Lipid Accumulation in Hypoxic Tissue Culture Cells

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Lipid droplets have long been recognized by light microscopy to accumulate in hypoxic cells both *in vivo* and *in vitro*. In the present tissue culture experiments, correlative electron microscopic observations and lipid analyses were performed to determine the nature and significance of lipid accumulation in hypoxia. Strain L mouse fibroblasts were grown in suspension culture, both aerobically and under severe oxygen restriction obtained by gassing cultures daily with an 8% CO_2–92% nitrogen mixture. After 48 hours, hypoxic cells showed an increase in total lipid/protein ratio of 42% over control cells. Most of this increase was accounted for by an elevation in the level of cellular triglyceride from 12.3 ± 0.9 μg/mg cell protein in aerobic cultures to 41.9 ± 0.7 in the hypoxic cultures, an increase of 240%. Levels of cellular free fatty acids (FFA) were 96% higher in the hypoxic cultures. No significant changes in the levels of cellular phospholipid or cholesterol were noted. Electron microscopic examination revealed the accumulation of homogeneous cytoplasmic droplets. The hypoxic changes were reversible upon transferring the cultures to aerobic atmospheres with disappearance of the lipid. These experiments indicate that hypoxic injury initially results in triglyceride and FFA accumulation from an inability to oxidize fatty acids taken up from the media and not from autophagic processes, as described in other types of cell injury associated with the sequestration of membranous residues and intracellular cholesterol and phospholipid accumulation. (Am J Pathol 88:663–678, 1977)

THE APPEARANCE OF INTRACELLULAR LIPID is one of the classic responses in cellular pathobiology, commonly noted in the parenchymal cells of many organs following sublethal injury. Ischemia (or hypoxic injury) has long been known to result in lipid accumulation in the injured tissues.[1,2]

The accumulation of lipid droplets in tissue culture cells was generally accepted as a sign of injury, usually associated with crowding, aging, or degeneration of the cultures.[3] In recent years, however, several investigators have shown that uncrowded cultures of a variety of mammalian cells growing exponentially can accumulate lipid by various alterations of the culture media, including lowering the pH,[4] using "lipogenic" serum,[5] and adding small amounts of glycerol [6] or free fatty acids.[7-10] This lipid has been identified as primarily triglyceride in nature and accumulates in the cells in the form of non-membrane-bounded cytoplasmic droplets having the typical ultrastructural appearance of neutral fat.[9,10]

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Supported in part by Grant HL-13713 from the National Heart and Lung Institute, US Public Health Service.

Accepted for publication May 3, 1977.

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Removal of the cells from the altered media results in the utilization and eventual disappearance of the triglyceride droplets.[8,9] These findings have led Mackenzie *et al.*[6] to conclude that triglyceride droplets produced by these means do not represent cellular degeneration but are the structural unit for the accumulate and storage of lipid in healthy, growing tissue culture cells.

In contrast, other experiments [11,12] have shown that tissue culture cells from old, or stationary, phase suspension culture accumulate lipid particles that arise from autophagy and result in markedly increased cellular levels of phospholipid and cholesterol. Similar findings were also observed in tissue culture cells injured by agents which interfered with cell growth and protein synthesis, such as *p*-fluorophenylalanine [11] and chloroquine.[13]

Hypoxic injury has been shown to inhibit cell growth and protein synthesis in tissue culture cells.[14,15] However, previous light microscopic studies [16,17] have demonstrated the early accumulation of lipid droplets that have the staining and ultrastructural characteristics of neutral fat, or triglyceride, rather than the sequestered lipid deposition resulting from increased autophagy.[12] In the present study, the lipid accumulation produced by hypoxic injury in strain L cells grown in suspension culture was investigated by correlation of cell growth, protein and lipid content, and fine structural alterations occurring in these cells in order to determine the nature, source, and significance of the increased lipid occurring in hypoxia.

Materials and Methods

Tissue Culture

Strain L (Earle) mouse fibroblasts were grown in suspension tissue culture in 250-ml Erlenmeyer flasks agitated on a rotary shaker at 37 C as described elsewhere.[12,18] Each flask contained 80 ml of culture media composed of Eagle's minimum essential media supplemented with 10% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml). An appropriate number of cells from stock cultures in logarithmic growth were used to obtain an initial inoculum of approximately 250,000 cells/ml.

In the first set of experiments, the effect of hypoxia on cell growth was investigated over a 72-hour period. Replicate cultures were established and growth curves determined by daily cell counts, performed using a standard hemocytometer as described previously.[11] Half of the replicate cultures were control cultures that were gassed daily for 10 minutes with an 8% CO_2–92% air mixture at a regulated flow rate of 4 standard cubic feet per minute (SCFM). The experimental cultures were each gassed upon establishment and daily thereafter for 10 minutes with an 8% CO_2–92% nitrogen gas mixture at the same regulated flow rate of 4 SCFM. Small aliquots for determination of medium pO_2, pCO_2, and pH were taken 1 hour after the daily gassing to allow for equilibration of the medium with the flask atmosphere. Determinations were made using a standard IL model 113 pH/gas analyzer. Aliquots for the daily cell counts and cell viability determinations were taken prior to each daily gassing. Cell viability was estimated by the trypan blue dye exclusion test.[19]

In another group of experiments, correlative studies of cellular lipid content and ultrastructural morphology were performed. Control aerobic cultures and experimental hypoxic cultures were allowed to incubate for 48 hours, at which time the growth inhibitory effect of the hypoxia was marked, and then harvested for cell protein and lipid analyses and electron microscopy. Aliquots of 5 ml for protein determinations and 50 ml for lipid studies were taken from each culture. The remainder of the culture was prepared for electron microscopy.

