

Mitochondrial oxidative phosphorylation: Pitfalls and tips in measuring and interpreting enzyme activities

D. CHRETIEN and P. RUSTIN*

*Unité de Recherches sur les Handicaps Génétiques de l'Enfant (INSERM U393),
Hôpital Necker-Enfants Malades, Paris, France*

**Correspondence: INSERM U393, Tour Lavoisier, 2^o Etage, Hôpital
Necker-Enfants Malades, 149 rue de Sèvres, 75743 Paris Cedex 15, France.
E-mail: rustin@necker.fr*

Summary: Mitochondrial oxidative phosphorylation (OXPHOS) is fundamental in all aspects of cellular life in aerobic cells and organisms. It is therefore not surprising that a variety of diseases have been attributed to dysfunction of the OXPHOS enzymes. Assessment of OXPHOS in human samples has proved to be a difficult task over years, even when relying on well-established methods. The complexity and the flexibility of the mitochondrial organization in cells account for a large part in the difficulties encountered in assessing OXPHOS activity. Nevertheless, a careful and detailed analysis of OXPHOS enzyme activity in cells or biopsy samples from patients at risk provides diagnosis of potential OXPHOS deficiency. Problems inherent in the use of human material, mostly the small size of the samples to be analysed, are difficult to resolve. However, cautious handling of these samples permits reasonable confidence to be reached in the interpretation of the data.

Mitochondrial oxidative phosphorylation (OXPHOS) *sensu stricto* refers to a coupled three-step process that takes place in the mitochondrial inner membrane. The process starts with the electrons from respiratory substrates being fed to the respiratory chain (RC) by a number of dehydrogenases, located on either the inner or the outer surfaces of the inner membrane (Figure 1) (Tzagoloff 1982). Along with the electron flow from substrates to molecular oxygen, protons are pumped from the mitochondrial matrix to the intermembrane space, resulting in membrane polarization with the formation of a membrane potential ($\Delta\Psi$) and a proton gradient (ΔpH) (Nicholls 1982). The ATPase makes use of these gradients to condense ADP and mineral phosphate into ATP that is subsequently distributed in the cell cytosol. The OXPHOS process takes place in all

