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Utilization of Fatty Acid Supplements by Cultured Animal Cells†

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ABSTRACT: Animal cells cultured in serum-free minimal essential medium (Eagle) containing a biotin antagonist can effectively utilize fatty acids from Tweens (sorbitan–fatty acid esters) provided as medium supplements. The fatty acid composition of the major phospholipids, phosphatidylethanolamine and phosphatidylcholine, was determined after mouse fibroblasts (LM cells) were grown in medium supplemented with Tweens synthesized to specifically contain an odd chain, unbranched, saturated fatty acid (C_{15}–C_{21}), an even chain, unbranched, saturated fatty acid (C_{16}–C_{22}), or an unsaturated or polyunsaturated fatty acid. The per cent of fatty acid in cellular lipids derived from various Tween supplements provided at the same concentration was demonstrated to be independent of fatty acid chain length for Tween preparations containing the following straight chain saturated fatty acids: C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, and C_{21}. When the medium concentration of the C_{19} fatty acid bound to Tween was increased, there was a concomitant increase in the extent to which C_{19} and its metabolic products were incorporated into cellular phospholipids, and this increase appeared to follow saturation kinetics. Cells grown in serum-free medium without a Tween supplement had a phospholipid fatty acid composition approximately 26% saturated. The fatty acid content of the cellular phospholipids was not significantly altered when cells were grown with Tween supplements that contained even chain saturated fatty acids ranging from C_{16} to C_{22}. The lipids derived from cells grown with the C_{20} or C_{22} acids did not contain significantly more C_{20} or C_{22} fatty acids in phospholipids than cells grown with Tween containing hexadecanoic or octadecanoic acids. The same per cent of saturated fatty acids was observed in the lipids of cells grown with Tween–heptadecanoate as was observed for cells grown with even chain saturated fatty acid supplements. However, the lipids of cells grown with Tweens containing C_{15}, C_{19}, and C_{21} saturated fatty acids were more extensively saturated. When high levels of Tween–nonadecanoate supplement were used, over 60% of the fatty acids in cellular phospholipid were saturated. The extent of utilization of unsaturated fatty acids from Tween supplements was approximately half that observed with saturated fatty acid Tween supplements. Though the per cent of unsaturated fatty acids in phospholipids did not increase when cells were grown with unsaturated or polyunsaturated supplements, the number of unsaturated sites was greater when polyunsaturated supplements were used. These data indicate that the degree of unsaturation in phospholipids of animal cells grown in culture can be manipulated experimentally. Cells treated in this fashion provide an ideal laboratory system for studying structure–function relationships in animal cell membranes.

The influence of membrane lipid physical state on membrane functions and membrane assembly processes has been studied extensively in microbial unsaturated fatty acid auxotrophs (Machtiger and Fox, 1973). With these microorganisms, the investigator can manipulate the fatty acid composition of the membrane lipids simply by changing the fatty acid supplement in the growth medium. Membranes derived from a bacterial unsaturated fatty acid auxotroph grown with a given fatty acid supplement, and phospholipids derived from these membranes, have been shown to exhibit two characteristic temperatures, each of which describes a phase boundary. The membrane lipids begin to freeze at the upper characteristic temperature, and become totally frozen at the lower one. [For more thorough treatments of phase transitions in membranes and in model mixed lipid systems, see articles by Phillips *et al.* (1970), Shimshick and McConnell (1973), and Linden *et al.* (1973).] Physiological processes such as transport show marked fluctuations at one or both of these characteristic temperatures (Linden *et al.*, 1973); membrane assembly and cellular growth have a critical temperature at the lower of these (Overath *et al.*, 1970; Tsukagoshi and Fox, 1973a,b; McElhaney, 1974).

Although these phase transition related phenomena are of considerable basic interest, the consequences of events occurring at phase boundary temperatures may have no physiologi-

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‡ Supported by Postdoctoral Fellowship DRF-749 from the Damon Runyon Fund.

§ Supported by Postdoctoral Fellowship PF-886 from the American Cancer Society.

¶ Recipient of Research Career Development Award GM-42,359 from the U. S. Public Health Service.

cal significance in homeothermic organisms. From a purely practical consideration, however, the events that occur at the lower phase boundary can be exploited as a tool to examine biologically significant phenomena, such as membrane assembly in bacteria (Tsukagoshi and Fox, 1973a,b). To extend the range of phenomena that can be studied with this tool, we have developed a technique for manipulating the fatty acid composition in lipids of animal cells grown in culture (Wisnieski *et al.*, 1973). In order to do this we first had to overcome a number of problems inherent in working with these cells. First, animal cells are normally grown in media containing serum, and consequently derive almost all their lipids from the serum in the growth medium (Bailey, 1967). We therefore restricted our initial efforts to cell lines that could be grown in serum-free medium. Second, nonesterified fatty acids are often toxic to animal cells growing in culture (Moskowitz, 1967). We discovered that fatty acid supplements were not toxic when added to the medium as sorbitan esters (Tweens). Third, we had to find some way to prevent desaturation if we were to be able to significantly increase the degree of saturation of membrane lipids over that normally encountered. Here, we took advantage of the fact that the mixed function dehydrogenase activity responsible for desaturation of fatty acids in animal cells has a narrow chain length specificity (Brett *et al.*, 1971). Fourth, animal cells growth in serum-free medium are, and indeed must be, capable of endogenous fatty acid synthesis. This was minimized by including desthiobiotin, a biotin antagonist, in the medium.

In our previous report, we showed that mouse LM cells grown in serum-free medium containing Tween-19:0[1] readily incorporated 19:0 and its metabolic products into cellular phospholipids. Since 19:0 is not readily desaturated in these cells, supplementation with 19:0 led to an increase in the degree of saturation of the cellular phospholipids. In the present report, we describe the results obtained when LM cells were grown with a wide variety of Tween supplements containing fatty acids of different chain length and degree of unsaturation.

Materials and Methods

Cell Line. Mouse LM cells, derived from NCTC clone 929 (L cells) and adapted to growth in medium 199 plus 0.5% peptone, were obtained from the American Type Culture Collection.

