CHAPTER 1

Isolation and Subfractionation of Mitochondria from Animal Cells and Tissue Culture Lines

Francesco Pallotti* **and Giorgio Lenaz†**

* Department of Neurology
Columbia University
New York, New York 10032

† Dipartimento di Biochimica "G. Moruzzi"
Universita' di Bologna
40126 Bologna, Italy

METHODS IN CELL BIOLOGY, VOL. 65

I. Introduction

Past and current mitochondrial research has been performed on mitochondria prepared from rat liver, rat heart, or beef heart because these tissues can be obtained readily and in quantity. Toward the end of the 1980s, a new branch of human pathology began with the discovery of human disorders linked to mitochondrial dysfunction (mitochondrial encephalomyopathies). Therefore, it became necessary to investigate and to study the status of mitochondria in human tissue. As it is not always possible to obtain large amounts of human tissue for extracting mitochondria, interest arose in developing smaller scale mitochondrial isolation methods to determine mitochondrial enzyme profiles.

This chapter describes the methods for mitochondrial isolation used in experiments from both human and animal tissues. Further manipulations of isolated mitochondria give the possibility for better investigations of some enzymatic pathways related to the mitochondrial function.

II. General Properties of Mitochondrial Preparations

Almost all preparation methods present major similarities, but unfortunately the details are often tissue or laboratory specific (Nedergaard and Cannon, 1979). All the steps for mitochondrial isolation should be performed on ice or, if possible, in a cold room.

A. Isolation Medium

Principal osmotic support:

a. Nonionic, e.g., sucrose, mannitol, or sorbitol; usually about 0.25 *M* for mammalian tissues.

b. Ionic, e.g., KCl (100–150 m*M*) for those tissues that assume a gelatinous consistency upon homogenization. For special preparations (e.g., mitoplasts), hypotonic KCl buffers can also be used for swelling mitochondria as a valid alternative method to incubation in digitonin.

Possible additions:

a. EDTA (1 m*M*), in order to chelate Ca^{2+} ions, which can function as uncouplers and which are also cofactors for certain phospholipases.
b. Bovine serum albumin (BSA, 0.1–1%). It binds free fatty acids, acyl-CoA esters, lysophospholipids, or other detergents.
c. Buffer: Usually Tris–HCl or Tris–acetate (5–20 m*M*).

B. Homogenization

The initial destruction of intercellular connections, cell walls, and plasma membranes is necessary for mitochondrial isolation. The choice of a destruction technique should be dictated by the type of cells and intercellular connections, and is either mechanical or enzymatic. For soft tissue homogenization of a tissue mince, the use of a Dounce hand homogenizer or a power-driven Potter–Elvehjem glass–Teflon homogenizer is suitable. For more fibrous tissues, the use of a tissue blender (Waring, UltraTurrax) is necessary. The ratio of tissue to isolation medium is usually 1:5 to 1:10 (w/v); the use of proteases softens the tissue, especially muscle, prior to homogenization. The homogenization step is always carried out on ice in order to inactivate cellular ezymes that could damage mitochondria.

C. Differential Centrifugation

Differential centrifugation consists of two-step centrifugation carried out at low and at high speed, consecutively. The low-speed centrifugation is necessary to remove intact cells, cellular debris, and nuclei.

It is likely that mitochondria in the lower part of the tube could remain entrapped in the pellet, therefore resuspension of the latter and recentrifugation at low speed increases the mitochondrial yield. The two supernatants obtained from the low-speed centrifugation undergo a subsequent high-speed centrifugation to sediment mitochondria.

The low-speed centrifugation is usually carried out at 1000*g* for 10 min and the subsequent high-speed centrifugation at 10,000*g* for 10 min. In many tissues, a light-colored pellet or "fluffy" layer may sediment over the darker brown mitochondrial pellet after high-speed centrifugation. This layer consists of broken mitochondrial membranes and of mitochondria with structural alterations. The fluffy layer can be removed by gentle shaking in the presence of a few drops of medium and discarded. In some tissues, two distincts mitochondrial fractions can be isolated. Usually, the heavier pellet contains the metabolically most intact mitochondria.

D. Gradient Centrifugations

The pellet obtained from Section II,C is usually considered "crude," and in some cases a purification based on size and density is necessary. This can be achieved by using discontinuous gradients (sucrose, Ficoll, or metrizamide).

E. Storage

Mitochondrial pellets should be suspended in a minimal volume of isolation buffer. It is preferable to use nonionic media as they prevent the loss of peripheral proteins. Prior to storage or to use, the protein concentration of the mitochondrial preparation should be determined using either the biuret method (Gornall *et al.*, 1949) or the Lowry method (Lowry *et al.*, 1951). The first method is recommended if high yields of mitochondria are obtained, whereas the second method is more sensitive for determining small protein concentrations. It is recommended to store mitochondria at concentrations no lower than 40 mg protein per milliliter of resuspension buffer and kept frozen at −70°C.

F. Contaminants of Mitochondrial Fraction

The nature of the contaminants that may be present in a mitochondrial preparation depends on the biological material from which they are isolated (de Duve, 1967). Except for erythrocytes, few cells are small enough to escape sedimentation during the low-speed centrifugation. However, it is possible to find pinched-off cell fragments; these can be eliminated by gradient centrifugation. Because nuclei are often damaged by homogenization, they also represent a possible contaminant. Nuclei are observed as a grayish sediment, often associated with red blood cells, at the bottom of the mitochondrial pellet. Care should be taken in analyzing mitochondrial DNA from mitochondrial fractions because phagocytized nuclear DNA could also contaminate these preparations.

Secretion granules and Golgi vesicles can contaminate mitochondrial preparations derived from exocrine and endocrine glands (Palade *et al.*, 1962) and can be eliminated by gradient centrifugation.

Lysosomes are almost ubiquitous, but they are particularly abundant in liver, kidney, spleen, leukocytes, and macrophages. They contain a variety of substances at various stages of proteolytic and acidic digestion, as well as indigestible residues. In some cases, it is preferable to separate these contaminants from mitochondrial preparations by gradient centrifugation on a discontinuous metrizamide gradient (Genova *et al.*, 1994).

Peroxisomes (de Duve and Baudhuin, 1966) contain large amount of catalase, hydrogen-producing oxidases, and L-lactate. They are always present in mitochondrial fractions from liver, but having a higher median equilibrium density than mitochondria, they can be separated easily from the latter fraction by gradient centrifugation.

Melanosomes can also be separated from mitochondrial fractions by gradient centrifugation. Particulate glycogen may be present in mitochondrial fractions from liver of fed animals, but an overnight starving prior to sacrifice is sufficient to eliminate this contaminant from mitochondria.

Microsomes (typical endoplasmic reticular membranes with attached dense granules, cell membrane fragments, and Golgi membranes) are present in the "fluffy" layer that covers the mitochondrial pellet after the high-speed centrifugation. "Fluffy" layers are easily dislodged and washed away, but we usually retain this layer as it could contain fragments of mitochondrial membrane as well. The activity of the enzymatic complexes of the respiratory chain is usually assayed both in the absence and in the presence of a specific inhibitor for the complex examined, revealing the specificity for the mitochondrial assay.

G. Criteria of Purity and Intactness

Mitochondria free from contaminant membranes should have negligible activities of the marker enzymes for other subcellular fractions, such as glucose-6-phosphatase for endoplasmic reticulum, acid hydrolases for lysosomes, and catalase and D-amino acid oxidase for peroxisomes. However, mitochondria should be highly enriched for cytochrome *c* oxidase and succinate dehydrogenase. Citrate synthase seems to be a rather stable mitochondrial enzyme whose activity is not subjected to fluctuations and pathological changes; for this reason, when homogenates or impure mitochondrial fractions have to be used for enzymatic determinations, activities are best compared by normalization to citrate synthase in order to prevent artifacts due to differences in the content of pure mitochondria.

Intact mitochondria, when investigated in an oxygen electrode, should have high respiratory control ratios (RCR) with both NAD-linked substrates and succinate; ratios of state-3 to state-4 respiration higher than 4–5 are considered to be diagnostic of tightly coupled mitochondria. Coupled mitochondria usually exhibit ADP/O ratios higher than 2.5, and approaching 3 with NAD-linked substrates, and ratios higher than 1.8, approaching 2, with succinate, glycerol-1-phosphate, or acyl-carnitines as substrates. The ADP/O ratios with 2-ketoglutarate are usually higher than 3 because of substrate-level phosphorylation. Intact mitochondria also exhibit respiratory control at site 3 using ascorbate and tetramethyl-phenylenediamine (TMPD) to reduce cytochrome *c* and cytochrome oxidase in the presence of a complex III inhibitor, such as antimycin A; the RCR, however, is usually lower. The ADP/O ratio at site 3 is usually higher than 0.5, theoretically approaching 1.

III. Mitochondria from Beef Heart

Mitochondria prepared from heart muscle present some peculiar advantages over those from other mammalian tissues. They are stable for up to a week with respect to oxidation and phosphorylation when stored at 4°C and for up to a year at −20°C. Generally, mitochondria prepared from slaughterhouse material are functionally intact. While beef heart is the tissue of choice for this preparation, pig heart may also be used.

A. Small-Scale Preparation

The method used is essentially the one described by Smith (1967), with modifications to allow large-scale isolation (i.e., from one or two beef hearts).

Hearts are obtained from a slaughterhouse within 1–2 h after the animal is slaughtered and placed in ice. All subsequent procedures are carried out at 4°C.

The tissue is trimmed from fat and connective tissue and cut into small cubes. Two hundred grams of tissue is passed through a meat grinder and placed in 400 ml of sucrose buffer (0.25 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.8). The suspension is homogenized in a Waring blender for 5 sec at low speed, followed by 25 sec at high speed. At this stage, the pH of the suspension must be adjusted to 7.5 with 1 *M* Tris.

The homogenate is centrifuged for 20 min at 1200*g* to remove unruptured muscle tissue and nuclei. The supernatant is filtered through two layers of cheesecloth to remove lipid granules and is then centrifuged for 15 min at 26,000*g*.

The mitochondrial pellet obtained is resuspended in sucrose buffer and is homogenized in a tight-fitting, Teflon–glass Potter–Elvejhem homogenizer (clearance of 0.006 inch) and is then centrifuged at 12,000*g* for 30 min. The pellet is resuspended in the sucrose buffer and stored at −80°C, at a protein concentration of 40 mg/ml. The protein concentration is determined using the biuret method (Gornall *et al.*, 1949).

The heavy fraction of beef heart mitochondria obtained by the procedure just described was reported to have a high content of mitochondrial components with an excess of cytochrome oxidase over the other complexes (Capaldi, 1982) (Table I) and with high

Table I
Main Components of Oxidative Phosphorylation System in Bovine Heart Mitochondrial Membrane[a]

	Concentration range		Molecular mass (kDa)	Number of polypeptides	Prosthetic groups[c]
	nmol/mg protein	μ*M* in lipids[b]			
Complex I	0.06–0.13	0.12–0.26	700	42	FMN, 7Fe-S
Complex II	0.19	0.38	200	4–5	FAD, 3Fe-S
Complex III	0.25–0.53	0.50–1.06	250	12	2*b*, c_1, Fe-S
Complex IV	0.60–1.0	1.20–2.0	160	12	*a*, a_3, 2Cu
Cytochrome *c*	0.80–1.02	1.60–2.04	12	1	*c*
Ubiquinone-10	3.0–8.0	6.0–16.0	0.75	—	—
NADH-NADP transhydrogenase	0.05	0.1	120	1	—
ATP synthase	0.52–0.54	1.04–1.08	500	23[d]	—
ADP/ATP translocator	3.40–3.60	6.8–9.2	30	1	—
Phospholipids	440–587	—	0.7–1.0	—	—

[a] Modified from Capaldi (1982).
[b] Assuming phospholipids to be 0.5 mg/mg protein.
[c] Fe-S, iron–sulfur clusters; *b*, c_1, *c*, *a*, and a_3 are the corresponding cytochromes.
[d] Fourteen types of subunits.

Table II
Oxidation of Various Substrates by Beef Heart Mitochondria[a]

Substrate	Rate of oxidation [b]	P:O
Pyruvate + malate	0.234	2.9
Glutamate	0.181	3.1
2-Oxoglutarate	0.190	3.8
3-Hydroxybutyrate	0.123	3.0
Succinate	0.050	2.0

[a] Modified from Hatefi *et al.* (1961).
[b] Expressed as micrograms of atoms oxygen taken up per minute per milligram of mitochondrial protein.

respiratory activities accompanied by high RCR and by P/O ratios approaching the theoretical values (Table II).

In our experience, mitochondria obtained using the just-described isolation method do not exhibit good respiratory control. However, they have rates of substrate oxidation comparable to those described by Smith (1967). This is likely to be due to the quality of distilled water used for the preparation as well as to the extent of mitochondrial rupture by the harsh treatment. Therefore, we only use beef heart mitochondria (BHM) for manipulations that do not require coupled mitochondria.

When the activity of individual enzymes, and not overall respiration, has to be determined, impermeable substrates, such as NADH, should be allowed to cross the inner membrane, whereas cytochrome *c* should enter the intermembrane space. BHM preparations should undergo two to three freeze–thaw cycles in order to disrupt the outer and the inner mitochondrial membranes, thus allowing NADH to enter the matrix and cytochrome *c* to become available at the outer side of the inner membrane. Alternatively, small amounts of detergents, such as deoxycholate, may be added to break the permeability barrier. Many individual and combined activities can be assayed, such as NADH CoQ reductase (complex I), succinate CoQ reductase (complex II), ubiquinol cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV), NADH-cytochrome *c* reductase (complexes I + III), and succinate cytochrome *c* reductase (complexes II + III).

