The lack of a phospholipid-exchange-protein activity in soluble fractions of *Spinacia oleracea* leaves

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When ^{14}C -labelled liposomes prepared from *Spinacia oleracea* leaf lipids or ^{14}C -labelled microsomal fraction ('microsomes') prepared from *Spinacia oleracea* leaf protoplasts were incubated with unlabelled intact chloroplasts, there was a considerable transfer of label to the chloroplasts. This transfer occurred in the absence of added protein, but was stimulated by soluble protein fractions from *Spinacia oleracea* leaves. The stimulation was heat-stable and decreased after dialysis of the protein fractions. Salt solutions, containing no protein, stimulated lipid transfer proportionally to their conductivity. In all cases, the lipid transfer was not protein-dependent, but rather resulted from the fusion of ^{14}C - and ^3H -labelled liposomes or microsomes with chloroplasts. It is proposed that this photosynthetic tissue contains no detectable lipid-exchange activity between liposomes, microsomes and chloroplasts and that lipid transfer between these organelles is achieved by non-protein-dependent means.

Phospholipid-exchange proteins have been purified from such animal tissues as bovine heart (Johnson & Zilversmit, 1975; DiCorleto *et al.*, 1979), bovine liver (Kamp *et al.*, 1973), bovine brain (Helmkamp *et al.*, 1974), rat hepatoma (Dyatlovitskaya *et al.*, 1978), rat liver and rat intestine (Lutton & Zilversmit, 1976) and yeast cells (Cobon *et al.*, 1976). Phospholipid exchange has also been studied in several non-photosynthetic plant tissues, notably by Mazliak's group using potato tubers and cauliflower inflorescences (Ben Abdelkader & Mazliak, 1969, 1970). The exchange was found to be dependent on the addition of a soluble protein fraction from the cytosol of the plant cells. More recently, phospholipid-exchange proteins have been partially purified from potato tuber (Kader, 1975), cauliflower inflorescence and Jerusalem-artichoke tubers (Douady *et al.*, 1978) and castor-bean endosperm (Tanaka & Yamada, 1979). In each case, the protein has a molecular weight of about 20000 and preferentially exchanged phosphatidylcholine rather than phosphatidylethanolamine, phosphatidylglycerol or phosphatidylinositol. The exchange was bidirectional and occurred between microsomal fraction ('microsomes') and mitochondria, although Mazliak's group has also observed transfer of phospholipids from liposomes to plant mitochondria (Douady & Mazliak, 1975), nuclei

(Mazliak *et al.*, 1977), plasmalemma (Demandre *et al.*, 1978) and peroxisomes (Tchang, 1977).

Despite this considerable activity in characterizing plant phospholipid-exchange proteins, there have to date been no published reports of such exchange proteins in photosynthetic tissues. Tanaka & Yamada (1979) did report that, whereas castor-bean phospholipid-exchange protein readily transferred phospholipid from castor-bean microsomes to mitochondria from castor-bean endosperm, potato tuber, cauliflower inflorescence, pumpkin hypocotyl and even rat liver, there was no transfer to etioplasts from *Avena*, although transfer to chloroplasts from the same plant was not tested.

In photosynthetic tissues, the chloroplasts are the major, and possibly the only (Ohlrogge *et al.*, 1979), site of fatty acid biosynthesis *de novo* from acetyl-CoA, but most investigators have found that isolated chloroplasts will only synthesize saturated and monounsaturated fatty acids (Stumpf & James, 1963; Murphy & Leech, 1977, 1978; McKee & Hawke, 1979a,b). After experiments *in vivo* with leaf tissue, the endoplasmic reticulum was proposed as the major site of dienoic-fatty-acid synthesis from the monoene with (dioleoyl)phosphatidylcholine acting as the substrate (Roughan, 1970), 1975; Slack *et al.*, 1976, 1977). This proposal has been more directly supported by the observation that microsomes from several leaf tissues will convert oleoyl-CoA into linoleoyl phosphatidylcholine via oleoyl phosphatidylcholine *in vitro* (Slack *et al.*, 1976; D. J. Murphy & P. K. Stumpf, unpublished

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work). The site of the further desaturation to linolenate is unclear at present, but it is obvious that there must be a considerable traffic of acyl groups between the chloroplasts and other membranous organelles. In the present paper, we report our attempts to observe lipid transfer to and from chloroplasts, microsomes and liposomes prepared from spinach (*Spinacia oleracea*) leaves and to isolate a soluble protein to stimulate such an exchange.

