A new pathway for the synthesis of the plant sulpholipid, sulphoquinovosyldiacylglycerol

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A new pathway is proposed for the biosynthesis of the plant sulpholipid, sulphoquinovosyldiacylglycerol. The pathway begins at UDP-glucose and involves the formation therefrom of UDP-4-ketoglucose-5-ene to which is subsequently added sulphite (or its metabolic equivalent). Evidence consistent with this pathway, rather than with the previously proposed 'sulphoglycolytic' route, was obtained from experiments with pea chloroplast preparations. The evidence included the failure of potential inhibitors of the sulphoglycolytic pathway to alter the rate of synthesis of sulpholipid and the stimulation of the incorporation of $^{35}SO_4^{2-}$ into the latter by UTP. Radioactivity was effectively incorporated into sulpholipid from UDP-[^{14}C]glucose and this radiolabelling was stimulated by the addition of methyl α-glucose-enide or of an enzyme system known to be forming (although not accumulating) UDP-4-ketoglucose-5-ene. This new pathway is also consistent with other data in the literature.

INTRODUCTION

All organisms carrying out oxygenic photosynthesis contain the plant sulpholipid, sulphoquinovosyldiacylglycerol, as a major component of their thylakoid membrane [1]. The lipid was first discovered and identified by Benson and co-workers [2] and, in some marine algae, may represent up to half of the total lipids (e.g. Dembitsky et al. [3]). Thus the plant sulpholipid is a very significant component of biological membranes and makes a major contribution to the sulphur cycle [4,5].

In spite of its quantitative importance, attempts to elucidate the biosynthesis of sulphoquinovosyldiacylglycerol have so far proved fruitless. The experiments conducted have been reviewed [5–8]. Most early work concentrated on the so-called sulphoglycolytic pathway, which was first outlined in detail by Davies et al. [9]. Because Shibuya and Benson [10] found a number of possible intermediates for this pathway in *Chlorella*, Benson [2] suggested a sulphoglycolytic sequence involving, first, adenosine 3′-phosphate 5′-sulphatophosphate and then sulphopyruvate. Experiments with *Euglena gracilis* [9] and with germinating seeds of alfalfa and brussels sprouts [11] were consistent with the pathway, whereas later work with spinach seedlings [12] or isolated chloroplasts [13] was not. In any case, there are a number of theoretical problems with a sulphoglycolytic pathway, as pointed out previously [6,7].

Haines [14] proposed a modification of the sulphoglycolytic pathway. Notably, he suggested that adenosine 3′-phosphate 5′-sulphatophosphate was involved in lipid synthesis by a nucleophilic displacement reaction on *O*-acetylserine to yield cysteic acid. Another route for the chemical synthesis of sulphonic acids involves the addition of sulphite to double bonds (see [15]), and Lehmann and Benson [16] proposed that a 6-sulphoquinovose residue could be formed by such a reaction *in vivo*. Direct evidence for the involvement of sulphite in sulpholipid synthesis has been obtained in germinating seeds [11], with *Chlamydomonas* membranes [17] and in *Euglena* [18]. In the latter organism, sulphate activation and reduction occur in mitochondria which release sulphite (and cysteine) to be taken up by chloroplasts and incorporated into sulpholipid [19]. If sulphite (or an *S*-sulpho protein) is involved in sulpholipid formation, at what level could it be used? Instead of reactions at the 3-carbon level to give molecules such as D-lactaldehyde 3-sulphonate [5,9], a glucose-5-enide could add sulphite, as suggested by Lehmann and Benson [16], who used this reaction to synthesize methyl α-sulphoquinovoside *in vitro*. An analogous reaction in chicken liver has been proposed to generate the carbon–sulphur bond in cysteic acid [20] and may also account for the formation of sulphoacetic acid in plants [16]. Addition of sulphite at the 6-carbon level has the merit that the pathway for sulpholipid synthesis would be much shorter and use abundant substrates such as glucose derivatives. A study by Benning and Sommerville [21,22] with sulpholipid-deficient mutants of *Rhodobacter sphaeroides* identified four genes only which were involved in the biosynthetic pathway. One of these showed sequence similarity with UDP-glucose epimerase [22]. Although there is no guarantee that the pathway in anoxygenic photosynthetic bacteria is the same as in plants, such an observation fitted with our working hypothesis that sulphite added to a glucose-enide derivative, a possibility which we were already testing by using the pea chloroplast system described by Pugh and Harwood [23]. Accordingly, we have formulated details of a possible pathway for sulpholipid synthesis which takes into account observations from a number of laboratories, and provide here experimental evidence in its support.