Chemicals and Radiochemicals. All fatty acids except stearolic (9-octadecynoic) acid were obtained from the Hormel Institute (Austin, Minn.). Stearolic acid was a gift from Dr. Alec Keith, Pennsylvania State University. All fatty acids were more than 99% pure when analyzed by gas–liquid chromatography (glc). [9,10-^{3}H]Palmitic acid (500 Ci/mol), [9,10-^{3}H]oleic acid (2.2 Ci/mol), and [9,10-^{3}H]stearic acid (500 Ci/mol) were obtained from Amersham/Searle. [9,10-^{3}H]Elaidic acid (1.7 Ci/mol) was synthesized by Tsukagoshi and Fox (1973a).

Synthesis and Characterization of Tween–Fatty Acid Esters. Tween–fatty acid esters were chemically synthesized to contain a defined fatty acid moiety. The details of the methodology have been published (Wisnieski *et al.*, 1973; Brody and Allen, 1972). Briefly, Tween–fatty acid esters were synthesized (1) by transesterification of commercially available Tweens with excess fatty acid (approximately 1:40 molar ratio) and (2) by transesterification of Tween–acetate with a moderate excess of fatty acid (1:2 to 1:5 molar ratio Tween–acetate to fatty acid).

Method 1 was used to synthesize Tween–21:0, Tween–22:0, and Tween–18:2$\Delta^{9,12}$*cis,cis*. With this method, 4 g of fatty acid was reacted with 1 g of Tween 20 in 25 ml of *tert*-butyl alcohol plus 2.5 ml of 12 N HCl. The reaction mixture was refluxed under N_2 for 4 hr and solvents were removed by vacuum evaporation.

Method 2 was used to synthesize the other Tween–fatty acid esters used in this study. The first step in this method was the synthesis of Tween–acetate by reaction of 5 g of Tween 20 with 20 ml of glacial acetic acid in a solution containing 100 ml of *tert*-butyl alcohol and 10 ml of 12 N HCl. The reaction mixture was refluxed under N_2 for 4 hr. Solvents were removed by vacuum distillation at 50°. The purification procedure for all Tweens, including Tween–acetate, is described later. (The Tween–acetate preparation used in growth media was obtained from two successive transacetylations and purifications.) The next step in the synthesis of Tween–fatty acid esters *via* method 2 involved refluxing 1 g of Tween–acetate with 0.5 g of fatty acid in a solution containing 10 ml of *tert*-butyl alcohol and 1 ml of 12 N HCl. After 4 hr of refluxing under N_2, the solvents were removed by vacuum evaporation. In the preparation of Tweens containing radioactive fatty acids, [^{3}H]fatty acids were first diluted with unlabeled fatty acids to yield the specific activities shown in Table I.

Tweens were separated from free fatty acids by column chromatography. A detailed account has been published (Wisnieski *et al.*, 1973). Columns were packed with MN-silica gel, 70–325 mesh ASTM (10 g/g of sample), in diethyl ether and washed with three column volumes of ether. Samples were applied in ether containing sufficient methanol to solubilize the Tween portion of the sample. Free fatty acids were eluted with four column volumes of ether, and Tween–fatty acid esters with four column volumes of methanol. Solvents were removed by vacuum evaporation. Methanol-free Tween preparations were solubilized in glass-distilled water (100 mg/ml) and sterilized by autoclaving at 15 psi for 15 min.

The purity of the Tween preparations was determined by thin-layer chromatography (tlc) and glc. The absence of free fatty acids was assessed by tlc and purity in terms of final fatty acid composition by glc. Tlc plates (250 μm silica gel G; Analtech) were predeveloped with ether, dried, spotted with sample, and developed with ether. Tween–fatty acid esters remained at the origin and free fatty acids migrated with an R_F of approximately 0.5. If free fatty acids were detected in a preparation at the level of 0.5 μg/mg of Tween, it was repurified as above. Conditions for glc have been published (Wisnieski *et al.*, 1973).

The characterization of the Tween–fatty acid esters used in this study is presented in Table I. Tweens with fatty acid compositions less than 95% pure were all prepared by method 1. Method 2, which employed Tween–acetate instead of commercially available Tween 20 as an initial adduct, yielded Tweens with relatively homogeneous fatty acid compositions. Based on an average molecular weight of the Tween moiety of 2800 (calculated from the formula given by Osipow (1962)), a fully esterified preparation of Tween–fatty acid ester should consist of 9–10% fatty acid (C_{16}–C_{18}) by weight. All synthetic Tweens contained less than 4% fatty acid by weight, except for Tween–16:0 which contained 14% of 16:0 by weight, a value somewhat higher than the calculated maximum. Tween–acetate/Tween–fatty acid ester ratios were equalized by adding Tween–acetate to provide a final Tween concentration of 1.5 mg/ml unless otherwise indicated in the text.

[1] See Table I for explanation of abbreviations for fatty acids and synthetic Tweens.

TABLE I: Compositional Analysis of Tween–Fatty Acid Esters.

Synthetic Tween[a]	Symbol	Fatty Acid[b] (% by weight)	Purity[c] (%)	Specific Activity (Ci/mol)
Tween–pentadecanoic acid	Tween–15:0	0.54	98	
Tween–heptadecanoic acid	Tween–17:0	0.74	95	
Tween–nonadecanoic acid	Tween–19:0	3.46	98	
Tween–heneicosanoic acid	Tween–21:0	1.59	66	
Tween–palmitic acid	Tween–16:0	14.1	99	
Tween–arachidic acid	Tween–20:0	3.24	97	
Tween–behenic acid	Tween–22:0	0.79	73	
Tween–linoleic acid	Tween–18:2$\Delta^{9,12}$*cis,cis*	1.73	85	
Tween–linolenic acid	Tween–18:3$\Delta^{9,12,15}$*cis,cis,cis*	1.80	98	
Tween–stearolic acid	Tween–18:1$^{\equiv 9}$	0.80	96	
Tween–acetate	Tween–acetate	0.003		
Tween–[9,10-^{3}H]palmitic acid	Tween–[9,10-^{3}H]16:0	0.45	98	1.79
Tween–[9,10-^{3}H]stearic acid	Tween–[9,10-^{3}H]18:0	1.64	99	3.00
Tween–[9,10-^{3}H]oleic acid	Tween–[9,10-^{3}H]18:1Δ^9*cis*	2.25	98	9.96
Tween–[9,10-^{3}H]elaidic acid	Tween–[9,10-^{3}H]18:1Δ^9*trans*	1.16	98	0.12

[a] See Materials and Methods for synthetic procedures. [b] Per cent by weight of fatty acid in Tween–fatty acid esters. [c] Purity in terms of fatty acid composition. Tweens that were less than 95% pure in their fatty acid content contained the fatty acids indicated below in addition to the designated fatty acid. The per cent of total fatty acid as a given contaminant is indicated by the number in parentheses. Tween–21:0 contained 12:0 (11.6), 14:0 (12.2), 16:0 (7.0), 18:0 (3.5); Tween–22:0 contained 12:0 (11.9), 14:0 (7.8), 16:0 (5.6), 18:0 (1.7); Tween–18:2$\Delta^{9,12}$*cis,cis* contained 12:0 (4.1), 14:0 (5.6), 16:0 (3.8), 18:0 (1.8).

