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Metabolism of Tween-Fatty Acid Esters by Cultured Soybean Cells[1]

KINETICS OF INCORPORATION INTO LIPIDS, SUBSEQUENT TURNOVER, AND ASSOCIATED CHANGES IN ENDOGENOUS FATTY ACID SYNTHESIS

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ABSTRACT

Uptake of Tween-fatty acid esters and incorporation of the fatty acids into lipids by soybean (*Glycine max* [L.] Merr.) suspension cultures was investigated, together with subsequent turnover of the incorporated fatty acids and associated changes in endogenous fatty acid synthesis. Tween uptake was saturable, and fatty acids were rapidly transferred from Tweens to all acylated lipids. Patterns of incorporation into glycerolipids were similar in cells treated with Tweens carrying [1-^{14}C]-fatty acids and in cells treated with [1-^{14}C]acetate, indicating that exogenous fatty acids were used for glycerolipid synthesis essentially as if they had been made by the cell. In Tween-treated cells neutral lipids (which include Tweens) initially accounted for the majority of lipid radioactivity. Radioactivity was then rapidly transferred to glycerolipids. A transient pool of free fatty acids accounting for up to 10% of lipid radioactivity was observed. This was consistent with the hypothesis that fatty acids are transferred from Tweens to lipids by deacylation of the Tweens, creating a pool of free fatty acids which are then used for lipid synthesis. Sterols were only slightly labeled in cells treated with Tweens, but accounted for nearly 50% of lipid radioactivity in cells treated with acetate. This suggested very little degradation and reutilization of the radioactive fatty acids in cells treated with Tweens. In cells treated with either [1-^{14}C]acetate or Tween-[1-^{14}C]-18:1, 70% of the initial fatty acid radioactivity remained in fatty acids after a 100 hour chase. By contrast, fatty acids not normally present disappeared more rapidly, suggesting differential treatment of such fatty acids compared with those normally present. Cells which had incorporated large amounts of exogenous fatty acids altered fatty acid synthesis in three distinct ways: (a) amounts of [1-^{14}C]acetate incorporated into fatty acids were reduced; (b) cells incorporating exogenous unsaturated fatty acids increased the proportion of [1-^{14}C]acetate partitioned into saturated fatty acids, while the converse was true of cells which had incorporated exogenous saturated fatty acids; (c) desaturation of 18:1 to 18:2 and 18:3 was reduced in cells which had incorporated unsaturated fatty acids. These results suggest that Tween-fatty acid esters will be useful for supplying fatty acids to cells for a variety of studies related to fatty acid or membrane metabolism.

A system for modifying the membrane fatty acid composition of soybean cells in suspension culture by supplying Tween-fatty acid esters in their medium has been described (14). Fatty acids were incorporated into all acylated membrane lipids, with the possible exception of 'prokaryotic' lipids (see Roughan and Slack [8] for review of prokaryotic and eucaryotic lipids in plants). This system promises to be useful for studying many aspects of plant fatty acid metabolism and membrane physiology.

To assess the suitability of this system for studying such problems it was important to know the extent to which metabolism of exogenous fatty acids supplied as Tween esters resembled that of fatty acids made by the cells, and the response of endogenous fatty acid metabolism to such feeding. Accordingly, metabolism of ^{14}C-fatty acids in cells treated with Tween-[1-^{14}C]-fatty acid esters was compared with metabolism of ^{14}C-fatty acids synthesized by cells treated with [1-^{14}C]acetate. Results, reported here, demonstrate that metabolism of Tween-derived fatty acids is similar to that of endogenously synthesized fatty acids, that fatty acids are probably transferred from Tweens to lipids by Tween deacylation generating a pool of free fatty acids which are then used for lipid synthesis, and that cells treated with Tween-fatty acid esters alter amounts and types of fatty acids synthesized endogenously.

MATERIALS AND METHODS

Cell Growth. Soybean suspension cultures derived from an F1 hybrid of PI-290136 crossed with Minsoy (7) were grown as described (14).

Chemicals. Radioactive fatty acids were purchased from ICN, NEN, and Amersham. Other lipids were purchased from Sigma or Supelco. All solvents were reagent grade. Synthesis and characteristics of all Tween-fatty acid esters used in this study have been described (14).

Lipid Extraction and Analysis. Lipids were extracted and analyzed as described (14).

Uptake Kinetics and Pulse-Chase Experiments. Uptake kinetics were measured by harvesting cells onto GF/A filters at appropriate times after adding label. These were washed three times with medium containing an excess of nonradioactive substrate, then three times with distilled H_2O. Radioactivity on the dried filters was determined by scintillation counting. For experiments measuring kinetics of incorporation into lipids, cells were washed as above, then lipids were extracted and analyzed as described (14). In pulse-chase experiments, cells were labeled with the appropriate radioactive substrate for a suitable interval, then harvested onto sterile coarse sintered glass filters. Cells were washed several times with fresh medium containing nonradioactive substrate and transferred to flasks containing fresh medium and nonradioactive substrate. At appropriate times aliquots were harvested and treated as described above.

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RESULTS

Tween Uptake Kinetics. Tween uptake was measured using Tweens conjugated to ^{14}C-labeled fatty acids. Figure 1 shows the time-course of ^{14}C uptake by cells treated with Tween-[^{14}C]-16:0. Incorporation was initially linear and then the rate decreased (similar results were obtained with Tween-[^{14}C]-18:1; W. Terzaghi, unpublished data). Table I shows that the maximum rate of fatty acid uptake by cells treated with Tween-16:0 and Tween-18:1 was relatively constant over a wide range of concentrations. This indicates that the rate limiting step was saturable, possibly enzymic. Little Tween remained free in the medium after 30 h in cultures treated with up to 100 μM Tween-18:1, indicating that uptake decreased because of depletion of the medium. Greater amounts of Tween remained in the medium of cells treated with higher concentrations, presumably because these cultures were still taking up Tweens.