Materials and methods

Preparation of labelled liposomes

Purified [^{14}C]phosphatidylcholine was obtained from spinach leaves incubated for 3 h with [^{14}C]acetate by extraction in hexane/propan-2-ol (3:2, v/v), followed by repeated t.l.c. and elution until only one phospholipid band was obtained. To the purified [^{14}C]phosphatidylcholine, 10% by weight of the antioxidant 2,6-di-*t*-butyl-*p*-cresol was added. For some experiments, a small quantity of [^3H]triacylglycerol was also added at this stage. The solvent was then evaporated under N_2 and the lipid redissolved in $100\mu\text{l}$ of diethyl ether. The diethyl ether layer was dried under N_2 to leave a thin film of lipid at the bottom of the test tube. A buffer solution containing 0.25 M-sucrose, 1 mM-EDTA and 50 mM-Tris/HCl, pH 7.4, was added to give a final lipid concentration of 1.67 mg/ml. The suspension was mixed vigorously on a vortex-mixer for 10 min and the lipid was allowed to swell for 1 h under an N_2 atmosphere. Finally, the test tube containing the suspension was placed in a sonicating bath at 25°C for 30 min.

Isolation of intact chloroplasts

Young healthy leaves were selected from batches of market-bought spinach plants. The leaves were floated under illumination for 2 h before fractionation. After being de-ribbed, 40 g of leaves was homogenized for $2 \times 1\text{s}$ bursts in 150 ml of buffer containing 0.33 M-sorbitol, 0.2 mM- MgCl_2 and 20 mM-4-morpholine-ethanesulphonic acid, adjusted to pH 6.5 with Trizma base (Nakatani & Barber, 1977). The homogenate was rapidly filtered through eight pre-wetted layers of cheesecloth plus one layer of $20\mu\text{m}$ nylon mesh into 40 ml centrifuge tubes maintained at 4°C . Chloroplasts were sedimented by centrifugation in an angle-head rotor in a Sorvall RC2-B centrifuge at $3000g$ for 1 min. The pellets were gently resuspended in 1 ml of grinding buffer and carefully layered on top of 10 ml of buffer containing 0.33 M-sorbitol, 20 mM-4-morpholine-ethanesulphonic acid, 0.2 mM- MgCl_2 plus 40% (w/v) Percoll (Mills & Joy, 1979). Intact chloroplasts were sedimented by centrifugation in an HB-4, swinging-bucket rotor at $15000g$ for 5 min. Broken chloroplasts failed to pass through the 40%-Percoll layer.

After removal of the Percoll layer, the purified chloroplast pellet was resuspended in grinding buffer. The chloroplast suspension was 90–95% intact as judged by phase-contrast microscopy and ferricyanide-dependent O_2 evolution.

Isolation of labelled microsomes

Spinach leaf protoplasts were prepared from thin flat young leaves (4–8 cm in length) selected from market-bought plants. The young leaves were removed from the bunches, washed and de-starched overnight at 4°C in the dark. The lower epidermis of the leaves was abraded by brushing with a soft nylon brush and the leaves were plasmolysed by floating on 0.7 M-mannitol for 30 min.

