ISOLATION AND PROPERTIES OF A CELL FROM LIVER CHARACTERIZED BY LIPID-RICH PARTICLES

COSMO G. MACKENZIE, Sc.D., JULIA B. MACKENZIE, Sc.D., and OSCAR K. REISS, Ph.D.

From the Department of Biochemistry, University of Colorado School of Medicine, and The Webb Institute for Medical Research, Denver, Colorado

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

ABSTRACT

A cell has been isolated from explanted rabbit liver which contains, during all phases of its growth in culture, hundreds of lipid-rich particles with a distinct limiting membrane. The cell grows logarithmically with a generation time of 19 to 20 hours and during mitosis the particles are distributed between the daughter cells. Associated with the particles is the high total lipid content of the rabbit liver cell as compared with a rat liver cell, which contains few, if any, lipid-rich particles. This difference in lipid content between the two cells is due primarily to an increase in the triglyceride fraction, in contradistinction to small differences in the polar lipid and sterol ester fractions. The lipid-rich particles have been isolated and found to contain 90 per cent triglyceride on a dry weight basis. The "genetic" factors responsible for the high concentration of lipid-rich particles and triglycerides in the rabbit liver cell require for their full expression one or more factors which are present in much higher effective concentrations in rabbit serum than in horse serum. The hypothesis is advanced that the lipid-rich particles represent a normal state of the non-structural cell lipid. A procedure is described for the quantitative isolation of the lipid of cultured cells.

In a study of the effect of extracellular H^+ concentration on cell growth and morphology, we observed that reducing the pH to the acid range caused a marked increase in the number of cytoplasmic perinuclear particles in HeLa and Chang liver cells and in two newly isolated cell lines from rat liver and rat muscle while only moderately reducing the growth rate. These particles were isolated from cell homogenates by differential centrifugation, and on the basis of their physical properties, extraction with organic solvents, and staining reactions, were called lipid-rich particles (1). During the course of these studies we repeatedly isolated a cell from rabbit liver which contains a large number of similar particles irrespective of the environmental H^+ concentration (2). The present paper is a report of the isolation, growth, structure, and chemical properties of this lipid-rich cell as compared with the rat liver cell,[1] and as affected by the extracellular environment. As a result of these findings a hypothesis is advanced concerning the physical state of triglycerides of the cell sap.

METHODS

Media

The medium used in the isolation of the rabbit liver cell[1] and for growing the stock cultures had the

[1] The term "liver cell" is used throughout this paper to designate a cell which is derived from liver but whose precise origin within this tissue is unknown. The lipid content of the isolated rabbit cell described in this paper is compatible with, but does not prove, its derivation from the parenchymal cell of liver.

following composition: 20 ml of rabbit serum,[2] 5 ml of human cord serum [3] 1 ml of beef embryo extract ultrafiltrate (EE-50, Microbiological Associates, Inc., Bethesda, Maryland), 5 ml of NCTC-109 (3), 80 units of insulin (U-80 Regular, Lilly U.S.P.), twice the amino acid, glutamine, and vitamin concentrations used in our modification of Eagle's medium (1), and Earle's salt solution (4) to a final volume of 110 ml. The medium was sterilized by filtering through a Selas candle of 03 porosity, which also removed any insoluble materials present in the serum. Stock cells were grown in large Petri dishes (9 cm diameter) at 37.5°C in an incubator gassed with 5 per cent CO_2 in air.

In all the growth and metabolism experiments, the cells were grown on the modified Eagle's medium previously described (1) with the level of serum raised to 20 per cent at the expense of Earle's salt solution. Suspensions of stock cultures were prepared either by treatment with 0.1 per cent trypsin (1–300, Nutritional Biochemicals Corp., Cleveland, Ohio) in a buffered electrolyte solution (1), or by scraping into the experimental medium. Suitable aliquots were then transferred to Petri dishes of 6 cm diameter, and incubated at 37.5°C in desiccators gassed with 5 per cent CO_2 in air as described earlier (1). After 18 hours, the "zero time" population of attached cells in four dishes was determined by protein analysis, which was in good agreement with cell counts. The experimental medium was replaced in the other dishes and thereafter changed every day throughout the experiment.

Isolation of Total Cell Lipid

The Petri dishes were cooled to 0°C, the medium was aspirated, and the cells were washed three times with 3 ml portions of ice-cold distilled water. The washed cells were extracted twice with 1 ml of a 1:1 mixture of 95 per cent ethyl alcohol and anhydrous ethyl ether (Mallinckrodt Analytical Reagent) and three times with 1 ml of ethyl ether. Each extraction was carried out for approximately 20 seconds while rotating the Petri dish on a warm plate at 35°C. The combined extracts from each dish were collected in a 10 ml beaker and evaporated under an infrared lamp at the water pump. The beakers were then stored *in vacuo* over paraffin chips for 1 hour and over P_2O_5 for 1 hour or longer. Next, the contents of the beakers were extracted three times with 1 ml of petroleum ether (Mallinckrodt Analytical Reagent, 30–60°C) on the warm plate and filtered under reduced pressure through a medium porosity sintered glass funnel into a counterweighed 10 ml beaker. The petroleum ether extracts were evaporated and dried as described above. After storage overnight *in vacuo* over P_2O_5, the beakers were weighed against a counterweight on a Mettler M5 microbalance to ± 5 μg. The weight of the extracted lipid was corrected by the weight of a solvent blank determined in each analysis. The blank value value ranged from 15 to 30 μg. When the weight of the isolated lipid was expressed as a ratio to the weight of cell protein, the results obtained with triplicate dishes of cells agreed to within 5 per cent.

