Regulation of Cell Lipid Metabolism and Accumulation. IV. The Isolation and Composition of Cytoplasmic Lipid-Rich Particles*

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ABSTRACT: A procedure is described for the isolation of a new particulate fraction from animal cells growing in culture. The fraction consists of spherical particles, with a mean diameter of 1 μ, which, by virtue of their low density, are separated completely by ultracentrifugation from the other formed elements of the cell. The twice-washed particles contain *ca.* 3% protein and 90% lipid.

The lipid in turn contains 90% triglycerides plus small amounts of diglycerides, monoglycerides, cholesterol esters, and polar lipids. It contains negligible amounts of free cholesterol. The chemical composition and physical properties of the particles indicate that they consist of a lipid matrix surrounded by a protein-containing membrane. Sufficient protein is present in the particles to provide a membrane containing two monolayers of protein. Electron micrographs show that *in situ* most of the the particles are surrounded by a unit membrane 80 A wide. The remainder of the particles are surrounded by a single membrane 25 A wide. The triglyceride content of the cell is directly related to the number of lipid-rich particles. The polar lipids and free cholesterol of the cell, on the other hand, are located in the pellet fraction and are unrelated to the number of particles. The cell sap contains little or no lipid. It is concluded that the cytoplasmic lipid-rich particles play an important role in the compartmentalization of cell lipids.

Recently we have found that either acidification of the extracellular environment (Mackenzie *et al.*, 1961) or the addition of a macromolecular factor present in rabbit serum (Mackenzie *et al.*, 1962, 1964) causes a great increase in the number of perinuclear granules in a variety of growing mammalian cells. These granules are stained in living cells by Janus green B and in fixed cells by lipid-soluble dyes (Mackenzie *et al.*, 1961). In the electron microscope they appear as dense bodies surrounded by a limiting membrane (Mackenzie *et al.*, 1962). In preliminary experiments with homogenates, the particles were found to possess a lower density than other cell organelles. On the basis of their microscopic and physical properties they were called lipid-rich particles. Similar particles were observed in parenchymal cells freshly isolated from rat and rabbit liver (Mackenzie *et al.*, 1962).

The present paper describes the isolation and chemical analysis of the lipid-rich particles and their contribution to the compartmentalization of lipid within the cell. Cultured cells were chosen for this purpose for several reasons. First, they provide homogeneous cell populations. Second, unlike tissue cells, they can be washed free of lipids present in the extracellular environment. Third, the disruption of particle-containing cells can be observed under the phase contrast microscope, thereby permitting the origin of the lipid-rich particles in the homogenate to be determined with certainty.

Materials and Methods

Cells. Stock cultures of the rabbit liver cell were grown on stock medium containing 20% rabbit serum and 5% human cord serum (Mackenzie *et al.*, 1962). Stock cultures of Earle's L cell (McQuilkin *et al.*, 1957) were grown on modified Eagle's medium (Mackenzie *et al.*, 1961) containing 10% horse serum. Experimental cultures were prepared by dispersing the cells with trypsin and plating in prescription bottles containing 40 ml of the respective stock medium. Sixteen hours was allowed for cell attachment and the resumption of growth. At the end of this time (time zero) both media were replaced with the modified Eagle's medium containing 20% rabbit serum. This medium maintains the high level of lipid-rich particles in the rabbit liver cell and produces numerous lipid-rich particles in the L cell within 24 hr (Mackenzie *et al.*, 1964). The cells were grown at 37.5° in an humidified atmosphere of 5% CO_2 in air. The medium was changed daily. When the cell protein per bottle had reached a level of 10–15 mg, the cells were used for the isolation of the lipid-rich particles. This was usually 2 days, occasionally 3 days, after time zero.

Chemical Analysis. Protein was measured by the method of Oyama and Eagle (1956). Lipid was determined gravimetrically after extraction from lyophilized cell fractions by the procedure described earlier for intact cells (Mackenzie *et al.*, 1962). The extracted lipid was fractionated on a column of silicic acid by the

