TECHNICAL

# Properties of the Glycerol Acylating Enzymes in Microsomal Preparations from the Developing Seeds of Safflower (*Carthamus tinctorius*) and Turnip Rape (*Brassica campestris*) and their Ability to Assemble Cocoa-Butter Type Fats.

**Maureen Bafor**[1], **Allan Keith Stobart**[2] and **Sten Stymne***
Department of Plant Physiology, Swedish University of Agricultural Sciences, P.O. Box 7047, S-750 07 Uppsala, Sweden

**Microsomal membrane preparations from the developing seeds of safflower (*Carthamus tinctorius*, var. Gila) and turnip-rape (*Brassica campestris*, var. Bele) catalyzed the assembly of triacylglycerols (triglycerides) from sn-glycerol 3-phosphate and acyl-CoA. The membrane preparations were used to assess the acyl specificity properties of the initial acylating enzymes—glycerol 3-phosphate acyltransferase (GPAT) and 1-acylglycerol 3-phosphate acyltransferase (lysophosphatidic acid acyltransferase, LPAAT)—that are responsible for the fatty acids at positions sn-1 and sn-2 of the sn-triacylglycerol, respectively. In spectrophotometric assays it was possible to evaluate, to some extent, how these enzymes will utilize unusual and foreign fatty acids that are not normally found in these particular plant species. The acylating enzymes from both plants used, to varying extents, a comprehensive range of acyl-CoA donor species and some kinetic properties of the substrates involved are presented. The enzymes from safflower, however, were generally the more selective, whereas the turnip-rape was less particular and could utilize a range of acyl substrates. The enzymes from both plants hardly utilized erucate (C22:1), and the significance of this is discussed in terms of mechanisms which have evolved in order to exclude certain, perhaps detrimental, fatty acids from structural membrane lipids and dedicate them to storage lipid assembly.**

**The ability of the microsomal preparations, from the developing seeds of both plants, to synthesize cocoa-butter type fats was investigated. Microsomal membranes were incubated with glycerol 3-phosphate and equimolar amounts of palmitate, oleate and stearate. Safflower preparations catalyzed the construction of sn-triacylglycerol with largely palmitate, oleate and stearate in positions sn-1, 2 and 3, respectively. The selectivity for acyl species in rape was less pronounced, however, substantial saturated-unsaturated-saturated oils were still produced. The results are discussed in terms of the acyl selectivity properties of the glycerol acylating enzymes. It is evident that given the correct composition of fatty acids, the plant can produce cocoa-butter or other exotic fats.**

Research worldwide is attempting to elucidate the regulatory mechanisms of the fatty acid synthetase (FAS) enzyme-complex in plants and to understand how chain termination is regulated. A consequence of this could be the ability to manipulate the fatty acid products in oil seeds through genetic engineering, and hence some of the constituent enzymes have been characterized molecularly (1-3). It is, however, also necessary to establish whether the enzymes for the assembly of the triacylglycerol (triglyceride) in the recipient plant will utilize the appropriate fatty acids being synthesized by the modified FAS and yield the desired oils in a realistic quantity.

A case in point is that of cocoa-butter. Cocoa-butter is characterized by being particularly rich in the saturated fatty acids palmitate (C16:0) and stearate (C18:0), and the monounsaturated fatty acid oleate (C18:1) (4). The major species of triacylglycerol is predominantly (>70%) saturated in positions sn-1 and sn-3, and with the oleate in position sn-2 (5). The triacylglycerol palmitate-oleate-stearate (POS) constitutes nearly 50% of the total with significant proportions of palmitate-oleate-palmitate (POP; 16%) and stearate-oleate-stearate (SOS; 33%). It is this spectrum of triacylglycerol fatty-acid quality which endows the fat with it's unique physical and chemical properties useful for the manufacture of chocolate and the production of high premium confectionary fats. The development of new varieties with similar constituents to cocoa-butter, perhaps through recombinant DNA technology, has been speculated upon (6,7). With this in mind, it is necessary to consider the possible changes which would have to be brought about in the seeds of the appropriate plant species. In many plants a significant alteration in the quality of the fatty acids being synthesized in the seed would have to take place. The ability of the system to utilize these fatty acids in a correct fashion for the assembly of cocoa-butter triacylglycerols will also be a necessary prerequisite for the successful outcome of such projects.

It is now possible to ascertain the latter for a number of oleaceous crops. Microsomal membrane preparations from the developing seed-cotyledons of some species will catalyze the assembly of triacylglycerol from the substrates, acyl-CoA and sn-glycerol 3-phosphate at sufficient rates to give rise to fat droplets in the reaction mixtures (6,8). These *in vitro* model systems can also provide important information on whether a particular plant species will be able to efficiently synthesize oils containing foreign fatty acids. This is particularly pertinent in the future design of strategies necessary for the transformation of conven-

[1]Present address: Biochemistry Division, Nigerian Institute for Oil Palm Research, P.M.B. 1030, Benin City, Nigeria.
[2]Present address: Department of Botany, University of Bristol, Bristol BS8 1UG, U.K.
*To whom correspondence should be addressed.

tional crops that will produce unusual fatty acids in their storage products.

The assembly of the triacylglycerol occurs through the participation of reactions in the so-called Kennedy pathway (9), and involves the acylation of the glycerol backbone in three steps. The sequence is specific and initially the acylation occurs at positions sn-1 and 2 by the enzymes, glycerol 3-phosphate acyltransferase (GPAT) and 1-acyl-glycerol 3-phosphate acyltransferase (LPAAT), respectively (10). The final acylation that occurs at position sn-3 is unique to triacylglycerol biosynthesis and is catalyzed by diacylglycerol acyltransferase (DAGAT) (10). Therefore, these acyltransferase enzymes regulate the stereochemical distribution of fatty acids in the final triacylglycerol. In this paper, we assess the acylating properties of the enzymes involved in triacylglycerol assembly, in the microsomal membranes prepared from the developing seeds of safflower and turnip-rape, in order to establish their ability to utilize unusual fatty acids and to catalyze the formation of cocoa-butter type fats.

## MATERIALS AND METHODS

*Chemicals.* [1-$^{14}$C]Palmitic acid (hexadecanoic acid), [1-$^{14}$C]-stearic acid (octadecanoic acid), [1-$^{14}$C]oleic acid (octadeca-9-enoic acid) and [1-$^{14}$C]linoleic acid (octadeca-9,12-dienoic acid) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. bovine serum albumin (fraction V, fatty acid free), CoASH, phospholipase $A_2$ [from Indian-cobra (*Naja naja*) venom], L-sn-glycerol 3-phosphate, 1-oleoyl-sn-glycerol 3-phosphate (lysophosphatidic acid), and the various fatty acids were purchased from Sigma Chemical Company (St. Louis, MO).