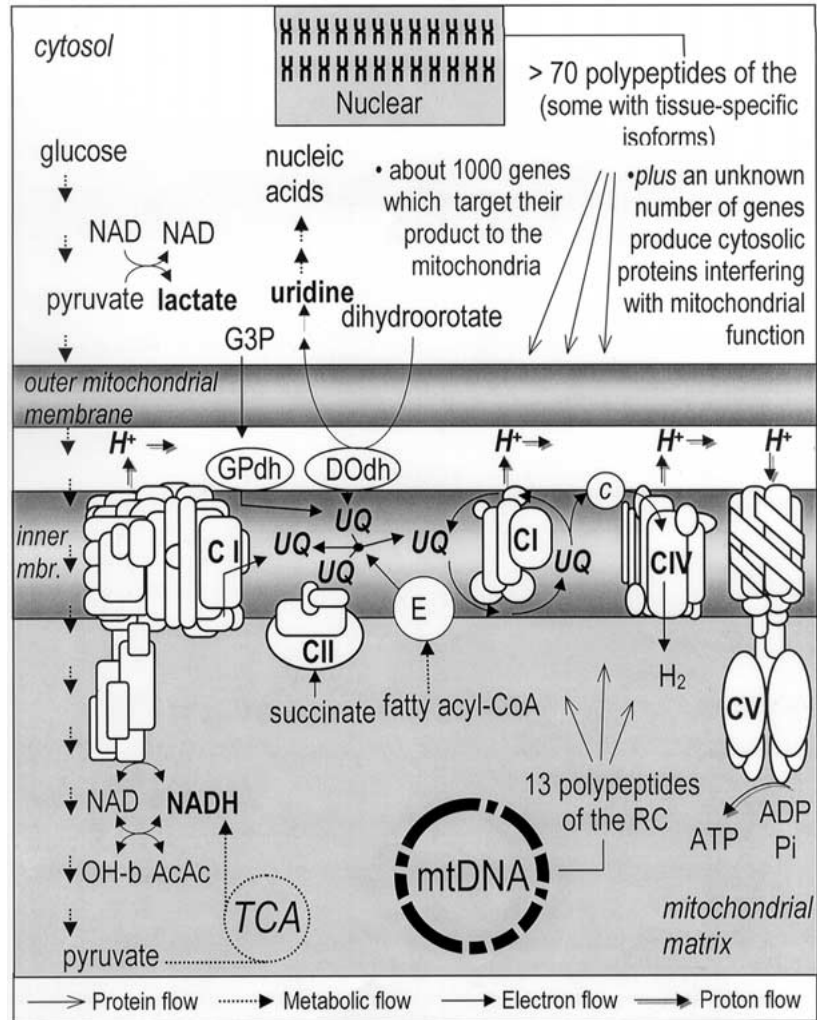


Figure 1 An oversimplified scheme of mitochondrial biogenesis and functioning. The synthesis (indicated as protein flow) of the respiratory chain (RC) components requires the coordinated expression of the mitochondrial genome (mtDNA) and of a number of nuclear genes. OXPHOS functioning, closely associated with cell metabolism (metabolic flow), results in both electron transfer from respiratory substrates (electron flow) to oxygen and proton extrusion (proton flow) from the mitochondrial matrix space. Note that lactate production and redox equilibria (lactate/pyruvate and hydroxybutyrate (OH-b)/acetoacetate (AcAc) ratios) will be affected in the case of OXPHOS deficiency. Nucleic acid synthesis depends on the activity of the dehydroorotate dehydrogenase (DODh) feeding electrons to the mitochondrial ubiquinone (UQ) pool. A blockade of the cytochrome segment of the respiratory chain might thus affect nucleic acid synthesis. AcAc, acetoacetate; CI–V, the various respiratory chain complexes; DODh, dihydroorotate dehydrogenase; G3P, glycerol 3-phosphate; GPdh, glycerol 3-phosphate dehydrogenase; mbr., membrane; OH-b, hydroxybutyrate; TCA, tricarboxylic acid

human cells, except red blood cells, and is a very efficient process. It has been inferred from the oxygen consumed by an active man that up to 70 kg of ATP can be produced and used up in a day. However, the mitochondrial content (and in most cases the mitochondrial activity) is highly variable according to cell type, being reduced to a few hundreds in skin fibroblasts but being as high as several thousands in mature oocytes (Tyler 1992).

About 80 protein components constitute the machinery supporting the OXPHOS process, mostly organized into five large complexes. However, on one hand, the machinery requires much more to actually generate ATP, e.g. import of substrates into the mitochondria, and activity of matrix dehydrogenases to produce reduced cofactors such as NADH. In keeping with this, about 15% of total cell protein can be found in the mitochondria, the function of a number of these proteins predictably affecting, or being affected by, the OXPHOS activities. On the other hand, OXPHOS also ensures much more than only ATP production. Indeed, besides interfering with cell homeostasis of a variety of cations (Ca^{2+} , Fe^{2+} , etc.), it carries out the difficult task of handling oxygen for the cell. By doing so, it tightly controls the fate of the oxygen used up by the cell, producing either water, through a set of two-electron redox reactions, or superoxides, by single-electron exchange reactions between semireduced components (mostly ubiquinone and flavin radical species) of the RC and molecular oxygen. Because the level of superoxides appears to play a central role in the decision taken by the cell to proliferate or to die, its control by the OXPHOS machinery is presumably of major importance (Rustin 2002). If only through ATP and superoxide production, the OXPHOS machinery thus controls a determinant part of the cellular activity. It is therefore not a surprise that OXPHOS deficiencies have been suspected, and actually shown, to be at the origin of a striking number of cellular dysfunctions resulting in a large spectrum of human diseases, the so-called mitochondrial diseases (Munnich and Rustin 2001). As a result, determination of OXPHOS activity has become a recognized necessity in an increasing number of at-risk patients. But it still represents a true challenge for biochemists owing to the complexity of the mitochondrial machinery and function.

