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Research Article

This study related ATP levels with membrane damage, lipid abnormalities, and cell death in energy-depleted LLC-PK1 cells. Oxidative phosphorylation was inhibited by antimycin A, and glycolysis was regulated by graded glucose deprivation to achieve stepwise ATP depletion. Over a range of ATP levels down to approximately equal to 5% of normal, over 5 h, cells were altered only minimally, or injured reversibly. Such cells maintained mitochondrial potential, and retained more K⁺ than cells without an energy source. Over the same duration, cells without an energy source were lethally injured. Treatment with antimycin induced increments of triglycerides and decreases of phospholipids. With severe ATP depletion (approximately equal to 5-10% of normal after 5 h), decrease of phospholipids was marked. Cells in which ATP was not measurable (or was less than 5% of normal) showed comparable phospholipid declines but, in addition, showed massive and progressive increase of unesterified fatty acids. The results identified a low threshold of ATP, at best 5-10% of normal, which preserved viability in LLC-PK1 cells despite major loss of membrane phospholipids. This threshold also determined the ability of cells to maintain their normally low levels of unesterified fatty acids. Failure of energy-dependent mechanisms that normally metabolize unesterified fatty acids may be a correlate of the extent of energy depletion that determines lethal injury.

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Energy Thresholds That Determine Membrane Integrity and Injury in a Renal Epithelial Cell Line (LLC-PK₁)

Relationships to Phospholipid Degradation and Unesterified Fatty Acid Accumulation

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Abstract

This study related ATP levels with membrane damage, lipid abnormalities, and cell death in energy-depleted LLC-PK₁ cells. Oxidative phosphorylation was inhibited by antimycin A, and glycolysis was regulated by graded glucose deprivation to achieve stepwise ATP depletion. Over a range of ATP levels down to $\approx 5\%$ of normal, over 5 h, cells were altered only minimally, or injured reversibly. Such cells maintained mitochondrial potential, and retained more K⁺ than cells without an energy source. Over the same duration, cells without an energy source were lethally injured.

Treatment with antimycin induced increments of triglycerides and decreases of phospholipids. With severe ATP depletion ($\approx 5\text{--}10\%$ of normal after 5 h), decrease of phospholipids was marked. Cells in which ATP was not measurable (or was $< 5\%$ of normal) showed comparable phospholipid declines but, in addition, showed massive and progressive increase of unesterified fatty acids.

The results identified a low threshold of ATP, at best 5–10% of normal, which preserved viability in LLC-PK₁ cells despite major loss of membrane phospholipids. This threshold also determined the ability of cells to maintain their normally low levels of unesterified fatty acids. Failure of energy-dependent mechanisms that normally metabolize unesterified fatty acids may be a correlate of the extent of energy depletion that determines lethal injury.

Introduction

Cells that show resistance to the noxious effects of respiratory arrest do so either because they are facultative anaerobes (i.e., they are able to generate ATP anaerobically), or because they are able to conserve ATP by reducing its consumption (1). Apart from lower animals, these principles may apply to mammalian cells also (1); thus, the capacity to utilize substrates under anaerobic conditions confers resistance to anoxia, and reduction of cellular workload by pharmacologic intervention blunts the injury caused by respiratory arrest (1–7). When oxidative phosphorylation fails, and if glucose is either not available or cannot be fermented, mammalian cells show decreases of ATP due to consumption by ion pumps and

metabolic events requiring phosphorylation (1). These are associated with decreased Na⁺-K⁺ pump activity and reversal of the cell Na⁺/K⁺ ratio (1, 8, 9); Cell calcium homeostasis may be affected as well (10, 11). In such circumstances, increased cell Ca²⁺ has been invoked as a pathogenetic factor that might cause membrane damage and cell death. However, direct measurement of free cytosolic Ca²⁺ using in vitro models of impaired energy supply have yielded conflicting results (12–15). In any case, a strong association exists between impaired energy supply and membrane damage, at least in myocardial cells (16). In several models, the membrane damage associated with energy depletion is thought to be caused by breakdown of membrane lipids (17–22). However, there is little quantitative information that relates ATP levels, membrane lipid abnormalities, and cell damage. These considerations led us to examine whether there are definable thresholds of cell ATP above which membrane integrity is maintained and below which membranes break down and compromise viability. Our experimental design was to metabolically inhibit respiration in cultured kidney epithelial cells and provide them with glucose in graded amounts to obtain cells with predictably different amounts of ATP. Membrane integrity was assessed functionally by monitoring the ability of cells to mount and maintain ion gradients, biochemically by studying the composition and metabolism of lipids, and morphologically by electron microscopy.

Methods

Cells. LLC-PK₁ cells were grown in DME with 450 mg/liter glucose and 10% calf serum at 37°C in 95% air and 5% CO₂. Culture dishes (60 or 100 mm diam) were plated with 0.65×10^6 or 1.8×10^6 cells, respectively. Cells were used at confluence 46 h later. The LLC-PK₁ cell line is derived from the tubular epithelium of pig kidney (23) and was between the 50th and 150th passages of a seed stock obtained from American Type Culture Collection, Rockville, MD.

Protocols. The cells were rinsed with, and exposed to, DME without serum, containing 3 g/liter NaHCO₃ and 10 mM Hepes at pH 7.45–7.5, at 37°C in 95% air and 5% CO₂. Cells were incubated up to 5 h in medium with 0–450 mg/dl glucose, without glutamine or pyruvate, either with, or without 1.5 μM antimycin A, a complex III inhibitor of mitochondrial electron transport (24). The cells were then studied directly or after reexposure to complete DME without serum without antimycin for 5 or 18 h to allow recovery.

Radiolabeling protocols. Lipids were radiolabeled by including 3 μCi [5,6,8,9,11,12,14,15-³H(N)]-arachidonic acid (100 Ci/mmol) in 3 ml of medium for 20 h, or to steady state with 5 μCi [1-¹⁴C]-acetate (10.8 mCi/mmol) in 4 ml of medium for 40–44 h. After separation, radioactivity in lipids was measured by scintillation spectrometry, and expressed as in disintegrations per minute after quench correction.

Chemical analyses. Cell protein and medium lactate were measured as described (25, 26). ATP was measured in neutralized perchloric acid extracts fluorimetrically (27). Acid extracts which were kept at

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23°C for 48 h or 37°C for 24 h to allow complete hydrolysis of ATP (28) were neutralized and used as blanks. Adenine nucleotides (ATP, ADP, AMP) were also measured by HPLC. TCA extracts of cells (as in cell K^+ , see below) were neutralized and extracted with equal volumes of 0.5 M tri-*n*-octylamine in Freon-113, and analyzed by HPLC on a Beckman Instruments, Inc. (Palo Alto, CA) C_{18} ion-pairing reverse-phase column isocratically with 19% acetonitrile, 40 mM KH_2PO_4 , 10 mM tetrabutylammonium dihydrogen phosphate, pH 3.5, at 24°C at 1 ml/min. Peaks were detected at 254 nm and compared with pure nucleotide standards. Free inorganic phosphate (P_i) was measured in the same extracts by a micromethod modified from that of Goldenberg and Fernandez (29).

Lipid analyses and chromatography. Methanol was added directly to cells and medium (including detached cells and debris) to terminate the experiment. Scraped cells and medium were transferred to tubes and chloroform added to extract lipids (30) using 2M KCl instead of water for phase separation. Extracts were filtered through Sephadex G 25-150 (in $CHCl_3$) to remove impurities and water. Carrier lipids were added to extracts with radiolabel. With some exceptions, lipids were analyzed by thin layer and gas liquid chromatography as described (22). Phospholipids were separated in one dimension by TLC on Silica Gel H using chloroform/methanol/water/28% NH_4OH (65:25:4:1 vol/vol/vol/vol) and in two dimensions on 0.25-mm silica gel H plates with 10% magnesium acetate, using chloroform/methanol/28% NH_4OH (70:25:5.5 vol/vol/vol) and chloroform/acetone/methanol/acetic acid/water (30:40:10:10:5 vol/vol/vol/vol/vol) for the first and second dimensions. After spraying with 6-*p*-toluidino 2-naphthalene sulfonic acid, the spots were visualized under UV light (31). For measurement of phospholipid mass, pooled lipid extracts were prepared from two 100-mm culture dishes. After TLC, phosphorus was measured in scrapings (32). To measure free fatty acids, cells were cultured for 46 h in serum free DME (supplemented with insulin, transferrin, selenium, and vasopressin), to avoid adsorption of serum fatty acids to plastic substrata.

Morphology. Cells were fixed with 2.5% glutaraldehyde in 0.1 M Na cacodylate-HCl buffer, pH 7.4, postfixed in 1% OSO_4 in water, and prepared for electron microscopy. Attached cells were counted by a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) after trypsin-EDTA treatment.

Mitochondrial distribution of rhodamine 123. Cells grown on glass coverslips were exposed to experimental medium. Rhodamine 123 (Rh 123),¹ in DMSO, was then added to a final concentration of 0.25 or 0.5 $\mu g/ml$. After 30 min, coverslips were rinsed three times in experimental medium without dye, and mounted in the same medium for intravital fluorescence microscopy (33, 34). Cells were compared to (a) sham-treated controls and (b) cells treated with 10 μM carbonyl cyanide *m*-chlorophenyl-hydrazone which abolishes mitochondrial potential (24).

Cell K^+ . Cultures were rinsed three times with 0.27 M sucrose and extracted with 6% TCA, and K^+ was measured by atomic absorption spectroscopy (35).

Statistical analysis. Multiple groups were compared by analysis of variance followed by the Newman-Keuls test; two groups were compared by Student's *t* test.