Fatty Acid Enrichment. The procedures used for enriching cultured animal cells with a fatty acid provided as a medium supplement are essentially those described by Wisnieski *et al.* (1973). Stock cultures of LM cells were maintained in Eagle's minimal essential medium with Earle's salts (MEM) plus 0.5% bactopeptone (MEM + P). All cultures except those described in the section on tracer analysis were inoculated from the same pooled cell suspension (see below) of MEM + P grown cells. Cells were grown in 75-cm^2 Falcon tissue culture flasks. Each flask contained 15 ml of a chemically defined medium containing no biotin and was inoculated with approximately 10^7 cells. This basal medium consisted of MEM modified to contain twice the normal glutamine and vitamin concentrations and 2 mg of *dl*-desthiobiotin/l. (MEM + GVdB medium). All cultures were incubated at 37° for 72 hr. At this point, the medium was removed by aspiration and replaced with 15 ml of MEM + GVdB supplemented with a Tween–fatty acid ester. The cultures were then incubated for an additional 72 hr. The contents of three 75-cm^2 Falcon flasks were combined for each analysis.

Preparation of Cell Suspensions for Inoculation and for Biochemical Analysis. At confluency (approximately 5×10^7–10^8 cells/flask), cells were removed by washing the monolayer with 3 ml of 0.005% trypsin (Sigma: Type 2, pancreatic; 1500 BAEE units/mg) diluted with Tris-saline (Tris (3 g/l.)–NaCl (8 g/l.)–Na_2HPO_4 (0.1 g/l.)–12 N HCl (1.75 ml)) at pH 7.4. Trypsin was removed by aspiration after 15 sec of washing, and the flask was incubated for an additional 5 min. Then, 3 ml of MEM was added and the monolayer was dispersed by agitation.

Analysis of Cellular Growth and Viability. After growth in medium supplemented with Tween–fatty acid ester, the cell suspensions (see above) from three flasks were pooled (yielding 9 ml total) and a 0.5-ml aliquot was removed and diluted 1:10 with Tris-saline for protein determination (Lowry *et al.*, 1951) and cell count (hemacytometer). A 10-μl sample was removed for cell count determination, and the remainder of the aliquot was pelleted and the supernatant solution discarded. The pelleted cells were washed with 5 ml of Tris-saline prior to protein determination. Most cultures had undergone two to three cell doublings in cell mass during the 72-hr growth time. (During the 72-hr period in which the cells were incubated in MEM + GVdB not supplemented with a Tween–fatty acid ester, there was a 30–35% increase in the number of cells that adhered to the flask.)

Lipid Analysis. The bulk of the trypsinized cell suspension (8.5 ml) was diluted with 30 ml of Tris-saline, pelleted by centrifugation (12,500*g* for 15 min), washed once with Tris-saline, and pelleted again. The cells were then extracted with chloroform–methanol (2:1 v/v). The extract was filtered through a plug of glass wool to remove debris, dried under N_2, and suspended in solvent before application to tlc plates (250 μ of silica gel G; Analtech; predeveloped with ether). Chloroform–methanol–ammonium hydroxide (65:35:10 by volume) was used to resolve the phospholipids. Tweens migrated with the solvent front.

Lipids were visualized by exposing an edge of the plate to iodine vapors. Areas of silic acid containing phosphatidylcholine and phosphatidylethanolamine were scraped from the plate, and the lipids were eluted with three volumes of methanol followed by one volume of chloroform–methanol (2:1, v/v). Lipids were transmethylated with BF_3 (14%) in methanol. Glc of methylated fatty acids was performed as described by Wisnieski *et al.* (1973).

Analysis of Radioactivity. All radioactive, methylated fatty acid samples were dried under N_2 and dissolved in 50 μl of hexane. A 5-μl aliquot was dissolved in 15 ml of the toluene–Triton (3:1 v/v) liquid scintillation solution of Patterson and Green (1965) for determination of radioactivity by scintillation counting. Another 5-μl aliquot of the fatty acid–hexane solution was subjected to glc to determine total fatty acid mass and the mass of each individual fatty acid species. To estimate the per cent of the cellular fatty acids derived from the supplement, the specific activity of the fatty acids in the

TABLE II: Effect of Added Tween–Acetate on Cellular Incorporation of Odd Chain Fatty Acids from Tween–19:0 into Phospholipids.[a]

Tween–Acetate[b] (mg/ml)	Enrichment for 19:0 (%)	Enrichment for Odd Chain Fatty Acids (%)[c]
none	25.5	30.7
0.25	23.2	28.4
0.50	23.4	27.9
0.75	21.6	26.8
1.00	24.5	28.1
2.25	24.7	30.6

[a] Phosphatidylcholine plus phosphatidylethanolamine. [b] All growth media contained 0.25 mg/ml of Tween–19:0 (containing 8.68 μg/ml of 19:0) plus Tween–acetate at the designated concentration. [c] 19:0 plus metabolic products of 19:0.

phospholipids (PE + PC) was first calculated by dividing the total radioactivity of a 5-μl aliquot of lipid in hexane by the total mass of fatty acids in this given aliquot. This ratio was then divided by the specific activity of the fatty acid in the Tween–fatty acid ester, and the quotient was multiplied by 100.

