	NAD	UTP	α -D-glucose-1-phosphate	α -D-galactose-1-phosphate	ATP	MgCl ₂	Cysteine	Enzyme VIII	Time (hr)
		0.8		1.5	0.2	1	0.3	0.1	1½
0.3				0.75	0.2	1	0.3	0.1	2
0.3	0.15				0.2	1	0.3	0.1	6
				2.2	0.6	1	0.3	0.1	2/3
			2.2		0.6	1	0.3	0.1	2/3

After incubation at 35°, an equal volume of ethanol was added to each digest and the products examined on paper chromatograms (solvent E). Acid hydrolysis of the nucleotide-sugar derivatives was effected with IR 120 (H⁺ form) resin (100° : 5 min); neutralization was achieved with IR 4B (OH⁻ form) resin. Chromatograms of the liberated sugars were developed with solvent A and spray a used to locate the spots.

16. Detection of β -fructofuranosidase, α -galactosidase and alkaline β -fructofuranosidase in V. Faba seeds at different stages of development

(a) β -fructofuranosidase.

The following Enzymes were tested for this activity:-

I; IV; VI; X; XII (all at pH 4.8). The enzyme (1 ml) was incubated with sucrose (0.1 g) for 18 hr. at 35°. The digest was then examined on a chromatogram (solvent A and spray a).

(b) α -galactosidase.

The same enzymes as (a) above were tested for this activity. The enzyme (1 ml) was incubated with melibiose (0.1 g) as above and the digest again examined on a chromatogram as in (a).

(c) Alkaline β -fructofuranosidase.

The following enzymes were tested for this activity:-

III; V; VII; IX; XI; ~~XV~~ XII (all at pH 7.8).

The experimental conditions were as in (a).

17. Activity-time plot for α -galactosidase.

Enzyme I (9 ml) was incubated with a solution of

melibiose (5% w/v: 1 ml) at 35°. Samples (0.1 ml) were withdrawn at zero time and at 15 min. intervals and the reducing power of each determined by the Somogyi method. 0.23 g melibiose \equiv 0.15 g glucose.

18. Optimum temperature curve for α -galactosidase.

Five samples (2 ml) of Enzyme I were preheated at temperatures varying from 20° to 60°. Melibiose solutions (5% w/v) were also preheated at the above temperatures. At zero time melibiose solution (1 ml) was pipetted into each extract and after 90 min. a sample (0.1 ml) was withdrawn from each digest and the reducing power determined by the Somogyi method. Knowing the reducing power of melibiose the increase ~~increase~~ in reducing power could be calculated.

19. Determination of pH activity curves for α -galactosidase and β -fructofuranosidases (acid and alkaline).

pH's (3.0 - 9.0) were used for these experiments. Reaction mixtures were incubated at 35° for 90 min. and the reducing power measured by the Somogyi method. The substrate (sucrose or melibiose) concentration used was 0.5%.

20. Inhibition of α -galactosidase.

The final concentration of each inhibitor used (2:3 dimercaptopropanol, phenyl mercuric acetate, iodoacetic acid and cysteine) was 1×10^{-3} M and the melibiose concentration was 0.5% w/v. The system was buffered with M-sodium acetate (pH 4.8) and incubation carried out at 35° for 90 min. After this time the monosaccharide content was determined as in (17). Blank experiments were carried out in the absence of melibiose to determine the reducing power of the inhibitor.

21. Determination of K_m values for α -galactosidase with various substrates.¹¹⁷

Five samples (0.5, 1.0, 1.5, 2.0 and 2.8 ml) of a 0.017 M solution of the substrate dissolved in M-sodium acetate buffer were each made up to 5 ml with buffer. These solutions were preheated to 35° . Enzyme I was also preheated and at zero time 3 ml of the Enzyme were added to each substrate solution. After 90 min. at 35° the increased reducing power was determined as in (17).

22. Synthesis of raffinose using α -galactosidase.

Five solutions were prepared as shown below. After 18 hr. incubation at 35° , the products were

examined chromatographically (solvent A; spray a).

<u>Enzyme I</u> (ml)	<u>Melibiose</u> (mg)	<u>Sucrose</u> (mg)	<u>Raffinose</u> <u>produced</u>	<u>Other compounds</u> <u>produced</u>
A 1.0	10	-	-	Oligosaccharide $R_G = 0.088$ also galac- tose and glucose.
B 1.0	-	50	-	-
C 1.0	10	50	+	Oligosaccharides $R_G = 0.088$ and 0.094 also galactose and glucose.
D 1.0	10	100	++	" " "
E 1.0	10	250	+++	" " "
F 1.0	10	500	+++	" " "

Table 1

Digest (F) was repeated on a large scale using sucrose (50 g) and melibiose (10 g) and Enzyme I (100 ml) and the mixture incubated for 18 hr. at 35° . Chromatography using solvent A, spray a showed as in Table 1, the formation of compounds which co-chromatographed with raffinose, galactose and glucose, (in addition to the original melibiose and sucrose); higher oligosaccharides had been formed, but as these were present when Enzyme I had been incubated with melibiose alone, they were presumed to be manninotriose and its homologues (Fig. X).

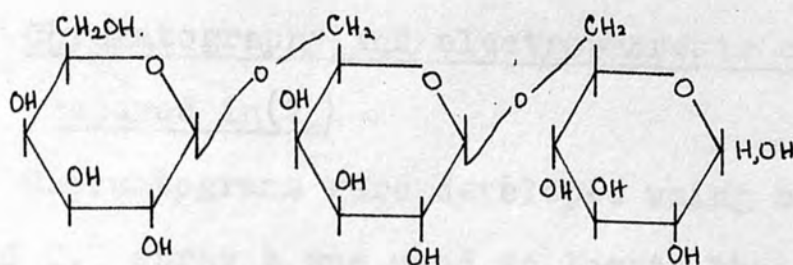


Fig.X: Manninotriose.

23. Separation of compounds from (22) on a charcoal column.

A squat charcoal column was prepared¹¹⁸. The solution from (22) was poured onto the column and the monosaccharide washed through with water (2L). Aq. ethanol (10% w/v: 2L) was used to remove the disaccharides and aq. ethanol (15% w/v : 4L) used to elute off the trisaccharide fraction. (Eluates were checked by paper chromatography : solvent A spray a). Chromatographic examination of the concentrated 15% aq. ethanolic eluate revealed the presence of two trisaccharides:- R_2 (the major one), which co-chromatographed with raffinose and P a slower moving compound which co-chromatographed with planteose and was only present in small amounts. The mixture of R_2 and P was resolved on Whatman No. 3 paper using solvent G. The resulting bands were eluted with water and the solutions concentrated under reduced pressure.

24. Chromatography and electrophoresis of raffinose prepared in (23).

Chromatograms were developed using solvents A, B and C, spray a was used to locate the spots. Electrophoresis was carried out using buffer I. The electrophoretogram was exposed to HCl fumes for 10 min. before being sprayed with spray a.

25. Acid hydrolysis of raffinose from (23).

Raffinose (approx. 2 mg) was treated with $\text{N-H}_2\text{SO}_4$ (3 ml) for 1 hr. on a boiling water bath. After neutralization with barium carbonate the products were chromatographically examined (solvent A; spray a).

26. Reaction of raffinose (from 23) with β -fructofuranosidase and α -galactosidase.

β -fructofuranosidase was used as in (11). Diluted 'yeast invertase concentrate' (0.5 ml) was incubated with raffinose (2 mg), also Enzyme I (0.5 ml) was incubated with raffinose (2 mg), both at 35° overnight. Chromatographic examination of the products was carried out using solvent A, (spray a).

27. Periodate oxidation of raffinose from (23).

Periodate oxidation of raffinose was carried out with sodium periodate using the method of Aspinall and Ferrier.¹¹³

28. Acetolysis of raffinose from (23).¹¹⁹

Raffinose (5 mg) was dissolved in an acetic anhydride (0.5 ml) - pyridine (1.5 ml) solution and the mixture poured onto ice. The crystalline compound formed was recrystallized from aq. ethanol (90% w/v).

mp = 97° (lit.m.p. = 98°).

Mixed mp (with authentic undeca-acetate of raffinose) = 97°.

29. Chromatography of planteose from (23).

Chromatography was carried out using solvent G, (spray a).

30. Action of β -fructofuranosidase on planteose.

Diluted 'invertase concentrate' (0.02 ml, diluted as in (11)) was incubated at 35° overnight with planteose (0.1 mg). Chromatographic examination of the products was carried out using solvent G (spray a).

31. Partial acid hydrolysis of planteose ⁽¹³⁾.

Prepared and standard planteose (1.0 mg each) were separately dissolved in water (1 ml) and 0.6 N-H₂SO₄ (0.3 ml) added to each. After incubation at 35° for 52 hrs. the solution was neutralized with barium carbonate and demineralized with Biodeminrolit resin (CO₃⁻ form). Chromatography of the products was carried out using solvent A (spray a).

32. Isolation of disaccharide from (31).

The disaccharide was purified by separation of the products from (32), on Whatman No.3 paper using solvent A. The disaccharide band was located with marker strips (spray b).

33. Chromatography and electrophoresis of disaccharide from (32).

The disaccharide was examined chromatographically in solvents A and G and electrophoretically using buffer II. Spray b was used to locate the spots on the chromatograms and spray d to locate the spots on the electrophoretogram.