The reversibility of the hypoxic effect was studied in other experiments by first exposing a group of cultures to the anaerobic atmosphere for 48 hours. The cultures were then centrifuged, combined, and resuspended in fresh media. Half of these resuspended cultures were reexposed to the 8% CO_2–92% nitrogen atmosphere, while the others were gassed with the normal 8% CO_2–92% air mixture. After an additional 24 hours' incubation, both sets of cultures were harvested for lipid studies and electron microscopy as described above. All experiments were done in triplicate and repeated twice so that the reported data on cell growth, lipid analysis, and ultrastructure represents the findings of six separate cultures. Statistical analyses for significance were performed using the Student *t* test.

Cell Protein and Lipid Studies

The aliquots for cell protein and lipid studies were immediately centrifuged at 400*g* for 3 minutes. The cells were rapidly resuspended in ice cold buffered Earle's balanced salt solution and recentrifuged. This was repeated for a total of three washes. Proteins were determined by the modified Lowry method of Oyama and Eagle.[20] Lipids were extracted by a modification of the method of Folch *et al.*[21] The cells were disrupted by sonication in cold chloroform/methanol (2:1). After centrifugation, the supernatant was collected and an additional overnight chloroform/methanol extraction of the residue was performed. The extracts were then combined and washed in separatory funnels with 0.05% $CaCl_2$. The washed extracts were filtered through fat-free filter paper and collected in weighing bottles. The solvent was removed by evaporation under a stream of nitrogen. The lipid was dried to constant weight and weighed on a semi-micro analytical balance to ± 10 μg to give a gravimetric determination of the total cellular lipid. The lipid residue was then redissolved in diethyl ether/hexane (1:1). Portions of this solution were used for determinations of triglycerides by the method of Fletcher,[22] free fatty acids (FFA) by the method of Novak,[23] phospholipids by the method of Bartlett,[24] and total cholesterol by the method of Chiamori and Henry.[25]

In addition, the lipid content of the culture media was determined in samples of the media taken prior to initiating the experiment and from the supernatant media of hypoxic and control cultures after 48 hours' incubation. Total lipids were estimated by the colorimetric method of Frings and Dunn.[26] Medium triglyceride [22] and FFA [23] levels were also analyzed.

Electron Microscopy

Suspension fixation of the L cells was performed as described previously.[27,28] Cold 2% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.3, was added immediately in equal amounts to the aliquots of the cultures taken for electron microscopy. After 10 minutes at 5 C, the cells were gently centrifuged and the supernatant discarded. Additional glutaraldehyde fixative was then layered over the cell buttons, which were allowed to fix overnight in the cold. The cell buttons were then washed in 0.1 M cacodylate buffer containing 0.2 M sucrose, sliced into 1 cu mm blocks, and postfixed in veronal-buffered 1% osmium tetroxide.[29] Dehydration through alcohol and propylene oxide was followed by embedding in Araldite.[30] Sections were cut with an LKB ultramicrotome and stained with uranyl acetate [31] and lead citrate [32] prior to examination in a Philips EM 300 electron microscope.

Results

Effect of Hypoxia on Culture Growth and Viability

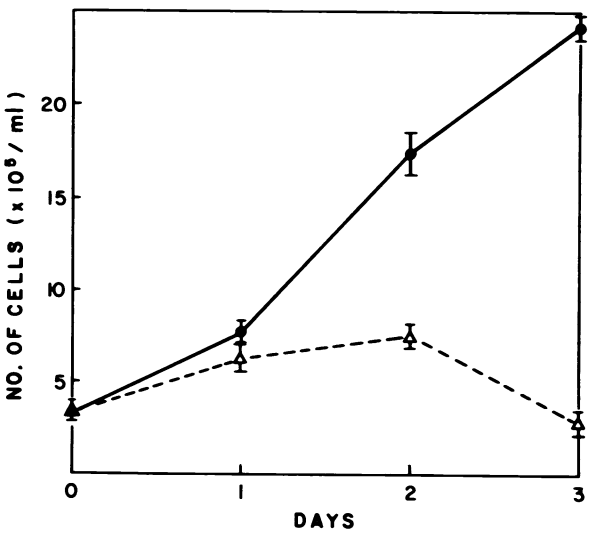
Daily gassing with a mixture of 8% CO_2–92% nitrogen resulted in a marked hypoxia of the strain L cell cultures as shown in Table 1. Measured pO_2 values of approximately 20 mm Hg were maintained in the experimental hypoxia cultures over the course of the experiment. This is well below the levels of 40 to 100 mm reported by Kilburn and Webb [33] as necessary for optimal growth of L-cell suspension cultures. The control values remained in the normal range of approximately 80 to 90 mm Hg (Table 1). The pCO_2 remained fairly constant throughout the 48 hours of culture in both experimental and control cultures (Table 1). A slight acidosis occurred in the nitrogen cultures after 48 hours of culture, probably as a result of increased lactic acid production due to increased glycolysis.