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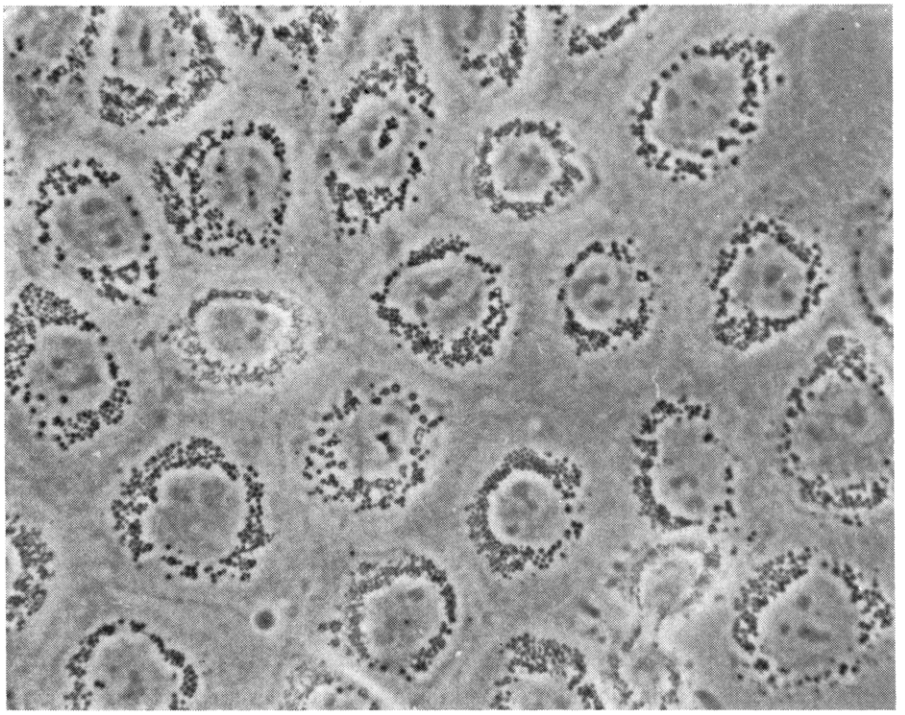

FIGURE 1: Rabbit liver cells growing in culture. Lipid-rich particles surround the nuclei of the polygonal cells; phase contrast; ×670.

procedure of Barron and Hanahan (1958) as modified and adapted to microquantities of material (Mackenzie *et al.*, 1964).

The lipid fractions obtained by column chromatography were chromatographed in the ascending direction on an upright layer of silica gel G (Merck and Co., Inc.) *ca.* 250-μ thickness. The solvent system consisted of hexane–ethyl ether–acetic acid in a ratio of 80:20:1. The solvent front was run to 10 cm and the chromatogram was developed by spraying with dichromate in 50% sulfuric acid and charring. The approximate R_F values were as follows: cholesterol esters 1.0, triglycerides 0.83, fatty acid 0.43, cholesterol 0.14, diglycerides 0.11, and monoglycerides 0. When the ratio of the solvents was changed to 50:50:1, monoglycerides migrated with an R_F value of *ca.* 0.18.

Cholesterol was determined by the method of Abell *et al.* (1952) with reduction of the reagent volume to 0.3 ml. Lipid phosphorus was determined by the method of King (1932).

Results

Isolation of Lipid-Rich Particles. Preliminary experiments showed that trypsinization of the cultures followed by homogenization in the Potter–Elvehjem apparatus damaged or destroyed many of the lipid-rich particles because of the grinding required to break the monodispersed cells. Furthermore, when the particles were concentrated by centrifugation, turbidity measurements suggested that distilled water was a better suspending medium than either 0.3 M sucrose or 0.15 M KCl. In view of these observations the following method was devised for the routine isolation of the lipid-rich particles.

Bottles containing the cells were cooled in the refrigerator at 3° for 10 min and then placed on a tray of cracked ice. The medium was removed by aspiration and the monolayer of rabbit liver cells was washed rapidly three times with 12-ml portions of ice-cold distilled water. Because of their greater fragility, the L cells were first washed twice with cold 0.3 osmolar saline and then only once with distilled water. As shown previously (Mackenzie *et al.*, 1964), these washing procedures free the cells completely of medium without a detectable loss of cell protein. Next, 2.5 ml of cold water was added and the surface was scraped with a wing-shaped rubber policeman. The shearing force together with the osmotic shock disrupts upward of 90% of the cells and the remainder are detached from the glass in a greatly swollen condition.

The homogenate was removed with a Pasteur pipet, 1.5 ml of water was added to the bottle, and the surface was again scraped with the rubber policeman. The combined homogenate and wash were drawn up in a 10-ml syringe and dispersed five times through a 25-gauge hypodermic needle. Sufficient pressure was applied to the plunger to maintain a steady stream from the orifice. Finally the homogenate was delivered into a 10 × 77 mm lusteroid centrifuge tube (Lourdes, 5 LT) and the volume was adjusted to 4.8 ml with cold water.