A third 5-μl aliquot was used to assess the radioactivity in the individual fatty acid species. It was mixed with sufficient standard, unlabeled fatty acid methyl esters to permit visualization after stream splitting. Effluent from the chromatograph was split 15:1 with a Perkin-Elmer stream splitter accessory. The larger portion was collected for analysis of radioactivity and fractionated using a Packard Model 852 gas fraction collector attached to the Perkin-Elmer 990 gas chromatograph. Each peak was collected in a Packard glass fraction collection tube packed with a cellulose filter and glass wool, and the tubes were placed directly into scintillation vials containing 15 ml of scintillation fluid. The per cent of total radioactivity in each individual fatty acid species was determined by dividing the cpm per individual species by the sum of cpm in all species from the fraction collection effluent and multiplying the quotients by 100.

Results

We previously showed that the fatty acid composition of cultured animal cells was grossly altered when growth occurred in a chemically defined, serum-free medium containing Tween–19:0 (Wisnieski *et al.*, 1973). As the first step in a systematic evaluation of the optimal conditions for enriching animal cells with fatty acid supplements supplied as Tweens, we have examined the efficiency of fatty acid uptake when the concentration of the Tween–fatty acid was held constant and the concentration of Tween containing no esterified fatty acid (*i.e.*, Tween–acetate) was varied. Under these conditions, we could differentiate between the response to detergent concentration and the response to Tween–fatty acid concentration. In the experiment described in Table II, the concentration of Tween–acetate was increased from 0 to 2.25 mg/ml, while the concentration of a Tween–19:0 preparation was held constant

TABLE III: Fatty Acid Composition of Phospholipids[a] from Cells Grown[b] with Odd Chain Saturated Fatty Acid Supplements.

	Tween–Fatty Acid Supplement[c]							
	15:0		17:0		19:0			21:0[d]
Fatty Acid	4 μg/ml	8 μg/ml	4 μg/ml	8 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml	4 μg/ml
15:0	*4.7*	*16.5*	4.0	0.6	0.4	0.6	0.8	0.7
15:1	0.2	0.5	0	0	0	0	0	0
17:0	3.5	6.0	*4.8*	*9.3*	1.0	1.7	3.2	2.2
17:1	9.5	13.0	8.5	16.8	1.9	3.1	1.6	1.2
19:0	1.3	0	0	0.1	*13.4*	*27.0*	*45.8*	3.6
19:1	1.1	0.7	3.2	2.3	3.1	5.4	0	0.8
21:0	0	0	0	0	0	0	0	*13.0*
21:1	0	0	0	0	0	0	0	0
% odd chain	20.3	36.7	20.5	29.1	19.8	37.8	51.4	21.5
14:0	0.7	0.6	3.5	1.5	1.1	1.3	0.6	3.1
16:0	9.7	6.8	9.7	12.1	11.4	8.8	7.2	9.1
16:1	8.6	8.8	8.8	7.7	9.4	7.4	5.9	4.8
18:0	8.2	5.6	6.5	4.9	6.3	4.4	4.5	10.1
18:1	50.9	40.6	49.1	43.2	49.5	38.7	30.2	46.5
20:1	1.5	0.6	1.8	1.4	1.8	1.4	0	3.3
Other[e]	0.1	0.3	0.7	0.1	0.7	0.2	0.2	1.6
% even chain	79.7	63.3	79.5	70.9	80.2	62.2	48.6	78.5
% saturated	28.1	35.5	27.9	28.5	33.6	43.8	62.1	41.8
Doublings of cell number	3	2	3	2.5	2.5	2.5	1[f]	1[g]
Mg of protein/10^8 cells	2.4	1.9	1.7	2.6	2.4	2.2	2.6	2.8

[a] Phosphatidylcholine plus phosphatidylethanolamine. [b] All data determined after 3 days of growth with the indicated supplements (see Materials and Methods). [c] The concentrations given are those of the fatty acid moiety of the Tween–fatty acid supplements. The total concentration of Tween–fatty acid supplement plus Tween–acetate was 1.5 mg/ml in all cases. [d] 8 μg/ml of Tween–21:0 was cytotoxic. [e] Fatty acids grouped under other are unsaturated and either $\leq$14:1 or $\geq$22:1. [f] No floating cells or debris were observed in the medium. [g] Numerous cells were floating in the medium above the monolayer at the time of harvesting. These were aspirated with the medium and discarded.

TABLE IV: Fatty Acid Composition of Phospholipids[a] from Cells Grown[b] with Even Chain Saturated Fatty Acid Supplements.

		Tween–Fatty Acid Supplements[d]			
		16:0	[^{3}H]-18:0[e]	20:0	22:0
Fatty Acid	Control[c]	8 μg/ml	8 μg/ml	8 μg/ml	8 μg/ml
14:0	1.4	2.4	2.1	2.5	2.8
15:0	1.2	2.8	0.4	1.7	2.3
16:0	14.7	16.3	13.1	14.4	12.3
16:1	9.1	13.7	10.6	12.3	13.9
17:0	0.2	1.1	0.4	1.3	0.8
17:1	0.2	1.4	0.5	1.9	1.7
18:0	10.0	7.8	12.2	8.1	7.6
18:1	59.5	50.0	58.7	53.9	54.8
20:0	0	0	0	0.8	0
20:1	2.2	2.6	1.5	1.6	2.5
22:0	0	0	0	0	0
22:1	0	0	0	0	0
Other[f]	1.5	1.9	0.5	1.5	1.3
% saturated	27.5	30.4	28.2	28.8	25.8
Doublings of cell number	2.5	3	2.5	2.5	2.5
Mg of protein/10^8 cells	2.2	2.0	2.3	2.0	2.2

[a] Phosphatidylcholine plus phosphatidylethanolamine. [b] All data determined after 3 days of growth with the indicated supplements (see Materials and Methods). [c] LM cells grown in MEM + P medium (see Materials and Methods). [d] The concentrations given are those of the fatty acid moiety of the Tween–fatty acid supplements. The total concentration of Tween–fatty acid supplement plus Tween–acetate was 1.5 mg/ml in all cases. [e] Fatty acids exogenously derived from Tween-[9,10-^{3}H]18:0 accounted for 28.7% of the total fatty acids in PC + PE. This was assessed by monitoring the specific activity of the fatty acids obtained from these phospholipids (see Materials and Methods for details). [f] Fatty acids grouped under other are unsaturated and either ≤14:1 or ≥22:1.

TABLE V: Fatty Acid Composition of Phospholipids[a] from Cells Grown[b] with Even Chain Unsaturated Fatty Acid Supplements.