The mannitol was replaced with a cell-wall-digesting solution (15 ml/g fresh wt. of leaf tissue). The solution consisted of 2.0% Cellulysin (Calbiochem), 1.0% Macerace (Calbiochem), 0.5% dextran sulphate (Calbiochem), 50 mM-4-morpholine-ethanesulphonic acid, pH 6.0, 0.7 M-mannitol, and a B5 salt solution without glucose as described by Gamborg & Wetter (1975). The leaves were vacuum infiltrated for 3 min and incubated for 4 h in the dark at room temperature, with gentle shaking. The protoplasts were filtered through a $73\mu\text{m}$ nylon mesh and pelleted by centrifugation at $140g$ for 4 min at room temperature. The pelleted protoplasts were washed twice by resuspension in 0.7 M-mannitol and centrifugation at $140g$ for 4 min. Filtration of the resuspended protoplasts through a $50\mu\text{m}$ nylon mesh removed xylem elements and druses (large crystals). The final pellet was resuspended to a known volume with 0.7 M-mannitol and the chlorophyll content was determined by the method of MacKinney (1941).

The protoplast suspensions were incubated with $50\mu\text{Ci}$ of [^{14}C]acetate in a slowly shaking (30 cycles/min) illuminated water bath at 22°C . The incubation medium contained 0.7 M-mannitol, B5 medium without sucrose and 10 mM- NaHCO_3 . After 3 h, the protoplasts were lysed by three passages through a 10 ml syringe with a 25-gauge needle fitted with a Swinnex filter (13 mm) and two layers of Miracloth and a single layer of $20\mu\text{m}$ nylon mesh. Extent of lysis was estimated by light microscopy. A protoplast lysate (1–2 mg of chlorophyll) containing less than 10% intact protoplasts was layered on to a 10–40% (w/w) linear sucrose gradient. The gradients were centrifuged for 3 h at $100000g$ in an SW-27 rotor. The microsomal membrane fraction was localized on the gradient by means of its NADH-cytochrome *c* reductase activity (assayed by the method of Masters *et al.*, 1965). The ^{14}C -labelled microsomal membranes were washed and concentrated by centrifugation to a specific radioactivity of $>5000\text{d.p.m.}/\mu\text{l}$ of resuspended

microsomal fraction ('microsomes'). The microsomal fraction had a density of 1.08 g/ml and contained very low amounts of galactolipid, which indicated that there was relatively little contamination by chloroplast envelope membranes.

Assay system

The assay system contained three components: a donor membrane preparation (normally either liposomes or microsomes), which was radioactively labelled, an acceptor membrane system (normally chloroplasts) and a soluble protein fraction. Control assays were always run in which assay medium was substituted for the soluble protein fraction. The chloroplast and soluble protein fractions were mixed and the assay was begun by adding the labelled donor membrane preparation in a total volume of 1 ml of 0.25 M-sucrose / 1 mM-EDTA / 50 mM-Tris / HCl, pH 7.4, in a Microfuge tube. The tubes were shaken in a water bath at 35°C and assays were halted by sedimenting the chloroplasts at 15 000 *g* for 2 min in a Beckman Microfuge. The pellets, containing the chloroplasts, and the supernatants, containing the donor membrane system, were either counted for radioactivity directly in a Beckman LS-230 scintillation counter, or extracted in chloroform/methanol (2:1, v/v) for lipid analysis.

Preparation of soluble protein fraction

Washed spinach leaves (3.2 kg) were homogenized for 10 s in 4 litres of 0.33 M-sorbitol / $\text{MgCl}_2 / \text{Na}_2\text{P}_2\text{O}_7$ / 1% polyvinylpyrrolidone at pH 6.5. The homogenate was strained through eight layers of cheesecloth and centrifuged at 5000 *g* for 15 min to pellet most of the membranes. Further membranous material was removed by centrifuging the resulting supernatant at 23 000 *g* for 30 min and finally at 100 000 *g* for 5 h. All of the pellet fractions were retained. In some cases the final supernatant fraction was adjusted to pH 5.1 with HCl by the technique of Wirtz & Zilversmit (1961). The mixture was left for 30 min at 4°C before the acid-insoluble material was sedimented at 15 000 *g* for 15 min. The pH of the supernatant was re-adjusted to 7.4 with concentrated NaOH.

