Ribeiro, A. A., & Dennis, E. A. (1975b) *Biochemistry 14*, 3746–3755.
Rosch, M. (1967) in *Non-Ionic Surfactants* (Schick, J. J., Ed.) pp 753–773, Marcel Dekker, New York.
Satto, H., & Shinoda, K. (1967) *J. Colloid Interface Sci. 24*, 10–15.
Schmidt, C. F., Barenholz, Y., & Thompson, T. E. (1977) *Biochemistry 16*, 2649–2656.
Seiter, C. H. A., & Chan, S. I. (1974) *J. Am. Oil Chem. Soc. 95*, 7541–7553.
Shah, D. O., & Schulman, J. H. (1976) *Lipids 2*, 21–27.
Sheetz, M. P., & Chan, S. I. (1972) *Biochemistry 11*, 4573–4581.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Chapter 6, Wiley, New York.
Yedgar, S., & Gatt, S. (1976) *Biochemistry 15*, 2570–2573.
Yedgar, S., Barenholz, Y., & Cooper, V. G. (1974a) *Biochim. Biophys. Acta 363*, 98–111.
Yedgar, S., Hertz, R., & Gatt, S. (1974b) *Chem. Phys. Lipids 13*, 404–414.

Cytoplasmically Inherited Respiratory Deficiency of a Mouse Fibroblast Line Which Is Resistant to Rutamycin†

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ABSTRACT: Mouse fibroblasts resistant to the drug rutamycin were isolated and found also to be respiratory deficient. These cells produce large amounts of lactic acid, and oxygen consumption data indicate that the first complex of the electron transport chain, NADH–coenzyme Q reductase, is defective. Levels of rotenone-sensitive NADH–cytochrome *c* reductase and pyruvate decarboxylase of the pyruvate dehydrogenase complex are markedly depressed in the mutant cells. Other components of the electron transport chain appear to be fully functional. The mutant cells were enucleated and fused with another cell line, and the resulting cybrid demonstrated a similar pattern of respiratory deficiency as did the original mutant. These results indicate that this defect in respiration is a cytoplasmically inherited characteristic in this cell line.

Respiratory deficiency has recently been studied in several mammalian cell lines (DeFrancesco et al., 1976; Soderberg, et al., 1977). One has been shown to be defective in NADH–coenzyme Q reductase (DeFrancesco et al., 1976) and another lacks succinate dehydrogenase activity (Soderberg et al., 1977). These cell lines all exhibit a high rate of glycolysis during growth and consume oxygen at significantly lower rates than do the parental cell lines. We have recently isolated a line of mouse fibroblasts resistant to the drug rutamycin (Lichtor & Getz, 1978), an inhibitor of mitochondrial ATPase. Four subclones of this mutant were derived from the initial population, and all of these were also found to be respiratory deficient. The mutagenesis procedure involved selectively introducing BrdUrd (5-bromodeoxyuridine) into the mitochondrial genome of a line of mouse fibroblasts (clone 1 D) lacking cytoplasmic thymidine kinase activity. The ATPase activity of mitochondria isolated from these cells was resistant to rutamycin. The rutamycin resistant mutants were enucleated with cytochalasin B and fused with mouse A 9 cells resistant to 8-azaguanine and sensitive to rutamycin. Cytoplasmic hybrids or cybrids were selected as cells resistant to rutamycin and 8-azaguanine and appeared at a high frequency. ATPase activity of mitochondria isolated from these cybrid cells was also resistant to rutamycin. Other fusions between rutamycin-resistant nucleated cells and A 9 resulted in many fewer resistant colonies, and only a small number of these continued to grow for any length of time. Finally, fusions between enucleated clone 1 D cells and A 9 cells produced no rutamycin-resistant colonies. These results indicate that rutamycin resistance is a cytoplasmically inherited characteristic in this cell line.

In this paper the respiratory characteristics of both the original mutant and the cybrid cells are described. The capacity to grow respiratory-deficient cells in tissue culture in reasonable yield indicates the possibility for the isolation and study of mitochondrial mutants.

Experimental Procedures

Growth Conditions. Cells were grown on Corning flasks or glass roller bottles in RPMI medium 1640 supplemented with 10% fetal calf serum, 2.0×10^{-3} M L-glutamine, 100 IU/mL penicillin, and 0.1 mg/mL streptomycin in a 37 °C incubator. The cultures were periodically checked for mycoplasma, and any contaminated cultures were discarded. It was determined that clone 1 D is capable of growing in the presence of rutamycin, an analogue of oligomycin, at concentrations up to 1.0×10^{-8} g/mL; however these cells were not able to divide even once in the presence of rutamycin at a concentration of 1.0×10^{-7} g/mL.

Growth Curves. A number of 25-cm^2 Falcon flasks were seeded with 1.5×10^5 cells each. On the following day varying concentrations of antibiotics were added. Cells from two flasks containing each concentration of the drug being tested were harvested and counted in a Coulter counter at intervals of several days.

Isolation of Mitochondria. The harvesting of cells, their storage, and isolation of mitochondria have been described (Lichtor & Getz, 1978).

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Succinate Cytochrome c Reductase Assay. Succinate cytochrome *c* reductase activity was determined by measuring the change in optical density at 550 nm by the procedure of Rabinowitz & de Bernard (1957). Activity was measured in both whole homogenate and isolated mitochondria and was expressed as micromoles of cytochrome *c* reduced per minute per milligram of protein used in the assay.

Lactate Determination. The lactate concentration of the growth medium was measured by the procedure of Mattenheimer (1969).

Oxygen Uptake. Oxygen consumption was measured with a Gilson Oxygraph while the suspension was stirred with a magnetic stirrer. Cells were suspended in air-saturated PBS buffer (0.0085 M Na_2HPO_4, 0.0016 M $NaH_2PO_4 \cdot H_2O$, and 0.15 M NaCl, pH 7.5) while freshly harvested mitochondria were suspended in air-saturated isotonic buffer (0.225 M sucrose, 5 mM $MgCl_2$, 20 mM KCl, 10 mM KPO_4, pH 7.4, and 20 mM triethanolamine, pH 7.4). The full scale of the instrument was set to correspond to the range of oxygen contained in either PBS or isotonic buffer. These solutions were initially saturated with air.

Malate Dehydrogenase Assay. Isolated mitochondria were sonicated 4 times for 15 s at 6 A in a Branson Sonifier prior to being assayed. Malate dehydrogenase was measured according to the method of Roodyn et al. (1962). One unit of enzyme converts 1 μmol of substrate per min at 25 °C.

Glutamate Dehydrogenase Assay. Glutamate dehydrogenase was measured with sonicated mitochondria by using the procedure of Martin & Denton (1970). One unit of enzyme converts 1 μmol of substrate per min at 25 °C.

Pyruvate Decarboxylase Assay. Pyruvate decarboxylase of the pyruvate dehydrogenase complex was assayed with isolated mitochondria according to the procedure of De-Francesco et al. (1976). Released $^{14}CO_2$ was trapped and counted. One unit of enzyme releases 1 nmole $^{14}CO_2$ per min at 30 °C.

