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Growth of Novikoff Hepatoma Cells in the Presence of Long-chain Fatty Acids (38211)

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Bloch-Frankenthal *et al.* (1) demonstrated that well differentiated hepatomas can oxidize long- and short-chain fatty acids, whereas poorly differentiated tumors such as the Novikoff hepatoma, are unable to do so. Spector (2) has shown that the rate of uptake of free fatty acid by Ehrlich ascite cells is determined by the chain length as well as by the degree of unsaturation. His results showed that longer chain fatty acids became adsorbed to the cells in greater quantity than shorter chain fatty acids, and that introduction of a double bond reduced the quantity of fatty acids adsorbed.

Thus, it should be possible to alter the growth rate of Novikoff hepatoma cells to differing degrees by adding fatty acids of different chain length or degree of unsaturation to the medium. Our preliminary investigations showed that this hypothesis was correct and, therefore, the investigations were extended to include experiments where fatty acids were added to the growth medium in different esterified forms. Accordingly, the following experiments were designed.

In the first series of experiments, palmitic (16:0), stearic (18:0), oleic (*cis*-9-18:1) and petroselenic (*cis*-6-18:1) fatty acids were added to the growth medium as their sodium salts, combined with fatty acid-free bovine albumin. In 2 further sets of experiments, the fatty acids were incorporated into the growth medium as either their methyl esters or as triglycerides. The results of these experiments are reported at this time.

Materials and Methods. Novikoff hepatoma N1S1-67 cells were obtained from Dr. P. G. Plagemann (3), University of Minnesota. The cells were grown in Swim's 67 G medium (Grand Island Biological Co., Grand Island, NY) as previously described (4). The fatty acids, methyl esters and triglycerides were obtained from the Lipids Preparation Laboratory, The Hormel Institute, Austin, MN. The methods for the preparation of the sodium salts of the fatty acids have already been described (4).

Difficulty in dispersing the methyl esters (ME) and triglycerides (TG) in the medium was encountered, especially with the TG of 18:0. The appropriate quantities of the various forms of the fatty acids were placed in incubation flasks which were fitted with tight-fitting screw caps and sterilized at 121° for 15 min in an autoclave. After sterilization, the temperature of the flasks was kept above the highest melting point of the fatty acid used in the experiment. Enough bovine plasma albumin (Fraction V, fatty acid free, Pentex, Inc., Kankakee, IL) solution (100 mg/ml) was added to each flask to bring the final concentration to 2 mg/ml medium. The solutions were subjected to pressure homogenization using a preheated syringe and needle until no fat droplets were visible. Dispersion of triolein was further aided by sonication at 20 KC for three—1 min intervals. The starting cell population was 2×10^5/ml. All incubations were carried out at 37° in a water bath shaker. Each cell treatment was carried out in triplicate. A minimum of 2 independent experiments were performed using each treatment.

Cell numbers were determined with the

TABLE I. Growth of Novikoff Hepatoma (N1S1-67) Cells in Swim's 67-G Medium Containing Various Concentrations of Sodium Petroselenate (*cis*-6-18:1).

Time (hr)	*cis*-6-18:1 (μg/ml)							
	0	5	25	50	100	125	150	250
2	0.25[a]	0.25	0.29	0.25	0.29	0.29	0.24	0.10
24	0.75	0.66	0.62	0.74	0.62	0.48*	0.30***	0.04***
48	2.29	2.35	2.42	2.21	1.80	1.05*	0.54***	0.06***
60	2.91	3.02	3.65	2.79	1.76*	0.99**	0.69***	0.08***
72	2.56	2.32	3.03	2.00	1.83	1.72	0.67*	0.04***
96	1.17	1.39	1.62	0.70	0.67	1.68	1.04	0.02***
120	ND[b]	ND	ND	ND	ND	ND	1.00	ND
144	ND	ND	ND	ND	ND	ND	0.22	ND

[a] $\times 10^6$ cells/ml medium.

[b] ND = not done.

*, **, *** Probability that differences as large as these could have arisen by chance, $P < 0.05$, < 0.01, < 0.001, respectively. Other differences not significant ($P > 0.05$).

aid of a haemocytometer (5). The results were analyzed for significant differences by the statistical methods of Snedecor and Cochran (6).