Daily gassing of the control cultures with 8% CO_2–92% air produced growth curves that are similar to those reported previously for L cell suspension cultures.[11,12] However, hypoxia induced by daily gassing with 8% CO_2–92% nitrogen resulted in a marked inhibition of cell growth (Text-figure 1). Inhibition of cell growth was slight during the first 24 hours, during which the normal lag phase was seen in the control cultures. By 48 hours (Day 2), when the control cultures were in logarithmic growth, the experimental hypoxic cultures exhibited marked growth inhibition, with cell counts on the average of 43% of control cultures. At this time, examination of the cultures, both by light and electron microscopy, showed virtually 100% intact cells with little cellular debris. Viability

Table 1—Effect of Daily Gassing With 8% CO_2–92% Air or 8% CO_2–91% N_2 on the pO_2, pCO_2, and pH of L Cell Cultures

Gas mixture	pO_2 (mm Hg)	pCO_2 (mm Hg)	pH
Day 0			
8% CO_2–92% air	79.1	55.7	7.23
8% CO_2–92% N_2	18.6	55.1	7.24
Day 1			
8% CO_2–92% air	92.6	59.5	7.20
8% CO_2–92% N_2	23.5	59.6	7.16
Day 2			
8% CO_2–92% air	81.5	65.0	7.12
8% CO_2–92% N_2	19.0	58.2	6.90

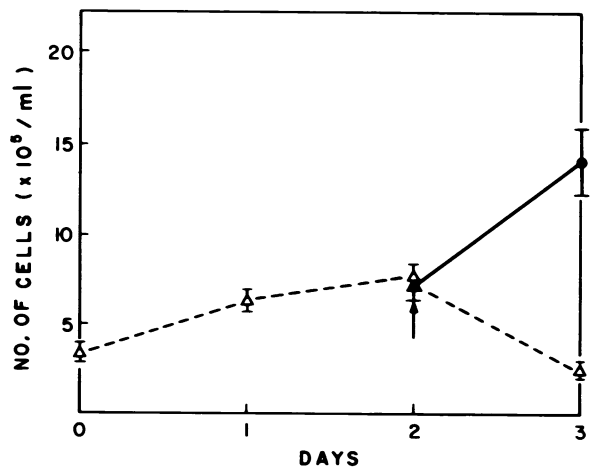
Cultures were gassed daily for 10 minutes at a flow rate of 4 SCFM with appropriate gas mixture. Each value represents average of six cultures.



TEXT-FIGURE 1—The effect of hypoxia on cell growth of strain L cells in suspension tissue culture. Hypoxic cultures (*dotted line*) were achieved by daily gassing with 8% CO_2–92% N_2. Controls (*solid line*) were gassed with 8% CO_2–92% air. Each symbol represents the mean ± SE of six replicate cultures.

counts using trypan blue demonstrated less than 3% cells dead in all cultures, with no significant difference between the experimental or control cultures. However, by 72 hours of culture (Day 3), cell death increased rapidly in the nitrogen-treated cultures, with a marked decline in the number of cells present in the cultures; 75% of these cells exhibited nonvital staining with trypan blue.

The inhibition of cell growth caused by incubation in a hypoxic atmosphere is reversed when sublethally injured cells are reexposed to normal aerobic conditions. As shown in Text-figure 2, cultures exposed to the



TEXT-FIGURE 2—Reversibility of hypoxic effect on growth of L cells. Cultures were initially made hypoxic by daily gassing with 8% CO_2–92% N_2; on Day 2 (*arrow*), they were transferred to fresh media, and half of the cultures were placed in an aerobic atmosphere by gassing with 8% CO_2–92% air (*solid line*). The remaining cultures were continued in the nitrogen atmosphere (*dotted line*).

CO_2–nitrogen atmosphere for 48 hours displayed the expected inhibition of growth. Incubation of the cells at this time in fresh medium gassed with the normal CO_2–air mixture resulted in the cells entering the logarithmic growth phase over the next 24 hours. However, cells transferred to fresh medium but continued under hypoxic conditions showed the expected decline in cell numbers and increase in nonvital staining. As might be expected, transferring cells to an aerobic atmosphere after 72 hours (Day 3) of severe hypoxia did not result in a noticeable reversibility of the growth inhibition, since over 75% of these cells exhibited nonvital staining prior to reaeration.