NADH–Cytochrome c Reductase Assay. NADH–cytochrome *c* reductase activities were measured spectrophotometrically with sonicated mitochondria by following the reduction of cytochrome *c* at 550 nm according to a modification of the procedure described by Rabinowitz & de Bernard (1957). The assay mixture contained 0.23 mM NADH, 0.03 mM cytochrome *c*, 0.5 mM KCN, and 40 mM phosphate buffer, pH 7.4. Rotenone-insensitive activity was determined by preincubating the enzyme at room temperature for 5 min with 1 μg of rotenone per 20 μg of mitochondrial protein. Both the enzyme with and without rotenone were incubated with the assay mixture for 3 min at room temperature before the addition of NADH in order to insure that the initial rate of reduction of cytochrome *c* was maximal as well as linear. All assays were done at least in duplicate. Activity was expressed as micromoles of cytochrome *c* reduced per minute per milligram of protein.

P/O Ratio Determination. A total of 3.0–5.0 mg of mitochondrial protein was suspended in 1.4 mL of buffer (0.225 M sucrose, 0.5 M glucose, 0.67 mg/mL defatted bovine serum albumin, 20 mM KCl, 5 mM $MgCl_2$, 10 mM KPO_4, pH 7.4, and 20 mM triethanolamine, pH 7.4) in an oxygraph cell. After 5 min of equilibration, succinate was added to give a final concentration of 3 mM. The phosphorylation reaction was initiated by the addition of 8 IU of hexokinase and ADP to give a final concentration of 0.3 mM. The rate of oxygen uptake was then measured for 20–30 min. The reaction was terminated with the addition of 0.1 mL of 35% w/v $HClO_4$. The oxygraph cell was rinsed several times with buffer. The wash solutions and reaction mixture were combined and centrifuged at 3000*g* for 5 min to remove precipitated protein. The supernatant was neutralized with saturated KOH, and this mixture was kept at 4 °C for 30 min. The precipitate was removed by centrifugation at 3000*g* for 5 min, and the supernatant was assayed for glucose 6-phosphate (Hohorst, 1963). From the amount of glucose 6-phosphate formed and the corresponding amount of oxygen consumed, the P/O ratio was calculated.

Transmission Electron Micrographs. Samples for ultrastructural studies were prepared according to a modification of the procedure of Hruban et al. (1976). The medium was first removed from the cultures, and a fixative containing 1% osmium tetroxide buffered with *s*-collidine at pH 7.4 was applied for 10 min at 25 °C. The cells were then scraped off the flasks and spun at 1000 rpm in a clinical centrifuge. The pellet of cells was embedded in Epon, and sections were cut on a Porter-Blum microtome and stained with uranyl acetate and lead citrate. Selected areas were photographed in a Siemen's Elmiskop IA electron microscope.

Phospholipid Analysis. A number of 75-cm^2 Falcon flasks were seeded with 5.0×10^5 cells each and grown in 30 mL of medium containing 0.5 mCi of $^{32}P_i$. The cells were allowed to proliferate for 6 days, and the medium was replaced with fresh medium containing the same amount of $^{32}P_i$ after 3 and 5 days. Phospholipids were analyzed by using a two-dimensional thin-layer chromatographic procedure (Getz et al., 1970), and the spots were detected with a spray (Vaskovsky & Kostetsky, 1968). The spots were then scraped off and counted.

Results

Lactic Acid Production. The pH of the medium in which the rutamycin-resistant cells are grown falls much more rapidly than does that of normal cells. This is attributable to the much greater lactic acid production by the rutamycin-resistant cells than by clone 1 D (Figure 1). This was the case regardless of whether or not the mutants were grown in the presence of rutamycin. Each of the four rutamycin-resistant subclones produced more lactic acid than clone 1 D. The cybrid cells produced by the fusion of the cytoplasm of mutant subclone 1 and A 9 produce amounts of lactic acid that greatly exceed either of the two parental (mutant or A 9) lines; this is especially notable since the rate of growth of the cybrids is clearly slower than that of the other cell lines and yet they produce lactic acid significantly more rapidly. A 9 also produces more lactic acid than clone 1 D; but, in contrast to the mutant cells and cybrid, this is clearly not attributable to a defect in respiration (see below).

Whole Cell Oxygen Consumption. The endogenous respiration rate of the rutamycin-resistant cells, measured in PBS buffer, was roughly 6% that of the respiratory-competent cells (Table I). This was the case regardless of whether the cells were grown in rutamycin or not. The addition of the uncoupler DNP (2,4-dinitrophenol) stimulated the respiration of mutant subclone 3 and clone 1 D by a ratio of 1.6 and 2.0, respectively, but even in the presence of DNP the respiration rate of the mutants was only about 5% that of clone 1 D. However, the mutant cell respiration could be stimulated about 10-fold with the addition of 3.0 mM succinate such that these cells were consuming oxygen within the range of clone 1 D; 3.0 mM succinate increased the oxygen consumption of clone 1 D by only 20%. Interestingly, the endogenous respiration rate of the cybrid was significantly higher than that of the mutant subclones, and this respiration was stimulated about 50% with the addition of 3.0 mM succinate. The rate of endogenous

Table 1: Whole Cell Oxygen Consumption Data

cell type	medium in which cells were grown (for a period of at least 2 weeks)	nmol of oxygen consumed per min per million cells: PBS buffer	+succinate (3.0×10^{-3} M)	+2,4-dinitrophenol (7.5×10^{-5} M)
clone 1 D	normal medium	2.60	3.17	5.20
A 9	normal medium	0.85	0.99	
mutant subclone 1	normal medium	0.16		
mutant subclone 1	+ 1.0×10^{-7} g/mL rutamycin	0.17		
mutant subclone 2	+ 1.0×10^{-7} g/mL rutamycin	0.16	1.58	
mutant subclone 3	+ 1.0×10^{-7} g/mL rutamycin	0.15	1.74	0.24
enucleated mutant subclone 1 × A9	+ 1.0×10^{-6} g/mL rutamycin	0.91	1.30	

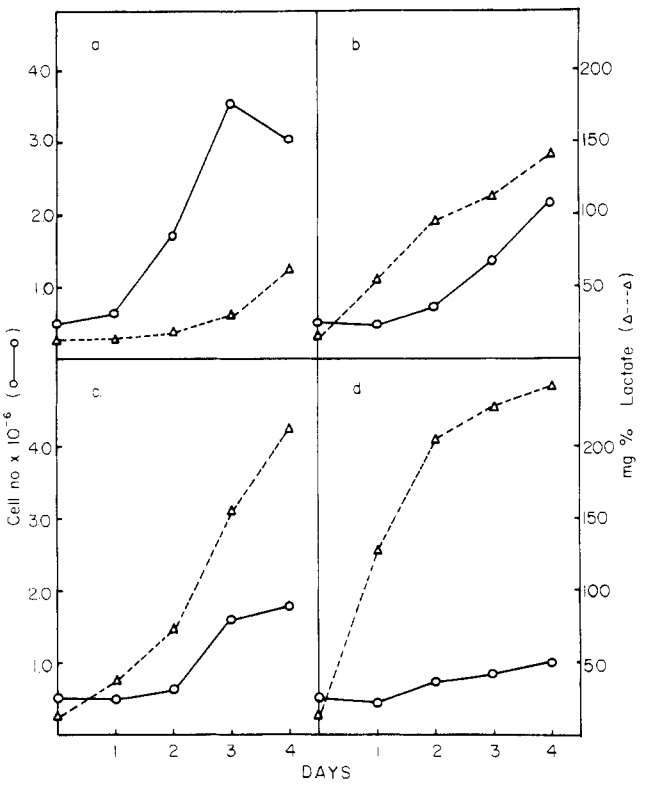


FIGURE 1: Lactic acid production of cell lines grown in normal medium. Solid lines refer to the number of cells per flask (O). Dashed lines refer to the milligram percent lactate in the medium (Δ). (a) Clone 1 D. (b) A 9. (c) Mutant subclone 1. (d) Enucleated mutant subclone 1 × A 9.

respiration of cybrid cells resembles that of A 9, the recipient cell in the formation of the cybrid. However, the cybrid respiration is stimulated to a greater extent by succinate than is that of A 9. In this respect the cybrid resembles the mutant subclones, which respire in the presence of succinate at about the same rate as the cybrid cells.

