Lipid Labelling in Intact Chloroplasts from Exogenous Nucleotide Precursors

Margrit Bertrams, Käthe Wrage, and Ernst Heinz

Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln 41

De novo-synthesis of glycerolipids in chloroplasts is initiated by a stroma enzyme which catalyzes the formation of lyso-phosphatidic acid from glycerophosphate and acyl-CoA. When these substrates are added to isolated, intact chloroplasts, only glycerophosphate can readily pass through the chloroplast envelope which represents a permeation barrier for acyl-CoA, although higher thioester concentrations destroy this membrane system. At low concentrations of acyl-CoA, which do not impair the envelope, intact chloroplasts metabolize exogenous acyl-CoA in two ways to give free fatty acids and labelled phosphatidyl choline. This indicates that the envelope thioesterase can use exogenous substrates.

Isolated, intact chloroplasts fixing radioactive CO_2 label free fatty acids and acylglycerols but not galactolipids, since they cannot convert 3-phosphoglycerate into UDP-galactose which *in vivo* is supplied by the cytoplasm. This cooperation was simulated *in vitro* by adding all enzymes and cofactors necessary for conversion of 3-phosphoglycerate into UDP-galactose to intact chloroplasts which then formed labelled monogalactosyl diacylglycerol from labelled CO_2.

The time required to transfer envelope-made galactolipids from the envelope into thylakoids was studied by incubating intact chloroplasts with radioactive UDP-galactose, subsequent osmotic disruption of organelles with concomitant enzymatic degradation of UDP-galactose followed by separation of envelopes and thylakoids. Only after short times (< 1 min) appreciable proportions (20 – 30%) of radioactive galactolipids were recovered in the envelope fraction. This in combination with previous results indicates a rapid galactolipid export from envelopes into thylakoids.

Introduction

Chloroplasts contain two different membrane systems, a yellow envelope and green thylakoids. Apart from pigments both membrane systems show only minor differences in the composition of lipids among which galactolipids predominate. Available evidence suggests that a whole series of enzymes catalyzing many steps in the final assembly of these lipids from water-soluble and membrane-bound precursors is concentrated in the envelope [1]. This localization does not require entrance of cytoplasmatically made UDP-galactose [2] into the chloroplast interior but necessitates galactolipid export into thylakoids which have no or very little galactolipid-forming activity [3]. *In vivo* labelling experiments intended to demonstrate formation of galactolipids in the envelope and subsequent transfer into thylakoids showed that the transport process was unexpectedly fast, at least in relation to the time scale of these experiments [4]. Although the shortest labelling time was 5 min, additional time was required to isolate and purify chloroplasts, before they could be separated into envelopes and thylakoids for the actual interruption of the transport process. In continuation of these investigations we now incubated isolated, intact chloroplasts with radioactive UDP-galactose and analyzed the distribution of galactolipids between envelopes and thylakoids after further reduction of incubation times to find out the time required for this transport process. In addition we were also interested in the question, whether intact chloroplasts can use exogenous acyl-CoA for lipid synthesis which starts in the chloroplast stroma by acylation of glycerol phosphate [5, 6]. This question is relevant in view of the possibility that not only galactose but also acyl residues of chloroplast galactolipids may be derived from extraplastidic sources (various possibilities summarized in ref. [1]).

Materials and Methods

General

Spinacia oleracea L. cv. "vital" was grown in the garden, *Pisum sativum* L. cv. "Kleine Rheinländerin" and *Vicia faba* L. cv. "Diana" in the greenhouse.

Percoll was obtained from Pharmacia, Uppsala, Sweden, betain and precoated Kieselgel G plates from Merck, Darmstadt, Germany. [1-^{14}C]palmitoyl-CoA (59 µCi/µmol) and [1-^{14}C]oleoyl-CoA (48 µCi/µmol) was purchased from NEN, Dreieichenhain, Germany; NaH^{14}CO$_3$ (50 µCi/µmol), UDP-[U-^{14}C]galactose (320 µCi/µmol) and UDP-[6-^{3}H]galactose (10 mCi/µmol) from the Radiochemical Centre, Amersham, England. Intact chloroplasts were isolated on isotonic density gradients made up with Percoll in resuspension medium as recently described [7]. For the isolation of spinach chloroplasts sorbitol in the media was replaced by betain as osmoticum [8]. The isolation medium contained betain (0.33 M), Na$_4$P$_2$O$_7$ (10 mM) and MgCl$_2$ (5 mM) the pH being adjusted with HCl to pH 7.6. The resuspension medium contained betain (0.33 M), Hepes (50 mM), EDTA (2 mM), MgCl$_2$ (1 mM) and MnCl$_2$ (1 mM) the pH being adjusted to 7.6 with KOH. After recovery from gradients chloroplasts were washed three times by sedimentation (90 sec at 1400×g) from resuspension medium to remove residual Percoll. Organelle integrity was routinely checked by their inability to use exogenous ferricyanide as Hill reagent for oxygen evolution [9]. Envelopes and thylakoids were separated by osmotic shock in swelling medium (10 mM Tricine pH 7.6 and 2 mM MgCl$_2$) and isolated by centrifugation on stepped sucrose gradients [10]. Details of lipid analysis such as extraction, washing, chromatography, hydrolysis and radioactivity counting have repeatedly been described before [11].