Cell Protein and Lipid Studies

Analysis of the total cellular lipid in the control and experimental hypoxic cultures after 48 hours' incubation demonstrated a marked increase of 54% in the cell lipid/cell protein ratio in the hypoxic cells. The control cells contained 166.2 ± 12.9 μg lipid/mg cell protein, while the experimental cultures contained 235.8 ± 11.4 μg lipid/mg, an increase of 42% in the hypoxic cells. If the lipid content is expressed as micrograms lipid per 10^6 cells, a similar increase of 42.5 ± 1.7 μg to 60.4 ± 8.4 μg was noted. Analyses of the individual lipid fractions (Table 2) showed that the bulk of the increase in cellular lipid was in the triglyceride fraction. The amount of cellular triglyceride increased from 12.3 μg/mg of cell protein in the control cultures to 41.9 μg/mg in the hypoxic cultures (Table 2), an increase of over 240%.

A smaller but significant increase in cellular FFA content was also found from 9.6 μg/mg cell protein to 18.6 μg/mg cell protein (Table 2). There was no significant change in cellular phospholipids or cholesterol content (Table 2). Similar increases in triglyceride (234%) and FFA (95%)

Table 2—Cellular Lipid Content in L Cells After 48 Hours' Growth in Media Gassed With 8% CO_2–92% Air or 8% CO_2–92% N_2

Lipid fraction	Lipid content (μg/mg cell protein)	
	8% CO_2–92% air	8% CO_2–92% N_2
Phospholipid	115.4 ± 2.2	114.7 ± 5.0
Cholesterol	15.0 ± 0.8	16.4 ± 0.9
Triglyceride	12.3 ± 0.9	41.9 ± 0.7*
FFA	9.6 ± 0.9	18.6 ± 0.7*

Each value represents the mean of six replicate cultures ± SE.
* $P < 0.001$ (paired Student t test).

levels were found when the values were expressed on a cellular basis as micrograms per 10^6 cells.

Samples of the media analyzed for total lipid content exhibited a decrease of total medium lipid of approximately 50% after 48 hours incubation occurring in both the nitrogen-treated experimental cultures and the control cultures, indicating similar lipid uptake by both control and hypoxic cells. Medium triglycerides and FFA also decreased from Day 0 to Day 2 with no noticeable difference between hypoxic and control cultures.

Total cellular lipid determination on cells that had been reexposed to an aerobic atmosphere after experimental hypoxic incubation for 48 hours showed reversal of the lipid accumulation, with a decline of 24% in the total cellular lipid at the end of a 24-hour period of reaeration. Cellular FFA levels returned to normal, and only a slight increase in cellular triglyceride content remained.

Electron Microscopy

In these experiments, strain L fibroblasts from control cultures grown in an aerobic atmosphere exhibited a normal electron microscopic appearance as previously described.[12,27] They usually appeared as oval, or nearly spherical, mononuclear cells with a diameter of 20 to 30 μ (Figure 1). A prominent Golgi zone largely filled the nuclear indentation, and mitochondria, present throughout the cytoplasm, tended to accumulate near the Golgi zone (Figures 1 and 4). They averaged 300 to 500 nm in average width, as estimated by measurements of 100 consecutive mitochondria on micrographs of randomly selected cells. The mitochondria presented the typical configuration of the cristae as described previously.[15,34] Ribosomes were present as clusters in the cytoplasm and also in association with scattered elements of the rough endoplasmic reticulum (ER). A few small cytoplasmic membrane-bounded dense bodies and small vacuoles were also present.

The marked increase in cellular triglyceride in the hypoxic cells is reflected in the differences in the ultrastructural appearance of the cells as compared to the control cells. Cells from the 48-hour hypoxic cultures contained numerous cytoplasmic droplets (Figures 2 and 3) with the typical electron microscopic appearance of neutral fat.[9] The droplets were fairly uniform in size, averaging 0.5 to 1 μ in diameter. They were not bound by a unit membrane, although an irregular dense line was often seen at the droplet–cytoplasm interface, probably representing heavy metal staining artifact. These droplets were homogeneous in appearance and of the type previously found to be associated with marked cellular

triglyceride accumulation.[10] The mitochondria (Figure 5) were noted to be approximately 50% smaller in measured average width when compared to those in aerobic cells (Figure 4) and had a dense mitochondrial matrix. Many of the mitochondria exhibited abnormal arrangements of their cristae, often being longitudinal to the long axis of the mitochondria (Figure 3). Abnormal ring forms of mitochondria were also seen, as were mitochondria with tubular cristae (Figure 5). Some abnormally dilated elements of the rough ER were present in the hypoxic cells (Figure 2). After 48 hours of incubation, there was no noticeable increase in autophagic vacuoles or other autolysosomal structures.[12,35,36] These did increase in number in longer incubations (Day 3), and other evidence of increased cell injury, including numerous necrotic cells and increased cellular debris, was seen.

Electron micrographs of L cells from cultures incubated aerobically for 24 hours after a previous 48-hour incubation in an hypoxic atmosphere showed a reversion to a more normal morphology (Figures 6 and 7). The mitochondria showed some evidence of swelling, but the cristae appeared normal and the inner mitochondrial matrix was no longer condensed as in the hypoxic cells. A moderate number of small lipid droplets remained (Figure 7). However, many areas could be identified in the cytoplasm which appeared free of cytoplasmic organelles and ribosomes and contained only a slight amount of loose amorphous material (Figure 6). These rarefied or empty areas of cytoplasm may represent previous sites of triglyceride droplets that have undergone lipolysis and reutilization during the period of reaeration.

