Triglyceride Utilization by Human HeLa and Conjunctiva Cells in Tissue Culture

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UNLIKE other major groups of nutrients such as carbohydrates, amino acids, and inorganic ions, lipids have thus far received relatively little attention in nutritional and biochemical studies employing cells maintained in continuous culture in vitro. It has been rather general procedure to avoid lipemic sera, often using fasted or at least preprandial donors. Toxic manifestations of unesterified fatty acids and their soaps are well known, and the surface activity of phosphatides, monoglycerides, and diglycerides has not favored their intentional inclusion in tissue culture media. In view of the fact, however, that most such media contain fresh or dialyzed serum, lipids at least in the form of lipoproteins are present. Recently, Cailleau *et al.*,[1] in a study of the reasons underlying the toxicity of certain sera, analyzed both "toxic" and nontoxic sera for their lipid concentrations and compositions. Their data revealed a higher lipid content for toxic sera and especially an elevation in free fatty acid content. Several investigators have included lipids in their media, especially where natural products such as serum have been omitted. A medium employed fairly extensively by Evans and associates[2] contained three unsaturated fatty acids: methyl linoleate, methyl linolenate, and methyl arachidonate. More recently, the three fatty acids have been omitted without noticeable effect.[3] Sato, Fisher, and Puck[4] have presented data showing the requirement for cholesterol by the S3 clonal strain of HeLa cells.

In the present studies, human HeLa and conjunctival cells have been incubated with a number of fat emulsions and similar preparations, and the effect on net cell multiplication has been determined. Emulsions containing glyceryl triolein-1-C^{14} and glyceryl tripalmitin-1-C^{14} have also been employed, and utilization of these triglycerides has been shown.

EXPERIMENTAL

The human cells used were of the HeLa and conjunctival (Chang) strains derived from carcinoma and normal conjunctiva, respectively.‡ Stocks of these cells were grown in Eagle's medium[5] containing 10 per cent horse serum and were transferred routinely about every eight days. The cells were checked at frequent intervals for possible contamination by pleuropneumonia-like organisms.§ Roller tube cultures were prepared from the stocks when approximately 3 to 5 $\times$ 10^6 cells were present, using the trypsinization technic previously described.[6] An inoculum of about 25 $\times$ 10^3 cells was used and the tubes were incubated on stationary racks at 35° C for 24 to 48 hours and were then rotated at a drum speed of 1 rpm at the same temperature. Eagle's medium was employed containing 10 per cent horse serum which had been dialyzed at 4° C. Additions of lipid preparations were made at times shown in the appropriate table. At the end of the experiments, cell counts were done as previously described.[7] Frequent micro-

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This work was supported in part by a research grant from the American Cancer Society and by the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health.

‡ Clonal strains of these cells were kindly furnished by Dr. R. S. Chang of the Department of Microbiology.

§ The authors are indebted to Dr. E. Murray of the Department of Microbiology for examining the slides in these tests.

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TABLE I
Composition of Emulsion Preparations

Preparation No.	Component and Per Cent*					
	Coconut oil	Cottonseed oil	Corn oil	Pluronic-F68	Phosphatide	Ethyl alcohol
1	10	—	—	2.5	—	8
2, 3, 4	—	10	—	2.5	—	8
5	—	10	—	—	2	—
6	—	10	—	2.5	2	8
7	—	—	10	2.5	—	8
8	—	—	—	2.5	—	8
9†	—	15	—	0.3	1	—
10	—	—	—	—	—	8

* Per cent expressed as weight per volume. All preparations were made to final volume with 5% dextrose solution.

† Lipomul I.V., Upjohn Company.

scopic examinations were done throughout the experimental period.

The nonradioactive emulsions used were prepared by high-pressure homogenization and sterilized as has been described.[8] Except where otherwise noted, the composition was as follows:

	Weight/volume, %
Triglyceride	10.0
Pluronic-F68*	2.5
Ethyl alcohol†	7.0
Dextrose‡	4.7
Pyrogen-free H_2O§	to 100 ml.

* Furnished by Mr. Phelps Trix of the Wyandotte Chemical Corporation, Wyandotte, Michigan. This material is prepared by the condensation of ethylene oxide with polyoxypropylene, and has a molecular weight of approximately 8,000. In vivo tests have shown this material to be of extremely low toxicity.[9,10]

† Redistilled shortly before use.

‡ Merck and Company, Rahway, New Jersey.

§ Obtained from the Massachusetts General Hospital Pharmacy.

The emulsions have few fat particles greater than 1 μ and the average visible under the phase microscope is well below this. A large portion of the particles are beyond the resolving power of the light microscope.

The triglycerides used were a purified cottonseed oil,* coconut oil (Cobee 76)† and corn oil.‡ In addition to the Pluronic-stabilized emulsions, two phosphatide-stabilized emulsions were also tested. One of these was a commercial product§ and the other was a 10 per cent cottonseed oil emulsion prepared in this laboratory and contained 2 per cent of the soybean phosphatide¶ as the sole stabilizer. For control purposes, some preparations were prepared without fat. All materials were stored at 5° C. Media containing the various preparations were prepared every two days, and all media were changed every two days.

