The Biosynthesis of Raffinose

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1. Reaction of UTP and α -D-galactose 1-phosphate with [U-¹⁴C]sucrose in the presence of a *Vicia faba* dormant-seed preparation yielded the trisaccharide raffinose. 2. UTP- α -D-galactose 1-phosphate-uridylyltransferase activity has been demonstrated in the bean preparation and evidence for the participation of UDP-galactose in the trisaccharide synthesis is presented. 3. UDP-galactose 4-epimerase is present in the dormant seed. 4. The biosynthesis of raffinose in relation to the metabolism of other carbohydrates in plants is discussed.

During the past 15 years it has become apparent that much of the carbohydrate metabolism in both plants and animals involves nucleoside diphosphate sugar derivatives (e.g. Feingold, Neufeld & Hassid, 1964; Ginsburg, 1964).

Sucrose is the major oligosaccharide found in plants and the formation of this sugar by the transfer of D-glucose from UDP-glucose to D-fructose has been demonstrated with transferases from several different species (e.g. Turner, 1954; Cardini, Leloir & Chiriboga, 1955; Bean & Hassid, 1955; Burma & Mortimer, 1956; Rorem, Walker & McCready, 1960). In addition, Leloir & Cardini (1955) showed that a wheat-germ preparation catalysed the formation of sucrose phosphate from UDP-glucose and D-fructose 6-phosphate. Trehalose, the non-reducing disaccharide found in yeasts and fungi, appears to be formed by the action of a specific phosphatase on trehalose 6-phosphate, the latter being derived from UDP-glucose and D-glucose 6-phosphate (Leloir & Cabib, 1953; Cabib & Leloir, 1958). After sucrose, the next most abundant oligosaccharide found in plant tissues is raffinose [α -D-Gal_p-(1→6)- β -D-Glc_p-(1→2)- β -D-Fru_p] (French, 1954; Courtois, 1959). The biosynthesis of this compound has already been achieved *in vitro* by allowing sucrose to react with α -D-galactopyranosides, such as phenyl α -D-galactoside and melibiose, in the presence of α -D-galactoside galactohydrolase (e.g. Courtois, Petek & To Dong, 1961; Pridham & Walter, 1964). This type of simple transferase reaction is, however, of doubtful biological importance and it is perhaps more reasonable to assume that *in vivo* the D-galactopyranosyl residue in raffinose is derived from a nucleoside diphosphate D-galactose derivative. The investigation described in this paper strongly supports this suggested mechanism. A preliminary account of the results

has already been published (Bourne, Pridham & Walter, 1962).

METHODS

General methods. Absorption spectra were measured in alkaline solution with a Perkin-Elmer model 137 u.v. spectrophotometer (1 cm. cell). All solutions were concentrated with a rotatory evaporator at temperatures not exceeding 40°.

Paper chromatography. Whatman no. 1 paper and the following solvent systems were used for the examination of carbohydrates: *A*, ethyl acetate-acetic acid-water (9:2:2, by vol.); *B*, ethyl acetate-pyridine-water (2:1:2, by vol.). *p*-Anisidine hydrochloride (spray *a*) (Hough, Jones & Wadman, 1950) was used to detect reducing sugars and galactosylsucrose derivatives. Silver nitrate-NaOH (spray *b*) (Trevelyan, Procter & Harrison, 1950) was used to locate reduced mono- and di-saccharides. Fructose-containing compounds were detected with urea-phosphate reagent (spray *c*) (Wise, Dinbew & Davies, 1955). Nucleotides were chromatographed with ethanol-m-ammonium acetate (7:3, v/v, pH 7.5) (solvent *C*) and located by u.v. absorption by using a Chromatolite lamp (Hanovia Ltd., Slough, Bucks.).

Paper electrophoresis. Carbohydrates were examined by this procedure with Whatman no. 3 paper and 0.2M-sodium borate, pH 10.0, and 8mM-ammonium molybdate, pH 5.2, solutions as electrolytes. Before treatment with spray *a*, borate electrophoretograms were exposed to HCl fumes for 10 min. Whatman no. 1 paper with 0.1M-ammonium formate buffer, pH 3.7, was used for the electrophoresis of nucleotides.