Incubation of intact chloroplasts with [^{14}C]acyl-CoA

A final volume of 0.3 ml isotonic resuspension medium contained the following components: intact chloroplasts equivalent to 150 µg of chlorophyll, *sn*-glycerol-3-phosphate (1 mM) and [1-^{14}C]acyl-CoA (3×10^5 dpm=7.5 µM of either palmitoyl-CoA or oleoyl-CoA or a 1:1-mixture of both). After incubation for 25 min at 24 °C in the dark, resuspension medium (2 ml) was added, the mixture placed on a Percoll gradient and centrifuged for 20 min at 3300×g to separate intact chloroplasts from any thylakoids which could have been released by organelle breakage during the incubation. Usually there was only very little organelle desintegration. The intact chloroplasts were removed from the gradient and washed twice in resuspension medium. These mani-

pulations resulted in a loss of 10−20% of the originally incubated chloroplasts. The final chloroplast pellet was extracted with CHCl$_3$/MeOH (1/1, 4 ml) and washed with acidified salt solution (1 M KCl in 0.2 M H$_3$PO$_4$ according to ref. [12], 2 ml). Phase separation was achieved by short centrifugation and the lipid phase, after solvent removal, chromatographed on Kieselgel G plates in CHCl$_3$/pyridine/formic acid 50/30/7 [13].

Galactolipid labelling with radioactive UDP-galactose

The following is a representative experiment for this kind of incubations. Intact spinach chloroplasts containing 15.5 mg of chlorophyll were sedimented in a 70 ml centrifuge tube and the supernatant resuspension medium was poured off. A solution of UDP-[6-^{3}H]galactose (3 µCi in 100 µl of resuspension medium) was added directly onto the sediment which was kept on ice. After swirling for 3 sec swelling medium (4 ml) containing nucleotide pyrophosphatase EC 3.6.1.9 (1 mg, from Sigma, St. Louis, MO, USA) was added and the mixture sucked up several times into a Pasteur pipette to disrupt the chloroplasts with concomitant degradation of UDP-galactose. The resulting mixture was loaded on a sucrose gradient and centrifuged for 1 h at 65 000 × g using a SW 25.1 rotor in a Beckman centrifuge. Fractions representing thylakoids, envelopes and stroma were removed and subjected to lipid extraction. Washed lipids were first chromatographed on Kieselgel G plates in diethyl ether to separate pigments from galactolipids which hardly move from the start in this solvent. They were recovered by scraping off and reextraction. Aliquots of these extracts were used to measure total radioactivity without interference by colour quenching. The main part was used for chromatographic separation of mono-, di- and trigalactosyl diacylglycerol in CHCl$_3$/MeOH/H$_2$0 70/30/4 and subsequent scintillation counting.

Galactolipid labelling from UDP-galactose formed outside of intact chloroplasts from products released during ^{14}CO$_2$ assimilation

In three parallel experiments a, b, c, various components and solutions were mixed to result in a final volume of 2 ml of isotonic resuspension medium. All three solutions a, b, c contained intact spinach chloroplasts with 3 mg of chlorophyll and NaHCO$_3$ at a

concentration of 8 mM. The solutions were enclosed in reaction vessels (5 ml) capped by air-tight teflon seals to allow further addition or withdrawal of aliquots with the aid of a syringe. Solution a did not contain any further additions. Solution b received a set of enzymes and substrates which were obtained from Boehringer, Mannheim, Germany. The enzymes were received in 3.2 M ammonium sulfate solution, which at the quantities required would decouple photosynthetic electron transport from photophosphorylation [14–16]. Therefore the enzyme mixture (see below) was placed in a Kollodium bag (SM 13 200 from Sartorius, Göttingen, Germany) and dialyzed for 3 h against 5 changes of resuspension medium (100 ml). The following enzymes were used, volumes of original suspension in μl and total activity of this quantity in μmoles of substrate convertable per min are given in brackets: Triosephosphate isomerase EC 5.3.1.1 (1 μl, 100 μmol/min), 3-phosphoglycerate kinase/glyceraldehyde 3-phosphate dehydrogenase EC 2.7.2.3/1.2.1.12 (10 μl, 4 μmol/min), aldolase EC 4.1.2.13 (30 μl, 2.7 μmol/min), fructose-1,6-diphosphatase EC 3.1.3.11 (50 μl, 2 μmol/min), phosphoglucose isomerase EC 5.3.1.9 (10 μl, 7 μmol/min) and phosphoglucomutase EC 2.7.5.1 (10 μl, 4 μmol/min). After dialysis the starting volume of 111 μl had increased to 300 μl, from which 135 μl were added to solution b and 165 μl to solution c (see below). Apart from these enzymes the following substrates were mixed to give a solution being 200 mM of each NADH, ATP, UTP and 100 μM of glucose-1,6-diphosphate. From this mixture 50 μl were included into solutions b and c resulting in final concentrations of 2 mM of NADH, ATP, UTP and 1 μM of glucose-1,6-diphosphate. Solution c received in addition to the components of solution b two other enzymes: UDPG pyrophosphorylase EC 2.7.7.9 (0.5 mg solids = 2 μmol/min) and UDPG 4-epimerase EC 5.1.3.2 (0.5 mg solids = 2 μmol/min, from Sigma, St. Louis, MO, USA). A control experiment showed that inclusion of enzymes and substrates at the concentrations and proportions listed above resulted in 17% reduction of CO_2-dependent oxygen evolution. Solutions a, b, c were placed in a water bath at 24 °C, illuminated with 3000 Lux and reactions started by injection of NaH^{14}CO$_3$ (100 μCi in 100 μl of resuspension medium). At 15 min intervals 300 μl samples were withdrawn and injected into 3 ml of CHCl$_3$/MeOH/7 M formic acid 5/12/13 [17]. An aliquot of this mixture was used for count-

ing of total CO_2-fixation. After washing with NaCl-solution the lipid subphase was analyzed for lipid labelling after chromatographic resolution in the usual way.