[1-$^{14}$C]Palmitoyl-CoA-g[1-$^{14}$C]stearoyl-CoA (sp. radioactivity 1450 d.p.m./nmol), [1-$^{14}$C]oleoyl-CoA (sp. radioactivity of 1450 d.p.m./nmol for the *in vitro* oil synthesizing experiments and 2850 d.p.m./nmol for the lysophosphatidic acid acyltransferase measurements), [1-$^{14}$C]linoleoyl-CoA (sp. radioactivity 2850 d.p.m./nmol) and various nonradioactive acyl-CoAs were synthesized from their mixed anhydrides as described by Sanchez *et al.* (11).

*Plant material and microsomal preparations.* Safflower (*Carthamus tinctorius,* var. Gila) and turnip-rape (*Brassica campestris*, var. Bele) plants were grown from seed in a 16 hr photoperiod at 25°C and an 8 hr night at 18°C. Seeds were harvested at a stage of mid-maturity (14–18 and 22–28 days after flowering for safflower and rape, respectively), and the cotyledons removed and stored on ice. The cotyledons were ground in a mortar with two parts (w/v) of 0.1 M-potassium phosphate buffer, pH 7.2, containing 0.1% bovine serum albumin and 0.33 M-sucrose. The homogenate was filtered through a double layer of Miracloth, diluted 10-fold with fresh grinding media and centrifuged at 20,000 × *g* for 10 min. The supernatant was filtered through Miracloth and centrifuged at 105,000 × *g* for 90 min. The resulting microsomal pellet was resuspended in 0.1 M-potassium phosphate buffer, pH 7.2, and either used immediately or stored at −80°C until required. No significant loss in any of the glycerol acylating activities was observed upon storage of the suspension for up to three weeks.

*Protein determination.* Protein was measured with the BCA protein-assay reagent of Pierce Chemical Company (Rockford, IL) after treatment of the sample with 0.1% sodium lauryl sulphate, and with bovine serum albumin as standard.

*Enzyme assays.* Glycerol 3-phosphate acyltransferase (GPAT) and the 1-acyl-glycerol 3-phosphate acyltransferase (lysophosphatidic acid acyltransferase; LPAAT) were measured spectrophotometrically by the coupling of the free CoA (liberated from acyl-CoA) with 5,5-dithiobis-(2-nitrobenzoicacid) (DTNB) by methods modified from (12). The increase in absorbance was followed at 405 nm in a Shimadzu UV-160 double-beam spectrophotometer at a temperature of 20°C.

In standard assays of the GPAT, the sample cuvette contained microsomal membranes (60–80 μg of microsomal protein), 50 nmol DTNB and 1 μmol glycerol 3-phosphate in 0.1 M-phosphate buffer, pH 7.2, in a total volume of 950 μl. The reference cuvette contained all the additions as the sample cuvette but with the omission of glycerol 3-phosphate. The reaction was initiated by the addition of 7.5 nmol acyl-CoA (dissolved in 50 μl water) to both cuvettes and with a thorough mixing of the reaction mixtures. After a time lag of 30–60 seconds (depending on the amounts of the enzyme present) the reaction proceeded at an essentially linear rate for 4 min. The increase in absorbance at 405 nm was monitored, and the activity calculated using a molar extinction coefficient of 13600. The LPAAT was measured similarly to the GPAT, but with 20–60 μg microsomal protein and the replacement of the glycerol 3-phosphate with 50 nmol of 1-oleoyl-glycerol 3-phosphate in the sample cuvette. The reaction was initiated by the addition of 5 nmol acyl-CoA, and was, after a lag of about 15 seconds, linear with time up to 3 min.

The activity of the LPAAT was also measured with a radio-assay method. Microsomal preparations (20–40 μg of protein) were incubated at 30°C for up to 15 min with [1-$^{14}$C]acyl-CoA, 1-oleoyl-glycerol 3-phosphate and with other additions at concentrations stated in the Results section, in a total volume of 8 ml with phosphate buffer at pH 7.2. The reactions were terminated by the addition of 1 mg bovine serum albumin and 100 μl of glacial acetic acid followed by 3.75 ml of methanol/chloroform (2:1) and 1.25 ml of chloroform. The accumulated phosphatidic acids were recovered in the lower chloroform phase (total volume 2 ml), whereas the acyl-CoA partitioned into the upper methanol/water phase. The lipids in the chloroform phase were further purified by thin-layer chromatography (TLC), and the radio-labelled phosphatidic acids were isolated, quantified and, in the case of the mixed [$^{14}$C]acyl-CoA substrates, methylated for radio- gas liquid chromatography (GLC) analyses as described below. The formation of phosphatidic acid was totally dependent on the addition of exogenous lysophosphatidic acid, and was linear with time up to 15 min. The presence of DTNB (50 μM) stimulated the initial rate of acylation about two-fold, but linearity was only observed over the first 5 min.

*In vitro* oil synthesis in the microsomal prepara-

tions was carried out at 30°C in incubation mixtures containing 2.4 μmol of glycerol 3-phosphate, 10 mg of bovine serum albumin, 10 μmol $MgCl_2$ and microsomal membranes (0.2 and 0.5 mg microsomal protein for safflower and rape, respectively) in 0.1 M phosphate buffer, pH 7.2, in a final volume of 1 ml. At the start of the incubation, 300 nmol of an equimolar mixture of (1-$^{14}$C)-labelled palmitoyl-, stearoyl- and oleoyl-CoA was added (sp. activity 1450 d.p.m./nmol). At 30 min intervals an additional 200 nmols of the mixed acyl-CoA substrate were added. The reactions were terminated by extraction in methanol/chloroform according to Bligh and Dyer (13) at the times stated in the tables. The distribution of radioactivity among the lipid classes, as well as the identity of the [$^{14}$C]acyl-groups, were determined as described below.

*Analytical procedures.* Chloroform phases, from the extracted incubation mixtures, were evaporated to dryness under $N_2$. The residue was dissolved in a small volume of chloroform and the polar and neutral lipids were purified by TLC on precoated silica gel plates (Merck, Darmstadt, FRG; silica-gel 60) with chloroform/methanol/acetic acid/water (170:30:20:7, by vol) or hexane/diethyl ether/acetic acid (70:30:1, by vol), respectively. Lipid areas, located by lightly staining with $I_2$ vapour, were removed from the plates and either assayed for radioactivity or methylated *in-situ* with methanolic HCl (14) for the analysis of the fatty acids. The radioactive fatty acid methyl esters were analyzed by radio-GLC with a glass column (2.5 m × mm) containing 10% BDS on Chromosorb W (HP, 80–100 mesh).

Positional stereochemical analysis of the fatty acids in the phosphatidic acid fraction and phosphatidylcholine were performed by treatment of the purified phospholipid with phospholipase $A_2$ as previously described (15).