34. Composition of the disaccharide.

The remaining disaccharide was divided into two portions.

- (a) was heated on a boiling water bath with $\text{N-H}_2\text{SO}_4$ (1 ml) for 30 mins.
- (b) was incubated with Enzyme I (1 ml) for 24 hr. at 35° . The products of both experiments were examined chromatographically (solvent A, spray a).

35. Synthesis of stachyose (Compound S) using α -galactosidase.

Three reaction mixtures were prepared as shown below. After 18 hr. incubation at 35° , the products were examined chromatographically (solvent A, spray a).

	Enzyme I (ml)	Raffinose (g)	Stachyose produced
A	1	.1	+
B	1	.2	++
C	1	.3	+++

A reaction mixture (D) was prepared of raffinose (3g) in Enzyme I (10 ml) and incubated as above.

36. Isolation of stachyose from (35).

Products of reaction mixture D were separated on Whatman No.3 paper using solvent A. Stachyose was located by marker strips (spray a) and eluted from the paper with water.

37. Chromatography and electrophoresis of stachyose.

Chromatography was carried out using solvents A, B, G, F electrophoresis using buffer I. Spray a was used to locate the spots, the electrophoretogram being exposed with HCl for 10 min. before spraying.

38. Action of β -fructofuranosidase and α -galactosidase on prepared stachyose.

Two samples of stachyose (each 0.4 mg) were incubated separately with diluted 'yeast invertase' (diluted as in (11):1 ml) and Enzyme I (1ml) at 35° for 18 hr. The products were examined chromatographically (solvent A, spray a).

39. Periodate oxidation of prepared stachyose.

Periodate oxidation was carried out on the prepared stachyose according to the method of Aspinall and Ferrier.¹¹³

40. Partial Acid Hydrolysis of prepared stachyose.

Stachyose (approx 0.5 mg) was treated with 0.4N - H₂SO₄ (0.5 ml) for 1 hr on a boiling water bath. After neutralization with barium carbonate, the products were chromatographically examined (solvent A; spray a).

41. Qualitative determination of possible galactose donors in V. Faba seeds.

1. Aqueous extract.

Whole dormant seeds (33 g) were powdered in a

vibratory ball mill for 2 hr, followed by maceration with distilled water (70 ml) in an M.S.E. homogenizer. The mixture was centrifuged (3000 r.p.m., 6°, 30 min.) and the supernatant liquid divided into three fractions:-

1st fraction Chromatography using solvent A (spray a) to detect galactose - containing oligosaccharides.

2nd fraction Chromatography using solvent A, utilizing spray c to detect phosphate compounds.

3rd fraction This fraction was added to three times its own volume of ethanol and the mixture centrifuged as above.

The remaining oligosaccharides were removed by reprecipitation with ethanol. Precipitate (mainly polysaccharide : 3g) was dried by washing with ethanol and ether. Acid hydrolysis was carried out by heating the polysaccharide (2 g) overnight with N-H₂SO₄ (10 ml) in a sealed tube immersed in a boiling water bath.

The following reaction mixtures were incubated at 35° for 18 hrs.

(a) Polysaccharide (0.1 g) with Enzyme I (1 ml).

(b) As in (a) but sucrose (0.5 g) was added.

Polysaccharide of the amylose or amylopectin type was detected with a solution of iodine in potassium iodide (1% w/v).

II. Alcoholic extract.

Conditions for powdering beans, macerating and centrifuging as in (I), but ethanol was used instead of distilled water. The ethanolic extract was evaporated to dryness and the residue (1.2 g) hydrolysed with $\text{N-H}_2\text{SO}_4$ (5 ml) on a boiling water bath. After neutralization with barium carbonate the products were examined chromatographically (solvent A, spray a).

42. Detection of alkaline β -fructofuranosidase using buffers other than phosphate.

Dormant bean extracts were prepared at pH 7.8 as in (5) but using tris¹¹⁵ and diethylbarbiturate¹¹⁶ buffers. Alkaline β -fructofuranosidase activity was determined as in 16 (c).

43. Reaction of alkaline β -fructofuranosidase with raffinose and stachyose.

Enzyme XI (1 ml) was incubated with raffinose (0.1 g) at 35° for 18 hr. The products were examined chromatographically (solvent A, spray a).

The experiment was repeated for stachyose but using one-tenth the quantities as for raffinose.

44. Transference of fructose to acceptors other than water by alkaline β -fructofuranosidase.

The following reaction mixtures were incubated at 35° for 48 hr. then examined chromatographically (solvent A, spray b).

<u>Enzyme XI (ml)</u>		<u>Sucrose (g)</u>
A	1	0.1
B	1	0.2

(B) showed the formation of three new compounds, two of which co-chromatographed with glucose and fructose and a third which had a $R_G = 0.2$ and co-chromatographed with 1'- β -fructosylsucrose (1-kestose) K_2 .

45. Separation of 1-kestose formed by the action of alkaline β -fructofuranosidase on sucrose.

Enzyme XI (10 ml) was incubated for 48 hr. with sucrose (2 g) at 35° . Chromatography as in the previous experiment confirmed the formation of 1-kestose which was separated from other saccharides by the use of a squat charcoal column.

Aq. ethanol (10% w/v : 4L) was used to remove the disaccharides and aq. ethanol (15% w/v : 5L) used to remove the trisaccharide fraction. Eluates were checked by paper chromatography (solvent A, spray b).

46. Chromatography and electrophoresis of 1^F - β -fructosylsucrose isolated in (45).

In all experiments 1^F - β -fructosylsucrose, (1-Kestose) 6^F - β -fructosylsucrose 6^G - β -fructosylsucrose (Fig. VI) were used as standards. Chromatography of 1-Kestose was carried out in solvents A and D (spray b). Electrophoresis was carried out in buffer I (spray b).

47. Elution chromatography and electrophoresis of compound K from (7).

Compound K was eluted from the chromatogram of reaction mixture (A) and that of reaction mixture (C) as compound R in (8). After evaporation an equal weight of 1-kestose (2 mg) was added to the prepared compound and the whole dissolved in water (1 ml).

Paper chromatography of the mixture was carried out using solvents A and C (spray b). Paper electrophoresis was carried out using buffer I (spray b). In all cases colour intensity and radioactivity were determined as with compound R.

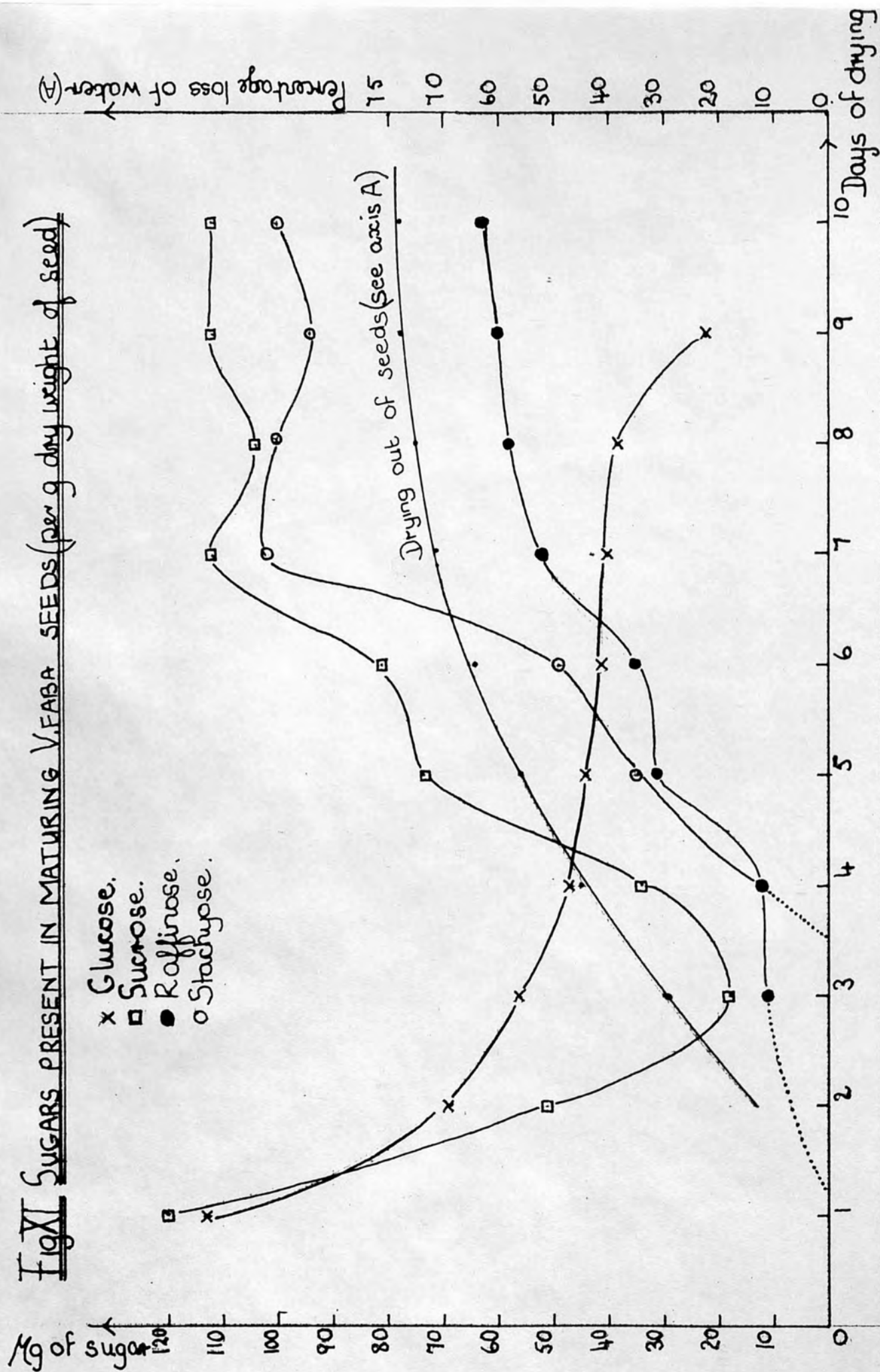
48. Hydrolysis of compound K with yeast β -fructofuranosidase.

