FEBS Letters 440 (1998) 46–50 FEBS 21180

Effect of MI-D, a new mesoionic compound, on energy-linked functions of rat liver mitochondria

Silvia M.S.C. Cadena[a], Eva G.S. Carnieri[a], Aurea Echevarria[b], Maria Benigna M. de Oliveira[a,*]

[a] *Departamento de Bioquímica, Universidade Federal do Paraná, C.P. 19046, C.E.P. 81531-990, Curitiba, Paraná, Brazil*
[b] *Departamento de Química, Universidade Federal Rural do Rio de Janeiro, Rio de Janeiro, Brazil*

Received 3 September 1998; received in revised form 5 October 1998

Abstract MI-D (4-phenyl-5-(4-nitro-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride), a new mesoionic compound, depressed the phosphorylation efficiency of liver mitochondria as deduced from an accentuated decrease of the respiratory control coefficient and ADP/O ratio. Analysis of segments of the respiratory chain suggested that the MI-D inhibition site is further on than complex I and between complexes II and III. The transmembrane electrical potential ($\Delta\psi$) was collapsed dependent on MI-D concentration. ATPase activity was dramatically increased by MI-D in intact mitochondria, but inhibited in carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled mitochondria. These results suggest that MI-D acts as an uncoupler agent, a property closely related to its structural characteristics.

Key words: Mesoionic compound; Mitochondrial uncoupling; ATPase

1. Introduction

Mesoionic compounds such as sydnones, sydnonimines, isosydnones, and mesoionic 1,3,4-thiadiazoles possess structural features, which confer on them potential biological activity and pharmaceutical use [1–5]. Their structures having well separated regions of positive and negative charge, associated with a polyheteroatomic system, enable them to interact with biomolecules, such as DNA and proteins. Although the molecules are internally charged, they are neutral overall, and therefore can cross biological membranes in vivo [6].

A number of mesoionic compounds have also been examined in the search for anticancer agents [7–9]. Recently, Grynberg et al. [10] synthesized a new one, the 4-phenyl-5-(4-nitro-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride (MI-D; Fig. 1), that was shown to enhance survival of Ehrlich and S-180 tumor-bearing mice, preventing the growth of the tumor, with no significant concomitant alterations in the hematological parameters in test animals.

In spite of the different types of biological activity that have been assigned to mesoionic compounds, much of their biological and toxicological action mechanisms at molecular and cellular levels remain to be elucidated. Considering that the liver is a target organ for toxic effects caused by several xenobiotics and the important anti-tumoral effect of MI-D demonstrated by Grynberg et al. [10], amplification of knowledge about this mesoionic compound is of interest. We therefore describe here the effect of MI-D on some energy-linked functions in isolated rat liver mitochondria.

2. Materials and methods

2.1. Materials

Safranine *O*, carbonyl cyanide *m*-chlorophenylhydrazone (FCCP), sodium succinate, sodium glutamate, rotenone, bovine serum albumin (BSA), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), mannitol, sucrose, phosphoenolpyruvate (PEP), pyruvate kinase, lactate dehydrogenase and NADH were obtained from Sigma (St. Louis, MO, USA). All other reagents were commercial products of the highest available purity grade. MI-D (4-phenyl-5-(4-nitro-cinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride) was synthesized in the Department of Chemistry of the Rural Federal University of Rio de Janeiro, Brazil, as described [10]. Its structure was confirmed by ^{1}H-NMR, ^{13}C-NMR and mass spectrometry [10]. For use in mitochondrial assays, MI-D was dissolved in dimethylsulfoxide (DMSO) and then further diluted with the assay medium.

2.2. Animals

These were male albino rats (Wistar strain, 220–300 g), which received a standard laboratory diet (Purina). All animals were starved 12 h before being sacrificed; none of them were found to have any gross pathological lesions.

2.3. Preparation of rat liver mitochondria

Mitochondria were isolated from rat liver by the method of Voss et al. [11], using an extraction medium consisting of 250.0 mM mannitol, 10 mM HEPES-KOH, pH 7.2, 1.0 mM ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) and 0.1 g% BSA. Only mitochondrial preparations, with respiratory control above 4.0, were used. Disrupted mitochondria were obtained by a freeze-thawing treatment and used to determine the activities of enzymes linked to the respiratory chain and ATPase. MI-D was incubated for 2 min with the mitochondrial preparations before initiation of the assays. For each one to be valid, duplicate solvent controls with DMSO were included. DMSO, in the concentrations used in these experiments, had no effect on mitochondrial properties.

