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Some Properties of a Microsomal Oleate Desaturase from Leaves

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1. When [1-^{14}C]oleoyl-CoA was incubated with a pea-leaf homogenate oleate was both incorporated into microsomal 3-*sn*-phosphatidylcholine and released as the unesterified fatty acid. The proportion of oleate incorporated into this phospholipid was dependent on the relative amounts of thiol ester and microsomal preparation present in reactions. 2. At the concentrations of microsomal preparation and [^{14}C]oleoyl-CoA used to study oleate desaturation the metabolism of the thiol ester was essentially complete after 5 min incubation, but the loss of label from 3-*sn*-phosphatidylcholine oleate and the concomitant increase in radioactivity in the linoleate of this phospholipid proceeded at approximately linear rates over a 60 min period. The kinetics of labelling of unesterified linoleate was consistent with the view that this labelled fatty acid was derived from 3-*sn*-phosphatidylcholine. 3. Oleate desaturation required oxygen and with unwashed microsomal fractions was stimulated either by NADPH or by the 105000*g* supernatant. Washed microsomal preparations did not catalyse desaturation, but activity was restored by the addition of NADPH, 105000*g* supernatant or Sephadex-treated supernatant. NADPH could be replaced by NADH or $NADP^+$, but not by NAD^+. 4. Microsomal fractions from mature and immature maize lamina and expanding spinach leaves also rapidly incorporated oleate from [^{14}C]oleoyl-CoA into 3-*sn*-phosphatidylcholine, but desaturation of 3-*sn*-phosphatidylcholine oleate was detected only with microsomal preparations from immature maize lamina. 5. It is proposed that leaf microsomal preparations possess an oleate desaturase for which 3-*sn*-phosphatidylcholine oleate is either the substrate or an immediate precursor of the substrate.

On the basis of labelling studies *in vivo* we proposed that linolenate of the chloroplast galactolipids is derived, at least in part, from oleate esterified to the 3-*sn*-phosphatidylcholine of a low-density membrane fraction, containing microsomal fragments (Slack & Roughan, 1975), and that this phospholipid was probably involved in the initial desaturation of the oleate to linoleate (Roughan, 1970, 1975; Slack & Roughan, 1975). Oleate desaturases have been identified associated with the microsomal fraction of *Neurospora crassa* (Baker & Lynen, 1971), the yeast *Torulopsis utilis* (Talamo *et al.*, 1973) and with a 28000*g* pellet from *Chlorella vulgaris* (Gurr *et al.*, 1969) for which 3-*sn*-phosphatidylcholine oleate does appear to be the substrate. However, it is generally accepted that oleoyl-CoA is the substrate of the microsomal desaturases studied so far from higher plant tissues (Vijay & Stumpf, 1971; Abdelkader *et al.*, 1973; Tremolières & Mazliak, 1974). In the present paper we describe studies of the metabolism of oleoyl-CoA and oleate desaturation by leaf microsomal preparations which strongly suggest that oleate is incorporated into 3-*sn*-phosphatidylcholine from oleoyl-CoA before desaturation.

Experimental

Materials

Seedlings of pea (*Pisum sativum* cultivar Victory) and maize (*Zea mays* cultivar Wisconsin 436) were grown in trays of pumice–peat in a heated, naturally lit glasshouse and watered daily with nutrient solution (Hoagland & Arnon, 1938).

Sodium methoxide was prepared by dissolving sodium metal (1.2 g) in anhydrous methanol (100 ml) containing 2,2-dimethoxypropane (5%, v/v) (Dow Chemical Co., Midland, MI, U.S.A.). Diazomethane was prepared by the method of Schlenk & Gellerman (1960).

[1-^{14}C]Oleoyl-CoA (10 μCi/μmol) was prepared by the modified method of Goldman & Vagelos (1961) as described by Talamo *et al.* (1973). [1-^{14}C]-Oleic acid (57 μCi/μmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) and oleic acid, prepared from chromatographically pure methyl oleate (Nu Check Prep, Elysian, MN, U.S.A.) by saponification, were used in the appropriate proportions to give the above specific radioactivity. CoA (lithium salt) was from Sigma Chemical Co., St. Louis, MO, U.S.A.

Tetrahydrofuran was refluxed with $LiAlH_4$ and then distilled immediately before use, and 2,4,6-collidine was refluxed with KOH pellets before distillation. The oleoyl-CoA, dissolved in 1ml of 50mM-acetic acid, adjusted to pH5.0 with NaOH, was passed through a column (6cm×1cm) of Sephadex G-25, equilibrated with the above buffer, and stored in 0.2ml samples under N_2 at −25°C. The purity of the oleoyl-CoA was monitored by chromatography on thin-layer plates of silica gel G with chloroform/methanol/acetic acid/water (85:15:10:3, by vol.) as the solvent; this separated oleoyl-CoA, which remained at the origin, from unesterified oleate, which travelled with the solvent front. The proportion of label in unesterified oleate varied from 2 to 10% in the different preparations. When aqueous solutions of these preparations were extracted with chloroform/methanol (2:1, v/v) as described below, between 85 and 92% of the label remained in the aqueous phase indicating that most of the oleoyl-CoA partitioned into the aqueous phase. The concentration of oleoyl-CoA was determined by the E_{232} (Morris & Redfearn, 1969) and by quantitative g.l.c. of the methyl oleate prepared from oleoyl-CoA by transmethylation with 2ml of sodium methoxide solution for 15min at 25°C. The methyl oleate was extracted into light petroleum (b.p. 40–60°C), dried under N_2 and chromatographed at 180°C on 10% (w/w) Silar 10C on Gas-Chrom Q (100–120 mesh; Applied Science Lab Inc., State College, PA, U.S.A.). The response of the chromatograph was determined by using a standard mixture of fatty acid methyl esters (Nu Check Prep). Estimates of oleoyl-CoA concentration by the two methods differed by about 10%. The radioactive purity of oleoyl-CoA was determined by co-chromatography of the methyl ester with a standard mixture of fatty acid methyl esters as above, but with a stream splitter attached to the chromatograph. The effluent gas containing individual esters was collected and counted for radioactivity as described by Slack & Roughan (1975). Approx. 98% of the radioactivity co-chromatographed with methyl oleate and the remainder with methyl linoleate. The specific radioactivity of the oleoyl-CoA was measured by determining the mass and radioactivity of the methyl oleate derivative.