Yeast β -fructofuranosidase was diluted as in (11). Solution (K + 1-Kestose : 0.5 ml) was incubated at 35°

with diluted 'yeast invertase' (0.1 ml), the solution being made up to 1 ml. with M-sodium acetate buffer (pH 4.8). Samples were taken at intervals (1 hr; 6 hr; 6 day) and analysed on paper chromatograms (solvent A; spray b). In all cases colour intensity and radioactivity were determined as with compound R.

MAIN SECTION

Fig XI SUGARS PRESENT IN MATURING VEABA SEEDS (per g dry weight of seed)



MAIN SECTION

A preliminary investigation of the galactosyl-sucrose derivatives in the immature seeds of four different species of Leguminosae showed that in all cases there was an increase in these sugars when the seeds were allowed to dry out in the air for a period of ten days. A high percentage of these oligosaccharides were also observed in other mature Leguminosae seeds which had been taken straight from the pod and examined (Table 2).

It was not possible to show from these results whether raffinose was formed at the same time, or prior to stachyose and the higher oligosaccharides.

To determine this, the experiment was carried out quantitatively using samples of Vicia faba seeds (Fig. XI). It was shown that when seeds were allowed to dry out in the dark, the formation of raffinose and stachyose (and higher oligosaccharides) appeared to take place concurrently, after an early start to raffinose formation, and that the concentration of these sugars remained constant when the seeds no longer continued to lose water. These results confirm observations made with other seeds.²³ The rapid decrease in the concentration of sucrose may signify that this sugar is the

Plant species Examined	Per- centage Loss in weight	Immature		Dormant	
		Raffinose and higher oligosa- ccharides	Sucrose Reducing Sugars	Raffinose and higher oligosa- ccharides	Sucrose Reducing Sugars
Collutea aborescens	78.5	++	+	++	+
Cytisus scoparius	74.8	++	+	++	+
Selegia orientalis	64.3	+	++	+	+
Lupinus Polyphillus	80.1	+	+	++	+
Spartium juncium				++	+
Pisum sativum				++	+
Vicia cracca				++	+

Table 2

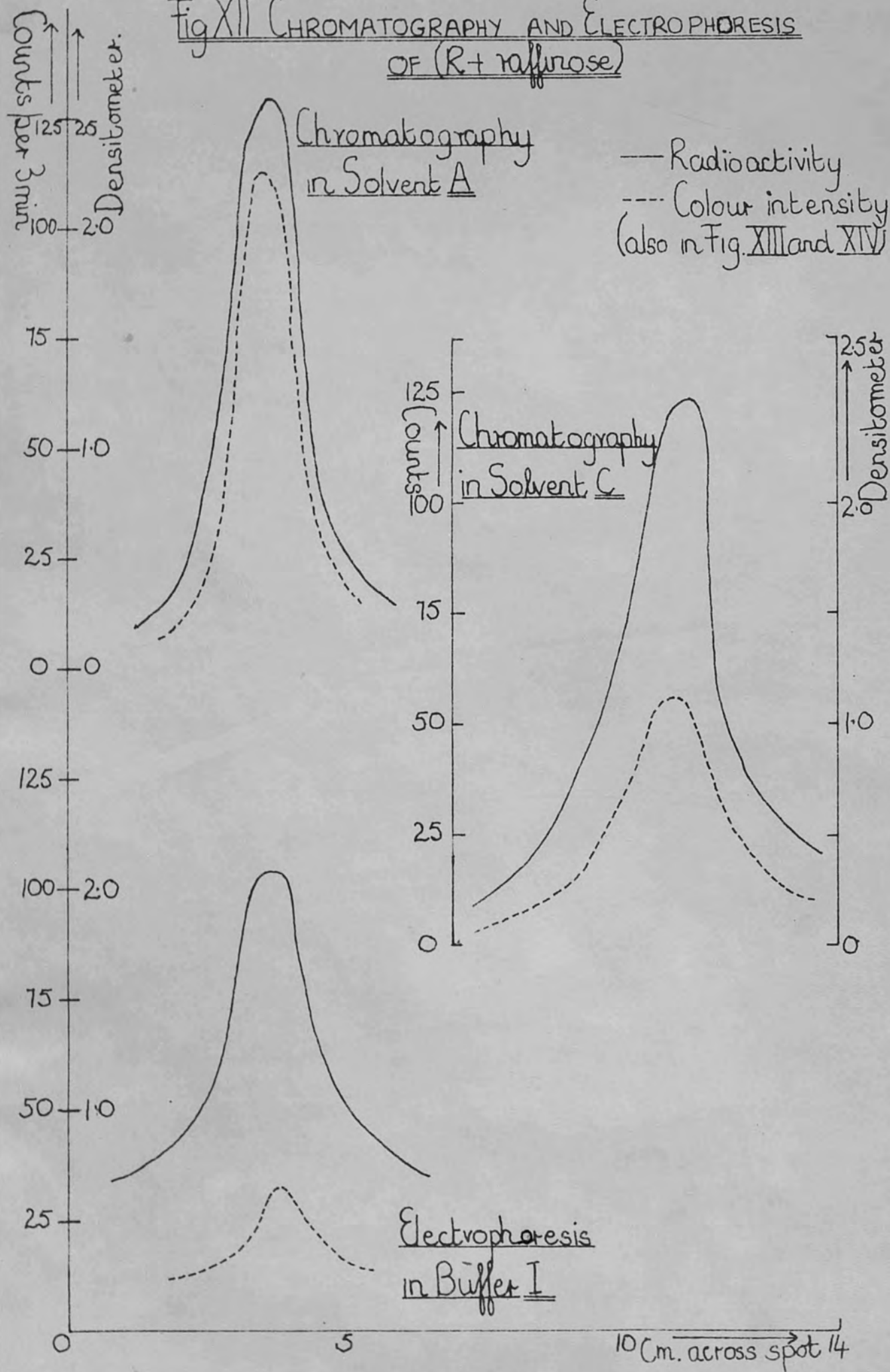
precursor of raffinose and perhaps also the higher oligosaccharides. The increase in sucrose concentration after 3 days is, however, difficult to explain. The drop in the concentration of reducing sugars during maturation has been reported by other workers²³ and is probably due to the fact that at this stage reducing sugars are being converted to suitable storage compounds such as starch, in the seed. It should be remembered, however, as the beans were allowed to dry out under non-physiological conditions, that these results may not apply exactly to seeds in vivo which are maturing in the pod and which dry out very much more slowly under the influence of the parent plant.

If sucrose does in fact act as an acceptor molecule for galactose in V. faba, formation of raffinose can be envisaged either involving UDP-galactose or a 'low energy' derivative of galactose, such as an oligosaccharide or monophosphate.

Synthesis of raffinose via UDP-galactose

Uridine compounds have been shown to act as intermediates in the synthesis of sucrose^{52,53} and it seemed probable that a uridine compound might also act as a galactose donor for the formation of raffinose.

Fig XII CHROMATOGRAPHY AND ELECTROPHORESIS
OF (R+ raffinose)



A trisaccharide (R) co-chromatographing with raffinose was synthesized enzymically from UTP, α -D-galactose-1-phosphate and ^{14}C -sucrose in the presence of ATP at pH 7.2. Mature V. faba seeds were used as a source of the enzyme preparation. Also formed in this reaction was another trisaccharide : K. The structure and formation of this compound will be discussed later (P9).

The identity of the oligosaccharide R produced was confirmed by the following methods. It was first diluted with an equal weight of authentic raffinose and the mixture shown to be chromatographically and electrophoretically homogeneous in a number of systems (Fig. XII). This mixture was used for all the following structural determinations. The distribution of the radioactivity across the chromatographic and electrophoretic spots corresponded exactly with the distribution of the sugar. An absence of this correspondence would have indicated the presence of more than one oligosaccharide.*

* The distribution of radioactivity and sugar concentration across the chromatographic and electrophoretic spots was measured in all the degradative experiments with labelled oligosaccharides.