Results and Discussion

Incorporation of Acyl-CoA

A prerequisite for the experiments described in this paper are physiologically intact chloroplasts which are easily obtained by using Percoll gradients. In contrast, chloroplasts purified on sucrose gradients retain only high molecular weight compounds, whereas low molecular weight intermediates can freely pass through the envelope. This is demonstrated in Fig. 1a using oxygen evolution in the Hill reaction with ferricyanide as test of envelope impermeability for low molecular weight compounds [9]. Ferricyanide cannot permeate the envelope of Percoll chloroplasts, which therefore do not evolve oxygen, whereas sucrose chloroplasts are freely permeable and therefore show the same rate of oxygen evolution as shocked Percoll chloroplasts. When Percoll chloroplasts (according to Hall [18] type A), sucrose chloroplasts (type B) and free thylakoid systems (type C) are mixed and recentrifuged on a Percoll gradient made up in 0.25 M sucrose, three separate bands are obtained at densities of 1.08, 1.04 and 1.05 g/ml, respectively, as measured by the use of reference density beads.

In addition Fig. 1b demonstrates the effect of increasing acyl-CoA concentrations on envelope impermeability. The detergent character of this substrate becomes evident at higher concentrations as seen by the onset of oxygen evolution which results from envelope destruction and thylakoid accessibility for originally exogenous ferricyanide. Very similar results were obtained using an enzymatic integrity assay based on the impermeability of the envelope for NADPH by measuring the stroma enzyme GAPDH in intact chloroplasts [19]. In both cases acyl-CoA at acyl-CoA: chlorophyll ratios up to 1:5 did not open the envelope. These results were confirmed when chloroplasts incubated for 15 min at various acyl-CoA concentrations were recentrifuged on sucrose gradients (Fig. 2). At low acyl-CoA: chlorophyll ratios chloroplasts survived this incubation and were recovered as a single band of intact organelles. Increasing acyl-CoA resulted in additional and increasing proportions of free thylakoids, until
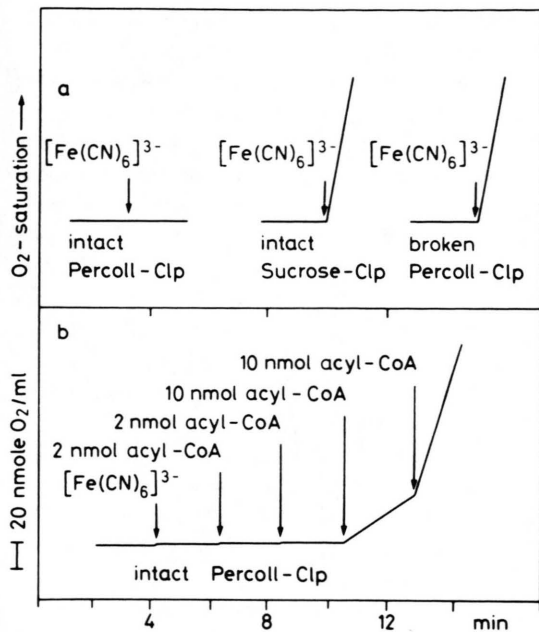
Fig. 1. Ferricyanide Hill reactions with: a) pea chloroplasts (28 μg chlorophyll) purified on Percoll (Percoll-Clp) or sucrose gradients (Sucrose-Clp) and resuspended in isotonic medium (1.5 ml) additionally containing 3 mM NH_4Cl, 0.5 mM Na_2HPO_4 and catalase; ferricyanide (2 mM) was added as indicated; b) intact Percoll-Clp, assay as in a, but palmitoyl-CoA, dissolved in suspension medium, was added as indicated.

at very high acyl-CoA concentrations even thylakoids were dissolved and appeared as green supernatant fraction which did not enter the gradient. The deleterious effects of acyl-CoA could be reduced by addition of bovine serum albumin which combines with acyl-CoA [20]. These introductory experiments demonstrate the intactness of the chloroplast preparations used in subsequent experiments. Their integrity is preserved in the presence of acyl-CoA up to ratios of acyl-CoA/chlorophyll of 1:5. In the following experiments chloroplasts were incubated with radioactive acyl-CoA at acyl-CoA/chlorophyll ratios of 1:50, which are well below critical values.

Incubation of intact chloroplasts with radioactive acyl-CoA, subsequent separation of intact organelles on Percoll gradients, lipid extraction and scanning of thin layer chromatographic separations gave the pictures shown in Fig. 3. In spinach and pea chloroplasts only two products were labelled: free fatty acids and phosphatidyl choline. Released fatty acids contained 10–26% and phosphatidyl choline 1–6%

of the radioactivity offered, depending on the acyl-CoA ($C_{18:1}$, $C_{16:0}$) and chloroplast preparations used. When oleoyl- and palmitoyl-CoA were offered in an equimolar mixture, both acyl groups were present in the released fatty acids in ratios close to 1. These results indicate that the chloroplast envelope contains a thioesterase which is accessible for substrates coming from the outside and which does not show fatty acid selectivity under the conditions used. This confirms previous observations on acyl-CoA hydrolysis by intact plastids [21, 22] ascribed to the action of a thioesterase, which has recently been investigated in more detail [23].

