A Respiration-deficient Chinese Hamster Cell Line with a Defect in NADH-Coenzyme Q Reductase*

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We have recently described a Chinese hamster cell line with a greatly reduced rate of respiration. In this report we conclude that the defect is in NADH-coenzyme Q reductase (NADH oxidase), the first part of the electron transport chain. The conclusion is based on the following observations. (a) In this and in the earlier report we determined that the relevant enzymes of the Krebs cycle are present and active. (b) Oxygen consumption by isolated mitochondria is normal when driven by succinate and α-glycerophosphate. (c) Difference spectra between reduced and oxidized forms indicate that all cytochromes are present and functional. (d) In contrast, substrates such as malate, glutamate, α-ketoglutarate, and isocitrate which generate NADH do not stimulate oxygen consumption in mutant mitochondria. (e) A direct assay of the rotenone-sensitive NADH oxidase in Lubrol-treated mitochondria from mutant cells revealed less than one-tenth of the activity when compared with wild type mitochondria. (f) The treatment of wild type cells with rotenone, a specific inhibitor of NADH-CoQ reductase, yielded an exact phenocopy of the mutant by several criteria. This is the first report of a respiration-deficient mammalian cell mutant in tissue culture.

The study of mitochondrial biogenesis and functions in lower eukaryotes, in particular yeast, has been facilitated by the existence of a large number of mutants. Mutants have been described in both cytoplasmic and nuclear genes (for a review, see Kovac (21)). Mammalian cell mutants defective in mitochondrial functions have not yet been found, although there are now a number of reports of mammalian cell mutants resistant to drugs which specifically block mitochondrial protein synthesis (3–5). At least in one such case the mutation has been located on a mitochondrial genome (6). The functional capacity of the mitochondria of these mutants has not been assessed and there is no reason a priori to think that the mitochondria would be nonfunctional.

It appears that mammalian cells are obligate aerobes, i.e. they cannot proliferate indefinitely under a nitrogen atmosphere (for a review see Gregg (7)), but one may question whether oxygen is absolutely required for respiration and oxidative phosphorylation, or for other oxidative reactions in the cell. On the other hand, it has been known for some time that tumor cells (and most likely, therefore, established cell lines in culture) are primarily defective in respiration. This idea, which has been critically reviewed by Racker (9) is no longer tenable, and it has been shown that respiration and mitochondria in cultured cells are normal in many respects (7, 10, 11).

Tissue culture media normally contain high concentrations of glucose and the high rate of glycolysis may be a response of cells to the external conditions, while the capacity for electron transport and oxidative phosphorylation is still present and essential for survival under glucose starvation. It would be of interest therefore to know whether mutations in electron transport and oxidative phosphorylation can be tolerated as long as glucose is abundant.

In this report we will describe a mutant of a Chinese hamster cell line which has an abnormally low rate of respiration, ~8% of the rate observed in the parental cell line, and is critically dependent on an ample supply of glucose for glycolysis. A partial characterization of this mutant cell line has already been published (12, 13), in which we established that the Krebs cycle was operating at a greatly reduced rate due to an apparent block between α-ketoglutarate and succinate. The experiments to be described here indicate that the enzymes of the Krebs cycle are present in normal amounts, suggesting that the observed block was perhaps due to feed-back inhibition of α-ketoglutarate dehydrogenase by elevated levels of NADH in the mitochondria. We provide evidence that the defect has occurred in the electron transport chain, at complex I, or NADH-coenzyme Q reductase.

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EXPERIMENTAL PROCEDURES

Cells and Culture Conditions—The parental cells (CCL16) are fibroblasts from the lung of a male Chinese hamster, and they were obtained from the American Type Culture Collection. The respiration-deficient mutant, res- (CCL16-B2 res-) was isolated by chance, and it was initially characterized as an auxotroph for carbon dioxide (aux-C02) (12). The biochemical characterization was extended by DeFrancisco et al. (13). Cells were routinely cultured as monolayers on plastic tissue culture plates (Falcon or Lux), or in glass roller bottles, in Dulbecco’s Modified Eagle’s Medium (DME, from Gibco), supplemented with 10% fetal calf serum, and asparagine (3 mM) (13). It was also found that increasing the glucose concentration 2- to 5-fold over that in normal salt concentration, to prevent excess salt from interfering with the subsequent chromatography. The cells were killed with 1 ml of 80% methanol, then scraped into the alcohol and the plates rinsed with an additional 0.5 ml of methanol. The cell suspension and wash were reduced in volume under nitrogen to 0.2 ml, and sonicated to produce a fine suspension. Chromatography, autoradiography, and counting were performed as described by Bassham et al. (14).

