THE SYNTHESIS OF GALACTOSYLDIACYLGLYCEROLS BY CHLOROPLAST ENVELOPES

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1. Introduction

The first model for the biosynthesis of galactolipids in higher plants was proposed [1] following the scheme:

UDPGal + diacylglycerol→monogalactosyldiacylglycerol + UDP (I)

UDPGal + monogalactosyldiacylglycerol→ digalactosyldiacylglycerol + UDP (II)

In contrast with the proposed reaction II it was shown in our laboratory that there are 2 distinct enzymic activities in galactolipid formation:
(1) The coupling of galactose to diacylglycerol and
(2) The intergalactolipid galactosyltransfer, which does not need any external cofactors and proceeds probably according to the scheme:

2 monogalactosyldiacylglycerol→ digalactosyldiacylglycerol + diacylglycerol (III)

The diacylglycerol may then be galatosylated again according to (I). Since reaction (III) proceeds only after some monogalactolipid has been formed, it is possible with short-time incubations to investigate reaction (I) separately. In these short-time incubations envelope preparations incorporate UDPGal according to the Michaelis-Menten theories [2]. Therefore we decided to examine reaction (I) further in order to investigate the reaction mechanism and, at the same time, to find more evidence in support of our theory with regard to reaction (III). A useful tool in studying the incorporation of galactose from UDPGal into galactolipids is NMR, because, apart from the information from the spectra, this method permits chemical analysis of the incubation mixture afterwards.

2. Materials and methods

2.1. *Materials*

UDP[^{3}H]Gal and UDP[^{14}C]Gal were obtained from the Radiochemical Centre, Amersham. UDPGal (Sigma grade) was obtained from the Sigma Chemical Co. Nucleotides, uridine, uracil, UDPG, galactose-1-P, and phospholipase C were obtained from Boehringer, Mannheim. [^{14}C]Dioleoyllecithin was a gift from Dr E. van Zoelen. All other reagents were from Merck, Darmstadt.

2.2. *Isolation procedure*

Chloroplast envelopes were isolated according to [3] with only minor differences. Contamination of envelopes by thylakoids was measured with a Beckman spectrophotometer model 25 after extraction with ethanol [4].

2.3. *Reaction mixtures*

The reaction mixture contained typically 100 μg/ml of envelope proteins, measured according to [5], 0.2 mM UDPGal, (2.5×10^5 cpm if ^{3}H-labelled and 10^5 cpm if ^{14}C-labelled), 0.1 M tricine/KOH buffer (pH 7.2) and 10 mM $MgCl_2$, all final concentrations. For NMR the mixture was altered as follows: 2 mg/ml of envelope proteins, 10 mM UDPGal, 1 mM EDTA and 50% D_2O, all final concentrations. Incubations, at 30°C, were started by addition of envelope material and stopped by addi-

tion of 3 vol methanol For pulse labelling experiments the first incubation was stopped by centrifugation of the reaction mixture in a Beckman Airfuge for 15 min at 10^6 m/s^2 The pellet was resuspended in incubation buffer by light sonification

2 4 *NMR*

Spectra were made with a Varian XL 100 operated at ^{31}P FT mode at 40 5 MHz (proton decoupled) with at least 3000 accumulations/spectrum

2 5 *Chromatography and quantification of label*

Lipids were extracted according to [6], dried and counted in a Philips PW 4540 LSA with Lipoluma (Baker) Lipids were separated by thin-layer chromatography (Kieselgel 60 plates, Merck) with $CHCl_3$ CH_3OH H_2O (65 25 4, v/v/v) Spots with radioactivity, identified with Kodirex X-ray film (Kodak), were scraped off and counted as mentioned

3 Results and discussion

^{31}P NMR spectra of UDPGal, UDP and the incubated mixture showed that during the incubation UDP is formed (fig 1) The quantities of UDP formed agree very well with the lipid incorporation of galactose from UDP[^{14}C]Gal When kept for a few days at room temperature in the absence of envelopes, part of the UDPGal is split into UMP and Gal-1-P, but no UDP is detectable from the NMR spectra So UDP is the result of enzymic activity in envelopes These results support scheme (I) More detailed information was obtained from the effect of several inhibitors on this reaction

UDP was found to be a competitive inhibitor, when the steady state velocities were plotted according to Lineweaver and Burk (fig 2) [7] The K_I of UDP was calculated from fig 2 to be 10 μM In the same experiment the K_m of UDPGal was calculated to be ~45 μM This suggested that UDP could be an important moiety of the substrate molecule for recognition by the enzyme This idea was strengthened by the results of tests with other effectors resembling UDPGal