A solution of unlabelled oleoyl-CoA was prepared and its concentration determined as described above.

Methods

Microsomal preparations. Expanding leaflets (20g) from 11-day-old pea seedlings were chilled on ice, sliced into approx. 3mm thick pieces and homogenized for 3s at one-half maximum speed with a Polytron PT20 homogenizer (Kinematica, Lucerne, Switzerland) in 150ml of N_2-saturated 100mM-Tris/HCl, pH7.5, containing 5mM-$MgCl_2$, 2mM-EDTA, 0.1% bovine serum albumin, 0.33M-sorbitol and 10mM-cysteine/HCl, adjusted to pH5.0 with NaOH and added to the media immediately before homogenization. The homogenate was filtered through three layers of miracloth and the filtrate centrifuged at 2000*g* for 15s. The supernatant was then centrifuged at 15000*g* for 10min and the pellet discarded. This supernatant was centrifuged at 105000*g* for 1h and the pellet suspended in 5ml of 50mM-Hepes*/KOH, pH7.5, containing 5mM-$MgCl_2$ and 0.1% bovine serum albumin in a Ten-Broek homogenizer. In some experiments the 105000*g* pellet was resuspended in 30ml of the buffer mixture containing, in addition, 1mM-dithiothreitol and again centrifuged at 105000*g* for 1h, and in others 1ml portions of the suspended pellet and 105000*g* supernatant were separately passed through columns (10cm×0.8cm) of Sephadex G-25 pre-equilibrated with the resuspension media. The 105000*g* pellets were similarly prepared from the immature lamina, the most recently emerged 10cm-long segment of the emerging sixth leaf, and from mature lamina of maize seedlings except that the laminae were homogenized for 10s to obtain adequate cell breakage. Protein was measured by the method of Schacterlle & Pollack (1973) with bovine serum albumin (fraction V) as a standard. All procedures were performed at between 0° and 4°C.

Determination of 3-sn-phosphatidylcholine in 105000g pellet. The resuspended pellet (0.5ml) was extracted with 2.5ml of chloroform/methanol (2:1, v/v) and the chloroform layer was evaporated to dryness under vacuum at 40°C. The extract was dissolved in chloroform and transferred to a screw-cap tube, dried under O_2-free N_2 and dissolved in 0.4ml of chloroform containing 0.005% butylated hydroxytoluene (3,5-di-t-butyl-4-hydroxytoluene). Samples were chromatographed on thin-layer plates of silica gel G in chloroform/methanol/acetic acid/water (85:15:10:3, by vol.) and the lipids stained with I_2 vapour. The phosphate content of the 3-*sn*-phosphatidylcholine region was determined by the method of Rouser *et al.* (1966).

Assay of oleate desaturase. All assays were performed with a freshly prepared microsomal fraction. Unless stated otherwise in the text each reaction mixture contained 50mM-Hepes/KOH, pH7.5, 5mM-$MgCl_2$, 1.8mM-NADPH, 1% bovine serum albumin, [1-^{14}C]oleoyl-CoA (0.010μmol, 2.5×10^5d.p.m.) and 0.15ml of the 105000*g* pellet in a tube (120mm×1.3mm) in a total volume of 0.4ml. The mixtures were kept on ice. Oleoyl-CoA was added to start the reaction. The tubes were then incubated at 25°C with shaking at 100 strokes/min. The reaction was stopped by the addition of 5vol. of chloroform/

* Abbreviation: Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

methanol (2:1, v/v), The radioactivity in the chloroform and aqueous-methanol layers was counted on a gas-flow counter at 20% efficiency. The lipids were separated and detected as described above and the plates dried for 1h at room temperature in a stream of N_2. The fatty acids of phosphatidylcholine were methylated by incubating the silica gel from the chromatogram containing this lipid with 3.0ml of sodium methoxide for 15min at 20°C. Light petroleum (5ml) (b.p. 40–60°C) was added followed by 2.0ml of water and the methyl esters were extracted into the light petroleum by shaking. The methyl esters were dried under N_2, redissolved in the light petroleum and separated by gas chromatography as described above. The mass of the individual esters was determined by comparison with standard methyl esters and the radioactivity in each determined, as described above, at a counting efficiency of 55%. Silica gel, from the region of the solvent front, from the thin-layer chromatograms was transferred to screw-cap tubes and the lipids were saponified under O_2-free N_2 with 10% KOH in methanol (w/v) for 1h at 95°C. The saponifiable material was methylated with diazomethane and the mass and radioactivity in the fatty acid methyl esters were determined as described above. Alternatively, silica gel from the region of the solvent front was extracted with 1ml of water containing 1.6ml of methanol and then 3.3ml of chloroform was added and the lipids were extracted into the chloroform layer. This was dried under N_2 and the extract chromatographed on thin-layer plates of silica gel G in light petroleum (b.p. 60–80°C)/diethyl ether/acetic acid (75:25:1, by vol.) to separate free fatty acids and mono- and di-acyl glycerols. The chromatograms were either radioautographed or the region containing unesterified fatty acids was extracted with diethyl ether, the fatty acids were methylated with diazomethane and the distribution of radioactivity in their methyl esters, separated by gas chromatography, was determined as above. The total radioactivity in individual lipids was measured by transferring the individual lipid-containing regions (separated by t.l.c. as above) into scintillation vials containing 0.5% *p*-terphenyl in xylene/Triton X-114/water (15:5:2, by vol.) and counted at 50% efficiency.