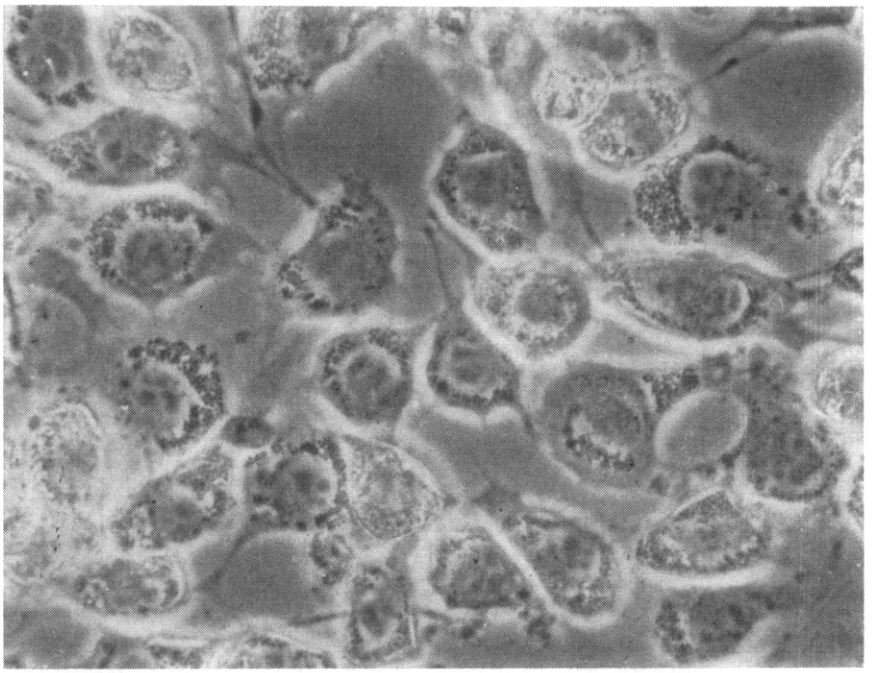

FIGURE 2: Mouse fibroblast cells growing in culture. Dark lipid-rich particles are in focus, others are outside of the plane of focus; phase contrast; ×670.

The tubes were stoppered with polyethylene stoppers and the homogenate was centrifuged in a Spinco No. 40 rotor, equipped with extruded polyethylene adapters, at 30,000 rpm for 20 min in the Spinco Model L ultracentrifuge. The centrifugation separated the homogenate into three distinct fractions: a sharp top band *ca.* 1 mm in depth which contained the lipid-rich particles, an intermediate water-clear layer which contained the soluble components of the cells, and a compact pellet which contained the nuclei, mitochondria, and other insoluble elements.

The lipid-rich particle layer was removed with a fine-tipped pipet, 0.5 mm in diameter, which was inserted into the particle layer and pressed gently against the side of the centrifuge tube. Although the volume of the undisturbed particle layer was only 0.07 ml, its rapid dispersion required the removal of a total volume of 0.6–1.0 ml to obtain the maximum yield. The resulting suspension of lipid-rich particles was washed by adjusting the volume to 4.8 ml with cold water and recentrifuging as described above. Again the particles formed a sharp band at the top of the centrifuge tube, and they were withdrawn in a volume of 0.6 ml for further washing or analysis.

By growing cells in petri dishes instead of bottles, it was possible to follow the isolation procedure under the phase contrast microscope starting with the living cells (Figures 1 and 2). When the cells were broken by scraping, the particles were seen to pass into the water without changes in their size or shape. Moreover, during the initial centrifugation and first wash the particles retained their spherical shape with diameters ranging from 0.5 to 1.0 μ. However, during the second, and particularly during the third wash, both smaller and larger spheres as well as angular shaped particles appeared in the preparation. These changes were due presumably to the fission, fusion, and fragmentation of the original particles. During the third wash, clumps comprising five to ten particles of various sizes and shapes were also formed.

The absence of contamination of the isolated lipid-rich particles with nuclei, mitochondria, and microsomes was verified in electron micrographs of the unwashed particle fraction at 14,000 and 40,000×.

Protein and Lipid Content of Lipid-Rich Particles. After each wash the protein was measured in an aliquot of the particle suspension and in an equal volume of the underlying wash water. The latter value was used to correct for dissolved protein present in the particle suspension. No correction was required for the space

TABLE 1: Protein and Lipid Content of Lipid-Rich Particles.[a]

Cell	Protein (%)	Lipid (%)
Rabbit liver	2.4 ± 0.1	91.2 ± 0.4
Mouse fibroblast (L)	3.7 ± 0.4	90.3 ± 1.9

[a] Isolated particles were washed twice, lyophilized, and dried to constant weight *in vacuo* at 60°. Mean values and standard errors are for 5–7 experiments each.