Chemicals and supplies. Culture media were obtained from Irvine Scientific, Santa Ana, CA. TLC supplies were obtained from Analtech, Inc., Newark, DE. [¹⁴C]acetate was obtained from ICN Biochemicals, Plainview, NY. [³H]arachidonic acid was obtained from New England Nuclear, Boston, MA. Rh 123 was obtained from Eastman Kodak Co., Rochester, NY. Tri-*n*-octylamine was obtained from Aldrich Chemical Co., Milwaukee, WI. Freon 113 was obtained from Matheson Div., Searle Medical Products, E. Lyndhurst, NJ. Other special reagents were obtained from Sigma Chemical Co., St. Louis, MO. LLC-PK₁ cells were obtained from the American Type Culture Collection, Rockville, MD.

1. Abbreviation used in this paper: Rh 123, rhodamine 123.

Results

Cell ATP and medium lactate. Fig. 1 shows ATP in cells exposed over 5 h to media with different concentrations of glucose with or without antimycin. With antimycin, cell ATP reflected the availability of glucose, and declined to undetectable levels in the absence of glucose (Fig. 1, *a* and *b*, *solid bars*). In contrast, glucose-deprived cells without antimycin showed minor changes only, suggesting the generation of ATP by the use of other substrates (Fig. 1 *a*, *empty bars*). Medium lactate paralleled the availability of glucose in the absence of antimycin, indicating aerobic glycolysis; with antimycin, increase of lactate was seen for each glucose concentration (shown for 0–25 mg/dl in Table I) indicating a Pasteur effect (1). With higher levels of glucose, medium lactate increased correspondingly, but plateaued between 100 and 450 mg/dl glucose (data not shown).

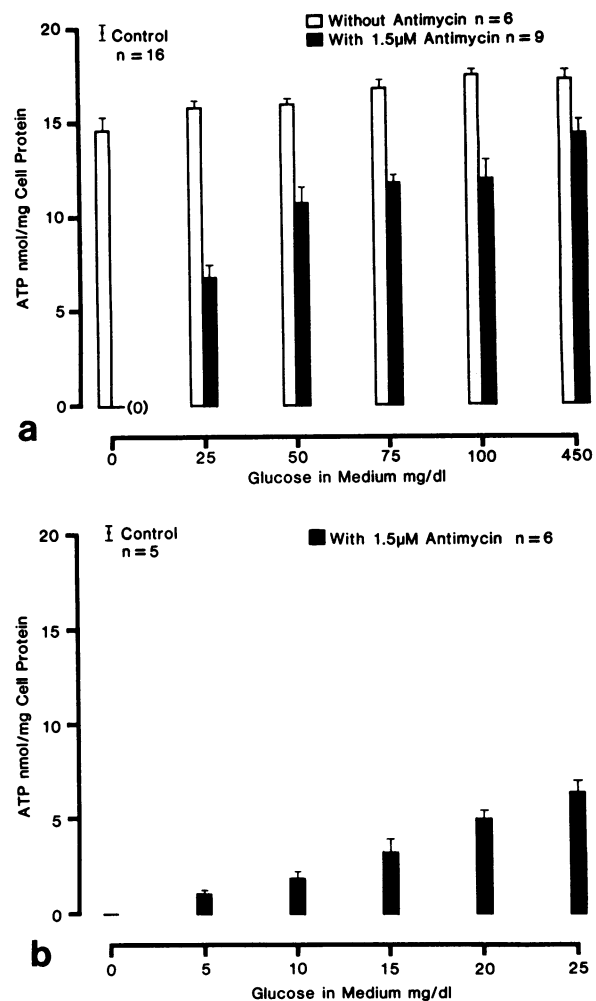


Figure 1. (a) ATP in cells. Cells were treated for 5 h without antimycin (*empty bars*), or with antimycin (*solid bars*) and different concentrations of glucose (*x*-axis). ATP is expressed in nmol/mg cell protein (*y*-axis). Mean \pm SEM (+ antimycin, *n* = 9) (– antimycin, *n* = 6). ANOVA showed all relevant differences to be significant $P < 0.001$. (b) ATP in cells. As in *a*, cells were treated for 5 h with antimycin, and 0–25 mg/dl glucose. Mean \pm SEM, *n* = 6. Differences between cells with 0–15 mg/dl glucose and 25 mg/dl glucose were significant, $P < 0.001$.

Table I. Lactic Acid in Medium

Glucose (mg/dl)	0	5	10	15	25
Without antimycin	5 \pm 3	78 \pm 6	140 \pm 1	186 \pm 2	260 \pm 4
With 1.5 μM antimycin	36 \pm 3	118 \pm 1	224 \pm 1	326 \pm 4	696 \pm 21

Lactic acid accumulated in medium after 5 h of treatment with or without 1.5 μM antimycin and 0–25 mg/dL of glucose. Values given as \pm SEM ($n = 4$). $P < 0.001$ for the effect of glucose on lactic acid levels, $P < 0.01$ for each comparison with and without antimycin.

Fig. 2 depicts ATP in cells treated with antimycin without glucose (Fig. 2 *a*) or with 10 mg/dl glucose (Fig. 2 *b*). After 1–5 h of incubation, cells were extracted immediately (*line graph*), or after an additional 5 h of recovery in complete DME with 450 mg/dl glucose and no antimycin (*bars*). Without glucose, ATP declined steeply over the 1st h and then slowly over the next 4 h; ATP was not detectable by 3 h (Fig. 2 *a*). In contrast, the fall in ATP was slower, and ATP remained measurable even after 5 h of incubation, in cells with 10 mg/dl glucose (Fig. 2 *b*). When cells were treated with antimycin without glucose, recovery of ATP during a 5-h period in complete antimycin-free DME was progressively less if the cells had been previously subjected to correspondingly longer periods in antimycin containing glucose-free medium (*bars*, Fig. 2 *a*). If glucose had been present in the original incubation medium (10 mg/dl), ATP regeneration was greater (*bars*, Fig. 2 *b*).

ATP, ADP, and AMP were also measured by HPLC, and P_i by spectrophotometry, in cells with and without antimycin, and 450, 10, or 0 mg/dl glucose (Table II). Measurements were made at 3 h, at which time ATP had been shown to be unmeasurable in cells with antimycin and no glucose (Fig. 2). With antimycin, ATP declined; the decrease was modest with 450 mg/dl glucose, and of large magnitude in cells with 10 mg/dl glucose. In contrast, ADP and AMP were elevated. In cells with antimycin and no glucose, ATP was nearly zero and ADP declined, whereas AMP showed major increases. Increased AMP corresponded to lower ATP levels; levels of P_i showed a similar pattern, being highest in cells with antimycin and no glucose. These data, which are consistent with augmented hydrolysis and limited synthesis of ATP, were used to compute the “adenylate energy charge” ($\text{ATP} + 1/2 \text{ADP}/\text{ATP} + \text{ADP} + \text{AMP}$), and “phosphorylation potential” ($\text{ATP}/\text{ADP} \times \text{P}_i$). These indices, shown in Table II, are thought to reflect the regulatory efficiency of the generation and utilization of energy (36, 37). In cells with antimycin and 10 mg/dl glucose, energy charge showed major declines, but was nevertheless much higher than in cells with antimycin and no glucose (lower box in Table II). Cells with antimycin showed even steeper declines of phosphorylation potential; however, the values were much higher in cells with 10 mg/dl glucose than in cells with no glucose. Mainly, this reflected a difference in the numerator of the index (ATP) between the two groups (cf. upper box in Table II).

Morphology. Cells were exposed to medium with or without antimycin, and with or without glucose (450, 25, or 10 mg/dl). After 5 h, cells were fixed for electron microscopy. In each case, 50 cells from two experiments were evaluated with-

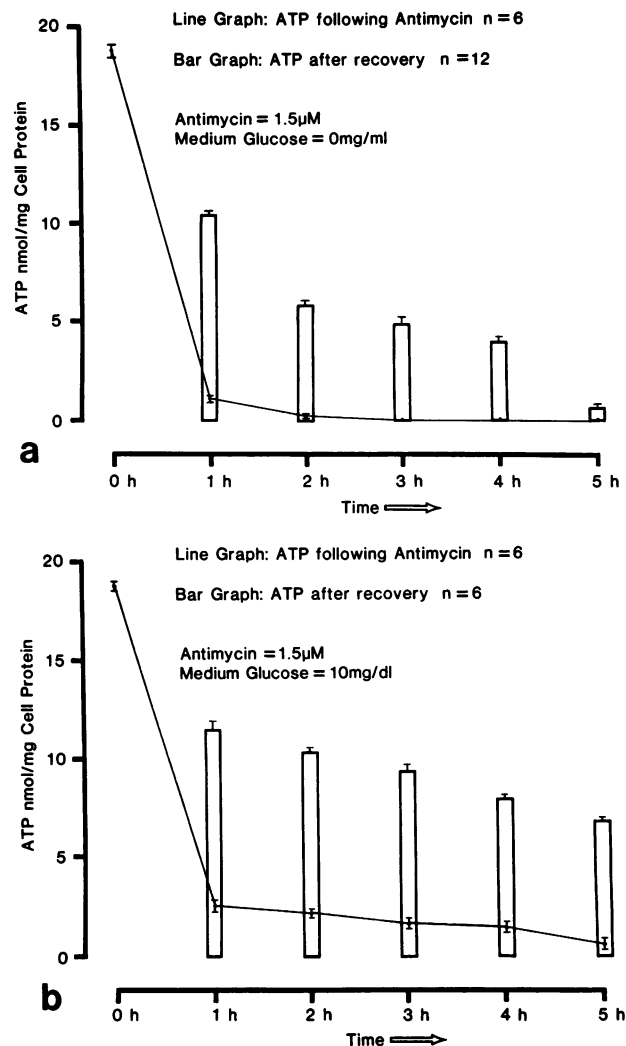


Figure 2. (a) ATP in cells. Cells were treated for 1–5 h with antimycin, without glucose. ATP was measured at the end of 1–5 h (*line graph*), or after an additional 5 h of recovery in medium with 450 mg/dl glucose without antimycin (*bars*). Mean \pm SEM + antimycin, $n = 6$; recovery, $n = 12$. (b) ATP in cells. As in *a*, except that cells were treated with antimycin and 10 mg/dl glucose. Mean \pm SEM + antimycin, $n = 6$; recovery, $n = 6$. With antimycin, all differences between groups with 0 and 10 mg/dl glucose were significant, $P < 0.001$. After recovery, all differences between groups originally treated with antimycin and 0 and 10 mg/dl glucose for 2 h and beyond were significant, $P < 0.001$.