On the other hand, incubation of acyl-CoA with intact chloroplasts in the presence of additional CoA and ATP reduced greatly the apparent hydrolysis of acyl-CoA. Similar observations were made with chromoplasts [24]. In control experiments with isolated envelopes (experiments not shown) this effect could be ascribed to a concomitant resynthesis of acyl-CoA from released fatty acids and excess ATP and CoA, due to the activity of the previously described thiokinase [6, 25]. We have not yet demonstrated that a combination of these two reactions explains the apparently reduced hydrolysis of exogenous acyl-CoA with intact chloroplasts. It would have the consequence that not only hydrolysis but also synthesis of acyl-CoA in envelopes of intact organelles occurs with substrates coming from the outside. Recent experiments on the formation of acetyl- and acyl-CoA by intact chloroplasts from exogenous
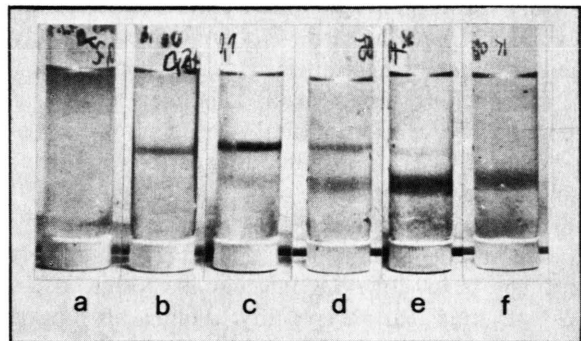

Fig. 2. Effect of acyl-CoA on intact spinach chloroplasts. Intact chloroplasts (65 μg chlorophyll) were incubated for 15 min at 24 °C in isotonic medium (0.15 ml) with various acyl-CoA concentrations and recentrifuged on sucrose gradients. Acyl-CoA concentrations: a and b 460 μM, c and d 230 μM, e and f 115 μM (46 μM resulted in the same gradient as shown in f). Assays b, d and f contained also 0.5 mg bovine serum albumin.
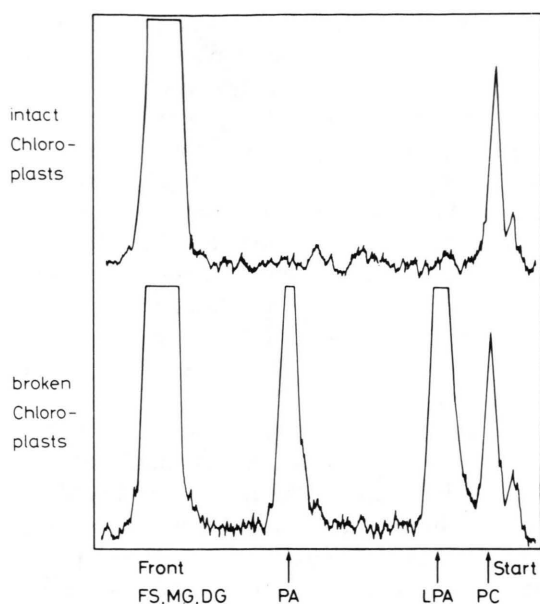
intact
Chloro-
plasts

broken
Chloro-
plasts

Front   ↑   ↑   ↑Start
FS,MG,DG   PA   LPA PC

Fig. 3. Labelling patterns of lipid fractions formed during [14C]acyl-CoA incubations with intact and broken pea chloroplasts. The organelles (150 μg chlorophyll) were incubated for 25 min at 24 °C in 0.3 ml of isotonic medium with 3.5 μM palmitoyl-CoA, 3.5 μM oleoyl-CoA and 1 mM *sn*-glycerol 3-phosphate. (PC = phosphatidyl choline, LPA = lyso-phosphatidic acid, PA = phosphatidic acid, MG = monoacyl glycerol; DG = diacylglycerol, FS = free fatty acids.

acetate, CoA and ATP point into the same direction [26]. In fact, it has not been shown that the well known thiokinase in the envelope can use all three substrates, when they come from the stroma side, and we do not know which of the two envelope membranes contains thiokinase and thioesterase activities and how these enzymes are related spatially and functionally in this membrane system.

The labelling of phosphatidyl choline was low but reproducible, confirming similar results from different plastid types [21, 24]. In our experiments this labelling was not increased by adding CoA and ATP to the assay system. Since chloroplasts isolated by the technique described in the experimental part did not incorporate UDPG into steryl glucoside [27] we think that phosphatidyl choline labelling is not due to contaminating microsomal membranes. In both plants oleoyl-CoA was a better acyl donor (up to 6% incorporated) than palmitoyl-CoA (up to 2% incorporated) either when offered individually or in equimolar mixture. Phosphatidyl choline labelled during incubation with this mixture contained about 86% of oleic acid and 14% of palmitic acid in both

spinach and pea chloroplasts. The positional analysis of phosphatidyl choline labelled in spinach chloroplasts from three exogenous acyl-CoA substrates (Table I) revealed a pronounced selectivity: $C_{16:0}$ was largely excluded from the C-2 position, whereas $C_{18:1}$ was preferentially incorporated into this location, particularly when offering an equimolar mixture. For $C_{16:0}$ similar results were obtained with pea chloroplasts, whereas $C_{18:1}$ was evenly distributed between C-1 and C-2 positions. Comparable patterns, *i. e.* $C_{18:1}$ and $C_{16:0}$ at C-1 and no $C_{16:0}$ at C-2, were observed in phosphatidyl choline from *in vivo*-labelled spinach [4] and pea leaves [28]. An enzymatic activity from spinach thylakoids differs from that described above and localized in the envelope, since the thylakoid activity catalyzed a specific acylation of C-2 in phosphatidyl choline, regardless of the acyl-CoA offered [29]. Silver nitrate thin layer chromatography of phosphatidyl choline labelled in intact chloroplasts showed many molecular species. Therefore an exact identification of the acyl acceptor molecule or its fatty acids is difficult, since labelling of phosphatidyl choline may result from acylation of preexisting lyso-phosphatidyl choline or involve acyl exchange reactions with phosphatidyl choline.