Densitometer method for determination of sugars. Known volumes of standard solutions of D-galactose, sucrose, raffinose and stachyose were spotted on to Whatman no. 1 papers, which were developed with solvent *A*. The papers were then treated with spray *a* and heated at 120° for 5 min. The chromatograms were cut into strips and the coloured spots measured with an EEL absorption densitometer (Evans Electro Selenium Ltd., Halstead, Essex). A comparison of the areas under the absorption curves with the sugar concentrations showed that there were linear relation-

ships with all four compounds and that the errors did not exceed $\pm 7\%$ over the range 20–90 μg .

Examination of sugars in leguminous seeds. The seeds were removed from the pods in July and extracted with cold aq. 75% (v/v) methanol. The extracts, after concentration, were examined on paper chromatograms with solvent *A* (sprays *a* and *c*). Other seed samples from the same species were left for 10 days in the air at room temperature before examination.

Ten samples of green *Vicia faba* seeds (ten beans/sample) were weighed and placed in a laboratory cupboard in an open Petri dish. At 24 hr. intervals samples were reweighed and extracted with aq. 75% (v/v) methanol (25 ml.). The extracts were concentrated to 5 ml., and samples were taken with a micrometer syringe and spotted on to Whatman no. 1 papers. These were developed with solvent *A* and the amounts of D-glucose, sucrose, raffinose and higher galactosylsucrose derivatives (the latter expressed as μg . of stachyose) present were determined by the densitometer method.

Enzyme preparation. The testas were removed from dormant *V. faba* (var. Johnson's long pod) seeds and the cotyledons and embryos powdered at room temperature in a vibratory ball mill. Extraction of the powder was achieved by maceration in a homogenizer (Measuring and Scientific Equipment Ltd., London) with 0.5 M-sodium phosphate buffer, pH 7.0 (1.5 ml. of buffer/g. of powder), for 1 min. at 0–5°. The gross tissue residue was removed by centrifugation at 1600g for 30 min. at 0° and the supernatant dialysed against 20 vol. of phosphate buffer at 5°.

Synthesis of raffinose. The enzyme (0.3 ml.) was incubated with ATP (10 μmoles), UTP (3 μmoles), α -D-galactose 1-phosphate (5 μmoles), [U- ^{14}C]sucrose (4 μmoles ; 20 μC), MgCl_2 (10 μmoles) and L-cysteine (3 μmoles) at 35°. Identical reaction mixtures, but (a) with boiled enzyme and (b) with UTP and ATP omitted, were also set up. After 2.5 hr., ethanol (0.3 ml.) was added to each reaction mixture and the solutions were streaked on to Whatman no. 3 papers. These were developed with solvent *A* for 24 hr. and radioautographs prepared (Ilford industrial G X-ray film; 4 days exposure). In the reaction mixture that contained boiled enzyme the [U- ^{14}C]sucrose appeared to be unchanged, and the one containing no exogenous nucleotides showed labelled bands corresponding to sucrose, glucose, fructose and a trisaccharide (*K*; R_{G10} 0.25; see Pridham & Walter, 1964). The complete reaction mixture exhibited a similar chromatographic pattern but an additional labelled compound (*R*) with R_F identical with that of raffinose was also observed. A scan of the radioactivity on the chromatogram of the latter reaction mixture is shown in Fig. 1. This was constructed by passing a 1 cm. strip of the paper under an aluminium plate possessing a 0.5 cm. $\times$ 1.0 cm. slit and measuring the radiation with a Geiger-Müller tube (2 mg./cm.² window) connected to a conventional scaler.

Isolation and examination of R. The band containing *R* was eluted with water and the solution concentrated to a syrup. Unlabelled raffinose (4 mg.) was added and the whole (*R'*) dissolved in water (1.5 ml.). Appropriate volumes of this solution were then subjected to further paper-chromatographic (solvents *A* and *B*, spray *a*) and electrophoretic (borate buffer, spray *a*) analysis. [Paper chromatograms and electrophoretograms were in all cases scanned for radioactivity, as described above, and then the coloured spots resulting from treatment with the carbohydrate-

Fig. 1. Scan of the ^{14}C -labelled compounds present after paper-chromatographic fractionation of the raffinose-synthesizing system described in the text. *R*, Raffinose; *K*, 1-kestose; *S*, sucrose; *G*, glucose; *F*, fructose.

locating reagent examined with an absorbance densitometer (see Fig. 2). This procedure was also used to investigate all the products of the degradative reactions described below.]