out knowledge of the identity of the experiment and cells were classified as normal, showing mild or moderate injury, or severe injury. Normal morphology was defined as an appearance which could not be distinguished from control cells that had not been subjected to any treatment. Mild or moderate injury was defined as changes including cell swelling, swelling of intracellular organelles, and clumping of nuclear chromatin. Severe injury was defined as all of the above changes plus high amplitude swelling of mitochondria, flocculent mitochondrial densities, or discontinuities in cell membranes (9). Cultures treated without antimycin, or with antimycin in the presence of 25, or 450 mg/dl glucose showed > 98% of cells in the normal category; none showed severe injury. Cultures treated with antimycin and 10 mg/dl glucose showed 60% normal, 36% moderately injured, and 4% severely injured cells. With

Table II. Adenine Nucleotides, Inorganic Phosphate, Energy Charge, and Phosphorylation Potential

	Control 0 time	Glucose (mg/dl)	450	10	0	450	10	0
		Antimycin 1.5 μ M	+	+	+	-	-	-
nmol/mg cell protein								
ATP	21.20 \pm 1.37	14.07 \pm 1.26 [†]	2.27 \pm 0.37*	0.03 \pm 0.01	19.41 \pm 2.44	18.50 \pm 0.79	17.88 \pm 1.12	
ADP	5.02 \pm 0.38	8.98 \pm 1.11	7.14 \pm 0.58*	3.05 \pm 0.11	4.57 \pm 0.64	4.92 \pm 0.62	4.97 \pm 0.86	
AMP	0.23 \pm 0.03	1.69 \pm 0.41*	4.95 \pm 1.02 [†]	8.88 \pm 0.58	0.20 \pm 0.03	0.23 \pm 0.06	0.33 \pm 0.13	
Total adenine nucleotides	26.44 \pm 1.67	24.74 \pm 1.96 [†]	14.36 \pm 1.25	11.95 \pm 0.57	24.18 \pm 3.06	23.66 \pm 1.23	23.18 \pm 2.07	
Inorganic phosphate	46.8 \pm 3.3	87.0 \pm 7.3 [†]	126.01 \pm 6.2	189.9 \pm 24.1	50.3 \pm 2.3	59.5 \pm 5.4	68.9 \pm 7.6	
Energy charge	0.90 \pm 0.01	0.75 \pm 0.03 [†]	0.41 \pm 0.04 [†]	0.13 \pm 0.01	0.90 \pm 0.01	0.89 \pm 0.01	0.88 \pm 0.01	
Phosphorylation potential	95.1 \pm 14.0	20.5 \pm 4.3*	2.6 \pm 0.6*	0.04 \pm 0.02	87.2 \pm 9.1	69.7 \pm 10.6	60.9 \pm 12.0	

Cells were exposed to experimental conditions for 3 h. ATP, ADP, AMP, and P_i expressed in nmol/mg cell protein ($n = 4$). Energy charge = ATP + 1/2 ADP/ATP + ADP + AMP. Phosphorylation potential = ATP/ADP $\times P_i$. For ease of expression, (ATP/ADP $\times P_i$) $\times 1,000$ is shown. All comparisons between groups with and without antimycin at the same glucose concentration were significant except for total adenine nucleotides at 450 mg/dl. * $P < 0.05$ relative to next lower glucose concentration. [†] $P < 0.01$ relative to next lower glucose concentration.

antimycin and no glucose, cultures showed no normal cells, 24% moderately injured, and 76% severely injured cells. Examples of these differences are shown in electron micrographs in Figs. 3, *a-d*. At the end of 5 h, virtually all cells without antimycin, and all cells with antimycin and 10 mg/dl glucose or more, retained the capacity to exclude vital dyes (trypan blue, ethidium bromide). Greater than 90% of cells with antimycin and no glucose also excluded vital dyes. Thus, energy-deprived cells showed alterations of ultrastructure and membrane lipids (see below), but rupture of the plasma membrane occurred only as a very late event.

Mitochondrial uptake and retention of Rh 123. The cationic lipophile and fluorochrome Rh 123 distributes across membranes according to transmembrane potential (33, 34, 38). Cultured cells partition Rh 123 into the cytosol and mitochondria; this results in bright mitochondrial fluorescence accounted for by electrical potential across the inner mitochondrial membrane (33, 34, 38). Normally this potential is maintained by H^+ extrusion using energy provided by electron transport. If electron transport is inhibited, but ATP is available, mitochondrial ATP synthetase works in reverse as an H^+ ATPase, and maintains potential by extruding protons (24). We have used this property as a bioassay to detect anaerobically generated ATP in the cytosol of antimycin treated cells, and also as an indicator of the ability of the inner mitochondrial membrane to maintain an ion gradient, a measure of its integrity (24).

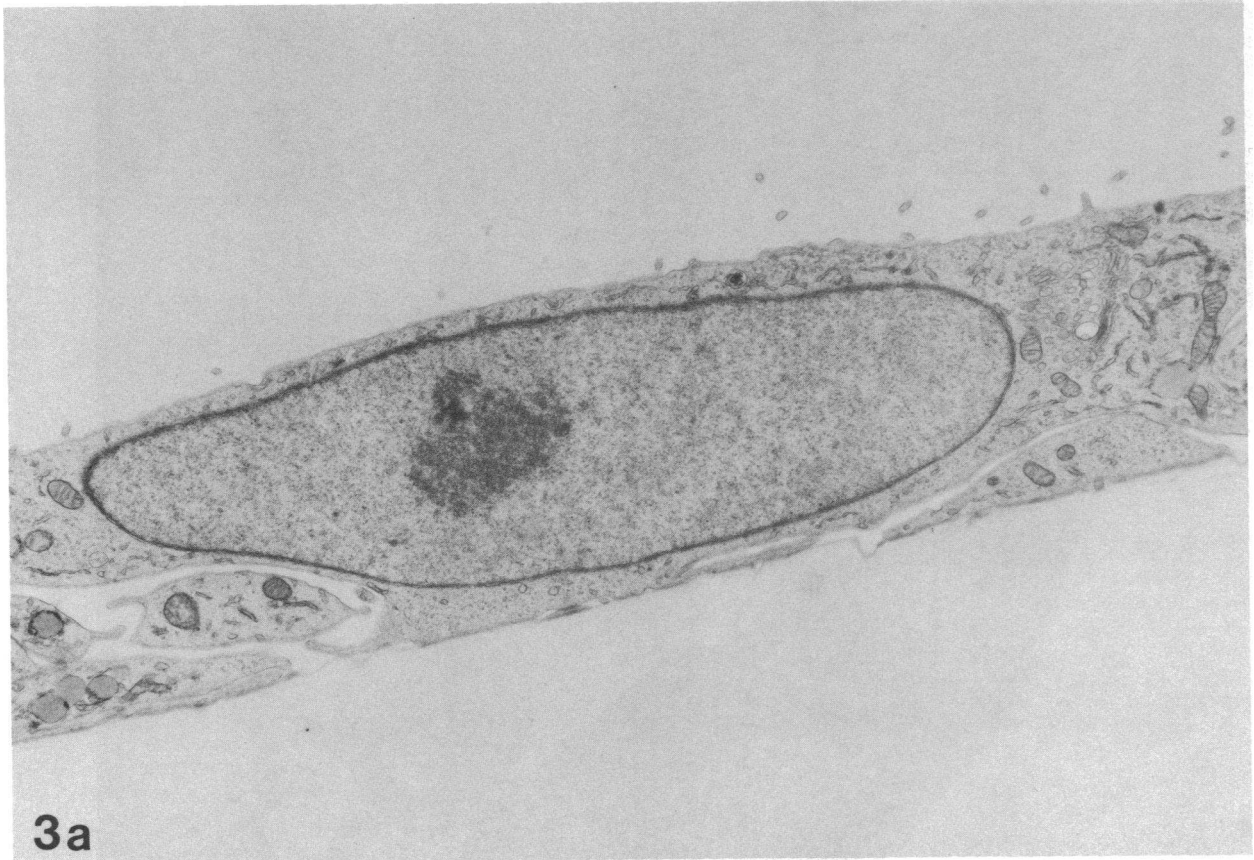
Figs. 4, *a-c* summarize the results obtained with cells which were incubated for 5 h with experimental medium (with or without antimycin, 0–450 mg/dl glucose) containing 0.5

μ g/ml of Rh 123 during the final 30 min. All cells that were exposed to medium without antimycin showed mitochondrial fluorescence. All cells treated with antimycin in medium containing 25–450 mg/dl glucose also showed a similar pattern of fluorescence (Fig. 4 *a*). This appearance could not be distinguished from what was seen in untreated control cells incubated for 30 min in complete DME with Rh 123 (not shown). Cells exposed to antimycin and 10 mg/dl glucose showed a variable pattern of fluorescence. They were either similar in appearance to control cells, or showed partial smudging of the fluorescent mitochondrial outlines and crowding of mitochondria around the nuclei (Fig. 4 *b*). In any case, mitochondria-based fluorescence was observed in every cell. In contrast, cells exposed to antimycin in glucose-free medium were markedly shrunken, and showed either faint or negligible fluorescence, or exhibited diffuse smudgy fluorescence throughout the cytoplasm without identifiable mitochondrial profiles (Fig. 4 *c*). This indicated that there was no longer a selective accumulation of Rh 123 in mitochondria, and that mitochondrial potential was lost (38).