Solid $(\text{NH}_4)_2\text{SO}_4$ was added to the remaining 3.8 litres of the supernatant fraction to 25% (w/v) and the mixture was stirred overnight. The precipitate was spun down, resuspended in a minimum volume and dialysed against 40 vol. of 10 mM-sodium pyrophosphate / 10 mM-iso-ascorbate / 10 mM- β -mercaptoethanol, pH 6.5. The procedure was repeated with 50%, 75% and 100% (w/v) $(\text{NH}_4)_2\text{SO}_4$. Conductivity of fractions was determined by using a model 31 conductivity bridge (Yellow Springs Instruments Co., Yellow Springs, OH, U.S.A.).

Lipid analysis

Chloroform/methanol (2:1, v/v) was added to the

assay fractions until they were rendered monophasic. Non-lipid material was partitioned by washing the organic phase with 0.2 vol. of 0.1 M-acetic acid / 0.1 M-KCl. The remaining total lipid mixtures were resolved by two-stage single-dimensional t.l.c. The neutral lipids were initially separated in light petroleum (b.p. 35–60°C) / diethyl ether / acetic acid (70:30:1, by vol.), which resolved the mixture into bands containing triacylglycerol, non-esterified fatty acid, 1,2-diacylglycerol, 1,3-diacylglycerol, monoacylglycerol and polar lipids. The polar-lipid band was removed from the plate and eluted in the presence of chloroform/methanol/formic acid/water (97:97:4:2, by vol.) and concentrated under N_2 . Polar lipids were then resolved in chloroform/methanol/acetic acid/water (170:30:20:7, by vol.) into bands containing diacylmonogalactosylglycerol, phosphatidic acid, phosphatidylethanolamine, diacylmono(digalactosyl)glycerol, phosphatidylglycerol, phosphatidylcholine and diacylsulphoquinovosylglycerol. The lipid bands were located by means of a 0.05% dichlorofluorescein in 80% methanol spray, and ^{14}C radioactivity was localized with a Packard model 7201 radiochromatogram scanner or by radioautography (Murphy & Stumpf, 1979).

Quantitative radioactivity determinations were made by eluting lipid bands as previously described and adding portions of the eluate to a counting vial. After removal of the elution solvents, the samples were taken up in a PCS (Phase Combining System; Amersham/Searle) / xylene (2:1, v/v) 'cocktail' and counted for radioactivity in a Beckman LS 230 liquid-scintillation counter.

Methyl esters of total lipid mixtures or separated lipid bands were prepared by alkaline hydrolysis in KOH followed by acidification and partitioning of non-esterified fatty acids into light petroleum and esterification with diazomethane as previously described (Murphy & Stumpf, 1979). Alternatively, the separated lipid bands were scraped directly into tubes containing 5 ml of either methanol/ H_2SO_4 (20:1, v/v) for polar lipids or benzene/methanol/ H_2SO_4 (10:20:1, by vol.) for neutral lipids. Boiling chips were added and the mixture was refluxed at 110°C for 4 h. After cooling, 7 ml of water was added to each tube and the methyl esters were extracted twice in 4 ml of light petroleum. The light-petroleum extracts were washed with 2 ml of water and 'blown down' under N_2 for separation on a 10% DEGS-PA (Supelco, Bellefonte, PA, U.S.A.) column (4.8 mm $\times$ 1.83 m) at 170°C. Radioactivity of the column effluent was monitored with a Nuclear-Chicago Biospan model 4998 radioactivity detector.

The ^{14}C -labelled liposomes used as donor membranes contained over 98% of the ^{14}C as phosphatidylcholine, and most of this ^{14}C was present as oleate (40%), linoleate (30%) and palmitate (20%).

After transfer of the ^{14}C to the chloroplasts, there was evidence of some hydrolytic breakdown, since [^{14}C]diacylglycerols, ^{14}C -labelled fatty acids and [^{14}C]lysophosphatidylcholine were all detected in the chloroplast fractions. From 10 to 20% of the transferred [^{14}C]phosphatidylcholine was hydrolysed in this manner. In some incubations, ^{14}C from the transferred phosphatidylcholine was found in diacylmonogalactosylglycerol, but this conversion could not be consistently observed. Similarly, the fatty acids of the transferred ^{14}C -labelled lipids were sometimes found to contain a higher proportion of linoleate and 5–10% α -linolenate that was not present in the original liposomes, but this effect could not always be reproduced. In general, the bulk of the transferred ^{14}C remained as phosphatidylcholine in the chloroplasts despite the very low endogenous concentrations of phosphatidylcholine in this organelle.