Separation of cell organelles either labelled in vitro with [1-^{14}C]oleoyl-CoA or in vivo with [1-^{14}C]acetate. Expanding pea leaflets (2g) were homogenized as described above in 10ml of 100mM-Hepes/NaOH containing 2mM-$MgCl_2$, 2mM-EDTA, 5mM-dithiothreitol, 10mM-KCl and 0.33M-sorbitol and filtered through three layers of miracloth. The filtrate (3ml) was incubated at 25°C with 0.1μmol of [1-^{14}C]-oleoyl-CoA under N_2 for 20min and the reaction mixture chilled to 0°C. The reaction mixture was centrifuged at 2000*g* for 15s and the lipids were extracted as described above. The supernatant was layered on to a sucrose density gradient consisting of 16ml of a linear gradient from 28 to 40% (w/w) sucrose below 3ml of 20% (w/w) sucrose and centrifuged in a MSE rotor (no. 59590) at 53000*g* for 4h at 2°C. The sucrose solutions also contained 50mM-Hepes/NaOH, pH7.5, 1mM-$MgCl_2$ and 3mM-EDTA. The gradient was pumped through a flowcell (Uvicord II, LKB Bromma, Sweden) and the transmission at 280nm recorded; fractions (0.7ml) were collected. The sucrose concentration was measured with a refractometer and the lipids were extracted as above. The radioactivity in lipids of individual fractions was determined in a gas-flow counter and the lipids in alternate samples were separated by chromatography and counted for radioactivity as described above.

Detached pea seedlings (10 days old) were fed with [1-^{14}C]acetate under the conditions described by Slack & Roughan (1975), homogenized and the organelles fractionated by differential and sucrose-density-gradient centrifugation and the distribution of radioactivity amongst the lipids was as determined above.

Results

Comparison of the metabolism of oleoyl-CoA by leaf homogenates with the metabolism of acetate in vivo.

After incubating a pea-leaflet homogenate for 20min with [1-^{14}C]oleoyl-CoA most of the label (85%) was in unesterified oleic acid, 14% was present in 3-*sn*-phosphatidylcholine and 1% in 3-*sn*-phosphatidylethanolamine. One-fifth of the radioactivity was associated with cell components, mainly chloroplasts, that were pelleted at 2000*g*, of which only 7% was in phosphatidylcholine. Most of the radioactivity in this glycerolipid was found to be associated with a low-density fraction when the 2000*g* supernatant was separated by sucrose-density-gradient centrifugation (Fig. 1*a*). This cell fraction, probably of microsomal membranes (Kagawa *et al.*, 1973, 1975; Slack & Roughan, 1975), also contained the bulk of the labelled 3-*sn*-phosphatidylcholine in pea leaflets supplied *in vivo* with labelled acetate for 20min (Fig. 1*b*). Since oleate is the fatty acid of 2-*sn*-phosphatidylcholine most rapidly labelled *in vivo* (Slack & Roughan, 1975) it would appear that oleate is preferentially incorporated into the 3-*sn*-phosphatidylcholine of the same cell component both *in vivo* and *in vitro*. The higher-density zone that contained a considerable proportion of the labelled unesterified oleic acid, derived from [1-^{14}C]-oleoyl-CoA (Fig. 1*a*), contained chloroplast lamellae.

Metabolism of oleoyl-CoA by pea leaflet microsomal fractions

The results described in Fig. 1(*a*) suggested that the pea-leaf homogenates contained both a thiol