Respiration of Isolated Mitochondria. As shown in Figure 2 the electron transfer pathway of the various cell lines was examined with the substrate mixture of pyruvate plus malate, which generates NADH, and this respiration was then terminated with rotenone, an inhibitor of NADH dehydrogenase. Succinate was next added as a substrate which reduces FAD and this oxygen consumption was inhibited with antimycin. Finally, ascorbate plus TMPD (*N,N,N',N'*-tetramethyl-*p*-phenylenediamine) was employed for the transfer of electrons to cytochrome *a*. Maximum respiration was seen with all substrates in the presence of 0.035 mM DNP and 0.6 mM ADP. Clearly, the rutamycin-resistant and cybrid cells respire quite poorly in the presence of pyruvate and malate while respiration is normal or even elevated with the other substrates.

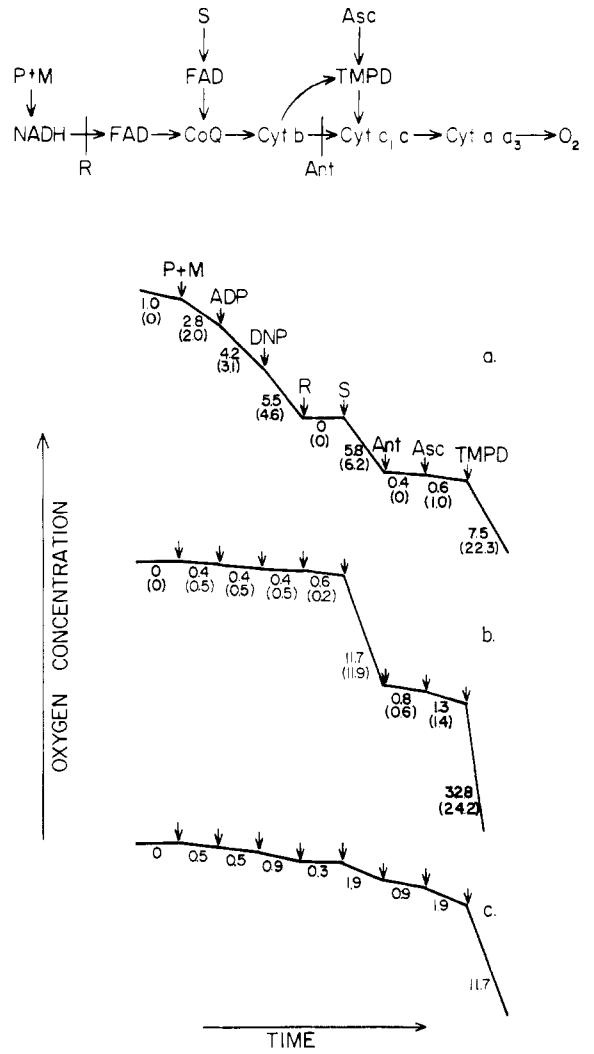


FIGURE 2: Oxygen consumption by isolated mitochondria with various substrates. Respiration is first stimulated by 6 mM pyruvate and malate (P + M) and then followed by the addition of 0.6 mM ADP and 35 μM 2,4-dinitrophenol (DNP) at the times indicated by the arrows. In all assays 2–5 mg of mitochondrial protein was added to the respiration chamber. Rotenone (R) (10 μg/mL) is then used to block this respiration. Succinate (3 mM) (S) is the second substrate to be added, and this respiration is inhibited by 1 μg/mL antimycin (Ant). Finally, 3 mM ascorbate (Asc) and 0.3 mM TMPD are used as substrates. The numbers along the traces refer to the calculated rates of O_2 consumption in nanomoles per minute per milligram of protein. (a) Clone 1 D (numbers in parentheses refer to the results of a similar tracing done with A 9 mitochondria). (b) Mutant subclone 1 (numbers in parentheses refer to the results of a similar tracing done with mutant subclone 2 mitochondria). (c) Enucleated mutant subclone 1 × A 9.

It was also found that respiration of clone 1 D mitochondria supported by 3.0 mM succinate was strongly inhibited by

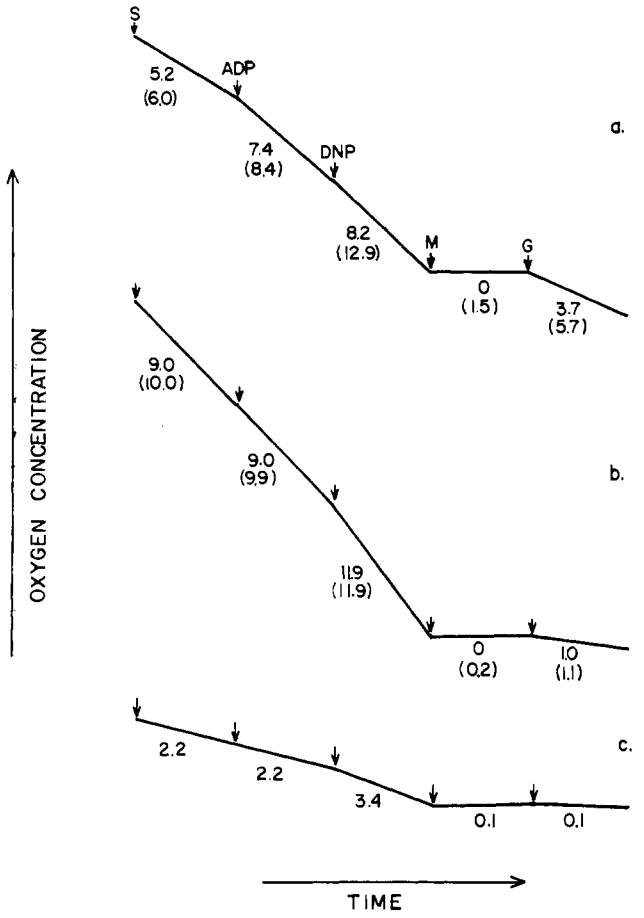


FIGURE 3: Respiration of mitochondria of rutamycin-sensitive and rutamycin-resistant cells with succinate and glutamate as substrates. Respiration is first driven by 3 mM succinate and then followed with ADP and DNP at concentrations of 0.6 mM and 35 μM, respectively. This respiration was then blocked by 5 mM malonate and stimulated again by 6 mM glutamate. The numbers along the traces refer to the calculated rates of O_2 consumption in nanomoles per minute per milligram of protein. The mitochondria used to generate traces a, b, and c are the same as those referred to in the legend of Figure 2.

rutamycin at concentrations ranging from 0.1 to 0.001 μg/mL, whereas respiration with mitochondria from the rutamycin-resistant cells was virtually unaffected by these concentrations of rutamycin.