Results

When ^{14}C -labelled liposomes were incubated with unlabelled chloroplasts, there was an apparent transfer of ^{14}C to the chloroplasts. Although this transfer was stimulated by the addition of the soluble protein fraction remaining after the acid precipitation (pH 5.1) step and rose with increasing protein concentration (Fig. 1a), transfer also took place in the absence of added soluble protein (Fig. 1b) at a slightly lower rate. The transfer rates in both the presence and absence of the soluble protein fraction were unexpectedly high when compared with similar studies on non-photosynthetic tissue by Ben Abdelkader & Mazliak (1970) and Kader (1975). There appeared to be at least two distinct phenomena involved in the transfer of ^{14}C from the liposomes to chloroplasts. Firstly, there was a non-protein-dependent transfer, possibly owing to fusion of liposomes with chloroplasts, and secondly there was a stimulatory effect of the added soluble protein extract.

The non-protein-mediated transfer was investigated by incubating liposomes labelled with [^{14}C]phosphatidylcholine, and also containing a small quantity of [^3H]triacylglycerol, with intact chloroplasts. There have been no published reports of protein-mediated transfer of triacylglycerol between liposomes, mitochondria and microsomes, and radioactively labelled triacylglycerol is commonly used to control for non-specific membrane fusion or sedimentation (Johnson *et al.*, 1975; Johnson & Zilversmit, 1975; DiCorleto *et al.*, 1979). Zero-time controls showed that less than 5% of the free liposomes sedimented with the chloroplasts, and no liposomes would sediment in the absence of chloroplasts. However, with time, an increasing amount of both ^{14}C and ^3H was found in the chloroplast fractions. The ratio of $^3\text{H}/^{14}\text{C}$ in the chloroplast

Fig. 1. Effect of protein concentration on (a), and stimulation of (b), transfer of [^{14}C]phosphatidylcholine from liposomes to chloroplasts

A pH 5.1 supernatant fraction of soluble spinach leaf extract was used for proteins (a) and stimulation (b) experiments.

fraction remained approximately constant, suggesting that whole liposomes were fusing with or adhering to chloroplasts rather than specifically transferring phospholipid. As Fig. 1(b) shows, this non-specific fusion of liposomes with chloroplasts resulted in the transfer of over 30% of the [^{14}C]phosphatidylcholine to chloroplasts in 60 min and may be due to a charge effect.

To study the putative protein-mediated transfer of phospholipid, a large-scale protein purification was undertaken starting with 3.2 kg of spinach leaves. Membrane proteins were removed by successive centrifugation steps, with little apparent lipid-exchange activity found in any of the pellet fractions (Table 1). Soluble proteins were then precipitated in increasing concentrations from 25–100% $(\text{NH}_4)_2\text{SO}_4$. Relatively little apparent exchange was found in any of the $(\text{NH}_4)_2\text{SO}_4$ pellets, despite the

Table 1. *Purification procedures used in investigation of possible lipid-exchange protein in spinach leaves*

Exchange activity units are measured as the percentage of ^{14}C transferred to chloroplast $\times$ total volume (litres) of the fraction. Assays were run for 40 min and contained 0.1 ml of the protein fraction, 0.1 ml of intact chloroplast suspension, 0.05 ml of ^{14}C -labelled liposomes and 0.75 ml of buffer to give a total volume of 1 ml of 0.25 M-sucrose/1 mM-EDTA/50 mM-Tris/HCl, pH 7.4.

