Increase in cell lipid and cytoplasmic particles in mammalian cells cultured at reduced pH

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ABSTRACT The hydrogen ion concentration of the medium has been shown to exert a regulatory effect on the lipid content of cultured mammalian cells. Reduction of the pH of the medium from 7.4 to 6.9 causes a significant increase in cell lipid, relative to cell protein, within 2–3 days. Triglycerides are increased twofold and account for 75% of the additional lipid. Polar lipids, on the other hand, remain nearly constant in concentration.

Concurrent with the increase in lipid, particles with an average diameter of 1 μ appear in the cytoplasm. Because the density of these particles is low, ultracentrifugation of the cell homogenate separates the particles completely from the other subcellular structures. The amount of lipid in the particle fraction is approximately equal to the increase in total cell lipid. As shown by silicic acid column chromatography, the particle lipid contains about 75% triglycerides, 15% diglycerides plus an unknown substance, and smaller amounts of material in the monoglyceride and sterol ester–hydrocarbon fractions. The quantitative results indicate that the lipid accumulated at low pH is assembled into discrete cytoplasmic particles.

KEY WORDS pH · lipid accumulation · lipogenic effect · triglycerides · composition · lipid-rich particles · isolation · cultured cells · mammalian cells

When mammalian cells are incubated in a medium resembling extracellular fluid, an increase in the hydrogen ion concentration of the medium results in a striking increase in the number of perinuclear granules present in the cytoplasm (1). This response is observed whether the increase in hydrogen ion concentration is caused by an increase in pCO_2, a decrease in HCO_3^- concentration, or an accumulation of lactic acid. The particles, which number 20–60 per cell after growth for 2 days at pH 6.9, have a mean diameter of 1 μ and stain with Janus green and lipid-soluble dyes. They are electron-opaque and under the electron microscope appear to be surrounded by a limiting membrane. These properties, together with the low density of the granules, led us to name the granules "lipid-rich particles" (1).

Since our original observations were made, methods have been devised for the quantitative determination of the lipid content and composition of cultured cells (2) and for the isolation of lipid-rich particles free from other cell organelles (3). The present paper describes the changes in the lipid content and composition of cells exposed to an acidotic environment and the relation of these changes to the newly formed lipid-rich particles.

MATERIALS AND METHODS

The rat liver cell was from a clone isolated in our laboratory. The mouse fibroblast was kindly provided by Dr. Virginia Evans (National Cancer Institute) from the L clone of strain NCTC 2071, and the human liver cell (Chang) was obtained from Microbiological Associates, Inc., Bethesda, Md.

In all experiments cells were grown in the modified Eagle's medium described earlier (1) except that the horse serum (Microbiological Associates, Inc.) was increased to 20% with compensatory reductions in inorganic constituents. The medium was sterilized by filtering through a 03 porosity Selas candle (Selas Flotronics, Spring House, Pa.). The cells were plated in 6-cm Petri dishes containing 5 ml of medium, pH 7.4, and incubated at 37.5°C in a constant atmosphere of 5% CO_2 in air. Two different cell populations were plated, each in sets of 8–12 dishes, in expectation of different growth rates at pH 7.4 and 7.0 (1). After 18 hr, when the cells had entered the log phase of growth, protein was measured in

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TABLE 1 EFFECT OF HYDROGEN ION CONCENTRATION ON THE LIPID CONTENT OF MAMMALIAN CELLS

Cell	pH of Medium*		Cell Protein		Cell Lipid	μg Cell Lipid
	Initial	Final	Initial	Final	Final	μg Cell Protein
			μg		μg	
Mouse fibroblast (L)†	7.4	7.3	164	1475	270	0.183
	7.0	6.9	334	1965	397	0.202
Rat liver‡	7.4	7.3	168	1225	221	0.180
	7.0	6.9	282	1300	264	0.203

Cells were grown for 3 days in medium containing 20% horse serum. Results are expressed as cell protein and lipid per dish and are averages for four to eight dishes. Each dish contained approximately 1 mg of cell protein.

* The slight decline in initial pH levels was due to the metabolic production of acid.

† Mean protein content per cell was 3.2×10^{-4} μg.

‡ Mean protein content per cell was 4.1×10^{-4} μg.

four dishes of each set of cells. This marked the zero time of the experiment.

The replicate dishes that had the smaller number of cells were left in the 5% CO_2 atmosphere and those with the higher population were transferred to a 15% CO_2 atmosphere. The medium was replaced daily with fresh medium, pH 7.4. Equilibration of the fresh medium with the 15% CO_2 was complete within 30 min. The pH was determined as described earlier (1).

Cells were washed free of medium and their total lipid was quantitatively extracted and weighed to ±5 μg by the procedures described previously (2). Protein was measured in the extracted cell residue by the method of Oyama and Eagle (4). The lipid-rich particle fraction was prepared by disrupting washed cells in distilled water and centrifugation in a Spinco No. 40 rotor at 30,000 rpm for 20 min in the Spinco model L ultracentrifuge (3).