A different sequence of respiratory substrate additions demonstrates the same pattern (Figure 3). In this case respiration was initiated with succinate and then terminated with malonate, a competitive inhibitor of succinate dehydrogenase. Then the substrate glutamate, which drives respiration by reducing NAD, was added. Again, both the original subcloned mutants and cybrid cells respire with succinate but consume very little oxygen in the presence of glutamate. This phenomenon was seen regardless of whether or not the cells were grown in the presence of rutamycin.

Though the cybrid mitochondria are capable of using succinate as a substrate for respiration, this capacity is limited when compared with that of either the rutamycin-sensitive parent cells or the rutamycin-resistant mutant clones. This is partly attributable to the lower specific activity of cybrid mitochondrial succinate cytochrome *c* reductase compared to that of A 9 and mutant subclone mitochondria (Table II). Whole cell respiration of cybrids is nevertheless as well supported by succinate as is the respiration of mutant subclones. This is partly accounted for by a larger total succinate cytochrome *c* reductase activity presumably representing more total mitochondrial protein present in the cybrid (deduced from

Table II: Succinate Cytochrome *c* Reductase Activity of Mitochondria and Homogenates from Rutamycin-Resistant and Rutamycin-Sensitive Lines

cell type	nmol of Cyt *c* reduced: from isolated mitochondria[a]	nmol of Cyt *c* reduced: from whole cell homogenate[b]	mg of mitochondrial protein per 10^{10} cells
clone 1 D	25.5	0.91	357
A 9	56.8	1.67	294
mutant subclone 1	66.4	2.57	387
mutant subclone 2	79.0	1.81	229
enucleated mutant subclone 1 × A9	27.6	4.81	1743

[a] Nanomoles of cytochrome *c* reduced per minute per milligram of protein. [b] Nanomoles of cytochrome *c* reduced per minute per 10^6 cells.

Table III: NAD-Dependent Enzyme Assays of Mitochondria from Rutamycin-Sensitive and Rutamycin-Resistant Cells

sample	μmol of substrate reduced per min per mg of protein: malate dehydrogenase	μmol of substrate reduced per min per mg of protein: glutamate dehydrogenase	nmol of $^{14}CO_2$ released per min per mg of protein, pyruvate decarboxylase (dehydrogenase)
clone 1 D	0.33	0.18	0.224
A 9	0.14	0.09	0.267
mutant subclone 1	1.11	0.14	0.074
mutant subclone 2	0.94	0.23	0.048
enucleated mutant subclone 1 × A9	1.14	0.17	0.076

data presented in Table II). However, this is not the full explanation of whole cell succinate oxidation in all cells. For example, both whole cell and mitochondrial succinate oxidation by clone 1 D is more effective than that by the cybrid, despite the similar succinate cytochrome *c* reductase specific activity of the mitochondria from these two sources and the lower total enzyme activity in clone 1 D. Also A 9 and clone 1 D mitochondria oxidize succinate at comparable rates, despite a threefold difference in the oxidation of succinate by the whole cells of these two lines. It is possible that clone 1 D cells are more permeable to exogenous succinate than A 9 cells, though we have no direct evidence implicating this as the basis for the discrepancy in their succinate oxidizing capacity. Clearly, however, regulation of succinate oxidation, other than the maximal activity of succinate cytochrome *c* reductase, must account for these discrepant observations.

The P/O ratio and respiratory control index (state 3/state 4 respiration) for the mitochondria of rutamycin-resistant cells and rutamycin-sensitive parent cells were also determined by using succinate as a substrate. The P/O ratio for the control cells was in the range of 1.0–1.2, while that for the rutamycin-resistant cells was between 0.5 and 0.6. The respiratory control index gave a similar pattern, with the normal cells yielding a value of around 3.0 while the mutant cells were found to have an index of about 1.5. These data indicate that the mutants are somewhat uncoupled in their respiration.

NAD-Dependent Enzymes. In order to ensure that the lack of respiration with substrates which reduce the first complex of the respiratory chain was not due to a depression of their respective dehydrogenases, we assayed these enzymes and the results are presented in Table III. Both malate and glutamate

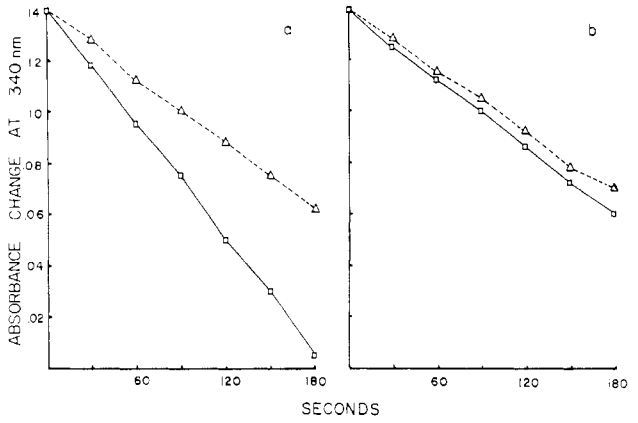


FIGURE 4: Assay of total and rotenone-resistant NADH oxidase activities in clone 1 D (a) and mutant subclone 1 (b) mitochondrial preparations (0.05 mg/assay). Solid lines (□—□) represent assays done in the absence of rotenone. Dashed lines (△---△) represent assays which were preincubated with rotenone (1 μg of rotenone per 20 μg of protein) for 5 min prior to the addition of the NADH substrate.

Table IV: NADH–Cytochrome *c* Reductase Assay in Mitochondria of Rutamycin-Sensitive and Rutamycin-Resistant Cells

	μmol of cytochrome *c* reduced per min of mg of protein	
source of mitochondria	total act.	rotenone-sensitive act.
clone 1 D	11.8	4.4
A 9	57.6	24.2
mutant subclone 1	7.3	0.8
mutant subclone 2	11.0	2.8
mutant subclone 3	12.6	1.0
enucleated mutant subclone 1 × A9	3.5	0.7

dehydrogenase activities in the rutamycin-resistant mitochondria are either the same or higher than those of normal mitochondria. However, the pyruvate decarboxylase activity of the pyruvate dehydrogenase complex is somewhat depressed in the mutant cell mitochondria.

NADH–Cytochrome c Reductase Activity. The crude mitochondrial preparations from the various cell lines were assayed for NADH–cytochrome *c* reductase activity. One of the problems with this assay is that there are multiple NADH oxidase activities found within such preparations. Because of the relatively small amount of material that was available from these cultured cells, it was not possible to isolate inner mitochondrial membranes, which would have eliminated all but respiratory chain associated activity. However, the specific inhibitor rotenone can be used to distinguish among the various activities that are specific for the electron transport chain. Also, since mitochondria are impermeable to NADH, the preparations were sonicated first to disrupt the mitochondrial membranes.