Assay of Krebs Cycle Enzymes—For assaying Krebs cycle enzymes, mitochondria were first isolated by the method of Nass (15). Cells were homogenized in “SM” buffer which contained 0.25 M sucrose, 2 mM EDTA, 50 mM Tris HCl (pH 7.4), and 1% bovine serum albumin. Mitochondrial pellets were prepared as described above and washed several times before use. Usually 0.1 ml of mitochondrial suspension was added to an electrode chamber (Gilson) containing 2 ml of prewarmed, air-saturated buffer (0.25 M sucrose/0.2 mM EDTA/1 mM MgCl2/10 mM KPi, pH 7.2) according to Gregg (7). Substrates were added to a final concentration of 2.5 to 5.0 mM. The amount of oxygen remaining in solution was measured with a Clark oxygen electrode connected to the amplifier as described by Carr et al. (21) and the signal was recorded continuously on a chart recorder. All measurements were made at 37° while the suspension was stirred with a magnetic stirrer. The instrument was calibrated by using buffer, air-saturated at 37° to set the deflection corresponding to 100% O2, which was assumed to be 0.199 mA (22). The zero per cent O2 was set by turning off the electrode.

Concentrated solutions of rotenone and antimycin A (0.1 to 1.0 mM) were made in 95% ethanol and stored at -20°. Prior to use, they were diluted 100-fold into 50% ethanol, such that between 5 to 10 μl were required to achieve the desired concentration in the electrode chamber. The effects of these amounts of ethanol alone on respiration were measured and found to be negligible.

mitochondria were prepared by differential centrifugation as described above, for respiration measurements. The final washed pellet was resuspended in a small volume of “SM” buffer and layered on top of a 40-ml linear sucrose gradient of 0.8 to 1.9 mM sucrose in 2 mM EDTA/25 mM Tris HCl (pH 7.4), and centrifuged for 2 hours at 25,000 rpm in a Beckman rotor SW-27 at 4°. One-millionth fractions were collected from the bottom of the tubes and the mitochondria were located by visual inspection or by cytochrome oxidase assays.

mitochondria were treated with Lubrol WX, supplied by Dr. M. White, Cancer Research Laboratory, University of California at Berkeley, for 10 min at room temperature (0.02 to 0.2% Lubrol) followed by three cycles of freezing and thawing in a dry-ice acetone bath. The conditions for assay were according to Green and Ziegler (23), 100 mM phosphate buffer (pH 7.4/0.1 mM EDTA/0.01% cytochrome c/0.5% bovine serum albumin/0.12 mM NADH. The decrease in absorbance at 340 nm at 34° was followed with a recording spectrophotometer (Varian model 635) coupled to a Heath-Schlumberger potentiometric amplifier and strip chart recorder. To measure inhibition by rotenone, the complete reaction mixture minus the substrate (NADH) was preincubated with 6 μg of rotenone for about 5 min.

RESULTS

In experiments described previously (13) we found that the rate of evolution of 14CO2 from 14C-labeled precursors entering the Krebs cycle via acetyl coenzyme A was less than 1% in the mutant compared to wild type cells. Similarly, the rate of oxidation of α-ketoglutarate (measured by evolution of 14CO2 from [1-14C]glutamate) was reduced at least 20-fold in the mutant cells. On the other hand, [14C]succinate and [14C]lactate were converted to [14C]O2 at appreciable rates. All of these results could be explained by postulating a block in the Krebs cycle between α-ketoglutarate and succinate, involving two enzymes; α-ketoglutarate dehydrogenase and succi-
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nyl-CoA synthetase (13). The reduced rate of respiration could therefore be explained either by existence of a defective enzyme within the Krebs cycle, or by a defect in the electron transport chain leading to a feedback inhibition of one or more enzymes of the Krebs cycle.