Fig XIII ACID HYDROLYSIS OF (R + raffinose)

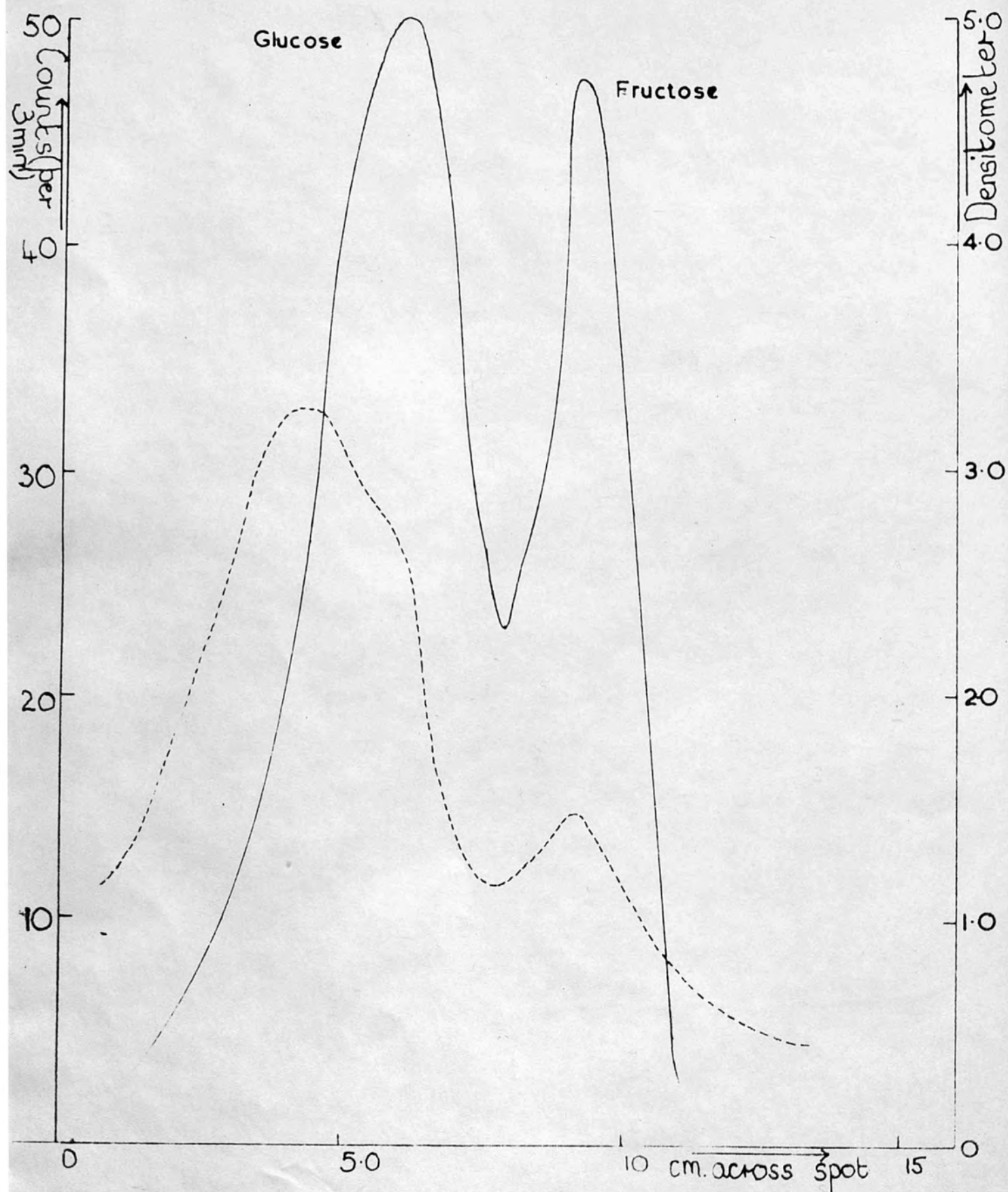
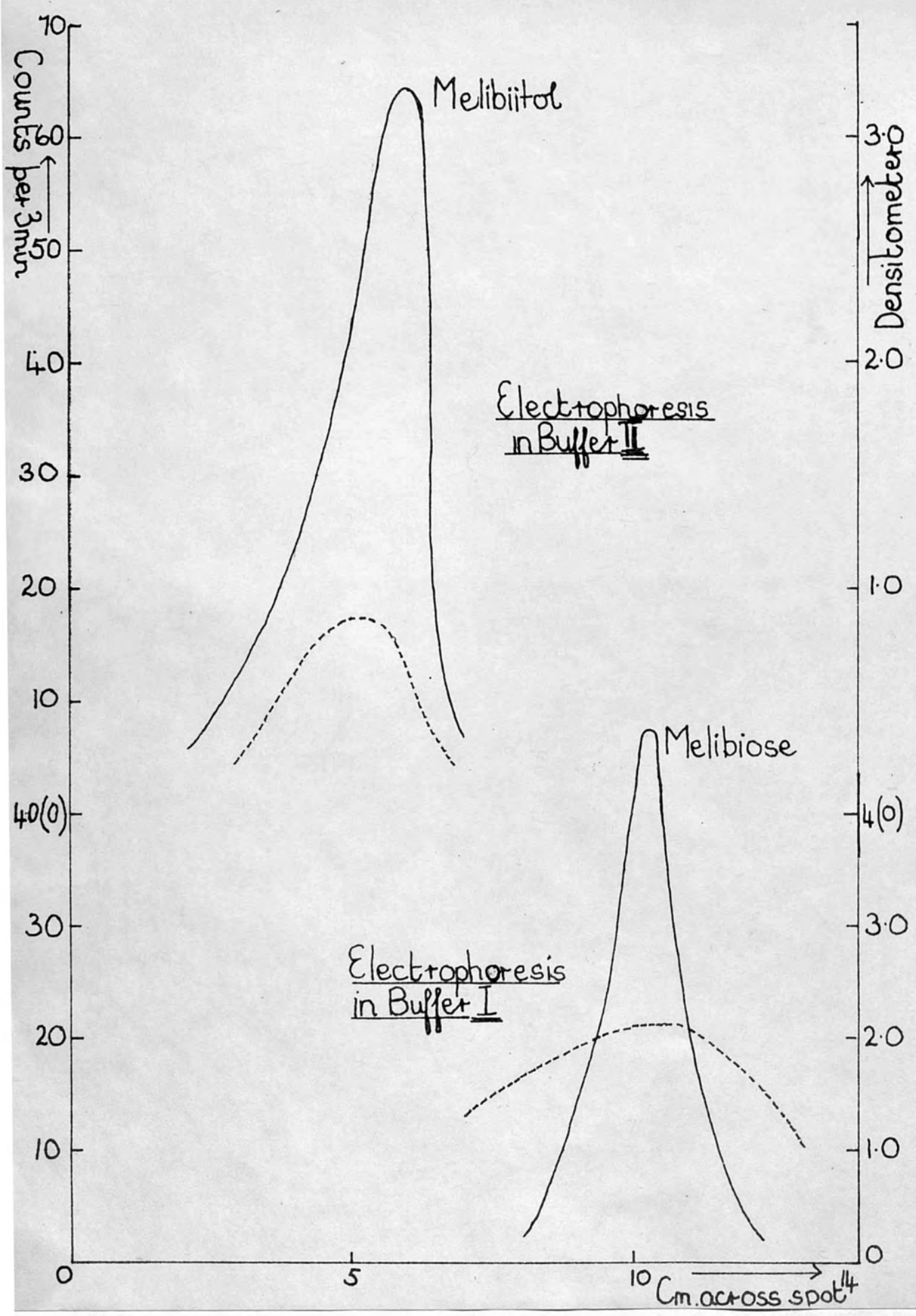


Fig XIV CHARACTERIZATION OF MELIBIOSE



Acid hydrolysis of the oligosaccharide yielded three monosaccharides : galactose, glucose and fructose, only the latter two showing radioactivity (Fig. XIII). Reaction of the oligosaccharide with yeast β -fructofuranosidase gave a disaccharide which co-chromatographed and co-electrophoresed with melibiose (Fig. XIV).

It has been shown that^{120,104} combining the results of paper electrophoresis in molybdate (pH 5.0) and borate (pH 10.0) buffers, the glycosidic linkage in a disaccharide can be determined.

Table 3.

Disaccharide	Linkage	Mobility in Buffer I (Mglucose)	Reduced disaccharide	Linkage	Mobility in Buffer II (Msorbitol)
Sophorose	$\beta(1;2)$	0.24	Sophoritol		0.9
Nigerose	$\alpha(1;3)$	0.69	Nigeritol		0
Laminaribiose	$\beta(1;3)$	0.69	Laminari- bitol.		0
Maltose	$\alpha(1;4)$	0.32	Maltitol		0.4
Cellobiose	$\beta(1;4)$	0.28	Cellobitol		0.4
Lactose*	$\beta(1;4)$	0.38	Lactitol		0.4
Isomaltose	$\alpha(1;6)$	0.69	Isomaltitol		0.8
Gentiobiose	$\beta(1;6)$	0.75	Gentiobitol		0.8
Melibiose*	$\beta(1;6)$	0.80	Melibitol		0.8
Disaccharide from (11).		0.81	Reduced disaccharide from (12).		0.8

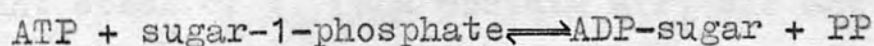
* These compounds contain galactose and glucose, the latter being the reducing moiety.

The results shown in Table 3 strongly suggest that the disaccharide obtained by the action of yeast β -fructofuranosidase contained an (α 1;6) linkage. Final confirmation was obtained by hydrolysing the reduced disaccharide which yielded galactose and labelled sorbitol.