The completeness of the lipid isolation was checked as follows. The extracted cells and the petroleum ether–insoluble residue from the alcohol and ether extracts were saponified in 6.5 M methanolic KOH in sealed tubes for 2 hours at 95°C. Each solution was acidified with H_2SO_4 and extracted three times with ethyl ether. The ether was evaporated and the residue was extracted with petroleum ether and weighed. In a typical experiment in which 527 μg of lipid were isolated from the rabbit liver cells, the saponified cell residue yielded 14 μg of petroleum ether–soluble material, and the saponified alcohol-ether residue yielded none. It appears, therefore, that the procedure employed is satisfactory for the quantitative isolation of lipids from glass-attached cells.

Chemical Analysis

The isolated lipids were separated on a silicic acid column by the method of Barron and Hanahan (5), modified for use on a microgram scale, into three parts as follows: a hydrocarbon and sterol ester fraction eluted with benzene-hexane, 15:85, a neutral lipid fraction (free of P) eluted with ethyl ether–hexane, 50:50; and a polar lipid fraction eluted with absolute methanol.

The neutral lipid fraction was chromatogramed on silicic acid–impregnated paper, prepared according to Marinetti and Stotz (6), in an ethanol-hexane system, 1:99, and the chromatogram was developed with a 0.006 per cent solution of rhodamine B in water. The polar lipid fraction was subjected to thin layer silicic acid chromatography in chloroform-methanol-water, 65:25:4, by the procedure of Wagner *et al.* (7) and sprayed with the ammonium molybdate–perchloric acid reagent.

Lipid phosphorus in the total isolated lipids was measured by the method of King (8), and glycerol in the hydrolized neutral lipid fraction by the method of Van Handel and Zilversmit (9). Cholesterol was measured by adapting the method of Abell *et al.* (10) to microquantities. Total lipid in the filtered medium was measured by the procedure of

[2] Prepared by Colorado Serum Co., Denver, from fasted adult rabbits and filtered through a Selas 02 porosity candle.

[3] Kindly supplied by Dr. E. Stewart Taylor of the Department of Obstetrics and Gynecology, University of Colorado School of Medicine.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

Bloor (11), and cell protein by the procedure of Oyama and Eagle (12).

EXPERIMENTAL RESULTS

Isolation of Rabbit Liver Cell

Adult rabbits are exsanguinated by heart puncture and thin slices of the liver are removed aseptically. Blocks of 1 cubic mm or less are cut from the slices and placed in 6 cm Petri dishes which have been previously coated with 0.05 to 0.1 ml of rabbit serum to provide a sticky surface. Ten to fifteen blocks are added per dish. 5 ml of stock medium are carefully added to each dish, which is then placed immediately in a 37.5°C incubator gassed with a continuous flow of 5 per cent CO_2 in air. The stock medium is changed every 48 hours.

Within 1 to 4 days cells containing many perinuclear particles grow out from the explant and form colonies. The location of several such colonies is marked on the bottom of the dish and the original explants are removed with forceps. The cells in the center of a marked colony are loosened with a fine probe, a drop of stock medium is added from a micropipette, and the loosened cells are drawn into the tip and transferred to a new dish. After a colony of cells containing the perinuclear particles has been formed, the cell is cloned by the direct method of Sanford *et al.* (13) or by the dilution procedure of Puck *et al.* (14). Stock cultures derived from the clones are transferred every 3 days. Clones possessing the chemical and cytological properties to be described below have been isolated from the livers of rabbits in fourteen consecutive experiments.