Discussion

The present tissue culture studies demonstrate that incubation in a hypoxic atmosphere causes an inhibition of cell growth, confirming earlier reports.[14,15] The inhibition of growth is accompanied by a marked accumulation of triglyceride, and to a lesser extent FFA, in the hypoxic cells. This lipid accumulation is manifested morphologically by the presence of large numbers of homogeneous non-membrane-bounded cytoplasmic droplets. The other morphologic changes such as dilated ER and smaller mitochondria with longitudinal cristae and matrix condensation have been previously reported in monolayer L cell cultures after prolonged oxygen deprivation and associated with reduced respiratory enzyme activity.[15,34] The finding that hypoxic injury produces a triglyceride accumulation is in contrast with earlier studies [11-13] which indicate that other injuries resulted in the accumulation of sequestered membranous lipid, high in phospholipid and cholesterol content, resulting from increased

autophagy.[35-37] For up to 48 hours of hypoxia at the levels achieved in this experimental system, no evidence of increased autophagic vacuoles or other forms of secondary lysosomes were noted, and cellular phospholipid and cholesterol levels remained normal.

These results appear to be different than the electron microscopic observations of Paule *et al.*,[38] who reported the accumulation of large numbers of secondary lysosomes in hypoxic tissue cultures of aortic smooth muscle cells. However, these observations were made after an incubation of 5 days, and no study of cellular growth, viability, protein synthesis, or lipid composition was performed. Our experiments with the suspension-cultured L cells indicate that hypoxic injury initially produces a triglyceride accumulation in the form of non-membrane-bounded homogeneous cytoplasmic droplets. This type of lipid accumulation is not associated with decreased viability or increased phagocytosis of cellular debris. The inhibition of growth and the accumulation of triglyceride is readily reversible at this stage if reaeration occurs. Only with more prolonged hypoxia (after 72 hours) did irreversible cell injury occur, with an increase in complex lysosomal structures, resulting from either autophagy or phagocytosis of cellular debris.

This *in vitro* evidence of the reversibility of hypoxic lipid accumulation is in agreement with classic pathologic observations that, in ischemic injury, neutral fat accumulated in reversibly injured cells and not in the dead cells in the center of infarcts.[1,2] It is also in keeping with the current concept that triglyceride storage results from an imbalance of cellular uptake and utilization of FFA.[2,6,39] There is no evidence at present to indicate that hypoxia causes increased uptake of lipid by tissue culture cells,[38] although it is known that hypoxia and other injuries can cause increased arterial endothelial permeability.[40,41] In cultured human arterial smooth muscle cells, hypoxia did not affect lipoprotein uptake but did cause impairment of lipoprotein degradation.[42]

In the present experiments, there was no noticeable change in medium lipid depletion in either the hypoxic or the control cultures, suggesting no striking differences in lipid uptake. Studies on hypoxic liver by Chiodi [43] and by Soler-Argilaga *et al.*[44] have shown that hypoxia does not modify the uptake of FFA by the liver cells but does cause decreased fatty acid oxidation with the resultant accumulation of triglyceride. This same mechanism is postulated for the accumulation of triglyceride in these experiments with hypoxic L cells. Fatty acid oxidation takes place mainly in the mitochondria and requires energy from oxidative phosphorylation.[45] The impairment of this utilization of FFA by hypoxia results in increased esterification and storage as triglyceride, since the intracellular

level of FFA is closely regulated.[39] The finding of significantly increased cellular FFA in the hypoxic cells is important in this regard. Although it is not elevated to the intracellular levels observed in fatty acid toxicity produced by excess saturated FFA,[10] the FFA accumulation caused by hypoxia might lead to increased saturated FFA toxicity. Indeed, studies from this laboratory [46] have shown that hypoxia potentiates saturated FFA cytotoxicity in tissue culture cells. This may be of great significance because several clinical and experimental studies [47,48] have implicated the combination of myocardial hypoxia and increased circulating FFA in the production of ventricular arrhythmias and sudden death.