The proposed pathway for sulpholipid biosynthesis

Our proposal for the pathway of sulpholipid biosynthesis is shown in Scheme 1. Supporting evidence from the literature can be summarized as follows. (1) Barber [24] suggested (although without experimental evidence) that sulphoquinovose might be formed from a compound related to the UDP-6-deoxy-α-D-*xylo*-hex-4-ulose (UDP-6-deoxy-4-ketoglucose), which is an intermediate in the synthesis of L-rhamnose by tobacco. (2) One of the

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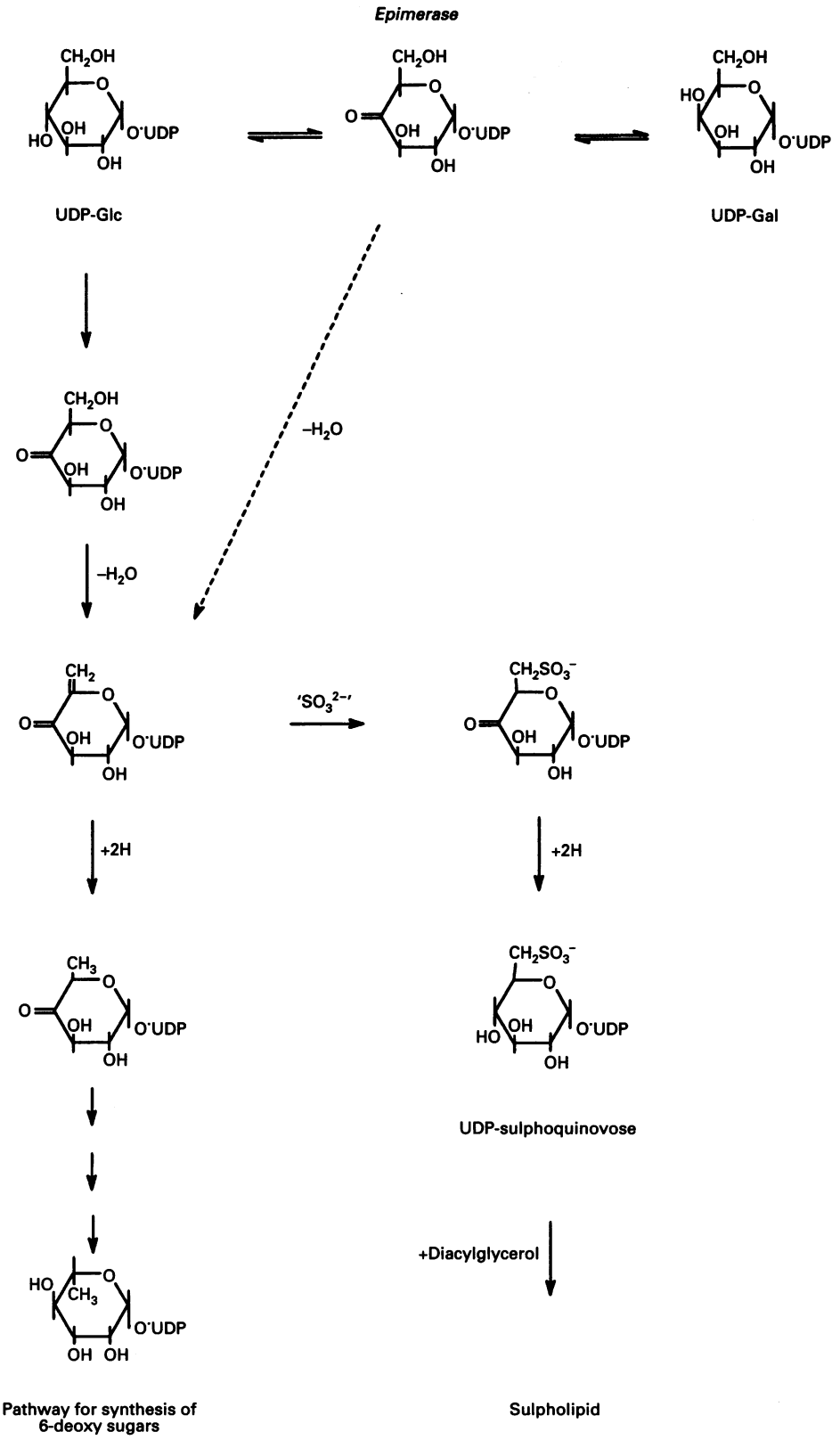

Scheme 1 Proposed pathway for the biosynthesis of the plant sulpholipid (sulphoquinovosyldiacylglycerol)

Two possible routes for the generation of UDP-4-keto-6-deoxyglucose are illustrated. Furthermore, the addition of 'sulphite' could occur either to tightly bound UDP-4-ketoglucose-5-ene or to free UDP-glucose-5-ene (see the text for discussion).