Figure 2 illustrates that amounts of incorporated exogenous fatty acids (as percent of total membrane fatty acids) fluctuated dramatically in cells treated with Tweens (note that Figure 2 presents the fatty acid composition of bulk phospholipids and galactolipids, as NL[3] were discarded together with any unmetabolized Tweens [14]). Exogenous fatty acid levels peaked within 1 d after transfer to fresh medium and then declined (although maximum exogenous fatty acid levels were not achieved until at least the second transfer in all sublethal treatments (15). This was observed in 32 trials with a number of different Tweens, although incorporation rates varied according to the fatty acid used (Fig. 2). Maximum amounts of exogenous fatty acids incorporated were proportional to amounts of Tween added (15), suggesting that the incorporation in Figure 2 leveled off due to depletion of the medium.

Figure 2 presents exogenous fatty acid content as percent of total membrane fatty acids. This is proportional to exogenous fatty acid content per cell, since fatty acid content per cell does not vary greatly in these cultures (provided they are transferred regularly so as to maintain exponential growth; W Terzaghi, unpublished data). Therefore, the exogenous fatty acid content per culture at a given time may be calculated by multiplying the proportion of exogenous fatty acids in membrane lipids by the cell density. This was done for a number of cultures at their maximum and subsequent minimum exogenous fatty acid levels (*e.g.* at d 1 and 3, respectively, using data as presented in Fig. 2). Results are presented in Table II. This shows that exogenous fatty acid contents per culture declined significantly after peaking in cells treated with alien fatty acids (fatty acids not normally present). For example, exogenous fatty acid content per culture of cells treated with 50 μM Tween-15:0 peaked on d 1 after transfer to fresh medium, but 2 d later this had been reduced to 63% of the maximum level. Similar reductions were observed in cultures treated with other concentrations of Tween-15:0, and other alien fatty acids (Table II). By contrast, cells treated with 18:1 did not decrease their 18:1 content per culture, and fatty acids turned over slowly in cells treated with [^{14}C]acetate or Tweens carrying radioactive 16:0 or 18:1 (see below, Fig. 5). These data indicate that cells turn over alien fatty acids more rapidly than those normally present. Consequently, in cells treated with fatty acids normally present the decline in exogenous fatty acid content per cell after peaking is mainly caused by dilution with newly synthesized fatty acids, whereas in cells treated with alien fatty acids turnover may also be involved.

[3] Abbreviations: NL, neutral lipids; FA, total fatty acids (free and bonded to lipids); ST, sterols; PG, phosphatidylglycerol; CL, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SL+CER, sulphur lipids and cerebrosides; MGDG, monogalactosyldiglyceride; PA, phosphatidic acid; LPC, lysophosphatidylcholine; SG, sterol glucosides; ESG, esterified sterol glucosides; FFA, free fatty acids; DGDG, digalactosyldiglyceride; ACP, acyl carrier protein.

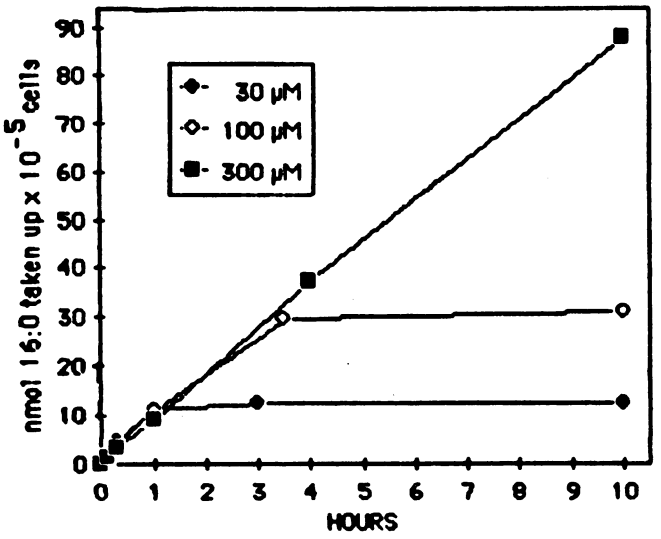

FIG. 1. Kinetics of Tween-[1-^{14}C]-16:0 uptake. Cells were incubated with a fixed aliquot of Tween-[^{14}C]-16:0 diluted with unlabeled Tween-16:0 to the appropriate final fatty acid concentration. At given timepoints aliquots were harvested onto filters and amount of nonexchangeable radioactivity was determined by scintillation counting after washing and drying the filters as described in "Materials and Methods." Values are means of two replicates. During washing 25 to 35% of the radioactivity added to the culture was displaced from the cells. This was presumably loosely bound in an exchangeable compartment, most likely the cell wall. A preliminary study comparing distribution of radioactivity between lipids and Tween plus free fatty acids (which comigrated in the TLC systems used) 1 h after treatment with varying concentrations of Tweens failed to detect any significant differences between treatments (data not shown). This implies that cells treated with higher concentrations of Tweens do not accumulate Tweens or free fatty acids in a nonexchangeable compartment.

Table I. *Uptake of Radioactivity by Cells Treated with Varying Concentrations of Tween-[1-^{14}C]-16:0 or Tween-[1-^{14}C]-18:1*

Cells were incubated with a fixed aliquot of either Tween-[^{14}C]-16:0 or Tween-[^{14}C]-18:1 diluted with unlabeled Tween to the appropriate final fatty acid concentration. At appropriate timepoints aliquots were harvested onto filters and amount of nonexchangeable radioactivity was determined by scintillation counting after washing and drying the filters as described in "Materials and Methods." cpm/h, Maximum observed rate of uptake (cpm/culture·h); nmol/h, nmol fatty acid taken up/100,000 cells/h; rem, % cpm initially added remaining free in medium after 30 h. All values are means of two replicates.

