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Effect of Petroselinic and Stearic Acids on the Alkyl Diacyl Glycerides of Novikoff Hepatoma Cells[1] (39818)

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Wood and Snyder (1) have shown that a number of rapidly growing tumors which they examined contained large amounts of alkyl diacyl glycerides (ADG). In common with these rapidly growing tumors Novikoff hepatoma cells also contain elevated levels of ADG (2), and Steele and Jenkin (2) found that the quantity of this compound could be reduced by including large amounts of unsaturated fatty acids in the growth medium of these cells. A possible explanation for the change may be that long-chain fatty alcohols are believed to be the precursors of ADG (3, 4) and that unsaturated fatty acids are less readily reduced to the corresponding alcohols (5). If this assumption is correct, then the amount of ADG in hepatoma cells grown in medium supplemented with saturated fatty acids should be of the same order of magnitude as, or greater than, that found in cells grown in unsupplemented medium. Since the major portion (about 60%) of the alkyl chains of ADG in Novikoff hepatoma cells grown in unsupplemented medium is composed of stearyl alcohol (2), then addition of stearic acid (18:0) to the culture medium should ensure that the synthesis of ADG is at a maximum.

Bloch-Frankenthal *et al.* (6) have shown that Novikoff hepatomas are unable to oxidize long-chain fatty acids when they are incubated for periods of 1 hr. However, it is not known whether these cells can be induced to catabolize long-chain fatty acids if they are allowed to multiply in the presence of high concentrations of them.

To investigate this question, balanced experiments were designed to compare the changes in the amount of ADG in cells which may be caused by adding a saturated (18:0) and an unsaturated (petroselinic, n12-18:1) fatty acid to the growth medium of Novikoff hepatoma cells and the results are herein reported. This study extends our results of a comparison of two octadecenoic acids, petroselinic acid (n12-18:1) and oleic acid (n9-18:1) (2).

Materials and methods. Novikoff hepatoma cells (NISI-67) were obtained from Dr. P. G. Plagemann, University of Minnesota (7), and were grown in suspension culture in Swim's 67G medium which contains 5% calf serum (Grand Island Biological Co., Grand Island, N.Y.) as previously described (2). The fatty acids were obtained from Nu-Chek Preparations, Elysian, Minnesota. The methods for their preparation as sodium salts and the method for the incubation of the cells have already been given (2).

The fatty acids were dispersed in the media using bovine serum albumin (Pentex Inc., Kankakee, Ill.). The molar ratio of the added fatty acid to albumin was 4:1 and the control flasks contained the same concentrations of albumin as the experimental flasks.

Cells were enumerated with the aid of a hemocytometer (8), harvested by centrifugation, and the lipids were isolated by the method of Bligh and Dyer (9). Further separation and characterizations of the lipids were performed by column (10), thin-layer (11), and gas-liquid chromatography (12).

The dry weight of the cells was determined gravimetrically and the amount of protein was determined by the method of Lowry *et al.* (13).

Each incubation flask contained a total of 500 ml of medium and the cells were seeded at a concentration of 2×10^5 cells/ml. The media contained either no supplement or a

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supplement of 100 μg of n12-18:1 or 100 μg of 18:0/ml. All fatty acids were added to the medium as their sodium salts bound to fatty-acid-free bovine albumin (Pentex Corp., Miles Laboratory, Kankakee, Ill.). After 48 hr the cells were harvested by centrifugation from each flask for further analysis.

The experiment was performed a total of three independent times and on each occasion each treatment was applied to triplicate samples. The results were analyzed statistically by the methods outlined by Snedecor and Cochran (14).

Results. Novikoff hepatoma cells growing in media which had been supplemented with either n12-18:1 or 18:0 had slower growth rates than those growing in unsupplemented media (Table I). However, microscopic examination showed that the cells appeared to increase in size. The protein or lipid in each cell was sharply increased in the presence of these fatty acids (Table I). The decrease in cell numbers and increase in cell size was greater when the medium contained 18:0. Nevertheless, the increase in cell size did not completely restore the total amounts of cellular dry weight or protein in the flasks to the same amounts found in the flasks containing the unsupplemented medium. The amount of total cellular lipid in the flasks containing added n12-18:1 was more than that found in the control flasks, while that in the flasks containing 18:0 was less after 48 hr incubation.