genes identified to be involved in sulpholipid biosynthesis in *Rhodobacter sphaeroides* [23] shows sequence similarity with UDP-glucose epimerase from various organisms. (3) Gabriel [25] pointed out common features between TDP-glucose oxidoreductase, UDP-galactose 4-epimerase and UDP-glucuronate carboxylyase. In each the initial step is abstraction of an axial hydride equivalent from C-4. In the oxidoreductase the postulated intermediate is stabilized by the elimination of water, in the epimerase by tight binding to the enzyme and in the carboxylase by elimination of CO_2. (4) In the reaction catalysed by dTDP-glucose oxidoreductase the formation of dTDP-6-deoxy-α-D-*xylo*-hex-4-ulose-5-ene (dTDP-4-ketoglucose-5-ene) from dTDP-4-ketoglucose appears to have been established (e.g. [26]). (5) The double bond in methyl 6-deoxy-α-D-*xylo*-hex-5-enopyranoside (methyl glucose-5-enide) readily accepts sulphite *in vitro* (in a free-radical reaction) to form methyl sulphoquinovoside [16]. (6) This addition of sulphite is a general reaction with hexose-5-enides [27–29] and would be expected to occur with UDP-6-deoxyglucose-5-ene. (7) The last intermediate in the postulated pathway is UDP-sulphoquinovose, which has been shown by Seifert and Heinz [30] to be used by the enzyme catalysing the final stage of sulpholipid formation in plants.

In Scheme 1, an alternative route for the generation of the UDP-4-ketoglucose-5-ene is to utilize UDP-4-ketoglucose directly from UDP-galactose epimerase, although the former may be too tightly bound to the enzyme to be of use. Moreover, there are at least two theoretical routes of sulphite addition. First, this could occur to an enzyme-bound UDP-4-ketoglucose-5-ene. Internal reduction by enzyme-bound NAD(P)H would then generate UDP-sulphoquinovose and this product would be released. Alternatively, reduction at C-4 of the UDP-4-ketoglucose-5-ene leads to UDP-glucose-5-ene as a free intermediate. A separate enzyme then catalyses the free-radical addition of sulphite. However, in the absence of specific evidence, it seems fruitless to discuss the detailed chemistry of these variants, especially the mechanism of sulphite addition.

Our new proposed pathway has the merit of brevity when compared with the sulphoglycolytic sequence [6] and, because of the published evidence described above, we designed experiments in order to obtain supporting biochemical data.

MATERIALS AND METHODS

Materials

Pea (*Pisum sativum* cv. Feltham First) seeds were obtained from Asmer Seed, Leicester, U.K., and germinated in Levington compost at 20 °C with a 16 h day/8 h night cycle with illumination of 650 $\mu E \cdot s^{-1} \cdot m^{-2}$. Leaves were used from 8–10-day-old plants for chloroplast preparations and labelling experiments.

UDP-[U-^{14}C]galactose (7.4 GBq/mmol), UDP-[U-^{14}C]glucose (7.4 GBq/mmol) and $^{35}SO_4^{2-}$ (74–370 MBq/mmol) were obtained from Amersham International p.l.c., Aylesbury, Bucks., U.K.

Preparation of chloroplasts

Chloroplasts were isolated from pea leaves by the method of Mills and Joy [31]. They were assessed for intactness by phase-contrast microscopy and found to be > 90% intact.

Preparation and assay of the 'rhamnose-synthesizing' system

The crude system was prepared from pea leaves essentially as described by Barber [24] for tobacco by homogenizing the tissue in 0.15 M Tris/HCl, pH 7.5, containing 0.1 M 2-mercaptoethanol, removing the particulate material by centrifugation and precipitating the required activity between 40%- and 50%-saturated ammonium sulphate by the slow addition of saturated ammonium sulphate. The material from 60 g of leaves was dissolved in 1 ml of 0.025 M phosphate buffer, pH 7.5, containing 0.01 M 2-mercaptoethanol and dialysed overnight against 2 × 500 ml of the same buffer at 5 °C. After removal of small amounts of insoluble material, the protein concentration of the resulting solution was 35–55 mg/ml and it retained its activity for several weeks when stored at −20 °C.

In the absence of NADPH, this system accumulates UDP-6-deoxy-α-D-*xylo*-hex-4-ulose (UDP-6-deoxy-4-ketoglucose) [24] and its activity was determined by incubation of 2–3 mg of the above protein for 1 h at 37 °C in 0.15 ml of 0.025 M phosphate buffer, pH 7.5, containing 0.01 M mercaptoethanol and 0.7 mM UDP-[^{14}C]Glc (about 45 MBq/mmol). The reaction mixture was chilled and filtered through a Microcon 10 concentrator (Amicon Ltd., Stonehouse, U.K.). To 50 μl of the filtrate was added 5 μl of 4 M $NaBH_4$ and the mixture was kept at room temperature for 10 min to reduce any hexuloses present [32]. The solution was acidified with 5 μl of either trifluoroacetic acid or lactic acid (about 86%, w/w; Sigma Chemical Co.); in the former case the mixture was heated in a capped tube for 10 min in a boiling water bath and in the latter it was heated in a sealed capillary for 16 h at 105 °C. In either case samples of the hydrolysate were taken for TLC on silica gel 60 plates (Merck, Darmstadt, Germany) impregnated with sodium phosphate and using the solvent system acetone/propan-1-ol/0.1 M lactic acid (2:2:1, by vol.) as described by Hansen [33]. After development, the plates were dried, the silica gel scraped off in zones and the radioactivity associated with the deoxyhexoses expressed as a percentage of the total radioactivity recovered from each lane. Chromatography of the hydrolysates in trifluoroacetic acid gave a rather diffuse zone of deoxyhexoses, while the hydrolysates in lactic acid gave clearly resolved spots corresponding to the expected quinovose and fucose.