	Tween–Fatty Acid Supplement[c]				
	[^{3}H]-18:1-Δ^9*cis*	[^{3}H]-18:1-Δ^9*trans*	18:3$\Delta^{9,12,15}$*cis,cis,cis*		
Fatty Acid	8 μg/ml	8 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml
14:0	2.0	2.1	2.3	2.8	0.4
15:0	0.5	2.2	1.5	0.6	0.7
16:0	16.7	12.7	14.5	17.1	8.7
16:1	8.8	10.9	17.9	13.0	3.3
17:0	0.1	0.7	0.6	0	0.1
17:1	0.4	1.4	0.9	0.3	0.2
18:0	7.2	6.6	7.4	7.0	15.2
18:1	61.4	61.5	46.0	40.2	33.4
18:3	0	0	4.5	11.4	32.1
20:1	2.8	1.6	1.2	1.5	0
20:3	0	0	0.9	2.0	1.6
20:4	0	0	1.7	3.4	3.7
Other[d]	0.1	0.3	0.6	0.7	0.6
% unsaturated	73.5	75.7	73.7	72.5	74.9
% derived from supplement	15.3[e]	19.6[e]	7.1[f]	16.8[f]	37.4[f]
Doublings of cell number	3	2.5	3	3	*g*
Mg of protein/10^8 cells	1.6	2.4	2.0	2.0	7.4

[a] Phosphatidylcholine plus phosphatidylethanolamine. [b] All data determined after 3 days of growth with the indicated supplements (see Materials and Methods). [c] The concentrations given are those of the fatty acid moiety of the Tween–fatty acid supplements. The total concentration of Tween–fatty acid supplement plus Tween–acetate was 1.5 mg/ml in all cases. [d] Fatty acids grouped under other are unsaturated and either ≤14:1 or ≥22:1. [e] Assessed by monitoring the specific activity of the fatty acids obtained from PC + PE. See Materials and Methods for experimental details. [f] It was assumed that the fatty acids chromatographing as 20:3 and 20:4 were derived from the 18:3 supplement. [g] Cell number decreased by one-half.

at 0.25 mg/ml. The data clearly demonstrate that the uptake of 19:0 into cellular phospholipids was not affected by this tenfold variation in Tween–acetate concentration. This finding indicated that we could safely use high levels of Tween–acetate to normalize fatty acid and Tween concentrations for comparative experiments. The low fatty acid contents of several Tween preparations (Table I) determined the final Tween supplement concentration (1.5 mg/ml) necessary to provide all cultures with the same concentration of Tween–acetate plus Tween–fatty acid ester. In this experiment and in all others described in this report, we chose to analyze the fatty acid composition of the combined phosphatidylethanolamine and phosphatidylcholine fractions. We were thus assured that unesterified fatty acids, or fatty acids derived from Tween or neutral lipids would not interfere with our analyses. Our data derived from compositional analyses of these phospholipids should also be generally representative of the bulk cellular phospholipids since phosphatidylethanolamine and phosphatidylcholine account for approximately 80% of the total cellular phospholipids (Anderson *et al.*, 1969).

Uptake of Odd Chain Fatty Acids from Tween Supplements. Animal cell phospholipids normally contain a very low percentage of unbranched, odd chain fatty acids. For example, LM cells grown in serum-free medium under our conditions with no added odd chain supplement contain no more than 1–2% odd chain fatty acids (Wisnieski *et al.*, 1973). Since odd chain fatty acids can be easily distinguished from even chain fatty acids by gas chromatography, it is relatively simple to assess the ability of cells to utilize odd chain fatty acid supplements provided in the medium as Tweens. The results of fatty acid compositional analyses of cells grown in medium with Tween supplements containing 15:0, 17:0, 19:0, and 21:0 are shown in Table III. [*At this point we emphasize the fact that all data in Tables III–V can be compared directly. All the cells used to provide the data in these tables were grown from the same pooled inoculum, and were treated identically except for variation of the fatty acid supplement.*]

The utilization of odd chain fatty acids provided as Tween supplements was tested at two concentrations of each supple-

ment (4 and 8 μg/ml). In one case (Tween–21:0), comparison with the higher concentration of supplement was not possible since severe cytotoxicity was encountered. There was no indication of any chain length specificity for uptake for any of these supplements. All four were taken up to almost the same extent when supplied in the medium at the same concentration. Tween–19:0 was also tested at a third, higher supplement concentration, and the increase in utilization was directly proportional to supplement concentration for the three concentrations of Tween–19:0 that were tested. The reciprocal of the concentration of the fatty acid portion of Tween–19:0 supplement was plotted *vs.* the reciprocal of the per cent of odd chain fatty acids in phospholipid by the method of Lineweaver and Burk (1934). All three points fell on a straight line (plot not shown) indicating that some step in utilization may exhibit saturation kinetics.

The ability of LM cells to desaturate odd chain fatty acids probably resides in the specificity of the responsible enzyme system (Brett *et al.*, 1971). The saturated fatty acid content of cells grown with the Tween–17:0 supplement is not significantly different from that of cells grown in medium containing peptone, but no desthiobiotin or Tween (Table IV). With the fatty acid concentration of the Tween supplement at 8 μg/ml, 15:0 was desaturated less effectively than 17:0, and 19:0 was desaturated the least effectively of these three. The least effective desaturation at the lowest concentration of fatty acid supplement tested (4 μg/ml) was encountered with 21:0. Confirming our earlier studies with a Tween–19:0 supplement, virtually no desaturation of utilized 19:0 was encountered when cells were grown with high concentrations of the supplement. Chain elongation or β oxidation of the fatty acids derived from the supplements was encountered to the greatest extent with the shortest chain or longest chain supplements, respectively. The least modification of utilized supplement was encountered in lipids of cells deriving fatty acids from Tween–19:0.

It is apparent from the cellular growth data, *i.e.*, the doublings of cell number, that growth is not limited by the extent to which the cells derive fatty acids from the medium supplement. Neither was there any consistent pattern in the protein content per unit number of cells that was dependent on the concentration of the Tween supplements. Where no cytotoxicity was encountered, microscopic inspection of the cells adhering to the surface of the flasks revealed no morphological characteristic that was dependent on either the concentration or chain length of the fatty acid supplements provided as Tweens.