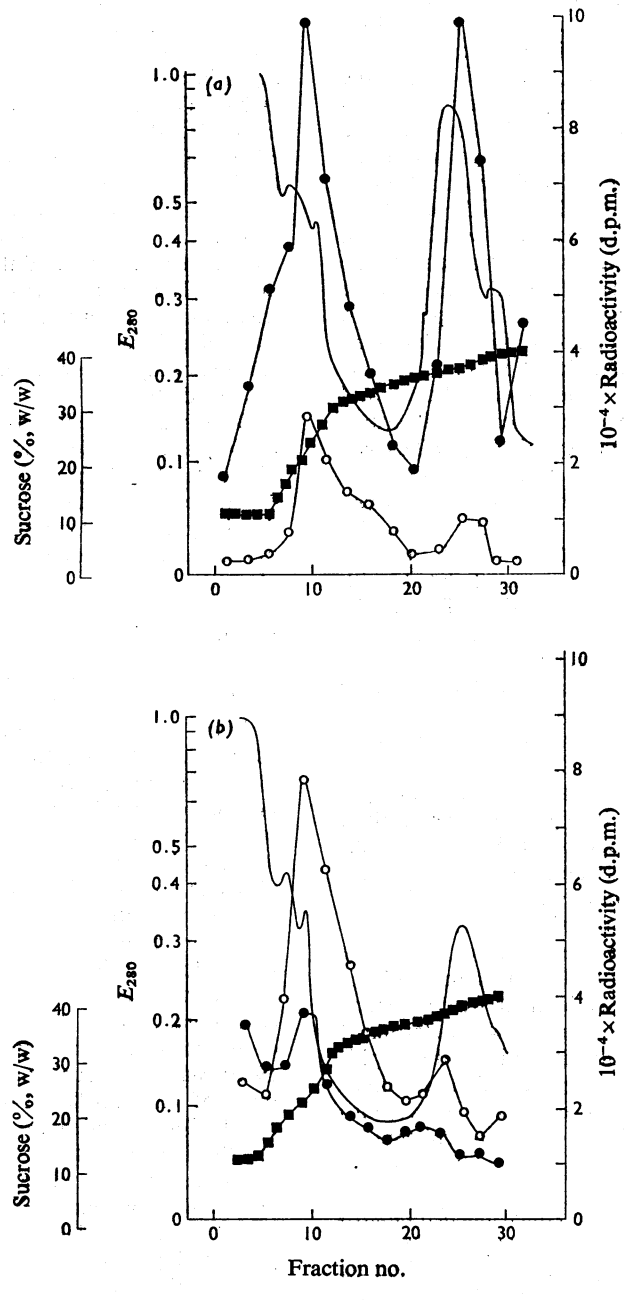

Fig. 1. *Comparison of the distribution of labelled 3-sn-phosphatidylcholine and free fatty acid in pea-leaf homogenates supplied with [1-^{14}C]oleoyl-CoA in vitro and [1-^{14}C]acetate in vivo*

The 2000*g* supernatants were subjected to sucrose-density-gradient centrifugation and the distribution of 280nm-absorbing material (——), radioactivity in 3-*sn*-phosphatidylcholine (○) and free fatty acids (●) and sucrose concentration (■) within the gradient were determined as described in the Experimental section. (*a*) Leaf homogenates were incubated with [1-^{14}C]-oleoyl-CoA for 20min and then fractionated. (*b*) Seedlings were supplied with [1-^{14}C]acetate for 20 min and then the leaflets were homogenized and fractionated.

ester hydrolase, that hydrolysed oleoyl-CoA to oleate and CoA, and lysolecithin acyltransferase (acyl - CoA – 1 - acylglycero - 3 - phosphocholine *O*-acyltransferase; EC 2.3.1.23). Since both oleoyl-CoA hydrolase and lysolecithin acyltransferase are associated with microsomal preparations (Devor & Mudd, 1971; Vijay & Stumpf, 1971), it appeared possible that the relative activities of the two enzymes could depend on the proportion of the microsomal fraction and oleoyl-CoA in the reaction mixture, since the former also contains the acceptor for the acyltransferase. The influence of various amounts of microsomal preparation at a constant oleoyl-CoA concentration and of various concentrations of oleoyl-CoA at a fixed microsomal preparation concentration on the reaction products are shown in Figs. 2(*a*) and 2(*b*) respectively. A 105000*g* pellet was used as the microsomal fraction; this preparation contained various amounts of chloroplast lamellae in different experiments and consequently microsomal fractions were quantified by their content of phosphatidylcholine, rather than protein, since microsomal fractions, but not the plastid lamellae, contain an appreciable quantity of this lipid (Douce *et al.*, 1973). The highest ratio of microsomal preparation/oleoyl-CoA gave the greatest percentage incorporation of oleate into 3-*sn*-phosphatidylcholine, and, at this, and similar ratios of microsomal preparation/oleoyl-CoA, the metabolism of the thiol ester was essentially complete within 5min. At lower microsomal preparation/oleoyl-CoA ratios the incorporation of oleate into 3-*sn*-phosphatidylcholine proceeded for a longer period, but the formation of free oleate, although initially rapid, soon ceased. At present we are unable to explain this apparent cessation of hydrolysis of oleoyl-CoA concomitant with the continued incorporation of oleate into 3-*sn*-phosphatidylcholine.

Reactions described in Fig. 2 were performed under conditions found necessary for oleate desaturation by microsomal preparations isolated from other organisms (Baker & Lynen, 1971; Abdelkader *et al.*, 1973; Talamo *et al.*, 1973). At the end of the incubation radioactivity was present in the linoleate of 3-*sn*-phosphatidylcholine (Table 1) but not in free linoleic acid. No significant quantity of radioactivity was present in 3-*sn*-phosphatidylcholine linolenate or free linolenic acid. The proportion of the radioactivity in linoleate bore little relationship to the

