Isolation and Lipid Analysis of Lipoid Granules from Ehrlich Ascites Tumor Cells.* (28278)

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A variety of cells from different sources, upon injury or cessation of growth, are known to accumulate substances which absorb dyes that also stain lipids. A relationship has been established between protein deficiency or imbalance and deposition of fat in liver(1), blood vessels(2), cells in tissue culture(3,4), and Ehrlich ascites tumor cells(4). In essence the excess lipid may arise from both extracellular lipoprotein and from *de novo* synthesis.

In our laboratory, with a dark contrast oil immersion lens of a phase contrast microscope, Ehrlich ascites tumor ELD cells have been noted to possess refractile lipoid granules. These granules appear as immobile bright spheres against a dark cytoplasmic background. The number of these granules in each cell increases as the ascites tumors grow in the peritoneum of the mouse. The discreteness and peripheral location of these granules can be demonstrated by their shifting in the peripheral cytoplasm under centrifugation when the viscosity of the tumor cells is decreased(5,6).

In supravital preparations with Nile blue sulphate stain, the large sudanophylic droplets stain red, indicating that they may contain saturated or unsaturated neutral lipid (7). The present investigation was undertaken to analyze chemically the lipid content of the large discrete lipoid granules of Ehrlich ascites tumor cells.

Materials and methods. ELD hyperdiploid Ehrlich ascites are maintained by weekly transfer in ICR/Ha Swiss mice. The mice used in these experiments are injected intraperitoneally with 0.2 ml of undiluted ascitic fluid and cells. On the tenth day after tumor inoculation, the mice are sacrificed by cervical dislocation, the peritoneal membrane exposed, and the cells collected by means of a syringe with a #20 needle. At this time mice have distended abdomens, very few blood cells, and approximately 10 lipoid granules per cell as indicated by phase microscopy. The ascitic fluid and cells are placed in test tubes kept chilled in beakers of crushed ice.

After centrifugation of the cells for 10 minutes at 100 $\times$ *g* at 5°C, the supernatant is decanted and the cells washed with physiological salt solution. The centrifugation is repeated, the saline decanted and the packed tumor cells frozen if there is insufficient material to warrant continuing the collection of the lipoid granules. Cells to be broken are suspended on a volume basis (1:10) in cold, distilled water to facilitate the breaking of the cells. Clumps are dispersed, the mixture stirred until the suspension is uniform and the suspension refrigerated for 30 minutes. Aliquots of the suspension are transferred to an Omni-mixer cup immersed in an ice bath and homogenized for intervals of 20 seconds to a maximum of 2 minutes. A drop of cell suspension is examined using phase microscopy after each 20 second interval to determine condition of the cells and relative number of floating granules. If cells are mixed for more than 2 minutes the number of granules decreases even though the mixing container is kept chilled in a salt-ice bath. Total volume is calculated and 0.086 g of sucrose/ml is dissolved to give a 0.25 molar sucrose solution. The mitochondria and cell debris are eliminated after centrifuging the suspension at 9,000 $\times$ *g* for 30 minutes. The supernatant is collected and centrifuged for 2 hours in a Spinco ultracentrifuge at 145.000 $\times$ *g* at tube tip at 0°C. The low specific gravity of these granules causes them to collect and form a white band at the top of the centrifuge tube. This material is removed with a syringe and may average 5 ml/tube. The refractile nature and homogeneity of the granules is confirmed again with phase microscopy.

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Until sufficient granules were ready for extraction they were frozen at −25°C. The lipids were extracted from the Ehrlich ascites granules by refluxing with an ethanol-ether mixture and extracting with petroleum ether. The solvents were removed by vacuum distillation under N_2 and the lipids dissolved in petroleum ether. The neutral lipid fraction was separated into triglycerides, the steroid esters, cholesterol and phospholipids by column chromatography on silicic acid(1). The triglyceride fraction and the fraction eluted with the steroid esters were stored under N_2 at −25°C until ready for use. The triglycerides and ester fraction were interesterified to the methyl esters with methanolic HCl(8). The methyl esters of the fatty acids were then separated from the unsaponifiables or other impurities by chromatography on silicic acid by the method of Luddy *et al.*(9). The fatty acid composition of the methyl esters was determined by gas-liquid chromatography on an Aerograph Hy-Fi, model 600 Chromatograph with a hydrogen flame ionization detector. The column was 5 ft × ⅛ in. stainless steel packed with 12% ethylene glycol adipate on 80/100 mesh Gas Chrom P.† The temperature of the column was 195°C. Usually 2 μl of the esters dissolved in CS_2 at a concentration of 0.5 to 1% were employed. Each sample was run 3 times and the average values were used. For more accurate measurements of trace components, 5 μl of the CS_2 solutions of the methyl esters were employed. Identification of the fatty acid methyl esters was based upon a comparison of the relative retention times (16:0 = 1) with known fatty acid methyl esters.‡ These esters at various concentrations and mixtures were also used for calibration of the GLC apparatus. The areas under the curves were measured with a Disc Integrator.