Where analysis of the acyl-CoA fraction was required, the methanol-water phase of the Bligh and Dyer (13) extraction was saponified by refluxing with 1 M KOH for 40 min, acidified with HCl and extracted with chloroform. The free fatty acids derived from the acyl-CoA were methylated with methanolic HCl and analyzed by GLC as described above.

Lipid samples were assayed for radioactivity in PCS (Amersham/Searle, Arlington Heights, IL)/xylene (2:1, v/v) scintillant in a LKB 1214 liquid-scintillation counter with an efficiency of 94% for $^{14}$C. All values were corrected for background and quenching.

*Reproducibility.* All single enzyme assays were carried out on duplicate samples with a standard error of the mean (SEM) of less than ± 10%. The data presented are mean values. The data from the *in vitro* oil synthesizing experiments are from single measurements which were repeated on separate occasions with different batches of plant material, and all gave essentially similar results.

## RESULTS

The safflower (var. Gila) used in the investigation contained a high level of linoleate (75%) in its seed triacylglycerols with 16% oleic acid. Palmitate and stearate were only present in small quantities and represented some 7% and 2%, respectively (16). The fatty acid composition of the triacylglycerols in turnip-rape, on the other hand, was more heterogeneous with oleate predominating (30%), followed by linoleate (18%), linolenate (10%), C20:1 (13%) and erucate (23%). Palmitate and stearate in the turnip-rape oil accounted for only 4% and 1%, respectively (6). Microsomal membrane preparations from the developing cotyledons of both the safflower and turnip-rape were capable of substantial Kennedy pathway activity and catalyzed the acylation of the glycerol backbone and the formation of phosphatidic acids and triacylglycerols (8,17).

*Acyl specificity of GPAT and LPAAT.* The microsomal membranes from the two plant species were used to assess the specificity of the GPAT and LPAAT enzymes towards a comprehensive range of acyl-CoA substrates using a spectrophotometric assay based on the coupling of the free thiol group with DTNB. The activity of the LPAAT for some species of acyl-CoA was 3–4 times greater than the corresponding GPAT. The apparent $K_m$ value of the LPAAT for the acyl acceptor 1-acyl-glycerol 3-phosphate was also low (1.5 μM). The observed activities of the GPAT might therefore, in some cases, be an overestimate of the real activity since some acylation of the formed lysophosphatidic acids would also occur. Considering, however, that the rates were essentially linear for 4 min, the overestimation would not exceed 50% of the actual value with any of the acyl-CoA species tested.

In safflower, the GPAT (Fig. 1A) was particularly active with the C18 unsaturated fatty acid substrates oleate (C18:1), linoleate (C18:2) and α-linolenate (C18:3 $^{\Delta 9,12,15}$). Gamma-linolenate (C18:3$^{\Delta 6,9,12}$), on the other hand, was utilized less efficiently at about 50% of the level found for the α-isomer. The longer chain fatty acid, C20:1, was used to a small extent, whereas eru-

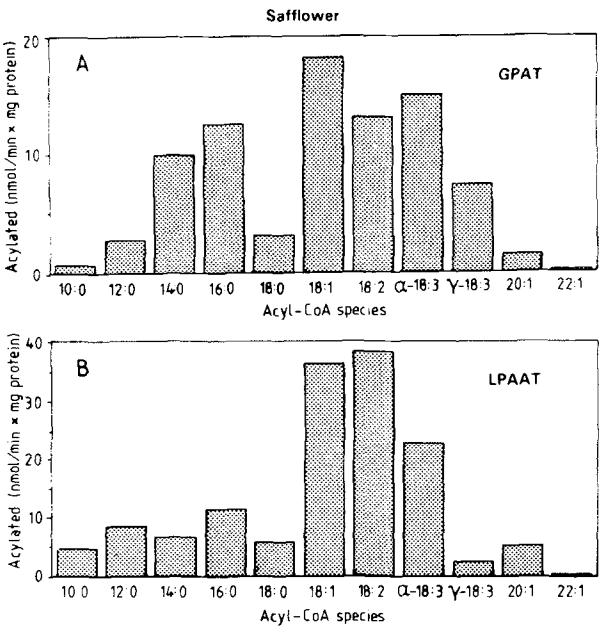


**FIG. 1. Acyl specificity of the glycerol 3-phosphate acyltransferase (GPAT) (A) and the 1-acyl-glycerol 3-phosphate acyltransferase (LPAAT) (B) in microsomal preparations from the developing cotyledons of safflower. Enzyme activities were measured spectrophotometrically with 79 μg and 40 μg microsomal protein and 7.5 μM and 5 μM acyl-CoA for the GPAT and the LPAAT, respectively.**

cate (C22:1) was the least effective of the acyl-CoA species tested. The utilization of the saturated, medium chain fatty acids exhibited a progressive increase from caproate (C10:0) up to palmitate (C16:0). Stearate (C18:0), however, was used substantially less than palmitate and oleate. The safflower LPAAT was active (with 1-oleoyl-sn-glycerol 3-phosphate as the acyl acceptor) with oleate, linoleate and $\alpha$-linolenate (Fig. 1B). Its efficiency, however, with the other acyl substrates was generally low, and almost negligible with erucate.

In the rape preparations, the specificity of the GPAT was less pronounced with the different acyl substrates (Fig. 2A). The efficiency of the acylation with linoleate was particularly high and the fatty acids, from laurate to C20:1, were all utilized to a reasonable extent. The enzyme, however, showed little preference for C10:0 or erucate. The LPAAT (with 1-oleoyl-sn-glycerol 3-phosphate as the acyl acceptor) exhibited little preference for fatty acid substrates ranging from laurate to $\alpha$-linolenate, and again erucate was only poorly utilized (Fig. 2B).

*Acyl selectivity in safflower.* The safflower microsomal system was used to assess acyl selectivity between oleoyl- and linoleoyl-CoA of the LPAAT as a function of the concentration of either acyl-CoA, bovine serum albumin or lysophosphatidic acid. The LPAAT exhibited an almost linear increase in activity up to 5 $\mu$M acyl-CoA with proportionately the same ratio of oleate:linoleate being utilized at each concentration (data not shown). The rate of acylation became constant with concentrations in excess of 5 $\mu$M acyl substrate. At all acyl-CoA concentrations the oleate was only slightly more selected for than the linoleate.