Although there was no net change in the quantity of total fatty acids in the flasks containing the unsupplemented medium during the observation period, there was a significant ($P < 0.001$) increase in the amount of 18:1 fatty acid and a corresponding reduction ($P < 0.001$) of 18:2 fatty acid during this time (Table II). The cells in the medium containing n12-18:1 fatty acid used approximately 30% of the total fatty acids in 48 hr, most of which was 18:1 fatty acid. This treatment also resulted in a small but highly significant ($P < 0.001$) increase in the quantity of 20:1 fatty acid. The cells growing in the flasks with added 18:0 fatty acid used up about 25% of the total fatty acid during the incubation period, most of the loss being accounted for by a disappearance of 18:0 fatty acid (Table II). During this time there was also a significant increase ($P < 0.01$) in the amount of 18:1 fatty acid in these flasks.

The amounts of neutral and polar lipids were increased in those cells cultivated in media supplemented with 18:0 and n12-18:1 fatty acids (Table III). The increase in the neutral lipid was greater in the presence of n12-18:1, whereas the increase in the polar lipid was greater in the presence of 18:0. The concentration of alkyl diacyl glycerides (ADG) in the cells was not significantly ($P > 0.05$) different in those cells that had been grown in the unsupplemented medium than those that had been grown in the medium with added 18:0 fatty acid. However, the presence of n12-18:1 fatty acid in the medium resulted in a highly significant ($P < 0.001$) reduction in the concentration of ADG in the cells.

The addition of n12-18:1 fatty acid to the medium reduced the concentration of all the fatty alcohols (Table IV) and fatty acids (Table V) in the ADG, including that of

TABLE I. EFFECTS OF LONG-CHAIN FATTY ACIDS SUPPLEMENTED IN GROWTH MEDIUM ON NOVIKOFF HEPATOMA CELLS.

	Cells grown for 48 hr in		
Parameter measured	Unsupplemented medium	Medium + n12-18:1	Medium + 18:0
Cell number per flask	14.5×10^8 $\pm 1.2 \times 10^8$[a]	8.4×10^8 $\pm 1.4 \times 10^8$	3.8×10^8 $\pm 1.2 \times 10^8$
Dry weight cells (mg/flask)	379 ± 29.6	316 ± 19.1	183 ± 23.6
Dry weight (pg/cell)	261 ± 20.4	376 ± 22.7	481 ± 62.0
Protein (mg/flask)	281 ± 21.9	196 ± 16.4	121 ± 10.5
Protein (pg/cell)	194 ± 15.1	233 ± 19.5	318 ± 27.6
Lipid (mg/flask)	40.6 ± 3.0	46.1 ± 2.9	34.0 ± 3.1
Lipid (pg/cell)	28.0 ± 2.1	54.9 ± 3.5	89.5 ± 8.2

[a] Flasks were seeded with 1×10^8 cells at 0 hr. Values are means ± standard errors of the means.

TABLE II. UTILIZATION OF THE MAJOR AND TOTAL FATTY ACIDS OF THE GROWTH MEDIUM BY NOVIKOFF HEPATOMA CELLS.[a]