Another very important result is the observation, that neither lyso-phosphatidic acid nor phosphatidic acid are labelled when intact chloroplasts are incubated in the presence of glycerol 3-phosphate with radioactive acyl-CoA (Fig. 3). The significance of this observation will be discussed below.

When incubations were carried out with sonicated chloroplasts or organelles recovered from sucrose gradients, different scans were obtained (Fig. 3). In

Table I. Positional distribution of radioactive fatty acids incorporated into phosphatidyl choline, when intact chloroplasts from pea or spinach were incubated with three different [14C]acyl-CoA substrates. When individual acyl-CoAs were incubated (first two lines), the data represent the proportion of radioactivity recovered at C-1 or C-2 in percent of the sum at both positions. When the 1 : 1-mixture was incubated, the table has to be read vertically to compare data in line three with those from line four, since for example radioactivities of $C_{16:0}$ and $C_{18:1}$ at C-1 sum up to 100%.

14C labelled acyl-CoA	*Spinacia*		*Pisum*	
	C-1	C-2	C-1	C-2
$C_{16:0}$	81.2	18.8	81.9	18.1
$C_{18:1}$	16.8	83.2	46.6	53.4
$C_{16:0}$ ⎫ 1 : 1	81.1	4.2	51.0	4.3
$C_{18:1}$ ⎭	18.9	95.8	49.0	95.7

addition to free fatty acids and phosphatidyl choline also lyso-phosphatidic acid and phosphatidic acid carried major proportions of label, lyso-phosphatidic acid up to 18% and phosphatidic acid up to 25% of the offered radioactivity. When comparing the labelling of these four products from incubations with intact or sonicated chloroplasts, the following ratios were obtained (labelling with intact:sonicated chloroplasts, followed by standard deviation and number of independent pairs of experiments): phosphatidyl choline $1:0.93 \pm 0.15$ ($n=11$); free fatty acids, $1:1.36 \pm 0.30$ ($n=6$); lyso-phosphatidic acid, $1:12.8 \pm 4.9$ ($n=11$); phosphatidic acid, $1:11.6 \pm 6.8$ ($n=11$). This ratio is close to unity for phosphatidyl choline, slightly increased in free fatty acids and raised dramatically by a factor of 10 in the case of lyso-phosphatidic acid and phosphatidic acid. We interpret these ratios in the following way. Use of exogenous acyl-CoA for labelling of glycerolipids by intact chloroplasts is restricted to labelling of phosphatidyl choline by enzymes located in the chloroplast envelope, and hydrolysis of exogenous acyl-CoA is catalyzed by a thioesterase also localized in the envelope. From glycerol 3-phosphate and acyl-CoA, both required for *de novo* synthesis of glycerolipid backbones, acyl-CoA cannot penetrate the envelope from the outside to be available for the soluble acyl-CoA: glycerol 3-phosphate acyltransferase. This enzyme operates in the chloroplast stroma [5, 6] and is supplied with exogenously added glycerol 3-phosphate via the phosphate translocator in the envelope [30, 31]. The physiological significance of these results in relation to the cooperation between subcellular compartments in supplying soluble precursors for lipid biosynthesis is the demonstration that *in vitro* extraplastidic pools of acyl-CoA cannot be used for *de novo* synthesis of glycerolipids by intact chloroplasts.

Simulated cooperation between chloroplasts and cytoplasm in galactolipid biosynthesis