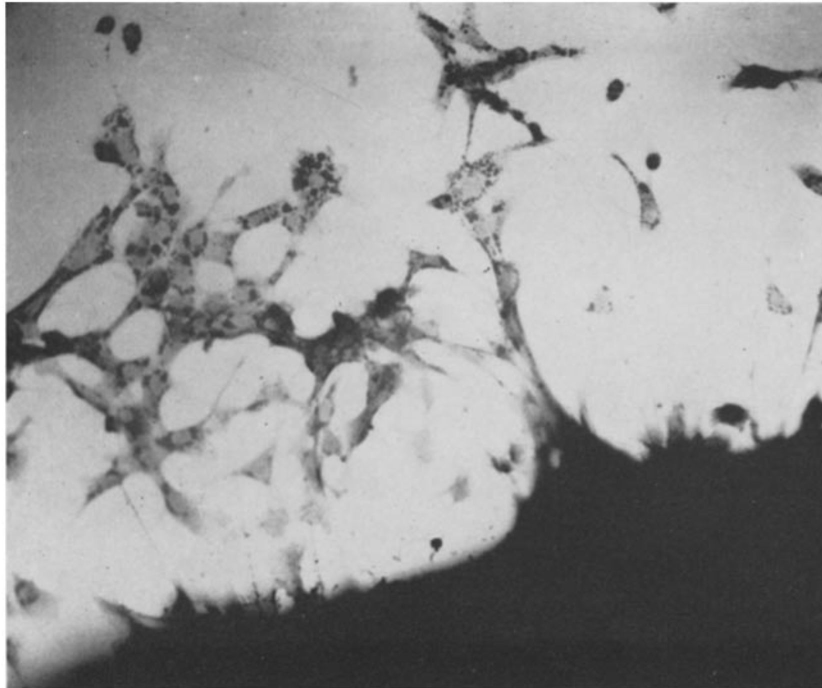

FIGURE 1

Rabbit liver cells at edge of explant. Fixed in Bouin's solution and stained with Janus green B and oil red O. The small dots are lipid-rich particles. Larger dark spheres are artifacts formed by fusion of the particles during fixation and staining. $\times$ 135.

Rabbit Liver Cell Morphology

The cells which emerge from the edge of the explant and migrate into the medium are "fibroblast-like" in appearance (Fig. 1), and contain numerous perinuclear particles approximately

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

1.0 μ in diameter. The width and length of the cells, exclusive of filamentous projections, range from 15 by 30 μ to 35 by 65 μ. As shown in Fig. 1, many of the cells at this stage appear to be connected by cytoplasmic bridges.

As the cells peripheral to the explant multiply, they lose their fibroblast appearance and torm colonies of four- and five-sided polygonal cells approximately 70 μ in diameter. The cells of clones obtained from these colonies continue to show the effect of the environment on cell morphology, *i.e.*, the proximity of the cells to one another. Thus, at each transfer the cells tend to assume a "fibroblast-like" shape which, with cell multiplication, is replaced by the polygonal shape of the cells in the colony (Fig. 2). At the periphery of the colony, however, it is not uncommon to find greatly stretched multi-armed cells with axes ranging from 100 to 250 μ. These cells, like those in the interior, contain perinuclear particles.

The morphology of the rabbit liver cell is also affected by the nature of the medium. For example, on the less complicated experimental medium, *i.e.*, modified Eagle's medium plus 20 per cent rabbit serum, a number of cells remain elongated even at high population densities. Consequently, groups of compact cells are found surrounded by "fibroblast-like" cells up to 200 μ in length. This picture is even more pronounced when horse serum is substituted for rabbit serum in the medium.

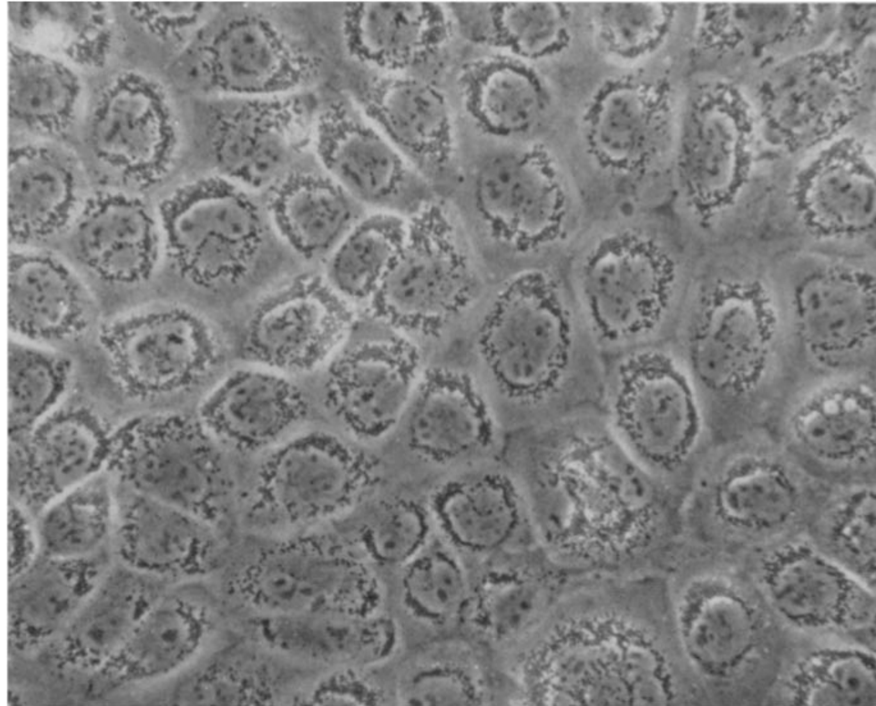

FIGURE 2

Interior of a colony of living rabbit liver cells in the stock medium. Lipid-rich particles surround the nuclei of the polygonal cells. Phase contrast. × 220.

Lipid-Rich Particles

The perinuclear particles are stained slowly with Janus green B by the procedure of Conn (15) or Cowdry (16). After the cells have been fixed with Bouin's solution, the particles stain brilliantly with oil red O dissolved in isopropanol. In these respects the perinuclear particles of the rabbit cell resemble the lipid-rich particles produced in four other cell lines by cultivation at acid pH (1).