A typical time course of this experiment is illustrated in Figure 4; the effects of rotenone on the activities from rutamycin-resistant and normal mitochondria are illustrated. A large proportion of the total activity with wild-type mitochondria is inhibited with rotenone while the mutant is barely affected. The complete results of this assay are shown in Table IV. The total amount of NADH–cytochrome *c* reductase activity was roughly comparable among the three rutamycin-resistant subclones assayed and clone 1 D mitochondria. Mutant subclones 1 and 3 had only 18 and 25%, respectively, of the rotenone-sensitive activity found in wild-type mitochondria, although mutant subclone 2 had somewhat more

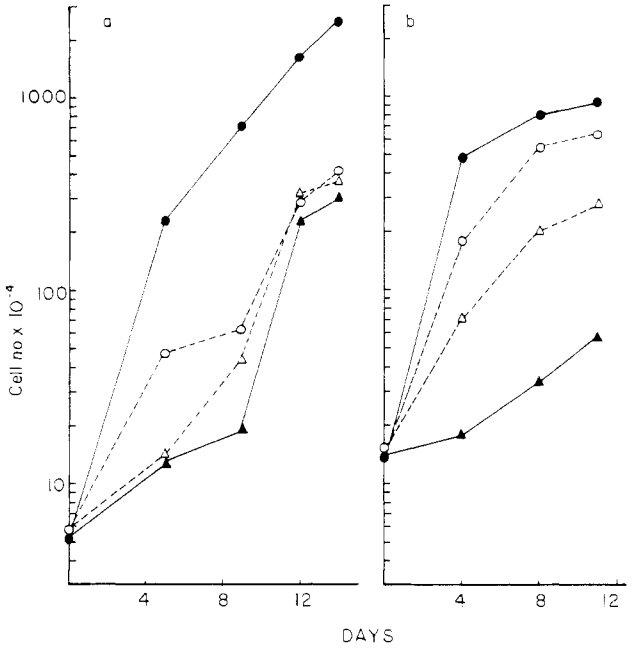


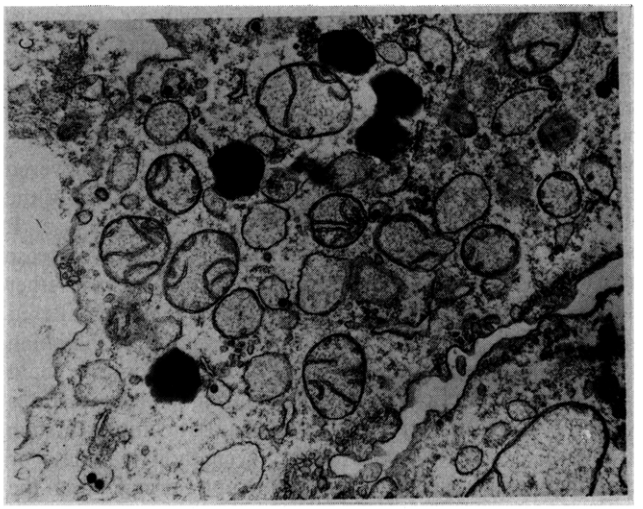
FIGURE 5: (a) Growth curves of clone 1 D and mutant subclone 2 in the presence and absence of rotenone. Solid lines refer to clone 1 D cells: (○—○) normal medium; (△—△) normal medium + 0.1 μg/mL rotenone. Dashed lines refer to mutant subclone 2 cells: (○---○) normal medium; (△---△) normal medium + 0.1 μg/mL rotenone. (b) Growth curves of clone 1 D and mutant subclone 1 in the presence and absence of antimycin. The symbols are the same as in Figure 5a except that 10 μg/mL antimycin is used in place of rotenone.

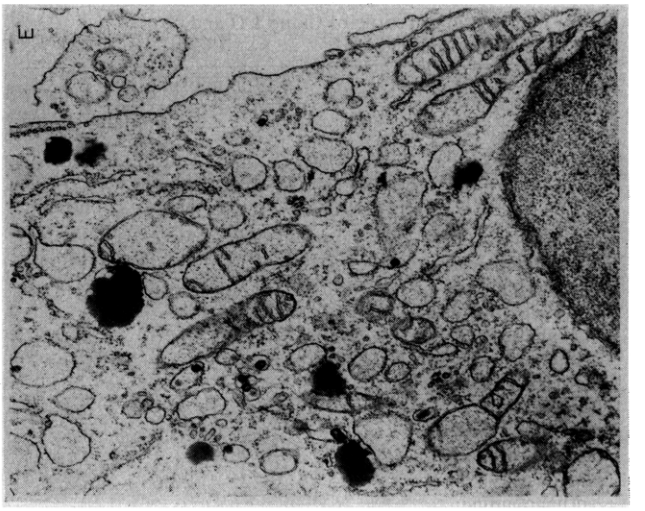
activity. Mitochondria from A 9 cells had higher levels of total NADH–cytochrome *c* reductase activity while mitochondria from the cybrid cells had rather low levels of this enzyme activity. Nevertheless, 42% of the total activity with A 9 mitochondria was rotenone sensitive while only 20% of that with cybrid mitochondria was inhibited by rotenone. The rotenone-sensitive activity with cybrid mitochondria was roughly the same as that of two mutant clones.

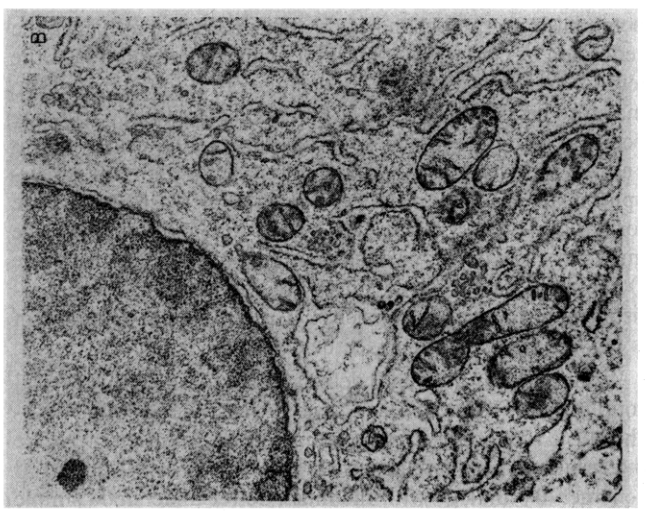
Effect of Respiratory Inhibitors on Cell Growth. Since the mutant cells survive and grow with a much reduced rate of respiration, it seemed of interest to determine whether the growth of these cells was relatively resistant to inhibitors of respiration. High concentrations of both antimycin and rotenone were lethal to both mutant and wild-type cells. However, as can be seen in Figure 5 there was a narrow concentration range of these two inhibitors at which the growth rate of clone 1 D cells was markedly depressed while that of the rutamycin-resistant cells was less affected.