B. Preparation of Coupled Submitochondrial Particles

BHM preparations obtained with the method just described are not maximally efficient for the coupling of oxidation to phosphorylation. However, further manipulations of BHM can yield a preparation of submitochondrial particles fully capable of undergoing coupling of oxidative phosphorylation. These are known as phosphorylating electron transfer particles derived from beef heart mitochondria (ETPH).

ETPH are inside-out vesicles formed by pinching and resealing of the cristae during sonication (Fig. 1) and are obtained using the methods of Beyer (1967) and Hansen and

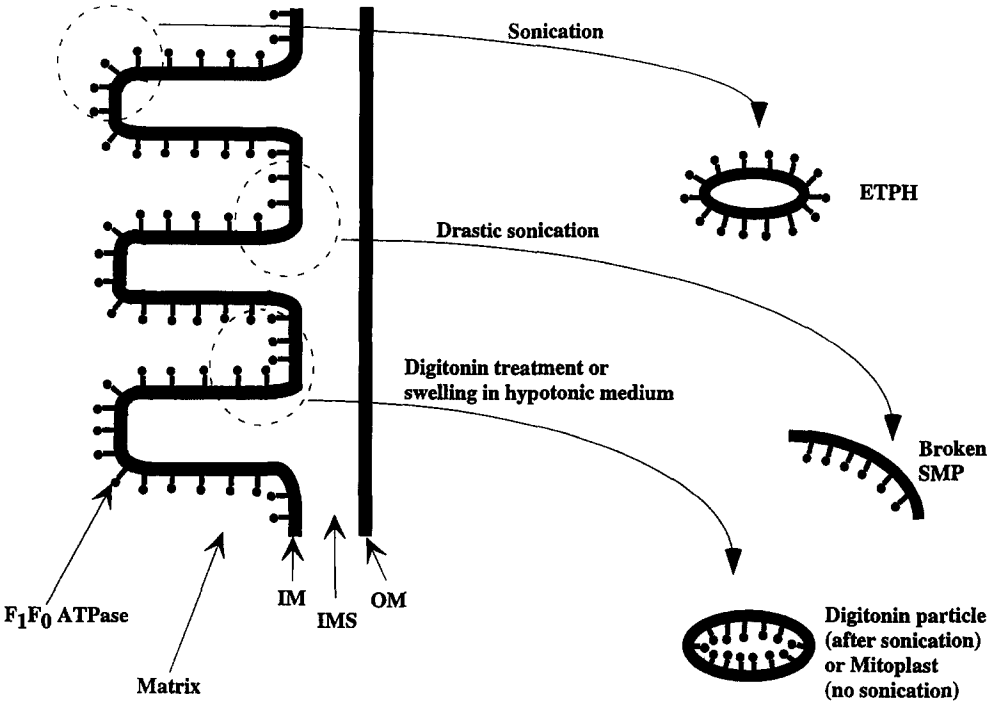

Fig. 1 Derivation of ETPH, digitonin particles, mitoplasts, and SMP from mitochondrial cristae (IM, inner membrane; IMS, intermembrane space; OM, outer membrane).

Smith (1964), with minor modifications. These particles are often better sealed, with a more intact permeability barrier, and therefore they have higher coupling capacity than their parent mitochondria.

BHM are prepared as described in Section II,A. However, the mitochondrial pellet, either freshly prepared or thawed from −80°C storage, is resuspended in STAMS buffer (0.25 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.8, 1 m*M* ATP, 5 m*M* $MgCl_2$, 10 m*M* $MnCl_2$, and 1 m*M* potassium succinate). The final protein concentration is adjusted to 40 mg/ml with STAMS. Aliquots of 20–25 ml of this mitochondrial suspension are subjected to sonic irradiation for 30 s using a probe sonicator (we use either the Branson sonicator at 20kc or the Braun sonifier set at 150).

The suspension is then centrifuged at 20,000*g* for 7 min in order to remove big particles. The supernatant is decanted and centrifuged at 152,000*g* for 25 min, and the pellet is rinsed with STAMS buffer and resuspended to a final protein concentration of 20 mg/ml in a preserving mix containing 0.25 *M* sucrose, 10 m*M* Tris–HCl, pH 7.5, 5 m*M* $MgCl_2$, 2 m*M* GSH, 2 m*M* ATP, and 1 m*M* succinate (Linnane and Titchener, 1960).

ETPH exhibit high rates of NADH and succinate oxidation, but no respiratory control: they are therefore loosely coupled. However, respiratory control can be shown in these particles by the addition of oligomycin; this ATPase inhibitor slows down respiration

rates, significantly, indicating that the lack of respiratory control is probably due to the backflow of protons through the ATPase membrane sector.

Coupled ETPH exhibit fluorescence quenching of 9-aminoacridine and derivatives, such as atebrine and ACMA (9-amino-6-chloro-2-methoxy acridine) in the presence of substrates and in the presence of valinomycin plus potassium (added in order to collapse the membrane potential), indicating the formation of ΔpH in closed inverted vesicles (the ΔpH usually obtained with succinate is 3.15) (Lenaz *et al.*, 1982). The precise determination of the pH difference across the external and internal compartment of ETPH is given by the distribution of 9-aminoacridine following the development of transmembrane pH differences, as only the uncharged species of the amine is freely permeable across the membrane (Casadio *et al.*, 1974). The fluorescence intensity of 9-aminoacridine is dependent on the concentration of the amine (Fig. 2).

C. Broken Submitochondrial Particles

Submitochondrial particles (SMP) are broken membrane fragments and therefore can react with exogenous by-added cytochrome *c*. They are not coupled, but they have good rates of individual enzymatic activities, such as NADH-coenzyme Q oxidoreductase and ubiquinol-cytochrome *c* oxidoreductase. SMP have been used in our laboratory for the kinetic characterization of complex I (Estornell *et al.*, 1993) and complex III (Fato *et al.*, 1993).

SMP are prepared by sonication of BHM, as obtained in Section III,A, in an MSE sonifier for 5 min, at 30-s intervals, thus allowing the preparation to cool down. Preparations are kept on ice and under nitrogen in order to avoid lipid peroxidation.

The mitochondrial suspension is then centrifuged at 20,000*g* for 10 min; the supernatant is collected and ultracentrifuged at 152,000*g* for 40 min. SMP are resuspended in sucrose buffer and kept frozen at $-80°C$ at a protein concentration of 40–50 mg/ml until needed.

In these particles, succinate evokes a slight quenching of 9-aminoacridine fluorescence that was roughly calculated to correspond to <5–8% sealed inverted vesicles (Casadio *et al.*, 1974). SMP can be used to assay NADH-, succinate-, and ubiquinol-cytochrome *c* reductase (or cytochrome *c* oxidase) without adding detergents (Table III).

The use of detergents (i.e., deoxycholate) is important in evaluating the status of the particles; if detergents stimulate cytochrome *c* reduction by upstream substrates, the preparation contains high levels of closed particles (Degli Esposti and Lenaz, 1982). However, ETPH must have very low cytochrome *c* reductase activities that are strongly stimulated by adding detergents.

D. Cytochrome *c*-Depleted and Cytochrome *c*-Reconstituted Mitochondria and Electron Transfer Particles Derived from Beef Heart Mitochondria

Cytochrome *c* is a mobile component of the respiratory chain and in order to estimate the amount of this component in mitochondria it is necessary to release it from the organelle. The quantitative extraction of cytochrome *c* can be accomplished with

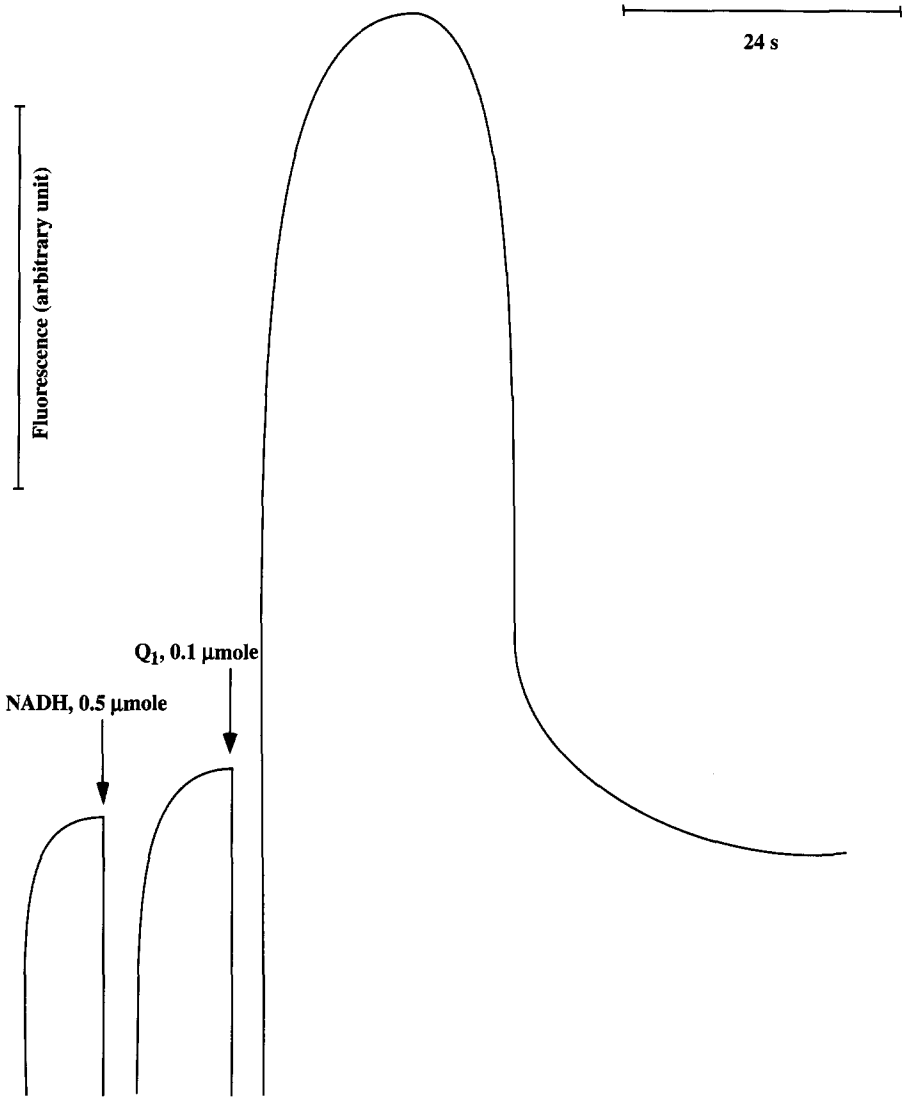

Fig. 2 Quenching of fluorescence of atebrine in an ETPH preparation induced by the oxidation of NADH by coenzyme Q_1(modified from Melandri *et al.*, 1974). The assay is performed in a final volume of 2.5 ml containing 100 μmol glycylglycine, pH 8.0; 1.25 nmol sucrose; 12.5 μmol $MgCl_2$; 250 μmol KCl; 2.5 μmol EDTA; 4 μg valinomycin; 10 nmoles atebrine; and ETPH corresponding to 480 μg of protein.

Table III
Some Individual Respiratory Chain Activities in Broken SMP

Activity	Substrates	V_{max} (μmol/min.mg)	k_{cat} (sec^{-1})
NADH-CoQ[a]	NADH, CoQ_1	0.90	380
	NADH, DB[d]	0.58	225
Ubiquinol-cytochrome *c*[b]	Ubiquinol-1, cytochrome *c*	2.77	220
	Ubiquinol-2, cytochrome *c*	4.65	370
Succinate-CoQ[c]	Succinate, CoQ_1	1.08	63

[a] From Fato *et al.* (1996).
[b] From Fato *et al.* (1993).
[c] Unpublished data from R. Fato and G. Lenaz.
[d] 6-Decylubiquinone.

salts, yielding a good relative amount of nondenatured mitochondria. The method for cytochrome *c* extraction from mitochondrial preparations has been described previously by MacLennan and colleagues (1966). It is summarized briefly in this section.

Suspensions of freshly isolated BHM at a concentration of 40 mg/ml are frozen at −20°C in a preserving medium containing 0.25 *M* sucrose, 0.01 *M* Tris–acetate, pH 7.5, 1 m*M* ATP, 1 m*M* $MgCl_2$, and 1 m*M* succinate. When thawed, the suspension is adjusted to pH 7.8 and centrifuged at 26,000*g* for 10 min. The supernatant and the residual light layer, composed of light mitochondria, are removed and the dark pellet is suspended in a solution of 0.015 *M* KCl (Jacobs and Sanadi, 1960), a hypotonic medium for intact mitochondria, to a protein concentration of 20 mg/ml. Mitochondria are allowed to swell for 10 min on ice before centrifugation at 105,000*g* for 15 min.

The colorless supernatant is discarded and the pellet is resuspended in 0.15 *M* KCl, an isotonic medium for intact mitochondria, and left on ice for 10 min. The last centrifugation step is carried out at 105,000*g* for 15 min. The resulting supernatant is red in color, representing cytochrome *c*. The mitochondrial pellet undergoes two further extractions in the isotonic medium.

The final centrifugation in the isotonic medium yields a fraction of light mitochondria with low phosphorylative capacity, which should be removed. The pellet of cytochrome *c*-depleted mitochondria is resuspended in 0.25 *M* sucrose and 0.01 *M* Tris–acetate, pH 7.5. This procedure usually removes about 85% of cytochrome *c* content from mitochondria, and the rates of substrate oxidation of these mitochondria are 15% of the rates of the mitochondrial preparation before the extraction procedure.

Preparation of cytochrome *c*-depleted ETPH is achieved by the sonication of cytochrome *c*-depleted mitochondria (Lenaz and MacLennan, 1967), using the conditions for sonication described in Section III,A. Usually the ETPH preparation undergoes two sonication cycles: after the first low-speed centrifugation (26,000*g*) the supernatant is collected and the pellet is resonicated and recentrifuged at low speed. The resulting supernatant is combined with the first one and centrifuged at high speed (105,000*g*). Cytochrome *c*-depleted ETPH preparations show reductions in substrate oxidation and in P/O ratios in comparison with normal ETPH.