Fraction	Protein		Total exchange activity
	Total (g)	(mg/ml)	
Total homogenate	16.79	3.65	94
5000 g { Pellet	1.96	13.1	9
Supernatant	12.41	2.9	70
23 000 g { Pellet	2.1	11.0	13
Supernatant	11.6	2.75	79
100 000 g { Pellet	0.6	5.8	8
Supernatant	7.6	2.0	69
25%-(NH_4) $_2\text{SO}_4$ { Pellet	0.26	1.45	13
Dialysed pellet	0.24	1.15	8
Supernatant	5.04	1.5	250
50%-(NH_4) $_2\text{SO}_4$ { Pellet	2.92	17.2	3
Dialysed pellet	2.58	15.4	1
Supernatant	1.68	0.45	307
75%-(NH_4) $_2\text{SO}_4$ { Pellet	1.05	4.4	13
Dialysed pellet	0.81	3.3	5
Supernatant	0.28	0.071	2000
100%-(NH_4) $_2\text{SO}_4$ { Pellet	0.005	0.23	>26
Dialysed pellet	0.002	0.056	7
Supernatant	0.071	0.059	2100

Table 2. *Effect of dialysis and boiling on the lipid-transfer activity of the 100%-(NH_4) $_2\text{SO}_4$ -supernatant protein fraction from spinach leaves*

Assays were run as described in the legend to Table 1.

Fraction	Additional treatments	Transfer (%)
100%-(NH_4) $_2\text{SO}_4$ supernatant	None	98.5
	Boiled	98.0
	Dialysed	10.4
	Dialysed and boiled	8.3
	Dialysed + 100% (w/v) (NH_4) $_2\text{SO}_4$	98.9
No protein	None	8.3
	+ 75% (w/v) (NH_4) $_2\text{SO}_4$	98.8
	+ 100% (w/v) (NH_4) $_2\text{SO}_4$	99.3

fact that these pellets together accounted for over 99% of the soluble protein collected from the leaves. The small amount of activity that was in the pellets was lowered further after dialysis overnight in 40 vol. of buffer. Additional dialysis resulted in even lower activities, which appeared to rule out a protein-mediated effect.

The (NH_4) $_2\text{SO}_4$ -supernatant fractions showed very high rates of lipid-transfer activity. In the absence of significant protein-dependent activity in the pellet fractions, we investigated the final (NH_4) $_2\text{SO}_4$ supernatant, which had the highest lipid-transfer activity of any fraction while still containing a small amount of protein. A sample of this fraction was repeatedly dialysed using dialysis tubing of very small pore size (molecular-weight

cut-off = 3500), since the proteins remaining in 100% (NH_4) $_2\text{SO}_4$ would be likely to have low molecular weights. Table 2 shows that the lipid-transfer activity of this fraction decreased markedly after dialysis. In addition, 2 min at 100°C failed to decrease the activity either before or after dialysis. The re-addition of 100% (NH_4) $_2\text{SO}_4$ to the dialysed extract resulted in the full restoration of apparent lipid-transfer activity, which may thus be attributed to fusion of membranes owing to an increase in the ionic strength of the buffer as noted by Barber & Chow (1979).

The above data indicate that there was no protein-mediated lipid-transfer activity in the (NH_4) $_2\text{SO}_4$ supernatants, and the decrease in the activity in the pellet fractions after dialysis made it

unlikely that these latter activities were protein-mediated either. The results presented in Table 3 show no decrease in any of the lipid-transfer activities after the dialysed protein fractions were maintained at 100°C for 2 min. The rate of lipid-transfer activity in any of these fractions could only be lowered by repeated dialysis and could be readily increased by the re-addition of $(\text{NH}_4)_2\text{SO}_4$.

The possibility that all of the lipid-transfer activity observed was due to ionic-strength-dependent membrane aggregation was investigated by incubating ^{14}C -labelled liposomes with chloroplasts in a variety of NaCl concentrations. No soluble protein was added to these treatments. The results given in Fig. 2 demonstrate a marked increase in apparent transfer of ^{14}C from liposomes to chloroplasts when the ionic strength of the assay medium, as measured by its conductivity, was raised. The conductivity of the $(\text{NH}_4)_2\text{SO}_4$ -pellet extracts was 7–15 mS before dialysis and 3–5 mS after one dialysis. From Fig. 2, the ionic strength of the protein fractions and assay media, both before and after dialysis, would be expected to increase lipid transfer significantly. The apparent transfer of ^{14}C from liposomes to chloroplasts was probably due to fusion of the membranes as ^3H from ^3H -triacylglycerol-labelled liposomes accumulated in the chloroplast pellet at the same rate as ^{14}C from ^{14}C -phosphatidylcholine.