The fixation in Bouin's solution and staining with oil red O causes many of the particles to fuse into large globules (Fig. 1), and hence distorts

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

the situation present in the living cells (Fig. 2). Better preparations are obtained when cells grown on coverslips are fixed in Bensley's solution, or Regaud's solution plus osmium, and stained with Altmann's anilin–acid fuchsin (17). However, clearing and dehydration in acetone or alcohol, followed by xylol, extracts the contents of the particles (Fig. 3). We have found that the perinuclear particles can be fixed and preserved for mounting by the following procedure. Cells attached to a coverslip are stained with a solution of Janus green B in 0.15 M NaCl, 1:1000, for 15 minutes, and then suspended over a 4 per cent solution of osmium tetroxide for 15 minutes. The fixed cells are washed with acetone, graded mixtures of acetone and xylol, and xylol for 1 minute each, and mounted in Permount (Fisher Scientific Co., New York). The lipid-rich particles are stained a greenish brown color. Without prior exposure to Janus green, the fixation with osmium does not prevent the extraction of the particles with xylol.

The particles are preserved in cells fixed in 2 per cent osmium tetroxide adjusted to a pH of 7.4 with veronal-acetate buffer and to isosmolarity with sucrose, and embedded in methyl and *n*-butyl methacrylate, 1:1, for electron microscopy. As shown in Fig. 4, they appear as electron-opaque bodies with a distinct limiting membrane.

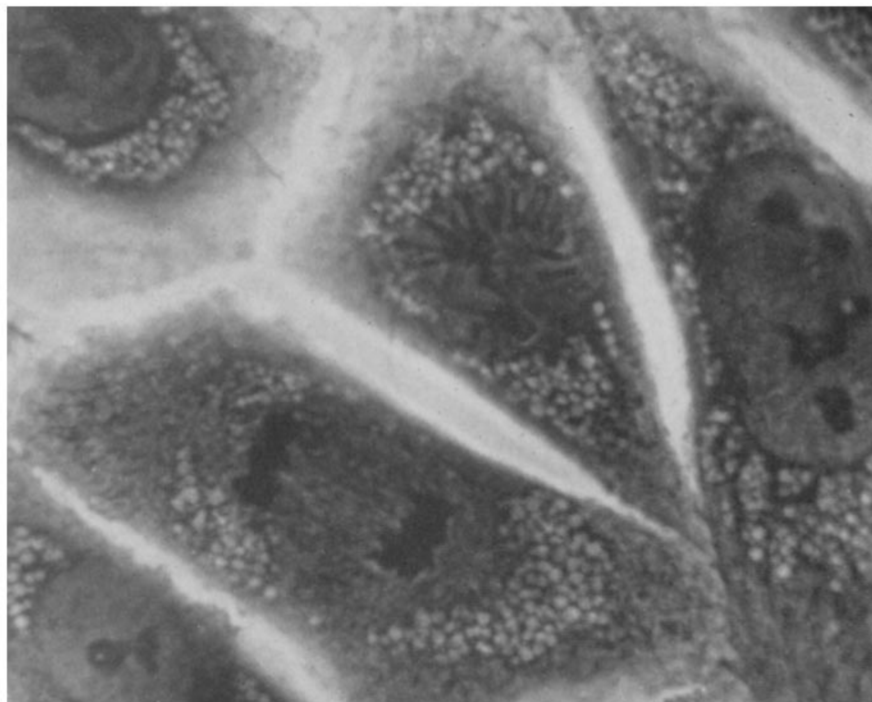

FIGURE 3

Cultured rabbit liver cells during mitosis. Fixed in Bensley's solution, stained with Altmann's anilin–acid fuchsin, and cleared with acetone and xylol. Although the lipid-rich particles were extracted by the xylol, their "ghosts" remain. The particles are excluded from the mitotic apparatus. $\times$ 1000.

Growth

The average protein content of the rabbit liver cell was 7.5×10^{-4} μg. When initial populations of 4 to 14×10^4 cells per dish were cultivated for 3 days on either the stock or the experimental medium, the increase in total cell protein corresponded to 3.7 generations. During the third day, the pH of the medium fell from 7.4 to 7.3. Cells cultivated under the experimental conditions for 9 days (with transfers at the end of the third and