Table IV
Oxidative and Phosphorylating Properties of Cytochrome c-Depleted and -Reconstituted Mitochondria and ETPH

A. BHM (data obtained from MacLennan *et al.*, 1966)

Substrate	Cytochrome *c* removed oxygen uptake[a]	P/O ratio	Cytochrome *c* added oxygen uptake[a]	P/O ratio
Pyruvate + malate	0.022	1.14	0.189	2.40
Succinate	0.035	0.71	0.136	1.06
Ascorbate + TMPD	0.037	0.24	0.169	0.63

B. ETPH (data obtained from Lenaz and MacLennan, 1966)

Treatment	Oxygen uptake with succinate[a]	P/O ratio
None	0.163	1.02
ETPH (− cytochrome *c*)[b]	0.064	0.77
ETPH (+ cytochrome *c*)[c]	0.155	1.15
ETPH (− cytochrome *c*) + cytochrome *c* added after sonication	0.093	0.55

[a] Expressed as μg atoms/min·mg protein.
[b] By sonication of cytochrome *c*-depleted mitochondria.
[c] Cytochrome *c* added after sonication.

Sonication of cytochrome *c*-depleted mitochondria can also be performed in isotonic KCl instead of in the classic medium for ETPH preparation (see earlier discussion) (Lenaz and MacLennan, 1966), with further reduction of both oxidation rates and P/O ratios.

Cytochrome *c* can be reincorporated in cytochrome *c*-depleted mitochondria through incubation in the presence of high levels of exogenous cytochrome *c*.

Reincorporation of cytochrome *c* into cytochrome *c*-depleted ETPH is complicated by the fact that ETPH are inside-out vesicles that cannot react with exogenous cytochrome *c*. For this reason, KCl-extracted BHM are resupended in the preserving mix for ETPH, with the exception that cytochrome *c* is added at a concentration of 10 μg per milligram of protein, before BHM are disrupted by sonication. The same procedure as for preparation of the cytochrome *c*-depleted ETPH is then followed.

Table IV summarizes the properties of cytochrome *c* depleted and reconstituted preparations.

E. Coenzyme Q-Depleted and Coenzyme Q-Reconstituted Mitochondria

Coenzyme Q (CoQ) can be extracted from BHM preparations using organic solvents. Coenzyme Q-depleted mitochondria are usually reconstituted with coenzyme Q homologues and analogs. Polar solvents, such as acetone, were first used for CoQ extraction, but they irreversibly damage complex I; thus nonpolar solvents are preferred, but they can extract neutral lipids, such as CoQ, only from dry material. The extraction is

performed on lyophilized BHM preparations following the method of Szarkowska (1966) with modifications.

The BHM preparation (Section III,A) is thawed on ice and diluted to a final protein concentration of 20 mg/ml in sucrose buffer (0.25 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.5). The suspension is centrifuged at 35,000*g* for 10 min (17,000 rpm in a Sorvall SS34 rotor). The pellet is resuspended in 0.15 *M* KCl, frozen at −80°C, and lyophilized.

The extraction of coenzyme Q is performed using pentane, homogenizing mitochondria in a Potter–Elvejhem homogenizer with a Teflon pestle, and centrifuging the suspension at 1100*g* for 10 min (3000 rpm in a Sorvall SS34 rotor). The supernatant, containing pentane, is removed and extraction–homogenization is repeated four times. Finally, pentane is removed from the extracted mitochondria, first in a rotary evaporator under reduced pressure at 30°C and then under high vacuum at room temperature for 2 h.

Lyophilized mitochondria are homogenized in sucrose buffer, centrifuged at 35,000*g* for 10 min, and resuspended in the same buffer. This suspension can either be used immediately or stored at −80°C for later use.

Reconstitution of coenzyme Q-depleted mitochondria is attained by treating the dry extracted mitochondrial powder with either the pentane extract or coenzyme Q homologues and analogs in pentane, following some modification in the method described by Norling *et al.* (1974).

Depleted particles are homogenized in a small volume of pentane (usually 2 ml) in the presence of a known amount of coenzyme Q. The amount of CoQ is usually adjusted to a final concentration between 0.4 and 25 nmol CoQ/mg mitochondrial protein. The protein content of CoQ-depleted mitochondria is estimated from the dry weight, considering that 3 mg dry weight corresponds to 1 mg mitochondrial protein, as measured by the biuret method.

The particle suspension is transferred in a rotary evaporator at 4°C under a slightly reduced pressure. Gradual removal of pentane is essential in order to get a good rate of incorporation (pentane should evaporate over a period of 30 min). After complete removal of the pentane, particles are dried at 4°C for an additional 30 min under vacuum and resuspended in sucrose buffer.

Coenzyme Q-depleted mitochondria have negligible contents of residual CoQ (less than 20 pmol/mg protein) and exhibit very low rates of both NADH and succinate oxidation in comparison with controls (either intact or lyophilized mitochondria); reconstitution with long-chain ubiquinones (CoQ_5 to CoQ_{10}) reconstitutes maximal rates of both enzymes, whereas short-chain homologs are not able to recover NADH oxidation and behave as complex I inhibitors. Some properties of these mitochondria are shown in Table V.

IV. Mitochondria from Rat Liver

Liver mitochondria prepared from rats are usually suitable for biochemical assays in studies on the pharmacological effects of different drugs or on effects of specific diets on mitochondrial membrane composition. We used rat liver mitochondria in studies after

Table V
Kinetic Constants of NADH- and Succinate-Cytochrome *c* Reductase in Lyophilized and Pentane-Extracted Beef Heart Mitochondria Reconstituted with Some Representative CoQ Homologues Having Different Lengths of the Isoprenoid Side Chain

	NADH-cytochrome *c* reductase		Succinate-cytochrome *c* reductase	
Quinone	V_{max} [a]	K_m [b]	V_{max} [a]	K_m [b]
CoQ_{10}	0.64 ± 0.46	1.53 ± 1.16	0.26 ± 0.17	0.42 ± 0.57[c]
CoQ_3	0.12 ± 0.04	1.42 ± 0.92	0.20 ± 0.01	0.53 ± 0.33[c]
CoQ_5	0.15 ± 0.02	1.46 ± 0.38	0.13 ± 0.05	0.37 ± 0.24[d]
6-Decylubiquinone	0.55	25	0.52	3.8

[a] Expressed as μmol/min/mg protein.
[b] Expressed as nmol quinone/mg protein, except for DB, whose K_m was calculated as m*M* quinone in the phospholipids.
[c] Statistically significant with respect to the corresponding K_m of NADH-cytochrome *c* reductase, $p < 0.05$.
[d] As above, $p < 0.001$.

perfusion and in studies where quinones were incorporated in liver fractions. Rat liver contains a considerable amount of mitochondria and is easier to manipulate than skeletal muscle. Generally, a high mitochondrial yield is obtained from rat liver.

A. Standard Preparation

Our method used for the extraction of mitochondria from rat liver is a modification of the method described by Kun and colleagues (1979). This method is based on the typical differential centrifugation procedure used for other mitochondrial preparations.

Fresh tissue is chilled on ice and washed in 0.22 *M* mannitol, 0.07 *M* sucrose, 0.02 *M* HEPES, 2 m*M* Tris–HCl, pH 7.2, and 1 m*M* EDTA (solution A). Subsequently, it is minced with scissors and washed three times in solution A with 0.4% BSA to remove blood and connective tissue and weighed in a prechilled glass petri dish.

The suspension is then homogenized in a prechilled Potter–Elvehjem glass homogenizer using a Teflon pestle and filtered. The homogenate is centrifuged at 3000*g* for 1.5 min (5000 rpm in a Sorvall SS34 rotor); the supernatant is decanted and the pellet is resuspended in solution A and is subjected to a second centrifugation step.

The two supernatants are combined and centrifuged at 17,500*g* for 2.5 min (12,000 rpm in a Sorvall SS34 rotor). The resulting pellet undergoes a wash in solution A and then it is centrifuged at 17,500*g* for 4.5 min. The pellet is resuspended in 0.22 *M* mannitol, 0.07 *M* sucrose, 0.01 *M* Tris– HCl, pH 7.2, and 1 m*M* EDTA (solution B) and centrifuged at 17,500*g* for 4.5 min. The pellet is finally resuspended in solution B at a ratio of 10 ml solution B per 7 g of starting material in order to standardize the protein content of the mitochondrial fraction.

Mitochondria obtained with this method also contain a lysosomal fraction, but if used immediately they show a good respiratory control with glutamate–malate and with

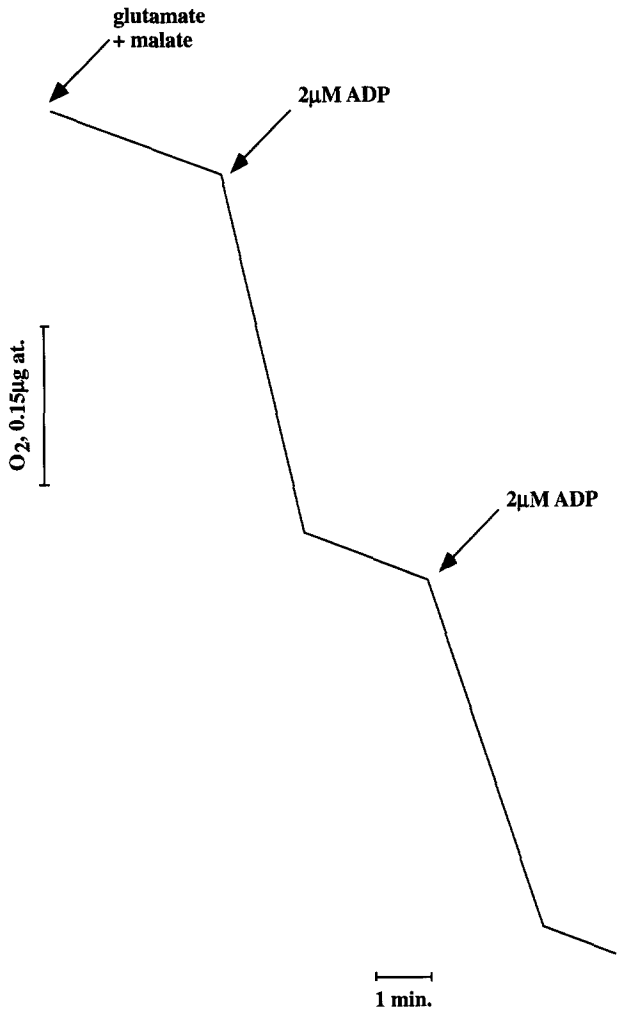

Fig. 3 Respiratory control in the presence of glutamate + malate in a mitochondrial preparation from rat liver. This preparation presented a respiratory control ratio (RCR) equal to 11.

succinate (Fig. 3) and good enzymatic activities of the four mitochondrial complexes (after membrane permeabilization by freeze–thaw cycles or by detergents).

B. Gradient-Purified Rat Liver Mitochondria

The preparation of rat liver mitochondria free from lysosomal contamination involves the purification of mitochondria in metrizamide gradient. The method described here is a modification of the one described by Kalen *et al.* (1990). This procedure for purification

of mitochondria has been used for monitoring the incorporation of exogenous CoQ by rat liver fractions (Genova *et al.,* 1994).

The liver is removed, chopped, weighed, and washed at least 10 times in ice-cold solution A (0.33 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.4, 1 m*M* EDTA, 0.4% BSA). The tissue is then homogenized in a prechilled Potter–Elvehjem glass homogenizer with a Teflon pestle to a ratio 1:4 (w/v) tissue to solution A.

The homogenate is centrifuged at 310*g* for 10 min; the supernatant is decanted and the pellet is resuspended in solution B (0.33 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.4, 1 *M* EDTA) and recentrifuged in order to obtain a clean nuclear fraction. The supernatant, collected previously, is centrifuged at 2800*g* for 20 min, and the pellet (washed twice) is recovered. This pellet contains a fraction of mitochondria defined as "heavy mitochondria" (HM). The supernatant is then centrifuged at 11,400*g* for 20 min, thus giving a pellet of mitochondria defined as "light mitochondria" (LM) containing lysosomes. The resulting supernatant, containing microsomes and cytosol, is discarded. The LM and HM fractions are resuspended in solution B at a volume three times the weight of the starting rat tissue.

Aliquots of HM and LM are then added to 2 volumes of metrizamide solution as described (Wattiaux *et al.,* 1978), usually 0.4 ml of mitochondria in 0.8 ml metrizamide 85.6% (w/v).

One milliliter of HM/LM-metrizamide solution is then layered at the bottom of a 5-ml ultracentrifuge tube, and a discontinuous metrizamide gradient is stratified on it by adding 0.6 ml 32.82% metrizamide, 0.6 ml 26.34% metrizamide, 0.6 ml 24.53% metrizamide, and 0.6 ml 19.78% metrizamide. Centrifugation is then performed in a swinging bucket rotor at 95,000*g* for 2 h at 4°C.

Mitochondrial subfractions can be collected using a Pasteur pipette. Usually, three subfractions from HM preparations and four subfractions from LM preparations (Genova *et al.,* 1994) are observed.

Mitochondria prepared with this procedure preserve good activities of the respiratory chain complexes, but little respiratory control.

C. Liver Submitochondrial Particles (SMP)

It is also possible to obtain submitochondrial particles from rat liver. Usually, SMP from rat liver are prepared after intensive sonication of mitochondria; thus they are not coupled. However, they can be used for studying NADH oxidation because intact mitochondria are not permeable to NADH.

The method used in our experiments is essentially the one described by Gregg (1967). Mitochondria are prepared from 20–30 g of rat liver and the buffer used is the same as that used for the preparation of broken SMP particles from BHM (Section III,C), with the addition of 0.4% BSA from the beginning of the isolation procedure.