Liposomes are a convenient donor membrane system and are widely used in routine assays for lipid-exchange proteins (Johnson & Zilversmit, 1975; Zilversmit, 1978; DiCorleto *et al.*, 1979), but the natural biological membranes are more complex, containing substantial amounts of protein in addition to lipid. To determine whether lipid exchange was possible between two different endogenous leaf-membrane systems, we prepared a quantity of ^{14}C -labelled microsomes from spinach-

leaf protoplasts. The ^{14}C -labelled lipid contained in the microsomes was mainly (dioleoyl)phosphatidylcholine. Control experiments demonstrated that the microsomes alone would not sediment during the $15\,000\text{g} \times 2\text{min}$ centrifugation used to terminate assays. However, when ^{14}C -labelled microsomes were incubated with unlabelled chloroplasts, increasing amounts of ^{14}C accumulated in the chloroplast pellets with time. This transfer of ^{14}C was unrelated to the presence of any soluble protein fraction (Table 4), and all the ^{14}C -labelled lipid species present in the microsomes were transferred with equal ease to the chloroplasts. The transfer may have been due to a non-specific fusion of the microsomal vesicles with the chloroplasts that was, if anything, even more rapid than the fusion of liposomes with chloroplasts.

We were able to observe the transfer of ^{14}C -labelled lipids from chloroplasts incubated with $[1\text{-}^{14}\text{C}]\text{acetate}$ in the light for 30 min to unlabelled

Fig. 2. Effect of conductivity of the assay medium on apparent transfer of $[^{14}\text{C}]\text{phosphatidylcholine}$ from liposomes to chloroplasts

Table 3. Effect of boiling on the lipid-transfer activities of the pellet and supernatant fractions after $(\text{NH}_4)_2\text{SO}_4$ treatment of a soluble protein extract from spinach leaves
Assays were run as described in Table 1.

Fraction	Heat	Transfer (%)
50%-(NH_4) ₂ SO ₄ dialysed pellet	—	7.0
	+	15.4
50%-(NH_4) ₂ SO ₄ dialysed supernatant	—	18.4
	+	19.2
75%-(NH_4) ₂ SO ₄ dialysed pellet	—	9.9
	+	11.9
75%-(NH_4) ₂ SO ₄ dialysed supernatant	—	15.9
	+	21.1
100%-(NH_4) ₂ SO ₄ dialysed pellet	—	12.9
	+	13.2
100%-(NH_4) ₂ SO ₄ dialysed supernatant	—	17.2
	+	15.5

Table 4. Comparison of the transfer of lipids from liposomes and spinach chloroplasts

Abbreviations used: TH, total leaf homogenate; S₁, dialysed supernatant from pH 5.1 treatment; S₂, dialysed supernatant after 50% $(\text{NH}_4)_2\text{SO}_4$ step. Assays were run as described in Table 1.

^{14}C -labelled donor membrane	Soluble protein fraction	^{14}C transferred to chloroplasts (%)
Microsomes	TH	36.1
	S ₁	46.3
	S ₂	46.6
	None	38.3
Liposomes	TH	34.3
	S ₁	37.3
	S ₂	36.1
	None	32.1

microsomes incubated with the chloroplasts for a further 30 min in the dark. ^{14}C -labelled lipids left the chloroplasts and accumulated in microsomes with time, independently of added soluble protein fractions. A dialysed acetone-dried-powder fraction from spinach leaves had no significant effect on lipid transfer from either liposomes or microsomes to chloroplasts or from chloroplasts to microsomes.