UDPG, UMP and UTP were also competitive inhibitors but less effective than UDP (fig 3) For UDPG and UMP K_I was calculated to be 100 μM The

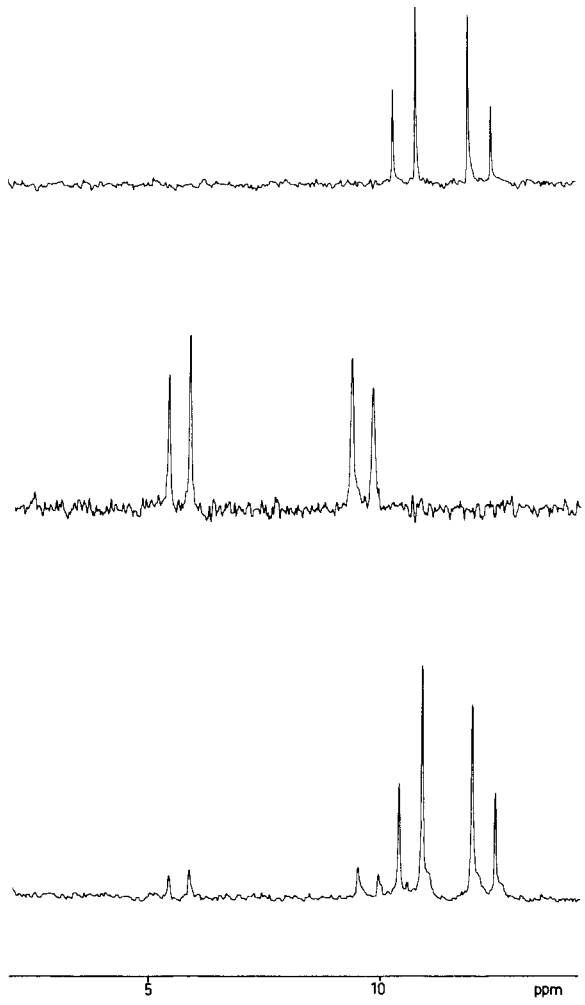


Fig 1 ^{31}P NMR spectra of UDPGal (top), UDP (centre) and incubation mixture (bottom) The NMR spectrum of the incubation mixture was taken after 1 h incubation of 0 8 mg chloroplast envelopes with 10 mM UDPGal at 30°C in 0 4 ml The mixture was cooled to 4°C and measured for 1 h Reference spectra were made from 10 mM UDPGal and 10 mM UDP All spectra were made in the presence of 50% D_2O Chemical shifts are reported relative to 30% H_3PO_4 in D_2O The proton decoupled ^{31}P NMR spectrum of UDPGal shows two doublets arising from the two homonuclear-coupled phosphorus nuclei The galactose-linked phosphorous nucleus resonates at 12 2 ppm (upfield) and shifts to 5 6 ppm upon hydrolysis of the ester bond The shift of the ribose bound phosphorus is much smaller, from 10 6–9 7 ppm Since the peak area changes approximately with the amount of nuclei present, it is possible to calculate from the spectrum of the incubation mixture the amount of UDP generated during the incubation

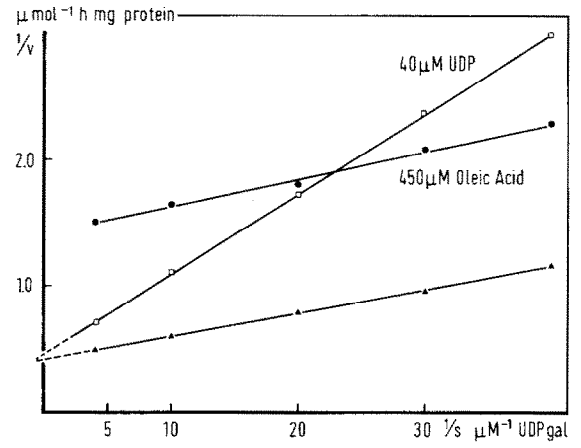


Fig.2. Inhibition of galactose incorporation by UDP and oleic acid. On the ordinate the reciprocal reaction velocity is plotted; on the abscissa reciprocal values of UDPGal concentrations are given for a reference incubation (▲), an incubation with 40 µM UDP (□) and an incubation in the presence of 450 µM oleci acid (●). The oleic acid was added in 1 µl alcoholic solution. The reaction was started by addition of 25 µg envelope proteins to 250 µl final vol. Incubation: 5 min at 30°C, pH 7.2.