The effect of lysophosphatidic acid on the selection of oleoyl-, and linoleoyl substrates at optimum acyl-CoA concentration is shown in Figure 3. At low concentrations of lysophosphatidic acid (3 $\mu$M), the enzyme was strongly selective for linoleate. With increasing lysophosphatidic acid concentration, however, the enzyme used progressively more oleate until, at 25 $\mu$M and above, the fatty acid substrates were utilized in almost equal proportions. Similar effects of lysophosphatidic acid on acyl selectivity was found for the LPAAT in microsomal preparations from spinach leaves (18). Hares and Frenzen (18) also reported that the quality

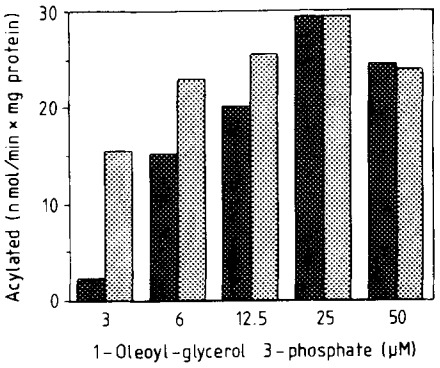


**FIG. 3. The effect of 1-oleoyl-glycerol 3-phosphate (lysophosphatidic acid) on the selectivity of the 1-acyl-glycerol 3-phosphate acyltransferase (LPAAT) in microsomal preparations of safflower. Microsomes (equivalent to 79 $\mu$g microsomal protein) were incubated with an equimolar mixture of [$^{14}$C]oleoyl-CoA (5$\mu$M), [$^{14}$C]linoleoyl-CoA (5$\mu$M) and 1-oleoyl-glycerol 3-phosphate at the concentrations given in the Figure. After 15 min incubation, the reactions were terminated and the lipids extracted. The purified phosphatidic acid was methylated and analyzed by radio-glc. Symbols used: black bars, oleate; grey bars, linoleate.**

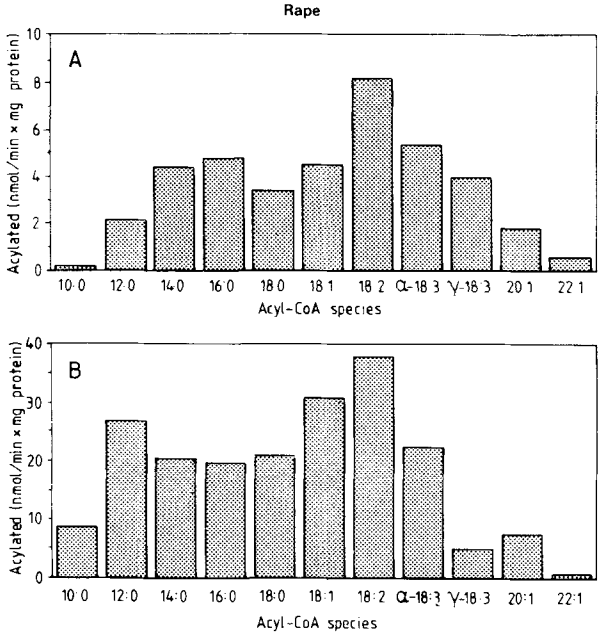


**FIG. 2. Acyl specificity of the glycerol 3-phosphate acyltransferase (GPAT) (A) and the 1-acyl-glycerol 3-phosphate acyltransferase (LPAAT) (B) in microsomal preparations from the developing cotyledons of rape. Enzyme activities were measured spectrophotometrically with 72 $\mu$g and 36 $\mu$g microsomal protein and 7.5 $\mu$M and 5 $\mu$M acyl-CoA for the GPAT and the LPAAT, respectively.**

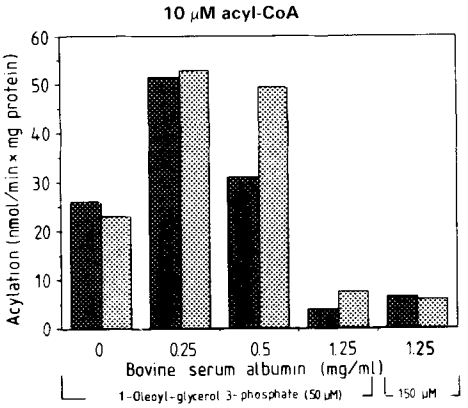


**FIG. 4. The effect of bovine serum albumin on the selectivity of the 1-acyl-glycerol 3-phosphate acyltransferase (LPAAT) in microsomal preparations of safflower. Microsomes (equivalent to 79 $\mu$g microsomal protein) were incubated with an equimolar mixture of [$^{14}$C]oleoyl-CoA (5$\mu$M) and [$^{14}$C]linoleoyl-CoA (5$\mu$M), and with bovine serum albumin and 1-oleoyl glycerol 3-phosphate at the concentrations given in the Figure. After 15 min incubation, the reactions were terminated and the lipids extracted. The purified phosphatidic acid was methylated and analyzed by radio-glc. Symbols used:black bars, oleate; grey bars, linoleate.**

of the acyl moeity in the acyl acceptor influenced the acyl-CoA selectivity. It is probable that the acyl selectivity of the LPAAT *in vivo* may be influenced by the properties of the first acylating enzyme in the glycerol 3-phosphate pathway, the GPAT, and the formation of lysophosphatidic acid.

The activity and acyl selectivity of the LPAAT were investigated in the presence of bovine serum albumin and optimum levels of acyl-CoA and lysophosphatidic acid (Fig. 4). The results show that the rate of acylation of the lysophosphatidic acid increased up to two-fold at 0.25 mg/ml bovine serum albumin, and at an acyl-CoA concentration of 10 $\mu$M (an acyl-CoA to bovine serum albumin molar ratio of 2.7). At higher concentrations of bovine serum albumin (0.5 mg and 1.25 mg/ml), however, there was a decrease in the acylation rate that was not overcome by increasing the acyl-CoA (data not shown) or the lysophophatidic acid concentrations. It may be that acyl-CoAs, which are bound to albumin, are more efficient substrates for the enzymes (19).

Bovine serum albumin, at 0.25 mg/ml, did not alter the acyl selectivity of the enzyme (20). At higher concentrations, however, there was a shift in selectivity, and a preference for linoleate was evident (Fig. 4). The shift was reversed, however, by increasing the amount of acyl acceptor (lysophosphatidic acid) in the reaction mixture (Fig. 4). This may indicate that the lysophosphatidic acid, which is bound to the bovine serum albumin, is unavailable to the enzyme. The lowering of the effective lysophosphatidic acid concentration may, therefore, alter the acyl selectivity in a similar way to that observed when varying the lysophosphatidic acid in the absence of albumin (Fig. 3).

It is relevant to consider the possible role of fatty acid binding proteins (FABP) in lipid metabolism. The FABPs are major cytosolic proteins in adipose tissue in animals (21). They also occur in plant tissues and are capable of binding acyl-CoA esters (22). Unfortunately, there appears to be no reports on the levels of FABP in developing seeds. However, phospholipid transfer proteins (PLTF) are major cytosolic proteins in developing maize seeds (23). Such proteins in significant amounts could play an important role in complexing the acyl-CoA substrate for interaction with the glycerol acylating enzyme-protein.

