We report on the loss of mitochondrial nicotinamide adenine dinucleotides in human cultured cells along with cell culture and acidification of the culture medium. This was established both by the direct measurement of the decrease in the mitochondrial NAD content and by the alteration of the oxidative properties of the mitochondria. In situ, this loss could be reversed in less than 2 hours by changing the culture medium or by readjusting the pH of the medium at physiological pH values.

By studying the oxidative properties of intact, but NAD-depleted, mitochondria in digitonin-permeabilized cells, we found that a rapid influx of NAD could replenish the mitochondrial NAD pool. This allowed the restoration of an active NAD+-dependent substrate oxidation. Depletion of mitochondrial NAD in cells grown under quiescent conditions was further confirmed by fluorimetric measurement of mitochondrial NAD, as was the influx of NAD into the mitochondrial matrix.

These data constitute the first evidence of rapid fluxes of NAD through mitochondrial membranes in animal cells. They also point to the possible confusion between a loss of mitochondrial NAD and a defect of respiratory chain complex I in the context of screening procedures for respiratory chain disorder in human.

The way exogenous NAD+ affects substrate oxidation differs between animal and plant mitochondria. In intact plant mitochondria, substrate oxidation by matrix NAD+-dependent dehydrogenases (malate dehydrogenase, NAD+-malic enzyme, α-ketoglutarate dehydrogenase, etc.) is often strongly stimulated by exogenous NAD+. The occurrence of a specific NAD translocator has been postulated (1–3). Although no definitive proof that this is the case has been provided, NAD+ has been shown to be actively accumulated from the external medium. This uptake being concentration-dependent, exhibiting Michaelis-Menten kinetics, and specifically inhibited by an azido derivative of NAD+ (4). On the other hand, a slow passive diffusion of NAD+ between intact isolated plant mitochondria and the suspending medium has been shown to gradually lead to NAD+-depleted organelles (1, 4).

So far, unlike their plant counterparts, animal mitochondria are considered to be impermeable to pyridine nucleotides (5, 6). Accordingly, no stimulation by exogenous NAD+ of matrix NAD+-dependent dehydrogenases or oxidation of exogenous NADH can be observed in isolated liver, heart, or muscle mitochondria. Thus, any influx of NAD has simply to be explained by the use of either improperly prepared mitochondria or osmotically unbalanced assay medium.

In this paper, we report on the following: (i) the decrease of intact cell respiration and of NAD+-linked substrate oxidation in digitonin-permeabilized cells during human B-lymphoblastoid cell line culture, concomitant with a decrease of the NAD+/NAD ratio (the mitochondrial); (ii) the in vitro restoration of an active oxidation of NAD+-linked substrates by exogenous NAD+, associated with an influx of NAD+ into the mitochondrial matrix; and (iii) the role of the culture medium pH in controlling NAD+ influx into the mitochondria. In order to rule out any artifact related to the isolation of mitochondria, we studied mitochondrial properties in digitonin-permeabilized cells that were shown, at the digitonin concentration used, to keep their mitochondria fully intact (7).

MATERIALS AND METHODS

Human skin fibroblasts and BCLL from controls were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% fetal calf serum (8). Harvested cells were washed twice in 30 ml of phosphate-buffered saline solution before use.

A mitochondria-enriched fraction was obtained from BCLL treated for 4 min by digitonin (0.05%, 0.1 μM/mL) at ice-melting temperature in 600 μM of 0.1 M sucrose, 5 mM succinate, and 10 mM phosphate buffer (pH 7.4). After a low speed centrifugation (2500 × g for 5 min), the supernatant was layered on top of a two-step gradient consisting of 600 μM of 0.3 M sucrose, 10 mM phosphate buffer (pH 7.4), 5 mM succinate, 100 mM NaCl and 1 mg/ml bovine serum albumin, topped with 800 ml of silicone oil (Versilube). After centrifugation (9000 × g for 8 min), a mitochondria-enriched fraction was obtained essentially free from contaminating cytosolic lactic dehydrogenase. Intactness of the mitochondria, as estimated from the latency of cytochrome c oxidase (9), was higher than 95%.

Polaronographic studies of intact cell respiration and substrate oxidation by digitonin-treated cells were carried out at 37 °C in a thermostated cell fitted with a Clark electrode (Hansatech) and containing 250 μM of 0.3 M mannitol, 10 mM KCl, 5 mM MgCl2, 1 mg/ml bovine serum albumin, and 10 mM KH2PO4 (pH 7.4) (medium A), as described (9). Cytochrome c oxidase (9), lactic dehydrogenase (10), and citrate synthase (10) activities were spectrophotometrically measured.