	Input at 0 hr in flasks with Unsupplemented medium		After 48-hr growth in flasks with					
			Unsupplemented medium		Medium + n12-18:1		Medium + 18:0	
Fatty acid	Total	Cells	Total	Cells	Total	Cells	Total	Cells
16:0	5.8 ± 0.2[b]	0.20 ± 0.03	6.7 ± 0.5	2.9 ± 0.3	6.3 ± 0.6	1.6 ± 0.3	5.3 ± 0.7	0.7 ± 0.2
16:1	1.2 ± 0.1	0.04 ± 0.01	1.3 ± 0.1	0.5 ± 0.1	2.0 ± 0.1	0.9 ± 0.3	1.0 ± 0.1	0.1 ± 0.1
18:0	7.6 ± 0.3[c]	0.16 ± 0.02	8.1 ± 0.5	2.3 ± 0.3	7.3 ± 0.5	1.4 ± 0.3	27.4 ± 2.7	8.8 ± 0.9
18:1	9.5 ± 0.6[d]	0.57 ± 0.04	13.7 ± 1.5	8.3 ± 0.9	30.2 ± 2.6	14.7 ± 0.9	16.6 ± 1.5	6.5 ± 0.9
18:2	20.2 ± 0.6	0.13 ± 0.04	17.5 ± 1.6	1.9 ± 0.2	17.8 ± 1.9	1.4 ± 0.4	18.9 ± 1.6	1.0 ± 0.3
20:0	1.2 ± 0.1	tr	1.0 ± 0.3	0.2 ± 0.1	0.8 ± 0.4	0.1 ± 0.1	1.0 ± 0.3	0.2 ± 0.1
20:1	0.2 ± 0.1	tr	0.2 ± 0.1	0.2 ± 0.1	2.0 ± 0.2	1.7 ± 0.2	0.3 ± 0.1	0.3 ± 0.1
20:3	2.4 ± 0.3	tr	2.2 ± 0.3	0.3 ± 0.1	2.0 ± 0.2	0.2 ± 0.1	2.1 ± 0.4	0.3 ± 0.1
20:4	1.9 ± 0.3	tr	1.8 ± 0.3	0.5 ± 0.2	1.6 ± 0.3	0.4 ± 0.1	1.7 ± 0.4	0.2 ± 0.1
Total	54.0 ± 1.3[e]	1.2 ± 0.3	55.4 ± 2.9	18.2 ± 1.0	73.0 ± 8.2	22.7 ± 2.2	77.4 ± 9.6	18.1 ± 2.3

[a] All values are means expressed as milligrams ± standard errors of the means.

[b] Values in columns headed Total include the fatty acids in both the 500-ml medium in each flask and the cells contained in it. Values in columns headed Cells are the amounts of fatty acids in the cells recovered from 500-ml medium.

[c] At 0 hr, flasks supplemented with 18:0 fatty acid contained a total of 57.6 ± 1.6 mg of 18:0 and included the same amounts of all the other fatty acids as the flasks with unsupplemented medium.

[d] At 0 hr, flasks supplemented with n12-18:1 fatty acid contained a total of 59.5 ± 1.9 mg of 18:1 and included the same amounts of all the other fatty acids as the flasks with unsupplemented medium.

[e] Flasks supplemented with either 18:0 or n12-18:1 fatty acids contained a total of 104.0 ± 2.7 mg of total fatty acid at 0 hr.

TABLE III. EFFECT OF ADDING LONG-CHAIN FATTY ACIDS TO THE GROWTH MEDIUM ON THE CONCENTRATION OF LIPID COMPONENTS IN NOVIKOFF HEPATOMA CELLS.

	Cells grown in		
Lipid class	Unsupplemented medium	Medium + n12-18:1	Medium + 18:0
Alkyl diacylglycerides	2.40 ± 0.3[a]	0.74 ± 0.1	2.10 ± 0.3
Neutral lipid	24.4 ± 1.9	87.1 ± 5.9	65.0 ± 6.1
Polar lipid	120.0 ± 15.1	148.2 ± 13.3	215.7 ± 18.6

[a] Values are means expressed as micrograms of each cellular lipid component per milligram of cellular protein ± standard errors of the means.

TABLE IV. CONCENTRATIONS OF THE MAJOR AND TOTAL FATTY ALCOHOLS OF ALKYL DIACYL GLYCERIDES OF NOVIKOFF HEPATOMA CELLS GROWN IN MEDIA SUPPLEMENTED WITH LONG-CHAIN FATTY ACIDS (100 μg/ml).