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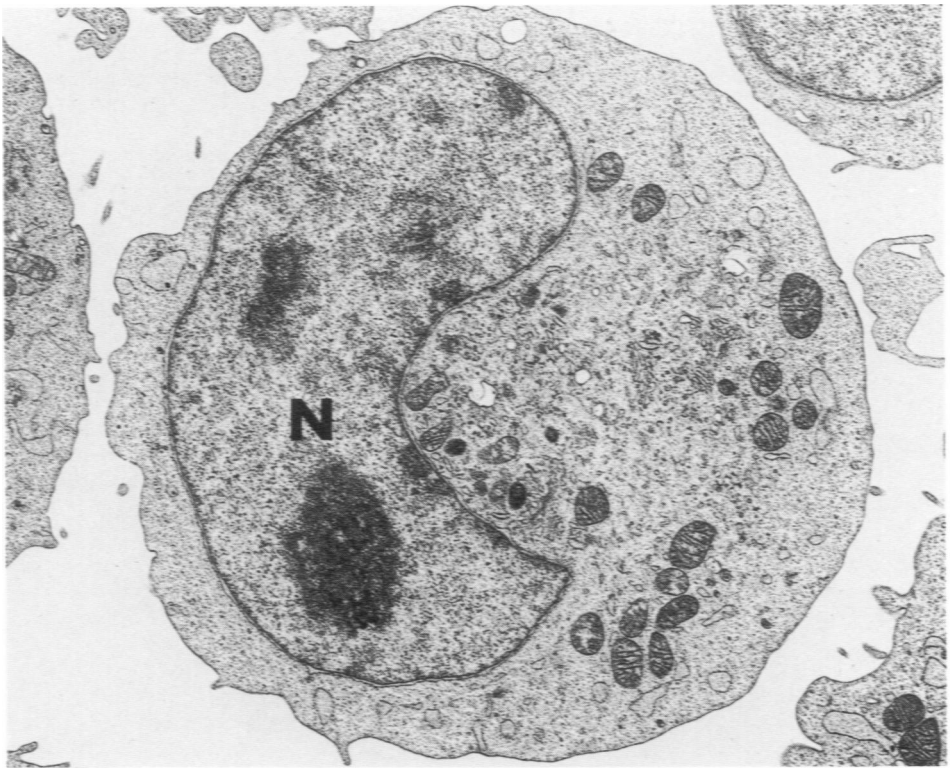
Acknowledgments

The authors wish to express their gratitude to Diane Thayer for her skillful assistance in the preparation of this manuscript. The valuable advice and helpful critical review of Dr. Rolla B. Hill is greatly appreciated.



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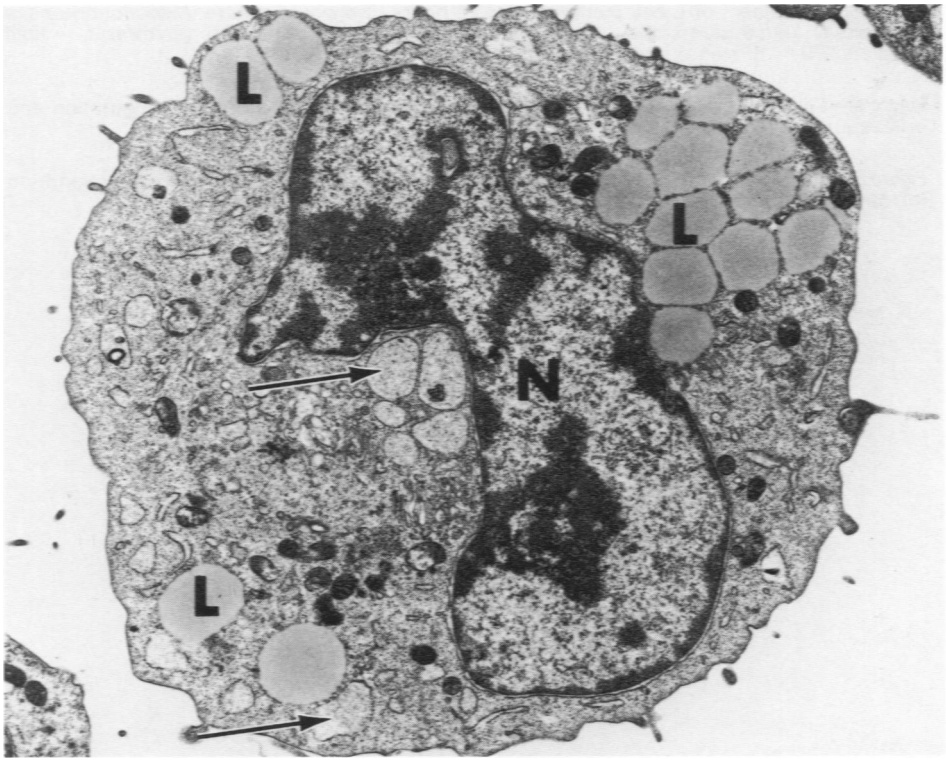