Lipid, isolated from whole cells and from particles, was separated into five fractions on a column of silicic acid by the procedure of Barron and Hanahan (5) as modified and adapted to microquantities of material (2). The amount of lipid in each fraction was measured gravimetrically. The composition of the fractions was determined by the distribution of test compounds (2) and by thin-layer chromatography on silica gel prepared according to Rouser, Galli, Lieber, Blank, and Privett (6). Two solvent systems were used, both of which, following Malins and Mangold (7), contained hexane–ethyl ether–acetic acid but in different ratios; i.e., 50:50:1 and 80:20:1. Cholesterol was measured in the appropriate eluate fraction by the method of Abell, Levy, Brodie, and Kendall (8).

The following compounds were employed as standards: cholesteryl stearate, cholesterol, tripalmitin (The Hormel Institute, Austin, Minn.), α,β-dipalmitin, α-monopalmitin (kindly supplied by Dr. E. P. Kennedy), and palmitic acid (Distillation Products, Industries, Rochester, N. Y.) three times recrystallized from hexane.

RESULTS AND DISCUSSION

Total Cell Lipid

As shown in Table 1, the reduction in extracellular pH from 7.4–7.3 to 7.0–6.9 caused an increase in total cell lipid relative to cell protein in both the mouse fibroblast and rat liver cell. The mean lipid:protein ratio for all dishes of cells grown at the higher pH was 0.182 ± 0.0013 and for cells grown at the lower pH, 0.203 ± 0.0011. This difference in means is highly significant as shown by the "*t*" test (9). The calculated value of *t* is 12.6 as compared with the value of 3.5 required for $P = 0.01$.

The increase in lipid was not due to the reduced growth of the cells at pH 7.0–6.9, for occasionally even slower growth has been encountered at pH 7.4 and in these instances cell lipid was not increased. Furthermore, since the initial cell populations at the two pH levels were adjusted to give comparable final populations (Table 1), the lipid accumulation was not due to cell crowding.

Composition of Cell Lipid

Approximately 1 mg quantities of lipid isolated from rat liver cells grown at the two pH levels were fractionated by silicic acid column chromatography. The material in the "triglyceride plus free fatty acid" fraction constituted 7% of the total lipid present in the cells grown at pH 7.4 and 15% in the cells grown at pH 7.0. In both cases thin-layer silica gel chromatography of this fraction gave only one spot, which corresponded in R_f to that produced by tripalmitin. Free fatty acids were either absent or below the level of detection.

The percentage composition of the cell lipid was used to calculate the amount of each lipid fraction present per μg of cell protein. As can be seen from Table 2, triglycerides accounted for most of the lipid increase in the cells exposed to the acidotic environment. On the other hand, the level of polar or structural lipids remained nearly con-

TABLE 2 EFFECT OF HYDROGEN ION CONCENTRATION ON CELL LIPID COMPOSITION

Lipid Fraction	μg Lipid in Fraction per μg Total Cell Protein	
	pH 7.4–7.3	pH 7.0–6.9
Total lipid	0.180	0.203
Sterol esters plus hydrocarbons	0.005	0.006
Triglycerides	0.013	0.030
Free cholesterol plus diglycerides	0.016*	0.018
Polar lipids	0.146	0.148

Rat liver cell lipid was fractionated by silicic acid column chromatography. Approximately 1 mg quantities of lipid were applied to the column. Total recovery ranged from 90 to 100%. Results at each pH are averages of two separate experiments. Values for all lipid subfractions were obtained gravimetrically and agreed to within 10%.

* Only cholesterol was detected when this fraction was analyzed by thin-layer chromatography on silica gel.

stant. Consequently, the increase in the cell lipid:protein ratio at pH 7.0–6.9 was not due to a leakage of protein but rather to a real increase in cell lipid.

Lipid Content of the Lipid-Rich Particle Fraction

Acidification of the medium was accompanied by the appearance of particles in the cytoplasm of both the mouse fibroblast and rat liver cell. When rat liver cells grown at pH 6.9–6.8 were disrupted in distilled water, the particles could be seen under the phase contrast microscope to pass into the water without change in size or shape. Centrifugation of the homogenate at 100,000 *g* concentrated the particles in a narrow band at the top of the supernatant fraction. The suspended particles, together with one-tenth of the supernatant solution, were transferred to a flask by means of a fine-tipped pipette. The remainder of the supernate, which was slightly contaminated with particles by the foregoing procedure, was decanted into a second flask and designated the soluble fraction of the cell. Both fractions were frozen in a thin layer, lyophilized, and extracted for lipid by the procedure used for intact cells. The lipid content of the particle fraction was approximately ten times greater than that of the soluble fraction (Table 3). In terms of μg lipid per ml of fraction it was 60 times greater. Similar results were obtained in experiments with human liver cells.

As a control, the same procedure was carried out on cells grown at pH 7.4. Only an occasional particle could be detected in these cells and the lipid present in the isolated particle fraction was only one-nineteenth of that found in cells grown at pH 6.9 (Table 3). It appears, therefore, that the cytoplasmic particles are indeed lipid-rich. Moreover, in these experiments, the increase in particle lipid was approximately the same as the increase in total cell lipid (Table 1).

