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Mitochondrial abnormalities in fibroblast line GM3093 defective in oxidative metabolism[1]

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Summary. Fibroblast line GM3093 deficient in the activity of the pyruvate dehydrogenase complex, was derived from a patient reported to have an inherited defect affecting the tricarboxylic acid cycle. Our results suggest a generalized defect consisting of few and abnormal mitochondria and low activities of all mitochondrial enzymes examined.
Key words. Mitochondria; mitochondrial abnormalities; mitochondrial enzymes; oxidative metabolism; fibroblast line GM3093.

Certain inherited neurological diseases have been attributed to defective oxidative metabolism. Thus, a defect in the pyruvate oxidation system has been shown in some patients with lactic acidemia and diffuse neurologic disease[5], a deficiency of mitochondrial malic enzyme in patients with Friedreich's ataxia[6], and a partial deficiency of the glutamate dehydrogenase in some patients with adult-onset olivopontocerebellar degeneration[7].

Fibroblast line GM3093 was derived from a now deceased 3-year-old girl with severe diffuse neurologic disease and persistent lactic acidosis[8]. Her fibroblasts oxidized radioactive citrate, palmitate, and pyruvate at a rate less than one third of that of the control cells. Cell homogenates had deficient activity of the pyruvate dehydrogenase complex (PDHC). It was concluded that the patient had an inherited defect affecting the tricarboxylic acid cycle. This is the only PDHC-deficient fibroblast line which is widely available and is used extensively as a reference. The objective of the present study was to further localize the defect in cell line GM3093. This led us to the measurement in cell homogenates of the activities of several mitochondrial and non-mitochondrial enzymes, and in electron microscopic examination of the mitochondria in cultured skin fibroblasts.

Materials and methods. GM3093 and control human skin fibroblast lines were obtained from the human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ. The cells were grown in McCoys 5A media with glutamine, supplemented with 10% fetal calf serum, 100 units/ml mycostatin, and 50 µg/ml gentamycin and were harvested at confluency. The harvested cells were subjected to three cycles of freeze-thawing and the homogenates were used immediately. Enzyme substrates, coenzymes, and all other chemicals were obtained from commercial suppliers at the highest grade available and were used without further purification.

Enzymic activities were assayed by published procedures adapted for this study. In particular, the activities of PDHC and α-ketoglutarate dehydrogenase complex (α-KGDHC) were measured radiochemically as follows: Radioactive substrates [1-C^{14}] pyruvic acid, sodium salt, 2–20 Ci/mol and [1-C^{14}] α-etoglutaric acid, sodium salt, 40–60 Ci/mol were purchased from New England Nuclear Corp. (Boston, MA) and were handled according to Blass et al.[9]. The reaction mixture (final volume 200 µl and pH 7.8) in 12 × 75 mm disposable glass test tubes, in an ice-bath, contained (final concentrations, mM), Tris buffer, 5, KCl, 25, $MgCl_2$, nicotinamide, 15, Na_2SO_3, 0.5, EDTA, 0.5, mercaptoethanol, 0.5, NAD^+, 0.3, CoA, 0.2, TPP, 0.1, pyruvate, 0.5 (5×10^5 cpm) or α-ketoglutarate, 0.1 (2×10^5 cpm), and fibroblast homogenate (50–100 µg protein). Cofactors were

Enzymic activities in GM 3093 and control fibroblast homogenates

Enzyme		GM 3093	Controls
		nmol/min/mg protein*	
Pyruvate dehydrogenase complex (non activated)		0.347 ± 151	1.45 ± 0.466
Pyruvate dehydrogenase complex (activated)		0.569 ± 0.110	2.66 ± 0.583
Dihydrolipoamide dehydrogenase	EC 1.6.4.3	17.4 ± 7	58.6 ± 12.1
α-Ketoglutarate dehydrogenase complex	EC 1.2.4.2	0.229 ± 0.043	0.912 ± 0.24
Isocitrate dehydrogenase[b] $NADP^+$	EC 1.1.1.42	17.1 ± 3.9	36.9 ± 14.3
Glutamate dehydrogenase[b] NADH	EC 1.4.1.3	887 ± 493	2554 ± 765
Cytochrome C oxidase[b]	EC 1.9.3.1	2.0 ± 0.9	10.45 ± 1.34
Malic enzyme[c] (mitochondria)	EC 1.1.1.40	0.5	1.21 ± 0.9
Lactate dehydrogenase[d, b]	EC 1.1.1.27	1137 ± 110	699 ± 91

*Values are mean ± SD; [a]Using the reverse reaction: lipoamide + NADH + H^+ → dihydrolipoamide + NAD^+, 30 °C; [b]Temperature 30 °C; [c]Data from Stumpf et al.[17]; [d]Utilizing the reaction: lactate + NAD^+ → pyruvate + NADH + H^+.