Treatment	Uptake of Radioactivity		rem
	cpm/h	*nmol/h*	%
Tween-16:0			
30 μM	51,000	11	ND[a]
100 μM	14,500	11	ND
300 μM	3,900	9	ND
Tween-18:1			
10 μM	160,000	18	7
30 μM	66,000	25	11
100 μM	14,300	16	13
300 μM	5,400	21	22
1000 μM	2,000	23	61

[a] Not determined

Kinetics of Fatty Acid Transfer between Lipids. Figure 3 shows that FA radioactivity peaked after 1 h in cells grown in 30 μM [^{14}C]acetate or 30 μM Tween-[^{14}C]-16:0 (at which time about 80% of the Tween-[^{14}C]-16:0 added to the medium had been

taken up; 50% had been incorporated into lipid while another 30% was displaced during washing. (W Terzaghi, unpublished data). After peaking, lipid and FA radioactivity remained constant in both treatments, therefore subsequent changes in labeling represent redistribution of radioactivity between lipids. In cells treated with Tweens nearly all lipid label was in FA (this refers to total fatty acid, *i.e.* both free and bonded to lipids), whereas in cells treated with acetate FA accounted for about half of lipid radioactivity, the remainder being in ST and ST derivatives.

Figure 4, A to D, illustrates that distribution of radioactivity between glycerolipids was similar in both treatments, with two exceptions: (a) there was a short lag before radioactivity appeared in lipids of cells treated with Tweens, and (b) PI and SL+CER were relatively more highly labeled in cells treated with Tweens.

Figure 4, E and F, shows that the major differences between these treatments were in labeling of NL and ST. In cells treated with Tweens most radioactivity was initially found in NL. The level of radioactivity in this fraction decreased in 3 h to the level observed in cells treated with acetate. Tweens co-migrate with NL in the TLC systems used, so these were not resolved, but this was interpreted as suggesting that by 3 h most Tween was deacylated. Moreover, in Tween-treated cells FFA transiently increased to 10% of lipid radioactivity, then essentially disappeared. Figure 4F shows that NL were also initially very radioactive in acetate-treated cells, and then rapidly decreased, but in this case FFA were negligible. Instead, label was transferred from NL to ST (W Terzaghi, unpublished data). After 10 h ST accounted for almost half of lipid radioactivity in cells treated with acetate, but were poorly labeled in Tween-treated cells (SG and ESG were minor components and were slowly labeled in both treatments, but ESG were the most radioactive members of the sterol group in Tween-treated cells).

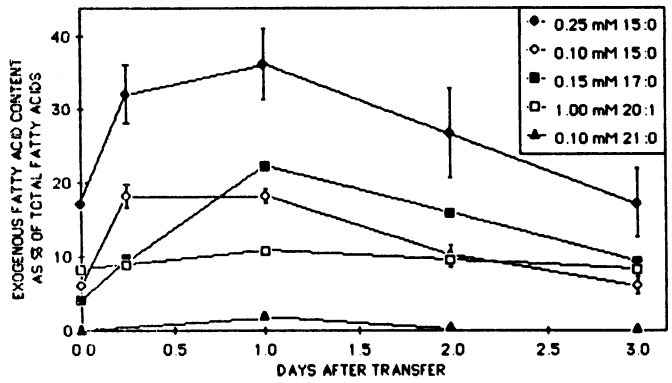

FIG. 2. Fluctuations in exogenous fatty acid content in cells treated with various Tweens. Lipids were extracted from cells at indicated times after transfer to fresh medium containing various Tweens, and fatty acid compositions were determined as described in "Materials and Methods." Exogenous fatty acid content is expressed as percent of total membrane fatty acids. Values are mean ± SD.

Effects of Tween treatment on persistence of fatty acids in individual lipids were determined by pulsing cells for 3 h with either Tween-[^{14}C]-18:1 or [^{14}C]acetate, then washing and chasing with the appropriate unlabeled substrate for 100 h. Tween-[^{14}C]-18:1 was used for this study as the maximum tolerable concentration of Tween-18:1 is much greater than that of Tween-16:0 (14), and was therefore more suitable for a chase. However, persistence of radioactivity in cells fed Tween-[^{14}C]-16:0 was also measured, with similar results to those described below (W Terzaghi, unpublished data).

Results are summarized in Figures 5 and 6. Neither treatment appreciably affected cell growth (Fig. 5A). Figure 5, B and C, illustrates that radioactivity decreased slowly; 70% of the radioactivity present after washing was still present in cells of either treatment after 100 h. Chasing did not affect persistence. Lipid cpm declined at the same rate as total cpm in both treatments, and essentially all lipid cpm were in FA in Tween-treated cells. In acetate-treated cells ST declined at the same rate as total lipids, but FA declined somewhat more rapidly.

Figure 6, A to F, shows that changes in lipid labeling were similar in both treatments, with three exceptions: (a) ST, SG, and ESG were highly labeled in cells treated with acetate but were poorly labeled in those treated with Tween, (b) FFA rapidly declined from 8% to less than 1% of lipid radioactivity in Tween cells, but were negligible in acetate cells, and (c) SL+CER were more highly labeled by growth in acetate.

Comparison of Figures 4 and 6 shows that PI and SL+CER were more highly labeled by growth in Tween-[^{14}C]-16:0 than by growth in Tween-[^{14}C]-18:1, whereas the converse was true of MGDG.