TABLE II: Fractionation of Particle Lipid by Silicic Acid Column Chromatography.[a]

Solvents	Distribution of Particle Lipid in Eluate Fractions: Liver Cell (%)	Fibroblast Cell (L) (%)	Compn of Fraction[b]
Hexane–benzene, 85:15	3.2	3.4	Cholesterol esters, *ca.* 50%;[c] hydrocarbons, *ca.* 50%
Hexane–ether, 95:5	90.0	92.0	Triglycerides
Hexane–ether, 80:20	2.6	3.4	Diglycerides, >95% Cholesterol, <5%
Hexane–ether, 15:85	0.9	0.6	Monoglycerides
Methanol, 100	2.0	1.1	Polar lipids

[a] Particle lipid (1–2 mg) was chromatographed on a silicic acid column using the indicated solvents, mixed on a volume basis. Average recovery of applied lipid was 98%. Each value is the average of 4–5 measurements made in different particle isolation experiments. [b] Composition of fractions was determined by comparison with the distribution of test compounds on the column plus subsequent thin layer chromatography and cholesterol analysis. [c] Calculated as cholesterol oleate. The remainder of this fraction is designated as hydrocarbons, after Barron and Hanahan (1958).

occupied by the particles themselves, since they made up <1% of the total volume.

A sharp decrease in the protein content of the particles occurred during the third wash. There was no comparable decline in the lipid content. This selective loss of protein was accompanied by changes in particle size and shape as described in the preceding section. Consequently, unless otherwise noted, all chemical analyses were made on twice-washed particles. To avoid turbidity, protein was usually determined after the removal of petroleum ether soluble material from the lyophilized particle fraction.

The results of the protein determination are shown in Table I. The difference in the mean protein content of the particles isolated from rabbit liver cells and L cells was significant at the 0.05 level, according to Fisher's (1932) t test. In both cells the lipid content of the particles was *ca.* 90% (Table I).

Composition of Particle Lipid. The particle lipid was subjected to silicic acid column chromatography followed by thin layer chromatography on silica gel. As shown in Table II, 90% of the particle lipid in both cells was identified as triglycerides. The lipid also contained small amounts of diglycerides and monoglycerides. Free fatty acids were not detected. In addition to glycerides, the particles contained small quantities of esterified cholesterol, traces of free cholesterol, and small amounts of polar lipids. In two determinations the latter fraction prepared from rabbit liver cell particles contained *ca.* 1% phosphorus.

In several experiments, lipid was also extracted from the unwashed particle fraction and chromatographed on the silicic acid column. In both cell lines the lipid composition of unwashed particles was found to be the same as that shown in Table II for twice-washed particles.

Distribution of Lipid in Cell Fractions. The lipid-rich particles and the soluble and pellet fractions prepared from rabbit liver, rat liver, and L cells were lyophilized and the lipid was extracted and weighed as described for intact cells (Mackenzie *et al.*, 1962). As shown in Table III, the lipid content of the particle fraction was in good agreement with the number of lipid-rich particles present in the living cells; *e.g.*, when grown on the horse serum medium, only an occasional rat liver cell contained particles, and analysis showed that the ratio of particle lipid–total cell protein (9 mg) was <0.001. However, when the rat liver cells were grown on the rabbit serum medium, most of the cells contained 10–15 particles and the ratio of particle lipid–cell protein (12 mg) was 0.009. Also the rabbit liver cell, which always contains more lipid-rich particles than the L cell, contained twice as much lipid in its particle fraction relative to total cell protein (Table III).

The soluble fraction of rat liver cells grown on horse serum contained a negligible amount of lipid. When either the rat liver cell or the other cell lines were grown on rabbit serum, the ratio of lipid in the soluble fraction–cell protein was 0.006. Even this low level of lipid was due in part to the visible contamination of the soluble fraction with some lipid-rich particles during their removal from the homogenate.

The pellet fraction from all cells contained a substantial amount of lipid. Moreover, the ratio of pellet lipid–cell protein was approximately the same in each of the cell lines studied. Furthermore, the pellet contained essentially all of the cell's free cholesterol and polar lipids. The phosphorus content of the latter fraction was 3.5%. In addition to the foregoing constituents, pellets prepared from both rabbit liver and L cells contained variable amounts of neutral glycerides. In different experiments, mono- and diglycerides com-

TABLE III: Distribution of Lipid in Cell Fractions.[a]

Cell	Serum	Lipid-Rich Particles/Cell[b]	Ratio of Lipid in Each Fraction to Total Cell Protein		
			Lipid Particle Fraction	Soluble Fraction	Pellet Fraction
Rat liver	Horse	Occasional	<0.001	0.001	0.144
Rat liver	Rabbit	10–15	0.009	0.007	
Mouse fibroblast	Rabbit	70–100	0.108	0.006	0.148
Rabbit liver	Rabbit	>150	0.226	0.006	0.160

[a] Cells were disrupted in water and centrifuged at 30,000 rpm for 20 min to obtain a top lipid-rich particle fraction, an intermediate soluble fraction, and a bottom pellet fraction. [b] Estimates based on the examination of cultures under the phase contrast microscope.

prised from 2 to 5% of the pellet lipid and triglycerides from 10 to 25%. It is possible that some of the triglyceride originated from lipid-rich particles and unbroken cells trapped in the pellet during centrifugation.