To gain insight into the existence of various metabolic pathways of glucose and to quantify some of the intermediates derived from it in wild type and mutant cells, a different set of experiments was performed under physiological conditions. Uniformly labeled glucose of high specific activity was added to the cells in normal tissue culture medium, and after incubating for 1 hour, the intracellular, low molecular weight compounds were extracted with methanol as described under "Experimental Procedures." Two-dimensional chromatography following the procedure of Bassham et al. (14) was used to separate the various labeled species which were then detected by autoradiography and counted quantitatively by semiautomated Geiger counter. A comparison of autoradiograms of wild type and mutant cells after a 1-hour incubation in [14C]glucose is shown in Fig. 1. The most striking difference is seen at locations on the chromatogram corresponding to Krebs cycle intermediates and amino acids derived from the Krebs cycle. While some of these metabolites were among the most heavily labeled species in wild type cells (for example, glutamate, citrate, and aspartate), corresponding to the largest pools, they were completely absent (unlabeled) in the mutant. This suggested either that the Krebs cycle was uniformly depressed in the mutant, with no evidence for the accumulation of any of the intermediates of the cycle preceding a single block, or that none of the carbons derived from glucose entered the Krebs cycle due to an inhibition of pyruvate dehydrogenase.

It was also significant that except for the dramatic absence of Krebs cycle intermediates, the pattern and the levels of intermediates corresponding to other metabolites derived from glucose (intermediates in glycolysis, hexose monophosphate shunt, glycogen synthesis, etc.) were essentially comparable in mutant and wild type cells. When variations were noted, these could be explained as secondary to the initial lesion (as will be explained later), or were due to differences in population density and growth rates of the two cultures. It has been shown previously that the level of intermediates of glycolysis, hexose monophosphate shunt, and glycogen synthetic pathways in cells in culture vary with the rate of glucose uptake, which in turn varies with population density and growth rates (24). It was also of interest that the pools of labeled amino acids which are not directly derived from the Krebs cycle, such as alanine, were comparable in mutant and wild type cells.

Assays of Krebs Cycle Enzymes—It was of obvious interest to measure the activity of Krebs cycle enzymes, and we initially concentrated on α-ketoglutarate dehydrogenase as it appeared to be the most likely site of the defect. As described in more detail under "Experimental Procedures," α-ketoglutarate dehydrogenase activity was determined in purified mitochondria either by measuring spectrophotometrically the reduction of NAD to NADH in mitochondria treated with phospholipase A, or by measuring the evolution of 14CO2 from α-keto[1-14C]glutarate in detergent-disrupted mitochondria. Fig. 2 shows an experiment with the radioactive substrate, in which the data have been normalized with respect to total protein added to the reaction mixture. It can be seen that the levels of enzyme in mutant and wild type mitochondria are comparable. The figure also shows that the decarboxylation reaction is dependent on the inclusion of NAD in the reaction mixture, which indicates that the complete reaction catalyzed by the α-ketoglutarate dehydrogenase complex is in fact occurring.

Table I shows the values for the specific activity of the enzyme α-ketoglutarate dehydrogenase obtained by both assay methods, which have been normalized with respect to the amount of protein or the amount of cytochrome oxidase present. It should be noted that, depending on the method of normalization, the level of the enzyme in the mutant could be either 150% or 65% of the wild type, neither of which we believe is significantly different from the parental cell line. This difference is a reflection of the fact that the specific activity of cytochrome oxidase was generally found to be 2 to 3 times higher in mutant mitochondria.

Other cellular dehydrogenases were also measured although they had not been expected to be inactive, based on previous results, in order to check the validity of our normalizations and more importantly, to help in the interpretation of measurements of oxygen consumption by isolated mitochondria (see below). A summary of the results is presented in Table I. The
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Fig. 2 (left). Assay of α-ketoglutarate dehydrogenase; formation of $^{14}CO_2$ from α-keto[1-$^{14}$C]glutarate. Details of the procedures are given under "Experimental Procedures." Filled symbols, 0.12 mM NADP added; open symbols, no NADP added; ○, ○ CCL16; △, △, res- mutant.