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

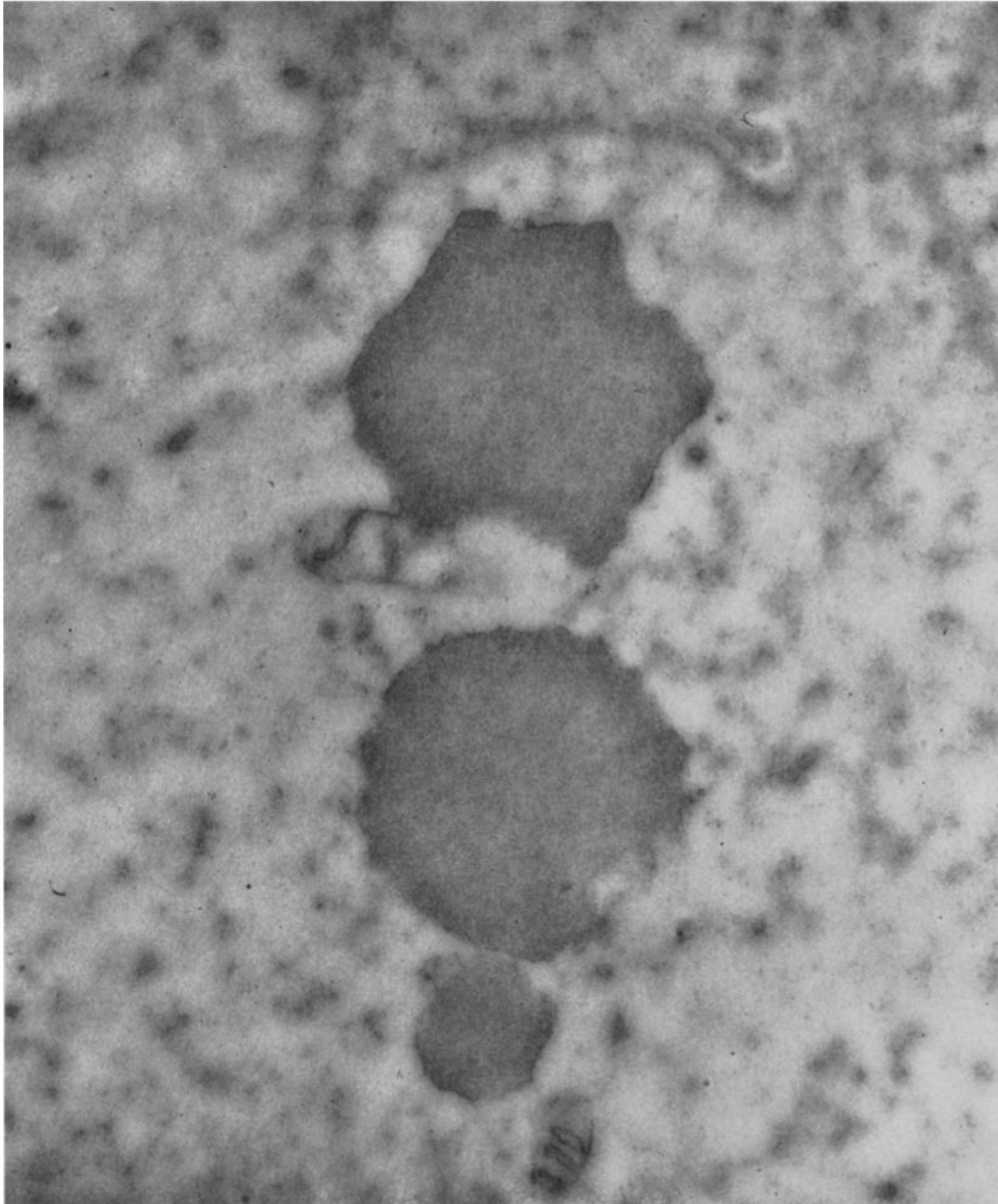

FIGURE 4

Electron micrograph of lipid-rich particles showing their distinct membrane. The cell was fixed *in situ.* × 40,500.

We are indebted to Dr. James F. Reger of the Department of Anatomy, University of Colorado School of Medicine, for this preparation and the electron micrograph.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

sixth day) grew logarithmically with an average generation time of 18.5 hours (Fig. 5).

Dialysis of the rabbit serum substantially reduced cell growth in a 3-day test period (Table I). The dialysate also exhibited poor growth-promoting activity. Recombination of the two fractions, however, resulted in a reasonably good growth response. It appears from these experiments that the rabbit liver cell requires two or more growth factors present in rabbit serum, but missing, or present in suboptimal concentrations, in the experimental medium.

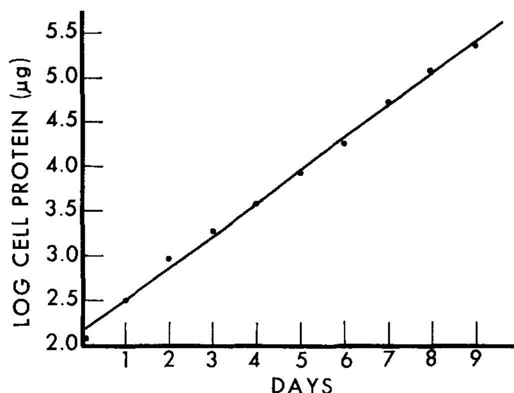

FIGURE 5

Growth of the rabbit liver cell on the experimental medium.

Total Cell Lipid and Subfractions of the Rabbit Liver Cell

In rabbit liver cells grown under the experimental conditions, the average total cell lipid, expressed as a ratio to cell protein, was 0.41 with a S.E. of ±0.01. This value was obtained with initial populations ranging from 7 to 18 × 10^4 cells per dish and with a generation time of 19.5 hours. The same value was also obtained when the generation time was increased to 40 hours by reducing the level of rabbit serum in the medium to 10 per cent.

The composition of the isolated cell lipid is given in Table II. More than half of the total cell lipid was present in the neutral lipid fraction. When this fraction was chromatographed on silicic acid–impregnated paper, only one spot was obtained by staining with rhodamine B. This spot migrated with the solvent front, as did a sample of a chromatographically pure triglyceride, tripalmitin. Samples of α-monopalmitin and α,β-dipalmitin exhibited R_f values of 0.084 and 0.50, respectively.[4] The neutral lipid fraction contained approximately 5 per cent cholesterol. After the removal of the cholesterol, the remainder of the fraction was found to contain 11 per cent glycerol (Table II). If it is assumed that all the glycerol was present as triglyceride, the average chain length of the fatty acids was C17.