Effects of Exogenous Fatty Acids on Endogenous Fatty Acid Synthesis. Previous results showed that cells incorporating exogenous fatty acids altered the proportions of the fatty acids normally present (14). Since results presented above indicated little turnover of fatty acids normally present, it was anticipated that these changes would be generated by changes in fatty acid synthesis. This was studied by measuring partitioning of [^{14}C] acetate between fatty acids and other acetate-derived compounds

Table II. *Disappearance of Exogenous Fatty Acids in Cells Treated with various Tween-Fatty Acid Esters*

Packed cell volumes and exogenous fatty acid content were determined as described in "Materials and Methods." Growth, packed cell volume at end of interval divided by packed cell volume at start (time at which maximum exogenous fatty acid content *per culture* was observed); [EX]max, maximum observed concentration of incorporated exogenous fatty acids per culture as μg/ml culture = packed cell volume × exogenous fatty acid content per cell (as proportion of total fatty acids) × 3.75 (a constant incorporating factors correcting for dry weight cells/ml culture as a function of PCV and for cellular fatty acid content); [EX]min, subsequent minimum concentration of exogenous fatty acids per culture; I, interval (days) between maximum and subsequent minimum exogenous fatty acid levels; R, ratios of maximum to minimum [EX] (%). Tween concentrations refer to concentration of fatty acid.

Treatment	Growth	[EX]max	[EX]min	I	R
		μg/ml	μg/ml	d	%
1 mM Tween-18:1c	2.0	15.6	16.2	2	104
50 μM Tween-15:0	1.8 ± 0.3 (n = 4)	4.3 ± 0.6	2.7 ± 0.6	2	63
100 μM Tween-15:0	1.8 ± 0.4 (n = 4)	8.7 ± 1.4	5.4 ± 0.9	2	62
150 μm Tween-15:0	1.8 ± 0.5 (n = 4)	10.8 ± 2.0	6.0 ± 1.2	2	56
250 μm Tween-15:0	1.6 ± 0.5 (n = 4)	12.8 ± 1.4	8.7 ± 1.1	2	68
200 μM Tween-17:0	1.0	10.0	6.6	3	66
100 μM Tween-21:0[a]	1.5	0.7	0.2	1	26

[a] Mixed with 100 μM Tween-18:1c.

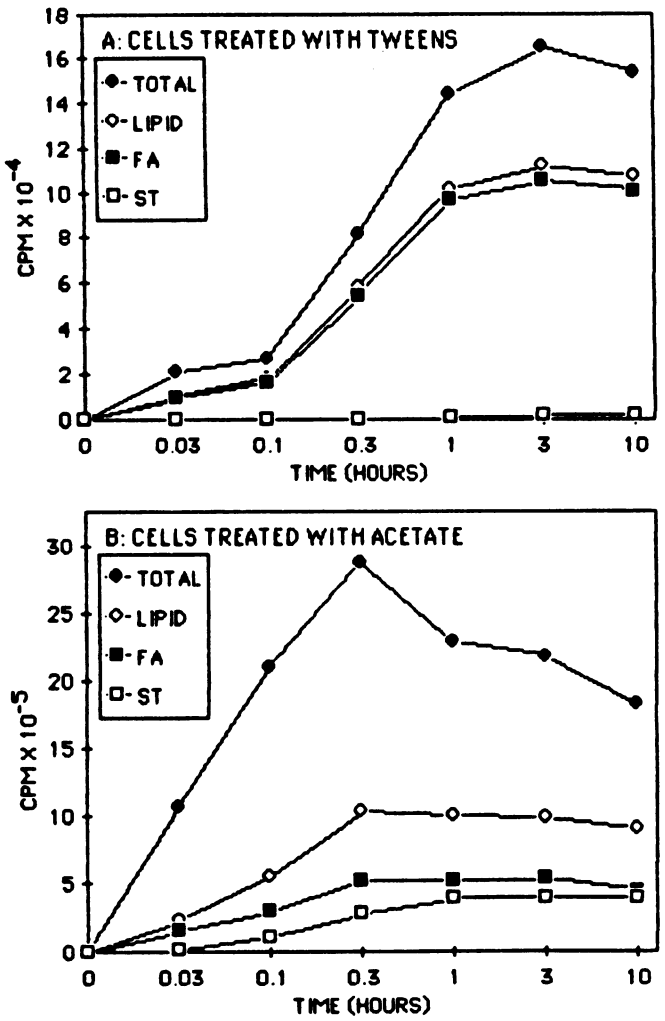

FIG. 3. [^{14}C]Acetate and Tween-[^{14}C]-16:0 uptake. Cells were harvested at indicated times after addition of [^{14}C]acetate or Tween-[^{14}C]-16:0 (to 30 μM final concentration in each case) and lipids were extracted as described in "Materials and Methods." Half of each extract was used to determine distribution of radioactivity between fatty acids and other lipids. Lipids were reacted with 0.5 M sodium methoxide in methanol, and fatty acid methyl esters and other nonpolar components were recovered as described in "Materials and Methods." These were fractionated by TLC on silica G in hexanes:diethyl ether:formic acid (80:20:2, v/v) and distribution of radioactivity between fatty acids, sterols, sterol derivatives, and other components was determined by scraping each lipid into vials and scintillation counting. Values are averages of two replicates. Note differences between panels in scales of vertical axes. Time is represented on a logarithmic scale for convenience.

in cells treated with various Tweens. It has been reported elsewhere that fatty acid synthesis was reduced up to 95% in cells treated with a mixture of Tweens which supplied both saturated and unsaturated fatty acids (13). To determine whether this response varied according to the fatty acid furnished, acetate metabolism by cells treated with Tweens carrying various fatty acids was compared. Table III presents results of the most extensive experiment of this type, and illustrates that acetate metabolism changed in cells which incorporated exogenous fatty acids.

Cells growing in the maximum tolerable concentration of the appropriate Tween were transferred to fresh medium, and [^{14}C] acetate was added 24 h later. This was done to allow the content of exogenous fatty acids to peak before testing its effect on fatty acid synthesis. Cells were then allowed to metabolize the acetate for 24 h before lipid extraction, because distribution of radioactivity between fatty acids may take this long to mimic cellular fatty acid composition (16). This protocol therefore does not distinguish between changes due to altered synthesis and enhanced degradation. However, the differences illustrated by Table III probably represent changes in patterns of *de novo* lipid synthesis, since Figures 3 and 5 indicate that both (normally present) fatty acids and sterols turn over slowly in cells treated with Tweens.