TABLE 3 EFFECT OF HYDROGEN ION CONCENTRATION ON LIPID-RICH PARTICLE FRACTION OF CELL

Cell Fraction	μg Lipid in Fraction per μg Total Cell Protein	
	pH 6.9–6.8	pH 7.4–7.3
Lipid-rich particles	0.019	0.001
Soluble	0.002	<0.001

Rat liver cells grown for 2 days at acid and alkaline pH were disrupted in water and the particle and soluble fractions prepared by ultracentrifugation. The number of cells used in each experiment provided approximately 8.0 mg of protein.

Composition of Particle Lipid

Particle lipid from rat and human liver cells was fractionated by silicic acid column chromatography and the major fractions were further analyzed by thin-layer chromatography on silica gel. The results are summarized in Table 4. Triglycerides constituted by far the greater part of the particle lipid in each of the cell lines. When subjected to thin-layer chromatography, this fraction exhibited a single spot with the R_f of tripalmitin. Free fatty acids were not detected.

The next largest eluted fraction was the one in which free cholesterol and diglycerides emerge from the column together. Both chemical analysis and thin-layer chromatography of the fraction gave negative tests for cholesterol. The presence of diglycerides, however, was verified by thin-layer chromatography. The fraction also contained material that remained at or near the origin on a number of plates. In this respect this material behaved like a polar lipid although it had emerged from the column well ahead of lecithin, cephalin, and sphingomyelin (2).

TABLE 4 FRACTIONATION OF PARTICLE LIPID BY SILICIC ACID COLUMN CHROMATOGRAPHY

Lipid Component	Rat Liver Cell	Human Liver Cell
	% *of total lipid*	
Sterol esters plus hydrocarbons	7	3
Free cholesterol	<1	<2
Triglycerides	70	75
Diglycerides*	18	15
Monoglycerides	4	3
Polar lipids	<2	<2

Particle lipid (300–500 μg) from cells grown for 2 days at pH 6.9–6.8 were fractionated by silicic acid column chromatography, with a recovery of 100%. Results at each pH are averages of two separate experiments. Values for all lipid subfractions were obtained gravimetrically and agreed to within 10%. Composition of eluate fractions was determined by the distribution of test compounds on the column plus subsequent thin-layer chromatography and cholesterol analysis.

* In addition to diglycerides this fraction contained an unidentified material that remained at or near the origin on thin-layer silica gel chromatography in hexane–ethyl ether–acetic acid 50:50:1.

The amount of the unknown compound(s) was insufficient for chemical analysis.

The composition of the particle lipid was in agreement with the composition of the lipid accumulated by cells grown at pH 7.0–6.9 (Table 2). Thus, triglycerides accounted for three-quarters of the particle lipid and for three-quarters of the increase in cell lipid. These findings, together with those of the previous section, indicate that the lipid-rich particles contained most or all of the additional lipid present in cells grown at the reduced pH. Since particles were not present in the medium, as shown by both ultracentrifugation and dark field examination, we concluded that they were assembled in the cell in response to the increase in its lipid content.

As we have recently reported (10), serum represses fatty acid synthesis in cultured cells and the fatty acids of the neutral and polar lipids are derived from exogenous sources; i.e., from the lipoproteins, including albumin, present in the medium. On the other hand, the glycerol moiety of both classes of lipids is derived from the endogenous metabolism of glucose. Whether hydrogen ions exert their lipogenic effect by increasing the availability of the fatty acids (nonesterified and esterified) of serum lipoproteins or by a direct action on the cell is at present unknown. Regardless of the mechanism, the hydrogen ion effect must be considered whenever lipid particles appear spontaneously in a viable cell system. This is not to say that the hydrogen ion concentration is the only chemical factor in the environment which can cause an increase in cell lipid. For example, rabbit serum proteins, and albumin in particular, have also been shown to cause a great increase in triglycerides in some cell lines even though the pH of the medium is maintained at 7.4 (2, 10). This increase in triglycerides is likewise accompanied by the formation of lipid-rich particles and these particles are surrounded by a protein-containing membrane (3). It should be noted that the lipid content of cells exposed to rabbit serum is further increased by increasing the external hydrogen ion concentration (G. C. Mackenzie, O. K. Reiss, and J. B. Mackenzie, unpublished work).

With respect to the intact animal, it may be recalled that pH levels of 6.8 and 6.6 have been reported for ischemic tissue (11) and for areas of inflammation (12). It has been known since the time of Virchow that fatty degeneration of the heart is characterized by the appearance of many small lipophilic particles in the cardiac muscle fibers (13). Several years ago Novikoff (14) observed the presence of lipophilic particles in an experimental tumor, and recently Apffel and Baker (15) have reported their presence in 95% of the human tumors they examined. Since fatty degeneration of the heart is often associated with severe anemia and tumors are noted for their high glycolytic activity, it is possible that local decreases in pH play an important role in the accumulation of lipophilic particles in these tissues.

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