D. Mitochondria from Rat Hepatocytes

Preparation of isolated rat liver cells is usually performed following the two-step collagenase liver perfusion technique of Seglen (1976). The purpose of this technique

is to obtain intact hepatocytes separated from nonparenchymal cells (up to 40% of total liver tissue); we have used this method to treat isolated cells with adriamycin in order to induce oxidative stress by reduction of this potent anticancer agent to semiquinone, thus releasing superoxide anion and hydrogen peroxide. In order to evaluate cellular integrity (or viability), the trypan blue exclusion test is performed. Mitochondria are prepared from hepatocytes (30×10^6 cells) incubated, at a starting concentration of 1×10^6 cells/ml, for 2 h (Wells *et al.*, 1987; Barogi *et al.*, 2000). Cells are suspended in a buffer containing 0.25 *M* sucrose, 0.01 *M* Tricine, 1 m*M* EDTA, 10 m*M* NaH_2PO_4, 2 m*M* $MgCl_2$, pH 8 (solution A) with 0.4% BSA, frozen at −80°C for 10 min to break the plasma membrane, and centrifuged at 760*g* for 5 min.

The supernatant is kept while the pellet undergoes a second homogenization step, using a UltraTurrax homogenizer for 10 min, followed by centrifugation at 760*g* for 5 min. The supernatants from the previous two steps are combined and centrifuged for a further 20 min at 8000*g*. The mitochondrial pellet is washed once with the same buffer in the absence of BSA and finally resuspended in the same buffer.

Mitochondria obtained with this method show good respiratory activities, but the NAD-dependent activities are rather low (Barogi *et al.*, 2000).

E. Isolation of Mitochondria from Frozen Tissues

Generally, mitochondrial isolations are performed on fresh tissue. Under these conditions, isolated mitochondria are pure and suitable for most of the biochemical assays and for further modifications of mitochondria used in particular assays. However, it is often necessary to prepare mitochondria from frozen tissues in cases where fresh tissues are not available.

The most important step in the procedure is the freeze–thawing of the tissue. Mitochondrial membranes are very sensitive to temperature variations, and the rupture of the membranes (cellular and mitochondrial) prior to homogenization not only makes it impossible to achieve separation of the subcellular fractions, but also modifies biochemical parameters.

In our experience, the most suitable method to preserve organs prior to extraction of mitochondria is that described by Fleischer and Kervina (1974) for liver tissue. We have also applied the procedure for extractions from other organs, such as heart, muscle, and kidney (Castelluccio *et al.*, 1994; Barogi *et al.*, 1995). This method allows for tissue storage for prolonged periods prior to the extraction of mitochondria.

After sacrificing the animal, organs are weighed and 1 volume of storage medium [0.21 *M* mannitol, 0.07 *M* sucrose, 20% dimethyl sulfoxide (DMSO), pH 7.5] is added. DMSO, used as an antifreeze agent, has the advantage over glycerol in that it is less viscous and penetrates the tissue rapidly. It is important to prevent ice crystal formation; in order to minimize this phenomenon, the organs should be frozen rapidly, and the thawing of them should be performed as quick as possible. Once in storage medium, the organ can be either diced into small pieces and homogenized or it can be maintained intact before quick freezing in liquid nitrogen. In our experience, intact organs can be stored in liquid nitrogen with no apparent alterations to mitochondrial function.

In soft tissues, such as liver, the storage medium diffuses through the organ rapidly. However, it is recommended to cut more fibrous tissues, such as kidney, heart, and muscle, into two to three pieces (1 cm^3) to facilitate medium penetration into the tissue. After rapid freezing in liquid nitrogen, the organs are stored in liquid nitrogen until mitochondrial isolation is required.

The thawing procedure should be performed quickly. It is necessary to preheat the thawing medium (0.25 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.5) at 45°C before adding it to the frozen tissue at a 4:1 ratio of medium to tissue. The thawing medium for liver should also contain 0.4% BSA. The extraction of mitochondria is then performed using the method described in Section IV,A, which is also suitable for heart, kidney, and muscle.

F. Phospholipid-Enriched Mitoplasts and SMP with/without Excess Coenzyme Q

The addition of phospholipid (PL) to mitoplasts or SMP increases the inner mitochondrial membrane surface area and dilutes intramembrane proteins. In the case of SMP, PL enrichment facilitates the study of diffusion control of electron transfer by ubiquinone.

Mitochondria are prepared from rat liver as described in Section IV,A. The mitochondrial pellet, however, is resuspended in 0.22 *M* mannitol, 0.07 *M* sucrose, 2 m*M* HEPES, pH 7.4 (solution A). After centrifugation at 20,500*g* for 20 min, the pellet is resuspended in 15 m*M* KCl and kept on ice for 10 min, thus allowing the external membrane to swell. The suspension is centrifuged at 31,000*g* for 45 min and resuspended in solution A. Alternatively, mitochondria from rat liver are washed in solution A in the presence of 0.5 mg/ml BSA, centrifuged at 4500*g*, and resuspended in solution A.

An alternative method for removing the outer membrane and for purifying the inner membrane–matrix fraction is given by the "controlled digitonin incubation" method (Schnaitman and Greenawalt, 1968). Digitonin is diluted in solution A at a concentration of 2% (w/v) and 0.5 mg/ml BSA is added after the digitonin is dissolved. The rat liver mitochondrial suspension is then treated with 1.1 mg of digitonin solution per 10 mg of mitochondrial protein. The suspension is stirred gently for 15 min and then diluted in 3 volumes of solution A. This suspension is then centrifuged at 31,000*g* for 20 min and then resuspended in solution A.

Unilamellar vesicles are prepared from soybean phospholipids (asolectin) by suspending the phospholipids in solution A at a concentration of 200 mg PL/ml solution A. The suspension is then sonicated using a MSE sonifier for 30 min with 30-s cycles.

Unilamellar PL vesicles enriched with coenzyme Q_{10} are prepared drying, under nitrogen atmosphere, both CoQ_{10} suspended in ethanol solution and phospholipids in chlorophorm/methanol. Dried PL and CoQ_{10} are then resuspended in solution A and sonicated with an MSE sonifier for 30 min with cycles of 30-s intervals.

Reincorporating mitoplasts or SMP with PL- or PL/CoQ_{10}-enriched vesicles is carried out as follows (Schneider *et al.*, 1980): 3 mg of mitochondrial protein is mixed with 1.5 ml PL or PL + CoQ_{10} vesicles (150 mg/ml). The mixture is frozen in liquid nitrogen and is then allowed to thaw at room temperature. This step is repeated three times. The suspension is then layered on a discontinuous sucrose density gradient (0.6, 0.75, 1, and 1.25 *M* of sucrose) and centrifuged in a swinging bucket rotor (SW 28) at 90,000*g* for

16 h. The fractions obtained are diluted in 0.25 *M* sucrose, 0.01 *M* Tris–HCl, pH 7.8 (SMP solution) for PL-enriched SMP and in solution A for PL-enriched mitoplasts. Fractions are then centrifuged twice at 160,000*g* for 45 min and resuspended in the appropriate buffers (SMP solution and solution A).

Protein content is assayed by the biuret method (Gornall *et al.*, 1949), and lipid content is measured by the method of Marinetti (1962).

V. Mitochondria from Muscle

Biochemical analysis of mitochondria isolated from muscle tissue provides valuable insights into the pathological and physiological characteristics of human diseases related to impaired mitochondrial metabolism (mitochondrial encephalomyopathies). Muscle tissue is a postmitotic tissue, and accumulation of exogenous and endogenous damage during the life span of the organism makes this tissue one of the best characterized tissues bioenergetically.

Skeletal muscle mitochondria are usually isolated from several grams of tissue. This may not be a problem if the animal is sufficiently large or if several muscles can be pooled. However, human samples are usually obtained from surgical biopsies and needle biopsies, the latter yielding less than 1 g of muscle tissue.

When dealing with experimental animals, the method described by Kun and colleagues (1979) is generally used for the isolation of skeletal muscle mitochondria. In our experience, this method is suitable for isolation from human muscle biopsies (Zucchini *et al.*, 1995) and from rat gastrocnemius (Barogi *et al.*, 1995).

The method is based on the use of mannitol and sucrose medium, BSA, and EDTA as a complexing agent. Essentially, muscle tissue is freed from collagen and nerves, weighed and homogenized in a Teflon–glass Potter–Elvehjem homogenizer in solution A (0.22 *M* mannitol, 0.07 *M* sucrose, 2 m*M* Tris, 1 m*M* EDTA, and 20 m*M* HEPES, pH 7.2) with 0.4% BSA, and centrifuged at 600 *g* for 80 s. The supernatant is collected and the crude nuclear fraction is reextracted by the same technique. The two supernatants are combined and centrifuged at 17,000*g* for 2.5 min. The mitochondrial pellet is then washed twice in solution A and resuspended in the same solution.

We have exploited this method to investigate some individual respiratory chain activities of muscle mitochondria from young and old individuals; the activities could be transformed into actual turnover numbers related to complex III content, established on the basis of the antimycin A inhibition titer (Zucchini *et al.*, 1995) (Table VI).

Lee and colleagues (1993) have described a valid alternative method for the isolation of mitochondria from 3 to 5 g of skeletal muscle using KCl medium and proteinase K to soften the tissue and facilitate cell disruption during homogenization.

The muscle is trimmed, minced, and placed in medium A (0.1 *M* KCl, 50 m*M* Tris–HCl, pH 7.5, 5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* ATP) in a proportion of 10 ml medium A/gram of muscle. Three to 5 mg proteinase K per gram of muscle tissue is then added to the muscle suspension. After 5 min of incubation (2 min are sufficient for human muscle) at room temperature, the mixture is diluted 1:2 with medium A and homogenized for 15 s

Table VI
Enzymatic Activities of Skeletal Muscle Mitochondria from Young and Old Individuals Expressed as Specific Activities and Turnover Numbers (TN)[a,b]

	NADH-DB reductase		Succinate-cytochrome *c* reductase		Ubiquinol-cytochrome *c* reductase	
Age range (years)	nmol/ min·mg protein	TN(s^{-1})	nmol/ min·mg protein	TN(s^{-1})	nmol/ min·mg protein	TN(s^{-1})
18–29 ($n = 5$)[c]	52.44 ± 25.35	8.78 ± 1.86	102.82 ± 57.97	16.44 ± 4.92	876.88 ± 437.93	142.16 ± 18.37
69–90 ($n = 9$)	49.31 ± 31.92	8.45 ± 7.04	90.79 ± 48.86	12.70 ± 8.31	939.72 ± 449.47	124.65 ± 37.29

[a] Mann–Whitney–U nonparametric text: $p > 0.05$ for all parameters evaluated.
[b] From Zucchini *et al.* (1995).
[c] Number of samples in the age range.

by UltraTurrax. The homogenate (pH 7.3) is centrifuged at 600*g* for 10 min; the pellet is discarded and the supernatant, filtered through a two-layer cheesecloth, is centrifuged at 14,000*g* for 10 min. The resulting pellet is resuspended in medium B (0.1 *M* KCl, 50 m*M* Tris–HCl, pH 7.5, 1 m*M* $MgCl_2$, 0.2 m*M* EDTA, 0.2 m*M* ATP) with 1% BSA and centrifuged at 7000*g* for 10 min. The pellet is resuspended in medium B, centrifuged at 3500*g* for 10 min, and resuspended in 0.25 sucrose to give an approximate content of 40–50 mg mitochondrial protein/ml.

Another method for isolation of mitochondria from 25 to 100 mg skeletal muscle tissue has been described by Rasmussen and colleagues (1997) using special equipment. Briefly, muscle tissue is weighed and incubated for 2 min in 500 μl proteinase medium (100 m*M* KCl, 50 m*M* Tris, 5 m*M* $MgSO_4$, 1 m*M* ATP, 1 m*M* EDTA, pH 7.4, 0.5% BSA, and 2 mg proteinase K/ml). The proteinase is then diluted with 3 ml ATP medium (100 m*M* KCl, 50 m*M* Tris, 5 m*M* $MgSO_4$, 1 m*M* ATP, 1 m*M* EDTA, pH 7.4, 0.5% BSA) and the liquid is discarded. ATP medium is added to the digested muscle and the tissue homogenized. The homogenate is centrifuged at 300*g* for 5 min (1600 rpm in a Sorvall SS34 rotor), and the supernatant obtained is centrifuged at 4500*g* (6000 rpm in a Sorvall SS34 rotor) for 10 min. The pellet is washed with 100 m*M* KCl, 50 m*M* Tris, 5 m*M* $MgSO_4$, 1 m*M* EDTA, pH 7.4, and centrifuged at 7000*g* for 10 min (6800 rpm in a Sorvall HB-4 swing-out rotor). The pellet is finally resuspended in 0.225 *M* mannitol, 75 m*M* sucrose. The relative yield is 40–50%, and mitochondria obtained with this method are well coupled and exhibit high rates of phosphorylating respiration.

VI. Synaptic and Nonsynaptic Mitochondria from Different Rat Brain Regions

Mitochondrial preparations from brain are often heterogeneous and a number of different methods have been used in the past to separate the populations of brain mitochondria. These methods, however, involve lengthy centrifugation in hypertonic sucrose gradients (Clark and Nicklas, 1970), thus limiting extensive metabolic studies.

The method for the isolation and characterization of functional mitochondria from rat brain was first described by Lai and colleagues (1977). Three distinct populations of mitochondria from rat brain can be isolated from a single homogenate preparation: two from the synaptosomal fraction (HM and LM, for heavy and light mitochondria, respectively) and one from nonsynaptic origin, the so-called "free mitochondria" (FM).