inhibition by UTP was mainly due to traces of UDP. When envelopes were incubated with UDP[^{14}C]G, no radioactivity was incorporated into galactolipids, demonstrating the absolute specificity for galactose in

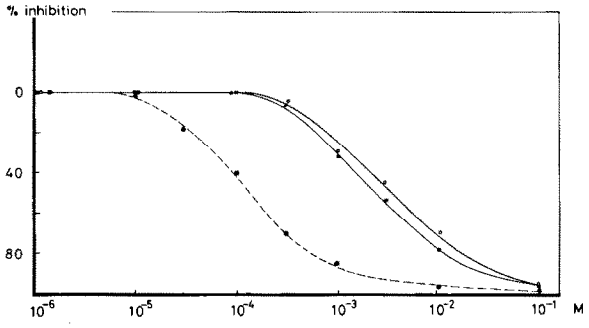
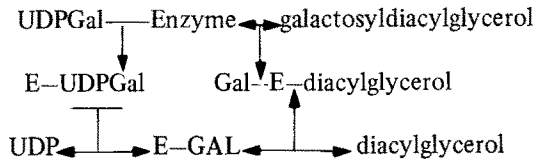


Fig.3. Dose–effect curves of uridine nucleotides. The abscissa indicates the concentration of UMP or UDPG (■), UDP (●) and UTP (○). Envelope material was incubated with 0.2 mM UDPGal, plus the indicated concentrations of effector, for 5 min at 30°C, pH 7.2. 50% Inhibition required a concentration of either 1.5×10^{-4} M UDP, 2.5×10^{-3} M UMP or UDPG or 4×10^{-3} M UTP.

the transfer reaction and the absence of epimerase activity. Uridine and uracil were without any effect.

The galactose part of the substrate molecule was also tested with analogues. Galactose, galactose-1-P and glucose-1-P were without effect, so in conclusion it appears very likely that the enzyme recognises UDPGal at its UDP moiety. Oleic acid proved to be an uncompetitive inhibitor (fig.2). This inhibition could be overcome by addition of albumin [8]. Independent of the quantity of fatty acids added, the albumin enhancement showed a broad optimum, its concentrations varying from 0.5–10 mg/ml. This effect could be produced to a lesser extent by prewashing the envelopes with albumin containing tricine buffer (pH 7.2). In both cases albumin produced an uncompetitive enhancement, suggesting the presence of free fatty acids in isolated envelopes. By gas–liquid chromatographic analysis we indeed found significant amounts of free fatty acids totalling ~4% of dry lipid weight. In a typical experiment the K_i for oleic acid was calculated to be 70 µM. After washing of the membranes for 20 min in a 2.5 mg/ml albumin containing tricine buffer, the K_i for oleic acid decreased to 15 µM. This low value suggests that fatty acids do not inhibit by a general effect on the membrane but interact specifically with an enzymic site. The fact of uncompetitive inhibition means that the fatty acids interact with the enzyme only, after the latter has combined with UDPGal.

A simple and elegant model which can amount for the inhibitory effect of oleic acid is a two step galactosylation reaction in which free fatty acids compete with diacylglycerol for the same binding site of the enzyme. Because diacylglycerol reacts with the enzyme–galactose complex, the competitive inhibition of oleic acid in the second reaction step is at the same time an allosteric inhibition on the enzyme–galactose complex with respect to the reaction with UDPGal. The model corresponding to the behaviour mentioned above is a double displacement or ping–pong reaction according to the following scheme [7]:



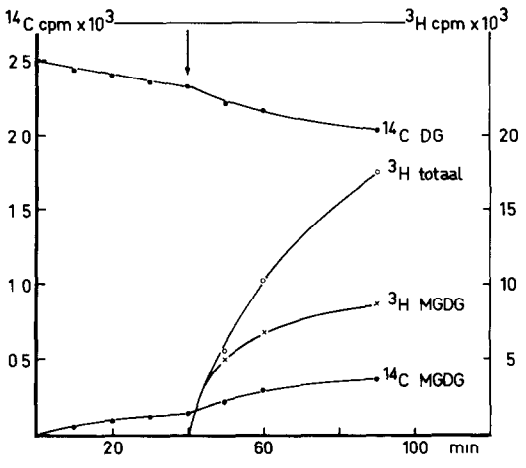


Fig 4 Incorporation of dioleoylglycerol into MGDG ^{14}C-labelled lecithin was sonified with chloroplast envelopes in 0 1 M tricine–KOH buffer (pH 7 2) in the presence of 20 mM $MgCl_2$ At time zero 0 8 I U phospholipase C was added and the mixture was incubated at 30°C After 40 min UDP[^{3}H]-Gal was added *Abbreviations* DG, *sn*-1,2 diacylglycerol, MGDG, monogalactosyldiacylglycerol