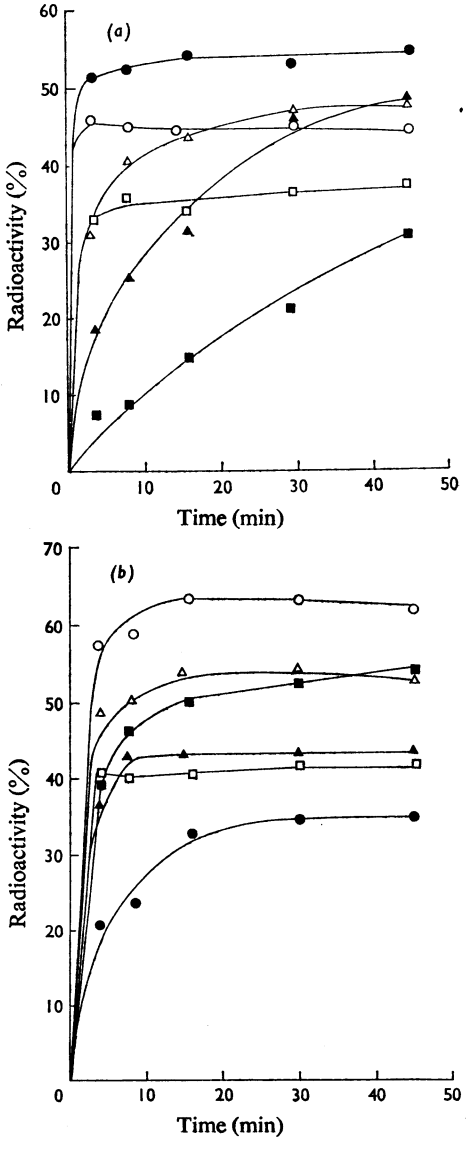

Fig. 2. *Influence of the relative concentrations of* [1-^{14}*C*]-*oleoyl-CoA and the microsomal fraction on the distribution of oleate between* 3-*sn-phosphatidylcholine and free acid*

Reaction mixtures (vol. 0.7ml) were incubated and samples (0.1ml) removed at intervals for analysis as described in the Experimental section. ▲, ■, ●, 3-*sn*-Phosphatidylcholine; △, □, ○, free acid. (*a*) Reaction mixtures contained 10nmol of [1-^{14}C]oleoyl-CoA and microsomal preparations containing: ■ □, 75nmol of 3-*sn*-phosphatidylcholine; ▲ △, 150nmol of 3-*sn*-phosphatidylcholine; ● ○, 580nmol of 3-*sn*-phosphatidylcholine. (*b*) Reaction mixtures contained microsomal preparations containing 580nmol of 3-*sn*-phosphatidylcholine and the following: ■ □, 16nmol of oleoyl-CoA; ▲ △, 66nmol of oleoyl-CoA; ● ○, 120nmol of oleoyl-CoA. Each reaction mixture contained 10nmol of [1-^{14}C]-oleoyl-CoA and various quantities of unlabelled oleoyl-CoA. Results are expressed as a percentage of the radioactivity present in the reaction mixtures.

Table 1. *Influence of the relative concentrations of the microsomal fraction and* [1-^{14}*C*]*oleoyl-CoA on the labelling of 3-sn-phosphatidylcholine linoleate*

The composition of reaction mixtures are described in Fig. 2. The distribution of radioactivity in fatty acids of 3-*sn*-phosphatidylcholine after 60min incubation was determined as described in the Experimental section, and the radioactivity in linoleate was expressed as a percentage of that in oleate plus linoleate. The quantity of 3-*sn*-phosphatidylcholine oleate that could have been desaturated and the amount of 3-*sn*-phosphatidylcholine oleate in each reaction were determined as outlined in the text.

Concentration of phosphatidylcholine in the microsomal fraction (μmol/reaction)	Oleoyl-CoA supplied (nmol/reaction)	Radioactivity in 3-*sn*-phosphatidylcholine linoleate (%)	3-*sn*-Phosphatidylcholine oleate (nmol/reaction)	Estimate of 3-*sn*-phosphatidylcholine oleate desaturated (nmol/reaction)
0.075	10	7.0	9	—
0.15	10	11.1	16	1.8
0.29	10	9.6	28	2.7
0.58	10	13.0	51	6.6
0.58	16	13.4	53	7.1
0.58	31	11.7	59	6.9
0.58	62	11.3	66	7.5
0.58	124	10.5	73	—

quantity of microsomal preparation present. If the labelled linoleate in 3-*sn*-phosphatidylcholine was derived from linoleyl-CoA which was produced by an oleoyl-CoA desaturase, as Vijay & Stumpf (1971) have proposed for oleate desaturation by an enzyme of safflower-seed microsomal preparations and Abdelkader *et al.* (1973) and Tremolières & Mazliak (1974) have inferred for desaturases of potato tuber and pea-leaf microsomal fractions respectively, then in most reactions the formation of labelled linoleate would have ceased after 5 min since little or no labelled oleoyl-CoA remained. However, if the oleate esterified to 3-*sn*-phosphatidylcholine was involved in the desaturation process, as has been suggested for other microsomal oleate desaturases (Gurr *et al.*, 1969; Baker & Lynen, 1971; Talamo *et al.*, 1973), then the data expressed as percentage radioactivity in linoleate would provide a false indication of the extent of desaturation since the amount of 3-*sn*-phosphatidylcholine oleate varied in the different reactions. We have estimated the amount of 3-*sn*-phosphatidylcholine oleate present during each reaction from the amount originally present in the microsomal preparation plus that incorporated from oleoyl-CoA (Fig. 2) and assumed that this equalled the quantity of oleate available for desaturation, i.e. that all 3-*sn*-phosphatidylcholine molecules, containing oleate, were equally good substrates for desaturation (Table 1). These estimates indicate an essentially linear increase in desaturation with increasing microsomal concentration.