Having proved that raffinose could be synthesized in vitro from UTP, ATP, α -D-galactose-1-phosphate and sucrose (and a V. faba extract), it remained necessary to determine the galactose donor in the above system. Incubation of α -D-galactose-1-phosphate with UTP and a V. faba extract at pH 7.2 yielded a compound which had an R_{AMP} of 1.2 (R_{AMP} of UDP-glucose = 1.38). This compound which gave galactose on acid hydrolysis and was shown to contain uridine by its UV spectrum

$\left\{ \frac{E_{250}}{E_{260}} = 0.80, \frac{E_{280}}{E_{260}} = 0.24 \right\}$ was thus proved to be UDP-

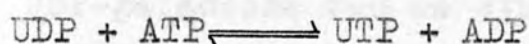
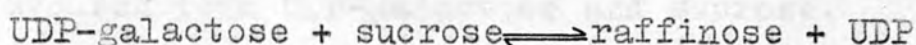
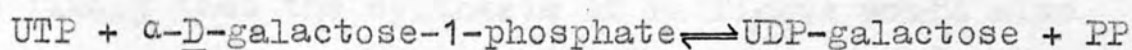
galactose. However the work of Espada⁸⁹ showed that ADP-sugars can be synthesized thus:-



As ATP was present in the digests and an ADP-sugar derivative might have been formed, attempts were made to synthesize ADP-galactose, by incubation of ATP, α -D-galactose-1-phosphate with a V. faba extract at pH 7.2. However no new compound appeared on the chromatogram of

this reaction mixture, Similar attempts were made to synthesize ADP-glucose (from ATP and α -D-glucose-1-phosphate), but these were also unsuccessful.

There seems little doubt, therefore, that in vitro raffinose is formed in the following way:-

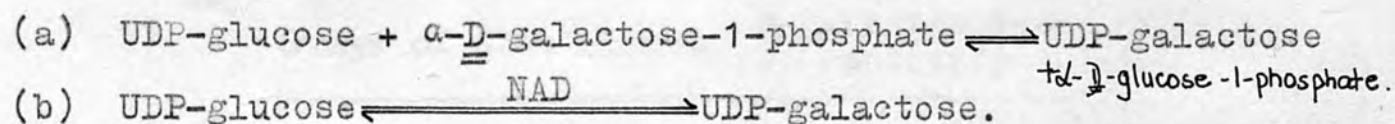


and in vivo similar mechanisms could operate as all the necessary enzymes and substrates are probably present by analogy with other plant tissues. As described on p 16 , in vivo examination of sucrose synthesis in wheat seedlings and sugar beet leaves has shown that α -D-glucose-1-phosphate, D-glucose-6-phosphate, fructose-6-phosphate, UDP-glucose are the probable precursors of this disaccharide and the 'glucosyltransferases' mentioned on p 17 occur in these plants.

Abdel Wahab and El Kinawaii^{121,122} have shown that dormant seeds of V. faba contain ATP, UTP and a nucleotide fraction UDPX which probably consists of UDP-glucose and UDP-galactose. Attempts to detect α -D-galactose-1-phosphate in V. faba seeds have been unsuccessful, probably owing to the small amount present.

Although the synthesis of raffinose from UTP, α -D-galactose-1-phosphate and sucrose has not been examined kinetically, other workers have shown that the synthesis of sucrose from UDP-glucose and fructose is kinetically favoured (45: K = 5) therefore it would seem likely that the synthesis of raffinose would also be favoured from UDP-galactose and sucrose.

UDP-galactose can be synthesized by other routes which are mentioned on p1.



These two reactions were attempted in the presence of a V. faba extract and in both cases they were successful. UDP-galactose was formed and characterized by its acid hydrolysis to give galactose and its UV spectrum to show the presence of the uridine moiety. Raffinose would then be formed from the UDP-galactose as above. It is conceivable that stachyose may also be synthesized from UDP-galactose by transfer of galactose to raffinose. This experiment however has not been attempted in this present study.

Synthesis of raffinose via a low energy galactose derivative

The synthesis of galactose-containing oligosaccharides by α -galactosidase catalysed transfer of galactose from

phenyl- α -D-galactoside to sucrose has already been discussed (p 10). In view of these results it was decided to investigate α -galactosidase activity in the Vicia faba and to examine the possibility that the synthesis of galactosylsucrose derivatives might take place by a similar mechanism in this plant.

An initial survey was made of the bean at different stages of development (Table 4).

Stage of development	α -galactosidase activity
Green bean	+
Dormant embryo	+
Dormant bean	+
Germinated embryo	+
Germinated bean	+

Table 4.

α -galactosidase activity was found in whole, dormant and germinating beans and in all cases the enzyme showed transferase as well as hydrolytic activity towards an α -galactosyl moiety.

The α -galactosidase in the dormant beans was examined in more detail and found to possess similar properties to that examined in Coffea arabica by Courtois et al.³¹ It hydrolysed melibiose to give galactose and