Results. In each experiment, it was found that when the cells were grown in the control medium to which no fatty acids had been added, the cells increased to their maximum number in 60 hr. The values presented in Tables I, II and III show that when sodium 18:0 was present in the growth medium at concentrations above 50 μg/ml, there was a reduction in the rate of cell growth as well as a delay in the time taken to reach maximum cell numbers. Similar delays were observed when sodium *cis*-6-18:1 or sodium *cis*-9-18:1 was present in the medium at concentrations above 100 μg/ml and 150 μg/ml, respectively.

When the sodium salt of the fatty acids was replaced by methyl esters, it was found that the growth rate of the cells could be improved. For example, when the cells were grown in the presence of 100 μg/ml of sodium *cis*-6-18:1, the time to attain a maximum number of cells was delayed by 12 hr and the maximum cell number was only 75% of that found in the control me-

TABLE II. Growth of Novikoff Hepatoma (N1S1-67) Cells in Swim's 67-G Medium Containing Various Concentrations of Sodium Oleate (*cis*-9-18:1).

Time (hr)	*cis*-9-18:1 (μg/ml)			
	0	50	100	150
2	0.25[a]	0.25	0.25	0.24
24	0.75	1.00	1.00	0.46
48	2.42	2.69	2.42	2.05
60	3.14	3.14	2.56	2.48
72	2.04	2.32	1.50	1.83
96	0.94	1.03	0.53	0.73

[a] $\times 10^6$ cells/ml medium. None of the differences are statistically significant ($P > 0.05$).

TABLE III. Growth of Novikoff Hepatoma Cells in Swim's 67-G Medium Containing Various Concentrations of Sodium Stearate (18:0).

Time (hr)	18:0 (μg/ml)				
	0	25	50	75	100
5	0.33[a]	0.33	0.35	0.32	0.32
24	0.73	0.72	0.56	0.36	0.31*
36	1.31	1.30	0.89	0.31*	0.31*
48	1.78	1.59	1.19	0.50**	0.43**
60	2.25	2.00	1.52	0.54**	0.48***
72	2.04	1.73	1.83	1.04*	0.93*
96	1.64	1.42	1.32	1.40	1.15
120	1.37	1.01	1.00	1.18	1.25
144	0.81	0.60	0.60	0.92	1.00

[a] $\times 10^6$ cells/ml medium.

*, **, *** Probability that differences as large as these could have arisen by chance, $P < 0.05$, < 0.01, < 0.001, respectively. None of the other differences are statistically significant ($P > 0.05$).

TABLE IV. Comparison of Growth of Novikoff Cells in Swim's 67-G Medium Containing Either Sodium (Na) or Methyl (Me) Petroselenate (*cis*-6-18:1).

Time (hr)	Supplements to Medium				
		Na-6-18:1		Me-6-18:1	
	0	100 μg/ml	600 μg/ml	100 μg/ml	600 μg/ml
12	0.31[a]	0.31	0.000***	0.31	0.24
24	0.74	0.62	"	0.55	0.17**
36	1.52	1.00*	"	1.25	0.20***
48	2.09	1.61*	"	2.24	0.16***
60	2.63	1.70*	"	2.36	0.11***
72	2.40	1.80*	"	1.98	0.09***
96	1.56	0.78*	"	1.39	0.00***

[a] $\times 10^6$ cells/ml medium.

*, **, *** Probability that differences as large as these could have arisen by chance, $P < 0.05$, < 0.01, < 0.001, respectively. Other differences not significant ($P > 0.05$).

dium. However, when the same amount of methyl ester as the sodium salt of *cis*-6-18:1 was included in the medium, the growth rate of the cells was approximately the same as in the control medium. The results of this experiment are shown in Table IV. The toxicity of the sodium salt of the fatty acid compared to the methyl ester is even more striking at very high concentrations. For example, when the medium contained 600 μg/ml of sodium *cis*-6-18:1, no cells could be found after 2 hr incubation, whereas the cells in medium containing 600 μg/ml of the methyl ester could be maintained at about 50% of the starting population for over 40 hr.

The results in Table V show that the inclusion at large amounts (up to 600 μg/ml) of the triglycerides of each fatty acid in the medium did not affect the growth characteristics of the cells when compared to cells cultivated in unsupplemented medium. This result is in sharp contrast to the results observed in medium containing the same

TABLE V. Comparison of Growth of Novikoff Hepatoma Cells in Swim's 67-G Medium Containing Various Concentrations of Tripetroselenate, Triolein and Tristearin.