The unwashed pellets contained from 40 to 50% of the cell protein. As can be calculated from the data in Tables I and III, lipid-rich particles accounted for <1% of the cell protein. Analysis showed that the remainder of the protein, 50–60%, was in the soluble fraction of the cells. In these experiments the mean protein content of the L cell was 3.3×10^{-4} μg and that of the rabbit liver cell was 4.5×10^{-4} μg.

Electron Microscopy. Cells were fixed for 15 min with a cold 2.5% solution of glutaraldehyde in acetate–Veronal buffer, pH 7.2, adjusted to an osmolarity of 0.3 with sucrose. The glutaraldehyde was removed by washing repeatedly with an 0.3 osmolar salt solution whose composition approximated that of the extracellular fluid (Mackenzie *et al.*, 1964). Next, the cells were post-fixed for 1.5 hr with a 2% solution of OsO_4 dissolved in the acetate–Veronal–sucrose mixture. After removal of the osmium, the cells were gradually dehydrated by immersion for periods of 10 min each in 25, 50, 75, and 95% acetone, followed by two 30-min periods in anhydrous acetone. The acetone was replaced with 1,2-propylene oxide and the cells were embedded in Araldite 502 (Ciba Co., Inc.) according to the procedure of Luft (1961). The sections were stained with uranyl acetate and lead citrate (Reynolds, 1963).

To obtain the maximum display of lipid-rich particles, glass-attached cells were converted to a suspension of spherical cells by incubation in a 0.1% solution of trypsin (Mackenzie *et al.*, 1961). The action of the trypsin was arrested by the addition of serum and the cells were centrifuged at low speed in the cold. The resulting pellet was fixed as outlined above.

Electron micrographs at 11,500× (Figure 3) showed the lipid-rich particles as electron-dense bodies surrounded by a membrane. Except for streaking caused by chatter of the glass knife, the interior of the particles was of uniform electron density. The limiting membrane, on the other hand, exhibited a higher electron density. This was the case for both free particles and particles which abutted against each other. Of particular interest was the close juxtaposition of many lipid-rich particles and mitochondria (Figure 3).

For examination of the fine structure of the particle membrane, glutaraldehyde solution at 0° was added directly to the medium of cell monolayers cooled to 5° (Figures 1 and 2). Since glass-attached cells are only several microns thick, fixation was instantaneous, and the living and fixed cells were indistinguishable under the phase contrast microscope. The glutaraldehyde was washed out and the cells were detached from the glass in monolayer sheets by gentle scraping with a rubber policeman. They were post-fixed with OsO_4, and after embedding, cut with a diamond knife.

In electron micrographs at 250,000×, the membrane of most of the lipid-rich particles was composed of two highly electron-dense lines separated by a less electron-dense space (Figure 4A). In both cells, the mean over-all width of the particle membrane was *ca.* 80 A. The dimensions of the constituent parts were 20–25 A for each of the dark lines and 30–40 A for the intervening space. As shown in Figure 4A the electron density of this space resembled that of the lipid matrix. Some of the particles lacked this unit membrane structure (Robertson, 1959) and were instead surrounded by a single 25 A line of high electron density. In appearance, the line was indistinguishable from the dark lines of the unit membrane structure.

Cells were also fixed in a 1% solution of $KMnO_4$ following Luft (1956). Exposure to $KMnO_4$ was limited to 1 min after examination under the phase contrast microscope showed that fixation was achieved with a minimum of distortion in this time. The $KMnO_4$ was washed out and the cells were detached in sheets into the isotonic saline as described above. Post-fixing with OsO_4 was omitted and the particle lipid was consequently extracted during the dehydration with acetone. In most of the resulting particle ghosts, the membrane appeared as a single 25 A electron-dense line. However, some of the ghosts were surrounded by a unit membrane which resembled in appearance and dimensions