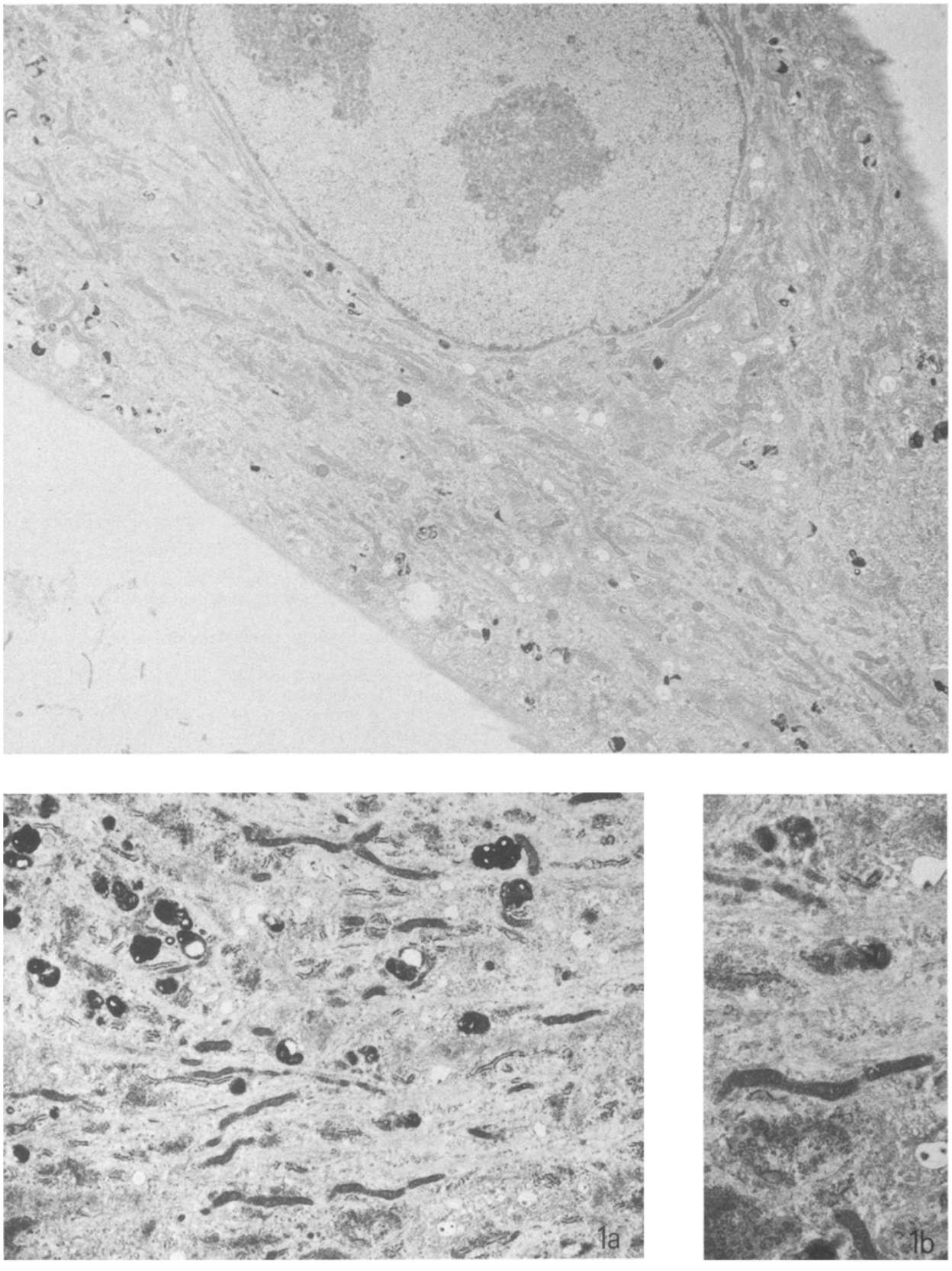

Figure 1. Transmission electron micrograph of control fibroblast line shows normal nucleus, RER, and normal configuration and distribution of mitochondria; ×4200. 1a, Control fibroblast; ×7400. 1b, Enlargement of mitochondria shown in 1a. ×15,500.