Time (hr)	Control[a]	Triglycerides								
		200 μg/ml			400 μg/ml			600 μg/ml		
		6-18:1[b]	9-18:1[b]	18:0	6-18:1	9-18:1	18:0	6-18:1	9-18:1	18:0
0	0.26[c]	0.33	0.32	0.36	0.30	0.28	0.32	0.31	0.29	0.32
20	0.58	0.42	0.52	0.61	0.64	0.40	0.54	0.48	0.51	0.65
25	0.75	0.74	0.71	0.79	0.67	0.81	0.70	0.62	0.65	1.02
44	2.03	1.49	1.42	1.49	2.03	1.33	1.85	1.60	1.66	1.52
50	1.89	1.75	1.74	2.13	1.83	1.43	1.99	1.67	1.71	2.18
68	1.89	1.94	1.77	2.15	1.81	1.53	2.09	1.58	1.80	2.13
73	1.81	2.04	1.57	2.17	1.79	1.78	2.04	1.54	2.06	1.98
92	1.73	1.67	1.30	1.42	1.41	1.27	1.57	1.42	1.52	1.39

[a] No lipid additions.

[b] The *cis* form of the unsaturated fatty acids were present in the triglycerides.

[c] $\times 10^6$ cells/ml medium. None of the differences are significant ($P > 0.05$).

amount of either the methyl ester or the sodium salt of each fatty acid (see Table IV).

Discussion. The results of the present investigations support the speculation that the rate of growth of Novikoff hepatoma cells can be limited by lower concentrations of 18:0 than of *cis*-9-18:1 acid. The data of Spector *et al.* (7) and Spector (2) show that the rate of binding of fatty acids to cells is greater for saturated than for unsaturated fatty acids. In order to explain the difference in the rate of growth of Novikoff cells in the presence of fatty acids of the same degree of unsaturation, such as *cis*-6-18:1 and *cis*-9-18:1, it may be assumed that the rate of binding of fatty acids to cells is related to their physical characteristics. The melting point of *cis*-6-18:1 is intermediate between that of 18:0 and *cis*-9-18:1 and, thus, it can be expected that the effects of *cis*-6-18:1 acid would be intermediate between 18:0 and *cis*-9-18:1. The results of our experiments support this hypothesis.

At present, it is impossible to determine the precise mechanism whereby the sodium salts of long-chain acids exert their effects on the growth rate of Novikoff hepatoma cells. Several possibilities exist; the sodium salts may act as detergents and cause lysis. Alternatively, the salts may be adsorbed onto the cell surface physically coating the cell surface and thus reducing the uptake of such essential nutrients as glucose. It is known that these cells cannot utilize fatty acids as an energy source (1). At very high levels of sodium salts of fatty acids in the medium, it seems likely that the detergent effect is the major one since the cells were actually lysed after a short period of contact with the fatty acid salt (Table I). However, in the presence of a lower amount of fatty acid salt, there was no evidence of cell lysis and the cells were in most cases able to grow albeit at a slow rate.

It is surprising that the Novikoff cells could grow at a normal rate in the presence of extremely high amounts of triglycerides in the growth medium. Although triglycerides are taken up by mammalian cells (8), they must first be hydrolyzed before being absorbed through the membrane. From the present experiments, it would seem that the cells do not hydrolyze more fatty acids than they can utilize. Novikoff cells cannot utilize fatty acids as an energy source (1), but they can use them in the synthesis of various lipid classes (4). Howard and Kritchevsky (9) presented evidence to show that serum lipase is probably responsible for triglyceride hydrolysis in the medium in which they grew WI-38 cells. This means the hydrolysis of triglycerides is not under the direct control of the cells. However, this is unlikely in the present experiments, otherwise the concentration of free fatty acids in the medium containing large amounts of triglycerides would quickly build up to toxic levels. Even when the concentration of triglycerides in the medium was 600 μg/ml, there was no reduction in cell proliferation.

These experiments do not resolve the question of whether fatty acids exhibit their effects on the cell surface or internally. This question will be investigated in future experiments.

Summary. The growth characteristics of Novikoff hepatoma cells growing in culture can be differentially altered by the addition of long-chain fatty acids to the growth medium. The greatest reduction in growth rate was obtained by adding sodium stearate to the growth medium and the least was when sodium oleate was added; the effect of sodium petroselenate was intermediate. The addition of the methyl ester to the growth medium caused a smaller reduction in cell proliferation than the corresponding sodium salt of the fatty acid.

When each of the long-chain acids in their triglyceride form was added to the growth medium, there was no reduction in the growth rate of the cells even at very high concentrations. Possible reasons for these changes are discussed.

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