Sulphoquinovosyldiacylglycerol labelling and analysis

Pea chloroplasts were incubated under conditions described elsewhere [34]. The reaction mixture used in all assays contained 0.33 M sorbitol, 33 mM Tricine, 2 mM $MgCl_2$, 2 mM NaH_2PO_4 and 5 mM dithiothreitol adjusted to pH 7.9 with KOH. For following the synthesis of sulpholipid from uridine diphosphohexoses the basic medium was supplemented with 2 mM ATP, 0.88 mM SO_4^{2-} and 0.1 mM UDP-[^{14}C]Glc or UDP-[^{14}C]Gal (about 75 MBq/mmol) as appropriate. For the synthesis from $^{35}SO_4^{2-}$ it was supplemented with 2 mM UTP and 0.1 mM $^{35}SO_4^{2-}$ (about 40 MBq/mmol). The reaction mixture contained about 120 mg of chloroplasts per ml and was incubated at 25 °C for 30 min.

The reactions were then stopped by extracting the lipids by the method of Garbus et al. [35]. Lipids were separated by TLC on silica gel 60 plates (Merck, Darmstadt, Germany) using chloroform/acetone/methanol/acetic acid/water (10:4:2:3:1, by vol.) as solvent. Lipid bands were revealed by spraying with 0.05% 1-anilino-8-naphthalenesulphonic acid in methanol and viewing under UV light. Radioactivity was detected with a spark chamber autoradiograph (Birchover Instruments, Hitchin, Herts., U.K.) and by exposure to X-ray film. Radiolabelled lipid bands were removed from the TLC plates and radioactivity was counted in a scintillation counter (Packard Emulsifier-Safe scintillant) using an external standard channels ratio method for quench correction. The identity of the radiolabelled sulphoquinovosyldiacylglycerol had previously been confirmed fully by

appropriate chromatographic, chemical and enzymic degradation procedures [34].

Investigation of the water-soluble fraction

In some experiments the aqueous phase remaining after the extraction of lipids (see above) from reaction mixtures with UDP-[^{14}C]Glc was freeze-dried and the residues dissolved in about 250 μl of water. This was applied to a column (4 cm × 0.6 cm) of Dowex-1 × 4(Cl^-) which was then washed sequentially with 6 ml portions of water, 0.01 M HCl, 0.1 M HCl and 1 M HCl; 1 ml fractions were collected. In two experiments the recoveries in the eluates of the applied radioactivity were 93% and 92%. Negligible amounts of radioactivity were eluted with 0.01 M HCl in these two experiments, but sharp peaks containing 44/37%, 23/12% and 26/43% of the total radioactivity were eluted with water (fraction 1; unabsorbed material), 0.1 M HCl and 1 M HCl (fractions 2 and 3 respectively). The appropriate fractions were combined and freeze-dried.

The residues were dissolved in small volumes of water and used for TLC by the method of Hansen [33] (see above). Chromatography was carried out with the untreated extracts and after hydrolysis in sealed capillaries with 0.1 M trifluoroacetic acid for 10 min at 100 °C or with 1 M trifluoroacetic acid for 24 h at 105 °C.

Chemical synthesis of methyl α-glucose-enide

Methyl 6-deoxy-α-D-*xylo*-hex-5-enopyranoside (methyl α-glucose-enide) was prepared essentially as described by Helferich and Himmen [36]. Its purity, assessed by proton magnetic resonance, was found to be > 98%.

RESULTS AND DISCUSSION

Experiments to characterize sulpholipid biosynthesis

A system active *in vitro* was optimized using pea chloroplasts [34]. This preparation catalysed sulpholipid formation at rates of up to 1.3 nmol/h per mg of chlorophyll, which is more than adequate for biosynthesis *in vivo*, and the rate of synthesis remained constant for at least 40 min. The effect of the addition of several reagents to the incubation is shown in Table 1. Cysteic acid, a compound potentially involved in one variation of the sulphoglycolytic pathway, had no effect on the incorporation of radioactivity from $^{35}SO_4^{2-}$. This was in contrast to results with *Euglena* [9] or germinating alfalfa seeds [11], but in keeping with the detailed studies using spinach seedlings [12] or chloroplasts [13]. Furthermore, effective inhibitors of metal-containing aldolase (phosphoglycolohydroxamate) and of enolase (phosphonoacetohydroxamate) had no effect (at 1 mM), whereas they would have been expected to inhibit a sulphoglycolytic pathway.