Fig. 3 (right). Absorption difference spectra of cytochromes of wild type (CCL16) and mutant (res-) cells (for details, see "Experimental Procedures"). A, dithionite reduced-oxidized; B, cyanide reduced-oxidized; C, anaerobically reduced-oxidized; maximum at $\Delta A_{460}$, cyt $\alpha$; shoulder at 555 to 560 nm, cyt $b$; maximum at $\Delta A_{437}$, cyt $c$.

Table I
Specific activities of NAD-dehydrogenases

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity</th>
<th>CCL16</th>
<th>Res-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol NADH/min-mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NAD-dependent isocitrate dehydrogenase</td>
<td>26.2</td>
<td>0.116</td>
<td>40.0</td>
</tr>
<tr>
<td>Malate dehydrogenase</td>
<td>729</td>
<td>1.97</td>
<td>744</td>
</tr>
<tr>
<td>α-Glycerol phosphate dehydrogenase</td>
<td>0.44</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogenase</td>
<td>1.9</td>
<td>2.9</td>
<td></td>
</tr>
<tr>
<td>α-Ketoglutarate dehydrogenase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvate decarboxylase (dehydrogenase)</td>
<td>1.0</td>
<td>0.0045</td>
<td>1.7</td>
</tr>
<tr>
<td>Lactate dehydrogenase*</td>
<td>1.3</td>
<td>0.75</td>
<td></td>
</tr>
</tbody>
</table>

*Co unit is an abbreviation for cytochrome oxidase unit, which is defined as the amount of extract required to oxidize 1 nmol of cytochrome c per min; cytochrome oxidase was assayed according to Smith (33).

*Assayed in crude extracts only.

results of these experiments suggested that contrary to our earlier expectations, the decreased activity of the Krebs cycle was not the result of a defective Krebs cycle enzyme.

**Cytochrome Difference Spectra**—Another feature of the mitochondria of obvious interest in light of the nonrespiring state of the mutant was the presence of respiratory chain linked cytochromes. Difference spectra recorded at low temperatures between oxidized and reduced cells are shown in Fig. 3. The cells were reduced in several ways: by adding a reducing agent to the cells before freezing, by incubating the cells in cyanide, or by allowing the cells to become anaerobic naturally by leaving them in a concentrated suspension. In all cases, the difference spectra obtained were identical in mutant and wild type cells. The difference spectra for both cell types are shown in Fig. 3 and peaks at 600 nm, 555 nm, and 548 nm corresponding to cytochromes $\alpha + \alpha_s$, $b$, and $c$, respectively, can be clearly distinguished. Although no quantitation of the absolute amounts of the different cytochromes was attempted, it can be concluded that the loss of a cytochrome is not the cause of the respiration deficiency in the mutant.

**Oxygen Consumption by Isolated Mitochondria**—Oxygen consumption by isolated mitochondria can be stimulated by the addition of suitable substrates (7, 10). These can be divided into two classes: substrates such as succinate and α-glycerophosphate are oxidized with the involvement of flavoproteins which transfer electrons directly to coenzyme Q; from these, electrons flow to oxygen via the cytochromes. On the other hand, substrates such as malate, isocitrate, α-ketoglutarate, and β-hydroxybutyrate are oxidized by their respective dehydrogenases generating NADH, which in turn is oxidized by the NADH dehydrogenase (complex I), before the electrons are transferred to coenzyme Q.

Mitochondria from mutant or wild type cells were prepared as described under "Experimental Procedures" and oxygen
consumption was measured with a Clark oxygen electrode in the presence of various substrates and inhibitors. A typical series of traces showing the amount of oxygen left in solution after the addition of succinate to mitochondria is shown in Fig. 4. These traces are not normalized with respect to the amount of mitochondrial protein present, which accounts for the different slopes of the traces: apparent background levels of oxygen consumption, caused either by instrument drift, or nonspecific reactions, have also not yet been subtracted. Fig. 4 illustrates that with succinate as a substrate, the mitochondria are coupled as shown by the increased rate of O₂ consumption upon the addition of ADP. From these kinds of data, P/O ratios can be calculated (25), and for both mutant and wild type mitochondria, the ratio was 2, as would be expected for succinate. Fig. 4 also shows the effect of the electron transport inhibitor antimycin A, which blocks the reaction with both kinds of mitochondria. In other experiments (not shown) malonate, a specific inhibitor of succinate dehydrogenase, was demonstrated to be an effective inhibitor of the succino-oxidase system.