For measurement of the NAD content, samples were treated by the addition of an excess of HClO4. After centrifugation (3000 × g for 10 min), the supernatant was neutralized by K2CO3, centrifuged (3000 × g for 10 min), and used for the measurement of NAD by an NAD+-regenerating microassay using 7 units of NAD+-dependent alcohol dehydrogenase (bakers' yeast enzyme; Sigma) (11). Accumulation of re-
**RESULTS**

Digitonin-permeabilized Cells Harbor Highly Intact Mitochondria—Intactness of mitochondria in dt-BLCL was first established by the measurements of marker enzyme latencies (Table I). In the absence of detergent, about 1.5% of the total lactic dehydrogenase (activity measured in the presence of 0.1% Triton X-100) of the fraction could be measured, whereas no citrate synthase or cytochrome c oxidase activities were detected. Upon addition of digitonin (0.002%, 15 μg/ml protein), the lactic dehydrogenase activity was fully released, whereas citrate synthase or cytochrome c oxidase were still undetectable (Table I). Only upon addition of Triton X-100 was citrate synthase activity measurable, whereas cytochrome c oxidase was made fully measurable after addition of lauryl maltoside. The intactness of the mitochondria in dt-BLCL was further polarographically established by (i) the measurement of a low proton permeability as measured by the RC and (ii) the inability to use exogenously supplied NADH as a substrate (see Fig. 3). RC associated with succinate oxidation was consistently higher than 7. Finally, no significant oxidation of exogenous NADH could be observed in dt-BLCL. Similar results were obtained whenever the time of cell harvesting (up to 8 days after changing cell culture medium).

Cell Respiration and Mitochondrial Oxidation of NAD⁺-Dependent Substrates Are Decreased in Cells Maintained under Quiescent Conditions—Oxidative properties were first investigated in cells harvested 1 day after changing the culture medium (Fig. 1). Cell respiration was highly rotenone-sensitive, and an active oxidation was triggered by both succinate or malate plus glutamate addition (Fig. 1, traces a and b). Addition of oligomycin, known to specifically inhibit the ATPase (5), strongly decreased the rate of succinate oxidation; this inhibition was fully released by the addition of an uncoupler such as m-CCP (Fig. 1, trace a). A subsequent addition of malonate, a competitive inhibitor of the succinate dehydrogenase (6), brought about a full inhibition of succinate oxidation. The RC associated with succinate oxidation was higher than 7 (Fig. 1, trace a), and adding exogenous NAD⁺ did not affect the rate of malate oxidation (Fig. 1, trace b). When similar experiments were carried out using cells harvested 6 days after changing the culture medium, a lower rate of cell respiration was measured (Fig. 1, trace c). Under these conditions, the pattern of succinate oxidation appeared quite similar to the one measured using 1dBLCL. Adding NAD⁺ did not affect the oxidation of succinate which was associated with a high RC. However, when studying malate plus glutamate oxidation, a low rate of oxidation was measured upon ADP addition (Fig. 1, in the absence of added NAD⁺).

Exogenously Supplied NAD⁺ Restores the Ability of Mitochondria to Oxidize NAD⁺-Dependent Substrates—When digitonin-permeabilized 6dBLCL oxidizing malate plus glutamate were added with exogenous NAD⁺, a strong stimulation of oxygen uptake was observed (up to 120%) that was fully inhibited by rotenone (Fig. 1, trace d). As shown by the high RC value measured, oxidation was tightly coupled to the phosphorylation process. The stimulation of malate plus glutamate oxidation by NAD⁺ was found maximal for an external NAD⁺ concentration of about 500 μM (Fig. 1, inset).

The stimulatory effect of NAD⁺ was similarly observed using α-ketoglutarate (Fig. 2, trace a) or pyruvate plus malate (Fig. 2, trace b) as substrates. This ruled out any involvement of the malate-aspartate shuttle in the stimulation of substrate oxidation by added NAD⁺.

It is noteworthy that NADH could be substituted to NAD⁺ to stimulate substrate oxidation (compare the final rate of malate oxidation in Fig. 3, traces a and b), although NADH alone did not sustain any significant rate of oxidation (Fig. 3, trace b). In contrast, NAD⁺ could not be substituted to NAD⁺ (Fig. 3, trace c).