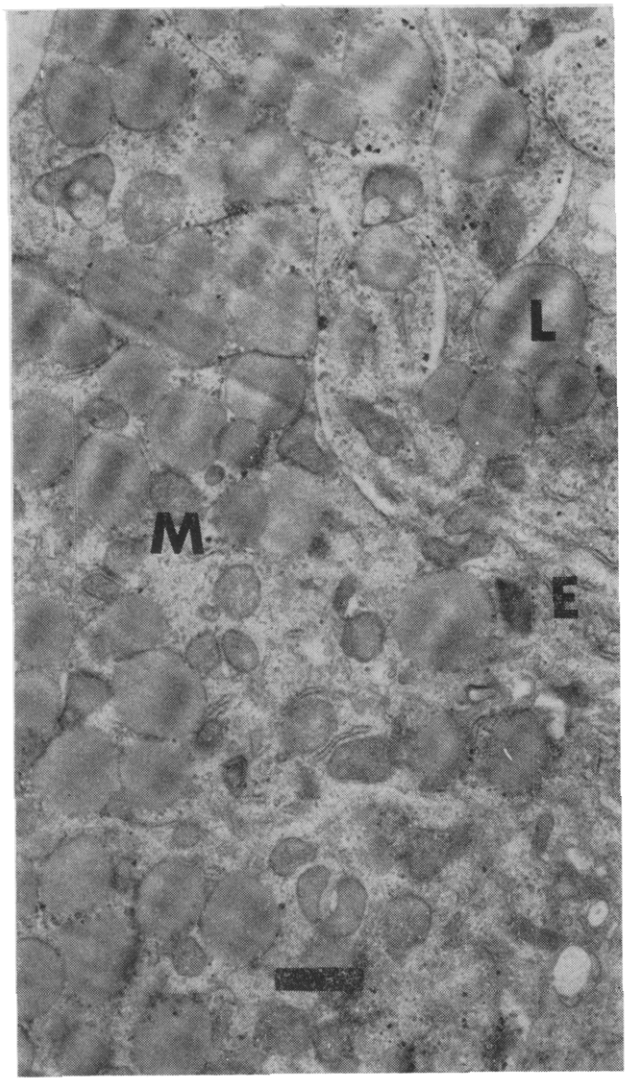

FIGURE 3: Portion of a rabbit liver cell fixed with glutaraldehyde and post-fixed with OsO_4. The numerous lipid-rich particles (L) are surrounded by a distinct membrane. Mitochondria (M) and endoplasmic reticulum (E) with attached ribosomes are also present. A number of the mitochondria abut on lipid-rich particles. The marker represents 1.0 μ; × 11,500.

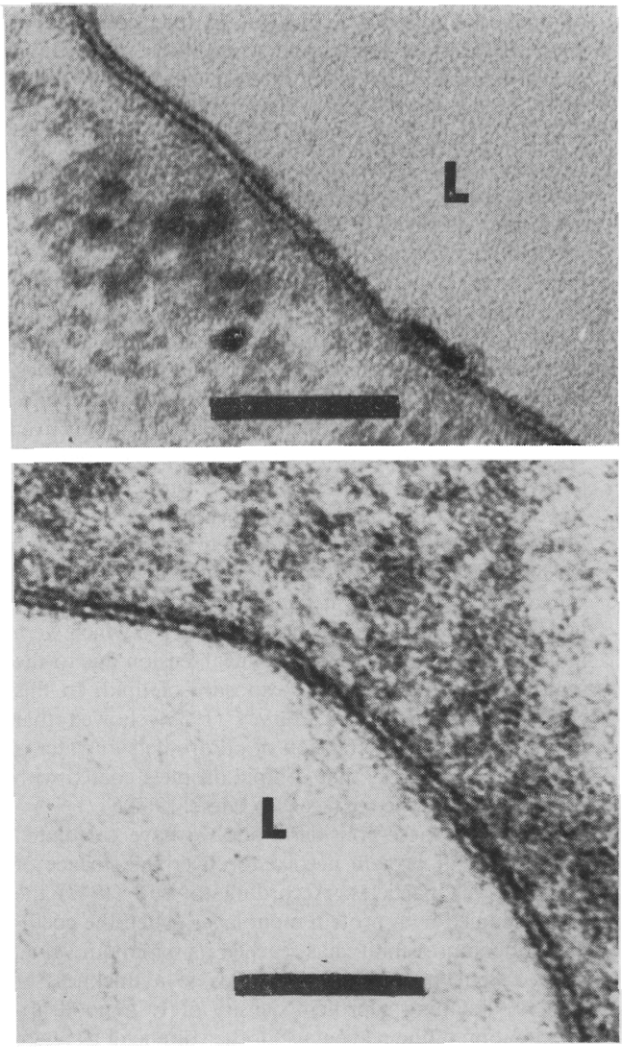

FIGURE 4: (A) The upper electron micrograph shows the membrane structure of a lipid-rich particle (L) in a cell fixed with glutaraldehyde and post-fixed with OsO_4. (B) The lower electron micrograph shows the membrane structure of a lipid-rich particle (L) in a cell fixed for 1 min with 1% $KMnO_4$. In this case the lipid in the particle matrix has been extracted with acetone. Markers represent 0.1 μ; ×250,000.