It is possible to separate the different types of mitochondria from the cerebral cortex, hippocampus, and striatum. The protocol followed in our experiments has been described by Battino and colleagues (1995) with further modifications (Genova *et al.,* 1997; Pallotti *et al.,*1998). The animal is sacrificed by decapitation and the skull is opened rapidly. The brain is placed on an ice-chilled glass plate and dissected according to the procedure described by Glowinski and Iversen (1966). First, the posterior region containing pons and cerebellum is eliminated. The reminder is divided into two hemispheres; the two brain cortexes are freed from the hippocampus and the striatum, and each one is cut into two pieces. Brain areas should be dissected rapidly (<20 s) and placed in buffer A (0.32 *M* sucrose, 1 m*M* EDTA, 10 m*M* Tris–HCl, pH 7.4). The tissue is then homogenized using a Teflon–glass homogenizer at 800 rpm by five up-and-down passes of the pestle (Villa *et al.,* 1989) and subsequently centrifuged. Centrifugation is carried out at 1000*g*, by gradually increasing speed for 4 min, and at the final speed for an additional 11 s. The pellet is resuspended in buffer A and centrifuged again; this step is repeated once again and the three supernatants are pooled and centrifuged at 15,000*g* for 20 min to obtain the "crude" mitochondrial pellet containing synaptosomes. Isolation of free mitochondria from synaptosomes is obtained by layering the pellet, resuspended in buffer A, on a discontinuous Ficoll–sucrose two-step gradient [12 and 7.5% (w/w) Ficoll in 0.32 *M* sucrose, 50 μ*M* EDTA, 10 m*M* Tris–HCl, pH 7.4]. The gradient is then centrifuged at 73,000*g* for 24 min (24,000 rpm in a swinging bucket rotor Sorvall SW 50.1), resulting in two bands (myelin and synaptosomes) and a pellet containing FM. The myelin is removed by aspiration, and the synaptosomal band at the 7.5–12% (w/w) Ficoll interphase is collected by aspiration, diluted in buffer A, and centrifuged at 15,000*g* for 20 min. In the original method (Battino *et al.,* 1991), buffer A at this stage also contained protease inhibitors. We omit protease inhibitor in our buffers because they interfere with complex I activity.

The pellet obtained is lysed by resuspension in 6 m*M* Tris–HCl, pH 8.1, homogenized, and centrifuged at 14,000*g* for 30 min. The pellet is resuspended in 3% Ficoll in 0.12 *M* mannitol, 30 m*M* sucrose, 25 μ*M* EDTA, 5 m*M* Tris–HCl, pH 7.4, and layered on a discontinuous Ficoll gradient consisting of two layers of 6 and 4.5% Ficoll in 0.24 *M* mannitol, 60 m*M* sucrose, 50 μ*M* EDTA, 10 m*M* Tris–HCl, pH 7.4. After centrifugation at 10,000*g* for 30 min, a pellet is obtained containing the HM fraction. The intermediate fraction is diluted in buffer A and centrifuged at 15,000*g* for 30 min to pellet the LM fraction. FM, LM, and HM pellets are resuspended in minimal volumes of 0.22 *M* mannitol, 0.07 *M* sucrose, 50 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.2, and stored at −80°C.

Several respiratory chain enzymatic activities from three different regions of the brain are reported in Table VII. In addition, we have characterized complex I activity in aging

Table VII
Respiratory Chain Activities of Nonsynaptic (FM) and Synaptic Light (LM) and Heavy (HM) Mitochondria from Rat Brain Regions[a]

	Cortex	Hippocampus	Striatum
Succinate-cytochrome *c*			
FM	0.196 ± 0.076	0.159 ± 0.048	0.134 ± 0.039
LM	0.171 ± 0.050	0.154 ± 0.051	0.159 ± 0.045
HM	0.075 ± 0.019	0.062 ± 0.011	0.063 ± 0.019
CoQ_2H_2-cytochrome *c*			
FM	2.397 ± 0.445	1.513 ± 0.175	1.776 ± 0.666
LM	2.647 ± 1.097	2.153 ± 0.995	2.028 ± 0.823
HM	1.137 ± 0.471	0.505 ± 0.129	0.766 ± 0.415
Cytochrome *c* oxidase			
FM	2.217 ± 0.342	1.677 ± 0.259	2.021 ± 0.380
LM	2.328 ± 0.498	1.870 ± 0.427	1.819 ± 0.688
HM	1.125 ± 0.307	0.698 ± 0.203	0.762 ± 0.219

[a] Enzymatic activities are expressed in μmol/min/mg mitochondrial protein. Results are means ±SD for number of rats >10.

in mitochondrial preparations from cerebral cortex (see Table VIII) and studied the flux control coefficient for NADH–CoQ reductase with respect to NADH oxidase (Lenaz *et al.*,1998) (Fig. 4). To assure availability of NADH, the mitochondrial fractions were pulse sonicated five times for 10 s/min at 150 W in an ice bath under nitrogen gas prior to enzymatic assays.

Table VIII
Biochemical Parameters in Nonsynaptic Mitochondria (FM) and in Synaptic Light (LM) and Heavy Mitochondria (HM) from Rat Brain Cortex

	FM		LM		HM	
	4 months	24 months	4 months	24 months	4 months	24 months
Mitochondrial yield (mg protein/g tissue)	4.46 ± 1.20	3.40 ± 1.82	3.06 ± 0.45	3.26 ± 0.82	5.83 ± 2.04	5.42 ± 1.58
NADH oxidase activity ($nmol \cdot min^{-1}mg^{-1}$)	203 ± 72 [b]	130 ± 51 [b]	109 ± 41	100 ± 55	104 ± 18 [b]	83 ± 18 [b]
NADH-ferricyanide reductase ($\mu mol \cdot min^{-1}mg^{-1}$)	4.29 ± 1.26	3.49 ± 0.73	3.91 ± 0.81	4.36 ± 2.36	3.89 ± 0.47	3.78 ± 0.58
I_{50} of rotenone (pmol rotenone/mg protein)	29 ± 15	41 ± 26	40 ± 14	33 ± 11	25 ± 8	26 ± 9
I_{50} of rotenone (corrected)[a]	6.8 ± 1.7 [b]	10.8 ± 4.8 [b]	9.4 ± 2.8	7.9 ± 4.6	6.3 ± 2.4	6.6 ± 2.4

[a] (pmol rotenone/mg protein)/NADH-ferricyanide reductase.
[b] $p < 0.05$.

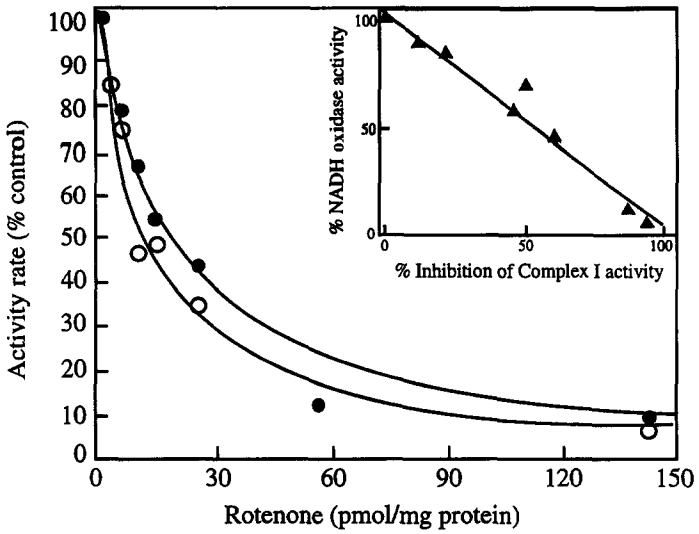

Fig. 4 Flux control of NADH oxidation in rat brain cortex nonsynaptic mitochondria [reprinted from Lenaz *et al.* (1999), with permission from Acta Biochimica Polonica]. The stepwise inhibition by rotenone of NADH-CoQ reductase (○) and of NADH oxidase (●) is shown. (Inset) A plot of NADH oxidase rates against inhibition of complex I activity after rotenone titration. Flux control coefficients were calculated as described elsewhere (Lenaz *et al.*, 1998).

VII. Mitochondria from Hamster Brown Adipose Tissue

The simplest branch of the mitochondrial respiratory chain connected with the CoQ pool is glycerol-3-phosphate dehydrogenase, which is tightly bound to the outer surface of the inner mitochondrial membrane. The amount of this enzyme varies in mitochondrial preparations from different tissues. The highest activity of this enzyme was found in insect flight muscle (Estabrook and Sacktor, 1958), but is also present in brown adipose tissue of either newborn or cold-adapted adult mammals (Chaffee *et al.*, 1964).

We have studied the activities of glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate cytochrome *c* reductase in brown adipose tissue mitochondria from cold-adapted hamsters (*Mesocricetus auratus*) (Rauchova *et al.*, 1992, 1997). Mitochondria are prepared following the method of Hittelman and colleagues (1969). Brown fat is excised rapidly, placed in ice-cold 0.25 *M* sucrose, 10 m*M* Tris–HCl, and 1 m*M* EDTA, pH 7.4 (STE buffer), and carefully cleaned of extraneous tissue. The tissue is homogenized in a Teflon–glass homogenizer followed by a high-speed centrifugation (14,000*g* for 10 min) (Smith *et al.*, 1966). The supernatant is carefully aspirated from beneath the overlying lipid layer, and the latter removed. The pellet is resuspended in STE buffer and centrifuged at 8500*g* for 10 min (Schneider, 1948). The final resuspension of mitochondrial pellet is then carried out in STE buffer.

Table IX
Glycerol-3-phosphate CoQ Reductase Activity in Hamster Brown Adipose Tissue Mitochondria with Different Acceptors at Saturating Concentrations of Both Donor and Acceptor Substrates[a,b]

Acceptor	Specific activity (nmol·min^{-1}mg^{-1}protein)
CoQ_1	225 ± 41
CoQ_2	109 ± 11
Duroquinone	121 ± 31
6-Decylubiquinone	133 ± 27
2,6-Dichlorophenol-indophenol	133 ± 16
Cytochrome *c*	367 ± 44

[a] Results are given as the mean ±SD. Activities are referred to two-electron reduction, except for cytochrome *c*, a one-electron acceptor. Single assays on NADH-cytochrome *c* and succinate-cytochrome *c* reductases gave specific activities of 405 and 265 nmol·min^{-1}mg^{-1}protein, respectively.
[b] From Rauchova *et al.* (1997).

Table IX reports the activities of mitochondrial glycerol-3-phosphate dehydrogenase and glycerol-3-phosphate cytochrome *c* reductase of brown adipose tissue mitochondria of cold-adapted hamster in comparison with the corresponding activities of NADH and succinate oxidation.

VIII. Mitochondria from Insect Flight Muscle

Mitochondria isolated from cockroach flight muscle have been studied for their high content in glycerol-3-phosphate dehydrogenase activity (Rauchova *et al.*, 1997). Cockroach (*Periplaneta americana*) flight muscle mitochondria are prepared according to Novak and colleagues (1979). Red metathoracic muscles are homogenized in a Teflon–glass homogenizer in 0.32 *M* sucrose, 0.01 *M* EDTA, pH 7.4, using 10 ml of medium per 3 g of tissue. Homogenization consists of 12 up-and-down strokes in a 1-min period. Fractions are then separated by low-speed centrifugation (2000*g* for 20 min) or by high-speed centrifugation (10,000*g* for 10 min). Fractions obtained with these centrifugations are composed mainly of mitochondria. Centrifugation of the homogenate at 18,500*g* for 20 min yields a fraction containing mitochondria and membranes of other origin.

IX. Mitochondria from Porcine Adrenal Cortex

Mitochondria from adrenal cortex contain three monooxygenase systems involved in corticosteroidogenesis: 11β-and 18-hydroxylation of deoxycorticosterone (DOC) to corticosterone and side chain cleavage of cholesterol to pregnenolone (Simpson and Boyd,

1971; Simpson and Estabrook, 1969). These involve a second electron transport chain: the primary electron donor is NADPH; electrons are then transferred to a flavoprotein, to adrenodoxin, and to cytochrome P450, where hydroxylation of the steroid precursor occurs. The two electron transport chains are not independent, and steroid hydroxylation can inhibit ATP synthesis.

Mitochondria are extracted from porcine adrenal cortex according to Popinigis and colleagues (1990). The adrenal glands are freed from all connective and fatty tissue. The central medulla is scraped away and the cortex is cut up and resuspended in 0.33 *M* sucrose, 20 m*M* Tris–HCl, pH 7.4, 2 m*M* EDTA, and 0.2% BSA. The suspension is homogenized using first a loose-fitting glass–glass homogenizer and then a tight-fitting, Teflon–glass homogenizer.

The homogenate is centrifuged at 600*g* for 8 min and the resulting supernatant is further centrifuged at 2000*g* for 8 min. The mitochondrial pellet is resuspended in 0.33 *M* sucrose, 20 m*M* Tris–HCl, pH 7.4, and 0.5 m*M* EDTA and washed three times. The final pellet is resuspended in the same washing medium at a final concentration of approximately 50–60 mg/ml.

Mitochondria prepared using the method just described have respiratory control with both succinate and glutamate, but ADP/O ratios are not very high (Popinigis *et al.*, 1990).