*Substrate affinity in safflower.* The spectrophotometric assay allowed an estimate of substrate affinity, as judged from apparent $K_m$ values, of both the GPAT and LPAAT (Table 1). The GPAT had a particularly low affinity for the acyl acceptor, with an apparent $K_m$ of 1.5 mM sn-glycerol 3-phosphate when measured with palmitate as the acyl-CoA substate. Ichihara (20) reports a corresponding $K_m$ value of 0.54 mM for the same enzyme in assays in the presence of bovine serum albumin. The apparent $K_m$ values for the GPAT and its acyl substrates indicated a greater affinity for the saturated fatty acid species when compared to the more unsaturated substrates (Table 1). On the other hand, the measurement of apparent $K_m$ values for the LPAAT towards the acyl substrates consistently gave somewhat surprising results. In acyl selectivity experiments, the enzyme exhibited a high selectivity for C18-unsaturated fatty acids, particularly linoleate, with little or no activity towards the saturated substrates palmitate and stearate (15). The apparent $K_m$ constants, as estimated here with single acyl substrates, indicate that the enzyme has a greater affinity for palmitate and stearate than for the C18-unsaturated fatty acids. It should be noted, however, that the rates of acylation (i.e., $V_{max}$ values) were substantially lower for the saturated fatty acids than observed for the unsaturated acyl substrates (Table 1). The enzyme had a particularly high affinity for the acyl acceptor 1-oleoyl-sn-glycerol 3-phosphate (apparent $K_m$ = 1.5 $\mu$M) when measured with linoleoyl-CoA as the acyl donor. A strong substrate inhibition occurred at acyl-CoA concentrations above 10 $\mu$M for both the GPAT and LPAAT (data not shown).

*Assembly of cocoa butter type fats in turnip-rape and safflower.* Microsomal membrane preparations from the developing cotyledons of safflower and rape were incubated with a ($^{14}$C)-acyl-CoA substrate containing equimolar amounts of palmitate, stearate, oleate and excess sn-glycerol 3-phosphate. The mixed acyl-CoA substrate was added to the incubation mixture at regular intervals (see Materials and Methods). The acyl-CoA, therefore, was kept at a sufficient concentration to overcome any competitive influence which could be exerted by the linoleate, that can enter the acyl-CoA pool from phosphatidylcholine via acyl-exchange (24). The incorporation of fatty acids into the complex lipids was analyzed at intervals and the relative distribution of each radioactive fatty acid species determined.

In the turnip-rape incubations the incorporation of radioactive fatty acids in phosphatidic acid and triacylglycerol proceeded in a linear fashion, and at the end of the incubation period the phosphatidic acid fraction contained some three times more radioactive fatty acid than the triacylglycerols (Table 2, A). The relative distribution of the radioactive acyl groups in each complex lipid is given in Table 2, B. Initially, palmitate predominated in the triacylglycerol. With the longer incubation times, however, the content of each labelled fatty acid became almost equal and therefore represented a proportional incorporation approaching 1:1:1. Among the phosphatidic acids, at the longer periods of incubation (2.5 and 4 hr) the proportion of palmitate, stearate and oleate became constant, and was almost 3:2:4, respectively. The very high proportion of labelled oleate in phosphatidylcholine largely represents acyl exchange of this fatty acid with position sn-2 of the complex lipid (25).

The stereospecific distribution of the labelled acyl groups in the sn-triacylglycerol was calculated from the distribution of radioactive fatty acids at positions sn-1 and -2 of the accumulated phosphatidic acids and the total labelled fatty acid content of the triacylglycerol itself. The assumption was made that the newly synthesized triacylglycerol would, therefore, strongly reflect the phosphatidic acid composition, from which it was being derived through the reactions of the so-called Kennedy pathway. The validity of this is supported by the observations that in both safflower and turnip-rape preparations little diacylglycerol accumulated (relative to the phosphatidic acids and triacylglycerol), indicating that the fatty acid species derived from the phosphatidic acid fraction is rapidly acylated

TABLE 1

**Apparent $K_m$ and $V_{max}$ Values for the Glycerol 3-Phosphate Acyltransferase (GPAT) and 1-Acyl-Glycerol 3-Phosphate Acyltransferase (LPAAT) in Microsomal Preparations of Safflower[a]**

| Enzyme | | acyl-CoA species | | | | |
|---|---|---|---|---|---|---|
| | | 16:0 | 18:0 | 18:1 | 18:2 | acyl acceptor |
| GPAT | $K_m$ (μM) | 4.2 | 3.4 | 6.4 | 6.6 | 1500 |
| | $V_{max}$ (nmol/mg protein × min) | 25 | 10 | 30 | 14 | 30 |
| LPAAT | $K_m$ (μM) | 0.5 | <0.5 | 2.7 | 2.9 | 1.5 |
| | $V_{max}$ (nmol/mg protein × min) | 13 | 9 | 45 | 55 | 40 |

[a]Rates of acylation were measured spectrophotometrically at different acyl donor and acyl acceptor concentrations. Affinity for the acyl-CoA substrate was measured for the GPAT at a fixed glycerol 3-phosphate concentration of 1 mM and the LPAAT at a fixed 1-oleoyl-glycerol 3-phosphate concentration of 50 μM. The constants for the acyl acceptors were measured at a fixed acyl-CoA concentration of 7.5 μM 16:0-CoA for the GPAT and 5 μM 18:2-CoA for LPAAT. $K_m$ and $V_{max}$ values were calculated from a double-reciprocal plot.

TABLE 2

***In-Vitro* Oil Synthesis in Microsomal Preparations from Turnip-Rape Seeds[a]**

A.

| Incubation time (hours) | [14]acyl groups incorporated (nmol) | | | |
|---|---|---|---|---|
| | Phosphatidic acid | Phosphatidylcholine | Diacylglycerol | Triacylglycerol |
| 1.5 | 100.6 | 27.0 | 2.7 | 33.6 |
| 2.5 | 190.5 | 38.2 | 4.2 | 69.6 |
| 4 | 335.8 | 48.5 | 5.3 | 100.9 |

B.

| Incubation time (hours) | [14C]acyl group | Relative distribution of [14]acyl groups (mol% of total radioactivity in the lipid) | | |
|---|---|---|---|---|
| | | Phosphatidic acid | Phosphatidylcholine | Triacylglycerol |
| 1.5 | 16:0 | 39.7 | 9.2 | 45.7 |
| | 18:0 | 17.2 | 4.8 | 25.5 |
| | 18:1 | 43.1 | 86.0 | 28.8 |
| 2.5 | 16:0 | 31.6 | 10.6 | 38.6 |
| | 18:0 | 22.4 | 10.6 | 27.6 |
| | 18:1 | 46.0 | 78.8 | 33.8 |
| 4 | 16:0 | 31.3 | 13.5 | 34.3 |
| | 18:0 | 24.8 | 17.3 | 34.8 |
| | 18:1 | 43.9 | 69.2 | 30.9 |

C.