Thin layer chromatography (silicic acid) of the polar lipid fraction produced two major spots and a weaker spot. The major spots had R_f values of 0.29 and 0.39, and the weaker spot an R_f value of 0.64. A sample of dipalmityl lecithin (Mann Research Laboratories, New York) gave two spots with R_f values of 0.28 and 0.38. It is of interest that when the lipid P is expressed as dipalmityl lecithin, a ratio of phospholipid to cell protein of 0.15 is obtained. This compares favorably with the experimentally determined ratio of polar lipid to cell protein of 0.16 (Table II).

TABLE I

Effect of Dialysis on Growth Factors in Rabbit Serum

Rabbit liver cells were grown for 3 days in Petri dishes (6 cm diameter) on the experimental medium plus the indicated supplements in a total volume of 5 ml. All media were changed daily. Atmosphere, 5 per cent CO_2; temperature, 37.5°C. Each result represents the average of 3 or more experiments.

Serum supplement	Generations in 3 days
20% whole serum	3.7
None	<1.0
20% dialyzed serum*	1.8
40% serum dialysate‡	1.0
20% dialyzed serum + 40% serum dialysate	3.0

* 20 ml of rabbit serum dialyzed at 0–3°C in a rocking dialyzer for 6 hours against 6 liters of running distilled water.

‡ 20 ml of rabbit serum dialyzed as above against consecutive volumes of 40, 30, and 30 ml of distilled water for 2 hours each. The total dialysate was lyophilized and dissolved in an appropriate volume of protein-free medium.

Relation of Cell Lipids to Serum and Species

In view of the invariable presence of large numbers of lipid-rich particles in the rabbit liver

[4] We are indebted to Dr. Eugene P. Kennedy for these compounds.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

cell grown on rabbit serum, and their paucity in four other cell lines grown on horse serum at pH 7.4, a comparative study wa; made of the effects of these two sera on the rabbit liver cell and the rat liver cell (1) grown under identical conditions. For this purpose sera with the same total lipid content were employed. As shown in Table III, the substitution of horse serum for rabbit serum in the experimental medium reduced the lipid-to-protein ratio in the rabbit cell from 0.43 to 0.23.

TABLE II

Lipid Composition of the Rabbit Livei Cell

All values were obtained by dividing micrograms of cell lipid by micrograms of cell protein and are the averages of 3 to 10 determinations. The cells were grown on the experimental medium containing 20 per cent rabbit serum at 37.5°C in an atmosphere containing 5 per cent CO_2.

Lipid fraction	Ratio of lipid to protein
Total lipid	0.41
Polar lipid	0.16
Lipid P*	0.006
Neutral lipid‡	0.23
Glycerol	0.025
Free cholesterol	0.01
Hydrocarbons + sterol esters	0.02
Esterified cholesterol§	0.008

* Obtained by analysis of the total cell lipid; no P was found in the neutral lipid fraction.

‡ Subdivided into triglyceride and free cholesterol fractions by elution from a silicic acid column with hexane-ether, 95:5, and hexane-ether, 80:20 (5). Glycerol was determined after hydrolysis of the triglyceride fraction.

§ Expressed as free cholesterol.

This decrease was due almost entirely to a fall in the neutral lipid fraction. At the same time, the lipid-rich particles were substantially reduced in number, though by no means eliminated.[5]

When the rat liver cell was grown on the two different sera, the neutral lipid content was in both instances well below the level always encountered in the rabbit cell grown on rabbit serum. However, some lipid-rich particles were readily discernible in the rat cells grown on rabbit serum, whereas none were seen in the cells grown on horse serum. Rat serum also failed to increase markedly the neutral lipid fraction of the rat liver cell (Table III). It is clear that, in these experiments, both the rabbit liver cell and rabbit serum were essential factors in the production of a high concentration of cell triglycerides. It is notable that this high concentration was not accompanied by high concentrations of free cholesterol or cholesterol esters (Table II).

With respect to the polar lipids, the concentration in both cell lines was slightly higher when they were grown on rabbit serum. Regardless of how this effect may be explained, it was small as compared with the effect of rabbit serum on the triglyceride fraction of the rabbit liver cell. Presumably the polar lipid fraction is made up predominantly of mitochondrial and microsomal phospholipids.

DISCUSSION

The appearance of microscopically visible lipid in cultured cells has usually been ascribed to crowding, aging, or degeneration of the culture. Recently, King and coworkers (18) have reported an increase in lipid staining material when Earle's L cell reaches the stationary phase of growth in suspension cultures. They observed a similar reaction when growth was inhibited by *p*-fluorophenylalanine.