Results. The lipid composition of the lipoid granules, by fractions, is indicated in Table I. Over 40% of the material is triglycerides. Of the 5.8% that appeared to be cholesterol, only 3.2%, more than half, could be determined colorimetrically by the method of Abell *et al.*(10). This constitutes a very small percentage of the total lipids. Other material eluted from silicic acid with the steroid esters constituted 37.8% of the total, and of this approximately 3% was steroid esters as determined colorimetrically, the remainder being of unknown composition. The phospholipids represent 11.4% of the total.

TABLE I. Lipid Composition of Ehrlich Ascites Fat Granules.*

Fraction	%
Steroid esters LB†	
Negative	37.8 ± 7.7
Positive	2.9 ± 1.0
Triglycerides	41.6 ± 8.9
Cholesterol LB†	
Negative	2.6 ± 1.1
Positive	3.2 ± 1.4
Phospholipids	11.4 ± 3.1
Total	99.5

* Results are mean ± standard deviation of the mean of 4 samples and are based upon amount of material eluted from the column.
† LB—Libermann-Burchard reaction.

The fatty acids (Table II) of the fraction eluted with the steroid esters were 82% unsaturated. Oleic and linoleic acids constitute the bulk of these: 67% of the total. The amount of oleic exceeds linoleic by a factor greater than 2.5. Palmitic and arachidonic acids are present in approximately the same amount, 8.5 and 9.9% respectively.

TABLE II. Fatty Acid Composition of Ehrlich Ascites Granule Fraction Eluted with Cholesterol Esters.*

C_8	1.9	Oleic	49.1
Myristic	4.1	Linoleic	18.3
Palmitic	8.5	Arachidonic	9.9
Palmitoleic	4.3	Other	.8
Stearic	3.0		

* Avg of 2 samples.

The fatty acid composition of triglycerides is summarized in Table III. Approximately

TABLE III. Fatty Acid Composition of Ehrlich Ascites Fat Granule Droplet Triglycerides.*

C_8	3.2 ±1.6	Oleic	29.2 ± .3
Myristic	1.6 ± .7	Linoleic	27.2 ± .3
Palmitic	18.6 ± .6	Linolenic	1.2 ± .5
Palmitoleic	2.0 ± .2	Arachidonic	2.3 ± .7
Stearic	12.5 ± .3	Other	2.0 ± .3

* Mean ± standard deviation of the mean of 4 samples.

† Applied Science Laboratories, Inc.
‡ The Hormel Foundation, Austin, Minn.

33% of the triglyceride fatty acids is saturated. The fatty acid composition of the triglycerides differs markedly from the fraction eluted with the steroid esters. Oleic and linoleic acids are nearly equal in amount and the level of arachidonic acid is appreciably decreased in comparison to the amount found in the fraction eluted with steroid esters.

Discussion. Evidence concerning the lipid composition of the lipoid granules has been presented. However no attempt has been made to show other materials which may be contained, such as carbohydrates or proteins. For the most part investigation of tumor cell fats has been limited to analysis involving extraction from whole cells(11) and consequently is not directly comparable to the present study even when Ehrlich ascites cells have been used. The inhibition of protein synthesis with increased acidosis occurs with decreased growth rate(12) and results in an increase in the number of lipoid granules. Chromatographic analysis of whole cell phospholipids from Ehrlich ascites cells by Wallach *et al.*(13) showed that total lipid was 16% of the dry weight, of which phospholipids were 58% and cholesterol was 11%. Yamakawa *et al.*(11) examined a variety of lipids of dried Ehrlich ascites cells by silicic acid chromatography and found relatively large amounts of phospholipids and cholesterol. In contrast the present study, based on the lipoid granules only, shows that the triglycerides form the largest single pool. Palmitic and stearic acids were the predominating saturated acids and oleic and linoleic acids the predominating unsaturated acids in the fraction eluted with the steroid esters. The triglyceride fraction was characterized by medium levels of palmitic and stearic acids and high levels of oleic and linoleic acids. However in other studies stearic acid was found to be the main component of the phospholipids in ascites tumor cells(13,14). Observations concerning the increase in number of these lipoid-rich granules have not led to any clues concerning metabolism of neoplastic tissue. The slow staining of these granules with Janus green B and their apposition to mitochondria have led MacKenzie *et al.*(12) to speculate that they may possess enzymes and furnish substrates for mitochondria.

Summary. The lipid composition of lipoid granules of Ehrlich ascites tumor cells was analyzed using silicic acid column and gas-liquid chromatography. Triglycerides formed the largest single pool. Oleic and linoleic acids formed the largest pools in the steroid ester fraction whereas palmitic, stearic, oleic and linoleic acids predominated in the triglyceride fraction.

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