NAD⁺ Fluxes Are Features Distinct from the Nonspecific Loss of Mitochondrial Integrity Observed in Aged Cells—Oxidative

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**Table I**

| Cell treatment | BLCL₁ = 1 day | BLCL₁ = 6 days |
|----------------|---------------|----------------|
|                | Lactate dehydrogenase | Citrate synthase | Cytochrome c oxidase | Lactate dehydrogenase | Citrate synthase | Cytochrome c oxidase |
| No detergent   | 17 ± 3 (1%)<sup>a</sup> | ND<sup>b</sup> | ND | 25 ± 4 (1.5%)<sup>a</sup> | ND | ND |
| 0.002% digitonin | 1610 ± 58 (100%) | ND | ND | 1630 ± 71 (100%) | ND | ND |
| 0.2% Triton X-100 | 1600 ± 62 | 173 ± 15 | NM<sup>c</sup> | 1640 ± 65 | 164 ± 13 | NM |
| 2.5 mM lauryl maltoside | NM | NM | 205 ± 21 | 212 ± 27 |

<sup>a</sup> Percent of total activity of the fraction as determined in the presence of 0.2% Triton X-100.

<sup>b</sup> ND, not detectable.

<sup>c</sup> NM, not measured.
properties of mitochondria were next studied in dt-BLCL that had been kept for 10 h at 37°C in phosphate-buffered saline solution. Then mitochondria showed (i) a significant decrease of oxidation rates of all substrates studied, (ii) a complete loss of RC, while (iii) exogenous NADH became actively oxidized through a rotenone-sensitive pathway (not shown). None of these features were found in the BLCL (up to 8 days after changing the medium) used to show the stimulation of NAD dependent substrate oxidations by exogenous NAD.

In Situ, the pH of the Culture Medium Controls the Influx of NAD—The effect of NAD was next plotted according to the cell harvesting days (Fig. 4a). Up to 3 days after changing culture medium, no significant effect of NAD on malate oxidation could be measured in dt-BLCL (grown at about 2 × 10⁶ cells/ml). After this period, marked by a gradual pH decrease of the medium (from 7.50 to 6.72, Fig. 4c), a progressive increase of the NAD⁻⁻ effect was observed, which was fully and rapidly (less than 2 h) reversed upon renewing the cell culture medium. Indicative of the preservation of the integrity of the inner mitochondrial membrane, no change in the RC associated with succinate oxidation could be observed during the duration of this experiment (Fig. 4b).

In order to determine the respective role of the components of the culture medium in reversing the effect of NAD⁻⁻, several of these were next individually tested. Under conditions in which mitochondria appeared as NAD⁻⁻ depleted (6d-BLCL), when a glucose addition (4 g/liter) alone was substituted to the change of the complete culture medium, no significant decrease in the NAD⁻⁻ stimulation of malate oxidation could be observed after 2 h (Table II). This ruled out the possibility that a limiting glucose concentration could act as a primary and/or unique factor controlling the influx of NAD⁻⁻ into mitochondria. In contrast readjusting the pH value of the culture medium (from 6.7 to 7.5 by adding 500 mM Hepes buffer) restored in 2 h normal oxidation of NAD⁻⁻ dependent substrate oxidation by exogenous NAD⁻⁻. Studying a whole range of pH values between 6.7 and 7.5, we observed that mitochondrial matrix NAD pool appeared fully replenished after 2 h incubation for pH values above 7.0 (Fig. 5).

At this point, one could hypothesize that, paralleling culture medium acidosis, mitochondria became gradually NAD-depleted and that the mitochondrial matrix NAD pool could be replenished in vitro by exogenously supplied NAD⁻⁻ to dt-BLCL or in situ by re-adjusting the pH of the culture medium to a physiological pH (7.0). To support further this hypothesis, we next looked for other matrix-mediated effects of NAD⁻⁻ on substrate oxidation and finally investigated the NAD content of the cells and of their mitochondria.

Exogenous NAD⁻⁻ Increases the Autoinhibition of Mitochon-

![Fig. 2.](http://example.com/figure2.png) The effect of exogenously supplied NAD⁺ on the oxidation of α-ketoglutarate (trace a) and pyruvate plus malate (trace b) by digitonin-permeabilized 6d-BLCL. Substrate concentrations and experimental conditions are as in Fig. 1. α-Ketoglutarate, 10 mM; Pyruvate, 5 mM; Malate, 200 μM. Numbers along the traces are nmol of O₂ consumed min⁻¹ mg⁻¹ protein.