Table III shows that cells with significant exogenous fatty acid content (cells treated with 16:1c, 18:0, 18:1c, and 18:1t) reduced incorporation of [^{14}C]acetate into fatty acids. Many treatments in Table III altered ratios of saturated to unsaturated fatty acids synthesized. Cells fed saturated fatty acids reduced proportions of saturated fatty acids made, while unsaturated fatty acids had the opposite effect. For instance, cells treated with Tween-18:1c had large increases in 18:1 content but mainly synthesized 16:0 and 18:0 (note that amounts of 18:0 made also increased). Therefore, their ratio of saturated to unsaturated fatty acids synthesized was 3-fold higher than in untreated cells. Table III also illustrates that certain treatments altered patterns of 18:1 desaturation. Ratios of 18:1 to 18:2 + 18:3 were not greatly affected by most treatments, but were much larger in cells treated with Tween-18:1c, while 16:1 and 18:1t had less dramatic effects. These results suggest that *de novo* fatty acid synthesis and desaturation may be subject to feedback regulation.

DISCUSSION

A system has been developed for altering the fatty acid composition of cultured soybean cells by adding Tween-fatty acid esters to their growth medium (14). In this paper metabolism of exogenous fatty acids by cells treated with Tweens was described. Tweens were rapidly taken up by a saturable mechanism (Fig. 1; Table I), and the fatty acids were rapidly transferred to lipids (Fig. 4). Similar patterns of glycerolipid labeling were observed in cells treated with [^{14}C]acetate or Tweens carrying ^{14}C-fatty acids (Figs. 4 and 6). Labeling of other lipids differed between cells treated with Tweens or acetate, but these appeared to be either intermediates in the transfer of fatty acids from Tweens to lipids, or lipids made from acetate that do not contain long-chain fatty acids (Figs. 4 and 6). Fatty acids normally present turned over slowly (Fig. 5), but alien fatty acids turned over more rapidly (Table II). The combination of rapid uptake, cell growth, and fatty acid turnover resulted in cyclic fluctuations in exogenous fatty acid content following transfer to fresh medium (Fig. 2). Cells treated with Tweens altered amounts and types of fatty acids synthesized endogenously.

Discussion of these results is divided into five parts: (a) mechanisms of Tween uptake, (b) pathways by which fatty acids are transferred from Tweens to lipids, and (c) specificities of fatty acid turnover, (d) effects on fatty acid synthesis, and (e) regulation of fatty acid composition.

Tween Uptake. It seems unlikely that plant cells normally take up lipids. Plants do not appear to transport lipids, as only trace amounts of lipid are found in either xylem (6) or phloem exudates (17), and even germinating oilseeds convert fatty acids to carbohydrate before exporting their carbon to growing tissues (1).

However, Tweens were rapidly taken up by a saturable mechanism (Fig. 1; Table I); by contrast, uptake of exogenous free fatty acids by soybean cell cultures was reported to be nonsaturable (12). Saturable Tween uptake might be mediated by a receptor for fatty acid or carbohydrate moieties of Tweens, although one can envisage a variety of reasons why passive uptake (*e.g.* by diffusion through the plasma membrane) might be saturable. Apparent uptake rates differed between Tweens (Fig. 2). This might be due to a number of factors, such as: (a) Tween solubility, which varies according to the fatty acid carried, (b) differences in fatty acid turnover, which also vary between fatty

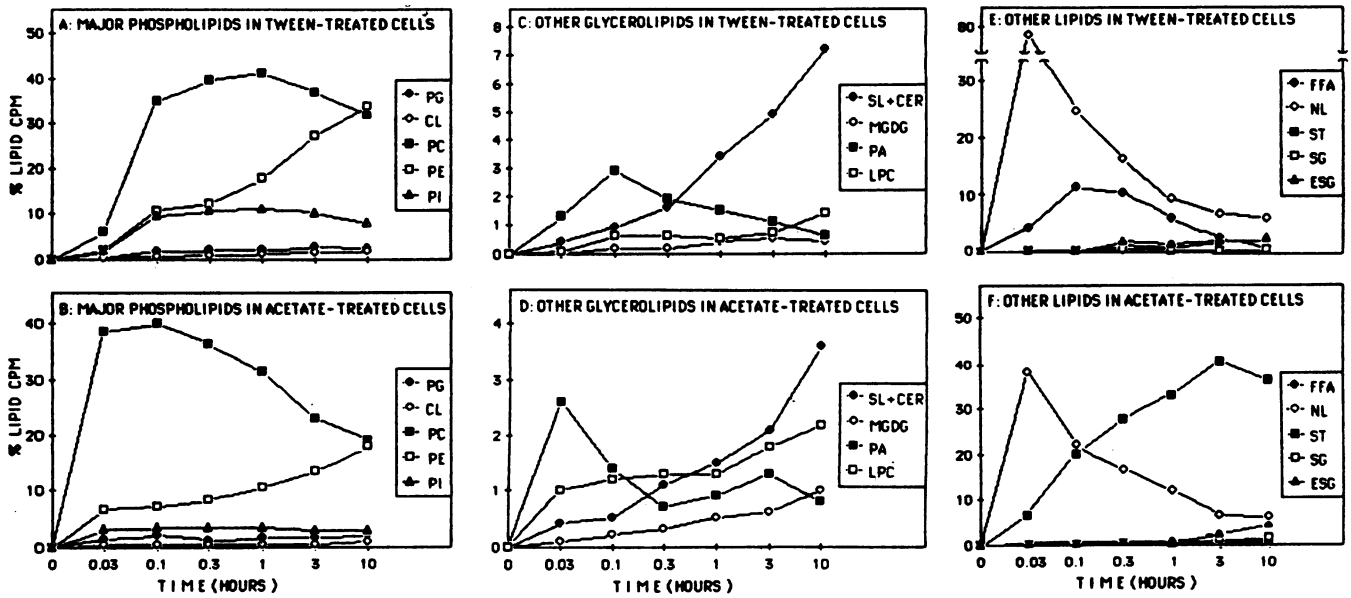

FIG. 4. Labeling of individual lipids in cells treated with [^{14}C]acetate or Tween-[^{14}C]-16:0. The remainder of each extract from Figure 3 was fractionated by two-dimensional TLC on Silica Gel G, individual lipids were scraped into vials, and their radioactivity was determined by scintillation counting. First dimension, chloroform:methanol:ammonium hydroxide (65:25:2, v/v); second dimension, chloroform:methanol:acetic acid:water (85:15:10:3, v/v). Note differences between panels in scales of vertical axes. Time is presented on a logarithmic scale for convenience.