To date, avoiding the biochemical detection of OXPHOS-deficient activities by direct sequencing of genes encoding proteins involved in this process does not constitute a realistic approach for a number of reasons.

1. The biogenesis, maintenance and functioning of the respiratory chain rely on the concerted expression of several hundred genes present in both the mitochondrial and the nuclear genome, and a complete mutational screening would represent an inconceivable investment. As a rough estimate, in a somatic cell, about 10 000 copies of mtDNA plus about 1000 nuclear genes (1/40 of all genes) are required for the normal functioning of mitochondria. In addition, an unknown number of nuclear genes also encode cytosolic proteins whose activity either interferes with or controls mitochondrial functioning (Larsson and Clayton 1995).
2. Only a subset of the genes encoding mitochondrial proteins has been identified in humans so far, a situation that hopefully will change in the near future.

3. The clinical presentations associated with a large proportion of identified mutations, even in different genes, are so closely related that they can hardly direct molecular screening towards a given gene (Munnich et al 2001).

As a result, without an initial biochemical characterization of the suspected deficiency, direct molecular screening is an unfeasible task, or at least a quite risky one. A few well-defined syndromes are an exception where common mutations in the mtDNA, or in a handful of nuclear genes, could be looked for on the basis of clinical considerations only. But even in these cases, negative results do not exclude, but may even rather imply, further progression to a complete biochemical investigation of OXPHOS. To date, the molecular bases are still unknown in a vast majority of the patients with proven respiratory chain deficiency.

Because abnormalities of OXPHOS should result in disturbance of organic acids, especially lactate and pyruvate (Figure 1), and amino acids in blood, urine or cerebrospinal fluid, quantification of these can provide useful information. However, it should be kept in mind that, because OXPHOS defects can produce identifiable defects in related metabolic pathways (pyruvate metabolism, Krebs cycle, protein catabolism, fatty acid oxidation), this quantification might be poorly discriminating. In addition, a number of pitfalls in this quantification have been described, which might result in either artefactual abnormality or, conversely, the overlooking of abnormality. It therefore has only partial predictive value: metabolic investigations are neither always specific nor always sensitive enough.

Other indications pointing to mitochondrial dysfunction may be found through morphological and histochemical studies of biopsy samples (Romero et al 1996). The presence of ragged-red fibres (RRF) resulting from abnormal accumulation of mitochondria presumably denotes a decrease of energy production in cells. However it should be kept in mind that RRF are seldom observed in muscle tissue from children with proven respiratory chain deficiency, while they have been reported in cases of excessive muscle exercise in nonaffected individuals. Histochemical study demonstrating either low activity of one respiratory chain enzyme throughout a tissue preparation or cell-to-cell variation in enzyme activity—typically resulting in a patchwork appearance—may constitute quite a good marker in some cases. However, on one hand, the pallor of a histochemical reaction can be confidently used to diagnose a case only if very severe and is poorly quantified. On the other hand, the presence of a few enzyme-negative cells is not necessarily related to a disease condition, particularly in the elderly. The occurrence of lipid droplets indicative of poor oxidation of lipid compounds is often observed in patients with an OXPHOS disease, but it may obviously originate from a number of other metabolic diseases. Finally, the rearrangements of mitochondrial ultrastructure that can be studied using electron microscopy are not consistently observed in cases of OXPHOS defects and these may be secondary to alterations of nonmitochondrial cellular functions as well.

All these investigations may thus sometimes provide strong indications of a mitochondrial dysfunction, but again these are not necessarily specific or sensitive. In most cases, investigating OXPHOS activity is therefore mandatory.