Cell K^+ . Fig. 5 shows cell content of K^+ at 1, 3, and 5 h after treatment with or without antimycin in medium with 0, 10, or 450 mg/dl glucose. Compared with unperturbed controls, cells treated without antimycin, and cells treated with antimycin and 450 mg/dl glucose showed either no changes, or minor declines only. Both with antimycin and no glucose, or 10 mg/dl glucose, steep declines of cell K^+ were seen. However, at 5 h, K^+ in cells with antimycin and 10 mg/dl glucose was significantly higher than in cells without glucose (266 \pm 28 vs. 186 \pm 20 nmol/mg cell protein, mean \pm SEM $P < 0.02$, $n = 5$).

Figure 3. Electron micrographs of cells treated for 5 h. (*a*) Without antimycin, with 450 mg/dl glucose; (*b*) with antimycin and 450 mg/dl glucose; (*c*) with antimycin and 10 mg/dl glucose; (*d*) with antimycin and no glucose. Panels *a* and *b* are similar in appearance and ultrastructurally normal. (*Opposite leaf, verso*) Panel *c* shows clumping of nuclear chromatin, but otherwise unremarkable struc-

ture. Panel *d* shows cells with distorted, rounded structure; clumped chromatin in shrunken nuclei; and swollen, intracellular organelles, including high-amplitude swelling of mitochondria. Cells were fixed in glutaraldehyde and osmium tetroxide, embedded in Epon, and stained with uranyl acetate and lead citrate. (*a*) $\times 8,100$; (*b*) $\times 8,400$; (*c*) $\times 10,500$; (*d*) $\times 6,200$.



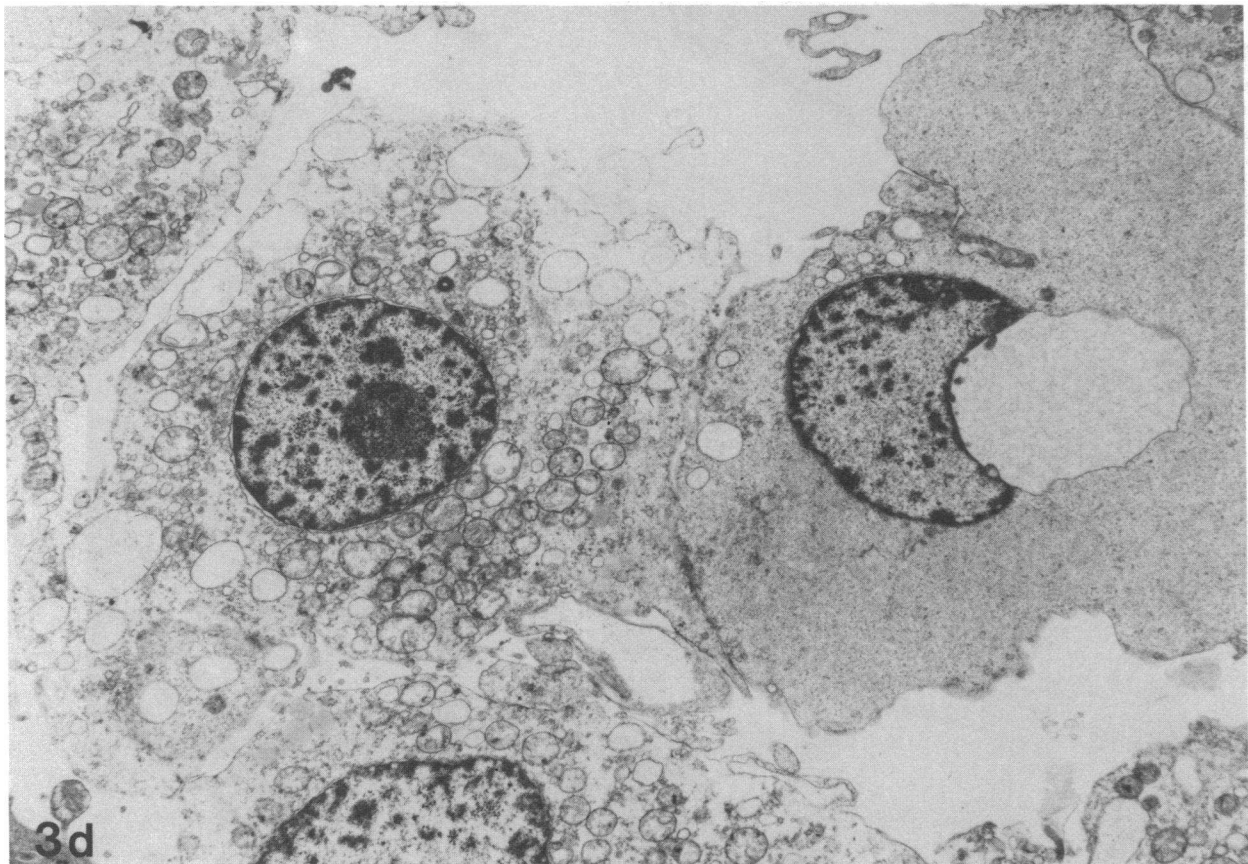
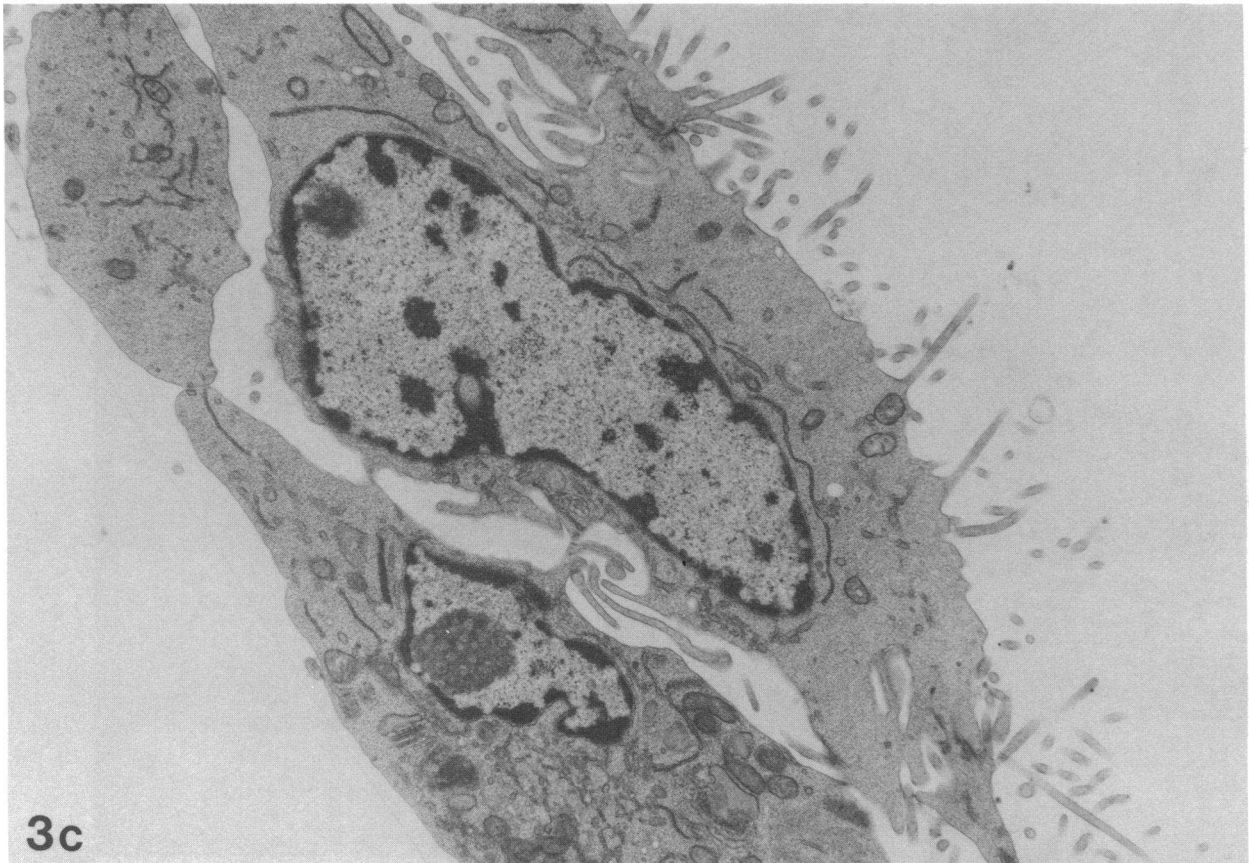


Figure 3 (Continued)