Uptake of Even Chain Fatty Acids from Tween Supplements. The fatty acid compositions of phospholipids in cells grown with Tween supplements containing even chain saturated fatty acids ranging from 16:0 to 22:0 are described in Table IV. These compositions are compared with the fatty acid composition in the phospholipids from cells of a control culture grown in medium containing peptone, but no Tween supplement or desthiobiotin. No truly distinctive difference in fatty acid composition was evident in phospholipids of cells grown with any of these Tween supplements. No increase in fatty acids with a chain length equal to or greater than C_{20} was detected in the phospholipids of cells grown with Tween–20:0 or Tween–22:0 supplements. Thus the cells are either unable to utilize 20:0 or 22:0 from the respective Tweens, or these fatty acids are utilized but then rapidly β oxidized to fatty acids with a chain length of C_{18} or less. The extent of utilization of supplements was tested only with Tween–18:0, which was provided as the 9,10-^{3}H-labeled fatty acid derivative. Approximately 29% of the fatty acids in phospholipids were derived from Tween–18:0. This corresponds quite favorably to the extent of utilization of fatty acids from Tween–15:0, Tween–17:0, and Tween–19:0 supplied in the medium at equivalent (8 μg/ml) fatty acid concentration (Table III).

Uptake of Unsaturated Fatty Acids from Tween Supplements. Several Tweens containing even chain unsaturated fatty acids were tested for their properties as supplements (Table V). Utilization of fatty acids from Tweens containing 18:1Δ^9*cis*, 18:1Δ^9*trans* and 18:3$\Delta^{9,12,15}$*cis,cis,cis* at a concentration of 8 μg/ml ranged from 15.3 to 19.6% of the total fatty acid in phospholipids. This is approximately half that observed with odd or even chain saturated fatty acid supplements (Tables III and IV), where utilization ranged from 29 to 37%. Apparently, fatty acids from unsaturated Tweens do not compete with endogenously synthesized saturated fatty acids for uptake into phospholipids. Though no significant change in the per cent of unsaturated fatty acids in phospholipids was observed with unsaturated supplements as compared with controls (Table IV), we expect that substitution with a trans-unsaturated or all cis-polyunsaturated fatty acid would lead to higher or lower critical temperatures, respectively, for the phase transitions in membrane associated lipids. Cytotoxicity was encountered in only one of the examples described in Table V. Incubation with a Tween supplement containing 16 μg/ml of linolenic acid led both to a reduction in cell number and to morphological abnormality. The surviving cells had a bloated appearance compared with cells of control cultures (Table IV) or cells grown with other concentrations of Tween–18:3; many of them were also characterized by a more nearly spherical shape, as compared with the spindle shape observed with other cultures. Severe cytoxicity was also observed with Tween–18:2$\Delta^{9,12}$*cis,cis* at a medium concentration of 8 μg/ml of linoleic acid. Cells grown with Tween–18:2$\Delta^{9,12}$*cis,cis* containing 4 μg/ml of fatty acid supplement had a normal appearance. At this concentration of supplement the cellular phospholipids contained 6.8% of 18:2 and 4.1% of 16:2. However, these cells also contained a large proportion of long chain ($\geq C_{20}$) unsaturated and polyunsaturated fatty acids. These accounted for approximately 25% of the total fatty acids in the phospholipids of these cells.

Cells were also grown with a Tween-containing stearolic acid (an acetylenic unsaturated fatty acid) at a medium concentration of 8 μg/ml. Stearolic acid was not detected in the phospholipids of these cells. These phospholipids had a fatty acid composition as follows: 14:0, 0.6%; 16:0, 17.3%; 16:1, 4.2%; 18:0, 17.5%; 18:1, 38.5%; and unidentified fatty acids, possibly metabolic products of stearolic acid, 21.9%.

Metabolism of Fatty Acids Derived from Tween Supplements. Tables VI and VII describe studies in which Tweens substituted with ^{3}H-labeled fatty acids were used to determine the course of metabolism of even chain saturated and unsaturated fatty acids. Studies with Tweens containing the saturated supplements, 16:0 and 18:0, are described in Table VI. Table VII describes experiments with cis- and trans-monoenoic unsaturated supplements.

The data in Table VI show that the saturated fatty acid supplements are affected by numerous routes of metabolism, *e.g.*, β oxidation, chain elongation and desaturation. Fatty acids derived from a Tween–16:0 supplement are apparently far more prone to be modified than those derived from a Tween–18:0 supplement. Over 77% of the phospholipid fatty acids derived from Tween–16:0 are modified by metabolism, whereas only 22% of those derived from a Tween–18:0

TABLE VI: Fatty Acid Composition of Phospholipids[a] from Cells Grown[b] with Radioactive Even Chain Fatty Acid Supplements.

	Tween–Fatty Acid Supplements			
	[9,10-^{3}H]16:0[c]		[9,10-^{3}H]18:0[d]	
Fatty Acid	Fatty Acid (%)	Cpm (%)	Fatty Acid (%)	Cpm (%)
14:0	4.4	0	4.6	0
14:1	0	0	0.6	0.2
15:0	1.2	0	1.8	0
16:0	13.5	22.8	12.5	0.1
16:1	15.0	28.3	15.6	1.7
17:0	0	0	0.6	0
17:1	0.4	0	1.8	0
18:0	6.2	7.7	12.6	8.4
18:1	51.6	23.0	44.2	78.1
20:0	0	1.9	0	0.6
20:1	2.7	5.2	1.6	5.1
22:0 + 24:0	3.8	3.2	1.3	0.9
22:1 + 24:1	0.9	4.2	1.4	4.7
Other[e]	0.3	3.7	1.4	0.2
% saturated	29.2	36.9	35.4	10.0
% unsaturated	70.8	63.1	64.6	90.0

[a] Phosphatidylcholine plus phosphatidylethanolamine. [b] All flasks were inoculated with 10^7 cells and were grown in the presence of the supplement for 4 days. Confluent cultures were obtained in all cases. [c] 0.25 mg/ml of Tween–[9,10-^{3}H]16:0 containing 1 μg/ml of [9,10-^{3}H]16:0. [d] 0.25 mg/ml of Tween–[9,10-^{3}H]18:0 containing 4.1 μg/ml of [9,10-^{3}H]18:0. [e] Fatty acids listed as other were unidentified, and therefore not considered in the calculation of % saturated/% unsaturated.