The enzyme reacts with UDPGal to form a covalently bound enzyme–galactose complex which in turn reacts with diacylglycerol to yield galactolipid and the original enzyme The two substrates react subsequently with the enzyme, UDPGal being the leading substrate

Besides competitive and uncompetitive inhibition a necessary condition to identify a ping–pong mechanism is the equilibrium exchange [7] In the model described above this means that, if monogalactolipid is labelled in its glycerol or fatty acid moiety, this label should appear in the diacylglycerol fraction and vice versa when envelopes are incubated in the absence of UDPGal This could indeed be demonstrated (fig 4) Di[^{14}C]oleoyllecithin was dried with N_2 and, after addition of the envelope suspension, the mixture was sonified for 30 s The lecithin was now degraded to diacylglycerol by phospholipase C, which did not influence the incorporation in reference incubations At various times aliquots were taken from the incubation mixture and analysed After 40 min UDP[^{3}H]Gal was added and the incorporation was measured similarly From fig 4 it is clear that

(i) An exchange of diacylglycerol molecules occurs in the absence of UDPGal
(ii) The incorporation of ^{14}C-label into monogalactolipid increases upon addition of UDPGal
(iii) The incorporation of ^{3}H-label demonstrates that in this respect it proceeds normally [2]

The same equilibrium exchange has been observed [9] in experiments in which galactolipid synthesis from [^{14}C]glycerol-3-P and acylCoA was studied in envelopes, although at the time the authors had no explanation for this shift of label

In this context we may recall the report [10] of an incorporation of labelled fatty acyl groups into monogalactolipid but not into digalactolipid, by cell-free extracts from *Euglena gracilis* supplied with fatty acyl thioesters and glycerol-3-P, but in the absence of UDPGal It is possible to explain these results as an equilibrium exchange between monogalactolipid and diacylglycerol, similar to that reported above The synthesis of diacylglycerol from glycerol-3-P and fatty acyl thioesters was already considered as a possibility [10] and has been observed in spinach envelopes [9] A stimulating effect of high concentrations of UDPGal was also reported [10] This may be compared to the experimental results of fig 4 supporting the double displacement reaction mentioned above

After the synthesis of monogalactolipid the galactose can be transferred from one galactolipid molecule to another in the absence of UDPGal [2] by a different enzyme [2,3,11] In pulse labelling experiments the influence of various effectors on this interlipid galactosyltransferase was investigated during the chase Neither UDP nor oleic acid altered the rate of transfer of label from monogalactolipid to higher homologues These data form additional evidence for the hypothesis that the galactosylation of monogalactolipid does not involve UDPGal and that in isolated envelopes digalactolipid is formed by dismutation of monogalactolipid according to (III)

Experiments [12] with several cell fractions from *Euglena gracilis*, seem to indicate the possibility of direct biosynthesis of digalactolipid by galactosyl-transfer from UDPGal Confirming [13] they observed a very rapid incorporation of label from UDPGal into both mono- and digalactolipid Both papers also show consistently higher activity in digalactolipid, after incubations from 2–90 min, with

no or only a faint decline in the ratio of mono- over digalactolipid, which is characteristic of all our experiments with spinach [2]. We may point to some differences in the *Euglena* experiments and those with spinach. *Euglena* chloroplast envelopes contain only 5% of the diacylglycerol found in spinach envelopes [3,12]. UDPGal was given to the *Euglena* chloroplasts [12] in very low concentrations, typically 0.17 nM, whereas the K_m was calculated to be 14 μM. Maximum incorporation in *Euglena* was quite low on a protein basis and amounted only to a modest percentage of the UDPGal offered. Future work will be required in order to establish whether different mechanisms for biosynthesis occur in *Euglena* and spinach or whether present discrepancies can be reconciled.

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