Fig XV OPTIMUM pH ACTIVITY CURVE FOR α -GALACTOSIDASE

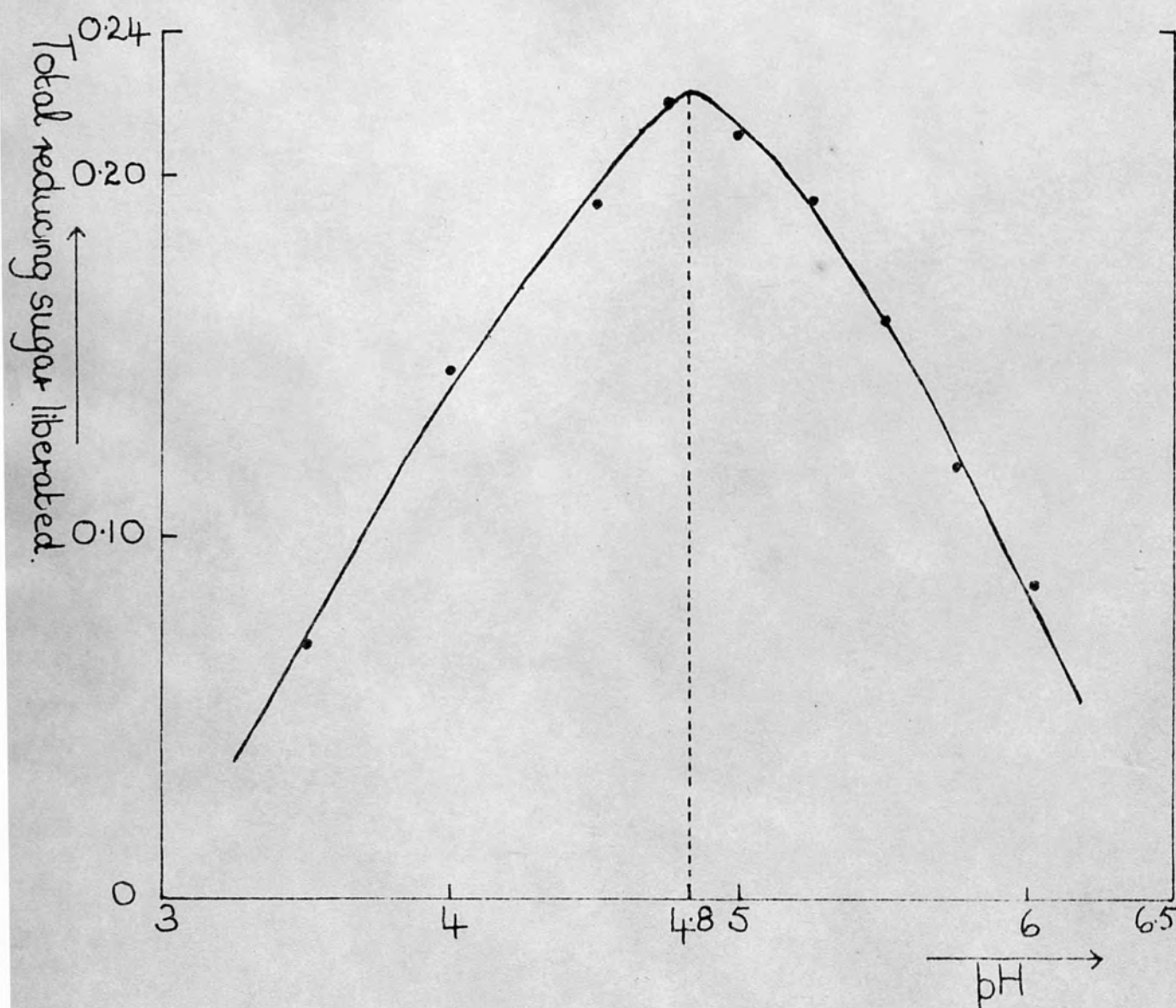


Fig XVI PROPERTIES OF α -GALACTOSIDASE

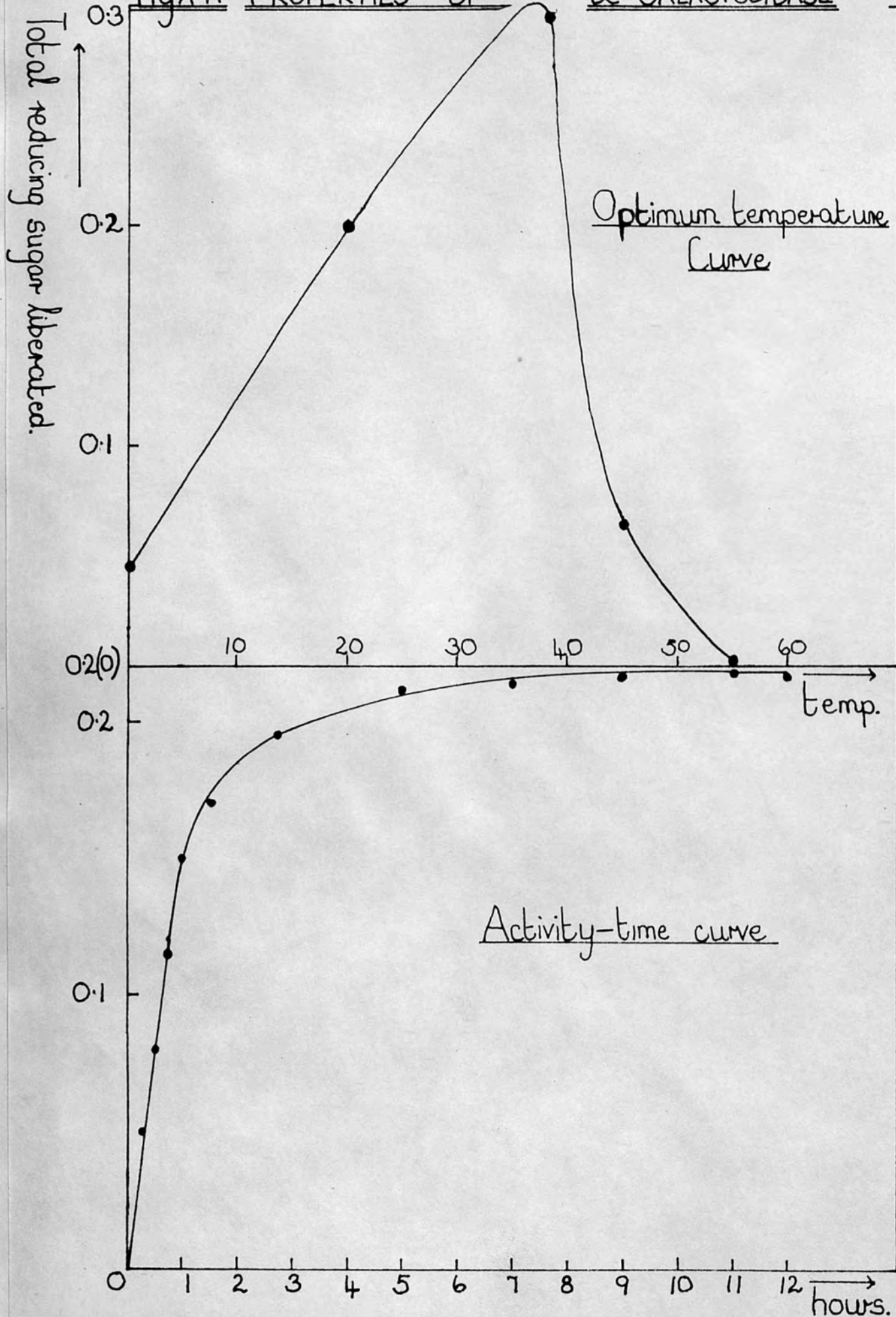


Fig XVII

LINEWEAVER - BURK

PLOT FOR α -GALACTOSIDASE

Raffinose.

Stachyose.

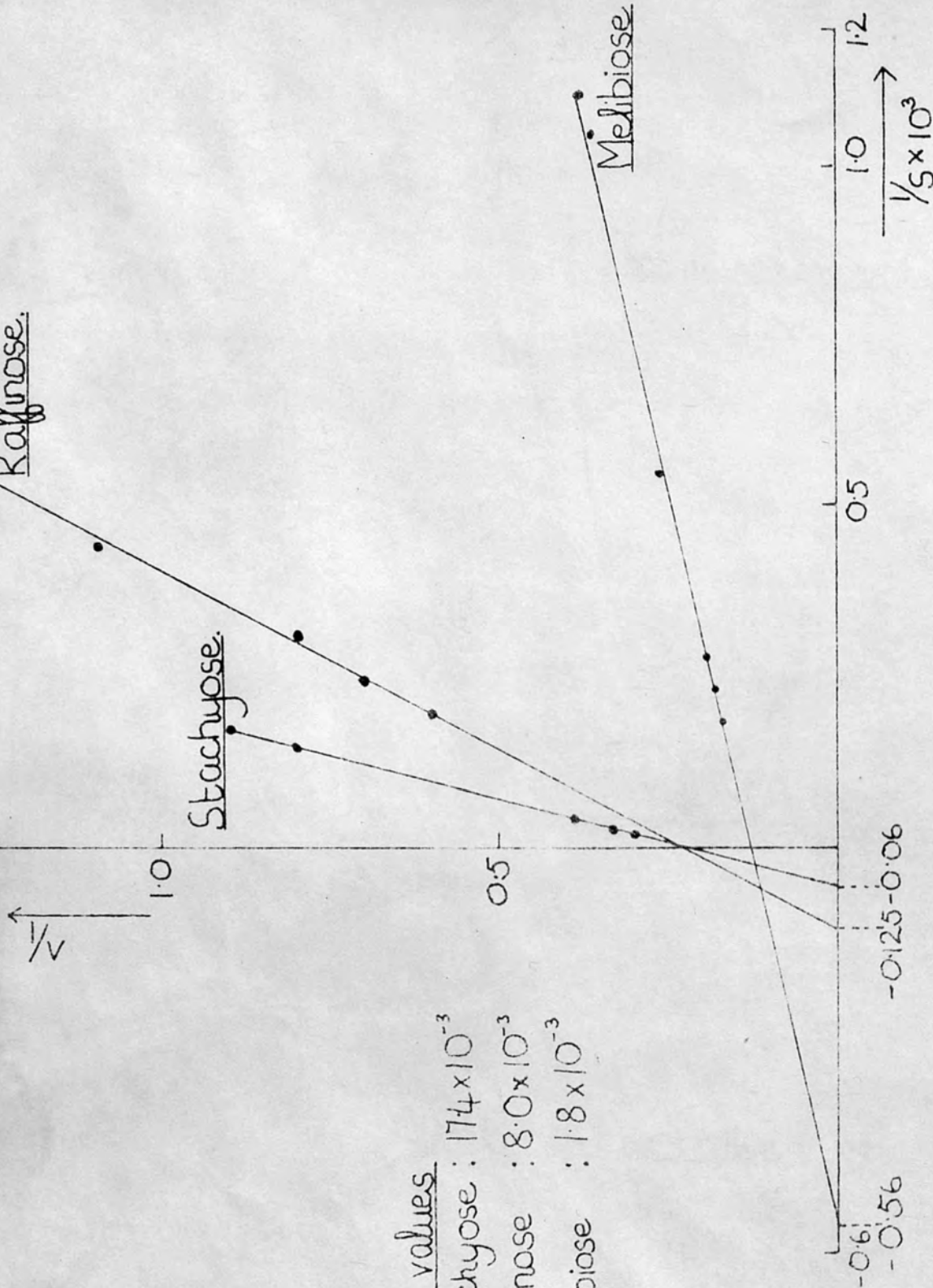
Melibiose

Km values

Stachyose : 174×10^{-3}

Raffinose : 8.0×10^{-3}

Melibiose : 1.8×10^{-3}



glucose but had no action on lactose (α -D-gal \rightarrow 4 β -D-glu). It showed a pH optimum of 4.8 using melibiose as a substrate (Fig. XV). It was inhibited by phenyl mercuric acetate and activated by 2:3 dimer_captopropanol and L-cysteine. Iodoacetic acid appeared to have only a small inhibitory effect on the enzyme (Table 5).

	% Inactivation.	
	I	II
Phenyl mercuric acetate.	100	100
Iodoacetic acid	15.2	14.2