the unit membranes seen in the glutaraldehyde, osmium-fixed cells. An example of a $KMnO_4$-fixed unit membrane is shown in Figure 4B. It is noteworthy that both the membrane space and the interior of the extracted particle exhibited electron densities comparable to that of the embedding plastic.

Discussion

The success of the procedure for the isolation of lipid-rich particles was based on the observation that glass-attached cells can be disrupted by scraping them in water. In our experience the use of a Potter–Elvejhem apparatus with a tight enough fit to break cells suspended in sucrose or saline also destroyed most of the particles. Disruption by scraping, on the other hand, as shown by following the procedure under the phase contrast microscope, allowed the particles to pass into the water without changing their size or shape. Furthermore, the microscopic appearance of the particles was unaltered when they were concentrated at the top of the homogenate by ultracentrifugation. It was clear, therefore, that the particles isolated for study were the cytoplasmic particles seen in the living cells (Figures 1 and 2); they were not artifacts produced by cell destruction or by centrifuging the homogenate.

With respect to their chemical composition, the twice-washed particles contained *ca.* 90% lipid on the basis

of their lyophilized weight. This in turn consisted of 90% triglycerides plus small amounts of other lipid components. Of particular interest was the low level of free cholesterol. The similar lipid distribution observed in unwashed particles is strong evidence that this was also the lipid composition of the particles in the living cells. Such a conclusion finds further support in the earlier observation that cells exposed to a particle-producing environment exhibit a great increase in their triglyceride content without major changes in their other lipid constituents (Mackenzie *et al.*, 1964).

The high lipid and low protein content of the particles suggests that they consist of a lipid matrix surrounded by a protein-containing membrane. Two additional lines of evidence favor such a structure. First, in rabbit liver cells viewed in the electron microscope the lipid-rich particles appear as uniform electron-dense bodies surrounded by a distinct limiting membrane (Mackenzie *et al.*, 1962). Second, as reported in this paper, the third washing of the isolated particles with cold water resulted in a sharp drop in their protein content. Simultaneously, changes in morphology occurred which were indicative of an increase in interfacial tension due to the removal of protein and the exposure of lipid. In this connection Danielli and Harvey (1935) showed that the interfacial surface tension of oil droplets in water is 15 dynes/cm whereas that of lipid droplets coated with protein is *ca.* 1 dyne/cm (see also Giese, 1962).

In view of these considerations we have calculated the amount of protein needed to cover the surface of the lipid-rich particles. According to Bull (1947) the area occupied by a protein monolayer, when the coefficient of compressibility has reached its minimum value, is *ca.* 0.8 m^2/mg. This corresponds to a thickness of 12.5 A, assuming a protein density of 1. A good approximation of the percentage of the volume of a sphere occupied by a surface layer of protein 1.25×10^{-3} μ thick is given by the following formula, 7.5×10^{-3} μ/(diameter of sphere in microns) $\times$ 100. Therefore, for lipid-rich particles with diameters of 0.5, 1.0, and 1.5 μ, a surface monolayer of protein would represent respectively 1.5, 0.75, and 0.5% of the total volume. As shown in Table I, the twice-washed particles from both cells contained more than enough protein to form such a surface layer. Indeed, it was sufficient to cover a particle population of 1-μ mean diameter with two layers of protein.

We have also estimated the amount of polar lipid required to cover the surface of the particles. According to van Deenen *et al.* (1962), a closely packed monolayer of lecithin at an air–water interface occupies about 60 A^2/molecule. For lecithin molecules with an average molecular weight of 760, the thickness of such a film is 20.8 A. Using this value in the derivation of the above formula, the numerator becomes 12.5×10^{-3} μ. Thus for lipid-rich particles 1 μ in diameter, a surface monolayer of lecithin would represent 1.25% of the total volume, and a bimolecular leaflet would presumably represent 2.5% of the volume. It can be calculated from Tables I and II that the rabbit liver cell particles contained 1.8% polar lipid, an amount sufficient to provide three-quarters of them with a bimolecular lipid leaflet. The L cell particles contained sufficient polar lipid to provide two-fifths of them with a similar structure. These estimates are probably minimal since they are based on the isolation of only 10–30 μg of polar lipid, and in addition, ignore cholesterol and triglycerides as membrane constituents.