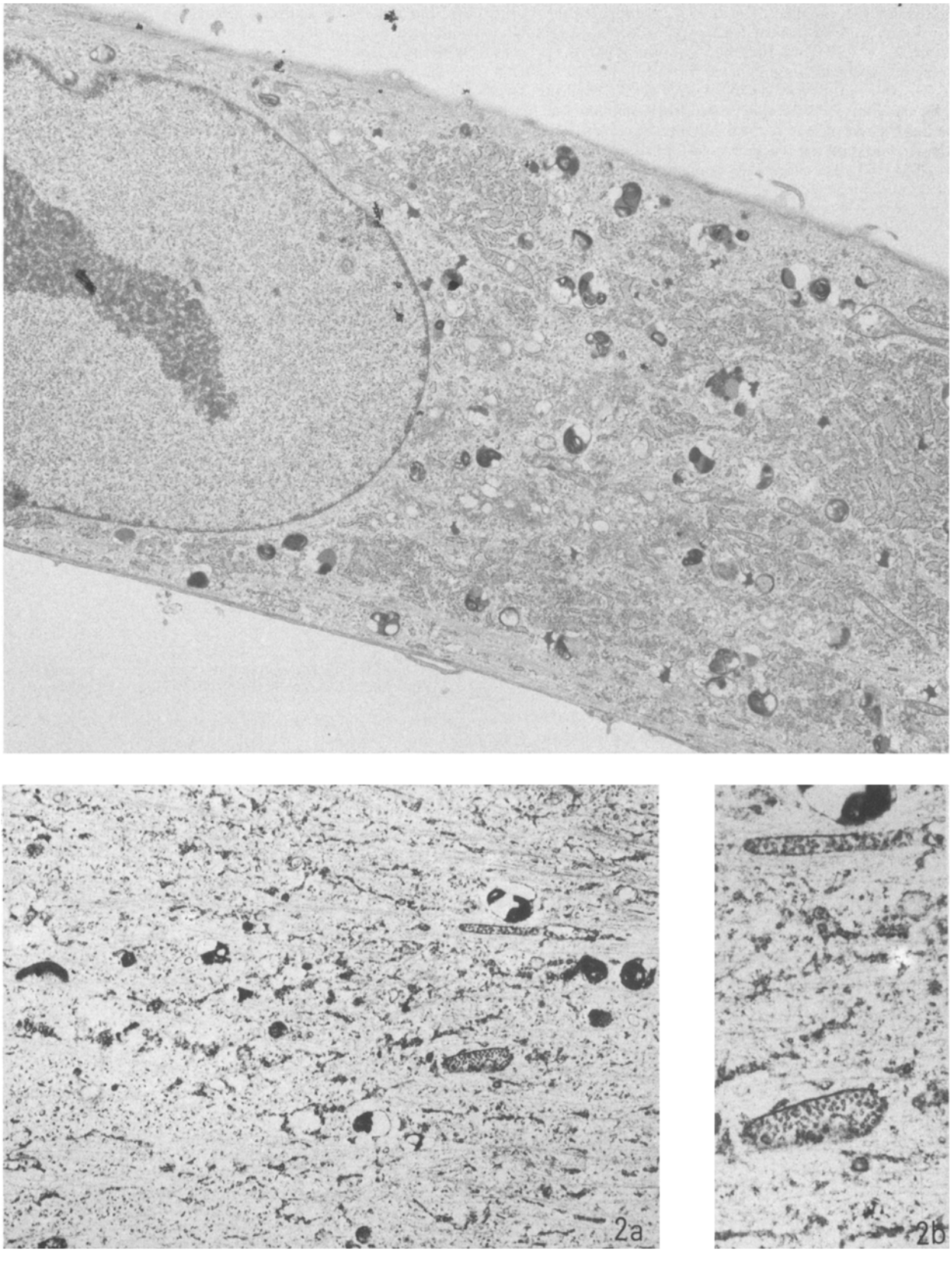

Figure 2. Transmission electron micrograph of fibroblast line GM3093. There is a paucity of mitochondria. Those present appear abnormal. Nucleus is normal. There are large pools of RER; ×5700. 2a, GM3093 fibroblast; ×7300. 2b, Enlargement of mitochondria shown in 2a; × 15,500.

omitted to serve as a blank. The tubes were stoppered with corks from which were suspended plastic wells (cellulose propionate tubes size 4.8 × 19.9 mm Beckman Cat. No. 341288). The wells contained paper wicks impregnated with 50 µl of 2.5 M NaOH in order to absorb the evolved CO_2. Assays were in triplicate. Two of the assay mixtures were incubated for 10 min and one for 30 min at 37 °C. At the end of the incubation the tubes were placed in an ice-bath and the reaction was stopped by the addition of 50 µl 3 M HCL. The tubes were stoppered again. Complete CO_2 collection was achieved by shaking the tubes for 2 h at 37 °C. The radioactivity was assessed by counting the contents of the wells by standard techniques. PDHC was activated by treating the harvested cells, with dichloroacetate[10]. However, the cell suspensions were incubated with 5 mM dichloroacetate, at 37 °C, for only 12 min.