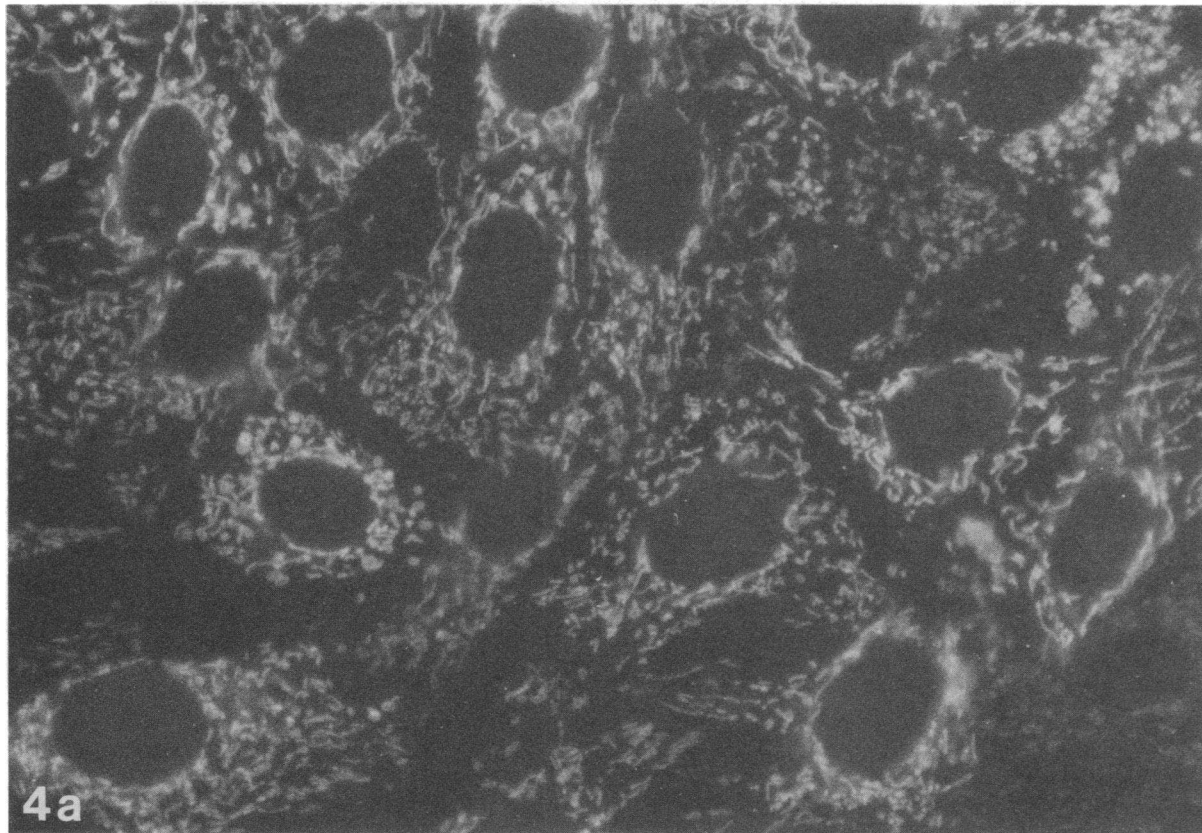


Figure 4. Intravital staining with Rh 123. Cells on glass disks were exposed to experimental medium for 5 h; during the last 30 min, 0.5 $\mu\text{g/ml}$ Rh 123 was included in the medium. Cells were mounted on shallow observation chambers for intravital fluorescence microscopy. (a) Cells treated with antimycin and 450 mg/dl glucose. This appearance could not be distinguished from that of unperturbed cells, from all cells treated without antimycin, and from all cells treated with antimycin and 25 mg/dl or more glucose. There is bright mitochondrial

fluorescence, indicating concentration of dye. (Overleaf) (b) Cells treated with antimycin and 10 mg/dl glucose. There is some clumping of mitochondria around nuclei; mitochondrial fluorescence is clearly recognized. (c) Cells treated with antimycin without glucose. Cells are rounded, and there is smudgy diffuse cytoplasmic fluorescence in some cells. Mitochondrial fluorescence cannot be recognized. All panels $\times 1,000$.

Cell survival. Cells were exposed to medium with antimycin and 0–450 mg/dl glucose for 5 h, and then reexposed to complete DME without antimycin to allow recovery for 18 h. At this time, in sets of dishes treated identically, ATP was measured in attached cells plus floating cells; in addition, attached cells were counted (Fig. 6). Recovery of ATP was a good correlate of cell survival as estimated by the number of attached cells. Thus, cells that had been treated with antimycin and no glucose showed negligible recovery of ATP; correspondingly, few of the cells remained attached to the substrate. In contrast, cells treated with antimycin and glucose (10 mg/dl or higher) showed recovery of ATP to levels $\geq 50\%$ of normal, and cell survival that approached control values. Attached cells had normal morphology, whereas floating cells showed marked cell injury and failed to exclude trypan blue. Fig. 7 shows the results for cell survival (attached cells) after 18 h of recovery in complete medium without antimycin, after treatment of cells with antimycin in glucose-free medium for 1, 2, 3, 4, or 5 h. Increasing durations of exposure to antimycin without glucose were associated with correspondingly decreased cell survival.

Lipids labeled with [^3H]arachidonic acid. Prelabeled cells were rinsed twice and chased in label-free medium to minimize unesterified label, and exposed to experimental condi-

tions. At this time $> 99.9\%$ of fatty acid label was esterified. Table III shows ^3H label in lipid classes after 5 h of treatment. The column *Control "0 time"* shows radiolabel in lipids prior to treatment. Cells without antimycin and with 450 mg/dl glucose (control 5 h), or without glucose, showed decreases of label in phospholipids compared with 0 time controls. With antimycin, cells that had been with 25 mg/dl of glucose or higher showed insignificant or negligible changes in total or individual phospholipids, with respect to the 5 h controls. In contrast, significant decreases of label in phospholipids occurred in antimycin-treated cells with 10 mg/dl of glucose or no glucose. These changes were accompanied by increases in diglycerides and triglycerides. Major increments of label in triglycerides occurred in all cells treated with antimycin, even with 450 mg/dl glucose. However, the most dramatic alteration observed was in the unesterified fraction of fatty acids. In cells with antimycin and no glucose, unesterified fatty acid label was ≈ 50 -fold greater than that present in cells at 0 time and ≈ 21 times that present in the 5-h time control. In contrast, cells with antimycin and 10 mg/dl of glucose or higher showed modest increments compared with the 0 time control and negligible or no increments compared with the 5-h time control. The time course of changes in the neutral glyceride and unesterified fatty acid fractions in a similar experiment is

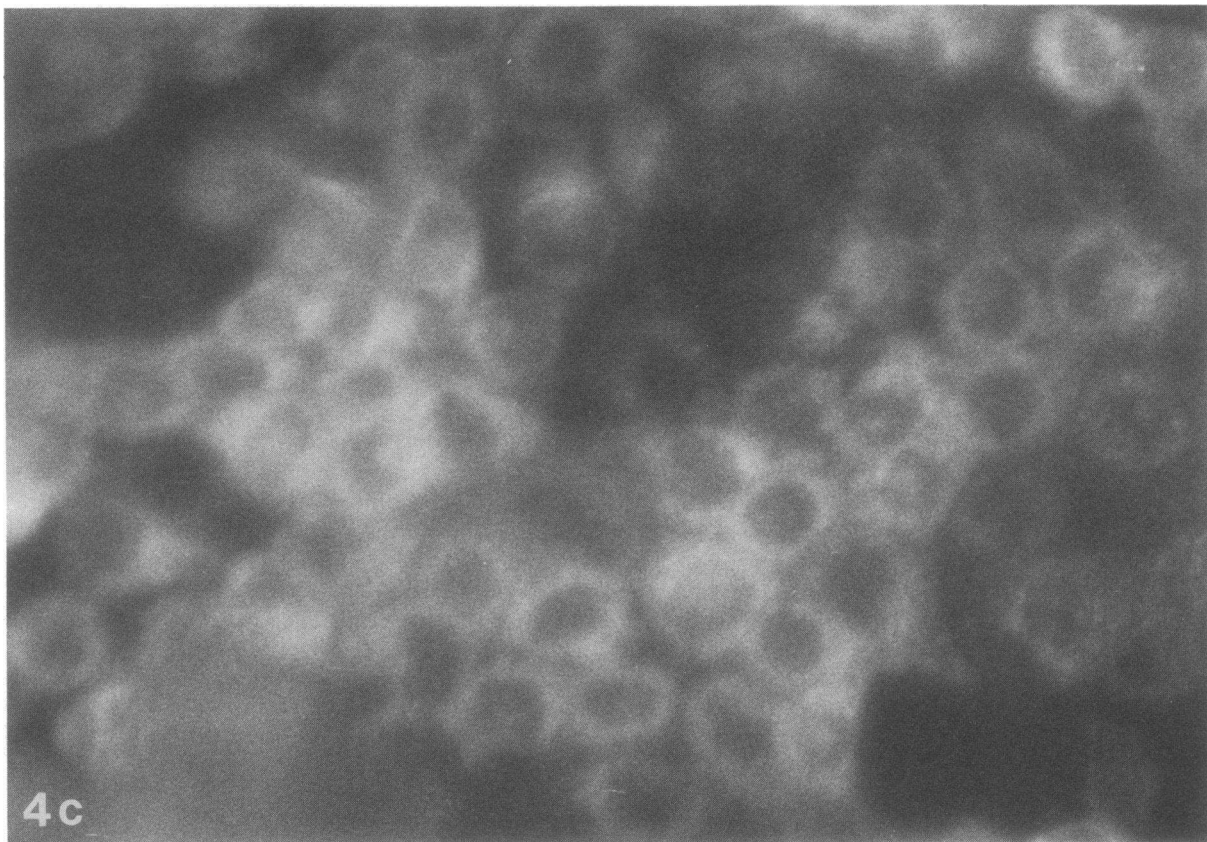
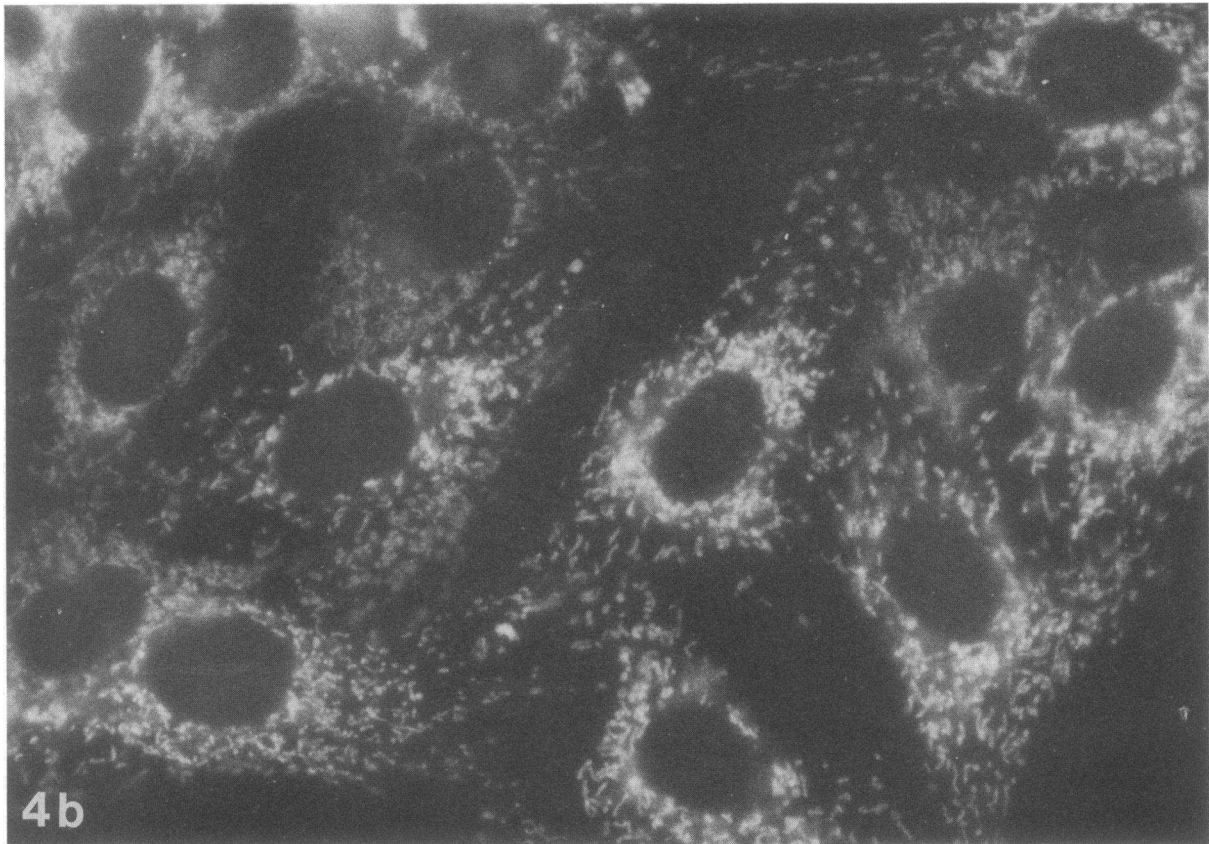