X. Mitochondria from Human Platelets

A. Crude Mitochondrial Membranes

Preparation of crude mitochondrial membranes from platelets has an advantage in that a large amount of blood is not required. The platelet-enriched fraction is prepared according to Blass and colleagues (1977). Blood is obtained by venipuncture. Forty to 60 ml of blood is sufficient for few respiratory activities (Merlo Pich *et al.*, 1996); for more complex experiments, involving kinetic detrminations or inhibitor titrations (e.g., rotenone titration), 100 ml of blood is required (Degli Esposti *et al.*, 1994). Erythrocytes (from 100 ml venous blood) are precipitated in 25 ml of 5% Dextran 250,000, 0.12 *M* NaCl, 10 m*M* EDTA, pH 7.4, at 4°C for 45 min (Blass *et al.*, 1977; Degli Esposti *et al.*, 1994). The upper phase is centrifuged at 5000*g* for 10 min. The pellet is resuspended in 3 ml H_2O for 60 s and adding 1 ml of 0.6 *M* NaCl blocks the osmotic shock. After centrifugation at 5000*g* for 10 min, the pellet undergoes a second lysis step. The pellet obtained after the second osmotic shock is resuspended in 3 ml 10 m*M* NaH_2PO_4, 10 m*M* Na_2HPO_4, 0.12 *M* NaCl, pH 7.4 (phosphate buffer), and is centrifuged twice at 15,000*g* for 20 min.

The platelet pellet is resuspended in 0.1 *M* Tris–HCl, 1 m*M* EDTA, pH 7.4, and sonicated five times for 10 s each (150 Hz) with intervals of 50 s. After sonication, the homogenate is centrifuged at 8000*g* for 10 min (10,000 rpm in a table-top Eppendorf centrifuge), and the supernatant, diluted 1:1 with 0.25 *M* sucrose, 50 m*M* Tricine–Cl, 2.5 m*M* $MgCl_2$, 40 m*M* KCl, 0.5% BSA, pH 8, is centrifuged at 100,000*g* for 40 min

Table X
Biochemical Parameters in Platelet Membranes from Young and Aged Female Individuals[a]

	Young ($n = 19$)[b]	Aged ($n = 18$)
NADH-DB reductase (nmol $min^{-1}mg^{-1}$protein)	4.6 ± 1.6	4.5 ± 0.9
Complex I turnover (s^{-1})	9.8 ± 4.6	7.4 ± 2.1
% rotenone sensitivity	76.1 ± 9.9	71.8 ± 10.0
I_{50} of rotenone (pmol mg^{-1} protein)	64.8 ± 44.8	126.1 ± 106.2[c]

[a] Reprinted from Merlo Pich *et al.* (1996), with permission from Elsevier Science.
[b] Number of pools (two individuals each).
[c] $p < 0.03$.

to pellet mitochondrial particles. The pellet is suspended in 0.125 *M* sucrose, 50 m*M* Tricine–Cl, 2.5 m*M* $MgCl_2$, 40 m*M* KCl, pH 8.

Alternatively (Merlo Pich *et al.,* 1996), 40 to 60 ml blood is mixed with 5% Dextran 70,000, 0.9% NaCl at room temperature for 30 min and the upper phase is centrifuged at 3000*g* for 3 min. The upper phase is collected and centrifuged at 4000*g* for 25 min and then washed in phosphate buffer. After sonication, as described earlier, platelet membrane fragments are diluted 1:1 in 0.25 *M* sucrose, 30 m*M* Tris, 1 m*M* EDTA, pH 7.7 (STE buffer), and then separated from the heavier cell debris by centrifugation at 33,000*g* for 10 min. The supernatant is then ultracentrifuged at 100,000*g* for 40 min and the pellet is resuspended in STE buffer.

This crude mitochondrial fraction has been used to study complex I activity in patients affected by Leber's hereditary optic neuropathy (Degli Esposti *et al.,* 1994; Carelli *et al.,* 1999) and in aged individuals (Merlo Pich *et al.,* 1996) (Table X).

B. Coupled Mitochondrial Particles from Platelet Mitochondria

This method involves the preparation of mitochondria followed by sonication in order to obtain coupled inside-out submitochondrial particles of the ETPH type (see Section III,B). Baracca and colleagues (1997) have described a method for preparation of coupled submitochondrial particles from horse platelets. This method is also suitable for human platelets with minor modification.

Horse platelets are isolated and purified from 200 to 500 ml of venous blood according to Blass and colleagues (1977). To isolate mitochondria, platelets are suspended in a hypotonic medium (10 m*M* Tris–HCl, pH 7.6) for 7 min; the osmotic shock is blocked by the addition of 0.25 *M* sucrose, and the suspension is centrifuged at 600*g* for 10 min in an Eppendorf microcentrifuge. The pellet undergoes a second osmotic shock, and the supernatant is collected, pooled with the one obtained from the first osmotic step, and centrifuged at 12,000*g* (12,000 rpm in a table-top Eppendorf centrifuge) for 20 min to sediment mitochondria. The mitochondrial pellet is then suspended in 0.25 *M* sucrose,

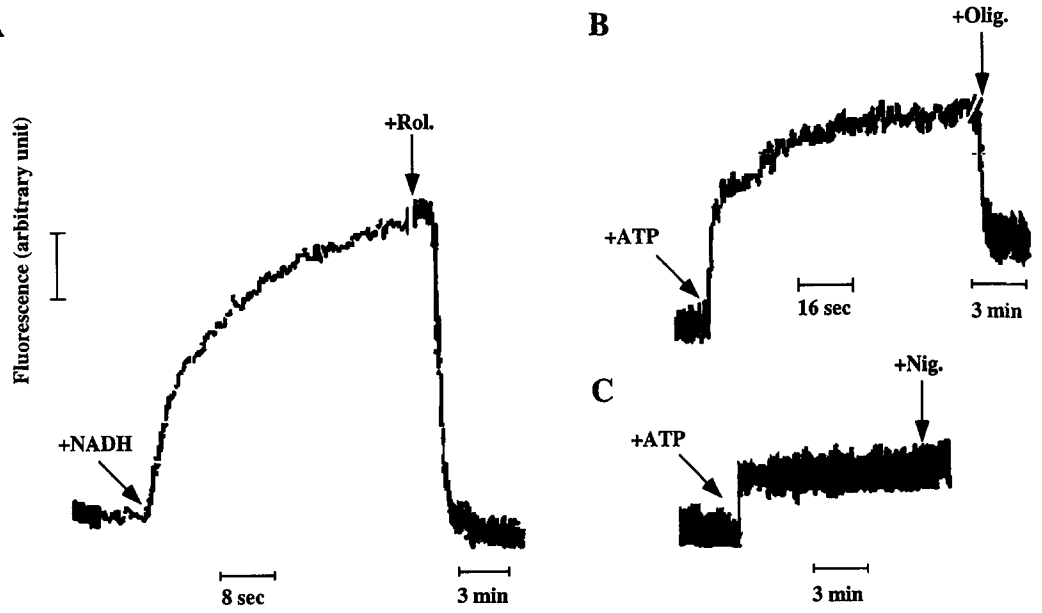

Fig. 5 Quenching of ACMA fluorescence induced upon addition of NADH (A) or ATP (B and C) to platelet-coupled submitochondrial particles (reprinted from Baracca *et al.* 1997). (A) Quenching induced by protophonoric activity of complex I in the presence of 100 μ*M* NADH; the addition of 1 μ*M* rolliniastatin-2 completely restores the initial ACMA fluorescence. (B) Quenching induced by the protonophoric activity of complex V in the presence of 0.8 m*M* ATP; the addition of 1 μ*M* oligomycin partially restores the initial ACMA fluorescence. (C) Addition of ATP to a sample containing 1 μ*M* oligomycin induces a significant quenching of ACMA fluorescence, which is insensitive to nigericin.

2 m*M* EDTA, pH 8, and subjected to sonic oscillation under partial N_2 atmosphere for 1 min at low output (40 W). The suspension is centrifuged at 12,000*g* for 10 min; the resulting supernatant is decanted and centrifuged at 105,000*g* for 40 min to precipitate the particles. The pellet is finally suspended in 0.25 *M* sucrose to give a protein concentration of 10 mg/ml as assayed by the Lowry method (Lowry *et al.*, 1951) in the presence of 1% deoxycholate. These inverted vesicles can be used to oxidize NADH and to measure ΔpH formation by fluorescence quenching of suitable acridine probes (Baracca *et al.*, 1997) (Fig. 5).

This method has been adapted for the preparation of coupled submitochondrial particles from human platelets. Platelets are isolated from 100 ml of venous blood; osmotic shock is kept for 4 min instead of 7 min, and the sonic oscillation step lasts 20 s rather than 1 min. In human platelets, we were not able to measure ΔpH formed by NADH, but the method is suitable for the assay of the protonophoric activity of ATP synthase. We have also applied this method for assaying ATP-dependent activities in patients with mutations in ATP synthase (Baracca *et al.*, 2000).

XI. Mitochondria from Fish Liver

Extraction of mitochondria from fish has been used for purification and characterization of different biochemical parameters. We applied the method for the preparation and characterization of eel (*Anguilla anguilla*) liver mitochondria and submitochondrial particles (Baracca *et al.*, 1992). It has also been applied to isolate mitochondria from sea bass (*Morone labrax*) liver (Ventrella *et al.*, 1982). The method is also suitable for extractions from other types of fish and amphibians (Degli Esposti *et al.*, 1992).

After blotting, the liver is immediately washed in 0.25 *M* sucrose, 0.01 *M* Tris–HCl, 1 m*M* EDTA, pH 7.4, and homogenized in 0.25 *M* sucrose, 24 m*M* Tris–HCl, 1 m*M* EDTA, pH 7.4, and 0.5 mg/ml BSA (homogenizing solution) using an UltraTurrax homogenizer.

The homogenate is then centrifuged at 750*g* for 10 min and the supernatant is filtered and centrifuged at 13,000*g* for 10 min. The pellet is washed once in the homogenizing solution and the suspension is centrifuged at 11,000*g* for 10 min. The mitochondrial pellet is resuspended in the homogenizing medium at a concentration of 40 mg mitochondrial protein/ml of solution.

XII. Mitochondria from Sea Urchin Egg

Mitochondria can be extracted from eggs of the sea urchin *Paracentrotus lividus* (Cantatore *et al.*, 1974). Eggs are collected and suspended in 0.25 *M* sucrose, 0.1 *M* Tris–HCl, pH 7.6, 1 m*M* EDTA, 0.24 M KCl (TEK buffer) and homogenized in Teflon–glass Potter–Elvehjem homogenizer. The homogenate is centrifuged at 600*g* for 10 min and the supernatant is centrifuged at 2600*g* for 10 min. Finally mitochondria are pelleted by centrifugation at 7500*g* for 10 min, suspended in TEK buffer, and centrifuged for 10 min at 15,000*g*. The pellet is suspended in TEK buffer and layered on a double 1.5–1 *M* sucrose discontinuous gradient in 0.1 *M* Tris–HCl, pH 7.6, 1 m*M* EDTA, 0.24 *M* KCl and centrifuged at 50,000*g* for 3 h. The band at the 1.5 *M* sucrose region is collected and centrifuged at 15,000*g* for 10 min. Finally, the pellet obtained is suspended in TEK buffer.

This method has been applied by Degli Esposti and colleagues (1990) to study the natural resistance of the sea urchin mitochondrial complex III to cytochrome *b* inhibitors.

XIII. Mitochondria and Kinetoplasts from Protozoa

Mitochondria from ciliate and trypanosome protozoans have been characterized regarding the sensitivity of ubiquinol:cytochrome *c* reductase to inhibitors (Ghelli *et al.*, 1992). Mitochondria of ciliates, such as *Tetrahymena piriformis,* are resistant to antimycin A and rotenone, whereas mitochondria of trypanosomes are quite resistant to stigmatellin. Both ciliates and trypanosomes are highly resistant to myxothiazol.

Mitochondria from *T. piriformis* are prepared according to Kilpatrick and Erecinska (1977). Cultured cells are harvested by low-speed centrifugation and washed once in cold 0.25 *M* sucrose. The pellet is then resuspended in 6 volumes of 0.25 *M* sucrose, 10 m*M* KCl, 5 m*M* MOPS, and 0.2 m*M* EDTA, pH 7.2 (solution A), and homogenized in a Teflon–glass Potter–Elvehjem homogenizer. The cell homogenate is centrifuged at 600*g* for 6 min and the supernatant is collected. The pellet is resuspended in half the previous volume, homogenized, and centrifuged again as described earlier. The supernatant collected from the previous two steps is centrifuged at 5000*g* for 10 min to pellet the mitochondria. The mitochondrial pellet is then washed twice in solution A containing 0.2% BSA and is finally sedimented by centrifugation at 8000*g* for 10 min. The colorless pellet overlaying the mitochondrial fraction and the black sediment that adheres firmly to the bottom of the centrifuge tube are discarded. Pellets from both extractions are suspended in solution A. According to Kilpatrick and Erecinska (1977), the pellet obtained from the second extraction yields more mitochondria with higher P/O ratios.

Mitochondria from *Paramecium tetraurelia* are prepared essentially as described by Doussiere and colleagues (1979). Cells are harvested by centrifugation at 20°C for 3 min at 100*g* and washed once in 0.5 *M* mannitol and 5 m*M* MOPS. Packed cells are then resuspended in 0.5 *M* mannitol, 5 m*M* MOPS, and 1 m*M* EDTA, pH 7.3, with 0.5% BSA (homogenization medium) and homogenized in a Potter–Elvehjem Teflon–glass homogenizer. The homogenate is centrifuged at 600*g* for 5 min. The supernatant is then collected and centrifuged at 600*g* for 10 min. The mitochondrial pellet is resuspended in the homogenization medium and centrifuged at 600*g* for 4 min to eliminate trichocystis. The supernatant is centrifuged at 5000*g* for 10 min to obtain the crude mitochondrial fraction. The latter is purified by centrifugation through a gradient of sorbitol (40–60%, w/w) containing 5 m*M* MOPS and 1 m*M* EDTA, pH 7.3, at 150,000 *g* for 1 h. After centrifugation, the interphase layer, corresponding to the mitochondrial particles, is removed and diluted in 10 volumes of homogenization medium. The suspension is then centrifuged at 10,000*g* for 15 min, and the mitochondrial pellet is finally resuspended in the homogenization medium.