Table 1 Effects of compounds potentially interacting with a sulphoglycolytic pathway on sulphoquinovosyldiacylglycerol labelling from $^{35}SO_4^{2-}$ in intact pea chloroplasts

Results are shown for duplicate determinations and each column represents a separate experiment. Incubation conditions and analysis of sulphoquinovosyldiacylglycerol (SQDG) are described in the Materials and methods section.

Addition	SQDG synthesis (nmol/h per mg of chlorophyll)		
	Expt. 1	Expt. 2	Expt. 3
None (control)	0.65, 0.72	0.46, 0.54	1.18, 1.02
Cysteic acid (1 mM)	0.69, 0.73	0.44, 0.50	–
Phosphoglycolohydroxamate (1 mM)	–	–	1.08, 0.96
Phosphonoacetohydroxamate (1 mM)	–	–	1.18, 0.98

Table 2 Effect of UDP-glucose or UDP-galactose on the synthesis of sulphoquinovosyldiacylglycerol (SQDG) by intact pea chloroplasts

Results show means of duplicate determinations for individual experiments. The concentration of the nucleotide was 0.1 mM in each case. For other details see Table 1.

Addition	SQDG synthesis (nmol/h per mg of chlorophyll)				
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5
None	1.30	0.51	0.79	0.99	1.25
UDP-Glc	1.48	0.59	1.00	1.68	–
HDP-Gal	0.82	0.33	–	–	0.74

According to our proposed pathway (Scheme 1), either UDP-glucose or UDP-galactose could have been expected to be precursors of the UDP-sulphoquinovose intermediate, shown by Seifert and Heinz [30] to be utilized. Indeed, we had shown previously [23], in agreement with others (e.g. Kleppinger-Sparace et al. [13]), that UTP could stimulate sulpholipid synthesis by intact chloroplasts. Therefore we tested the effect of UDP-glucose and UDP-galactose addition (Table 2). UDP-glucose caused a small but significant increase in sulpholipid labelling, consistent with a role in the latter's biosynthetic pathway. With UDP-galactose a marked inhibition was seen. At first sight, this might appear inconsistent with the pathway shown in Scheme 1. However, UDP-galactose is used for galactolipid synthesis by chloroplasts [37], and this reaction will compete with sulpholipid formation for the diacylglycerol which is the substrate for the final stage of the reaction. Thus an inhibition of sulpholipid labelling following the addition of UDP-galactose can be explained by competition for the diacylglycerol pool and has been observed before in experiments with spinach chloroplasts [38]. It is also possible that this competition for diacylglycerol limits any stimulation by UDP-glucose of sulpholipid labelling, since endogenous epimerase activity converts UDP-glucose to UDP-galactose at significant rates (results not shown).

In order to see whether competition for diacylglycerol by the sulpholipid versus the galactolipid pathway could explain the UDP-galactose results, we carried out further experiments. The simplest experiment, that is the direct addition of exogenous diacylglycerol, was not attempted because numerous previous examples with plant membrane fractions have shown that this method seldom gives reliable or effective substrate presentation. Instead, we choose to generate additional diacylglycerol *in situ* by the use of limited phospholipase C action (Table 3). The amount of phospholipase C was chosen so that it would be expected to cause less than 30% hydrolysis of the envelope phosphoglycerides during the time course of the incubation, in order to prevent possible enzyme inhibition caused by membrane digestion. Although sulpholipid labelling was not restored fully to control levels, phospholipase C did alleviate the inhibitory action of UDP-galactose addition. This was in keeping with the idea that UDP-galactose channels diacylglycerol into galactolipid synthesis, thus decreasing the amount available for sulpholipid formation. We also attempted to increase diacylglycerol availability by stimulation of the Kennedy pathway [37,39] with acyl-

Table 3 Effect of reagents which could alter the diacylglycerol supply on the synthesis of sulphoquinovosyldiacylglycerol (SQDG) from $^{35}SO_4^{2-}$ in intact pea chloroplasts

All experiments were carried out in duplicate. The concentration of UDP-Gal was 0.1 mM. Abbreviations: PLC, *Bacillus cereus* phospholipase C (at 0.32 μg/ml); acyl-CoAs, palmitoyl-CoA and oleoyl-CoA (50 μM); G3P, glycerol 3-phosphate (0.1 mM).

Additions	SQDG synthesis (nmol/h per mg of chlorophyll)			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
None (control)	1.27, 1.39	1.34, 1.41	1.26, 1.65	1.25, 1.25
UDP-Gal	0.75, 0.84	0.86, 0.88	0.87, 1.00	–
UDP-Gal + PLC	1.33, 1.20	–	–	–
Acyl-CoAs + G3P	–	–	–	1.30, 1.40

Table 4 Influence of UDP-galactose and phospholipase C on the synthesis of sulphoquinovosyldiacylglycerol (SQDG) from $^{35}SO_4^{2-}$ by broken pea chloroplasts

Results are means ± S.D. for three experiments. Phospholipase C (PLC) was used at 0.32 μg/ml.