Figure 4 (Continued)

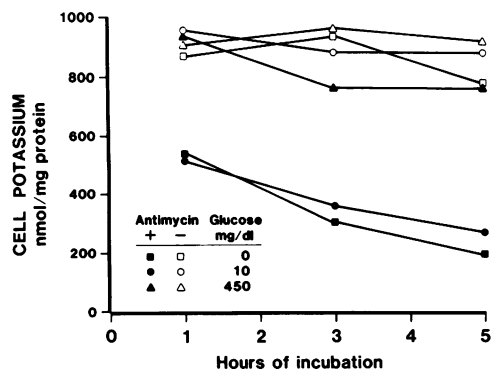


Figure 5. Cell potassium. Cells were exposed to medium with or without antimycin and 0, 10, or 450 mg/dl glucose, after which cell K⁺ was measured by atomic absorption spectrometry. Marked decreases of cell K⁺ are seen both in cells with antimycin and 10 mg/dl or no glucose. However, at 5 h, cells with antimycin and 10 mg/dl glucose have significantly more K⁺ than those without glucose (266±28 vs. 186±20 nmol/mg cell protein. Mean±SEM, n = 5, P < 0.02).

shown in Table IV for cells treated with antimycin in glucose-free medium. Progressive increases occurred in unesterified fatty acids, diglycerides, and triglycerides.

Lipids labeled with [¹⁴C]acetate. Cells were labeled to steady state with [¹⁴C]acetate and exposed to experimental conditions as before, either with radiolabel in the medium, without radiolabel, or without radiolabel after a 16-h chase in label-free medium. The results with all three protocols were similar, suggesting that changing specific radioactivity in intracellular acetate pools did not substantially affect the results. These results with a label that is incorporated into all fatty acids confirmed the observations presented in Tables III and IV. There was significant decline of label in phospholipids when cells were exposed to antimycin in medium without glu-

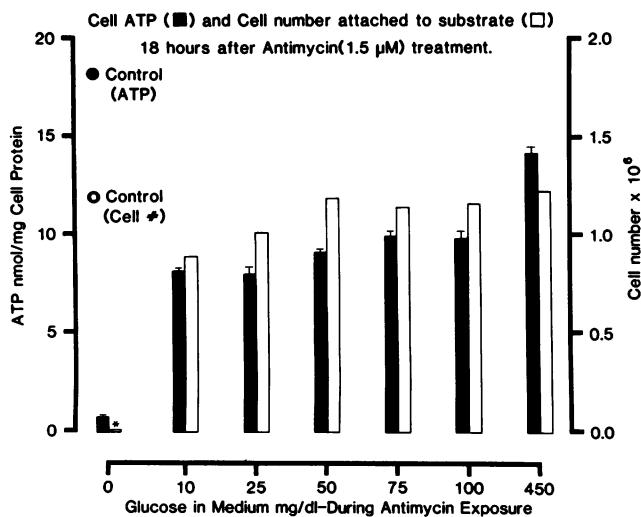


Figure 6. Cell ATP and number of attached cells 18 h after recovery in medium without antimycin after 5 h of treatment with antimycin and 0–450 mg/dl glucose. ATP was measured in attached cells + floating cells (solid bars, n = 4). Attached cells, which had normal morphology, were detached with trypsin-EDTA and counted (n = 2).

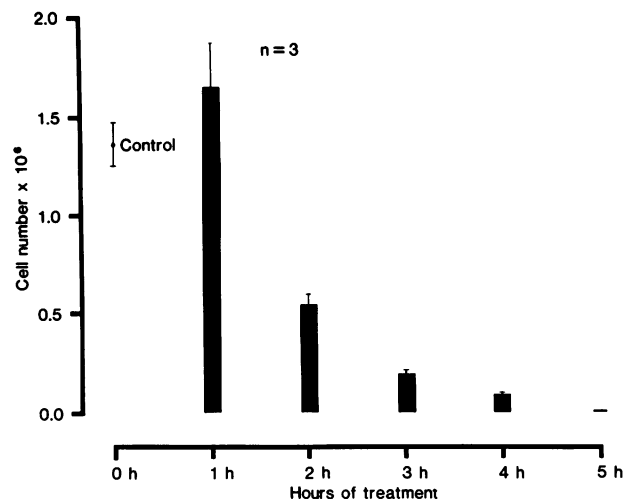


Figure 7. Number of attached cells 18 h after recovery in control medium, after treatment in medium with 1.5 μM antimycin and no glucose for 1–5 h. Number of attached cells after recovery decreases with increasing durations of experimental treatment.

cose or 10 mg/dl glucose, compared with cells in medium with 450 mg/dl glucose with, or without, antimycin (data not shown). There was a major time-dependent increase of label in unesterified fatty acids in cells treated with antimycin in glucose free medium, but not in any other group (Fig. 8).

Changes in lipid mass. Cells were exposed to medium with 450 mg/dl, 10 mg/dl, or no glucose, with or without antimycin for 5 h. The results are shown in Table V, for total phospholipids and phosphatidylcholine. Major declines of phospholipids of similar magnitude were observed in cells treated with antimycin and either 10 mg/dl or no glucose. Smaller declines were seen also in cells without antimycin and either 10 mg/dl or no glucose, and cells treated with antimycin and 450 mg/dl glucose.

Unesterified fatty acids were measured in cells grown in serum-free medium and exposed to experimental conditions as before. Cells grown with serum could not be used, as serum-derived fatty acids adsorbed to culture dishes and were measured in lipid extracts of cells (not shown). Fatty acids were quantified as methyl esters by gas chromatography. The results for total unesterified fatty acids as they relate to cell ATP content are shown in Fig. 9. Fatty acids of cells incubated without antimycin were not different from unperturbed controls at 0 time (not shown). Similarly, cells incubated with antimycin and 50, 100, or 450 mg/dl glucose (which had > 7 nmol ATP/mg protein), had normal levels of total unesterified fatty acids (Fig. 9). In contrast, cells with antimycin and 10 mg/dl glucose, or no glucose, both of which had unmeasurable ATP in this experiment, showed marked increments. The increments affected all individual fatty acids that could be identified and measured with accuracy (not shown). ATP in cells with antimycin and 25 mg/dl glucose was 0.812 nmol/mg protein, which is < 5% of control values, on the borderline of the threshold that appeared to determine viability. Correspondingly, there was a smaller increment of fatty acids. By microscopy, cells in this group showed evidence of severe cell injury (not shown). It may be noted that ATP in cells shown in Fig. 9 is lower (for each level of glucose in medium) than in similarly treated cells shown in Fig. 1. This is because cells had

Table III. Lipids in Cells Labeled with [³H]Arachidonic Acid

	Control 0 time	Glucose (mg/dl)		Antimycin 1.5 μM				
		450	0	450	25	10	0	
		-	-	+	+	+	+	
		<i>dpm × 10³/mg cell protein</i>						
Total phospholipids	2,268.1±41.9	2,054.8±11.2	2,142.8±31.8	1,936.4±67.7	2,200.1±41.7	1,950.7±15.0*	1,750.3±22.7*	
Phosphatidylcholine	1,353.3±18.8	1,153.0±11.4	1,222.8±31.2	1,085.3±38.8	1,207.4±16.7	1,052.3±11.0*	940.7±27.5*	
Triglycerides	456.5±41.7	476.7±18.5	404.5±16.2	808.5±25.3*	847.9±20.2*	957.1±52.7*	658.7±38.5*	
Diglycerides	18.8±0.7	33.5±1.4	32.4±0.3	31.8±0.9	34.0±0.9	41.1±1.4*	88.4±0.2*	
Unesterified fatty acid	3.1±0.2	7.3±0.3	10.5±0.3*	7.7±0.4	7.3±0.2	9.2±1.3	154.1±18.8*	

Cells were labeled with [³H]arachidonic acid as indicated in the text for 20 h, chased with fresh serum containing medium without label for 2 h, and studied. The first column, control 0 time, indicates the distribution of label in lipid classes immediately after chase. The other columns, with the indicated conditions, show distribution of label in lipids 5 h after treatment after the 2-h chase. Values given as ±SEM (n = 4). * P < 0.01 compared with the 5-h control, treated without antimycin and with 450 mg/dl glucose.

to be grown in serum-free medium which slowed their growth (not shown) and appeared to affect the utilization of glucose for ATP generation (as shown in Fig. 9).