To achieve such an investigation, a complete set of methods was devised several years ago and has been used extensively to progressively describe and understand mitochondrial functioning. However, most of these methods needed to be adapted to the particular conditions imposed by the use of human material, especially the small amounts of tissues or cells generally available (Rustin et al 1994). Both polarographic assays, allowing an overall estimation of OXPHOS activity, and spectrophotometric assays, allowing the measurement of activity of each of the different RC complexes, have been used successfully. Owing to the relative complexity of most of these assays, the combination of both approaches is probably the most efficient method to reach a confident diagnosis, even if incomplete information is actually obtained. It is undoubtedly a good idea to multiply experimental approaches to reinforce any indication of a potential deficiency in one or more of the OXPHOS components.

Notably, polarographic investigation—the measurement of oxygen consumption by the OXPHOS system—is only feasible when fresh material (biopsies or cells) is available: perfect integrity of the mitochondria is required for such investigations. It should also be noted that polarographic investigation gives information on more than the OXPHOS system per se. Indeed, abnormality in substrate carrier activity, oxidation cofactor (NAD⁺, CoA, Tpp) concentrations and Krebs cycle enzyme activity can be studied as well. Similarly, polarographic assay can determine some parameters (coupling between electron flow and phosphorylation, significant cytochrome *c* loss, etc.) that are not normally measured by regular spectrophotometric assays. Although less convenient, ATP measurement by substrate-supplemented mitochondria can sometimes be substituted for polarographic studies with similar limitations (availability of fresh material).

Spectrophotometric assays of RC complex activity can be performed on a number of human tissues and cell types. The principle of these measurements is based on the supplementation of tissue homogenates—or enriched mitochondrial fractions—by an electron donor and/or acceptor, the oxidation and/or the reduction of one of which is studied using a spectrophotometer. The assay of RC complex activity can be done on fresh or frozen material using relatively simple experimental conditions, resulting in a set of values for RC complex activities characteristic for a given sample and allowing diagnosis of most RC defects. It is, however, a general observation that measuring these activities in a cohort of nonaffected individuals results in a considerable scattering of absolute activities (up to two orders of magnitude!). Whatever the cause of this scattered distribution might be, it has been shown that these values are not normally distributed and that calculation of mean and standard deviation for control values is statistically meaningless (Rustin et al 1991). In addition, the null value is often found at less than two ‘standard deviations’ if calculated on such a cohort. This was initially a major problem in our diagnostic centre and still is of tremendous importance when trying to interpret results from these investigations. It should also be added that a RC defect often triggers mitochondrial accumulation, e.g. ragged-red fibres in skeletal muscle in adulthood. This accumulation results in an increase of overall mitochondrial activities, possibly including the partially defective ones. This may sometimes mask a deficiency, particularly if only partial. The solution to this major problem came when it was realized that, to sustain con-

tinuous and 'safe' substrate oxidation, a strict equilibrium between electron carriers must be respected. Any imbalance between components of the RC may have two major consequences.

First, imbalance between RC components may theoretically result in overreduction of several components (flavin, quinone), possibly leading to overproduction of superoxides with unpredictable consequences depending on tissue-specific antioxidant defences. The deficiency of one or more of the RC complexes has been shown to result in such superoxide overproduction, which can be an important factor in the development of disease (Raha and Robinson 2001). This overproduction is highly variable according to the type of deficiency. It will be favoured by high membrane potential and overreduction of RC carriers. In keeping with this, compared to deficiency affecting other respiratory chain complexes, ATPase deficiency (associated with the 8993T>G NARP mutation in the ATPase 6 gene harboured by the mtDNA) was found to generate a high level of superoxides, accompanied by superoxide dismutase induction and triggering of apoptosis in cultured skin fibroblasts (Geromel et al 2001). Second, the sustained co-oxidation of the various RC substrates requires a concerted activity of the several dehydrogenases that compete for the quinone pool reduction (Geromel et al 2002). Owing to this competition, any imbalance between dehydrogenases, even if partial, can lead to severely hampered access of one dehydrogenase to the RC and the subsequent decrease of the whole respiratory process.