Permeability problems similar to those discussed in the case of acyl-CoA incorporation by intact chloroplasts have also to be considered in relation to galactolipid biosynthesis. Chloroplasts are unable to synthesize UDP-galactose which is the galactosyl donor for initiation of galactolipid synthesis. UDPG pyrophosphorylase and UDPG 4-epimerase are localized in the cytoplasm [2, 27] providing an exogenous supply of UDP-galactose for chloroplasts. In the experiments shown in Fig. 4 we have confirmed and simulated this cooperation between chloroplasts and cytoplasm. A series of exogenous "cytoplasmic" enzymes and cofactors, unable to penetrate the chloroplast envelope, were added to intact chloroplasts which assimilated $^{14}CO_2$ in the light. Newly fixed [14C] carbon atoms are excreted from isolated chloroplasts into the surrounding medium mainly as triosephosphates and phosphoglycerate [32] via the phosphate translocator [30, 31]. Therefore a combination of enzymes and cofactors required to catalyze exogenous conversion of labelled phosphoglycerate into galactose-labelled UDP-galactose were added to the medium. The reaction sequence involves the following steps and requires 9 enzymes and 4 cofactors listed in the experimental part: 3-phosphoglycerate $\rightarrow$ 1,3-diphosphoglycerate $\rightarrow$ glyceraldehyde-3-phosphate $\rightarrow$ dihydroxyacetone phosphate $\rightarrow$ fructose-1,6-diphosphate $\rightarrow$ fructose-6-phosphate $\rightarrow$ glucose-6-phosphate $\rightarrow$ glucose-1-phosphate $\rightarrow$ UDP-glucose $\rightarrow$ UDP-galactose. Three parallel incubations were carried out: sample a contained only chloroplasts and radioactive $^{14}CO_2$, sample b contained additional enzymes and cofactors with the exception of UDPG pyrophosphorylase and UDPG 4-epimerase, whereas sample c contained all enzymes and cofactors necessary to convert 3-phosphoglycerate into UDP-galactose. Fig. 4a shows that addition of enzymes and cofactors increased the rate of $^{14}CO_2$-fixation compared to sample a without these additions, although from the total $^{14}CO_2$ fixed only a very small proportion (0.1–0.4%) was recovered in the lipid fraction. Thin layer chromatography showed that a substantial proportion of lipid labelling was in the apolar fraction when chromatographed with a polar solvent. In all samples a–c very similar proportions were recovered in this fraction (Fig. 4b), which contained mainly free fatty acids and diacylglycerols as shown by rechromatography in an apolar solvent in agreement with previous results [33, 34]. Great differences were found in the labelling of monogalactosyl diacylglycerol (Fig. 4c). As expected only sample c with the complete set of enzymes contained radioactive monogalactosyl diacylglycerol, which carried twice as much label as the apolar fraction. This confirms previous experiments [33, 34] and supports the suggestion [2] that UDP-galactose made in the cytoplasm is used for galactolipid biosynthesis at the chloroplast surface.
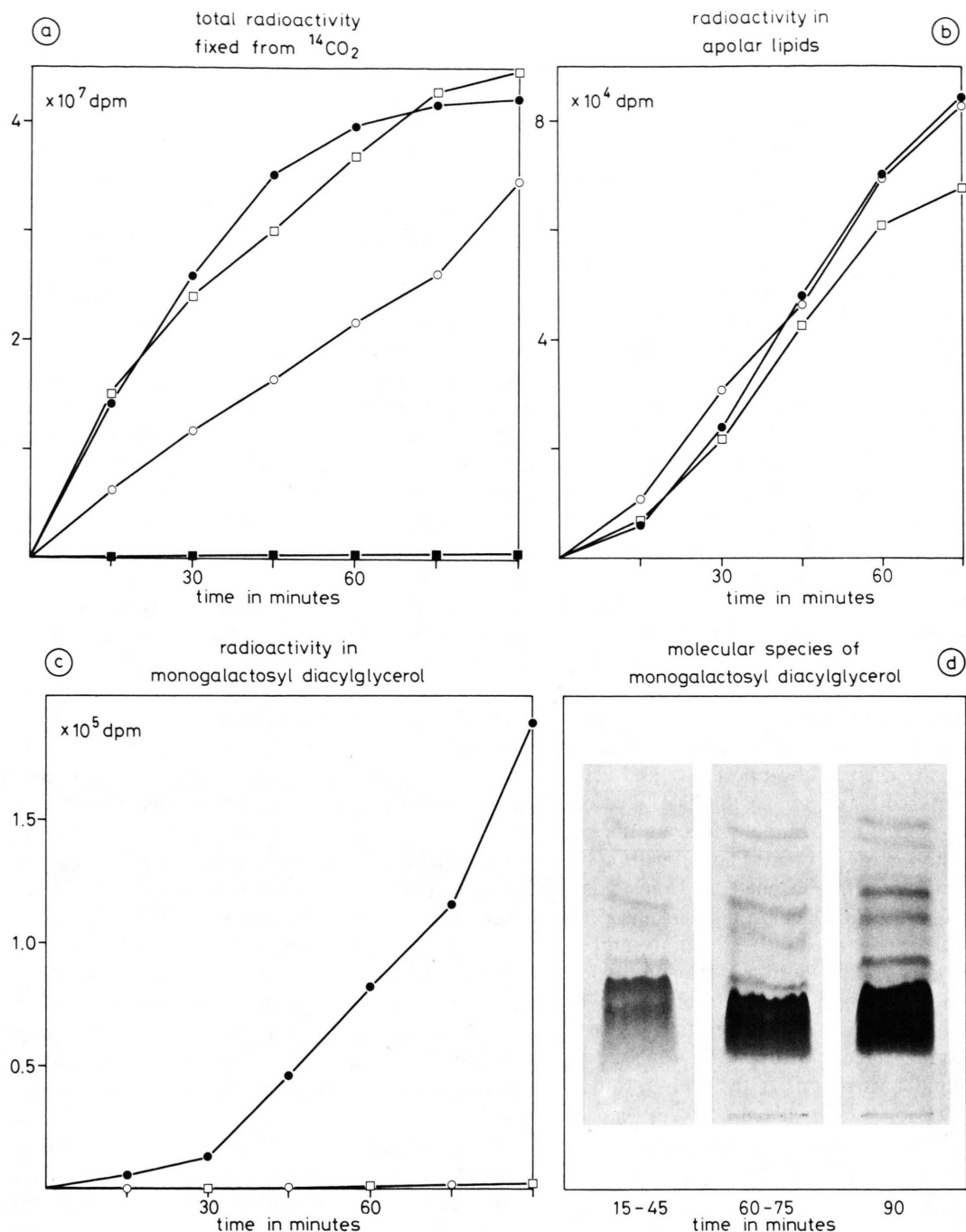
Fig. 4. Monogalactosyl diacylglycerol formation in isolated chloroplasts from radioactive CO_2 under conditions simulating the *in vivo* cooperation between chloroplasts and cytoplasm in the synthesis of UDP-galactose from 3-phosphoglycerate. Details are given in the experimental part. a. Incorporation of radioactivity from $^{14}CO_2$ into acid-stable products by isolated spinach chloroplasts incubated in resuspension medium without addition of "cytoplasmic" enzymes ○–○; □–□ plus cofactors and enzymes required to convert phosphoglycerate into UDP-galactose with the exception of UDPG pyrophosphorylase and 4-epimerase; ●–● plus the complete set of cofactors and enzymes; ■–■ radioactivity incorporated into total lipids in the presence of all cofactors and enzymes. b. Incorporation of radioactivity from $^{14}CO_2$ into the apolar lipid fraction by isolated chloroplasts under the same conditions as in a. c. Incorporation of radioactivity from $^{14}CO_2$ into monogalactosyl diacylglycerol by isolated chloroplasts under the same conditions as in a. ○–○ and □–□ were identical. d. Molecular species of monogalactosyl diacylglycerol formed in isolated chloroplasts from $^{14}CO_2$ in experiment c as separated by $AgNO_3$ thin layer chromatography in $CHCl_3/MeOH/H_2O$ 60/21/4. The two heavily labelled and overlapping bands close to the start (at the bottom) represent $C_{18:3}/C_{18:3}$- and $C_{18:3}/C_{16:3}$-fatty acid combinations [35]. The times given below each line represent sampling times and corresponding samples from experiment c which were used for separation of molecular species and subsequent radioautography.