Triglycerides were separated by TLC of neutral lipid fractions of lipid extracts and measured as total fatty acid methyl esters after saponification and methylation. Cells were treated with experimental media as before, for unesterified fatty acids. The results are shown in Table VI. Compared with unperturbed controls, and 5-h time controls without antimycin, there was an increase of the lipid in cells with antimycin and 100 mg/dl or less of glucose. With greater deprivation of glucose, there was progressively greater increase of the triglycerides.

Discussion

The requirements of ATP for cellular enzymatic reactions have previously been determined in vitro, using broken cell preparations or purified enzyme-substrate systems. However, little is known about the minimum levels of ATP needed for the energy-dependent reactions which maintain membrane integrity in living cells. This type of information is necessary for the understanding of how cells are damaged when energy metabolism is impaired in pathologic states (1). It might be assumed, a priori, that major reductions of ATP would be attended by breakdown of energy-dependent reactions in cells

and compromise cell integrity. These could include vital functions such as ion homeostasis and the reconstitution of membrane lipids lost by catabolism. We were surprised by our results. Although severe energy deprivation (manifested by ATP levels under 10% of control values and major reductions of energy charge and phosphorylation potential) did affect a number of parameters studied, LLC-PK₁ cells maintained their overall integrity for long periods of time. ATP levels had to be virtually unmeasurable, along with correspondingly severe reductions of energy charge and phosphorylation potential, before cell viability was compromised. It may be noted here that the evolution of damage in energy deprived LLC-PK₁ cells was slower than what is observed in vivo in energy-deprived parenchymal cells of ischemic organs. This may relate to differences in the rates of decline of ATP in the affected cells. These rates depend on how fast ATP is consumed by energy-dependent functions such as ion homeostasis; fundamental membrane properties such as ion permeability may therefore determine the sensitivity of cells to circumstances that decrease ATP synthesis (1). Of interest, even within the same organ, different types of cells showed major differences in the rates of ATP decline during ischemia (39).

Our experimental design was prompted by the high rates of glycolysis in LLC-PK₁ cells and the reproducibility with which stepwise glucose deprivation led to decrements of ATP in anaerobic cells. Another approach could have been to remove

Table IV. Lipids in Cells with [³H]Arachidonic Acid

Control 0 time	Incubation (h) with antimycin 1.5 μM and glucose 0 mg/dl					
	1	2	3	4	5	
	<i>dpm × 10³/mg cell protein</i>					
Unesterified fatty acids*	3.0±0.2	17.2±0.6	20.0±0.8	48.9±3.0	76.5±3.5	127.3±5.4
Diglycerides [‡]	17.4±0.6	20.3±0.6	29.8±0.6	45.4±2.1	52.3±2.0	63.9±2.7
Triglycerides [§]	182.0±8.4	321.1±1.5	349.5±3.4	404.9±12.1	410.2±18.0	568.9±5.2

Cells were labeled and chased exactly as in Table II, after which they were all exposed to 1.5 μM antimycin in glucose-free medium for 1–5 h. The data show the distribution of label in lipids 1–5 h after treatment compared to control 0 time immediately after chase period. Values given as ±SEM (n = 4). Significance levels for the increase of label in lipid with time, compared to control: * P < 0.001, ‡ P < 0.05, § P < 0.01.

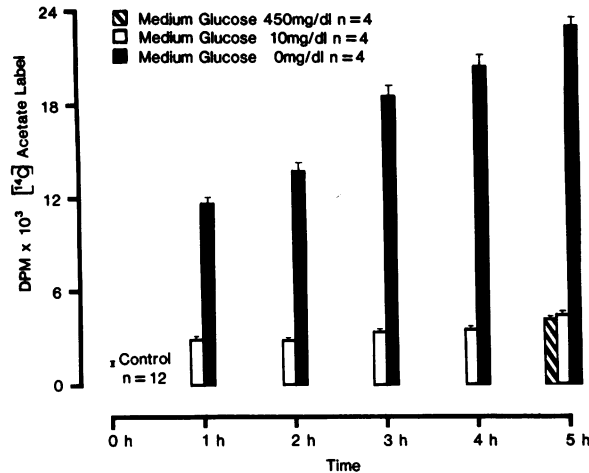


Figure 8. [¹⁴C]acetate label in chromatographically separated unesterified fatty acids from cells treated with antimycin and 450 mg/dl glucose, 10 mg/dl glucose, or no glucose for 1–5 h. Compared with unperturbed 0 time controls, significant, modest increases occurred with antimycin and 450 mg/dl glucose (*hatched bars*) or 10 mg/dl glucose (*empty bars*), and in cells treated without antimycin (*not shown*). With antimycin and no glucose, there were progressive, sustained, and major increments of unesterified fatty acid label (*solid bars*). Relevant differences were highly significant.

glucose from the medium and to regulate mitochondrial function by using graded doses of an inhibitor of electron transport. In this fashion, rotenone, a complex I inhibitor of oxidative phosphorylation (24), was used by Soltoff and Mandel (40) to study energy requirements for proximal tubule transport. A criticism of our design might be that there are functionally separate pools of ATP in cells related to oxidative and anaerobic modes of ATP generation, and that cells might be differentially susceptible to their depletion. However, it is debatable whether functionally separate pools of ATP exist. Moreover,

Table V. Phospholipids in Cells Measured as Lipid Phosphorus

Incubation	Total phospholipid μg/mg cell protein	Phosphatidylcholine μg/mg cell protein
Control (0 time)	4.87±0.03	2.46±0.03
No antimycin + 450 mg glucose	4.87±0.04 (0)	2.36±0.03 (-4)
No antimycin + 0 glucose	4.64±0.04* (-5)	1.99±0.08* (-19)
Control (0 time)	4.14±0.06	1.96±0.04
1.5 μM antimycin + 450 mg glucose	3.88±0.03* (-6)	1.72±0.02* (-12)
1.5 μM antimycin + 0 glucose	3.37±0.04* (-19)	1.36±0.02* (-31)
Control (0 time)	4.38±0.13	2.26±0.08
No antimycin + 10 mg glucose	4.10±0.03 (-6)	1.95±0.02* (-14)
1.5 μM antimycin + 10 mg glucose	3.75±0.10* (-14)	1.45±0.04* (-36)

Cells were treated as indicated for 5 h prior to lipid extraction, or extracted at the beginning of the 5-h period (control 0 time). The data are from three separate studies, done on three different days with 0 time controls for each experiment. Values given as ±SEM ($n = 4$; each data point = two 100-mm dishes). Figures in parentheses show percent deviations from control 0 time over 5 h.

* $P < 0.005$ vs. control.

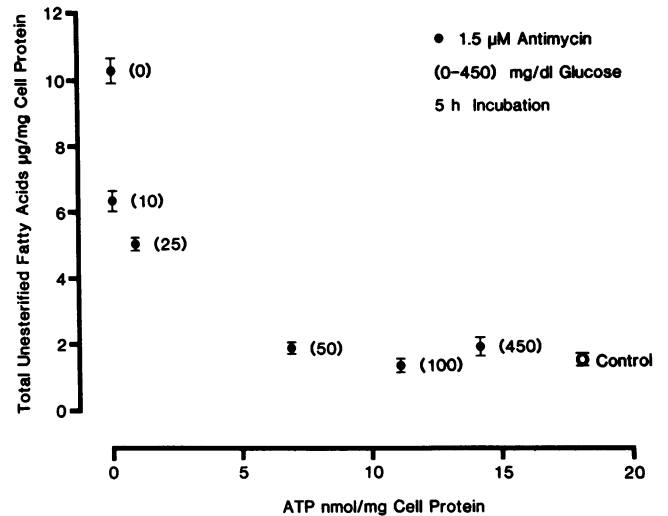


Figure 9. Unesterified fatty acid mass. Cells grown in serum-free DME were exposed to antimycin and 0–450 mg/dl glucose for 5 h. In identically treated dishes, cell ATP was extracted (*x*-axis), and unesterified fatty acids measured as fatty acid methyl esters by gas liquid chromatography (shown as total unesterified fatty acids, *y*-axis). Cells with unmeasurable ATP or marginal levels of ATP show elevations of unesterified fatty acid levels, $n = 4$. Relevant differences were highly significant.

in preliminary studies, we have found that graded suppression of ATP synthesis by rotenone in glucose deprived LLC-PK₁ cells led to virtually similar patterns of cell injury reported here (data not shown).

The effects of antimycin and glucose deprivation in LLC-PK₁ cells can be largely attributed to ATP depletion, rather than glucose deprivation or some effects of antimycin per se. First, glucose-deprived cells without antimycin maintained high ATP levels and normal ultrastructure, were viable, and showed only modest perturbations of lipid metabolism. Secondly, cells with antimycin and 450 mg/dl glucose, which had relatively high ATP levels, showed no changes, or only modest perturbations of structure, function, and lipid composition. The results also show that cell ATP needs to be reduced to very low levels (~ 5–10% of control) before injury can be detected by electron microscopy. Even in such cells with very low ATP, the majority of cells were either normal or showed only mild alterations which have been characterized as reversible (9). Because such cells were able to regenerate ATP, remained attached to plastic substrates, excluded trypan blue, and recovered normal morphology, the injury they suffered can be characterized as reversible. On the other hand, cells with unmeasurable ATP underwent injury which was progressive and irreversible with time (Figs. 2–6).