Mitochondria from *Crithidia lucilae* and *Leishmania infantum* are prepared following the method of Renger and Wolstenholme (1972). Protozoans of the order Kinetoplastida, which includes *Trypanosoma, Leishmania,* and *Crithidia* genera, all contain kinetoplasts. Because this organelle is a modified mitochondrion, mitochondria from trypanosomes are purified from kinetoplast-enriched fractions.

Cells are harvested by centrifugation at 1000*g* for 10 min and washed three times in SSC, pH 7.5. Cells are then resuspended in 0.3 *M* sucrose, 10 m*M* Tris–HCl, pH 7.4, and 1 m*M* EDTA (STE buffer), homogenized in a Waring blender for 15–20 s at high speed, and centrifuged at 700*g* for 10 min. This centrifugation step should be repeated until the supernatant becomes free of all cells. The supernatant is then centrifuged at 8000*g* for 10 min. The pellet is resuspended in STE buffer and incubated with 200 μg/ml of DNase I at 37°C for 30 min in the presence of 7 m*M* $MgCl_2$. The DNase is then removed by washing three times with 40 m*M* EDTA. The kinetoplast-enriched fraction is then resuspended in 0.15 *M* NaCl, 0.1 *M* EDTA, and 0.05 *M* Na_2HPO_4.

XIV. Mitochondria and Mitoplasts from Cultured Cells

Human cultured cells represent a valid experimental model for investigating different biochemical parameters, both in the physiology and in the physiopathology of different disorders. Cells are usually easy to cultivate and to obtain in large amounts; intact mitochondria are usually required for biochemical and genetic analyses.

Mitochondria from cell lines have been isolated as described by Yang and colleagues (1997) with minor modifications. Cells are harvested by centrifugation at 600*g* for 10 min, washed with phosphate-buffered saline, and resuspended with 5 volumes of solution A (0.25 *M* sucrose, 20 m*M* HEPES–KOH, pH 7.5, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* EGTA, 1 m*M* dithiotreithol, 0.1 m*M* phenylmethylsulfonyl flouride). The cellular suspension is homogenized with a Teflon–glass homogenizer with 20 up-and-down passes of the pestle. In our experience, the mitochondrial yield is increased by the use of a glass–glass pestle. The homogenate is then centrifuged at 750*g* for 10 min, the supernatant is collected, and the pellet is resuspended in solution A and recentrifuged at low speed. The pooled supernatants are then centrifuged at 10,000*g* for 15 min and the crude mitochondrial pellet is resuspended in solution A.

Purified mitochondrial fractions can be obtained by separation on a sucrose gradient. A valid method comprising modifications of the "two-step" procedure described by Tapper and colleagues (1983) has been described by Magalhães and colleagues (1998). Cells are harvested by low-speed centrifugation and resuspended in 10 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 10 m*M* Tris–HCl, pH 7.5. Cells are allowed to swell for 4–5 min on ice and briefly homogenized in a Teflon–glass Potter–Elvehjem homogenizer. Sucrose concentration is then adjusted to 250 m*M* by adding 2 *M* sucrose in 10 m*M* Tris–HCl and 1 m*M* EDTA, pH 7.6 ($T_{10}E_{20}$ buffer); the suspension is then centrifuged at 1300*g* for 3 min and the supernatant is recentrifuged at low speed. Mitochondria are then collected by centrifugation at 15,000*g* for 15 min and the pellet is washed three times with 250 m*M* sucrose in $T_{10}E_{20}$ buffer and resuspended in the same solution. The mitochondrial suspension is layered on a discontinuous sucrose gradient (1–1.7 *M*) in $T_{10}E_{20}$ buffer and centrifuged at 70,000*g* for 40 min. The mitochondrial fraction is recovered from the interface, diluted in an equal volume of 250 m*M* sucrose in $T_{10}E_{20}$ buffer, and washed twice in the same solution. The mitochondrial pellet is finally resuspended in 250 m*M* sucrose in $T_{10}E_{20}$ buffer and protein concentration is determined by the Lowry method.

An alternative method to this procedure consists of the "one-step" procedure described by Bogenhagen and Clayton (1974). Cells are harvested and resuspended as described earlier, but osmotic swelling is avoided by immediately adding 2 *M* sucrose in $T_{10}E_{20}$ buffer. Cell debris and nuclei are pelleted as described in the "two-step" procedure and the supernatant is layered on 3 volumes of 1.7 *M* sucrose in $T_{10}E_{20}$ buffer and centrifuged at 70,000*g* for 40 min. The mitochondrial fraction is then washed and resuspended as described earlier.

A "no gradient procedure" method is based on slight modifications to the method described by Schneider (1948). Instead of isotonic sucrose used in the original method, the buffer used consists of 0.21 *M* mannitol, 0.07 *M* sucrose, 5 m*M* Tris–HCl, 5 m*M* EDTA, pH 7.5 (mannitol–sucrose buffer) (Bogenhagen and Clayton, 1974). Cells are

homogenized and nuclei are pelleted as described in the "one-step" procedure. The mitochondrial fraction is collected by centrifugation at 20,000*g* for 20 min and washed twice in mannitol–sucrose buffer. Alternatively, cell debris and nuclei are pelleted by centrifugation at 400*g* for 4 min and the crude mitochondrial pellet is obtained from centrifugation of the supernatant at 27,000*g* for 10 min (Bestwick *et al.*, 1982).

Mitoplasts from gradient purified mitochondria can be obtained by modifications of the original method, as described by Magalhães and colleagues (1998).

In the modification of the "swell–contract" method (Murthy and Pande, 1987), gradient-purified mitochondria are resuspended in 20 m*M* potassium phosphate (pH 7.2) containing 0.02% BSA and are allowed to swell for 20 min on ice. After addition of 1 m*M* ATP and 1 m*M* $MgCl_2$, incubation is prolonged for an additional 5 min and mitoplasts are collected by centrifugation at 15,000*g* for 10 min.

The modification of the "digitonin method" for the production of mitoplasts (Greenawalt, 1974) consists of incubating the resuspended gradient-purified mitochondria with 0.1 mg digitonin/mg of mitochondrial protein. The suspension is then stirred on ice for 15 min, and 3 volumes of 250 m*M* sucrose in $T_{10}E_{20}$ buffer and mitoplasts are pelleted after centrifugation at 15,000*g* for 15 min. Mitoplasts are washed once in 250 m*M* sucrose in $T_{10}E_{20}$ buffer and are finally resuspended in 250 m*M* sucrose, 10 m*M* Tris–HCl, pH 7.6.

Acknowledgments

The authors thank Dr. J. Ojaimi for helpful comments and for critical revising of the manuscript. This work is supported by grants from the Muscular Dystrophy Association (to F.P.) and for "PRIN Bioenergetics and Membrane Transport" from MURST, Rome, Italy (to G.L.).

References

Baracca, A., Barogi, S., Carelli, V., Lenaz, G., and Solaini, G. (2000). Catalytic activities of the mitochondrial ATP synthase in patients with the mtDNA T8993G mutation in the ATPase 6 gene encoding for the a subunit. *J. Biol. Chem.* **275,** 4177–4182.

Baracca, A., Bucchi, L., Ghelli, A., and Lenaz, G. (1997). Protonophoric activity of NADH coenzyme Q reductase and ATP synthase in coupled submitochondrial particles from horse platelets. *Biochem. Biophys. Res. Commun.* **235,** 469–473.

Baracca, A., Degli Esposti, M., Parenti Castelli, G., and Solaini, G. (1992). Purification and characterization of adenosine-triphosphatase from eel liver mitochondria. *Comp. Biochem. Phys. B* **101,** 421–426.

Barogi, S., Baracca, A., Cavazzoni, M., Parenti Castelli, G., and Lenaz, G. (2000). Effect of oxidative stress induced by adriamycin on rat hepatocyte bioenergetics during ageing. *Mech. Aging Dev.* **113,** 1–21.

Barogi, S., Baracca, A., Parenti Castelli, G., Bovina, C., Formiggini, G., Marchetti, M., Solaini, G., and Lenaz, G. (1995). Lack of major changes in mitochondria from liver, heart, and skeletal muscle of rats upon ageing. *Mech. Ageing Dev.* **84,** 139–150.

Battino, M., Bertoli, E., Formiggini, G., Sassi, S., Gorini, A., Villa, R. F., and Lenaz, G. (1991). Structural and functional aspects of the respiratory chain of synaptic and nonsynaptic mitochondria derived from selected brain regions. *J. Bioenerg. Biomembr.* **23,** 345–363.

Battino, M., Gorini, A., Villa, R. F., Genova, M. L., Bovina, C., Sassi, S., Littarru, G. P., and Lenaz, G. (1995). Coenzyme Q content in synaptic and non-synaptic mitochondria from different brain regions in the ageing rat. *Mech. Ageing Dev.* **78,** 173–187.

Bestwick, R. K., Moffett, G. L., and Mathews, C. K. (1982). Selective expansion of mitochondrial nucleoside triphosphate pools in antimetabolite-treated HeLa cells. *J. Biol. Chem.* **257,** 9300–9304.

Beyer, R. E. (1967). Preparation, properties, and conditions for assay of phosphorylating electron transport particles (ETPH) and its variations. *In* "Methods in Enzymology" (R. W. Estabrook and M. E. Pullman, eds.), Vol. 10, pp. 186–194, Academic Press, San Diego.

Blass, J. P., Cederbaum, S. D., and Kark, R. A. P. (1977). Rapid diagnosis of pyruvate and ketoglutarate dehydrogenase deficiencies in platelet-enriched preparations from blood. *Clin. Chim. Acta* **75,** 21–30.

Bogenhagen, D., and Clayton, D. A. (1974). The number of mitochondrial deoxyribonucleic acid genomes in mouse L and human HeLa cells: Quantitative isolation of mitochondrial deoxyribonucleic acid. *J. Biol. Chem.* **249,** 7991–7995.

Cantatore, P., Nicotra, A., Loria, P., and Saccone, C. (1974). RNA synthesis in isolated mitochondria from sea urchin embryos. *Cell Differ.* **3,** 45–53.

Capaldi, R. A. (1982). Arrangement of proteins in the mitochondrial inner membrane. *Biochim. Biophys. Acta* **694,** 291–306.

Carelli, V., Ghelli, A., Bucchi, L., Montagna, P., De Negri, A., Leuzzi, V., Carducci, C., Lenaz, G., Lugaresi, E., and Degli Esposti, M. (1999). Biochemical features of mtDNA 14484 (ND6/M64V) point mutation associated with Leber's hereditary optic neuropathy (LHON). *Ann. Neurol.* **45,** 320–328.

Casadio, R., Baccarini-Melandri, A., and Melandri, B. A. (1974). On the determination of the transmembrane pH difference in bacterial chromatophores using 9-aminoacridine. *Eur. J. Biochem.* **47,** 121–128.

Castelluccio, C., Baracca, A., Fato, R., Pallotti, F., Maranesi, M., Barzanti, V., Gorini, A., Villa, R. F., Parenti Castelli, G., Marchetti, M., and Lenaz, G. (1994). Mitochondrial activities of rat heart during ageing. *Mech. Ageing Dev.* **76,** 73–88.

Chaffee, R. R. J., Allen, J. R., Cassuto, Y., and Smith, R. E. (1964). Biochemistry of brown fat and liver of cold-acclimated hamsters. *Am. J. Physiol.* **207,** 1211–1214.

Clark, J. B., and Nicklas, W. J. (1970). The metabolism of rat brain mitochondria. Preparation and characterization. *J. Biol. Chem.* **245,** 4724–4731.

de Duve, C. (1967). Criteria for homogeinity and purity of mitochondria. *In* "Methods in Enzymology" (R. W. Estabrook and M. E. Pullman, eds.), Vol. 10, pp. 7–18. Academic Press, San Diego.

de Duve, C., and Baudhuin, P. (1966). Peroxisomes (microbodies and related particles). *Physiol. Rev.* **46,** 323–357.

Degli Esposti, M., Carelli, V., Ghelli, A., Ratta, M., Crimi, M., Sangiorgi, S., Montagna, P., Lenaz, G., Lugaresi, E., and Cortelli, P. (1994). Functional alterations of the mitochondrially encoded ND4 subunit associated with Leber's hereditary optic neuropathy. *FEBS Lett.* **352,** 375–379.

Degli Esposti, M., Ghelli, A., Butler, G., Roberti, M., Mustich, A., and Cantatore, P. (1990). The cytochrome *c* of the sea urchin *Paracentrotus lividus* is naturally resistant to myxothiazol and mucidin. *FEBS Lett.* **263,** 245–247.

Degli Esposti, M., Ghelli, A., Crimi, M., Baracca, A., Solaini, G., Tron, T., and Meyer, A. (1992). Cytochrome *b* of fish mitochondria is strongly resistant to funicolosin, a powerful inhibitor of respiration. *Arch. Biochem. Biophys.* **295,** 198–204.

Degli Esposti, M., and Lenaz, G. (1982). Kinetics of ubiquinol-1-cytochrome *c* reductase in bovine heart mitochondria and submitochondrial particles. *Biochim. Biophys. Acta* **682,** 189–200.

Doussiere, J., Sainsard-Chanet, A., and Vignais, P. V. (1979). The respiratory chain of *Paramecium tetraurelia* in wild type and the mutant. C_1. I. Spectral properties and redox potentials. *Biochim. Biophys. Acta* **548,** 224–235.

Estabrook, R. W., and Sacktor, B. (1958). α-Glycerophosphate oxidase of flight muscle mitochondria. *J. Biol. Chem.* **233,** 1014–1019.

Estornell, E., Fato, R., Pallotti, F., and Lenaz, G. (1993). Assay conditions for the mitochondrial NADH: coenzyme Q oxidoreductase. *FEBS Lett.* **332,** 127–131.