Time-course of oleate desaturation

In the experiment described in Fig. 3, the proportion of radioactive counts in the oleate of 3-*sn*-phosphatidylcholine decreased linearly with time over a 40min period, whereas that in the linoleate of this lipid increased linearly with time. Despite the loss of radioactivity from oleate during the reaction, its specific radioactivity increased slightly, concomitant with the large increase in the specific radioactivity in the 3-*sn*-phosphatidylcholine linoleate. One interpretation of this observation is that the unlabelled oleate of phosphatidylcholine was desaturated somewhat more rapidly than the labelled oleate. As Table 1 shows there was no significant radioactivity in free linoleic acid. In certain experiments, however, some label did appear, with time, in the linoleate, which chromatographed with neutral lipids at the solvent front of the chromatogram (Fig. 4). The kinetics of labelling of this linoleate and of phosphatidylcholine linoleate were different and were consistent with the view that the label appearing in the former could have been derived from the latter. Approximately 90% of the label at the chromatogram solvent front was in the unesterified fatty acid and the percentage in linoleate was similar to that in the total neutral lipids. We assume, therefore, that at least most of the labelled linoleate, not in 3-*sn*-phosphatidylcholine, was in the unesterified fatty acid.

Factors affecting oleate-desaturation

As previously found with microsomal desaturases from other organisms (Vijay & Stumpf, 1971; Baker & Lynen, 1971; Abdelkader *et al.*, 1973; Talamo *et al.*, 1973) the enzyme from pea leaf was inhibited by the exclusion of O_2 from the reaction mixture (Table 2). The extent of desaturation was similar, however, in reactions equilibrated with air and with 100% O_2 and we assume, therefore, that the assay

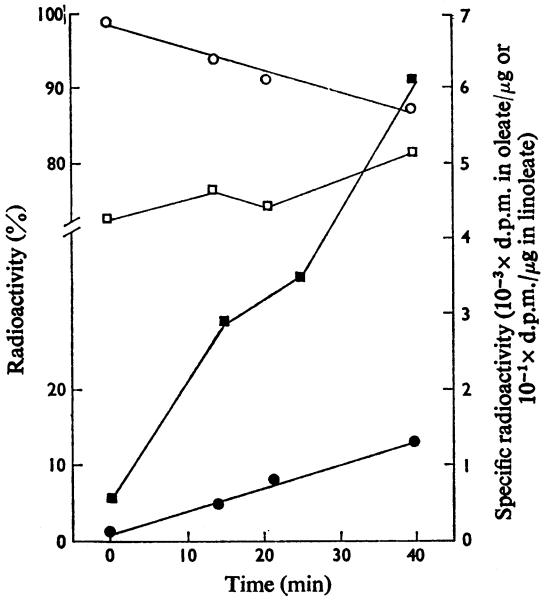

Fig. 3. *Time-course of changes in radioactivity in 3-sn-phosphatidylcholine oleate and 3-sn-phosphatidylcholine linoleate*

Microsomal preparations containing 0.9μmol of 3-*sn*-phosphatidylcholine were incubated with 80nmol of [1-^{14}C]oleoyl-CoA as described in the Experimental section, except that the diameter of the reaction tube was 25mm and the reaction volume was 2.0ml. The mixture also contained 0.5ml of the 105000*g* supernatant (1mg of protein). At the start of the reaction a 0.3ml sample was gassed with N_2 then incubated and analysed after 40min to provide a zero-time estimate of desaturation. The remaining mixture was incubated in air and 0.3ml samples were removed at intervals and analysed. Percentage radioactivities of 3-*sn*-phosphatidylcholine oleate (○) and 3-*sn*-phosphatidylcholine linoleate (●) and their respective specific radioactivities (□, ■) were determined.

conditions normally used provided non-limiting oxygenation. Dithiothreitol at concentrations above 1mM inhibited desaturation in aerated reaction mixtures. Bovine serum albumin at 1% (w/v) increased the amount of desaturation when present during assays, but its addition to the extraction media had no effect on activity.

Oleate desaturation was catalysed by the unwashed microsomal preparation without added nucleotides (Fig. 5), but the addition of NADPH (not shown) and of NADPH plus the crude 105000*g* supernatant increased activity. Gel filtration of the microsomal preparation and 105000*g* supernatant with Sephadex G-25 decreased, but did not eliminate activity, and the addition of either NADPH or crude supernatant enhanced the activity after Sephadex treatment.

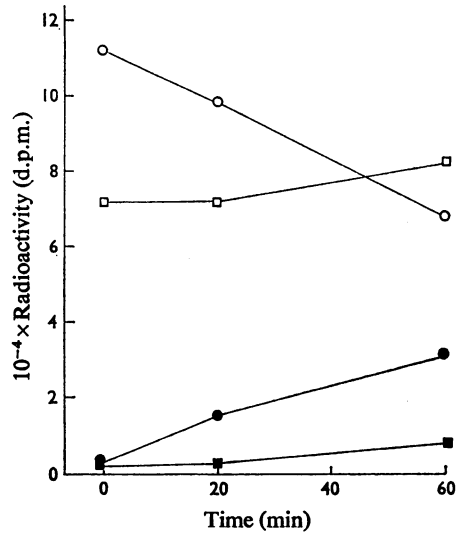

Fig. 4. *Time-course of changes in radioactivity in 3-sn-phosphatidylcholine oleate, 3-sn-phosphatidylcholine linoleate and free oleic and linoleic acids*

Reaction mixtures with microsomal preparations containing 0.56μmol of 3-*sn*-phosphatidylcholine, 0.2ml of 105000*g* supernatant (0.4mg of protein) and 19nmol of [1-^{14}C]oleoyl-CoA were incubated as described in the Experimental section, in 0.6ml total volume, either under N_2 to provide an estimate of zero-time distribution of radioactivity, or in air. Reactions were stopped at the times indicated and analysed as described in the Experimental section to determine the radioactivity in 3-*sn*-phosphatidylcholine oleate (○), oleate (□), 3-*sn*-phosphatidylcholine linoleate (●) and linoleate (■).