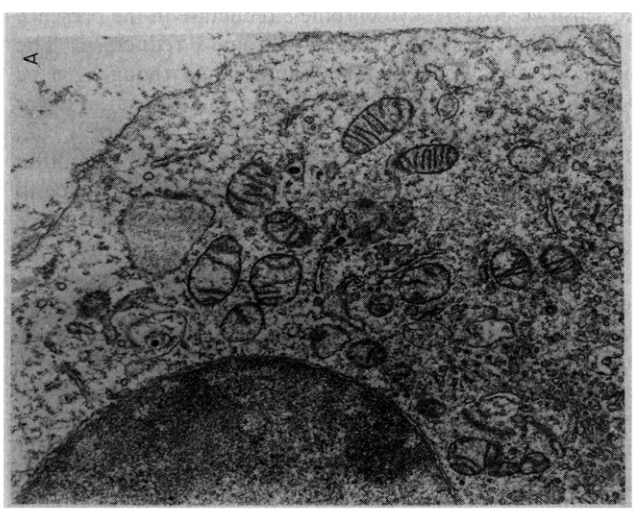
Phospholipid Profile. The phospholipid profile of the rutamycin-resistant and normal cells was examined to ascertain whether an impaired mitochondrial generation of ATP might preferentially interfere with phospholipid synthesis. Relative phospholipid content was determined by examining the distribution of radioactive phosphorus among phospholipid classes after long-term growth in radioactive phosphate medium. It is clear from Table V that the phospholipid spectrum of the whole cell homogenate and mitochondria is closely comparable between clone 1 D and mutant subclone 2.

Transmission Electron Micrographs. Transmission electron micrographs of the rutamycin-resistant cells and clone 1 D are illustrated in Figure 6. A great majority of the mitochondria in the mutant and cybrid cells appears swollen and possesses fewer cristae which are also somewhat less regularly arranged. A major difference between these cells, however, is the appearance of large storage vacuoles in the rutamycin-resistant









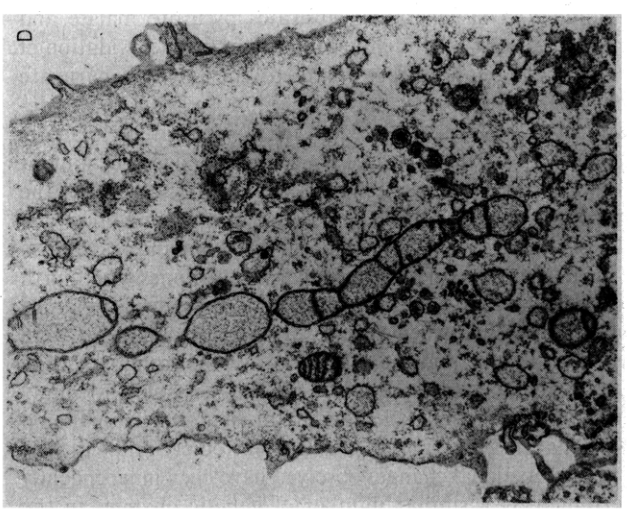


FIGURE 6: Transmission electron micrographs of wild-type and mutant cell lines at 26 400×. (A) Clone 1 D. (B) A 9. (C) Mutant subclone 1. (D) Mutant subclone 2. (E) Enucleated mutant subclone 1 × A 9.

Table V: Phospholipid Profile of Clone 1 D and Rutamycin-Resistant Subclone 2

	% of total phospholipid			
	clone 1 D		mutant subclone 2	
	homog-enate	mito-chondria	homog-enate	mito-chondria
cardiolipin	3.3	2.6	7.6	4.5
phosphatidic acid	0.4	0.4	1.0	0.5
dimethylphospha-tidylethanolamine	0.1	0.1	0.1	0.1
phosphatidylserine	4.0	3.6	4.0	4.3
lysophosphatidyl-ethanolamine	0.8	0.8	1.1	1.0
lysolecithin	0.4	0.6	0.5	0.4
phosphatidylglycerol	1.0	0.9	0.8	0.8
phosphatidylinositol	7.1	7.2	6.7	7.1
phosphatidyl-ethanolamine	28.5	29.2	23.6	26.2
lecithin	45.7	46.1	43.8	44.6
sphingomyelin	8.4	8.4	10.3	10.2

cells. These changes appeared regardless of whether or not the cells were grown in rutamycin. Both the normal cells and the mutant subclones contained similar intracellular viruses, which were comparable in numbers and morphology among normal and mutant cells. Their appearance is common in many mammalian cell lines (Panem et al., 1975).

Discussion

In this paper a defect in oxidative energy metabolism has been described in a mouse fibroblast isolated by virtue of its resistance to the cytoxicity of rutamycin, an inhibitor of mitochondrial ATPase. The resistance of the isolated mutants to rutamycin is manifested in vitro not only by the resistance of their mitochondrial ATPase (Lichtor & Getz, 1978) but also by the in vitro insensitivity of coupled respiration to the inhibitory effects of the rutamycin (this paper), i.e., a resistance of the ATP synthetase. Attention was attracted to the defect in respiration as a consequence of the rapid drop in pH of the medium in which the mutants were grown, and this is attributable to the excessive amounts of lactic acid produced by these cells. The respiratory defects are pleiomorphic. In addition to rutamycin resistance and markedly increased lactic acid production, the mutant cells also reveal an impaired oxygen consumption, reduced oxidative phosphorylation with succinate as substrate, reduced rotenone-sensitive NADH-cytochrome *c* reductase, and a reduced pyruvate decarboxylase activity. The isolated mitochondria from these mutants oxidized the NADH-generating substrates, pyruvate, malate, and glutamate, poorly. While the impairment in the oxidation of pyruvate and malate may relate to the lowered pyruvate decarboxylase activity, no reduction in glutamate dehydrogenase was noted to account for the decreased glutamate oxidation. In addition to these phenotypic changes, the mutant cells were relatively resistant to the cytotoxic effects of the respiratory chain inhibitors, rotenone and antimycin.

These alterations in mitochondrial function and respiration are evident in three of the four separate subclones (the fourth was not tested) and are all transmitted to the cybrids obtained from the cross between respiratory competent, rutamycin sensitive A 9 cells, and the cytoplasm derived from one of the mutant subclones (subclone 1). This suggests very strongly that all of the functional changes are attributable to an altered mitochondrial gene product(s). It is extremely unlikely that the many functional changes observed have been independently determined by a similar number of genetic changes in the mitochondrial genome. It is more probable that the pleiomorphic nature of the mitochondrial defect is attributable to a series of secondary consequences of one or perhaps two mitochondrial genetic changes. All the observed changes could be related to a defect either in the ATP coupling systems, manifested by a rutamycin-resistant ATPase and lowered efficiency of oxidative phosphorylation, or in complex I of the electron transport chain or NADH-coenzyme Q reductase. It is not clear from our data whether there is an absolute decrease in one or more elements of the respiratory complex or whether these are present at normal levels but are qualitatively altered so that the complex exhibits an unusual resistance to inhibition by rotenone. The alteration in this respiratory complex could account for the deficiency in the oxidation of the substrates which reduce the electron transport chain at complex I, such as malate, pyruvate or glutamate, even though malate or glutamate dehydrogenase is normal or supranormal. Even the marked reduction in pyruvate decarboxylase could be a secondary consequence of a complex I deficiency. The latter deficiency is likely to increase the mitochondrial ratio of NADH/NAD which may in turn decrease the proportion of the pyruvate dehydrogenase complex present in the active form (Pettit et al., 1975). The defect in mitochondrial oxidation of the NAD-dependent substrate and the increased reducing potential in the mitochondria can be expected to influence the reduction of cytoplasmic pyridine nucleotides and hence the level of lactate produced. The storage of fat in the cytoplasm may also be attributable to the increased cytoplasmic reducing potential with the consequent stimulation of fatty acid synthesis and the reduction of dihydroxyacetone phosphate to glycerophosphate, the circumstances known to increase triglyceride formation (Lieber, 1975). Also, fatty acid oxidation may be impaired. The defect in rotenone-sensitive NADH–coenzyme Q reductase could also account for the relative cellular resistance to rotenone cytotoxicity. The resistance to antimycin is less readily explained (see below).