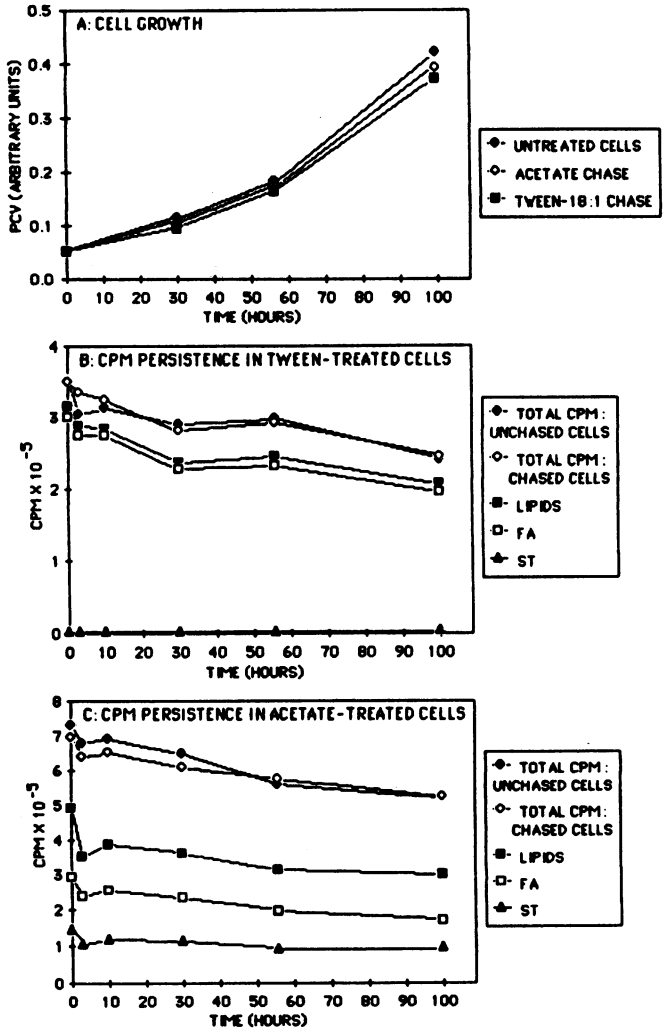

FIG. 5. Persistence of radioactive fatty acids in cells pulsed with [^{14}C] acetate or Tween-[^{14}C]-18:1 and chased with acetate or Tween-18:1c.

acids (Table II), or (c) differences in receptor affinity or turnover (if uptake is via a fatty acid receptor).

Pathways of Fatty Acid Incorporation. Kinetics of glycerolipid labeling were similar in cells treated with [^{14}C]acetate or ^{14}C-fatty acids bonded to Tweens, except that in cells treated with Tweens most label was initially in NL, followed by a transient pool of FFA (Figs. 4 and 6). This is consistent with the hypothesis that Tweens are deacylated at an unspecified location creating a pool of FFA which are then used for lipid synthesis. Patterns of glycerolipid labeling in these experiments resembled those reported in soybean cell cultures supplied [^{14}C]acetate or free ^{14}C-fatty acids (12, 16).

These results indicate that exogenous fatty acids were used for 'eucaryotic' lipid synthesis essentially as if they were made endogenously. All lipids labeled by [^{14}C]acetate were labeled by Tween-[^{14}C]-18:1 (14), and glycerolipids were labeled to about the same extent in cells treated with Tweens or acetate after correcting for relative amounts of ST (Figs. 4 and 6). Moreover, relatively saturated lipids such as PI became more radioactive in cells treated with Tween-[^{14}C]-16:0 than in those treated with

Cells growing at 28°C were pulsed for 3 h with either [1-^{14}C]acetate or Tween-[1-^{14}C]-18:1, then washed thoroughly with fresh medium and appropriate substrate. They were then resuspended in fresh medium with either 300 μM acetic acid or 500 μM Tween-18:1c and left shaking for 100 h. Three aliquots were withdrawn at indicated times, harvested onto filters, and washed thoroughly with medium and appropriate substrate then with distilled H_2O. Two filters were dried, and remaining radioactivity was determined by scintillation counting. Lipids were extracted from cells in the third aliquot as described in "Materials and Methods." Half of each extract was used to determine distribution of radioactivity between fatty acids and other lipids. Lipids were reacted with 0.5 M sodium methoxide in methanol, and fatty acid methyl esters and other nonpolar components were recovered as described in "Materials and Methods." These were fractionated by TLC on silica G in hexanes:diethyl ether:HCOOH (80:20:2, v/v) and distribution of radioactivity between fatty acids, sterols, sterol derivatives, and other components was determined by scraping each lipid into vials and scintillation counting. Values are averages of two replicates. Note differences between panels in vertical axes.