In practical terms, this provided us with a unique tool for analysing enzyme activity data, the controlled equilibrium necessary for the normal function of the mitochondria resulting in consistent activity ratios in the RC. In addition, the normal distribution of these in the control population allows mean and standard deviations to be calculated. Therefore, except for a general deficiency of the mitochondria, the comprehensive analysis of all the different activity ratios is certainly of major importance when attempting to demonstrate abnormality in the functioning of the RC (Figure 2) (Chretien et al 1997). Because of the inherent difficulty of these enzyme assays, we still consider these ratios to be abnormal only when differing by more than 3 SD from control mean values, if the anomaly is observed in only one tissue.

While the interpretation of measured values requires our greatest attention, there are several pitfalls to be avoided in obtaining the initial values. The most frequent ones are discussed below.

First, dealing with patients with mtDNA mutation, cells often harbour a mixture of wild-type and mutant mtDNA, a phenomenon referred as heteroplasmy (Larsson and Clayton 1995). Depending on the ratio between these two mtDNA species and/or the residual amount of wild-type mtDNA, a given tissue may or not present a biochemical defect. A threshold value of mutant mtDNA is then required for the observation of a biochemical phenotype, a threshold that may vary from tissue to tissue. However, it should be kept in mind that a given level of heteroplasmy may uncover two different types of distribution. For example, 60% mutant mtDNA in the total mtDNA of a cell population can correspond either to 60% of the cells with 100% of mutant DNA plus 40% harbouring only wild-type mtDNA. The former cells predictably will be unable to carry out OXPHOS processes and will easily be detected. Alternatively, all cells can harbour 60% of mutant mtDNA, a situation that may well not result