Acid hydrolysis of *R'*. The solution of *R'* (0.5 ml.) was heated with $\text{N-H}_2\text{SO}_4$ (3 ml.) at 100° for 3 hr. After neutralization with Amberlite IR-4B (OH^- form) resin the hydrolysate was examined on a paper chromatogram (solvent *A*, spray *a*).

Treatment of *R'* with α - and β -D-galactoside galactohydrolases. The raffinose-synthesizing enzyme from dormant *V. faba* seeds was used as a source of α -galactosidase (see Pridham & Walter, 1964), and an *Escherichia coli* β -galactosidase preparation was kindly provided by Professor K. Wallenfels. The enzymes (0.1 ml. volumes) were incubated with solutions of *R'* (0.1 ml.) at 35° for 12 hr. and the mixtures examined on paper chromatograms (solvent *A*, spray *a*).

Hydrolysis of *R'* with β -D-fructofuranoside fructohydrolase. Yeast invertase concentrate (British Drug Houses Ltd.; diluted 10-fold with 0.2 M-sodium acetate buffer, pH 4.8; total volume 0.1 ml.) was incubated with *R'* (0.1 ml.) at 35° for 3 hr. The reaction mixture was examined on paper chromatograms (solvent *A*, spray *a*) and electrophoretograms (borate buffer, spray *a*).

Reduction of melibiose. The solution remaining after the invertase hydrolysis was diluted to 1 ml. with water and KBH_4 (2 mg.) added. After 4 hr. the reaction mixture was deionized with Bio-Deminrolit (HCO_3^- form) resin and the radioactive product (melibiose) isolated by paper electrophoresis (molybdate buffer; side strip treated with spray *b*) followed by elution of the compound from the paper with water.

Fig. 2. (a) Paper chromatography with solvent *A*, (b) paper chromatography with solvent *B* and (c) paper electrophoresis in borate buffer of raffinose (*R'*). Details are given in the text. Continuous lines represent the radioactivity and broken lines the colour intensity of the spots produced by reaction with *p*-anisidine hydrochloride.

Hydrolysis of melibi-itol. The syrup remaining after concentration of the above eluate was heated with $\text{N-H}_2\text{SO}_4$ (0.5 ml.) at 100° for 3 hr. Electrophoresis of the hydrolysate (borate buffer, spray *b*) after neutralization with Amberlite IR-4B (OH^- form) resin revealed a radioactive spot with the same mobility as D-glucitol.

Biosynthesis of UDP-galactose. UTP (0.8 μmole), α -D-galactose 1-phosphate (1.5 μmole), ATP (0.2 μmole), MgCl_2 (1 μmole) and L-cysteine (0.3 μmole) were incubated with the dormant-bean preparation (0.1 ml.) for 1.5 hr. at 35° . The reaction mixture was then examined on a paper chromatogram (solvent *C*) and a u.v.-absorbing spot that co-chromatographed with UDP-galactose was eluted from the paper with water. The spectrum of the resulting solution was measured and a sample also subjected to paper electrophoresis (formate buffer). Hydrolysis of the nucleotide was effected by heating the aqueous solution with Amberlite IR-120 (H^+ form) resin at 100° (5 min.). The liberated sugar (galactose) was identified by paper chromatography (solvent *A*, spray *a*). A control reaction from which UTP had been omitted yielded no u.v.-absorbing band in the UDP-hexose region of the paper chromatogram.

The u.v.-absorbing band, corresponding to UDP-galactose on a paper chromatogram (solvent *C*), that resulted from the incubation (1 hr. at 35°) of UDP-glucose (0.3 μmole), NAD^+ (0.15 μmole), ATP (0.2 μmole), MgCl_2 (1.0 μmole) and L-cysteine (0.3 μmole) with the dormant-bean preparation (0.1 ml.) was also examined by the same methods.

Attempted biosynthesis of ADP-galactose. The bean preparation (0.1 ml.) was incubated for 2.5 hr. at 35° with ATP (0.6 μmole), α -D-galactose 1-phosphate (2.2 μmole), MgCl_2 (1 μmole) and L-cysteine (0.3 μmole). The reaction mixture was examined on a paper chromatogram (solvent *C*).