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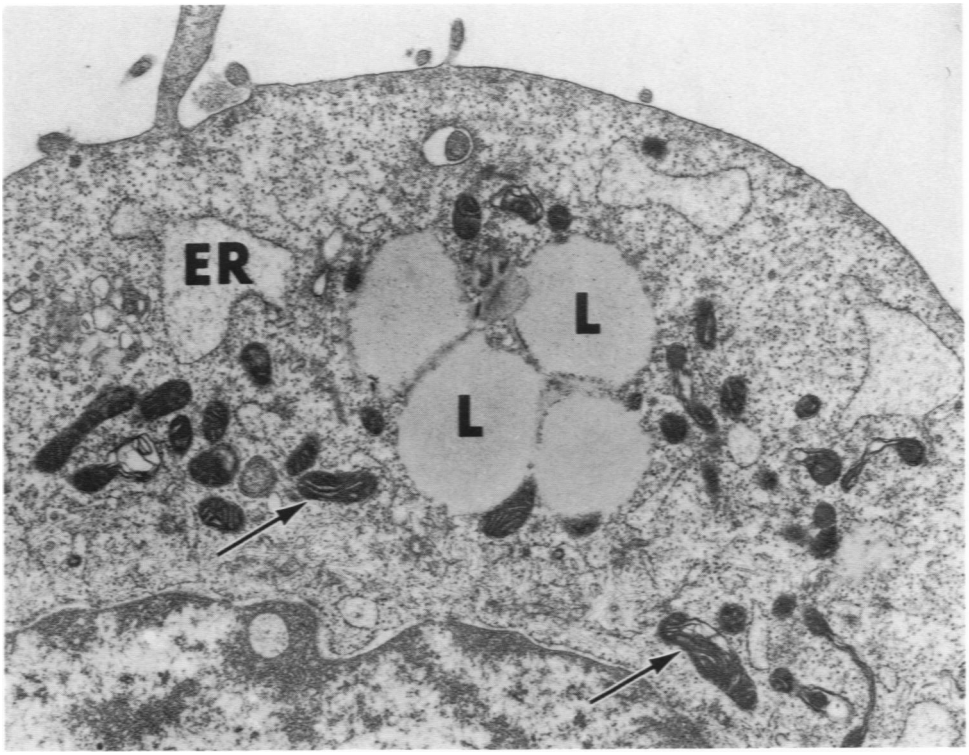
Figure 1—Electron micrograph of a control strain L mouse fibroblast grown in suspension tissue culture in an aerobic atmosphere of 8% CO_2–92% air. Cytoplasm contains scattered mitochondria and profiles of rough endoplasmic reticulum. A prominent Golgi region is present in indentation of nucleus (*N*). (× 8000) **Figure 2**—Strain L cell from a culture incubated for 48 hours under hypoxic conditions achieved by daily gassing with 8% CO_2–92% N_2. Cytoplasm contains scattered small condensed mitochondria, numerous lipid droplets (*L*), and dilated endoplasmic reticulum (*arrows*). *N* = nucleus. (× 12,000)

Figure 3—Cytoplasm of L cell incubated for 48 hours in hypoxic culture. Mitochondria have abnormally dense matrix and often longitudinal arrangement of cristae (*arrows*). *L* = lipid droplets, *ER* = dilated endoplasmic reticulum. (× 21,000)

Figure 4—Mitochondria of cell from control aerobic culture; note normal configuration and cristae arrangement (× 31,000).

Figure 5—Mitochondria of cell from hypoxic culture; note unusual ring-shaped mitochondrion (*arrow*) and tubular cristae (*double arrows*). *L* = lipid droplet. (× 31,000)



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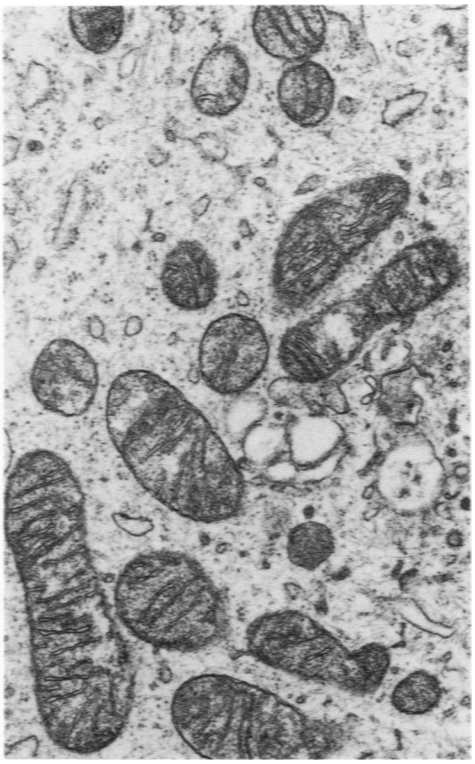

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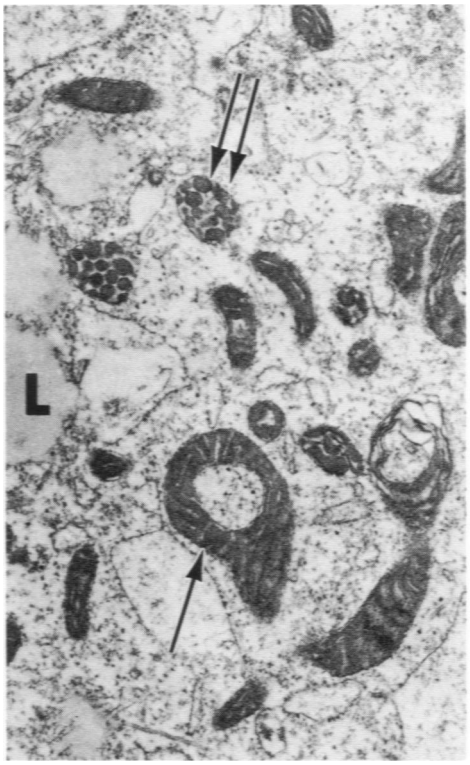