| Position | Deduced Relative distribution of [14C]acyl groups (mol% within each position) | | |
|---|---|---|---|
| | 16:0 | 18:0 | 18:1 |
| sn-1 | 59.6 | 35.1 | 5.3 |
| sn-2 | 14.7 | 16.1 | 69.3 |
| sn-3 | 40.4 | 54.8 | 4.9 |

[a]Microsomal preparations (equivalent to 0.5 mg of microsomal protein) were incubated with sn-glycerol 3-phosphate, $MgCl_2$, bovine serum albumin and [14C]acyl-CoA (equimolar amounts of [14C] 16:0-CoA, [14C] 18:1-CoA and [14C] 18:1-CoA), at 30°C. At intervals, the radioactive distribution among the different lipid classes, the identity of the [14C] acyl groups and the positional distribution of the [14C] acyl groups in phosphatidic acid, were determined. The radioactive distribution of acyl groups at the different sn-positions of the sn-triacylglycerol was calculated from the total distribution in that lipid and the distribution at position sn-1 and sn-2 of the phosphatidic acids in the fourth hour.

at position sn-3. Further evidence for these proposals also comes from the fatty acid profile of the phosphatidic acid fraction which, in both the plant species, remained similar throughout the incubation period. It should also be noted that the fatty acid composition of the diacylglycerol in the safflower experiments was similar to that of the phosphatidic acid fraction. The calculated stereospecific distribution in the triacylglycerols (Table 2, C), shows that the sn-1 position consisted of almost 2:1 palmitate and stearate, respectively, whereas the stearate was predominant in position sn-3. Some 70% of the radioactive fatty acid at position sn-2 was oleate, with the remainder being almost equally palmitate and stearate.

TABLE 3

***In Vitro* Oil Synthesis in Microsomal Preparations from Safflower Seeds[a]**

| A. Incubation time (hours) | [14]acyl groups incorporated (nmol) | | | |
|---|---|---|---|---|
| | Phosphatidic acid | Phosphatidylcholine | Diacylglycerol | Triacylglycerol |
| 2 | 23.0 | 41.0 | 5.9 | 72.1 |
| 4 | 191.9 | 45.6 | 10.6 | 105.6 |
| 5.5 | 226.9 | 50.2 | 14.9 | 120.6 |

| B. Time (hours) | [14C]acyl group | Relative distribution of [14]acyl groups (mol% of total radioactivity in the lipid) | | | | |
|---|---|---|---|---|---|---|
| | | Acyl-CoA | Phosphatidic acid | Phosphatidylcholine | Diacylglyerol | Triacylglycerol |
| 2 | 16:0 | 42.9 | 27.9 | 13.0 | 39.0 | 40.1 |
| | 18:0 | 40.2 | 23.2 | 14.6 | 22.0 | 30.7 |
| | 18:1 | 16.9 | 48.9 | 72.4 | 38.0 | 29.2 |
| 4 | 16:0 | 39.1 | 32.1 | 14.9 | 36.2 | 38.6 |
| | 18:0 | 37.1 | 18.9 | 16.5 | 17.2 | 32.2 |
| | 18:1 | 23.8 | 49.0 | 68.5 | 46.6 | 32.9 |
| 5.5 | 16:0 | 36.5 | 28.8 | 18.3 | 31.5 | 32.3 |
| | 18:0 | 35.9 | 17.9 | 14.1 | 12.9 | 39.1 |
| | 18:1 | 27.5 | 53.3 | 67.5 | 55.6 | 28.6 |

| C. Position | Deduced relative distribution of [14C]acyl group (mol% within each position) | | |
|---|---|---|---|
| | 16:0 | 18:0 | 18:1 |
| sn-1 | 70.4 | 24.2 | 5.4 |
| sn-2 | 13.3 | 6.6 | 80.1 |
| sn-3 | 13.2 | 86.5 | 0.3 |

[a]Microsomal preparations (equivalent to 0.2 mg microsomal protein) were incubated with sn-glycerol 3-phosphate, $MgCl_2$, bovine serum albumin and [14C]acyl-CoA (equimolar [14C]16:0-CoA, [14C]18:0-CoA and [14C]18:1-CoA), at 30°C. At intervals, the radioactive distribution among the different lipid classes, the identity of the [14C]acyl groups and the positional distribution of the [14C]acyl groups in phosphatidic acid were determined. The radioactive distribution of acyl groups at the different sn-positions of the sn-triacylglycerol was calculated from the total distribution in that lipid and the distribution at position sn-1 and sn-2 of phosphatidic acid at 5.5 hr incubation.

The results for a similar experiment with safflower microsomal preparations are given in Table 3. Again, substantial quantities of phosphatidic acids and triacylglycerols were formed in the incubations. Here, we also give the radioactive fatty acids present in the acyl-CoA fraction (Table 3, B), and show that after the initial periods of incubation the proportions of acyl substrate available for triacylglycerol assembly remain almost constant, but with slightly less oleate present than the other fatty acids. Small amounts of linoleate were also found in the acyl-CoA fraction (3.6, 1.1 and 0.7% of total acyl groups after 2, 4 and 5.5 hr of incubation, respectively). This linoleoyl-CoA has probably arisen through acyl exchange, which occurs between the acyl-CoA pool and the fatty acid constituents (largely linoleate in safflower) at position sn-2 of the sn-phosphatidylcholine (25). The calculated stereospecific distribution of each fatty acid in the sn-triacylglycerol (Table 3, C) shows that the palmitate was the preferred acyl substrate for the acylation of position sn-1 where it accounted for some 70% of the fatty acid present with the remainder being largely stearate. The fatty acids at the sn-3 position, on the other hand, were composed almost entirely of stearate (90%). At position sn-2 some 80% of the radioactive fatty acid present was oleate.

An analysis of the fatty acids in the safflower microsomal phosphatidylcholine at the 4 hr incubation period showed (data not given) that considerable incorporation of palmitate, oleate and stearate also had occurred.

## DISCUSSION

Microsomal preparations from the developing cotyledons of either safflower or turnip-rape were capable of sustained triacylglycerol assembly in the presence of acyl-CoA and sn-glycerol 3-phosphate. The initial acylations of the glycerol backbone, at the sn-1 and sn-2 positions, were particularly active and the enzymes responsible, GPAT and LPAAT, respectively, could be monitored without difficulty by spectrophotometric methods.

Although in safflower the specific activity of the acylation of glycerol 3-phosphate at position sn-1 (GPAT) was usually greater than observed in turnip-rape, it was evident, generally, that in both species the saturated fatty acid acyl donors were the poorer substrates. This became more marked at shorter carbon-chain lengths until at C10:0 (caproate), the acyl substrate was relatively little used. On the whole, however, it was apparent that the turnip-rape enzyme tends not to be so discriminating as that of the safflower. In both plant species the acylation of position sn-1 with erucate was extremely poor. In the second acylation step (LPAAT) and the formation of phosphatidic acid

the enzyme from turnip-rape was also the more tolerant of the acyl donor species, but again erucate proved to be little used.