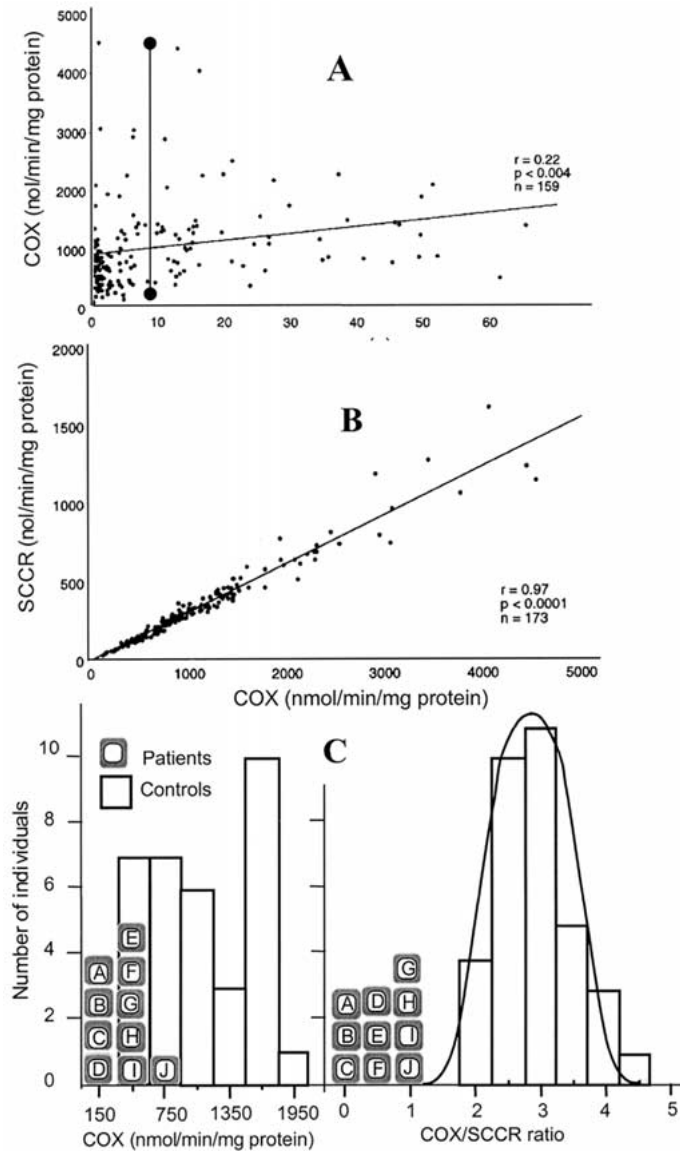


Figure 2 Distinguishing normal from defective OXPHOS activity. (A) The significant scatter of respiratory chain complex activity (here cytochrome-*c* oxidase, COX) in control population. The vertical black bar indicates the variation around 10 years of age. Note the comparatively very small variation with age. (B) The good correlation between respiratory chain complex activities (here COX and succinate:cytochrome-*c* reductase (SCCR) activities) in a control population. (C) In the left panel, COX absolute activity does not display normal distribution and patients' COX activity values overlap with control values. The normal distribution of COX/SCCR ratio in controls and the distinct distribution of values for COX-deficient patients are presented in the right panel

in any biochemical dysfunction. In this case, biochemical study will not detect significant deficiency. The study of a clinically affected tissue, if available, might help to reduce this problem.

Heteroplasmy of mtDNA can also result in problems when studying cultured cells. Indeed, changes in heteroplasmy levels can cause instability of the biochemical phenotype (Bourgeron et al 1993). Depending on the cell culture medium used, cells with defective OXPHOS may die, or multiply normally, or grow slowly as compared to cells with normal OXPHOS activity. The resulting biochemical phenotype will obviously be affected by change in the proportion of cells harbouring mutant or wild-type mtDNA. Two factors may allow cells with defective OXPHOS to survive and multiply. Pyruvate is one factor that will help in maintaining these cells alive because it helps to oxidize cellular NADH, which otherwise accumulates and hinders intermediary metabolism in OXPHOS-defective cells. In the case of a profound deficiency of the last segment of the respiratory chain (from the ubiquinone pool to the cytochrome-*c* oxidase), mitochondria will be unable to produce the uridine necessary for nucleic acid synthesis, because a key step in uridine biosynthesis is catalysed by the dihydroorotate dehydrogenase, an enzyme present at the outer surface of the inner membrane and feeding electrons to the quinone pool (Figure 1). Deficiency of the last segment of the chain will thus block uridine synthesis and survival of deficient cells will depend on an exogenous source of uridine. It therefore appears mandatory to culture skin fibroblasts from patients suspected of a RC deficiency in the presence of pyruvate (2 mmol/L) and uridine (200 μ mol/L).

Using cultured cells may also prove problematic because of the variable oxidative cofactor content of the mitochondria. This has been well illustrated for the mitochondrial pyrimidine nucleotide (NAD⁺/NADH) pool, which tends to decrease in quiescent cells for a number of different types of cultured cells (Rustin et al 1996). Decreased mitochondrial pyrimidine nucleotides may impair both respiration and complex I substrate oxidation (malate, pyruvate). Changing culture medium before OXPHOS determination by polarographic means therefore appears essential. Adding exogenous NAD⁺ during polarographic recording may also replenish the mitochondrial pyrimidine nucleotide pool.

Cell culture studies may not be the only ones to encounter problems. In all centres, spectrophotometric analyses have occasionally (or routinely) to be carried out on frozen samples that are highly sensitive to improper handling. Indeed, the large RC complexes embedded in the mitochondrial inner membrane and the electron exchanges between them are quite sensitive to membrane disturbance. A comparative study focused on the respective rates of loss of enzyme activity in the OXPHOS system shows that most activities that rely on the quinone pool rapidly lose function, together with complex III activity. Unfortunately, there is no means to differentiate such artefactual loss of activity from an actual deficiency. This is illustrated by the increased frequency in frozen samples of complex III deficiency, which is extremely rare in fresh biopsies. It should be kept in mind that performing OXPHOS studies by biochemical procedure is simply meaningless if samples have not been correctly frozen instantaneously and kept below -80°C .