Fig. 6A shows the average values for the rates of O₂ consumption (μmol/hour) observed with succinate and a-glycerolphosphate as substrates, which have been normalized with respect to the amount of protein present. When normalized in this way, the mutant appears to have more of this activity than the wild type. However, as mentioned above, the specific activity of cytochrome oxidase was also higher in mutant mitochondria preparations, and when this difference was taken into account, the values for the mutant were approximately the same as the wild type. This demonstrates that the electron transport chain from complex II to the end is functional in mutant mitochondria, which is not unexpected in view of the normal spectrum of cytochromes found in the mutant.

In contrast, very different results were obtained with substrates that generate NADH. In every case, the mutant showed substantially reduced rates of O₂ consumption compared to wild type mitochondria. It was found, however, that even with wild type mitochondria, the rates observed with these substrates were lower than those observed with succinate and a-glycerolphosphate. This has been observed with other preparations of mitochondria from cultured cells (10) and it is likely that the rate of oxygen consumption is limited by the rate of uptake of these substances by mitochondria. Shuttles and coupled carrier systems for di- and tricarboxylic acids have been described (e.g. Ref. 26), but we were not successful in our attempts to make use of such known carrier systems to increase the rates of respiration. We do not believe that our parental cells are particularly abnormal in this regard, but rather that we are dealing with a problem peculiar to isolated mitochondria. Respiration in intact, wild type cells was completely inhibited by rotenone, which blocks electron transport at complex I (“NADH oxidase”, “NADH-CoQ reductase”), indicating that this activity is indeed significant and essential.

Some actual traces with substrates which generate NADH are shown in Fig. 5. In wild type mitochondria, oxygen consumption was clearly stimulated by substrates such as α-ketoglutarate, glutamate, and malate, and furthermore, this respiration was inhibited by rotenone, as expected for a reaction involving complex I of the respiratory chain (Fig. 5). By contrast, in experiments with mutant mitochondria in which there was an apparent slow decrease in oxygen concentration, for example in the presence of α-ketoglutarate or malate (Fig. 5), this decrease was found to be insensitive to rotenone. This could either be ascribed to nonspecific oxidations or, we believe, to the generation of some succinate which is oxidized normally in the mutant. In support of this interpretation we have found that the addition of malonate (which inhibits succinate dehydrogenase) tends to suppress the background reaction.
Thus, in the presence of substrates such as malate, glutamate, isocitrate, β-ketoglutarate, and α-hydroxybutyrate the rates of oxygen consumption in mutant mitochondria are reduced 10-fold or more (for a summary, see Fig. 6), even though for a number of these substrates the corresponding dehydrogenases had been found in normal amounts in mutant mitochondria. These results point to a defect in the early part of the electron transport chain, i.e. in the NADH-coenzyme Q reductase.

**Assay of the Rotenone-sensitive NADH Oxidase**—A method for assaying the respiratory chain-associated NADH oxidase activity has been described for cultured cells (11). However, there are several problems that are encountered when attempting to assay this activity in crude mitochondrial preparations.

First, there are multiple NADH oxidase activities found within mitochondrial 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 the respiratory chain-associated activity. However, the existence of the specific inhibitor, rotenone, for the respiratory chain NADH oxidase, allows one to distinguish among the various activities. Second, as mitochondria are normally impermeable to NADH, it is necessary to employ some agent to destroy the permeability barrier. For this, Lubrol WX, a mild nonionic detergent has been suggested (27).

As described in more detail under “Experimental Procedures”, our approach was to purify mitochondria on sucrose gradients, which eliminated some of the non-mitochondrial contamination, treat with Lubrol, and assay the oxidation of NADH spectrophotometrically in the presence and absence of rotenone. In the absence of rotenone, the total NADH oxidase activity was measured; in the presence of rotenone, only the rotenone-insensitive activity was measured, and by subtraction, the amount of rotenone-sensitive NADH oxidase could be determined. Fig. 7 shows a typical time course of an experiment of this kind and it illustrates the different effects that rotenone had on the activities from mutant and wild type mitochondria. The data shown have not yet been normalized with respect to total protein added. With wild type mitochondria, it inhibited a large proportion of the total activity (usually 50%), while in the mutant, it barely affected the reaction. Table II presents the results of a series of these experiments, consistent with the above conclusions. It should be noted that although the proportion of the total NADH oxidase activity that was rotenone-sensitive varied from preparation to preparation, the actual amounts of rotenone-sensitive NADH oxidase calculated from the data varied much less in both the mutant and wild type preparations. The mutant had, on the average, only 9% of the activity found in wild type mitochondria which roughly corresponds to the relative levels of respiration observed in the two cell lines.