Radioactive emulsions were prepared by the following procedure: the radiolipide was dissolved with or without carrier triolein|| in 1 ml of redistilled acetone with warming. This solution was rapidly expelled from a small volume syringe through a #20 needle into a 1 ml volume of aqueous solution 2 per cent Pluronic-F68 and 5 per cent dextrose. Acetone from the resulting almost invisible emulsion was then removed by aeration with water pump N_2. The volume was adjusted to 1 ml. Sterile technic was employed throughout the procedure. The emulsions were used immediately after preparation. Radioactive triolein°

* Kindly supplied by Dr. Curtis Meyer of the Upjohn Company, Kalamazoo, Michigan.

† Obtained from the Drew Company, Boonton, New Jersey.

‡ Mazola Corn Products Refining Company, Argo, Illinois.

§ Lipomul-I.V., Upjohn Company, Kalamazoo, Michigan. This emulsion contains 15% purified cottonseed oil, 1% purified soybean phosphatides, 0.3% Pluronic-F68, and 5% dextrose to volume.

¶ Furnished by the Upjohn Company.

|| Hormel Institute, Austin, Minnesota.

° Prepared by Dr. Walter Gensler from oleic acid-1-C^{14} previously described.[11]

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TABLE II

Human Conjunctival Cell Multiplication in the Presence of Various Fat Emulsions

Expt. No.*	Days with substrate	Preparation		Cell population (cells/ml × 10^3)
		No.	Conc. (ml/100 ml medium)	
1	7	3	0.0	545
			0.1	486
			0.5	425
2	5	3	0.0	68
			0.1	65
			0.5	70
		10	0.3	74
		8	0.3	64
3	4	9	0.0	88
			0.3	42
			0.5	37
			1.0	40
		5	0.1	18
			0.3	10
			0.5	10
		6	0.1	30
			0.3	24.5
			0.5	30.5
			1.0	35.5
		5 + 8 (1:1)	0.1	30
			0.3	24.5
			0.5	30.5
			1.0	35.5
		4	1.0	67

* Comparisons of actual cell counts should be made only within experiments, not between experiments, since different absolute cell populations were purposely chosen in the various experiments.

TABLE III

Human HeLa Cell Multiplication in the Presence of Various Fat Emulsions

Expt. No.*	Days with substrate	Preparation		Cell population (cells/ml × 10^3)
		No.	Conc. (ml/100 ml medium)	
4	7	1	0.0	199
			1.0	270
			5.0	231
			10.0	206
	7	2	1.0	285
			5.0	231
			10.0	246
5	6	—	—	177
		10	0.3	169
		8	0.3	200
		3	0.1	186
		3	0.5	176
6	5	3	0.0	21.5
			0.1	24.5
			0.5	17.5
			1.0	14.5
			5.0	19.5
7	5	3	0.0	527.5
			0.1	627.5
			0.2	628.5
8	7	3	0.0	160.5
			0.1	125
			0.3	147
			0.5	109
9	6	—	—	101
		4	0.3	99
		5	0.3	50
		5 + 8 (1:1)	0.3	80
		9	0.3	95
		6	0.3	65
		7	0.3	78.5

* Comparisons of actual cell counts should be made only within experiments, not between experiments, since different absolute cell populations were purposely chosen in the various experiments.

was used without carrier and radiopalmitin[11] was dissolved in nonradiotriolein. The experiments with cells were conducted by incubating the radioactive materials with roller tube cultures containing approximately 2 × 10^5 cells and 1 ml of Eagle's medium containing 10 per cent horse serum. The tubes were equipped with glass ampules containing filter paper,[6] and after 18 to 23 hours of incubation, 0.2 ml of 10 per cent KOH was introduced into the filter paper by means of a syringe and the tubes were incubated for an additional 45 minutes. A 0.1 ml of 10 per cent H_3PO_4 was added to the medium by means of a syringe. After 15 minutes, the KOH ampule was removed and assayed for C^{14} by previously described procedures.[12]

[11] New England Nuclear Company, Cambridge, Massachusetts.

RESULTS AND DISCUSSION

Within the limits of these short-term experiments, the presence of fat emulsion did not greatly alter the net multiplication of either human HeLa or conjunctival cells. The emulsions and related preparations tested,

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TABLE IV

Conversion of Glyceryl Triolein-1-C^{14} and Glyceryl Tripalmitin-1-C^{14} to $C^{14}O_2$ by Human Cells of the HeLa and Conjunctiva (Chang) Strains