Previous studies on the utilization of erucoyl-CoA in the acylation of the glycerol backbone, by microsomal preparations of oil-seed species, have indicated little preference for this long chain fatty acid. The safflower microsomal LPAAT exhibited little activity toward erucate (26), and Sun *et al.* (27) found that no erucate or laurate entered diacylglycerols and triacylglycerols in membrane preparations from maize and rapeseed incubated with the appropriate substrates. There was some evidence, however, that laurate and erucate may have accumulated in lysophosphatidic acid but failed to enter phosphatidic acid. These authors, therefore, suggested that the acyl specificity of the LPAAT may regulate the fatty acid composition at position sn-2 of the sn-triacylglycerol, and is responsible for the exclusion of particular fatty acids from this position. In oil-seed rape, erucic acid is primarily utilized at the sn-1 and sn-3 position of the sn-triacylglycerol (28), and is similarly distributed in turnip-rape (6). In studies on the diacylglycerol acyltransferase (DAGAT), which is responsible for the acylation of position sn-3 in the sn-triacylglycerol (Fig. 1), however, little preference for erucoyl-CoA was observed in rape preparations (29). Microsomal membranes from the developing cotyledons of turnip-rape, when incubated in the presence of labelled sn-glycerol 3-phosphate and nonradioactive erucoyl-CoA, were also not capable of catalyzing acylation (6). Erucate species of acyl donor were not even utilized in the presence of linoleoyl-CoA (6), a result in accord with the present findings, on the negligible activity of both the GPAT and the LPAAT with erucoyl-CoA.

The observations with rape species, membrane preparations of which are highly active in triacylglycerol assembly given the appropriate acyl substrate, and, in the case of turnip rape, will synthesize large quantities of erucic acid from oleoyl-CoA and malonyl-CoA (unpublished observations), appear contradictory to what might be anticipated and merit further consideration. It may be that the situation in rape species, for the assembly of erucoyl-oils, differs from our basic concepts of triacylglycerol construction and may involve other mechanisms, such as acyl remodelling, as previously suggested (30, 31). If this is so, then plants, like rape, may have evolved separate reactions in order to exclude unusual, and perhaps detrimental, fatty acids from the assembly of membrane structural lipids, such as phosphatidylcholine. However, it is still unclear how this is achieved.

Acyl specificity studies, such as those reported, give some indication as to whether a particular plant species might be expected to utilize foreign fatty acids in the biosynthesis of storage oils. For instance, it seems probable that if chain termination in the FAS-complex was manipulated to occur at the level of C10/C12 then the formation of diacylglycerols, containing these fatty acids, might occur. The enzyme DAGAT, which catalyzes the acylation of position sn-3, appears, in rape, to be particularly efficient with laurate (29), and so lauric acid rich oils might accumulate. It should be noted, however, that Sun *et al.* (27), suggested that laurate may be utilized, to some extent, by the GPAT and not by the LPAAT in rapeseed. In the present study, it seems on the basis of direct enzyme assay that both these enzymes could utilize the shorter chain fatty acids under the appropriate conditions. In some preliminary studies, however, it was evident that the rape LPAAT may be much less active toward acyl acceptors (lysophosphatic acids) esterified with the more medium-chain fatty acids (unpublished observations). In any case, it seems quite likely that conventional crops which grow in the more temperate regions of the world would, if tailored to synthesize medium-chain fatty acids, incorporate them to some extent into their storage products. The effect of this on the well-being of the plant remains conjectural. If the C10–C12 fatty acids become incorporated to any great extent into the structural membrane-lipids of the developing seed, then they could adversely affect biochemical and physiological function, particularly under moderate fluctuations in temperature experienced by the plant. In this respect, it is interesting that in experiments in which safflower microsomal preparations were incubated with equimolar amounts of palmitate, oleate and stearate in the presence of glycerol 3-phosphate, these fatty acids were also substantially incorporated into the phosphatidylcholine. A fuller understanding, therefore, of the mechanisms whereby plants exclude 'non-membrane' fatty acids from their structural complex lipids and dedicate them to storage products is of importance to the basic understanding of oil biosynthesis and to any biotechnological advances which develop from it (32).

There would appear to be fewer problems associated with the synthesis of γ-linolenate in plants. γ-Linolenate rich oils are considered to be of some therapeutic value (33), and yet are only found in the seeds of a few species (34,35). The acid is synthesized from oleate via linoleate, through the action of a Δ-6 desaturase, which utilizes the acyl substrates associated with position sn-2 of microsomal sn-phosphatidylcholine, and it is, therefore, also a natural component of the membrane lipids (36,37). The transfer of the gene(s), for the Δ-6 desaturase enzyme to recipient plant species, which are already capable of linoleate formation in their seeds, should lead to γ-linolenate production. It is interesting to observe that if the recipient plant has Δ-15 desaturase activity, and contains α-linolenate, then the inclusion of the Δ-6 enzyme will more likely lead to the accumulation of octadecatetraenoic acid ($C18:4^{\Delta 6,9,12,15}$) (38). In any event, if γ-linolenic acid was synthesized in rape seed, then it would be used, perhaps to a reasonable extent, in the acylation of the glycerol backbone and the formation of triacylglycerols.

It was apparent from these experiments that if the appropriate fatty acids (palmitate, stearate and oleate) were synthesized in approximately equimolar amounts, and made available to the glycerol acylating enzymes of safflower and rape seeds, then triacylglycerols having a similar fatty acid composition to cocoa-butter might arise. This was particularly the case with safflower, where there was a high degree of selectivity for palmitate, oleate and stearate, as at positions sn-1, -2 and -3, respectively. The selectivity for these particular acyl species in the acylation of the glycerol back-

bone in safflower seed preparations is interesting in itself. The GPAT and LPAAT in safflower are, therefore, highly selective for palmitate and oleate, respectively, and this confirms, to some extent, previous observations (15). Ichihara and coworkers (20,26), however, consider that in safflower the acylation catalyzed by the GPAT is not so specific, and depends on the fatty acids in the acyl-CoA pool that are available for triacylglycerol assembly. The properties of the LPAAT, on the other hand, may play a more precise role in the nonrandom distribution of fatty acid species found in the final triacylglycerol (26), a suggestion confirmed in the present study and elsewhere (15). Also, it was reported that the DAGAT in safflower had a broad acyl specificity (39), and that it had no strict selectivity for acyl-CoA substrates when presented in mixtures (40). In the present study, however, it is evident that safflower microsomal membranes can catalyze the acylation of the glycerol backbone in a highly distinctive fashion when presented with mixtures of palmitate, oleate and stearate. In fact, under such conditions the DAGAT is highly selective for stearate. These results, and their variance with other work, bring into question the significance of direct *in vitro* enzyme assays in fully understanding the role of the glycerol acylating enzymes in regulating fatty acid content and acyl distribution in the triacylglycerol. Studies on the assembly of triacylglycerols as a function of acyl quality may be more pertinent when carried out in toto from glycerol 3-phosphate in the microsomal membrane. It is also evident that turnip-rape will synthesize saturated-unsaturated-saturated triacylglycerols, but without the more distinct acyl selectivity found in safflower. In any case, these observations indicate that provided the correct proportion of fatty acid species can be produced in oil plants by whatever means, cocoa butter type fats may result.