The activity of pyruvate dehydrogenase was measured radiochemically[11]. Spectrophotometric methods were used for the assay of dihydrolipoamide dehydrogenase (LAD)[12], isocitrate dehydrogenase[13], cytochrome C oxidase[14], and glutamate dehydrogenase[15]. The latter was measured with 85 µM NADPH or 175 µM NADH as coenzyme in the presence of 1 mM ADP. The activity of lactate dehydrogenase was measured spectrophotometrically utilizing the lactate to pyruvate reaction with reagents supplied by Sigma Chem. Co., St. Louis, MO. Protein was determined according to Lowry et al.[16], using bovine serum albumin as standard.

For electron microscopy, the cells were grown in six-well plates. Young cells (less than 20 passages) of approximately the same passage number were used. For transmission electron microscopy, the cells were washed in Hank's balanced salt solution. They were fixed in prewarmed Karnovsky's fixative (pH 7.4) for 1 h at 37 °C. After washing three times in 0.1 M cacodylate buffer (pH 7.4), they were osmicated with 2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 h at 25 °C. After dehydration through a graded series of methanols (each change for 5 min) and in 100% methanol (3 changes for 10 min each), the cells still in the Costar wells were embedded in Poly-Bed 812.

Following polymerization, the plastic discs in which the cells were now embedded, were removed from the wells. Sections, 60–70 nm thin, were cut on an LKB Ultratome IV. They were counterstained with saturated uranyl acetate and 0.5% lead citrate and were examined with a JEOL 100CX electron microscope.

Results and discussion. Our findings are given in the table. We, as others[10], easily confirmed the PDHC deficiency of fibroblast line GM3093. The activity of the untreated or dichloroacetate activated PDHC, was about 20–25% of control levels. Since no reliable assays are available for pyruvate dehydrogenase (enzyme 1 of PDHC complex) and dihydrolipoyl transacetylase (enzyme 2 of the complex), in cell homogenates, we measured the activity of LAD (enzyme 3 of the complex). Its activity was about 30% of control levels. α-KGDHC, which, like PDHC, depends on LAD for its total activity was also deficient in this line (about 25% of control levels). From that, one would suppose that the defect is localized in LAD. However the activity of cytochrome C oxidase which was used as a reference enzyme was very low. Similarly the activities of glutamate dehydrogenase and isocitrate dehydrogenase were only 35 and 45% of control levels respectively. In contrast, the activity of the cytosolic enzyme lactate dehydrogenase was increased. These findings and the recent observation by Stumpf et al.[17], that the activity of mitochondrial malic enzyme and cytochrome C reductase were low in fibroblast line GM3093, suggested a generalized defect in the mitochondria.

Electron microscopic examination of fibroblasts GM3093 revealed a paucity of mitochondria averaging 20% of the amount found in the control fibroblast lines of approximately same passage number. The mitochondria were elongated or round in shape and did not appear swollen (figs 1 and 2). Cristae were not aligned in parallel arrays. They appeared vesicular or broken and did not fill the entire mitochondria. Some mitochondria contained electron dense debris and few remnants of cristae. With the exception of a greater electron density in the cytoplasm of the control fibroblasts no other specific nuclear or cytoplasmic abnormality was observed.

Mitochondrial abnormalities in fibroblasts derived from patients with an inherited disease involving oxidative metabolism have not been reported previously. Ultrastructural changes of muscle mitochondria have been found in certain muscle diseases – the mitochondrial myopathies[18] – and also in spinocerebellar and macular degeneration[19]. Unfortunately, fibroblast mitochondria from patients with these diseases were not examined. In mitochondrial myopathies, the ultrastructural changes of mitochondria have been considered a sequel of the enzymic defect[18]. Regarding the low activities of mitochondrial malic enzyme and cytochrome C reductase in GM3093, Stumpf et al.[17] suggested that they could occur secondarily (down regulation) because of the PDHC deficiency or from the results of the present study, more precisely, because of the LAD deficiency. However the results of this study, particularly the deficiency of cytochrome C oxidase, suggest that all abnormalities are caused by a structural (or other) defect affecting the functional integrity of the mitochondrion. This hypothesis can be tested by examining the cellular and/or mitochondrial DNA.

Notwithstanding the underlying cause of the abnormalities, our results suggest that fibroblast mitochondria can be useful in the study and diagnosis of inherited disorders of oxidative metabolism.

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