Electron micrographs show that the lipid-rich particles are surrounded by a distinct limiting membrane (Figure 3). In most of the particles the membrane is 80 A thick and consists of two electron-dense lines separated by a less electron-dense space (Figure 4). It resembles, therefore, the structure that has been described for all other membranous elements of the cell (Robertson, 1959; Sjöstrand, 1963) which Robertson (1959) has designated the unit membrane. The chemical composition of the lipid-rich particles (Table I) indicates that the electron-dense lines of the unit membrane reflect the presence of protein. The persistence of these lines in acetone-extracted preparations supports this conclusion (Figure 4B). The less dense zone of the membrane, on the other hand, appears to reflect a localization of lipid since it exhibits the same electron density as the interior of the particle in both extracted and nonextracted preparations.

In the case of the lipid-rich particles which lack the unit membrane structure, the lipid matrix is surrounded by a single electron-dense line *ca.* 25 A wide. The considerations enumerated above indicate that this simple membrane is composed of protein.

In cells devoid of lipid-rich particles, triglycerides make up only a small part of the total lipid, *ca.* 5% (Mackenzie *et al.*, 1964). Moreover, this triglyceride is found in the pellet fraction together with almost all of the cell's cholesterol and polar lipids (Table III). In cells containing particles, however, triglycerides make up a substantial part of the total lipid, and this additional triglyceride is directly related to the number of particles. Furthermore, the bulk of the new triglyceride, 75–90%, can be recovered from the lipid-rich particle fraction. The remainder of the new triglyceride may also be present as particles, either free or in unbroken cells, which have been trapped in the pellet. We conclude, therefore, that the lipid-rich particle is the structural unit for the accumulation and storage of triglycerides in healthy, growing cells. As such, it represents the cell's most concentrated form of chemical energy. Accordingly, the molecular events responsible for particle formation and disappearance are of interest.

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Lipid Alterations after Cell Wall Inhibition. Fatty Acid Content of *Streptococcus pyogenes* and Derived L-Form*

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ABSTRACT: The fatty acid content of whole streptococcal and derived L-form (*i.e.*, cells without rigid cell walls) cells in the mid-logarithmic phase of growth, and their respective isolated membranes, was compared by capillary column gas chromatography. Oleic acid predominated in L-form whole cells and membranes, whereas *cis*-vaccenic acid was found to predominate within the cells and membranes of the parent *Streptococcus pyogenes*. This reversal of positional isomers was not noted in the hexadecenoic acid fractions. In addition, the per cent of total C_{18} acids was greater than the total C_{16} acids in the L-form, whereas the reverse was found in the coccus. Control studies with the streptococcus grown in L-form medium could not account for these changes in the L-form as due solely to an osmotic effect. It was observed that permanent loss of cell wall biosynthesis (*i.e.*, L-form) could be associated with fatty acid alterations in the relative proportions of the C_{18} positional isomers. The presence of minute amounts of: (a) straight chain fatty acids containing an odd number of carbon atoms; (b) monoenoic fatty acids containing odd and even numbers of carbon atoms; and (c) a series of branched methyl fatty acids from cells and membranes of both organisms was demonstrated. The presence of various positional isomers within the tetra-, hexa-, and octadecenoic acid fractions from the coccus, its derived L-form, and their respective membranes supports the validity of earlier concepts of the mode of lengthening of monounsaturated fatty acids in bacteria. These results on the content of long chain monoenoic acids, together with the observations of others on the content of cyclopropane ring containing fatty acids in L-forms, indicate that a plausible function for bacterial ring containing fatty acids may be as cell wall structural units. The high resolving capabilities of capillary column gas chromatography and its application to bacterial fatty investigations is demonstrated.

Some information has appeared concerning the lipid content of microbial forms lacking a rigid bacterial cell wall. More recently, the total lipid and nonsaponifiable lipid content of pleuropneumoniaelike (PPLO) and L-type organisms was compared (Smith and Rothblat, 1962). Of the various cell wall-less organisms examined (PPLO, salt requiring and nonrequiring L-forms), the salt-requiring L-forms contained the least amount of these two lipid classes. O'Leary (1962a) has determined the fatty acid content of a PPLO and noted certain over-all differences and similarities between it and those of many bacterial species. These included the presence of bacterial cyclopropane ring containing lipids but a lower proportion of unsaturated to saturated acids than found in bacteria. A comparison of the lipids and lipopolysaccharide from the bacillary and L-forms of a Proteus has also been documented (Nesbitt and Lennarz, 1965). The L-form was found to contain 1.5 times as much extractable lipid and considerably less

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