Finally, in addition to the problems mentioned above, two general observations should be made when dealing with biochemical investigations, which also apply to mitochondrial OXPHOS investigation. First, normal activities of the measured enzymes do not necessarily preclude dysfunction 'next door' affecting another enzyme. In keeping with this, very few mitochondrial enzyme activities are actually measured, and mostly under V_{\max} conditions. Second, deficiency of an enzyme activity does not imply primary impairment; for example, severe fatty acid oxidation defects may subsequently affect mitochondrial membranous enzymes.

CONCLUSION

Reflecting the central role of OXPHOS and mitochondrial functions in cells, defects in OXPHOS are recognized as being the source of a steadily increasing number of diseases. Consequently, OXPHOS deficiency has to be looked for in an increasing number of patients. However, it is reasonable to conclude that there is no unique approach that would detect 100% of the defects in patients at risk because (i) with few exceptions, clinical features are much too variable; (ii) metabolic analyses in body fluids are neither specific nor sensitive enough; (iii) a straight molecular approach is possible only in a few well-defined syndromes; (iv) histochemistry is often poorly sensitive, restricted to a few enzymes; and (v) enzyme assays may encounter numerous pitfalls, and never establish the primary nature of the defect.

REFERENCES

- Bourgeron T, Chretien D, Rötig A, Munnich A, Rustin P (1993) Fate and expression of the deleted mitochondrial DNA differ between heteroplasmic skin fibroblast and Epstein-Barr virus-transformed lymphocyte cultures. *J Biol Chem* **268**: 19369–19376.
- Chretien D, Gallego J, Barrientos A, et al (1997) The biochemical parameters for the diagnosis of respiratory chain deficiency in man and their lack of age-related changes. *Biochem J* **329**: 249–254.
- Geromel V, Kadhom N, Ceballos-Picot I, et al (2001) Superoxide-induced massive apoptosis in cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the ATPase-6 gene of the mitochondrial DNA. *Hum Mol Genet* **10**: 1221–1228.
- Geromel V, Darin N, Chretien D, et al (2002) Coenzyme Q₁₀ and idebenone in the therapy of respiratory chain diseases: rationale and comparative benefits. *Mol Genet Metab* **71**: 21–30.
- Larsson NG, Clayton DA (1995) Molecular genetic aspects of human molecular disorders. *Annu Rev Genet* **29**: 151–178.
- Munnich A, Rustin P (2001) Clinical spectrum and diagnosis of mitochondrial disorders. *Am J Med Genet* **106**: 4–17.
- Munnich A, Rötig A, Cormier-Daire V, Rustin P (2001) Clinical presentation of respiratory chain deficiency. In Scriver C, Beaudet AL, Sly WS, Valle D, eds; Childs B, Kinzler KW, Vogelstein B, assoc. eds. *The Metabolic and Molecular Bases of Inherited Metabolic Disease* 8th edn. New York: McGraw-Hill, 2261–2274.
- Nicholls DG (1982) *Bioenergetics: An Introduction to the Chemiosmotic Theory*. New York: Academic Press.
- Raha S, Robinson BH (2001) Mitochondria, oxygen free radicals, and apoptosis. *Am J Med Genet* **106**: 62–70.
- Romero NB, Lombes A, Touati G, et al (1996) Morphological studies of skeletal muscle in lactic acidosis. *J Inherit Metab Dis* **19**: 528–534.

- Rustin P (2002) Mitochondria, from cell death to proliferation. *Nature Genetics* **30**: 352–353.
- Rustin P, Chretien D, Bourgeron T, et al (1991) An improved representation of enzyme activities for assessment of the mitochondrial respiratory chain. *Lancet* **338**: 60.
- Rustin P, Chretien D, Gerard B, et al (1994) Biochemical and molecular investigations in respiratory chain deficiencies. *Clin Chim Acta* **228**: 35–51.
- Rustin P, Parfait B, Chretien D, et al (1996) Fluxes of nicotinamide adenine dinucleotides through mitochondrial membranes in human cultured cells. *J Biol Chem* **271**: 14785–14790.
- Tyler D (1992) *The Mitochondrion in Health and Disease*. New York: VCH.
- Tzagoloff A (1982) *Mitochondria*. New York: Plenum Press.