**Effect of Rotenone on Wild Type Cells** Since it appeared from the experiments described so far that the defect in the mutant could be localized in the NADH-coenzyme Q reductase complex, it seemed reasonable to ask whether the phenotype of the mutant could be reproduced in wild type cells treated with rotenone, since rotenone is a specific inhibitor of the same complex I in the electron transport chain. When rotenone was added at a concentration (5 × 10⁻⁴ M) such that oxygen consumption was ≥ 99% inhibited, the following kinds of experiments suggested that this was indeed the case: (a) the formation of ¹⁴CO₂ from [²⁻¹⁴C]pyruvate was completely inhibited in wild type cells in the presence of rotenone, while the formation of ¹⁴CO₂ from [¹⁻¹⁴C]aspartate was only partly affected, just as observed in mutant cells without rotenone (12, 13). (b) The addition of rotenone to wild type cells made these cells auxotrophic for asparagine and carbon dioxide, a phenotype which we have described for the mutant cells (13). This is because the intracellular production of carbon dioxide by the Krebs cycle is reduced and because the interconversion of

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**Table II**

<table>
<thead>
<tr>
<th>NADH oxidized/min/mg of protein</th>
<th>CCL16</th>
<th>Res *</th>
</tr>
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<tbody>
<tr>
<td>Total activity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL16</td>
<td>45.0</td>
<td>21.6</td>
</tr>
<tr>
<td>Res</td>
<td>75.2</td>
<td>57.2</td>
</tr>
<tr>
<td>Total activity</td>
<td>62.2</td>
<td>46.8</td>
</tr>
<tr>
<td>Rotenone-insensitive</td>
<td>17.5</td>
<td>17.2</td>
</tr>
<tr>
<td>CCL16</td>
<td>36.9</td>
<td>53.2</td>
</tr>
<tr>
<td>Res</td>
<td>37.8</td>
<td>46.8</td>
</tr>
<tr>
<td>Rotenone-sensitive</td>
<td>27.5</td>
<td>4.3</td>
</tr>
<tr>
<td>CCL16</td>
<td>39.2</td>
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<tr>
<td>Res</td>
<td>25.4</td>
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</table>

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**Fig. 6.** Summary and comparison of oxygen consumption by wild type and mutant mitochondria with different substrates. A, Non-NADH linked substrates; B, NADH-linked substrates (note the change in scale). Open bars, CCL16 mitochondria; crosshatched bars, res * mutant mitochondria.

**Fig. 7.** Assay of total and rotenone-resistant NADH oxidase activities in wild type (A) and mutant (B) mitochondrial preparations (for details, see “Experimental Procedures”). Open symbols, no rotenone present; filled symbols, the reaction mixture without NADH was pre-incubated with rotenone (40 μM) for 5 min before the reaction was started by the addition of substrate.
glutamine to oxaloacetate, aspartate, and asparagine via the Krebs cycle is blocked. (c) The pattern of intraacellular, acid-soluble metabolites derived from [U-14C]glucose as determined by two-dimensional paper chromatography and autoradiography (see “Experimental Procedures”) was very similar in mutant cells and wild type cells treated with rotenone as shown in Table III. In particular, all Krebs cycle intermediates and amino acids derived from them were absent or drastically reduced (see also Fig. 1). There were some variations in the relative levels of some other metabolites, but these were not reproducible and depended on the precise conditions of the experiment. Similar variations had been observed in the comparison of mutant and wild type cells. (d) A precise comparison of the amount of glucose consumed and lactate produced per milligram increment of total protein in logarithmically growing cultures showed no difference between mutant cells and rotenone-treated wild type cells, while untreated wild type cells consume approximately 40% less glucose.