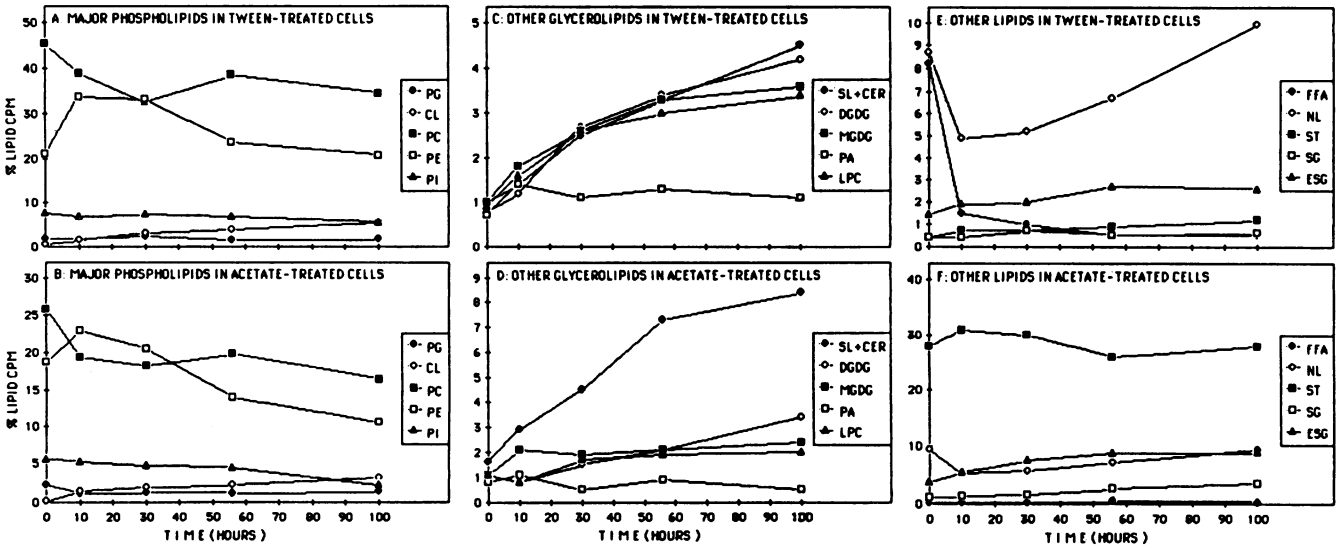

FIG. 6. Changes in labeling of individual lipids in cells pulsed with [^{14}C]acetate or Tween-[^{14}C]-18:1 and chased with acetate or Tween-18:1c. The remainder of each extract from Figure 5 was fractionated by two-dimensional TLC on silica G, individual lipids were scraped into vials, and radioactivity was determined by scintillation counting. First dimension, chloroform:methanol:NH_4OH (65:25:2, v/v); second dimension, chloroform:methanol:acetic acid:water (85:15:10:3, v/v). Note differences between panels in vertical axes.

Tween-[^{14}C]-18:1, whereas the converse was true of relatively unsaturated lipids such as MGDG (the greater labeling of SL+CER in Tween-[^{14}C]-16:0 cells may partly be due to incorporation of 16:0 into sphingosine backbones of cerebrosides).

Previous results indicated that exogenous fatty acids were probably not incorporated into prokaryotic lipids, but these are only minor components of the cultures used (14).

Major differences were observed between treatments in labeling of nonglycerolipids. ST and SG were major components of acetate-treated cells, but were poorly labeled in Tween-treated cells (Figs. 4 and 6). This is probably because ST and SG are made from acetate but do not contain long-chain fatty acids, so in cells treated with Tweens ST and SG were presumably labeled by degradation and reutilization of the radioactive fatty acids. However, little of this occurred, since nearly all lipid radioactivity remained in fatty acid moieties of Tween-treated cells (Fig. 5). By contrast, ESG may also be labeled by acylation, and much less discrepancy in ESG labeling was observed between cells treated with Tween and acetate (Figs. 4 and 6).

NL displayed similar trends in cells treated with acetate or Tweens (Figs. 4 and 6), but probably for very different reasons. NL as isolated in this study include Tweens, di- and triglycerides, and long-chain alcohols. In Tween-treated cells the initial rapid decline in NL reflects Tween deacylation, whereas in acetate-treated cells this is due to conversion of ST precursors to ST (W Terzaghi, unpublished data). By contrast, in both treatments the subsequent slow increase in NL (Fig. 6) may be due to conversion of phospholipids to triglycerides, as PC has been proposed to be a triglyceride precursor (8).

Results indicate that Tweens provide a relatively simple system for *in vivo* studies of many aspects of lipid metabolism, as presumably only diacylglycerol moieties made in the cytosol will be labeled. For instance, studies of lipid incorporation into particular membranes, especially incorporation of eucaryotic lipids into plastid membranes, will have few sources of label. The same is true for studies of precursor-product relationships between various lipids, or specificities of various enzymes involved in lipid synthesis for such things as acylation positions, species to desaturate, or molecular species of diacylglycerol to convert to particular lipids.

Fatty Acid Turnover. Major differences were observed in turnover rates of alien fatty acids and those normally present. Fatty acids normally present—even when exogenously supplied—turn over slowly (Fig. 5; Table II), whereas alien fatty acids turn over more rapidly (Table II). Total fatty acid turnover was not measured in cells treated with alien fatty acids, so it is unknown whether this difference is due to specific turnover of alien fatty acids or a general increase in fatty acid turnover in cells treated with alien fatty acids. Turnover seems most likely to be due to fatty acid degradation, but $^{14}CO_2$ production was not determined and alternatives such as release of incorporated fatty acids to the medium by reexport or cell lysis cannot be ruled out.

Effects on Fatty Acid Synthesis. Cells which incorporated exogenous fatty acids altered endogenous synthesis at three distinct levels: (a) amounts of fatty acids made, (b) ratios of saturated to unsaturated fatty acids made, and (c) subsequent desaturation of unsaturated fatty acids (Table III).

Cells treated with Tweens carrying 16:1c, 18:0, 18:1c, or 18:1t reduced incorporation of acetate into fatty acids (Table III). These differences were probably due to altered partitioning rather than reduced acetate uptake, as amounts of water soluble radioactivity were similar to those of control cells. Moreover, distribution of radioactivity between lipids and other compounds did not vary between cells treated with 3 and 100 μM acetate, suggesting that altered acetate pool sizes did not affect partitioning (W Terzaghi, unpublished data). These results therefore suggested that *de novo* fatty acid synthesis may be subject to feedback regulation. Ways in which this might be mediated are unknown in plants.