In an earlier experiment we found that lipid-rich particles increased markedly in four cell lines when the extracellular pH was reduced to 6.8 even though the rate of growth was still reasonably good (1). In the present experiments with the rabbit liver cell, growth inhibition, crowding, and extracellular acidity were all eliminated as causative factors in the appearance of cell lipid. The lipid-rich particles are just as prominent in cells dividing every 20 hours as in slower growing cells, and, as shown in Fig. 3, they are present during mitosis and are distributed between the daughter cells. It may be concluded, therefore, that the presence of an abundance of microscopically visible lipid is not necessarily an indication of crowding, degeneration, etc., and that other biochemical and environmental factors must always

[5] The addition of 10 per cent rabbit serum to medium containing 20 per cent horse serum produced a lipid-to-protein ratio of 0.40 in a 3-day test period. This experiment indicates that the failure of lipid to accumulate in cells grown on the horse serum medium was not due to the presence of inhibitors.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

be considered as possible causes of lipid appearance.[6]

The abundance of lipid-rich particles in the rabbit liver cell is associated with a higher total lipid content as compared with the rat liver cell (Table III), and in particular a higher triglyceride content. The isolation of the lipid-rich particles and their high lipid content has been described in a preliminary report (2). Recently, we have found that they contain approximately 90 per cent triglyceride on a dry weight basis. The presence of the particles, then, represents not an unmasking or molecular transformation of lipid, but a true increase in cell triglyceride concentration.

Lipid-rich particles are present in this rabbit liver cell when it emerges from the explant (Fig. 1). They are retained as the morphology of the cloned cell changes during successive transfers from "fibroblast-like" to polygonal (Fig. 2) and back again, and, as already mentioned, they are retained during cell division (Fig. 3). They are not eliminated by dilution of the original cell's molecular constituents by a factor of 2^{170}. They distinguish this cell not only from the rat liver cell, but also from another cell isolated from rabbit liver which retains a "fibroblast-like" morphology even on the stock medium and contains no visible particles. It appears, therefore, that the plethora of lipid-rich particles, which serves as a marker for this cell, is based on "genetic" factors. Of considerable importance is the fact that the cell has been isolated repeatedly from rabbit liver. It is not a tissue culture oddity or freak. Elucidation of the biochemical mechanisms responsible for the differences in lipid metabolism between this cell and other cells isolated from liver may prove useful in the study of cell differentiation in the organism.

As shown in Table III, an environmental factor(s) is also necessary for the *full* expression of the inherent capacity of the rabbit liver cell to accumulate lipid, and it is present in rabbit serum to a greater extent than in horse serum. Conversely, rabbit serum does not produce a great increase in lipid or hundreds of lipid-rich particles in the rat liver cell even after one month of continuous cultivation on the stock medium.

TABLE III

Relation of Cell Lipid to Serum and Cell Type

Cells were grown on the experimental medium containing 20 per cent serum under the conditions given in Tables I and II. Total lipid content of the rabbit serum medium was 49 μg per 100 ml, and of the horse serum medium 56 μg per 100 ml. The results are averages of 2 to 4 determinations.

Liver cell	Serum	Generation time	Ratio of lipid to protein			
			Total	Hydrocarbons + sterol esters	Neutral	Polar
		hr				
Rabbit	Rabbit	19	0.43	0.03	0.25	0.17
Rabbit	Horse	23	0.23	0.01	0.08	0.15
Rat	Rabbit	25	0.24	0.01	0.05	0.18
Rat	Horse	20	0.17	0.02	0.03	0.13
Rat	Rat	16	0.22	0.02	0.05	0.16

With respect to the origin of the lipid-rich particles, we have been unable to detect chylomicrons in the experimental medium containing rabbit serum, either by phase contrast or by dark field examination. Furthermore, no chylomicrons were present in the top layers after centrifugation at 100,000 *g*. It appears, therefore, that the particles are made or assembled inside the cell, and if one wishes to call them chylomicrons, then the cultured rabbit liver cell may be said to make chylomicrons.

The lipid-to-protein ratios observed in the rabbit liver cells grown on media containing horse

[6] Neither the addition of vitamin B_{12} to the experimental medium nor doubling the methionine and choline concentrations had any visible effect on the lipid-rich particles of the rabbit liver cell.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

serum or rabbit serum (Table III) lie within the range that is known to occur in the livers of normal animals. For example, in our laboratory the livers of rats fed a diet containing 18 per cent casein and 24 per cent fat, supplemented with choline and vitamin B_{12}, exhibit a lipid-to-protein ratio that ranges from 0.2 to 0.35 (19). Since in our tissue culture experiments a lipid-to-protein ratio of 0.23 or higher was invariably associated with the presence of lipid-rich particles, the hypothesis that these particles represent a normal state of non-structural lipid seemed a reasonable one. Fresh liver cells were therefore prepared by the light homogenization in water or 0.15 M NaCl of bits of liver from rats and rabbits fed commercial chow diets. These cells contained particles which resembled the lipid-rich particles of the cultured rabbit liver cells (Fig. 6). When the fresh cells were disrupted in distilled water and centrifuged at 100,000 *g* for 30 minutes, the particles rose to the top of the centrifuge tube and formed a distinct band. The particles isolated from rabbit liver were washed twice in water at 100,000 *g*. The purified particles contained 90 per cent lipid on a dry weight basis. Over 85 per cent of the lipid was triglycerides.