Table 2. *Influence of O_2 concentration, dithiothreitol and bovine serum albumin on desaturation of 3-sn-phosphatidylcholine oleate*

Control reactions were performed as described in the Experimental section; others were modified as indicated in the Table. The proportion of radioactivity in 3-*sn*-phosphatidylcholine linoleate was measured after 60min incubation.

Expt.	Conditions	Desaturation (%)
1	Control (20% O_2)	16.9
	100% O_2	16.8
	100% N_2	0
2	Control	15.4
	1mM-dithiothreitol	15.2
	5mM-dithiothreitol	1.0
	20mM-dithiothreitol	0
3	Control	27.4
	Minus bovine serum albumin	17.0

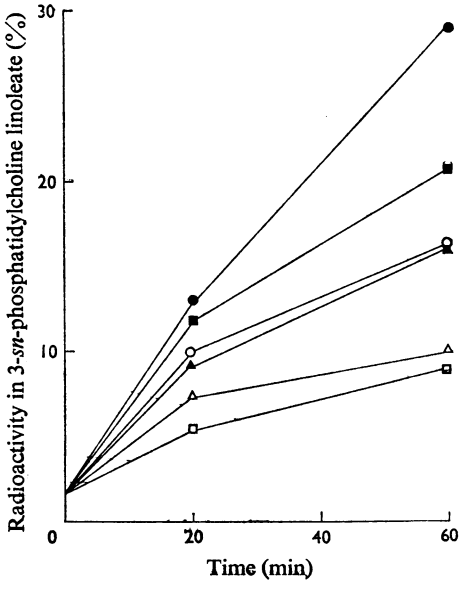

Fig. 5. *Desaturation of 3-sn-phosphatidylcholine oleate by microsomal preparations before and after gel-filtration*

The microsomal preparation and 105000*g* supernatant were used either directly or first passed through columns of Sephadex G-25. In all reaction mixtures the microsomal preparation contained 0.37 μmol of 3-*sn*-phosphatidylcholine, and where indicated 0.3 mg of supernatant protein. Assays were performed as described in the Experimental section and reaction mixtures contained: microsomal preparation (○); microsomal preparation, supernatant and NADPH (●); Sephadex-treated microsomal preparation (□); Sephadex-treated microsomal preparation and supernatant (■); Sephadex-treated microsomal preparation and Sephadex-treated supernatant (△); Sephadex-treated microsomal preparation, Sephadex-treated supernatant and NADPH (▲). The percentage radioactivity in 3-*sn*-phosphatidylcholine linoleate was determined as described in Table 1 and the zero-time percentage radioactivity in this fatty acid was estimated from reactions performed under N_2.

Though the centrifuge tubes were drained before resuspending the microsomal pellets, a small amount of supernatant was undoubtedly carried over into the resuspended pellet. The observation that gel-filtration decreased but did not eliminate activity could be interpreted as indicating that both high- and low-molecular-weight components of the supernatant were necessary for desaturation. Such an interpretation would be in accord with the observation of Talamo *et al.* (1973) who found an absolute requirement for supernatant, in addition to NADPH, for oleate desaturation by the microsomal preparation from the yeast *T. utilis*. However, this interpretation seems unlikely since NADPH alone restored

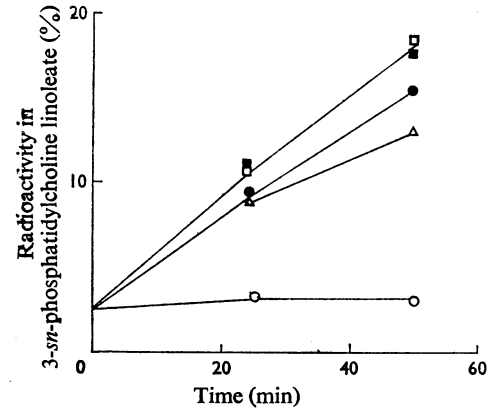

Fig. 6. *Desaturation of 3-sn-phosphatidylcholine oleate by washed microsomal preparation*

A 105000*g* pellet was resuspended and repelleted before use, and the supernatant obtained from the first centrifugation was used either directly or first passed through a column of Sephadex G-25. Assays were performed as described in the Experimental section in reaction mixtures with microsomal preparations containing 0.32 μmol of 3-*sn*-phosphatidylcholine and 0.01 μmol of [1-^{14}C]oleoyl-CoA. ○, Microsomal preparation; ●, microsomal preparation and supernatant (0.25 mg of protein); □, microsomal preparation and NADPH; △, microsomal preparation and Sephadex-treated supernatant (0.2 mg of protein); ■, microsomal preparation, Sephadex-treated supernatant and NADPH. The percentage radioactivity in 3-*sn*-phosphatidylcholine linoleate was determined as in Fig. 5.

desaturase activity of the pea-leaf microsomal fraction that had been repelleted after suspension in a large volume of media (Fig. 6). The observation that Sephadex-treated supernatant also restored activity suggests that some high-molecular-weight component may act in a manner similar to NADPH. NADH and $NADP^+$ were equally effective as NADPH in stimulating the desaturase activity of the washed microsomal preparation, but NAD^+ was ineffective.