Analysis of methanolysis products from labelled monogalactosyl diacylglycerol showed that radioactivity was only present in galactose, but not in glycerol or fatty acids. Silver nitrate chromatography of molecular species of monogalactosyl diacylglycerol labelled in experiment c (Fig. 4d) showed unchanged patterns throughout the experiment. These patterns are dominated by molecules, which have $C_{18:3}/C_{18:3}$- and $C_{18:3}/C_{16:3}$-fatty acid combinations in the diacylglycerol portion and therefore stay close to the start [35]. This indicates that newly synthesized diacylglycerols are hardly galactosylated, since the resulting monogalactosyl diacylglycerol molecules would contain more saturated and monoene fatty acids [35] (see below). Galactose is apparently combined with a preexisting pool of highly unsaturated diacylglycerols which in chloroplast envelopes may be derived from monogalactosyl diacylglycerol [36, 37]. In the experiments described above surprisingly little radioactivity was incorporated into digalactosyl diacylglycerol. Methanolysis of an aliquot of the total lipid extract followed by purification of fatty acid methyl esters and rechromatography on silver nitrate impregnated plates indicated label only in saturated and monoene fatty acids as was found before [38]. In summary these experiments show that application of exogenous UDP-galactose to chloroplasts reflects an *in vivo* situation resulting from the close cooperation between choroplasts and cytoplasm in providing this sugar nucleotide.
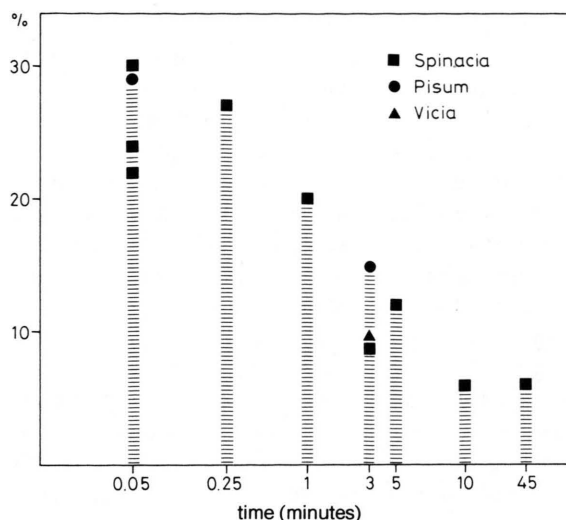

Fig. 5. Distribution of newly formed radioactive galactolipids between envelope and thylakoids as a function of the labelling time of intact chloroplasts with radioactive UDP-galactose. The ordinate represents radioactivity in envelope galactolipids as percent of total galactolipid radioactivity recovered in envelopes plus thylakoids. The time on the abscissa is plotted in a logarithmic way. Details of labelling, enzymatic degradation of UDP-galactose to stop the reaction and subsequent separation of labelled chloroplasts into envelope and thylakoid fractions are given in the experimental part. Experiments were carried out with chloroplasts obtained from ■−■ *Spinacia*, ●−● *Pisum* and ▲−▲ *Vicia* after purification on Percoll gradients.

Distribution of radioactive galactolipids between envelopes and thylakoids

To investigate the time scale of galactolipid transport from envelopes into thylakoids we incubated intact chloroplasts with radioactive UDP-galactose. The shortest incubations lasted 3 seconds which was the time required to add a small volume of isotonic solution of UDP-galactose to a chloroplast sediment followed by swirling and immediate addition of swelling medium which resulted in osmotic shock of the organelles. Inclusion of excess nucleotide pyrophosphatase in the swelling medium stopped any further incorporation of radioactivity into galactolipids of envelopes during subsequent handling of shocked organelle suspensions (loading of gradients and start of centrifuge), since this enzyme destroys UDP-galactose as shown in control experiments. Stroma, envelopes and thylakoids were separated by gradient centrifugation and all three fractions subjected to lipid extraction. Radioactivity was only found in the two membrane fractions which were analyzed for total radioactivity as well as for labelling of individual galactolipids. The results obtained with chloroplasts from three different plants are shown in Fig. 5. It is evident that the shorter the incubation time the higher the proportion of radioactive galactolipids in envelopes. After incubation times up to 1 min 20−30% of the galactolipids labelled in chloroplasts are present in the envelope fraction, whereas after times longer than 10 min only 6% are recovered in this fraction. This time-dependent loss of radioactivity from envelopes in favour of thylakoids was confirmed in pairs of experiments, in which a chloroplast preparation was divided and used for two simultaneous incubations, one lasting 3 seconds and the other 3 min. This kind of experiment repeatedly showed a higher proportion of ra-