	Cells grown in		
Fatty alcohol	Unsupplemented medium	Medium + n12-18:1	Medium + 18:0
14:0	6 ± 0.8[a]	4 ± 0.5	4 ± 0.7
16:0	272 ± 4.5	73 ± 2.1	70 ± 7.3
16:1	12 ± 3.0	19 ± 6.1	19 ± 2.0
18:0	288 ± 9.1	38 ± 4.0	489 ± 37.8
18:1	186± 12.1	96 ± 6.1	74 ± 9.3
Total	758 ± 94.7	236 ± 31.5	673 ± 86.8

[a] Values are means expressed as nanograms of fatty alcohol per milligram of cellular protein ± standard errors of the means.

18:1 fatty acid. In contrast, this treatment resulted in an increase in the amounts of all fatty acids in the cellular triglycerides (Table VI). The supplementation of the growth medium with 18:0 fatty acid brought about an increase in the concentration of both the 18:0 fatty acid and alcohol but produced decreases in the yields of all the other fatty acids and alcohols in the ADG compared to the unsupplemented medium (Tables IV and V). Supplementation of the growth medium with 18:0 fatty acid increased all the triglyceride fatty acids of the cells (Table VI).

Discussion. The hypothesis that long-chain fatty alcohols, which are the precursors of the alkyl moiety of ADG, are more efficiently synthesized from saturated than from unsaturated fatty acids appears to be substantiated by the results of the present experiments. Thus, supplementation of the growth medium with saturated fatty acids did not significantly alter the quantity of ADG in hepatoma cells, whereas supple-

TABLE V. CONCENTRATIONS OF THE MAJOR AND TOTAL FATTY ACIDS OF ALKYL DIACYL GLYCERIDES IN NOVIKOFF HEPATOMA CELLS GROWN IN MEDIA SUPPLEMENTED WITH LONG-CHAIN FATTY ACIDS (100 μg/ml).

	Cells grown in		
Fatty acid	Unsupplemented medium	Medium + n12-18:1	Medium + 18:0
14:0	132 ± 9.1[a]	42 ± 2.3	108 ± 13.6
16:0	503 ± 13.6	60 ± 6.1	148 ± 19.0
16:1	32 ± 9.1	12 ± 2.3	5 ± 2.7
18:0	165 ± 24.2	28 ± 4.2	731 ± 35.3
18:1	535 ± 50.0	244 ± 21.5	298 ± 44.7
18:2	33 ± 1.5	12 ± 0.5	8 ± 2.7
20:0	15 ± 1.5	2 ± 0.4	14 ± 1.4
20:1	53 ± 6.1	52 ± 5.6	7 ± 1.3
Total	1515 ± 189.3	467 ± 63.1	1356 ± 190.2

[a] Values are means expressed as nanograms of fatty acid per milligram of cellular protein ± standard errors of the means.

TABLE VI. CONCENTRATIONS OF THE MAJOR AND TOTAL FATTY ACIDS OF TRIGLYCERIDES IN NOVIKOFF HEPATOMA CELLS GROWN IN MEDIA SUPPLEMENTED WITH LONG-CHAIN FATTY ACIDS (100 μg/ml).

	Cells grown in		
Fatty acid	Unsupplemented medium	Medium + n12-18:1	Medium + 18:0
14:0	254 ± 24[a]	798 ± 109	322 ± 65
16:0	1264 ± 115	3553 ± 399	1870 ± 226
16:1	127 ± 18	1088 ± 109	290 ± 121
18:0	822 ± 97	3538 ± 218	14799 ± 742
18:1	2818 ± 248	23642 ± 1886	13638 ± 1773
18:2	133 ± 6	870 ± 36	548 ± 64
20:0	48 ± 6	73 ± 4	258 ± 32
20:1	254 ± 18	3408 ± 145	290 ± 32
Total[b]	6170 ± 1500	37000 ± 3200	32900 ± 5100

[a] Values are means expressed as nanograms fatty acid per milligram cellular protein ± standard errors of the means.