Assuming that the major defects reside in the mitochondrial coupling ATPase and the rotenone-sensitive NADH–cytochrome *c* reductase, it remains uncertain whether these are the results of separate genetic changes in the mitochondrial genome. By analogy with the much better understood simple eucaryotes, such as *Saccharomyces cerevisiae*, it is quite likely that one or more of the components, particularly of the rutamycin-sensitive ATPase, is specified by the mitochondrial genome (Schatz & Mason, 1974; Tzagoloff et al., 1973; Ebner et al., 1973). On the other hand there is no analogous precedent for a mitochondrial genomic specification of rotenone-sensitive NADH–cytochrome *c* reductase in the presence of normal levels of succinate cytochrome *c* reductase. The mitochondrial cytochrome *b* apoprotein is thought to be specified by the mitochondrial genome, at least in lower eucaryotes and insects (Weiss, 1972, 1976; Tzagoloff et al., 1973; Schatz & Mason, 1974), and an absence or alteration of this apoprotein could account for a relative resistance to antimycin cytotoxicity. However, NADH–cytochrome *c* reductase and succinate cytochrome *c* reductase are both thought to employ cytochrome *b* of the same structure. In the recently derived maps of the yeast mitochondrial genome, the cytochrome *b* gene is assigned a single locus (Slonimski & Tzagoloff, 1976; Molloy et al., 1975; Sriprakash et al., 1976; Colson et al., 1977). However, there do appear to be at least two spectroscopically distinct cytochromes *b* in mammalian mitochondria (Rieske, 1976; Wikstrom, 1973), but it is yet to be shown that the apoproteins of these two *b* cytochromes are chemically distinct (Weiss, 1976). The two cytochromes

b are not apparently selective in their functional reduction by electrons from NADH (complex I) or succinate (complex II) (Rieske, 1976). Both are apparently mitochondrial translation products (Weiss, 1976). Furthermore coenzyme Q–cytochrome *c* reductase is a highly organized complex (complex III), whose physiological conformation is required for high-affinity antimycin binding (Rieske, 1976), so that the antimycin resistance may reflect an altered conformation of complex III within the mitochondrial membrane. This may also apply to complex I and the rotenone resistance.

We have hitherto been unsuccessful in separating the rotenone-sensitive NADH–cytochrome *c* reductase defect and the rutamycin-sensitive ATPase defect, although recently we have cloned the cybrid line, and currently these clones are being studied to determine if they all express the same phenotype as the originally derived rutamycin-resistant mutants. An alternate explanation to the separate genetic determination of these two defects is that the one defect is the physiological consequence of the other, determined by a single genetic change in the mitochondrial genome. Thus, the putative single genetic change could result in an altered conformation of the inner mitochondrial membrane which could in turn be responsible for the rutamycin resistance of the mitochondrial ATPase, a reduction in the activity of the NADH–coenzyme Q or –cytochrome *c* reductase, the antimycin resistance, and perhaps the reduction in pyruvate decarboxylase activity. The altered morphology of the mutant and cybrid cell mitochondria is consistent with this interpretation, as is a reduction in the efficiency of oxidative phosphorylation using a well oxidized substrate (succinate). The fact that the mitochondrial ATPase of the mutant cells was resistant to leucinostatin [see note added in proof to Lichtor & Getz (1978)], an inhibitor of ATPase which binds to a membrane site different from rutamycin, is also consistent with this interpretation (Lichtor & Getz, 1978). Further experiments should enable one to distinguish between the alteration in one or more mitochondrial genes.

It is noteworthy that despite the pleiomorphic nature of the functional mitochondrial changes observed in the mutants studied here, these mutant cells nevertheless display selective changes in their mitochondria and the functioning of their mitochondrial genomes. Thus, the mutant cells are fully competent to oxidize those substrates which reduce the electron transport chain at complex II or IV and probably III too. Elements of complex III and IV require input from the mitochondrial genome (Schatz & Mason, 1974; Tzagoloff et al., 1973; Slonimski & Tzagoloff, 1976). Succinate cytochrome *c* reductase and cytochrome oxidase activities are normal, and the cells are still sensitive to the cytotoxic effects of chloramphenicol, an inhibitor of mitochondrial protein synthesis (Lichtor & Getz, 1978). Thus, although the mutant cells seem to emphasize glycolysis and produce large quantities of lactic acid and appear to have many defects in their mitochondria, they nevertheless have a normal mitochondrial protein synthetic mechanism and depend upon the function of their mitochondria for growth. This is in contrast to respiratory-deficient or petite mutants in yeast (Perlman & Mahler, 1970; Schatz, 1968; Kovac & Weissova, 1968).

One of the respiratory-deficient mutants obtained in Chinese hamster ovary cells (DeFrancesco et al., 1976) by an entirely different selection procedure is strikingly similar to the mouse cell mutant described in this paper. Both are unable to oxidize pyruvate and glutamate, have normal malate and glutamate dehydrogenase and depressed pyruvate decarboxylase activities, and have reduced rotenone-sensitive NADH–cytochrome *c* reductase. It is not clear whether the Chinese hamster ovary mutant is a Mendelian or a cytoplasmic mutant. Also, no information has been reported on the mitochondrial ATPase or on its resistance to rutamycin. However, the derivation of two respiratory-deficient mutants by entirely different procedures and in two different cell lines may indicate that this type of genetically determined respiratory deficiency in mammalian cells is most compatible with growth and survival.

In conclusion, this mutant taken together with some of the other mammalian cell mutants defective in oxidative metabolism (DeFrancesco et al., 1976; Soderberg et al., 1977) should be useful in contributing to the understanding of the control of energy metabolism in mammalian cells. Furthermore, they may be helpful in elucidating the mechanisms of mammalian mitochondrial biogenesis and the interaction of nuclear and mitochondrial gene products in the assembly of this organelle. In particular, this mutant could contribute to establishing the physiological role of rutamycin-sensitive ATPase in mammalian cell mitochondria. These studies should be especially interesting in light of the fact that it has been known for some time that tumor cells frequently exhibit patterns of respiratory deficiency and high rates of aerobic glycolysis (Warburg, 1956a,b; Racker, 1975).

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References

Colson, A. M., The Van, L., Convent, B., Brignet, M., & Goffeau, A. (1977) *Eur. J. Biochem. 74*, 521–526.

DeFrancesco, L., Scheffler, I. E., & Bissell, M. J. (1976) *J. Biol. Chem. 251*, 4588–4595.

Ebner, E., Mason, T. L., & Schatz, G. (1973) *J. Biol. Chem. 248*, 5369–5378.

Getz, G. S., Jakovcic, S., Heywood, J., Frank, J., & Rabinowitz, M. (1970) *Biochim. Biophys. Acta 218*, 441–452.

Hohorst, J.-J. (1963) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., Ed.) pp 134–138, Academic Press, New York.

Hruban, Z., Wong, T.-W., Itabashi, M., & Lyon, E. S. (1976) *Virchows Arch. B. 20*, 91–102.