Discussion

The results presented in this paper show that no protein-dependent lipid-exchange activity was present in soluble extracts from spinach leaves. The very high rates of lipid transfer from liposomes and microsomes to chloroplasts were artefacts caused by non-specific membrane fusion or clumping. This could be observed even in the incubation buffer alone, where the conductivity of 2–3 mS was sufficient to cause some of the ^{14}C -labelled donor membranes to fuse or clump with the chloroplasts. The very high rates of apparent lipid transfer in the presence of protein extracts from the concentrated $(\text{NH}_4)_2\text{SO}_4$ treatments (Table 2) were probably due to fusion of the membranes caused by the high salt concentration. Under such conditions, a large population of small micelles and organelles would be thermodynamically unfavourable and the non-polar membranes would tend to partition out of the aqueous phase.

At the lower conductivities in dialysed protein extracts, there was still considerable apparent lipid transfer to chloroplasts (Tables 3 and 4) that was stable to boiling. This effect may be due to actual membrane fusion, but could also be caused by the clumping of the organelles and micelles. It has been observed by Neuberger *et al.* (1977) that chloroplast envelopes are strongly negatively charged and can be readily agglutinated by yeast cationic ferricytochrome *c*. A similar effect has been reported for the high-molecular-weight sugar polymer Ficoll, which caused clumping of spinach chloroplasts at concentrations of 2.5% (w/w) (Murphy, 1977). In this laboratory, we have more recently observed that leaf protoplasts will readily clump together and will also aggregate with free chloroplasts and membrane fragments (Kuhn & Stumpf, 1980). The apparent transfer of lipids from microsomes and liposomes to chloroplasts in solutions of low conductivity may therefore be due to an electrostatic binding, rather than actual fusion of the membranes. Barber & Chow (1979) have observed a similar type of membrane aggregation in the presence of uni-, bi- or ter-valent cations with isolated pea thylakoids.

The presence of this artefact in all of the assay systems that we employed, using organelles derived from photosynthetic tissue, gives rise to a high background transfer of lipid. However, this non-

specific non-protein-dependent transfer could be minimized and controlled for. Under these circumstances, we were still unable to find any convincing stimulation of lipid transfer in any of the protein fractions assayed. Using similar techniques, many other workers have found and purified phospholipid-exchange proteins from other tissues, in some cases with non-protein-dependent background transfer rates as high as 18% (Cobon *et al.*, 1976). We therefore conclude that there was no phospholipid-transfer activity in this particular photosynthetic tissue.

Mazliak & Kader (1980) have commented on the gross uniformity of lipid composition among the organelles of non-photosynthetic plant cells, which are mainly composed of phospholipids. In particular, phosphatidylcholine and phosphatidylethanolamine together constitute over 70% of the total phospholipid by weight. Animal phospholipid-exchange proteins can cause a net transfer of lipid to an acceptor membrane, and their general specificity towards phosphatidylcholine and phosphatidylethanolamine means that these lipids would tend to equilibrate among various organelles. Such lipids as diphosphatidylglycerol (cardiolipin) would not be transferred by such specific exchange proteins. In the case of cardiolipin, the phospholipid would also be inaccessible to exchange proteins, since it is located on the mitochondrial inner membrane. This specificity on the part of the phospholipid-exchange proteins of non-photosynthetic plant cells may be responsible for the above-mentioned uniformity of composition of their organelles with regard to the principal phospholipids, phosphatidylcholine and phosphatidylethanolamine.

This contrasts sharply with the situation in photosynthetic tissue, and especially in the chloroplasts with their overwhelming preponderance of polyunsaturated galactolipids, very low amounts of phosphatidylcholine, and virtual absence of phosphatidylethanolamine. However, transfer of lipids, or at least acyl residues, to extra-chloroplastic membrane systems must occur in leaf cells such as in spinach, where the exclusive site of fatty acid synthesis is the chloroplast (Ohlrogge *et al.*, 1979). In these cells, it is possible that the acyl groups are exchanged between organelles independently of their head-groups as activated fatty acids, e.g. oleoyl-CoA, rather than as phospholipids, as occurs in non-photosynthetic tissues.

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