TABLE VII: Fatty Acid Composition of Phospholipids[a] from Cells Grown[b] with Radioactive Even Chain Fatty Acid Supplements.

	Tween–Fatty Acid Supplements			
	[9,10-^{3}H]18:1Δ^9*cis*[c]		[9,10-^{3}H]18:1Δ^9*trans*[d]	
Fatty Acid	Fatty Acid (%)	Cpm (%)	Fatty Acid (%)	Cpm (%)
14:0	2.4	0	3.5	0
14:1	2.4	0	0.3	14.7
15:0	1.2	0	1.4	0
16:0	11.5	0	12.0	0
16:1	15.3	7.7	14.0	21.8
17:0	0.3	0	0	0
17:1	1.1	0	0.2	0
18:0	4.1	0	8.8	0
18:1	58.3	67.5	55.6	31.1
20:0	0	0	0	0
20:1	2.7	10.1	2.9	17.3
22:0 + 24:0	0	0	0	0
22:1 + 24:1	1.5	7.9	1.1	13.0
Other[e]	1.6	6.8	0.2	2.1
% saturated	19.8	0	25.7	0
% unsaturated	80.2	100.0	74.3	100.0

[a] Phosphatidylcholine plus phosphatidylethanolamine. [b] All flasks were inoculated with 10^7 cells and were grown in the presence of the supplement for 4 days. Confluent cultures were obtained in all cases. [c] 0.25 mg/ml of Tween–[9,10-^{3}H]18:1-Δ^9*cis* containing 5.6 μg/ml of [9,10-^{3}H]18:1Δ^9*cis*. [d] 0.25 mg/ml of Tween–[9,10-^{3}H]18:1Δ^9-*trans* containing 2.9 μg/ml of [9,10-^{3}H]18:1Δ^9*trans*. [e] Fatty acids listed as other were unidentified, and therefore not considered in the calculation of % saturated/% unsaturated.

supplement share this fate. The fatty acids derived from Tween–16:0 are characterized by a slightly higher extent of saturation than are the total fatty acids in the cellular phospholipids. Those derived from a Tween–18:0 supplement, on the other hand, have a higher extent of unsaturation. These compositional characteristics are probably the metabolic consequences of the specificity of the fatty acid supplements and their metabolic products for the enzyme system responsible for desaturation.

Studies with Tween–18:1Δ^9*cis* and Tween–18:1Δ^9*trans* indicate that their fatty acid moieties, which differ only in the structure of the double bond, have widely divergent modes of metabolism prior to uptake into phospholipids. Two-thirds of the radioactivity from fatty acids of cells grown with the cis-unsaturated supplement was in fatty acid not modified by metabolism, whereas over two-thirds of the fatty acids from cells grown with the trans-unsaturated supplement was modified by β oxidation or chain elongation. Approximately 50% more modification by chain elongation was observed with the trans-unsaturated than with the cis-unsaturated supplement. A much greater difference was observed in β oxidation. Nearly fivefold more β-oxidized metabolic products (16:1 and 14:1) were incorporated into the phospholipids of cells grown with the trans-unsaturated supplement. This greater tendency for β oxidation may indicate a cellular response to the uptake of the higher melting trans isomer.

Viability of Cells Grown with Tween Supplements. All cell cultures grown with fatty acid supplements were routinely tested for viability. Cells from control cultures (MEM + P, Table IV) or cultures grown with fatty acid supplements were suspended by trypsin treatment (Materials and Methods) and inoculated in culture dishes at a density of approximately 1.5×10^5 cells/cm^2. Growth in MEM plus 5% calf serum was then monitored. Cells from all experimental cultures described in Tables I–VII of this report grew to confluency at the same rate as cells from control cultures.

Attempts to serially propagate LM cells under the experimental conditions described here have met with mixed success. Cells have been propagated in MEM + GVdB (Materials and Methods) medium supplemented with 50 μg/ml of Tween–16:0 (containing 7 μg/ml of 16:0; Table I) for over 40 serial passes. In the presence of Tween–19:0 at the highest concentration described in Table III, however, cells have not survived more than one serial pass.

Discussion

We have described a procedure for manipulating the fatty acid composition of animal cells grown in chemically defined, serum-free medium (Wisnieski *et al.*, 1973). The fatty acid supplements are added to the medium as sorbitan esters (Tweens), and uptake of fatty acid from Tween is enhanced by the addition of a biotin analog to the medium. In the present study we have characterized the enrichment procedure by determining the fatty acid composition of a pooled fraction

TABLE VIII: Comparison of Fatty Acid Desaturation by Cultured Cells and by Goat Mammary Gland Microsome Preparations *in Vitro*.

Fatty Acid Substrate or Supplement	% Desaturation	
	In Culture[a]	*In Vitro*[b]
15:0	2.9 (3)	5.0 (20)
16:0	55.4 (61)	15.5 (76)
17:0	64.4 (71)	20.3 (80)
18:0	90.3 (100)	25.5 (100)
19:0	10.3 (11)	2.5 (10)
21:0	0.1 (<0.1)	

[a] This study (Tables III and VI). Fatty acid concentration of 8 μg/ml. Per cent desaturation in culture is calculated for that portion of the supplement fatty acid that was incorporated into phospholipid with no alteration by chain elongation or β oxidation. The numbers in parentheses are a treatment in which the data are normalized to the per cent desaturation of 18:0 which has arbitrarily been set at 100. [b] Brett *et al.* (1971). Results obtained *in vitro* with a preparation of goat mammary gland microsomes. Per cent desaturation *in vitro* represents the actual per cent of the labeled fatty acid substrate converted to the monoenoic derivative. The numbers in parentheses are a data treatment as described under footnote *a* above.

of the major phospholipids from cells cultured with 13 different synthetic Tween supplements.