![Fig. 3.](http://example.com/figure3.png) The comparative effect of NAD⁺ (trace a), NADH (trace b), and NADP⁺ (trace c) on the oxidation of malate plus glutamate by digitonin-permeabilized 6d-BLCL. Substrate concentrations and experimental conditions are as in Fig. 1. NAD⁺, 1 mM; NADH, 1 mM; NADP⁺, 1 mM. Numbers along the traces are nmol of O₂ consumed min⁻¹ mg⁻¹ protein.

![Fig. 4.](http://example.com/figure4.png) Effect of cell harvesting time and of medium change on the stimulation of malate plus glutamate oxidation by exogenously supplied NAD⁺ in digitonin-permeabilized BLCL. a, time-dependent changes of the pH value of culture medium; b, values of respiratory control associated with succinate oxidation as measured in Fig. 2, trace a; c, time-dependent changes in percent stimulation of malate plus glutamate oxidation by exogenously supplied NAD⁺ (% of the rate measured in the absence of added NAD⁺) as measured in Fig. 2. Arrows indicate medium changes (RPMI 1640 added with 10% fetal calf serum).
TABLE II

| Experimental conditions | Percent stimulation of malate plus glutamate oxidation by exogenously added NAD⁺ |
|-------------------------|--------------------------------------------------------------------------------|
| No medium change        | 160 ± 20                                                                 |
| Medium change           | 0.5 ± 0.2                                                                  |
| Glucose added (4 g/l)   | 138 ± 35                                                                   |
| 500 mM Hepes added (pH 7.5, final concentration 150 mM) | 0.2 ± 0.1 |

*Percent stimulation of the rate measured in the absence of added NAD⁺.*

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Effects of medium change, glucose addition, and pH adjustment (pH 7.5) of culture medium on the stimulation of malate plus glutamate oxidation by exogenously supplied NAD⁺ in digitonin-permeabilized 6dBLCL

Culture medium pH was adjusted to the desired final values by adding 500 mM Hepes buffer. Measurements were carried out 2 h after the modifications of culture media. Experimental conditions are as described in Fig. 1 and under “Materials and Methods.” Each value is the mean ± S.D. of three to five replicates.

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![Diagram](a) Cells 1d
![Diagram](b) Cells 6d
![Diagram](c) Cells 6d

**FIG. 6.** Effects of cell harvesting time (a), exogenously supplied NAD⁺ (b), and rotenone (c) on the auto-inhibition of succinate oxidation by digitonin-permeabilized BLCL. Substrate concentrations are as in Fig. 2. Numbers along the traces are nmol of O₂ consumed min⁻¹ mg⁻¹ protein (n = 3).

We next estimated in situ the NAD content of mitochondria in dt-BLCL, still avoiding the isolation of the mitochondria. This was achieved by measuring the reduction of matrix NAD⁺ by reverse electron flow from succinate, which is known to reduce more than 90% of the matrix NAD⁺ pool, which then acts passively as an electron sink (4, 12). BLCL were initially incubated 5 min under aerobic conditions at room temperature in the presence of digitonin (15 µg/mg protein) in order to deprive mitochondria from any respiratory substrate.

When using digitonin-treated 1dBLCL, a rapid reduction of the matrix NAD⁺ was obtained in the presence of succinate and oligomycin, and adding exogenous NAD⁺ did not bring about any increase of fluorescence (Fig. 7, trace a). The addition of an uncoupler led to the rapid reoxidation of the NADH. This latter level of oxidation, which corresponds to a full oxidation of the NAD pool (4), was similar to the initial level measured before the addition of oligomycin, establishing that the NAD pool initially was fully oxidized. A further addition of rotenone did not affect the reduction of the NAD⁺. However, under these conditions, more NAD⁺ could be exogenously reduced by cytosolic malate dehydrogenase in the presence of malate plus glutamate (Fig. 7, trace a). In the absence of glutamate, this exogenously reduced NAD⁺ was instantaneously reoxidized by adding oxaloacetate (not shown). No reduction of NAD⁺ (before or after addition of exogenous NAD⁺) could be observed in the absence of succinate, or of oligomycin, or in the presence of rotenone (not shown).