Expt. No.	Radiosubstrate	Cell strain	Total radiosubstrate (mg)	Total no. cells (× 10^3)	Radioactivity recovered as $C^{14}O_2$	
					Total	Per 10^3 cells
1*	Glyceryl triolein-1-C^{14}	Conj.	1.25	4,670	10,365	22.0
		HeLa	1.25	2,500	10,490	42.0
2†	Glyceryl triolein-1-C^{14}	Conj.	0.25	125	511	4.1
			0.25	125	743	5.9
			0.5	125	1,880	15.0
			0.5	125	1,084	8.7
			0.5	125	400	3.2
		HeLa	0.25	300	2,555	8.5
			0.25	300	1,580	5.3
			0.25	300	890	3.0
			0.5	300	1,540	5.1
			0.5	300	1,300	4.3
			0.5	300	1,092	3.6
3‡	Glyceryl tripalmitin-1-C^{14}	Conj.	0.16	16	231	14.0
			0.16	18	250	14.0
			0.31	26	379	15.0
			0.31	30	190	6.3
			0.31	24	289	12.0
		HeLa	0.31	15	173	16.0
			0.31	16	509	32.0
			0.31	13	287	22.0

* Based on results with 1 stock bottle of each cell strain. Total fluid volume per bottle was 10 ml and total activity was 3.18 × 10^5 counts per minute (cpm).

† 1.0 mg of triolein furnished 2.54 × 10^5 cpm. Cell counts given are the mean of four comparable tubes on which no radioassays were performed.

‡ 0.3 mg of tripalmitin furnished 1.73 × 10^5 cpm. The radioglyceride was dissolved in 0.7 mg of triolein.

given in Table I, represent a rather diverse spectrum of lipids. These were chosen primarily to ascertain whether or not major changes in unsaturation and chain length (Prep. No. 1 vs. Nos. 2, 3, and 4), type of emulsifying agent (Prep. Nos. 2, 3, and 4 vs. No. 5), and so forth, would result in arrested multiplication and cell destruction. In no instance did the emulsions stabilized with Pluronic-F68 cause any adverse effects (Tables II and III), and the fat-free Pluronic preparation (Prep. No. 8, Table I) was also without effect. In connection with the lack of toxicity of this stabilizer may be mentioned the fact that even a final concentration of 1 per cent of Pluronic-F68 in Eagle's medium did not affect the appearance or multiplication of either human HeLa or conjunctival cells.

Emulsion No. 5 and No. 6, containing 2 per cent phosphatide, appeared to depress net multiplication to some extent (Expt. 4, Table II; Expt. 9, Table III), even at the lowest concentration of emulsion tested. Surface and interfacial tension measurements were not done, so the possibility of effects mediated through a reduction in such forces remains. A difference between the two stabilizers employed which is pertinent to the present discussion is that of chemical and enzymatic stability. Phosphatides have various kinds of ester bonds and are therefore labile to hydrolysis, yielding such diverse products as lysolecithins and fatty acids. Such materials could well give toxic manifestations. Pluronic-F68 on the other hand, being a polyether, is fairly inert to cleavage agents present in the systems studied. If any peroxides are formed, they are evidently not injurious. In related studies

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use had been made of Pluronic-F68 labeled in the ethylene moiety with C^{14}. The incubation of this material with either HeLa or conjunctival cells has yielded no $C^{14}O_2$, indicating lack of metabolism of the Pluronic-F68. Current in vivo experiments with dogs have shown no alteration in the molecular weight or structure of the Pluronic-F68. Thus, it can be concluded that this material is metabolically inert. No such studies have been done with labeled phosphatides. It should be pointed out that in several experiments the cells grew in a lacy pattern, but no consistent correlation with type of stabilizer or oil could be drawn.

In order to determine whether or not emulsified triglyceride could be used by these human cells, initial studies were done in which the turbidity of the medium was used as the criterion of utilization. No consistent decrease in turbidity upon incubation with the cells could be obtained. Therefore, studies were done with C^{14}-tagged triglycerides. As shown in Table IV, both radiotriolein and tripalmitin were converted to $C^{14}O_2$. In a 24-hour period, approximately 20 μg of triolein, or 28 μg of tripalmitin, was oxidized per 1×10^6 cells. It must be remembered that nonradioactive triolein was also present in the tripalmitin emulsion. Since the caloric requirement of these cells is still unknown, it is not possible to calculate the percentage of the caloric requirement furnished by these triglycerides. The extent to which lipids normally present even in dialyzed serum are utilized by such cells is not known, but it would appear that the capacity to utilize triglyceride and fatty acid is available. It is, of course, possible that enzymatic action of the serum, such as lipolysis, may convert some of the triglyceride to fatty acid and mono- and diglycerides and that these products are substrates for the cells. Further experimentation is necessary to resolve this point. In any event, these human cells can oxidize oleic and palmitic acids to carbon dioxide.

SUMMARY

Two types of fat emulsions have been studied in tissue culture of human HeLa and conjunctival cells. Soybean phosphatides or a synthetic material (Pluronic-F68) was used as the emulsifying agent. Cell multiplication was unaffected by the presence of Pluronic-stabilized emulsions and appeared to be somewhat decreased with a 2 per cent phosphatide-stabilized emulsion. Emulsions of glyceryl triolein-1-C^{14} and glyceryl tripalmitin-1-C^{14} were converted to $C^{14}O_2$ by both kinds of cells.

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