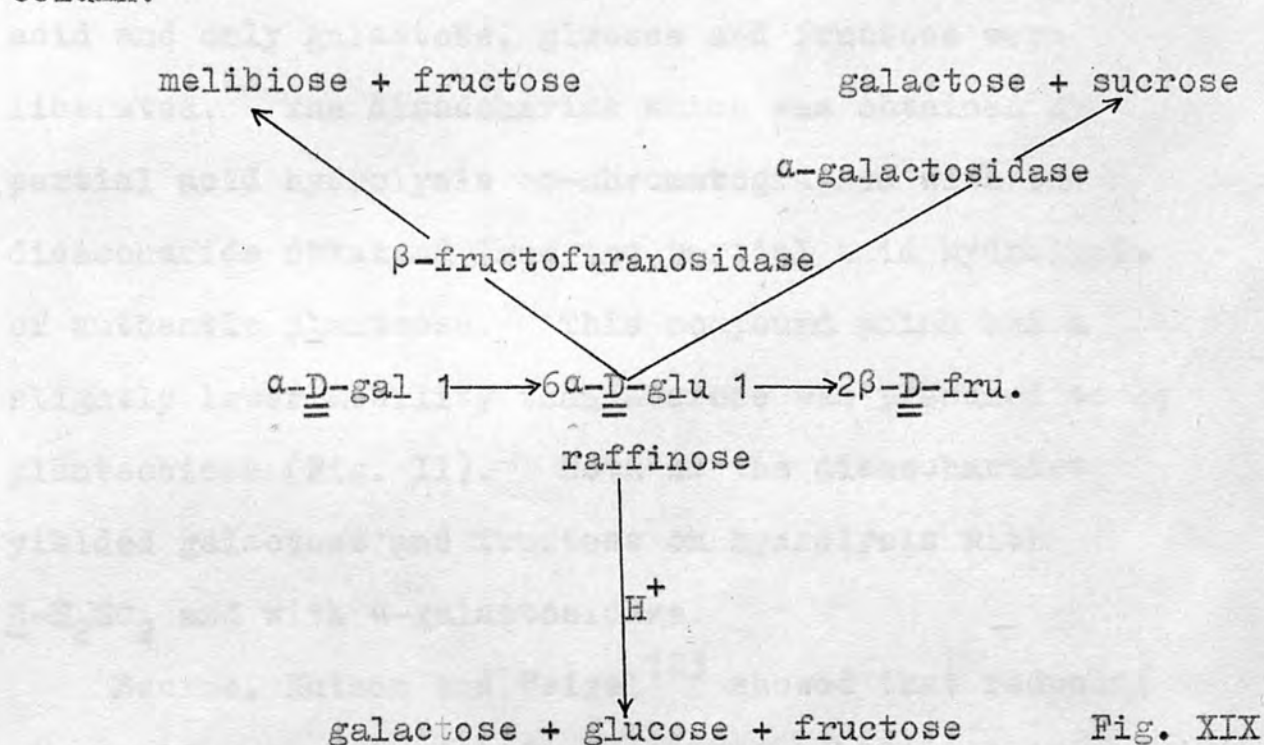
	% Activation	
	I	II
2:3 dimercapto-propanol.	128.2	140
<u>L</u> -cysteine	91.3	120

Table 5.

The optimum temperature for the activity of the enzyme again using melibiose as substrate, was found to be 37° (Fig. XVI). Measurement of the K_m values for α -galactosidase with melibiose, raffinose and stachyose showed the 'affinity' of the enzyme for these compounds was in the following order melibiose > raffinose > stachyose (Fig. XVII).

Incubation of the α -galactosidase preparation with melibiose in the presence of excess sucrose produced

compounds R_2 and P which were chromatographically identical to authentic raffinose and planteose, respectively. A compound was also formed which behaved like manninotriose on paper chromatograms. The experiment was repeated on a large scale and the oligosaccharides R_2 and P isolated using a charcoal-celite column.



In addition to paper chromatography and electrophoresis, R_2 was further characterized by incubation with $\beta\text{-fructofuranosidase}$ and $\alpha\text{-galactosidase}$ and by acid hydrolysis as shown in Fig. XIX. Final confirmation that R_2 was raffinose was obtained by periodate oxidation, (the uptake being 5 moles) and the preparation of an acetate, the mp of which was not depressed by mixing with authentic raffinose undeca-acetate.

The trisaccharide P which was formed in much smaller quantities than raffinose had an R_F value very similar to the latter on paper chromatograms in six different solvents (A-F: p26). Separation was however achieved using a butanol/pyridine/water solvent. The purified trisaccharide P was then hydrolysed with acid and only galactose, glucose and fructose were liberated. The disaccharide which was obtained by partial acid hydrolysis co-chromatographed with the disaccharide obtained from the partial acid hydrolysis of authentic planteose. This compound which had a slightly lower mobility than sucrose was presumed to be planteobiose (Fig. II). Both of the disaccharides yielded galactose and fructose on hydrolysis with $N-H_2SO_4$ and with α -galactosidase.

Bourne, Hutson and Weigel¹²³ showed that reducing glucosylfructose derivatives exhibit mobilities in molybdate buffer which are characteristic of the type of glycosidic linkage present. The non-reducing unit has little effect on the mobility in molybdate buffer. The disaccharides obtained from authentic planteose and P were observed to have the same mobilities which were equal to that of isomaltulose (α -D-glu 1 \rightarrow 6D-fru) This strongly suggested that the two disaccharides were

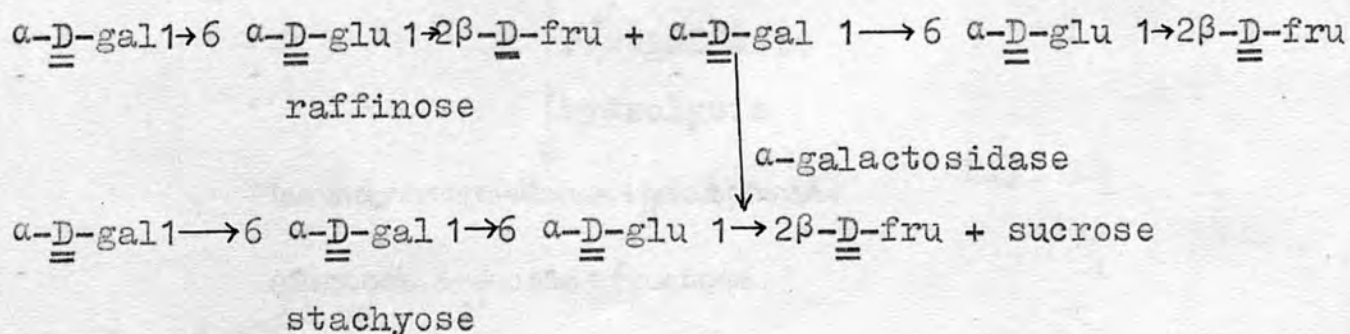
both 6-O-galactosylfructose. These electrophoretic results are additional evidence for the structure of planteose which was originally deduced by French et al.¹³

As mentioned previously (p 10), an enzyme preparation from Plantago ovata has now been resolved into two fractions by Courtois and his associates. It has been shown that while one α -galactosidase (pH optimum : 5.98) synthesizes raffinose from phenyl- α -D-galactoside and sucrose, the other (pH optimum : 5.12) synthesizes planteose from the same substrates. However the two α -galactosidases obtained from Coffea arabica by Petek and To Dong, which had pH optima at 5 and 6, both produced raffinose when incubated with sucrose and phenyl- α -D-galactoside. The V. faba α -galactosidase preparation has not been resolved, but if two enzymes (or isoenzymes) are involved in the synthesis of raffinose and planteose in vitro, it would appear that the planteose synthesizing enzyme is inactive in vivo as V. faba seeds do not contain planteose.

One argument against the hypothesis that raffinose is synthesized by α -galactosidase in vivo is the apparent absence of a suitable galactose donor. The water-soluble and alcohol-soluble extracts of V. faba seeds were examined. No galactose-containing oligosaccharides were

found (other than raffinose and its homologues) or other galactosides. The water-soluble polysaccharide was shown to be mainly a mixture of amylose and probably amylopectin. However when the polysaccharide fraction was hydrolysed with acid, galactose, mannose and arabinose were also detected in addition to glucose. Incubation of this polysaccharide fraction with sucrose and α -galactosidase did not appear to yield raffinose but a small amount of galactose was formed. Courtois *et al.* have shown³⁴ that the α -galactosidase of Trigonellum foenum graecum (p O) seeds can partially hydrolyse a galactomannan and transfer the galactose to sucrose with the formation of raffinose. It seems highly dubious that such a reaction is important for raffinose synthesis in vivo.

Stachyose, however, could conceivably be synthesized from raffinose in the bean. This was shown to occur in vitro by incubating an α -galactosidase preparation with raffinose. An oligosaccharide S co-chromatographing in 4 solvent systems with authentic stachyose, was formed.



No attempt was made to synthesize verbascose or higher galactosylsucrose derivatives but presumably these could also be prepared by an analogous method.