It was surprising that Tween-16:0 had so little apparent effect on fatty acid synthesis, as it is both an abundant fatty acid and the precursor to the C18 fatty acids. This lack of effect may have resulted because only low concentrations were administered, due to its cytotoxicity (Table III).

Cells treated with saturated fatty acids reduced proportions of saturated fatty acids made, while the converse was true of cells treated with unsaturated fatty acids with *cis* double bonds. Subsequent desaturation was also reduced in cells treated with certain Tweens (Table III); 18:1 is synthesized inside plastids (4, 10), while desaturation of 18:1 to 18:2 is mainly catalyzed by a membrane-bound enzyme in the ER (3, 5). Results suggest that both processes may be subject to feedback control.

Activity of the 18:2 desaturase might be modulated by changes in membrane fluidity associated with changes in fatty acid composition, as in other eucaryotes (2). Synthesis of 18:1 is probably regulated in some other way, as all the enzymes involved are

Table III. *Distribution of [1-^{14}C]Acetate between Fatty Acids and Other Sinks, and between Fatty Acid Species in Cells Grown in Various Tweens*

Cells adapted to growth in the maximum tolerable concentrations of the appropriate Tweens were transferred to fresh medium. Twenty-four h later [1-^{14}C]acetate was added, and left for 24 h. Lipids were then extracted and analyzed as described in "Materials and Methods." In part b) the top line for each treatment presents the distribution of radioactivity between fatty acids, whereas the bottom line presents the fatty acid composition of that treatment, as % by weight. Tween concentrations refer to concentration of fatty acid. TOT, total radioactivity taken up; LIP, lipid radioactivity as cpm and % of total radioactivity; WS, water soluble radioactivity as cpm and % of total radioactivity; FA, cpm in fatty acids; S/U, saturated fatty acid/unsaturated fatty acid cpm; 1/2 + 3, 18:1/(18:2 + 18:3) cpm, EX, exogenous fatty acid in membrane lipids (% by weight). All data are means of two replicate flasks.

a) Total Radioactivity

Treatment	TOT CPM $\times 10^{-3}$	LIP CPM $\times 10^{-3}$	%	WS CPM $\times 10^{-3}$	%	FA CPM $\times 10^{-3}$
Control	679	251	37	428	63	131
100 μM 16:0	707	239	34	468	66	122
150 μM 16:1c	600	141	24	459	76	68
30 μM 16:1t	771	299	39	472	61	173
1 mM 18:0	410	89	22	321	78	45
1 mM 18:1c	715	202	28	513	72	73
300 μM 18:1t	551	97	18	454	82	49
150 μM 18:2	606	246	41	360	59	125
250 μM 18:3	684	279	41	405	59	156

b) Fatty Acids

Treatment	EX	16:0	18:0	18:1	18:2	18:3	S/U	1/2 + 3
Control		27	10	6	30	28	0.59	0.10
	0	23	3	5	38	31		
100 μM 16:0		22	10	7	33	30	0.47	0.11
	"0"	23	7	6	40	25		
150 μM 16:1c		48	15	8	18	11	1.70	0.21
	10	23	4	12	39	12		
30 μM 16:1t		27	11	5	33	25	0.61	0.09
	1	23	4	7	41	25		
1 mM 18:0		16	8	9	44	23	0.32	0.13
	"24"	15	27	3	43	12		
1 mM 18:1c		46	19	15	12	10	1.86	0.68
	"43"	6	2	48	36	11		
300 μM 18:1t		25	10	10	33	24	0.54	0.18
	35	8	5	40	34	14		
150 μM 18:2		33	10	7	24	27	0.75	0.14
	"0"	31	8	5	36	21		
250 μM 18:3		27	9	6	31	28	0.56	0.10
	"0"	23	4	6	39	28		

soluble (4, 10, 11). Results indicated that regulation involved reductions in both elongation of 16:0 and desaturation of 18:0, as increased proportions of both 16:0 and 18:0 were made in cells treated with unsaturated fatty acids (Table III). This could be most simply mediated by increased activity of an acyl-ACP thioesterase on 16:0-ACP and 18:0-ACP, as both elongation of 16:0 (9) and desaturation of 18:0 (4) specifically require the acyl-ACP thioester as substrate.

Regulation of Membrane Fatty Acid Composition. Large variations in membrane fatty acid composition were previously observed in these cultures, suggesting that perhaps membrane fatty acid composition was not closely regulated (14). However, results reported here suggest that the cells were attempting to correct externally imposed imbalances in fatty acid composition (Table III). Results indicated that most changes in fatty acid composition in these cultures (other than those created by incorporation of exogenous fatty acids) were generated by changes in fatty acid synthesis, since this changed in cells with altered fatty acid composition (Table III) whereas little turnover of fatty acids normally present was observed (Figs. 3 and 5). Altering membrane fatty acid composition by changes in fatty acid synthesis is a slow process, and it will be of interest to determine whether fatty acid turnover may be enhanced under conditions, such as temperature shifts, where one might expect cells to attempt to rapidly alter their fatty acid composition.

Given the wide range of fatty acid compositions observed (14) one might anticipate large changes in membrane properties unless some counteraction is taken. It will therefore be of interest to determine whether large variations in membrane properties are tolerated, or whether changes in membrane fatty acid composition are accompanied by compensatory changes in other membrane components.

The system described in this and the previous publication (14) promises to be useful for many studies on fatty acid metabolism or membrane-related processes in plants. Extension of these studies to whole plants may now be possible, as incorporation of exogenous fatty acids into membrane lipids of both leaves and roots of plants treated with Tweens has recently been observed (W Terzaghi, unpublished data).

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