Addition	SQDG synthesis (nmol/h per mg of chlorophyll)
None (control)	1.23 ± 0.06
UDP-Gal (0.1 mM)	0.72 ± 0.02
UDP-Gal (0.1 mM) + PLC	1.17 ± 0.05

CoAs and glycerol 3-phosphate. No significant stimulation of sulpholipid labelling was seen (Table 3). This may have been due to the fact that the chloroplast-localized Kennedy pathway has distinct characteristics [40] and, therefore, exogenous acyl-CoAs cannot contribute to the synthetic route. Alternatively, sulpholipid labelling may be limited by factors other than the diacylglycerol concentrations in intact chloroplasts. In agreement with the latter suggestion we found that phospholipase C addition alone could not stimulate sulpholipid labelling from $^{35}SO_4^{2-}$ (results not shown), suggesting that diacylglycerol supply only became limiting if some were diverted into galactolipid formation.

The Percoll-purified chloroplasts that we used were substantially intact initially (see the Materials and methods section), but during the incubations breakage occurred. Because of this and the inability of intact chloroplasts to accept some exogenous intermediates of sulpholipid synthesis (e.g. UDP-sulphoquinovose [30]) we were interested to see if broken chloroplast preparations could catalyse good rates of sulpholipid labelling from $^{35}SO_4^{2-}$. Chloroplasts were damaged by freeze/thaw and their breakage was confirmed by refractive light microscopy. Such preparations were found to label sulpholipid at rates that were substantially the same as those for intact preparations (Table 4). These broken chloroplast preparations showed similar characteristics to the intact organelles, and the decrease in sulpholipid labelling with UDP-galactose together with the almost complete alleviation of this decrease by phospholipase C is illustrated in Table 4. The ability of broken pea chloroplasts to label sulpholipid at good rates (compare Tables 3 and 4) means that these preparations are potentially very useful for studies of the biosynthetic pathway.

Thermolysin has been used to inhibit the galactolipid:galactolipid galactosyltransferase, one of the key enzymes

Table 5 Effect of thermolysin on the incorporation of radioactivity from UDP-[^{14}C]glucose into glycosylglycerides by intact pea chloroplasts

Incubations were carried out as described in the Materials and methods section. Thermolysin (200 μg/ml) was used at 4 °C for a pre-treatment of 60 min as described by Douce et al. [43]. The results are means ± S.D. for a representative experiment performed in triplicate. Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; SQDG, sulphoquinovosyldiacylglycerol.

	Glycosylglyceride labelling (d.p.m.)		
	MGDG	DGDG	SQDG
Control	8976 ± 134	2260 ± 30	794 ± 94
+ Thermolysin	5608 ± 68	1338 ± 16	876 ± 80

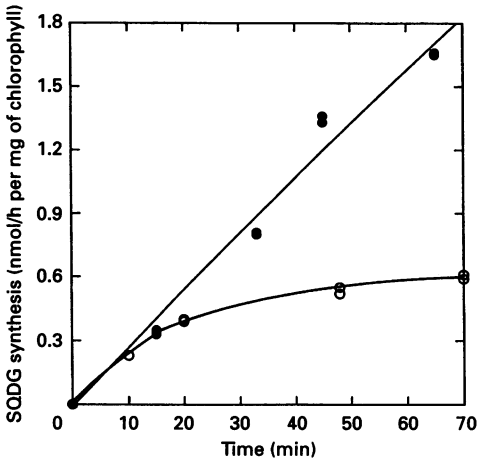

Figure 1 Time course of the labelling of sulphoquinovosyldiacylglycerol (SQDG) by pea chloroplasts

The standard conditions of assay were used (see the Materials and methods section). The concentrations of UDP-[^{14}C]glucose (●) and UDP-[^{14}C]galactose (○) were 0.1 mM and their specific radioactivities were identical.

of galactolipid formation [39], which also gives rise to much of the diacylglycerol pool in chloroplast envelopes. We used this proteinase in order to try to adjust the relative rates of sulpholipid and galactolipid formation. Thermolysin had no significant effect either on $^{35}SO_4^{2-}$ incorporation or on the incorporation of radioactivity from UDP-[^{14}C]glucose into sulpholipid (Table 5). With the latter precursor, thermolysin decreased galactolipid labelling, as expected, but any consequent changes in diacylglycerol availability clearly had no effect on sulpholipid biosynthesis. This result was consistent with the conclusions above that, in our experiments, sulpholipid labelling was not normally limited by diacylglycerol availability.