RESULTS AND DISCUSSION

Raffinose, stachyose and higher-molecular-weight α -D-galactosylsucrose derivatives are common constituents of the seeds of legumes (French, 1954;

Courtois, 1959), where they appear to be formed during the final stages of maturation (e.g. Korytnyk & Metzler, 1962; Gould & Greenshields, 1964). A qualitative chromatographic comparison of the sugars in the seeds of *Colutea arborescens*, *Cytissus scoparius*, *Selegia orientalis* and *Lupinus polyphyllus* at an immature stage and after dehydration at room temperature confirmed this. A quantitative illustration of the changes in sugar concentrations in green *V. faba* seeds that were taken from the pod and left in the air for several days is given in Fig. 3. This shows that the loss of water by the tissues is roughly paralleled by an increase in raffinose and higher homologues. Sucrose exhibited an initial rapid decrease in concentration followed by an increase after 3 days, and the glucose content of the tissues slowly declined. It should, however, be emphasized that Fig. 3 may not be an exact illustration of the changes that occur *in vivo* as the 'maturation' was probably accelerated by removal of the bean seeds from the pods, thus increasing the rate of dehydration.

Incubation of an extract of mature broad-bean seeds with $[\text{U-}^{14}\text{C}]$ sucrose, α -D-galactose 1-phosphate, UTP and ATP produced a labelled compound *R* (see Fig. 1) with chromatographic properties characteristic of raffinose together with a second radioactive trisaccharide *K*. When the nucleoside triphosphates were omitted from the reaction mixture *K* was still produced but not *R*. *K* has been identified as 1^F - β -D-fructosylsucrose (1-kestose) (Pridham & Walter, 1964) and is presumably formed by the action of an 'alkaline' β -D-fructofuranoside fructohydrolase on sucrose.

Compound *R* was mixed with unlabelled raffinose (mixture *R'*) and further examined on paper

Fig. 3. Changes in mono- and oligo-saccharide content during dehydration of *V. faba* seeds. Details are given in the text. ○, Sucrose; □, stachyose; ▽, loss of water; ●, raffinose; △, glucose.

chromatograms (two solvent systems) and by paper electrophoresis (borate buffer). By scanning the papers for radioactivity and by using a densitometer to examine the coloured spots produced by reaction with *p*-anisidine hydrochloride, an exact coincidence between colour and radioactivity was observed in all cases (Fig. 2). This procedure was also used to examine the products resulting from acidic and enzymic degradation of the mixture *R'*. In this way it was shown that complete acid hydrolysis yielded galactose and labelled glucose and fructose and that treatment with a *V. faba* α -D-galactoside galactohydrolase preparation produced galactose and [14 C]sucrose. No hydrolysis was observed on incubation of *R'* with *E. coli* β -D-galactoside galactohydrolase. A labelled disaccharide that was indistinguishable from melibiose on paper chromatograms and electrophoretograms was formed when yeast β -D-fructofuranoside fructohydrolase was used as the hydrolytic agent. The identity of this disaccharide was further checked by reduction with potassium borohydride followed by electrophoresis in ammonium molybdate solution. The resulting non-reducing derivative was indistinguishable from authentic melibi-itol on electrophoresis. The rate of movement of melibi-itol in this electrolyte ($M_{\text{melibi-itol}}$ 0.8) is characteristic of a 6-substituted hexitol derivative (Bourne, Hutson & Weigel, 1959). Acid hydrolysis of the melibi-itol yielded labelled glucitol. This degradative study therefore confirmed that *R* was raffinose and that it was formed by the transfer of D-galactose to 14 C-labelled sucrose.

Incubation of α -D-galactose 1-phosphate with UTP and the bean enzyme preparation produced a compound that behaved as a UDP-hexose deriva-