Our initial studies were based on earlier observations that animal cell lines that were grown in serum-free medium have a simple fatty acid composition in cellular lipids (Geyer *et al.*, 1962; Geyer, 1967; Bailey, 1967; Anderson *et al.*, 1969). Furthermore, the endogenous synthesis of cellular lipids, monitored by the uptake of radioactivity from labeled acetate or glucose, was strongly inhibited when lipid sources such as serum were added to the medium (Bailey, 1967). Bailey also observed that a preparation described as "Tween oleate" (presumably Tween 80) inhibited uptake of label into cellular lipid. However, he did not describe the amount of growth which occurred in the presence of Tween; nor did he demonstrate that fatty acid from Tween was actually incorporated into cellular lipid. Geyer (1967) analyzed the fatty acid composition of lipids from L cells grown in serum-free medium supplemented with nonesterified fatty acids provided as sonicated dispersions of the sodium or potassium salts. Cells grown with oleic acid were enriched for oleate and had an increased unsaturated/saturated fatty acid ratio, compared with cells grown with no fatty acid supplement. Cells grown with a linoleate supplement had an unaltered unsaturated/saturated fatty acid ratio, but a portion of the cellular 16:1 and 18:1 was replaced by 18:2. This indicated that nonesterified fatty acid supplements could alter the fatty acid composition of total cellular lipids, but the investigators did not determine if there was any alteration in the fatty acid composition of the structural lipids of the cell, *i.e.*, phospholipids. The fate of supplement fatty acid in cellular lipids is of particular significance since nonesterified fatty acid supplements caused extensive steatosis (*i.e.*, formation of lipid droplets within cells) (Geyer, 1967; Moskowitz, 1967). The steatosis encountered when nonesterified fatty acids were included in the growth medium led not only to gross alterations in cellular morphology, but also to cellular degeneration and death when saturated fatty acid supplements were used (Moskowitz, 1967). Cells grown with saturated fatty acid supplements (16:0 or 18:0) were characterized by needlelike inclusions, quite possibly composed of tripalmitin or tristearin. Moskowitz suggested that cellular death was encountered with saturated, rather than with unsaturated supplements because of the failure of a lipolytic response to steatosis in the former case. We have avoided both the steatosis encountered with nonesterified fatty acids, and the lethal effects of the saturated fatty acids, by employing synthetic Tweens as supplements. Toxicity has been observed with only a few of the Tween supplements. At concentrations greater than 4 and 8 μg/ml, respectively, linoleic and linolenic acids provided as Tweens were cytotoxic. Cytotoxicity was also encountered with Tween-21:0.

The data in Tables III–V indicate that in most cases the fatty acid composition of the major cellular phospholipids was altered significantly with little or no consequence to cellular integrity. There was no evidence of a cellular preference for any of the fatty acid supplements on the basis of chain length. Saturated supplements were utilized to a greater extent than unsaturated ones. Since the unsaturated/saturated fatty acid ratio in phospholipids was not altered by growth with unsaturated supplements, unsaturated fatty acids may act as negative feedback effectors of fatty acid desaturation. The effects of chain length of saturated fatty acid supplements on the unsaturated/saturated fatty acid ratio appear to be determined by the specificity of the cellular desaturation apparatus. In Table VIII, we have compared our data on desaturation of supplement fatty acids by cultured cells with the data of Brett *et al.* (1971), who studied the specificity of desaturation catalyzed by a goat mammary gland microsome preparation. There is a close similarity in chain length specificity; the only marked difference in activity was with 15:0. This could arise either from differences in species variation, or from differences in the *in vitro* and intact cell systems.

The supplement fatty acids were also modified by both chain elongation and β oxidation prior to their incorporation into phospholipids. The β oxidation of fatty acids was once thought to go to completion after being initiated (Weinman *et al.*, 1950). Elovson (1965), however, demonstrated that in the oxidation of stearic acid by intact rats, there was a constant "leak" in which shorter chain fatty acids were prematurely released from the oxidation system. Our experiments (Tables III and V–VII) indicate that a similar "leak" is characteristic of β oxidation in cells grown in culture.

Our primary objective in this study was to screen fatty acid supplements to determine the most likely candidates for altering the physical properties of membrane lipids. Three of the 13 supplements tested, 19:0, 18:1Δ^{9}*trans*, and 18:3$\Delta^{9,12,15}$-*cis,cis,cis*, should significantly change the lower characteristic temperature of the membrane phase transition, *i.e.*, the temperature at which all the membrane lipids become frozen. The freezing temperature of the membrane phospholipids of cells grown with a linolenate supplement should be depressed, compared with the freezing temperature of phospholipids in membranes of control cells (Table IV). Both elaidic acid and nonadecanoic acid supplements should increase the freezing temperature since elaidic acid replaces the lower melting oleic acid, and nonadecanoic acid decreases the unsaturated/saturated fatty acid ratio in the cellular phospholipids (Tables III and V). In the case of cells grown with a linolenic acid (18:3) supplement, the alteration of the fatty acid composition of the cell surface membrane phospholipids could be greater than that indicated by the composition of the bulk cellular

phospholipids (Table V). Weinstein *et al.* (1969) have reported a fivefold enrichment in the linolenic acid content of the plasma membrane over that of the bulk cellular phospholipids when L cells were grown in medium containing calf serum.

We have now studied a number of physiological phenomena that apparently respond to the freezing of membrane lipids. Noonan and Burger (1973a,b) have shown that both quantitative binding of concanavalin A and the agglutination of transformed 3T3 cells were decreased below what may be the lower characteristic temperature of the surface membrane phase transition. We have studied these phenomena in LM cells grown in control medium (MEM + P) and in medium supplemented with Tween–19:0 or Tween–linolenate. New characteristic temperatures were observed for these phenomena in cells grown with each supplement (Rittenhouse *et al.*, 1974). Since the characteristic temperatures for both processes increased in cells grown with Tween–19:0 and decreased in cells grown with Tween–linolenate, we are confident that the characteristic temperatures for the cell surface membrane phase transition are altered in the directions predicted by the changes in cellular phospholipid fatty acid composition. We have made similar findings for other phenomena occurring at the cell surface, *i.e.*, the transport of aminoisobutyric acid (Parkes *et al.*, 1974) and the penetration of the host cell membrane by infectious particles of Newcastle disease virus (Li *et al.*, 1974). The viral studies employed BHK_{21} cells subjected to the same supplementation procedures described here for LM cells. The results of these preliminary biological studies indicate that animal cells with altered phospholipid fatty acid compositions will be a valuable tool in the study of structure–function relationships in cell surface membranes.

Acknowledgments

We acknowledge the assistance of Sharon Taxer, Winifred Johnson, and Charlotte Miller.

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