The effective incorporation of radioactivity from UDP-[^{14}C]glucose into sulpholipids (Table 5) also shows the ability of chloroplasts to use this precursor. Incorporation of radioactivity was approximately linear for 65 min in the normal assay system (Figure 1). Radioactivity was also incorporated from UDP-[^{14}C]galactose. Initially, labelling from the latter was at a similar rate to that from UDP-[^{14}C]glucose (Figure 1), but incorporation rapidly fell with time, as expected because of the competition for this substrate from rapid galactosylglyceride biosynthesis (as discussed above). Thus both precursors were effective for sulpho-

Table 6 Effect of methyl α-glucose-enide on the synthesis of sulphoquinovosyldiacylglycerol (SQDG) from $^{35}SO_4^{2-}$ by preparations of pea chloroplasts

The results are means ± S.D. (where appropriate) with the numbers of independent experiments in parentheses. Intact chloroplasts were > 85% intact at the beginning of the incubations and approx. 60% intact at the end (estimated by phase-contrast microscopy). For broken chloroplasts, it was found that the maximum stimulation was caused by 100 μM methyl α-glucose-enide (results not shown). Results for broken chloroplasts show means of an experiment performed in duplicate.

	SQDG synthesis (nmol/h per mg of chlorophyll)
Intact chloroplasts	
Control	1.75 ± 0.08 (3)
+ Glucose-enide (1 mM)	2.42 ± 0.15 (4)
Broken chloroplasts	
Control	0.99 (1)
+ Glucose-enide (10 μM)	1.17 (1)
+ Glucose-enide (100 μM)	2.08 (1)

Table 7 Effect of the synthesis in the reaction mixture of UDP-6-deoxy-4-ketoglucose on the labelling of sulphoquinovosyldiacylglycerol (SQDG) formed from UDP-[^{14}C]glucose by intact pea chloroplast preparations

Two preparations capable of producing UDP-6-deoxy-4-ketoglucose were obtained as described in the Materials and methods section. These were added to the standard chloroplast incubation system and the reaction mixture was treated as usual. The results are given as means ± S.D. for expt. 1, where $n = 4$. For expt. 2, which was in duplicate, the individual results are shown.

	SQDG labelling	
	(d.p.m.)	(% of control)
Expt. 1		
Control	17184 ± 526	–
+ 6 units of enzyme	27323 ± 313	159
Expt. 2		
Control	2711, 3029	–
+ 4 units of enzyme	3713, 4163	137
+ 12 units of enzyme	4056, 4656	152

lipid formation but, because of the competition for UDP-galactose by galactolipid formation in the chloroplasts, our further experiments used UDP-[^{14}C]glucose as precursor.

Optimal rates of sulpholipid labelling from UDP-[^{14}C]glucose were obtained in the absence of UTP. In addition, [^{14}C]glucose itself was a poor precursor of labelled sulpholipid (results not shown). These results are in keeping with UDP-glucose (and/or UDP-galactose) being a more direct precursor for sulpholipid formation.

That the chloroplast system was capable of the interconversion of glucose and galactose, presumably in the form of their UDP derivatives, was shown by the investigation of the water-soluble fraction of the reaction mixtures. The chromatographs obtained from the three fractions (see the Materials and methods section) were complex; at least 18 compounds containing ^{14}C were resolved: three, nine and six in fractions 1, 2 and 3 respectively. Of these, only two could be identified with any certainty: glucose and galactose were present, in approximately equal amounts (2% of the total radioactivity), in fraction 2. These carbohydrates would not be absorbed by Dowex-1 and therefore should have appeared in fraction 1. However, it was shown that extensive hydrolysis of UDP-Glc occurred during freeze-drying of a solution of this nucleotide in 0.1 M HCl, so that the appearance of glucose in fraction 2 probably reflects the elution therein of UDP-Glc. A similar origin may be accepted for galactose. The results therefore show the interconversion of UDP-Glc and UDP-Gal during the incubation of the former with the chloroplast preparation, presumably through the action of epimerase. The approximately equal concentrations of the two sugars show, however, that there is insufficient epimerase to maintain their reported equilibrium concentrations, which gives an approximate 3:1 ratio in the direction of UDP-Glc [41].

Hydrolysis of the eluates in 0.1 M trifluoroacetic acid for 10 min made little difference to any of the chromatograms, apart from a 2-fold increase in the amounts of glucose and galactose, without any change in the ratio of their concentrations, in fraction 2. Hydrolysis in 1 M trifluoroacetic acid for 24 h caused extensive changes in the chromatograms. Again, the only identifiable compounds were glucose and galactose (60% and 10% of the total radioactivity respectively) in fraction 2. The precursor(s) of these sugars is/are unknown.

In the pathway proposed (Scheme 1), we envisage addition of sulphite to a glucose-enide derivative and, as discussed above, there is evidence for the use of sulphite in sulpholipid biosynthesis [11,16]. Accordingly, we synthesized methyl α-glucose-enide (see the Materials and methods section) and tested it directly for an effect on sulpholipid labelling. The choice of this derivative was made because of the instability of the hypothetical free glucose-5-ene which rapidly tautomerizes, at least in acid solution, to the corresponding ketone, 6-deoxy-D-*xylo*-hex-5-ulose [36]. Nevertheless, the methyl group must be removed at some stage in the utilization of the glycoside in order to free the oxygen at the anomeric carbon to allow formation of the purported nucleotide.