We obtained indexes (mitochondrial potential, cell K⁺) that would report on the functions of two membrane-based ion pumps (H⁺ ATPase, Na⁺-K⁺ ATPase) for two reasons. First, we examined the “functional” availability of energy in cells shown to have different levels of ATP by chemical measurement. Monitoring these functions provided a “bioassay” for the low levels of ATP found in some experimental cells. Secondly, we examined ATP driven functions that are dependent not only on the integrity of the enzyme proteins, but also of the lipid membranes in which they are embedded. Diminution or abolition of an ion gradient (K⁺, H⁺) implied that ATP

Table VI. Triglycerides in Cells Measured as Triglyceride Fatty Acids

Control 0 time ↓	Glucose (mg/dl)	450	0	450	100	50	25	10	0	
	Antimycin 1.5 μM	-	-	+	+	+	+	+	+	
μg/mg cell protein										
1.051±0.07	0.91±0.04	1.56±0.16*	1.09±0.10	2.39±0.10*	2.47±0.12*	2.58±0.12*	2.92±0.03*	3.06±0.12*		

Triglycerides were measured as the mass of total triglyceride fatty acids by gas chromatography. Cells were grown in serum-free medium and exposed to antimycin and different amounts of glucose exactly as in Fig. 9. At the beginning of the experiment (control 0 time), or at the end of 5 h, lipid extracts were prepared for analysis. Values given as ±SEM ($n = 4$). * $P < 0.01$.

was not available, the enzyme was damaged, or the barrier functions of the membranes had been compromised. Preserved function implied not only that ATP was available, but that the enzymes and their lipid domains were reasonably intact and that the membranes were undamaged to the extent that they could maintain ion gradients.

Examined in this light, our results provide further evidence which suggests that LLC-PK₁ cells maintain the integrity of their membranes to a large extent even in the face of a very low energy supply. Although the Rh 123 fluorescence technique is not quantitative, the data suggest that mitochondria performed a complex ATP-driven, membrane-based enzymatic function (H⁺ extrusion), mounted electrical potential, and maintained it without dissipation. When ATP was unmeasurable for a long period of time, mitochondria were unable to mount a potential (this might be explained by lack of ATP) or recover function in normal medium. It seems likely in the latter case that in addition to, or instead of, damage that may have affected the protein (ATP synthetase-H⁺ ATPase) itself, there was damage in the lipid membranes of the mitochondria. Consistent with this view, the cells showed ultrastructural abnormalities in mitochondrial membranes such as dissolution and loss of continuity, and the formation of membrane stacks. Previous studies have shown that depletion of ATP induces hydrolysis of phospholipids and the accumulation of unesterified fatty acids in the membranes of mitochondria incubated in vitro (41). Alternatively or in addition to membrane disintegration, substances with the properties of "uncouplers" might have accumulated in the mitochondria, causing dissipation of potential. In fact, there was a marked and progressive increase in the content of unesterified fatty acids in cells with unmeasurable ATP. Fatty acids are uncouplers of oxidative phosphorylation (38, 42-45). In this context, it is interesting to note that studies on mitochondrial function in anoxically injured cardiac myocytes revealed evidence for "leakiness" of mitochondrial inner membranes, and uncoupling of respiration (15).

The data on cell K⁺ are also consistent with a role played by a relatively small amount of ATP in the preservation of membrane integrity. Cells with very low ATP showed losses of cell K⁺, consistent with diminished Na⁺ pump activity, but not to the extent which occurred in cells without measurable ATP. Again, the results suggest that cells in which ATP is low, but measurable, maintained a degree of plasma membrane integrity that permitted not only a low level of ATP driven pump activity, but also a steeper K⁺ gradient, than in cells with unmeasurable ATP.

We undertook the study of cell lipid composition and metabolism in this model with the presumption that we might identify lipid defects associated with energy depletion, and more specifically, lipid defects associated with irreversible loss of membrane integrity. Our results show that treatment of cells with antimycin induced a decrease of cell phospholipids, compared to time controls without antimycin, as well as 0 time controls (Tables III and V). In cells labeled with [³H]-arachidonic acid, significant decreases were seen only in cells in which ATP was either very low (< 10% of control), or unmeasurable. Chemically quantitated as lipid phosphorous, marked declines (up to 19% total phospholipid, up to 36% phosphatidylcholine) occurred in both groups. Smaller but statistically significant declines were seen also in cells with antimycin and 450 mg/dl glucose and in cells without antimycin deprived of glucose (both groups would have relatively high levels of ATP). These decreases of phospholipids in energy-deprived cells were accompanied by increments of radiolabel and mass, in triglycerides. Increases of [³H]arachidonic acid label in triglycerides occurred in all antimycin-treated cells and increments of triglyceride mass were shown in all antimycin treated cells incubated with 100 mg/dl glucose or lower (Tables III and V).

The data also identify a lipid abnormality that occurs uniquely in cells without an energy source, i.e., in cells in which both oxidative phosphorylation and glycolysis had been inhibited. Thus, a massive increase of unesterified fatty acids occurred only in those groups of cells in which ATP either became unmeasurable eventually, or declined to values < 5% of normal. The increase equally affected saturated, monounsaturated, and polyunsaturated fatty acids. Large increments of unesterified fatty acids occurred early, within an hour of incubation in experimental medium, and was progressive with time (Table IV; Fig. 8). Therefore, the increments began to occur in viable cells as ATP levels were precipitously declining, even before they became unmeasurable (Fig. 2 a).

The contrasting patterns of inability to maintain phospholipid levels, but ability to increase triglycerides in energy poor cells, and the accumulation of unesterified fatty acids in cells without an energy source need to be explained. It is instructive to compare lipid alterations seen in cells with antimycin, and those in cells without the inhibitor. 5 h of incubation in label-free complete medium, without antimycin ("time control") resulted in significant loss of [³H]arachidonic acid label from phospholipids, when compared to controls at 0 time (Table III), but this was not accompanied by net loss of phospholipid mass (Table IV). In contrast to cells with antimycin, cells in-

cubated without the inhibitor and with 450 mg/dl glucose showed no changes in triglycerides with respect to unperturbed 0 time controls (Tables III and VI). These patterns of changes in radiolabel and lipid mass are consistent with the supposition that phospholipids were being deacylated during the experimental incubation period even in control cells, but that they were being reconstituted, presumably via reacylation or *de novo* synthesis. Moreover, it appears that fatty acids, derived by deacylation of phospholipids, were diverted for acylation onto triglycerides in antimycin-treated cells, whereas they may have been otherwise normally metabolized by oxidation. The data are also consistent with the supposition that in cells with low levels of ATP, there was a failure of energy-dependent mechanisms which reconstitute phospholipids (by reacylation or *de novo* synthesis), but that mechanisms responsible for triglyceride synthesis continued to be operative (perhaps because ATP requirements for triglyceride synthesis are lower).² In any case, unesterified fatty acids did not accumulate as long as cell ATP remained above a critical threshold. In cells without an energy source, all energy-dependent mechanisms that can dispose of fatty acids may have failed, accounting for their increase.

We are unable to comment on whether depletion of phospholipids from energy-deprived cells occurred exclusively by attrition (i.e., failure of reacylation or synthesis due to lack of energy), by accelerated hydrolysis due to phospholipase "activation," or both. It could be that both mechanisms operate under conditions of severe energy deprivation (46). Membranes in ATP-depleted mitochondria and cells have been shown to be susceptible to hydrolytic attack by phospholipases (41, 47–50). In this context, it is interesting to consider that depletion of phospholipids, to the extent which occurred in LLC-PK₁ cells with the lowest ATP levels, did not correlate with loss of viability. Phospholipid loss was similar in reversibly as well as lethally injured cells. Rather, massive increase of unesterified fatty acids, occurring over a prolonged period, stood out as a unique feature which was associated with severe cell injury, and loss of viability.

The present data do not indicate whether fatty acids were the mediators of energy deprivation-associated injury in LLC-PK₁ cells, and, if so, how they have may have been injurious. However, unesterified fatty acids are potentially injurious to membranes and cells by virtue of their properties as detergents, inhibitors of membrane based enzymes and uncouplers of oxidative phosphorylation (38, 42–45, 51–54). Treatment of lymphocytes and hepatocytes with unsaturated fatty acids uncoupled mitochondria and decreased ATP levels (38, 43). Exposure of LLC-PK₁ cells, and primary cultures of proximal tubules to unsaturated fatty acids *in vitro* led to blebbing of the plasma membrane, and cell death (22, 55). Of interest, accumulation of unesterified fatty acids has been observed in several models of organ ischemia and anoxia (19, 21, 22, 56, 57), and *in vitro* models which simulate anoxia (17).

In this context, the question may arise whether fatty acids

in energy-deprived cells increase to the levels which may prevail in cells injured by exogenously provided lipid. Although we can calculate intracellular concentrations of fatty acids in cells injured by energy deprivation (from the data in Fig. 9), this issue cannot be addressed until similar measurements can be made reliably in cells which have been injured by the provision of exogenous fatty acid. Technical difficulties in the preparation of cells to remove adsorbed extracellular lipid preclude such measurements at the present time. Moreover, being amphipathic, fatty acids partition into membranes and bind to proteins; their concentrations at the target sites would be the more relevant parameters to study. These questions remain to be addressed in future studies.

Acknowledgments

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2. Theoretically, triglyceride synthesis may also have been favored in cells with antimycin and provided glucose, due to augmented availability of reducing equivalents (required for fatty acid synthesis), under conditions where ATP was still being generated. Because fatty acids were being deacylated from phospholipids in such cells and were therefore available in abundance, *de novo* synthesis of fatty acids and their esterification onto glycerol need not be invoked as a mechanism to explain our findings.

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