Oleate desaturase activity of the microsomal preparation from lamina of other species

Microsomal preparations from expanding spinach leaf and from immature and mature maize lamina also rapidly converted [1-^{14}C]oleoyl-CoA into free oleate and 3-*sn*-phosphatidylcholine oleate. However, only with microsomal preparations from immature maize lamina was label detected in the linoleate of 3-*sn*-phosphatidylcholine (Table 3). No significant amount of radioactivity occurred in unesterified linoleate. The extent of [1-^{14}C]oleate desaturation with these microsomal preparations was considerably

Table 3. *Comparison of labelling of microsomal 3-sn-phosphatidylcholine linoleate from maize and pea leaf supplied with [1-^{14}C]oleoyl-CoA*

The microsomal fractions from immature and mature maize lamina were incubated and analysed as described in the Experimental section; reaction mixtures contained in addition 0.3 and 0.32mg of supernatant protein from the respective preparations. Data for pea-leaf microsomal preparations are from the experiment described in Fig. 4.

Microsomal preparation	Concentration of 3-*sn*-phosphatidylcholine in the microsomal fraction (μmol/reaction)	3-*sn*-Phosphatidylcholine oleate (nmol/reaction)	Radioactivity in 3-*sn*-phosphatidylcholine linoleate (%)	
			20min	60min
Immature maize lamina	0.72	123	3.8	6.8
Mature maize lamina	0.61	82		1.6
Immature pea leaflets	0.53	43	11.4	28

smaller than with those from pea. However, the differences in the actual amounts of desaturation obtained with these microsomal preparations may be less if, as discussed above, 3-*sn*-phosphatidylcholine oleate is involved in the desaturation process, since the molar percentage of oleate of the microsomal 3-*sn*-phosphatidylcholine of maize was greater than that of peas (Table 3) and hence the specific radioactivity of this fatty acid after incorporation of similar amounts of radioactive oleate would be expected to be lower in the maize 3-*sn*-phosphatidylcholine. In this regard, it is noteworthy that the molar percentage of oleate in 3-*sn*-phosphatidylcholine of spinach-leaf microsomal fractions was high (22.5) compared with pea microsomal fractions (2.9) and this may have contributed to our inability to detect desaturation.

Discussion

[1-^{14}C]Oleoyl-CoA was rapidly metabolized by leaf microsomal preparations and, at the microsomal and oleoyl-CoA concentrations used, the amount remaining after a few minutes incubation was not detectable. Despite the absence of substrate amounts of this thiol ester, the label in the linoleate of 3-*sn*-phosphatidylcholine increased and that in the oleate of this phospholipid decreased over a period of at least 60min. This observation suggests that the leaf microsomal oleate desaturase may, as previously proposed for oleate desaturases of *C. vulgaris* (Gurr *et al.*, 1969), *N. crassa* (Baker & Lynen, 1971) and *T. utilis* (Talamo *et al.*, 1973), utilize the oleate esterified to 3-*sn*-phosphatidylcholine, rather than to CoA, as substrate. Such a reaction is consistent with the extent of desaturation observed with various amounts of microsomal preparations and oleoyl-CoA (Table 1).

The rapid, and essentially complete, metabolism of oleoyl-CoA consistently observed in the present studies differs from that reported for safflower (*Carmanthus tinctorius*) seed microsomal preparation (Vijay & Stumpf, 1971), which, in contrast with the leaf microsomal preparation, contained no thiol ester hydrolase. These workers also found that the initial rate of oleate transfer to glycerolipid, mainly 3-*sn*-phosphatidylcholine, was rapid, but that the conversion of thiol ester into oxygen ester was not complete. We can offer no positive explanation for these differences. However, it is noteworthy that Nichols & Safford (1973) showed that reductive cleavage by $NaBH_4$, used by Vijay & Stumpf (1971) to distinguish acyl thiol esters and oxygen esters, forms fatty alcohols from 3-*sn*-phosphatidylcholine as well as from acyl-CoA and concluded that this procedure would overestimate the content of thiol ester present in a mixture of thiol and oxygen esters.

We were unable to demonstrate an absolute requirement for a soluble component in the oleate-desaturating system of pea-leaf microsomal fraction, though our results indicated that a high-molecular-weight component of the 105000*g* supernatant could be substituted for a pyridine nucleotide. On the other hand, Talamo *et al.* (1973) found that the yeast microsomal oleate desaturase required a supernatant factor, whereas no such factor was identified for oleate desaturases of the microsomal fraction from safflower seed (Vijay & Stumpf, 1971), *N. crassa* (Baker & Lynen, 1971) or potato tuber (Abdelkader *et al.*, 1973) even though the microsomal preparations in the latter two studies were washed before assay. Details of the similarities and differences of the oleate-desaturating system of the various organisms must await some means of solubilizing this enzyme system.

The observation that oleate was incorporated from oleoyl-CoA, *in vitro*, into the 3-*sn*-phosphatidylcholine of the same microsomal membrane fraction as was the oleate synthesized from [1-^{14}C]acetate, *in vivo*, supports our previous suggestion (Slack & Roughan, 1975) that the desaturation of oleate in

leaves is associated with a microsomal membrane. Since there is increasing support for the belief that plastids are the site of oleate biosynthesis both in non-photosynthetic tissue (Weaire & Kekwick, 1975) and leaves (Nakamura & Yamada, 1975) of higher plants, it follows that an interorganelle transfer of oleate between plastids and the microsomal fraction must occur. At present the mechanism by which this is achieved is not known, but since the present studies and those of Vijay & Stumpf (1971) indicate that plant microsomal fractions possess a highly active lysolecithin acyltransferase, it is possible that oleoyl-CoA is the immediate donor of oleate to the microsomal phospholipid.

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