When a similar experiment was carried out using 6dBLCL, a much lower amount of NAD⁺ was reduced in comparison with 1dBLCL (Fig. 7, comparison between traces b and a). Upon exogenous NAD⁺ addition, a significant increase of fluorescence was observed, which again could be abolished by adding an uncoupler. Finally, we found that the NAD⁺ reduced under such conditions was not available to external oxaloacetate, consistent with a matrix location of this NADH (Fig. 7, trace c). Using these 6dBLCL, the initial presence of rotenone (or of m-Cl-CCP) also abolished any reduction of NAD⁺, before or after the addition of exogenous NAD⁺ (not shown).

We finally isolated mitochondrial fractions from dt-BLCL and measured their NAD content. In good agreement with the estimation of NAD content through fluorimetric measurement (Fig. 7), a roughly 50% decrease of mitochondrial NAD content...
**Fluorescence** is only indicative, since based on the fluorescence of free mitochondrial matrix space to the cytosol and/or from an vested under quiescent conditions (6–8 days after medium dinucleotides. Our results show that human cultured cells have NAD+ amounts on the NAD+ and tested under strictly identical conditions, could be abolished by simply changing the cell culture medium.

As shown by the fluorimetric measurement of NAD+ reduction by reverse electron flow, external NAD+ uptake is relatively rapid (saturation after 20–30 s) and only catalytic amounts enter the mitochondrial matrix. These amounts roughly correspond to the replenishment of the mitochondrial NAD pool. They appear sufficient to saturate and stimulate the matrix dehydrogenases but remain insufficient to sustain any significant oxidation per se, as shown by the absence of oxidation triggered by the addition of NADH alone. How these catalytic amounts of NAD enter the mitochondrial matrix remains to be elucidated and is currently under investigation.

So far, a similar influx of NAD in the mitochondrial matrix has only been reported for plant mitochondria (1). In this latter instance, the varying sizes of the mitochondrial matrix NAD pool may play a general role in the coarse regulation of oxidative metabolism during the transition from dormancy to the stage of active plant growth and may be important during some processes such as seed germination (1). By analogy, it can be hypothesized that the observed decrease of the mitochondrial matrix NAD content in cultured cells plays a role in the slowing down of oxidative metabolism observed along with the impoverishment of culture medium, under quiescent conditions. This progressive and reversible decrease in the NAD content of the mitochondria appears to precede any other mitochondrial property change (decrease of membrane potential, of enzyme activity, of respiratory control, etc.).

Finally, any physiological significance of the effect of pH in controlling the size of the mitochondrial matrix NAD pool, as reported in our study, should be considered with circumspection. In human (if one discounts the peculiar conditions encountered by stomach and kidney cells), pH values in blood and body fluids are found to reach values below 7.0 only under exceptional and extreme conditions, i.e. ketoacidosis of insulin-dependent diabetes mellitus or lactic acidosis of noninsulin-dependent diabetes mellitus for example. Only localized, transitory, or intracellular pH values could perhaps be sufficiently low to favor a decrease of the mitochondrial NAD pool size, potentially contributing to the pathogenesis of diseases that are accompanied with significant acidosis.

The practical aspect of this study is related to the use made of circulating lymphocytes (15), BLCL (16), and cultured skin fibroblasts (17) to investigate patients suspected of mitochondrial disorders. Due to the difficulties encountered to assay complex I activity in these cells (17, 18), polarographic assays
tactness were all indicative of a high degree of integrity; (iii) when RC values associated with substrate oxidations were not changed; (iv) under conditions where exogenous NADH could not be quantitatively oxidized; and finally, (v) such a NAD influx, as estimated by the stimulation of substrate oxidation by NAD and tested under strictly identical conditions, could be abolished by simply changing the cell culture medium.

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of malate or pyruvate oxidations are widely used to indirectly detect potential complex I defects, which represent, according to our experience, about 35% (84/240) of the cases of respiratory chain disorder in children. Here we have shown that all these cell types, at some stage, potentially harbor NAD-depleted mitochondria with low ability to oxidize NAD$^+$-dependent substrates. The recognition of the variable size of the mitochondrial matrix NAD pool in human cells should allow avoidance of confusion between complex I deficiency and a low NAD content of the mitochondria, both impairing the ability of the mitochondria to oxidize NAD$^+$-dependent substrates.

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Fluxes of Nicotinamide Adenine Dinucleotides through Mitochondrial Membranes in Human Cultured Cells
Pierre Rustin, Béatrice Parfait, Dominique Chretien, Thomas Bourgeron, Fatima Djouadi, Jean Bastin, Agnès Rötig and Arnold Munnich

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