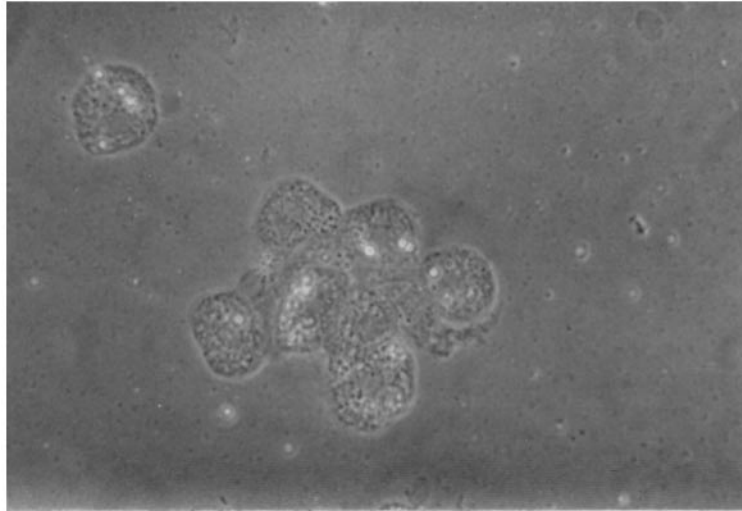

FIGURE 6

Parenchymal cells prepared by homogenizing fresh rabbit liver in water. Because of the thickness of the cells, only a few of the low density particles are in sharp focus at one time. Phase contrast. × 215.

Undoubtedly these lipid-rich particles would have appeared as electron-opaque bodies if the cells had been fixed in osmium and viewed in the electron microscope. However, when bits of fresh rabbit liver were fixed in Zenker-formalin, embedded in paraffin, and stained with hematoxylin and eosin, neither lipid-rich particles nor their "ghosts" could be detected. This is not surprising, for the lipid-rich particles of the isolated rabbit liver cell may also be obliterated by the fat solvents used in dehydration and clearing. In this connection, Dr. Robert W. Wissler has kindly sent us a section of liver from a monkey fed a diet containing 20 per cent coconut oil and ample quantities of lipotropic agents. The preparation had been fixed in neutral buffered formalin, frozen and cut on a freezing microtome, stained with Sudan IV, and mounted in glycerol jelly. The parenchymal cells contain numerous lipid-rich particles resembling those seen in our cultured rabbit liver cells that have been fixed in Bouin's solution and stained directly with oil red O. On the other hand, embedded sections did not reveal the presence of the particles or their "ghosts." The foregoing results indicate that the presence or absence of lipid-rich particles in tissues or cells cannot be established by an examination of the conventional dehydrated and cleared sections.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022

This work was made possible by a grant from The Muscular Dystrophy Associations of America, Inc.

We wish to thank Miss Myra Fuller and Mrs. Inger Christensen for their excellent assistance in the laboratory.

Received for publication, February 19, 1962.

REFERENCES

1. MACKENZIE, C. G., MACKENZIE, J. B., and BECK, P., *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 141.
2. MACKENZIE, C. G., REISS, O. K., and MACKENZIE, J. B., *Fed. Proc.*, 1961, **20**, 135.
3. EVANS, V. J., BRYANT, J. C., FIORAMONTI, M. C., MCQUILKIN, W. T., SANFORD, K. K., WESTFALL, B. B., and EARLE, W. R., *Cancer Research*, 1956, **16**, 87.
4. EARLE, W. R., *J. Nat. Cancer Inst.*, 1943, **4**, 165.
5. BARRON, E. J., and HANAHAN, D. J., *J. Biol. Chem.*, 1958, **231**, 493.
6. MARINETTI, G. V., and STOTZ, E., *Biochim. et Biophysica Acta*, 1956, **21**, 168.
7. WAGNER, H., HOERHAMMER, L., and WOLFF, P., *Biochem. Z.*, 1961, **334**, 175.
8. KING, E. J., *Biochem. J.*, 1932, **26**, 292.
9. VAN HANDEL, E., and ZILVERSMIT, D. B., *J. Lab. and Clin. Med.*, 1957, **50**, 152.
10. ABELL, L. L., LEVY, B. B., BRODIE, B. B., and KENDALL, F. E., *J. Biol. Chem.*, 1952, **195**, 357.
11. BLOOR, W. R., *J. Biol. Chem.*, 1929, **82**, 273.
12. OYAMA, V. I., and EAGLE, H., *Proc. Soc. Exp. Biol. and Med.*, 1956, **91**, 305.
13. SANFORD, K. K., EARLE, W. R., and LIKELY, G. D., *J. Nat. Cancer Inst.*, 1948, **9**, 229.
14. PUCK, T. T., MARCUS, P. I., and CIECIURA, S. T., *J. Exp. Med.*, 1956, **103**, 273.
15. CONN, H. J., Biological Stains, Geneva, New York, Biotech Publications, 1953.
16. COWDRY, E. V., Laboratory Techniques, Baltimore, Williams and Wilkins Co., 1948.
17. LILLIE, R. D., Histopathologic Technique and Practical Histochemistry, New York, Blackiston Division, McGraw-Hill Book Co., 1954.
18. KING, D. W., SOCOLOW, E. L., and BENSCH, K. G., *J. Biophysic. and Biochem. Cytol.*, 1959, **5**, 421.
19. MACKENZIE, J. B., and STRADER, R., unpublished results.

Downloaded from http://rupress.org/jcb/article-pdf/14/2/269/1291732/269.pdf by guest on 18 January 2022