Fato, R., Cavazzoni, M., Castelluccio, C., Parenti Castelli, G., Palmer, G., Degli Esposti, M., and Lenaz, G. (1993). Steady-state kinetics of ubiquinol-cytochrome *c* reductase in bovine heart submitochondrial particles: Diffusional effect. *Biochem. J.* **290,** 225–236.

Fato, R., Estornell, E., Di Bernardo, S., Pallotti, F., Parenti Castelli, G., and Lenaz, G. (1996). Steady-state kinetics of the reduction of Coenzyme Q analogs by Complex I (NADH:ubiquinone oxidoreductase) in bovine heart mitochondria and submitochondrial particles. *Biochemistry (US),* **35,** 2705–2716.

Fleischer, S., and Kervina, M. (1974). Long-term preservation of liver for subcellular fractionation. *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 31, pp. 3–6. Academic Press, San Diego.

Genova, M. L., Bovina, C., Formiggini, G., Ottani, V., Sassi, S., and Marchetti, M. (1994). Uptake and distribution of exogenous CoQ in the mitochondrial fraction of perfused rat liver. *Mol. Aspects Med.* **15,** (Suppl.), s47–s55.

Genova, M. L., Bovina, C., Marchetti, M., Pallotti, F., Tietz, C., Biagini, G., Pugnaloni, A., Viticchi, C., Gorini, A., Villa, R. F., and Lenaz, G. (1997). Decrease of rotenone inhibition is a sensitive parameter of complex I damage in brain non-synaptic mitochondria of aged rats. *FEBS Lett.* **410,** 467–469 .

Ghelli, A., Crimi, M., Orsini, S., Gradoni, L., Zannotti, M., Lenaz, G., and Degli Esposti, M. (1992). Cytochrome *b* of protozoan mitochondria: Relationships between function and structure. *Comp. Biochem. Phys. B* **103,** 329–338.

Glowinski, J., and Iversen, L. L. (1966). Regional studies of catecholamines in the rat brain. I. The disposition of [^{3}H]norepinephrine, [^{3}H]dopamine and [^{3}H]dopa in various regions of the brain. *J. Neurochem.* **13,** 655–669.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949). Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177,** 751–766.

Greenawalt, J. W. (1974). The isolation of outer and inner mitochondrial membranes. *In* "Methods in Enzymology" (S. Fleisher and L. Packer, eds.), Vol. 31, pp. 310–323. Academic Press, San Diego.

Gregg, C. T. (1967). Preparation and assay of phosphorylating submitochondrial particles: Particles from rat liver prepared by drastic sonication. *In* "Methods in Enzymology" (R. W. Estabrook and M. E. Pullman, eds.), Vol. 10, pp. 181–185. Academic Press, San Diego..

Hansen, M., and Smith, A. L. (1964). Studies on the mechanism of oxidative phosphorylation. VII. Preparation of a submitochondrial particle (ETP_H) which is capable of fully coupled oxidative phosphorylation. *Biochim. Biophys. Acta* **81,** 214–222.

Hatefi, Y., Jurtshuk, P., and Haavik, A. G. (1961). Studies on the electron transport system. XXXII. Respiratory control in beef heart mitochondria. *Arch. Biochem. Biophys.* **94,** 148–155.

Hittelman, K. J., Lindberg, O., and Cannon, B. (1969). Oxidative phosphorylation and compartmentation of fatty acid metabolism in brown fat mitochondria. *Eur. J. Biochem.* **11,** 183–192.

Jacobs, E. E., and Sanadi, D. R. (1960). The reversible removal of cytochrome *c* from mitochondria. *J. Biol. Chem.* **235,** 531–534.

Kalen, A., Soderberg, N., Elmberger, P. G., and Dallner, G. (1990). Uptake and metabolism of dolichol and cholesterol in perfused rat liver. *Lipids* **25,** 93–99.

Kilpatrick, L., and Erecinska, M. (1977). Mitochondrial respiratory chain of *Tetrahymena piriformis:* The termodynamic and spectral properties. *Biochim. Biophys. Acta* **460,** 346–363.

Kun, E., Kirsten, E., and Piper, W. N. (1979). Stabilization of mitochondrial functions with digitonin. *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 55, pp. 115–118. Academic Press, San Diego.

Lai, J. C. K., Walsh, J. M., Dennis, S. C., and Clark, J. B. (1977). Synaptic and non-synaptic mitochondria from rat brain: Isolation and characterization. *J. Neurochem.* **28,** 625–631.

Lee, C. P., Martens, M. E, and Tsang, S. H. (1993). Small scale preparation of skeletal muscle mitochondria and its application in the study of human disease. *In* "Methods in Toxicology" (D. Jones and L. Lash, eds.), Vol. 2, pp. 70–83. Academic Press, New York.

Lenaz, G., Bovina, C., Formiggini, G., and Parenti Castelli, G. (1999). Mitochondria, oxidative stress, and antioxidant defences. *Acta Biochim. Polon.* **46,** 1–21.

Lenaz, G., Cavazzoni, M., Genova, M. L., D'Aurelio, M., Merlo Pich, M., Pallotti, F., Formiggini, G., Marchetti, M., Parenti Castelli, G., and Bovina, C. (1998). Oxidative stress, antioxidant defences and aging. *Biofactors* **8,** 195–204.

Lenaz, G., Degli Esposti, M., and Parenti Castelli, G. (1982). DCCD inhibits proton translocation and electron flow at the second site of the mitochondrial respiratory chain. *Biochem. Biophys. Res. Commun.* **105,** 589–595.

Lenaz, G., and MacLennan, D. H. (1966). Studies on the mechanism of oxidative phosphorylation. X. The effect of cytochrome *c* on energy-linked processes in submitochondrial particles. *J. Biol. Chem.* **241,** 5260–5265.

Lenaz, G., and MacLennan, D. H. (1967). Extraction and estimation of cytochrome *c* from mitochondria and submitochondrial particles. *In* "Methods in Enzymology" (R. W. Estabrook and M. E. Pullman, eds.), Vol. 10, pp. 499–504. Academic Press, San Diego.

Linnane, A. W., and Titchener, E. B. (1960). Studies on the mechanism of oxidative phosphorylation. VI. A factor for coupled oxidation in the electron transport particle. *Biochim. Biophys. Acta* **39,** 469–478.

Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–275.

MacLennan, D. H., Lenaz, G., and Szarkowska, L. (1966). Studies on the mechanism of oxidative phosphorylation. IX. Effect of cytochrome *c* on energy-linked processes. *J. Biol. Chem.* **241,** 5251–5259.

Magalhães, P. J., Andreu, A. L., and Schon, E. A. (1998). Evidence for the presence of 5S rRNA in mammalian mitochondria. *Mol. Biol. Cell* **9,** 2375–2382.

Marinetti, G. V. (1962). Chromatographic separation, identification and analysis of phosphatides. *J. Lipid Res.* **3,** 1–20.

Melandri, B. A., Baccarini Melandri, A., Lenaz, G., Bartoli, E., and Masotti, L. (1974). The site of inhibition of dibromothymoquinone in mitochondrial respiration. *J. Bioenerg.* **6,** 125–133.

Merlo Pich, M., Bovina, C., Formiggini, G., Cometti, G. G., Ghelli, A., Parenti Castelli, G., Genova, M. L., Marchetti, M., Semeraro, S., and Lenaz, G. (1996). Inhibitor sensitivity of respiratory complex I in human platelets: A possible biomarker of ageing. *FEBS Lett.* **380,** 176–178.

Murthy, M. S. R., and Pande, S. V. (1987). Malonyl-CoA binding site and the overt carnitine palmitoyltransferase activity reside on the opposite sides of the outer mitochondrial membrane. *Proc. Natl. Acad. Sci. USA* **84,** 378–382.

Nedergaard, J., and Cannon, B. (1979). Overview: Preparation and properties of mitochondria from different sources. *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 55, pp. 3–28. Academic Press, San Diego.

Norling, B., Glazek, E., Nelson, B. D., and Ernster, L. (1974). Studies with ubiquinone-depleted submitochondrial particles: Quantitative incorporation of small amounts of ubiquinone and its effects on the NADH and succinate oxidase activities. *Eur. J. Biochem.* **47,** 475–482.

Novak, F., Novakova, O., and Kubista, V. (1979). Heterogeneity of insect flight muscle mitochondria as demonstrated by different phospholipid turnover *in vivo*. *Insect Biochem.* **9,** 389–396.

Palade, G. E., Siekevitz, P., and Caro, L. G. (1962). Structure, chemistry and function of the pancreatic exocrine cell. *In* "Ciba Foundation Symposium on the Exocrine Pancreas: Normal and Abnormal Function" (A. V. S. de Reuck and M. P. Cameron, eds.), pp. 23–55. Little, Brown, and Co., Boston.

Pallotti, F., Genova, M. L., Merlo Pich, M., Zucchini, C., Carraro, S., Tesei, M., Bovina, C., and Lenaz, G. (1998). Mitochondrial dysfunction and brain disorders. *Arch. Gerontol. Geriatr.* **6** (Suppl.), 385–392.

Popinigis, J., Antosiewicz, J., Mazzanti, L., Bertoli, E., Lenaz, G., and Cambria, A. (1990). Direct oxidation of glutamate by mitochondria from porcine adrenal cortex. *Biochem. Int.* **21,** 441–451.

Rasmussen, H. N., Andersen, A. J., and Rasmussen, U. F. (1997). Optimization of preparation of mitochondria from 25–100 mg skeletal muscle. *Anal. Biochem.* **252,** 153–159.

Rauchova, H., Battino, M., Fato, R., Lenaz, G., and Drahota, Z. (1992). Coenzyme Q-pool function in glycerol-3-phosphate oxidation in hamster brown adipose tissue mitochondria. *J. Bioenerg. Biomembr.* **24,** 235–241.

Rauchova, H., Fato, R., Drahota, Z, and Lenaz, G. (1997). Steady-state kinetics of reduction of coenzyme Q analogs by glycerol-3-phospate dehydrogenase in brown adipose tissue mitochondria. *Arch. Biochem. Biophys.* **344,** 235–241.

Renger, H. C., and Wolstenholme, D. R. (1972). The form and structure of kinetoplast DNA of *Crithidia*. *J. Cell Biol.* **54,** 346–364.

Schnaitman, C., and Greenawalt, J. W. (1968). Enzymatic properties of the inner and outer membranes of rat liver mitochondria. *J.Cell Biol.* **38,** 158–175.

Schneider, H., Lemasters, J. J., Hochli, and Hackenbrock, C. R. (1980). Fusion of liposomes with mitochondrial inner membranes. *Proc. Natl. Acad. Sci. USA* **77,** 442–446.

Schneider, W. C. (1948). Intracellular distribution of enzymes. III. The oxidation of octanoic acid by rat liver fractions. *J. Biol. Chem.* **176,** 259–267.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *In* "Methods in Cell Biology" (D. M. Prescott, ed.), Vol. 13, pp. 29–83. Academic Press, New York.

Simpson, E. R., and Boyd, G. S. (1971). The metabolism of pyruvate by bovine-adrenal-cortex mitochondria. *Eur. J. Biochem.* **22,** 489–499.

Simpson, E. R., and Estabrook, R. W. (1969). Mitochondrial malic enzyme: The source of reduced nicotinamide adenine dinucleotide phosphate for steroid hydroxylation in bovine adrenal cortex mitochondria. *Arch. Biochem. Biophys.* **129,** 384–395.

Smith, A. L. (1967). Preparation, properties, and conditions for assay of mitochondria: Slaughterhouse material, small-scale. *In* "Methods in Enzymology" (R. W. Estabrook and M. E. Pullman, eds.), Vol. 10, pp. 81–86. Academic Press, San Diego.

Smith, R. E., Roberts, J. C., and Hittelman, K. J. (1966). Nonphosphorylating respiration of mitochondria from brown adipose tissue. *Science* **154,** 653–654.

Szarkowska, L. (1966). The restoration of DPNH oxidase activity by coenzyme Q (ubiquinone). *Arch. Biochem. Biophys.* **113,** 519–525.

Tapper, D. P., Van Etten, R. A., and Clayton, D. A. (1983). Isolation of mammalian mitochondrial DNA and RNA and cloning of the mitochondrial genome. *In* "Methods in Enzymology" (S. Fleischer and B. Fleisher, eds.), Vol. 97, pp. 426–434. Academic Press, San Diego.

Ventrella, V., Pagliarani, A., Trigari, R., and Borgatti, A. R. (1982). Respirazione e fosforilazione ossidativa in mitocondri epatici di branzino (*Morone labrax*) e loro dipendenza dalla temperatura. *Boll. Soc. It. Biol. Sper.* **58,** 1509–1515.

Villa, R. F., Gorini, A., Geroldi, D., Lo Faro, A., and Dell'Orbo, C. (1989). Enzyme activities in perikaryal and synaptic mitochondrial fractions from rat hippocampus during development. *Mech. Ageing Dev.* **49,** 211–225.

Wattiaux, R., Wattiaux-De Coninck, S., Ronveaux-Dupal, M. F., and Dubois, F. (1978). Isolation of rat liver lysosomes by isopycnic centrifugation in a metrizamide gradient. *J. Cell Biol.* **78,** 349–368.

Wells, W. W., Seyfred, M. A., Smith, C. D., and Sakai, M. (1987). Measurement of subcellular sites of polyphosphoinositide metabolism in isolated rat hepatocytes. *In* "Methods in Enzymology" (P. M. Conn and A. R. Means, eds.), Vol. 141, pp. 92–99. Academic Press, San Diego.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: Release of cytochrome *c* from mitochondria blocked. *Science* **275,** 1129–1132.

Zucchini, C., Pugnaloni, A., Pallotti, F., Solmi, R., Crimi, M., Castaldini, C., Biagini, G., and Lenaz, G. (1995). Human skeletal muscle mitochondria in aging: Lack of detectable morphological and enzymic defects. *Biochem. Mol. Biol. Int.* **37,** 607–616.