Exogenous methyl α-glucose-enide was found to stimulate significantly the labelling of sulphoquinovosyldiacylglycerol formed from $^{35}SO_4^{2-}$ (Table 6), as expected if such a compound was an intermediate (or could generate an intermediate) in the biosynthetic pathway. These experiments were carried out with both intact and broken chloroplast preparations (Table 6). Similar data were obtained with each. For intact chloroplasts, methyl α-glucose-enide also caused an increase in the uptake of the precursor $^{35}SO_4^{2-}$ (albeit to a smaller extent than the stimulation of sulpholipid labelling) but, clearly, this could not explain the stimulation of sulpholipid labelling, since a similar increase in the latter was seen in broken chloroplasts. The results are consistent with the participation of methyl α-glucose-enide or a metabolic derivative thereof in the biosynthetic pathway to sulphoquinovosyldiacylglycerol.

Because our proposed pathway has features in common with biosynthetic routes to 6-deoxyhexoses, the effect was investigated of adding to the chloroplast system a crude enzyme preparation capable of carrying out these reactions (see the Materials and methods section), but under conditions causing the accumulation of UDP-6-deoxy-4-ketoglucose and, therefore, the formation of UDP-4-ketoglucose-5-ene as an intermediate. This caused a consistent increase in sulpholipid labelling (Table 7). Although we cannot exclude the possibility of other non-specific effects, these results are consistent with the concept that intermediates generated by the rhamnose-synthesizing system, such as UDP-4-ketoglucose or UDP-6-deoxy-4-ketoglucose-5-ene, could be used for sulpholipid biosynthesis.

Concluding remarks on the new pathway

The results we have described here are all consistent with the new pathway as depicted in Scheme 1. No data that we have yet

obtained with pea chloroplasts are inconsistent with the pathway and, together with the previous data discussed above, they provide a firm foundation for the proposal.

Interconversion of UDP-glucose and UDP-galactose is catalysed by UDP-galactose 4-epimerase, a reaction having a 4-keto intermediate suitable for use in sulpholipid biosynthesis. However, it is known that the intermediates of the epimerase reaction are very tightly bound to the enzyme, because the proton abstracted from glucose reduces an enzyme-bound NAD^+ molecule and is subsequently used quantitatively in the formation of galactose; exchange with the medium does not occur. Thus we do not think it likely that the keto intermediate in sulpholipid synthesis is formed by an epimerase. The sequence analogy for a gene involved in sulpholipid synthesis in *Rhodobacter* with the epimerase [22] could relate to a role of an epimerase in providing, for example, UDP-glucose or, more likely, to a similar binding site in another enzyme using UDP-derivatives and, perhaps, forming similar intermediates. Thus UDP-4-ketoglucose is an intermediate in the reaction of UDP-glucose-5,6-hydrolyase involved in the formation of UDP-rhamnose. Such an enzyme would be expected to show structural similarities to UDP-galactose 4-epimerase.

Whether or not an epimerase is directly involved in sulpholipid synthesis, its presence in our chloroplast fractions is of advantage in allowing both UDP-[^{14}C]glucose and UDP-[^{14}C]galacose to be used for sulpholipid labelling.

Conversion of UDP-4-ketoglucose to a 4-keto-5-ene intermediate occurs in, for example, fucose and rhamnose formation [42]. Addition of an enzyme system catalysing this reaction stimulated sulpholipid labelling (Table 7), consistent with this part of the proposed pathway. Instead of the UDP-4-ketoglucose-5-ene being, for example, reduced by hydrogen to form a 6-deoxyhexose (as in rhamnose synthesis), attack by sulphite allows a sulphonate residue to be introduced. This type of reaction was suggested by Barber [24], occurs readily *in vitro* [16] and is consistent with the involvement of sulphite [11] and with the stimulation by methyl α-glucose-enide (Table 6) of sulpholipid synthesis. Reduction would then generate UDP-sulphoquinovose, which is the substrate for the final reaction [30]. A detailed mechanism for the incorporation of sulphite (or its metabolic equivalent) cannot be given, but the results in Table 7 might suggest the operation of the second of the two alternative pathways mentioned at the end of the Introduction section, i.e. the formation of UDP-glucose-5-ene as a free intermediate, because it is difficult to envisage the entry of an enzyme-bound nucleotide into the chloroplast.

Our new pathway is relatively simple and only contains reactions for which there are estabished precedents. Available evidence from other laboratories, together with the present results, are consistent with its operation. However, final proof will need to be obtained by the isolation and/or measurement of the individual enzymes concerned.

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