## ACKNOWLEDGMENTS

The authors are grateful for financial support from Stiftelsen Karlshamns forskning (Karlshamns ab) and The Swedish Natural Science Research Council. M.B. thanks NIFOR (Nigeria) for study leave.

## REFERENCES

1. Slabas, A.R., C.M. Sidebottom, A. Hellyer, R.M.J. Kessel and M.P. Tombs, *Biochim. Biophys. Acta 877*:271 (1986).
2. Slabas, A.R., C. Davies, A. Hellyer, R.W. MacKintosh, P. Shelden, D.G. Hardie, R.G.O. Kekwick and R. Safford, in *Plant Lipids: Targets for Manipulation, Monograph 17*, eds. N.J. Pinfield and A.K. Stobart, British Plant Growth Regulator Group, Bristol, 1988, p. 1.
3. Slabas, A.R., A. Hellyer, C. Sidebottom, H. Bambridge, I.R. Cottingham, R. Kessell, C.G. Smith, P. Sheldon, R.G.O. Kekwick, J. deSilva, C. Lucas, J. Windust, C.M. James, S.G. Hughes and R. Safford, in *Plant Molecular Biology, Vol. 140*, eds. D. Wettstein and N. Chua, Plenum Press, New York, 1986, p. 265.
4. Fincke, A., *Gordian 76*:324 (1976).
5. Kattenberg, H.P., *Manuf. Confect.* 32 (1981).
6. Griffiths, G., S. Stymne and K. Stobart, in *Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry*, ed. T.H. Applewhite, American Oil Chemists' Society, Champaign, IL, 1988, p. 23.
7. Griffiths, G., I. Hakman, E. Tillberg, M. Hellman, S. Stymne and A.K. Stobart, in *Plant Lipids: Targets for Manipulation, Monograph 17*, eds. N.J. Pinfield and A.K. Stobart, British Plant Growth Regulator Group, Bristol, 1988, p. 11.
8. Stobart, A., S. Stymne and S. Höglund, *Planta 169*:33 (1986).
9. Kennedy, E.P., *Fed. Proc. Fed. Am. Soc. Exp. Biol. 20*:943 (1961).
10. Stymne, S., and A.K. Stobart, in *Biochemistry of Plants*, Vol. 9, ed. P.K. Stumpf, Academic Press, New York, 1987, p. 175.
11. Sanchez, M., D.G. Nicholls and D.N. Brindley, *Biochem. J. 132*:697 (1973).
12. Lands, E.W., and P. Hart, *J. Biol. Chem. 240*:1905 (1965).
13. Bligh, E.G., and W.J. Dyer, *Can. J. Biochem. Physiol. 37*:911 (1959).
14. Kates, M., *J. Lipid Res. 5*:132 (1964).
15. Griffiths, G., A.K. Stobart and S. Stymne, *Biochem. J. 230*:379 (1985).
16. Stymne, S., A.K. Stobart and G. Glad, *Biochim. Biophys. Acta 752*:198 (1983).
17. Stymne, S., G. Griffiths and A.K. Stobart, in *The Metabolism, Structure and Function of Plant Lipids*, eds. P.K. Stumpf, J.B. Mudd and W.D. Nes, Plenum Press, New York, NY, 1987, p. 405.
18. Hares, W., and M. Frenzen, *J. Plant Physiol. 131*:49 (1987).
19. Hershenson, S., and M.L. Ernst-Fonberg, *Biochim. Biophys. Acta 751*:412 (1983).
20. Ichihara, K., *Arch. Biochem. Biophys. 232*:685 (1984).
21. Haq, R.U., L. Christodoulides and B. Ketterer, *Biochim. Biophys. Acta 713*:193 (1982).
22. Rickers, J., I. Tober and F. Spener, *Ibid. 794*:313 (1984).
23. Grosbois, M., F. Guerbette and J-C. Kader, *Plant Physiol. 90*:1560 (1989).
24. Stobart, A.K., S. Stymne and G. Glad, *Biochim. Biophys. Acta 754*:292 (1983).
25. Stymne, S., and A.K. Stobart, *Biochem. J. 223*:305 (1984).
26. Ichihara, K., T. Asashi and S. Fujii, *Eur. J. Biochem 167*:339 (1987).
27. Sun, C., Y. Cao and A.H.C. Huang, *Plant Physiol. 88*:56 (1988).
28. Brockerhoff, H., *Lipids 6*:942 (1971).
29. Cao, Y., and A.H.C. Huang, *Plant Physiol 84*:762 (1987).
30. Mukherjee, K.D., *Ibid. 73*:929 (1983).
31. Mukherjee, K.D., and I. Kiewitt, *Phytochem. 23*:349 (1984).
32. Ohlrogge, J.B., in *Proceedings of the World Conference on Biotechnology for the Fats and Oils Industry*, ed. T.H. Applewhite, American Oil Chemists' Society, Champaign, IL 1988, p. 87.
33. Horrobin, D.F., and M.S. Manku, in *Fats for the Future*, eds. S.G. Brooker, A. Renkwick, S.F. Hannan and L. Eyres, Duromark, Auckland, New Zealand, 1983, p. 99.
34. Kleiman, G.R., F.R. Earle and I.A. Wolff, *J. Am. Oil Chem. Soc. 41*:459 (1964).
35. Jamieson, G.R., and E.H. Reid, *Phytochemistry 8*:1489 (1969).
36. Stymne, S., and A.K. Stobart, *Biochem J. 240*:385 (1986).
37. Griffiths, G., A.K. Stobart and S. Stymne, *Ibid. 252*:641 (1988).
38. Griffiths, G., E.Y. Brechany, W.W. Christie, S. Stymne and K. Stobart, in *Biological Role of Plant Lipids*, eds. P.A. Biacs, K. Gruuiz and T. Kremmer, Akadémiai Kiadó, Budapest, and Plenum Press Publishing Corporation, New York and London, 1989, p. 151.
39. Ichihara, K., and M. Noda, *Phytochemistry 21*:1895 (1982).
40. Ichihara, K., T. Takahashi and S. Fujii, *Biochim. Biophys. Acta 958*:125 (1988).

[Received August 8, 1989, accepted November 20, 1989]
[J5770]