**DISCUSSION**

In this paper we complete the characterization of a Chinese hamster cell line which had initially attracted attention because of the apparent auxotrophy for carbon dioxide (12). Subsequent studies (13) showed that oxygen consumption was severely depressed in the mutant, and that there was an apparent block in the Krebs cycle between α-ketoglutarate and succinate. As a result, these cells did not produce sufficient endogenous amounts of carbon dioxide, and they also required asparagine in the medium, which is normally derived from glutamine by a series of reactions involving the Krebs cycle.

Our earlier studies had shown at least some enzymes of the Krebs cycle to be active (13), and we now present direct measurements of relevant enzyme activities, of which the activity of α-ketoglutarate dehydrogenase was of the most interest. Since none of these enzymes were absent or defective, the electron transport chain itself was investigated.

A number of lines of evidence all support the conclusion that the basis of the respiration deficiency of the mutant is a block in the electron transport chain at complex I, or NADH-enzyme Q reductase. First, the presence of all the cytochromes and the finding of normal succino-oxidase activity suggest a block in the early portion of the electron transport chain. Second, the inability of substrates such as malate, α-ketoglutarate, isocitrate, and glutamate to stimulate oxygen consumption in mutant mitochondria is in agreement with a postulated block in NADH oxidase. Third, the amount of rotenone-sensitive NADH oxidase measured directly in mutant mitochondria was found to be less than one-tenth of the amount found in wild type mitochondria. Finally, the ability to produce a photocopy of the mutant by treatment of the wild type cells with the specific inhibitor rotenone supports our conclusion that all the known properties of the mutant can be attributed to a single block at complex I in the electron transport chain. It has also been shown in our laboratory (1) that revertants can be found in which all of the properties of the mutant return to normal.

Complex I (NADH-coenzyme Q reductase) is not a single protein, but is constituted of a ferroflavoprotein, a series of iron-sulfur proteins, and some mitochondrial structural protein (28). A further characterization of the defect and the identification of a defective peptide or protein would require the purification and analysis of a quantity of material which is at present hardly feasible for cells grown in tissue culture.

The existence of a mammalian cell mutant defective in electron transport and therefore in respiration may at first seem surprising, but studies in our laboratory have shown that the rate of glycolysis has to be increased by only about 60% to provide the necessary energy for cellular proliferation when respiration is inhibited either by mutation or by the inhibitor rotenone (29); that is to say, we have demonstrated that these particular wild type Chinese hamster fibroblasts from which the mutant was derived produce 40% of their total ATP requirement from electron transport and 60% from glycolysis. Under conditions where the rate of glycolysis is reduced, for example by the substitution of galactose for glucose, wild type cells increase their rate of respiration, while the mutant cells die within 6 hours. It is also of interest that 97% of the glucose used by wild type or mutant cells is converted to lactate and that glutamine plays a very important role in oxidative energy metabolism of wild type cells (29).

Numerous attempts to grow mammalian cells anaerobically seem to have failed, particularly when rigorous attention was paid to removing the last traces of oxygen (see Ref. 7 for a discussion). We speculate that either a small amount of the leakiness of the mutation and therefore some respiration is essential, or that oxygen may be absolutely required for oxidations other than those related to oxidative phosphorylation. It is of interest in this regard that the residual rate of oxygen consumption in the mutant cells was not found to be appreciably sensitive to the presence of the inhibitors, rotenone or antimycin, which may be an indication of oxidations unrelated to electron transport.

We should also point out that these cells have not lost their mitochondria, which appear to be morphologically almost normal (13). This is in contrast to certain respiration-deficient (petite) mutants of yeast (30, 31). One may speculate whether in mammalian cells mitochondria perform other indispensable functions, which are not immediately related to energy metabolism.

To our knowledge this is the first characterization of a mammalian cell line defective in respiration. It demonstrated the feasibility of isolating more such mutants. Although this particular mutant was isolated by chance, we have since made use of the properties of this mutant to devise a selection scheme which has yielded more than a dozen mutants with defects in oxidative energy metabolism (32). Several of these have been shown to be different from the one described here, based on preliminary biochemical data and on genetic experiments.

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1. G. Ditta, unpublished observations.
involving somatic cell hybridizations. Thus, a whole new class of somatic cell mutants is becoming available for detailed biochemical studies of energy metabolism in tissue culture, and for genetic experiments such as gene mapping.

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