[b] Includes other minor fatty acids.

mentation with unsaturated fatty acids resulted in a large reduction of this fraction. In earlier experiments (2) the quantity of ADG was expressed as a percentage of neutral lipid, however, as the addition of the fatty acids to the growth media increased the amount of neutral lipid in the present investigation, it was therefore decided to express the concentration of ADG relative to the amount of cellular protein. When this is done the total quantity of ADG in the cells grown in the medium supplemented with n12-18:1 is about 33% of that in the control, whereas this value would be only about 10% if it were expressed in relation to the neutral lipid. The value of 10% compares well with a value of 8.4% found in previous experiments (2).

Novikoff hepatoma cells (Table II), in common with a number of other animal cells, appear to be able to desaturate (15, 16) and elongate (17) fatty acids. However, the present experiments demonstrate that the products of these reactions do not appear to be used in the synthesis of ADG (Table V) but appear to be readily available for TG synthesis (Table VI).

It has previously been found (18) that increasing the concentration of unsaturated fatty acids in the growth medium did not significantly increase the amount of total lipid in Novikoff hepatoma cells. The results of the present experiment confirm this observation. However, those cells which were grown in the medium containing 18:0 fatty acid had a highly significant increase in their lipid concentration. At present, it is not known whether this difference between the effects of unsaturated and saturated fatty acids is due to a differential rate of uptake or a differential rate of catabolism.

From the present experiment it appears

that there is a direct correlation between the size of the cells and their lipid content. However, it is impossible to show at this time which factors are cause and which are effect.

The results of the present experiment (Table I) are in agreement with our earlier work (19), which showed that the inclusion of n12–18:1 or 18:0 at a level of 100 μg/ml of medium reduces the number of Novikoff hepatoma cells to approximately 50 and 25%, respectively, of that in unsupplemented medium. However, when the quantity of cells in the growth medium is expressed in terms of dry weight rather than numbers, the respective values are increased to about 83 and 48% for cells grown in unsupplemented medium.

The present experiments show that Novikoff hepatoma cells can utilize both saturated and unsaturated fatty acids from the growth medium for synthesis of polar and nonpolar lipids. The values for the quantities of fatty acids present at the beginning and end of the present experiment (Table II) show that from 25 to 30% of the fatty acids were catabolized in 48 hr. Although CO_2 was not collected, it is nevertheless tempting to speculate that the cells were in fact oxidizing the fatty acids. The cells which were grown in the unsupplemented medium (Table II) did not show any net changes in the total fatty acids; this is in agreement with the results of Bloch-Frankenthal *et al.* (6). However, Swim's 67G medium contains twice the concentration of glucose found in most other media, but only a small amount of unesterified fatty acid. Thus, it is to be expected that cells growing under these conditions would use glucose as their primary energy source. When the amount of unesterified fatty acid in the growth medium is increased from about 2 to more than 50 mg/liter, it is to be expected that cells growing under these conditions might adapt to use fatty acids as a primary energy source.

Summary. Differences in the metabolism of petroselinic (n12–18:1) and stearic (18:0) acids were compared in Novikoff hepatoma cells cultivated *in vitro*. Addition of 100 μg of 18:0/ml to the growth medium of the cells increased their size and the concentrations of polar, neutral, total lipid, and protein, as well as the dry weight. Inclusion of the same amount of n12–18:1 in the culture medium produced similar changes but of a smaller magnitude than those of 18:0. Cells grown in the presence of 18:0 contained an amount of alkyl diacyl glycerides (ADG) similar to that found in cells grown in unsupplemented medium, whereas the cells grown in culture with n12–18:1 contained only about 33% of this amount of ADG.

There was no change in the amount of total lipid with time of incubation when the cells were grown in unsupplemented medium, whereas when the cells were grown in the presence of n12–18:1 or 18:0, 30 and 25%, respectively, of the total fatty acids were utilized in 48 hr.

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