tive on paper chromatograms and electrophoretograms and had a u.v.-absorption spectrum characteristic of a uridine nucleotide (E_{250}/E_{260} , 0.8; E_{280}/E_{260} , 0.24). Acid hydrolysis of this compound liberated galactose. It appeared therefore that the bean preparation possessed UTP- α -D-galactose 1-phosphate-uridylyltransferase activity (cf. Neufeld, Ginsburg, Putman, Fanshier & Hassid, 1957) and hence that UDP-galactose served as the galactose donor for raffinose synthesis. Passeron, Recondo & Dankert (1964) have, however, demonstrated the presence of ATP- α -D-galactose 1-phosphate-adenylyltransferase activity in wheat germ, and it was conceivable that ADP-galactose was formed and acted as a galactose donor in the raffinose-synthesizing system. All attempts in the present work to synthesize ADP-galactose from α -D-galactose 1-phosphate and ATP in the presence of the bean enzyme failed, and it is therefore unlikely that ADP-galactose played any significant role in the synthesis of the trisaccharide. Further evidence of the participation of UDP-galactose in raffinose formation has also been obtained by allowing UDP-[1- 14 C]galactose to react with sucrose and the bean preparation. This resulted in approx. 39% incorporation of [1- 14 C]galactose after 2.5 hr. incubation at 37° (Pridham & Hassid, 1965).

It is probable that *in vivo* the same pathway is responsible for the synthesis of raffinose and possibly other α -D-galactosylsucrose derivatives. Rast, McInnes & Neish (1963) have come to the same conclusion from a study of 14 C-incorporation into the carbohydrates of spruce twigs.

UDP-galactose is, no doubt, widespread in plant tissues (e.g. Ginsburg, Stumpf & Hassid, 1956; Brown, 1962) and has actually been isolated from dormant and germinating *V. faba* seeds (J. B. Pridham & M. W. Walter, unpublished work). UDP-galactose can also be readily derived from UDP-glucose by the action of UDP-galactose 4-epimerase (cf. Neufeld *et al.* 1957) and this enzyme has now been shown to be present in bean seeds. Thus the incubation of UDP-glucose with NAD^+ and the bean enzyme preparation yielded a nucleotide that was identified as UDP-galactose by the same procedures described above for the examination of the product of UTP- α -D-galactose 1-phosphate-uridylyltransferase activity. A pathway from UDP-glucose to raffinose via UDP-galactose is therefore very likely to exist in *V. faba* (and other seeds), as all the necessary enzymes and their substrates are present in the tissues. Dupéron (1964) has observed that 14 C-labelled glucose rapidly gives rise to labelled sucrose and raffinose in *Phaseolus vulgaris* seeds. The nucleotide-dependent pathway would also allow raffinose formation to be closely linked to starch and sucrose metabolism.

The possible role of α -D-galactoside galactohydrolase in the formation of raffinose and its homologues should perhaps not be entirely dismissed at the present time. Pridham & Walter (1964) have shown that this enzyme from *V. faba* seeds will transfer galactose from melibiose to sucrose *in vitro* with the formation of raffinose and small amounts of planteose [α -D-Glc_p-(1→2)- β -D-Fru₇-(6←1)- α -D-Gal_p]; it may be significant that this latter sugar does not appear to be present in *V. faba* and that raffinose in the presence of this enzyme can act as both galactose donor and acceptor with the consequent formation of stachyose [α -D-Gal_p-(1→6)- α -D-Gal_p-(1→6)- α -D-Glc_p-(1→2)- β -D-Fru₇] (cf. Courtois *et al.* 1961). If such a pathway is responsible for the formation of raffinose *in vivo* then the galactose donor for the reaction will have to be established. No naturally occurring low-molecular-weight compounds that would be suitable substrates have so far been identified, although the possible existence of galactose donors of high degree of polymerization should be borne in mind. For example, Courtois & Pecheron (1961) have shown that an α -D-galactoside galactohydrolase from *Trigonellum foenum graecum* seeds can synthesize raffinose by transfer of galactose from a galactomannan to sucrose. The equilibrium constants for glycohydrolase-catalysed reactions normally favour hydrolysis rather than the synthesis of glycosidic bonds. Hence synthesis is normally forced by the use of high (non-physiological) substrate concentrations and this has led to scepticism about the part played by these enzymes in biosynthesis. There is, however, little information on the effective concentrations of potential substrates in the plant cell. In addition, most of the studies *in vitro* have been carried out with very crude enzymes. Clearly the role of α -D-galactoside galactohydrolase and other glycohydrolases in synthetic processes in the plant require further investigation but, on the basis of existing evidence, the nucleotide-dependent pathways for the formation of the principal oligosaccharides occurring in higher plants appear to be the more important. With sucrose there is virtually no doubt about the nature of the reaction; the enzymic synthesis of this disaccharide has never been achieved with higher-plant enzymes in the absence of a nucleoside diphosphate derivative of D-glucose.

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