dioactivity in envelopes from short time as compared to long time labelled chloroplasts. Labelling patterns of individual galactolipids from envelope and thylakoid extracts differed from each other, but did not show a time-dependent pattern variation. From nine different incubation experiments the following means were calculated for envelope and thylakoid galactolipid mixtures (data for thylakoids in brackets): monogalactosyl diacylglycerol = 36 (53) %, digalactosyl diacylglycerol = 39 (31) %, trigalactosyl diacylglycerol = 20 (16) %. Therefore, the radioactivity in the thylakoid fraction cannot be ascribed exclusively to contamination by envelopes. On the other hand, at present it is not possible to quantify this contamination leading to a similar uncertainty regarding the actual proportion of total envelope recovered

in the envelope fraction. Despite this uncertainty the data of Fig. 5 demonstrate that transport of galactolipids from envelopes to thylakoids or their equilibration between these two membrane systems is a process which *in vitro* requires time in the order of a few minutes to come to completion. This time scale confirms previous experiments on the distribution of labelled lipids between these two membrane systems, in which longer incubation times (5 min and more) had always resulted in envelopes which retained only about 7% of the lipids as compared to thylakoids [4, 7].

[1] R. Douce and J. Joyard, Adv. Bot. Res. **7**, 1–116 (1979).
[2] B. Königs and E. Heinz, Planta **118**, 159–169 (1974).
[3] J. Joyard, Thèse de Docteur ès Sciences Naturelles, University of Grenoble, Grenoble, France 1979.
[4] J. Joyard, R. Douce, H. P. Siebertz, and E. Heinz, Eur. J. Biochen. **108**, 171–176 (1980).
[5] M. Bertrams and E. Heinz, Planta **132**, 161–168 (1976).
[6] J. Joyard and R. Douce, Biochim. Biophys. Acta **486**, 273–285 (1977).
[7] R. Haas, H. P. Siebertz, K. Wrage, and E. Heinz, Planta **148**, 238–244 (1980).
[8] A. W. D. Larkum and R. G. W. Jones, Planta **145**, 393–394 (1979).
[9] U. Heber and K. A. Santarius, Z. Naturforsch. **25 b**, 718–728 (1970).
[10] J. Joyard and R. Douce, Physiol. Vég. **14**, 31–48 (1976).
[11] H. P. Siebertz, E. Heinz, M. Linscheid, J. Joyard, and R. Douce, Eur. J. Biochem. **101**, 429–438 (1979).
[12] A. K. Hajra, Lipids **9**, 502–505 (1974).
[13] G. Hauser and J. Eichberg, J. Biol. Chem. **250**, 105–112 (1975).
[14] G. Hind and C. P. Whittingham, Biochim. Biophys. Acta **75**, 194–202 (1963).
[15] J. W. Anderson and J. Done, Plant Physiol. **60**, 504–508 (1977).
[16] R. L. Heath and R. M. Leech, Arch. Biochem. Biophys. **190**, 221–226 (1978).
[17] R. L. Bieleski, Anal. Biochem. **9**, 431–442 (1964).
[18] D. O. Hall, Nature New Biol. **235**, 125–126 (1972).
[19] U. Heber, U. W. Hallier, and M. A. Hudson, Z. Naturforsch. **22 b**, 1200–1215 (1967).
[20] R. G. Lamb and H. J. Fallon, J. Biol. Chem. **247**, 1281–1287 (1972).
[21] A. A. Khan and S. S. Malhotra, Phytochemistry **17**, 1107–1110 (1978).

[22] B. Liedvogel, H. Kleinig, J. A. Thompson, and H. Falk, Planta **141**, 303–309 (1978).
[23] J. Joyard and P. K. Stumpf, Plant Physiol. **65**, 1039–1043 (1980).
[24] B. Liedvogel and H. Kleinig, Planta **144**, 467–471 (1979).
[25] P. G. Roughan and C. R. Slack, Biochem. J. **162**, 457–459 (1977).
[26] P. G. Roughan, T. Kagawa, and H. Beevers, Plant Sci. Lett. **18**, 221–228 (1980).
[27] R. Haas and E. Heinz, in Biogenesis and Function of Plant Lipids (P. Mazliak, P. Benveniste, C. Costes, and R. Douce, eds.), Elsevier/North-Holland Biomedical Press, Amsterdam, New York, Oxford, pp. 19–28 (1980).
[28] H. P. Siebertz, Ph. D. Thesis, University of Cologne, Cologne, Germany 1979.
[29] W. E. Shine, M. Mancha, and P. K. Stumpf, Arch. Biochem. Biophys. **173**, 472–479 (1976).
[30] H. W. Heldt and L. Rapley, Febs Lett. **10**, 143–148 (1970).
[31] R. Fliege, U. I. Flügge, K. Werdan, and H. W. Heldt, Biochim. Biophys. Acta **502**, 232–247 (1978).
[32] R. McC. Lilley, C. J. Chon, A. Mosbach, and H. W. Heldt, Biochim. Biophys. Acta **460**, 259–272 (1977).
[33] D. J. Murphy and R. M. Leech, Febs Lett. **77**, 164–168 (1977).
[34] J. W. A. McKee and J. C. Hawke, Febs Lett. **94**, 273–276 (1978).
[35] H. P. Siebertz, E. Heinz, J. Joyard, and R. Douce, Eur. J. Biochem. **108**, 177–185 (1980).
[36] A. van Besouw and J. F. G. M. Wintermans, Biochim. Biophys. Acta **529**, 44–53 (1978).
[37] A. van Besouw, Ph. D. Thesis, University of Nijmegen, Nijmegen, Netherlands 1979.
[38] D. J. Murphy and R. M. Leech, Febs Lett. **88**, 192–196 (1978).