Alkaline β -fructofuranosidase of *V. faba*

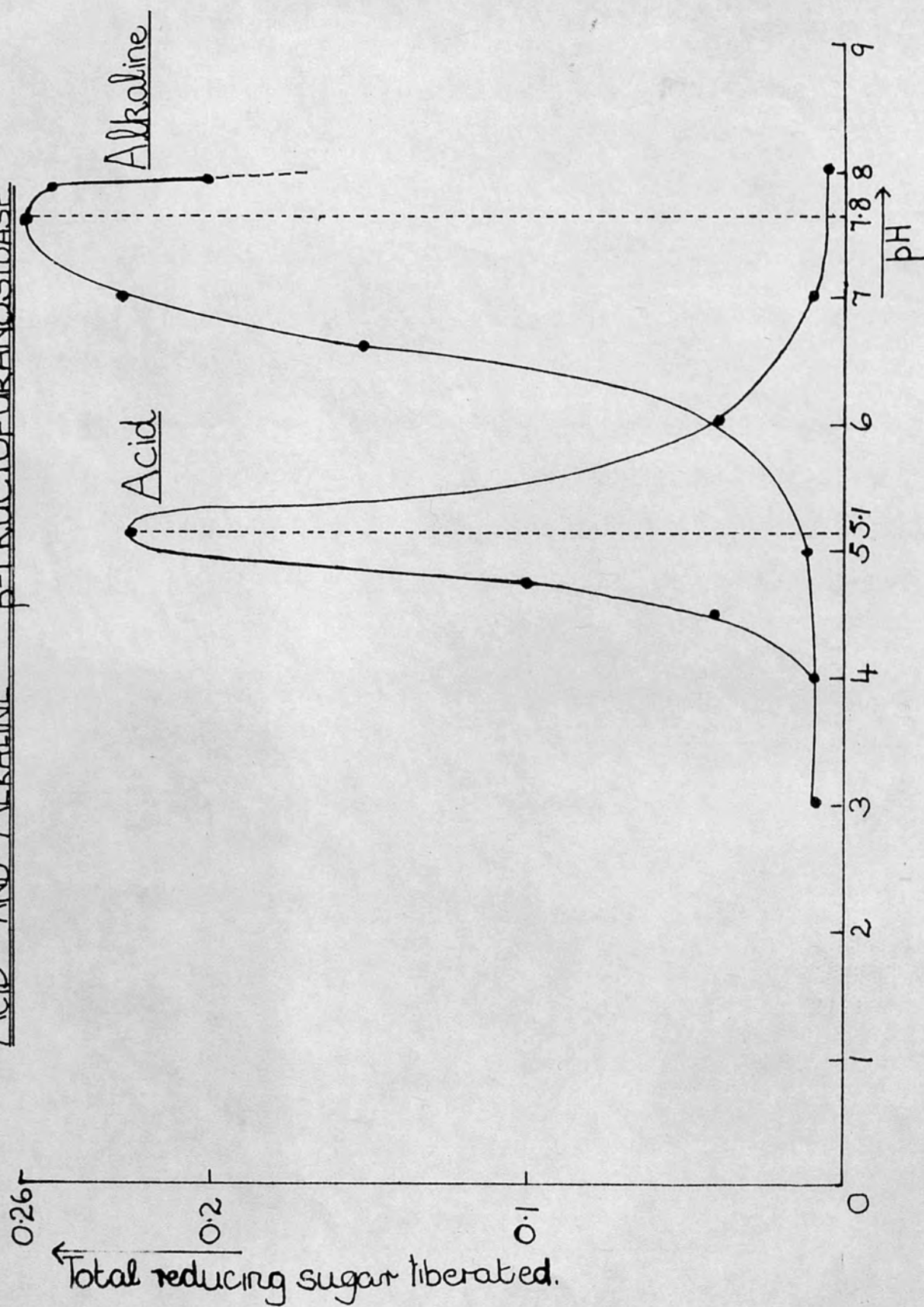
As this enzyme was present in the bean, it was decided to examine its properties in relation to the formation of the oligosaccharide K (see p56), and to see if the activity was in any way related to the presence of the galactosylsucrose derivatives in the seeds.

The enzyme was shown to be present only in dormant seeds and seeds that had been steeped in water for 12 hr. while acid β -fructofuranosidase activity was present only in green and germinated beans (Table 6).

Source of enzyme	Acid β -fructofuranosidase	Alkaline β -fructofuranosidase
Green beans	+	-
Dormant beans	-	+
Dormant embryo	-	+
Steeped beans	-	+
Germinated beans	+	-
Germinated embryo	+	-

Table 6

Fig XVIII OPTIMUM pH ACTIVITY CURVES FOR VFABA
ACID AND ALKALINE β -FRUCTOFURANOSIDASE



The pH activity curves for these two enzymes are shown in Fig. XVIII and clearly demonstrate that extracts from dormant and germinated seeds each possess only one type of β -fructofuranosidase activity.

The alkaline β -fructofuranosidase was able to hydrolyse sucrose and galactosylsucrose derivatives where fructose was an end group i.e. its broad specificity was similar to that of acid β -fructofuranosidase.¹²⁴ Alkaline β -fructofuranosidase was also shown to be capable of transferring fructosyl residues to sucrose at pH 7.8 with the formation of an oligosaccharide K_2 . Chromatography and electrophoresis showed these two compounds K and K_2 , were identical to 1^F - β -fructosylsucrose. Further proof of the structure of K was obtained by incubating it with yeast β -fructofuranosidase. This yielded labelled fructose and a labelled disaccharide which co-chromatographed with sucrose; on longer incubation, radioactive glucose and more fructose appeared to be formed. 1^F β -fructosylsucrose behaves in a similar way with this enzyme which brings about a stepwise cleavage of the two β -fructofuranosyl residues.

Alkaline β -fructofuranosidase differs from the alkaline sucrose prepared by Greenshields from Phaseolus vulgaris (p13) by this ability to transfer fructose.

The so-called neutral β -fructofuranosidase found in the mature seeds of Saccharum officinarum and S. spontaneum by Hatch and Glaziou¹²⁵ does however possess this property. A comparison of the various β -fructofuranosidases is given in Table 7. None of these enzymes are phosphorylases as they will all function in the absence of phosphate.

Reactions	<u>V. faba</u> acid β -fructo- furanosidase	<u>V. faba</u> Alkaline β -fructo- furanosi- dases.	<u>Saccharum</u> <u>officinarum</u> <u>S. spontaneum</u> Neutral β -fructo- furanosidase	<u>Phaseolus</u> <u>vulgaris</u> Alkaline sucrase.
Sucrose hydrolysis	+	+	+	+
Fructose transfer from sucrose.	1^F - β -fructosyl formed	1^F - β -fructo sylsucrose formed.	1^F - β -fructo sylsucrose formed.	-
pH of maximum activity.	4.8 (broad)	7.8 (also 7.2)	7.0 (broad)	7.8 (narrow)
Hydrolyse other substrates (e.g. galactosyl-sucrose derivatives).	+	+	+	-
Inhibited by Tris (hydroxy methyl) - aminomethane - HCl.		+	+	+

Table 7

As seen from the above table, V. faba alkaline and acid β -fructofuranosidase and S. officinarum and S. spontaneum neutral β -fructofuranosidase appear to have similar properties with the exception of the values for the pH of maximum activity.

Although alkaline β -fructofuranosidase will hydrolyse galactosylsucrose derivatives, it is unlikely that this reaction occurs in vivo as melibiose, manninotriose etc. have never been detected in the germinating bean.¹²⁷ It is conceivable however that these oligosaccharides could be rapidly metabolised.

It is interesting to note that the change in pH optimum of β -fructofuranosidase from an acid to an alkaline maximum during maturation is a feature of S. officinarum and S. spontaneum tissue as well as V. faba seeds. The reason for this is unknown, also if the two enzymes are separate proteins or a single protein which undergoes change in its tertiary structure.

General Conclusions

1. In theory there are two possible methods for raffinose synthesis, (i) via α -galactosidase, (ii) via UDP-galactose. It seems, at the present time that (ii) would be the more favourable route as (a), it is kinetically favoured (b), all the enzymes and the majority of the substrates for this reaction are present

in V. faba seeds or are known to occur in other plant tissues, (c), the process is intimately connected with other major metabolic processes, which is obviously important. Pathway (i), on the other hand, requires a suitable galactose donor which does not appear to be present.

In general, there is no evidence to suggest that type (i) reactions are important for the formation of oligosaccharides in plant tissues. In fact there appears to be no known function for carbohydrases in healthy, intact tissues and indeed, these enzymes and their potential substrates may never come together unless the cells are mechanically damaged or become diseased. In theory, however, α -galactosidase could be involved in raffinose biosynthesis in the presence of a suitable galactose donor and provided the necessary disturbance of the equilibrium constant for the synthesis occurred, possibly by coupling with other metabolic reactions.

2. Again the breakdown of galactosylsucrose derivatives could theoretically occur by the catalytic action of α -galactosidase or alkaline β -fructofuranosidase in vivo. However there is no evidence of any hydrolysis

products of such reactions in the tissues. Also by analogy with starch breakdown, a more favourable pathway for the breakdown of galactosylsucrose derivatives would involve a phosphorylase with the direct formation of α -D-galactose-1-phosphate, which could rapidly enter the general metabolism of the plant as shown in Fig. XXI. Raffinose could also be catabolysed by the reversal of the synthetic mechanism involving UDP-galactose. Such a reaction might be facilitated by other metabolic pathways involving the rapid utilization of the UDP-gal.

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The Biosynthesis of Galactosylsucrose Derivatives

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Galactosylsucrose derivatives are widely distributed in the plant kingdom (French, 1954; Courtois, 1959) although little is known about the biosynthesis or metabolism of these oligosaccharides. Members of the 'raffinose family' of oligosaccharides are formed in some seeds as the tissues mature and they are then rapidly metabolized when the seeds germinate (e.g. Pridham, 1958).

Incubation of α -D-galactose 1-phosphate with [14 C]sucrose in the presence of ATP, UTP and an enzyme preparation from *Vicia faba* seeds yielded an oligosaccharide which was indistinguishable from raffinose on paper chromatograms and electrophoretograms. This sugar was further characterized by hydrolysis with N-H₂SO₄ which yielded

labelled glucose and fructose and inactive galactose, and by treatment with yeast invertase which produced melibiose and fructose both labelled. The melibiose was identified by paper chromatography and electrophoresis and by reduction to melibi-itol with potassium borohydride. Labelled glucitol was produced by acidic hydrolysis of the melibi-itol.

Preliminary studies also suggest that *V. faba* α -galactosidase can transfer α -galactopyranosyl residues to sucrose acceptor molecules (cf. Anagnostopoulos, Courtois & Petek, 1955). The biosynthesis of galactosylsucrose derivatives *in vivo* was discussed with reference to the pathways described.

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