Kovac, L., & Weissova, K. (1968) *Biochim. Biophys. Acta 153*, 55–59.

Lichtor, T., & Getz, G. S. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 324–328.

Lieber, C. S. (1975) *Ann. N.Y. Acad. Sci. 252*, 63–84.

Lowry, O. H., Rosebrough, N. J., Farr, A. J., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Martin, B. R., & Denton, R. M. (1970) *Biochem. J. 117*, 861–877.

Mattenheimer, H. (1969) *Micromethods for the Clinical and Biochemical Laboratory*, p 45, Ann Arbor Science Publishers, Inc., Ann Arbor, MI.

Molloy, P., Linnane, A., & Lukins, H. (1975) *J. Bacteriol. 122*, 7–18.

Panem, S., Prochownik, E., Reale, F., & Kirsten, W. (1975) *Science 189*, 297–299.

Perlman, P. S., & Mahler, J. R. (1970) *J. Bioenerg. 1*, 113–138.

Pettit, F. H., Pelley, J. W., & Reed, L. J. (1975) *Biochem. Biophys. Res. Commun. 65*, 575–582.

Rabinowitz, M., & de Bernard, B. (1957) *Biochim. Biophys. Acta 26*, 22–29.

Racker, E. (1975) in *Energy Transducing Systems* (Racker, E., Ed.) pp 163–184, University Park Press, Baltimore, MD.
Rieske, J. S. (1976) *Biochim. Biophys. Acta 456*, 195–247.
Roodyn, O. B., Suttie, J. W., & Work, T. S. (1962) *Biochem. J. 83*, 29–40.
Schatz, G. (1968) *J. Biol. Chem. 243*, 2191–2199.
Schatz, G., & Mason, T. (1974) *Annu. Rev. Biochem. 43*, 51–87.
Slonimski, P., & Tzagoloff, A. (1976) *Eur. J. Biochem. 61*, 27–41.
Soderberg, K. L., Ditta, G. S., & Scheffler, I. E. (1977) *Cell 10*, 697–702.
Sriprakash, K. S., Molloy, P. L., Nagley, P., Lukins, H. B., & Linnane, A. W. (1976) *J. Mol. Biol. 104*, 485–503.
Tzagoloff, A., Rubin, M. S., & Sierra, M. F. (1973) *Biochim. Biophys. Acta 301*, 71–104.
Vaskovsky, V. E., & Kostestsky, E. Y. (1968) *J. Lipid Res. 9*, 396.
Warburg, O. (1956a) *Science 123*, 309–314.
Warburg, O. (1956b) *Science 124*, 267–270.
Weiss, H. (1972) *Eur. J. Biochem. 30*, 469–478.
Weiss, H. (1976) *Biochim. Biophys. Acta 456*, 291–313.
Wikstrom, M. K. F. (1973) *Biochim. Biophys. Acta 301*, 155–193.

Kinetics of Mg^{2+} Flux into Rat Liver Mitochondria†

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ABSTRACT: Unidirectional fluxes of Mg^{2+} across the limiting membranes of rat liver mitochondria have been measured in the presence of the respiratory substrate succinate by means of the radioisotope ^{28}Mg. Rates of both influx and efflux of Mg^{2+} are decreased when respiration is inhibited. A linear dependence of the reciprocal of the Mg^{2+} influx rate on the reciprocal of the external Mg^{2+} concentration is observed. The apparent K_m for Mg^{2+} averages about 0.7 mM. *N*-Ethylmaleimide, an inhibitor of transmembrane phosphate–hydroxyl exchanges, enhances the observed pH dependence of Mg^{2+} influx. In the presence of MalNEt, the apparent V_{max} of Mg^{2+} influx is greater at pH 8 than at pH 7, and there is a linear dependence of the Mg^{2+} influx rate on the external OH^- concentration. The K^+ analogue Tl^+ inhibits Mg^{2+} influx, while La^{3+}, an inhibitor of mitochondrial Ca^{2+} transport, has no effect on Mg^{2+} influx. Mg^{2+} competitively inhibits the flux of K^+ into rat liver mitochondria. The mechanism(s) mediating mitochondrial Mg^{2+} and K^+ fluxes appear to be similar in their energy dependence, pH dependence, sensitivity to Tl^+, and insensitivity to La^{3+}.

Rat liver mitochondria take up Mg^{2+} by an energy-dependent mechanism (Judah et al., 1965; Johnson & Pressman, 1969). It has been suggested that Mg^{2+} and K^+ may be transported into the mitochondria by a common mechanism (Judah et al., 1965). Depletion of endogenous Mg^{2+} has been shown to result in stimulation of K^+ uptake by both liver and heart mitochondria (Duszynsky & Wojtczak, 1977; Wehrle et al., 1976). Competitive inhibition by Mg^{2+} of K^+ flux into heart mitochondria has been reported (Jung et al., 1977). Other data suggest competitive inhibition by Mg^{2+} of Ca^{2+} transport in both liver and heart mitochondria (Hutson et al., 1976; Parr & Harris, 1976). Energy-dependent net Mg^{2+} efflux from rat liver mitochondria associated with Ca^{2+} uptake has been observed in the presence of added phosphate (Siliprandi et al., 1977).

Kun (1976a,b) has examined the kinetics of net Mg^{2+} uptake by mitochondria which have been treated with digitonin to remove lysosomal contaminants. The data fit a proposed kinetic model which assumes an active uptake of Mg^{2+} (Kun, 1976a).

Measurements of unidirectional K^+ flux into rat liver mitochondria have been carried out under conditions of approximately steady-state K^+ content, in which requirements for secondary counterion fluxes may be assumed to be minimal (Diwan, 1973; Diwan & Lehrer, 1977, 1978; Diwan et al., 1977). The reciprocal of the K^+ influx rate is a linear function of the reciprocal of the external K^+ concentration. K^+ influx is competitively inhibited by the K^+ analogue Tl^+. The V_{max} of K^+ influx increases when the pH of the medium is increased from 7 to 8, while the apparent K_m for K^+ remains approximately constant at about 5 mM. The pH dependence of the V_{max} of K^+ influx is increased in the presence of MalNEt[1] or mersalyl, each of which is known (Meijer et al., 1970) to block transmembrane phosphate–hydroxyl exchange. In the presence of MalNEt or mersalyl, a linear dependence of K^+ influx on external OH^- concentration is observed. On the basis of these results and evidence indicating lack of involvement of a membrane potential in driving K^+ influx (Diwan & Tedeschi, 1975), it has been postulated that K^+ may enter the mitochondria by a nonelectrogenic mechanism involving cotransport with OH^- (Diwan, 1973; Diwan et al., 1977; Diwan & Lehrer, 1978).

Brierley and co-workers have measured the dependence of unidirectional K^+ flux into beef heart mitochondria on external K^+ concentration (Jung et al., 1977). Linear Lineweaver–Burk plots are observed in agreement with the data obtained with rat liver mitochondria. However, the experiments with beef heart mitochondria indicate a higher K_m for K^+ of about 12 mM (Jung et al., 1977).

Steady-state Ca^{2+} fluxes, estimated indirectly from measurements of respiration by rat liver mitochondria in the

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[1] Abbreviations used: MalNEt, *N*-ethylmaleimide; EDTA, ethylenediaminetetraacetate.

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