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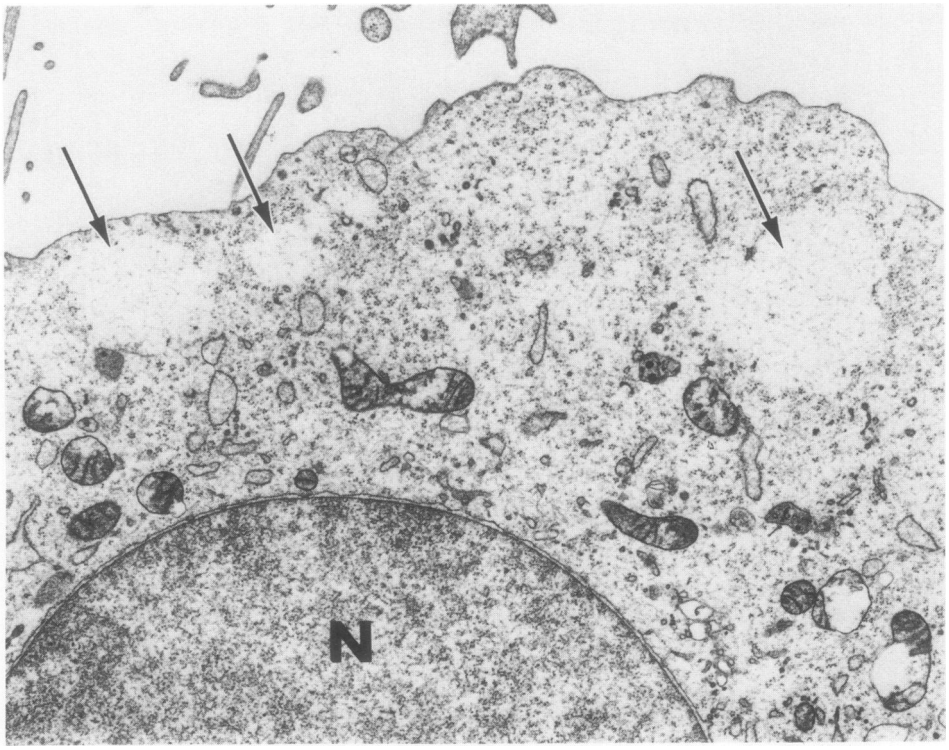

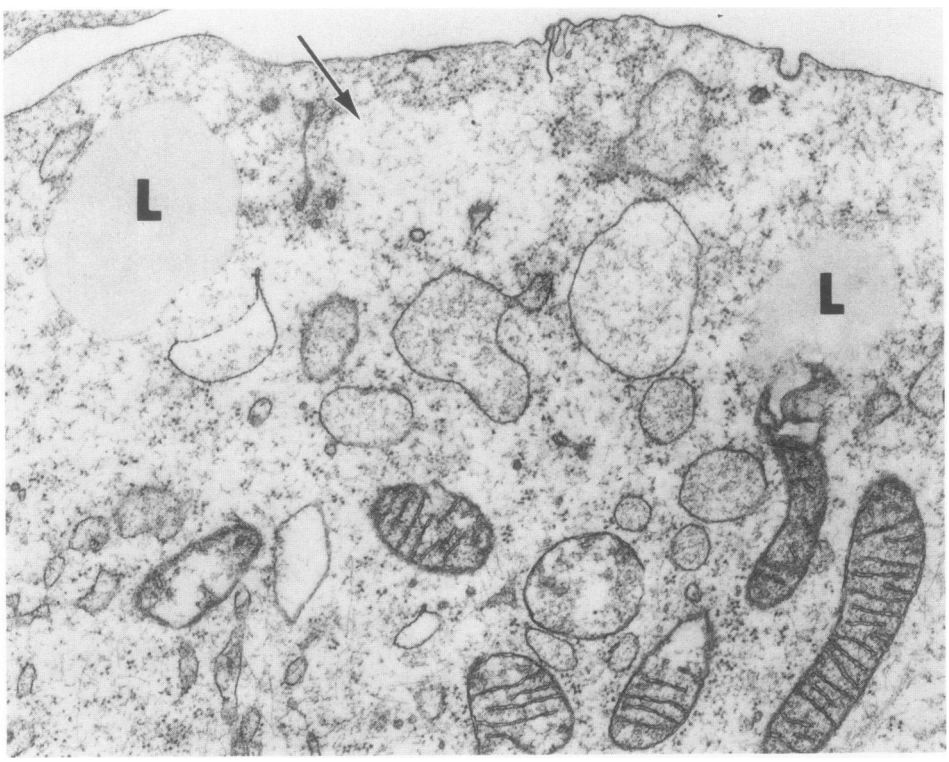

Figure 6—Portion of L cell after 24 hours of aerobic incubation following 48 hours of hypoxia. Electron-translucent areas of cytoplasm (*arrows*) devoid of organelles are present and presumed to represent previous location of lipid droplets. *N* = nucleus. (× 15,000) **Figure 7**—Cytoplasm of reaerated cell with residual lipid droplets *(L)* and electron-lucent area (*arrow*). Mitochondria have returned to more usual morphology. (× 27,000)