2.4. Oxygen uptake

Oxygen uptake and oxidative phosphorylation were evaluated at 30°C in a 1.3-ml thermostatically controlled water-jacketed closed chamber with magnetic stirring. Oxygen consumption was measured polarographically using a Clark-type electrode with a Gilson oxygraph [12,13]. The reaction medium containing 125.0 mM mannitol,

*Corresponding author. Fax: (55) (41) 2662042.
E-mail: mbmo@bio.ufpr.br

Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin; PMS, *N*-methyldibenzopyrazine methyl sulfate; Tris, tris(hydroxymethyl)aminomethane; PEP, phosphoenolpyruvate; CCCP, carbonylcyanide-*m*-chlorophenylhydrazone; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DNP, 2,4-dinitrophenol; $\Delta\psi$, transmembrane electrical potential; DMPC, dimyristoylphosphatidylcholine; DMSO, dimethylsulfoxide; DPIP, 2,6-dichlorophenolindophenol; Pi, inorganic phosphate

PII: S0014-5793(98)01427-6

65.0 mM KCl, 10.0 mM HEPES-KOH, pH 7.2, 0.1 mM EGTA and 0.1 g% BSA, was supplemented either with 10.0 μM rotenone, 0.8 mM inorganic phosphate (Pi), 0.08 mM ADP, 3.0 mM sodium succinate and 0.5 mg of mitochondrial protein, or 1.6 mM Pi, 0.16 mM ADP, 5.0 mM sodium glutamate and 2.0 mg of mitochondrial protein. The respiratory rate is expressed as nmols of oxygen consumed per min per mg of mitochondrial protein, according to Estabrook [14].

2.5. Measurements of activities of the enzymatic complex of the respiratory chain

NADH oxidase, NADH dehydrogenase and succinate dehydrogenase activities were assayed spectrophotometrically [15]. Succinate oxidase activity was evaluated polarographically according to Singer [15]. NADH-cytochrome *c* reductase and succinate cytochrome *c* reductase activities were measured by reduction of cytochrome *c* at 550 nm as described by Somlo [16]. The activity of cytochrome *c* oxidase was evaluated according to Mason et al. [17].

The activity of ATPase in broken and intact mitochondria was evaluated as described by Pullman et al. [18], but with modifications. In disrupted mitochondria the enzyme was assayed at 37°C, using a system consisting of 170.0 mM mannitol, 50.0 mM tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.4, 3.0 mM magnesium acetate, 30.0 mM potassium acetate, 1.0 mM PEP, 4.0 U pyruvate kinase, 3.0 U lactate dehydrogenase, 0.2 mM NADH, 3.0 mM ATP, and 100.0 μg of mitochondrial protein. Results are expressed as nmols of Pi liberated per min per mg, considering that 1.0 nmol of oxidized NADH corresponds to 1.0 nmol of Pi liberated. ATPase activity of intact mitochondria was assayed at 30°C in the presence or absence of 1.0 μM FCCP, the reaction medium consisting of 50.0 mM sucrose, 120.0 mM Tris-HCl, pH 7.4, 50.0 mM KCl, 3.0 mM ATP, and 3.0 mg of mitochondrial protein. Liberated orthophosphate was measured according to Summer [19] and the results expressed as nmols of Pi liberated per min per mg.

2.6. Measurements of mitochondrial transmembrane electrical potential ($\Delta\Psi$)

The transmembrane potential was evaluated at 30°C with safranine *O* [20]. The standard reaction medium consisted of 250.0 mM mannitol, 10.0 mM HEPES, pH 7.2, and was supplemented with 0.5 mM Pi, 10.0 μM safranine *O*, 1.5 mg of mitochondrial protein, 3.0 mM sodium succinate and 4.0 μM rotenone. When sodium glutamate (6.0 mM) was used as substrate, rotenone was omitted. Absorbance at 511–533 nm was registered in an Aminco Chance dual wavelength beam recording spectrophotometer, and the transmembrane electrical potential ($\Delta\psi$) estimated as described by Rossi and Azzoni [21].

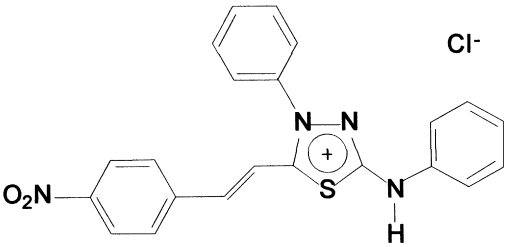

Fig. 1. Chemical structure of 4-phenyl-5-(4-nitrocinnamoyl)-1,3,4-thiadiazolium-2-phenylamine chloride.

2.7. Protein determinations

Mitochondrial protein was assayed by the method of Lowry et al. [22], using bovine serum albumin as standard.

2.8. Statistical analysis

Statistical analysis of the data was carried out as analysis of variance and test of Tukey for average comparison. Mean values ± S.D. were used.

3. Results and discussion

Fig. 2 shows the effect of various concentrations of MI-D

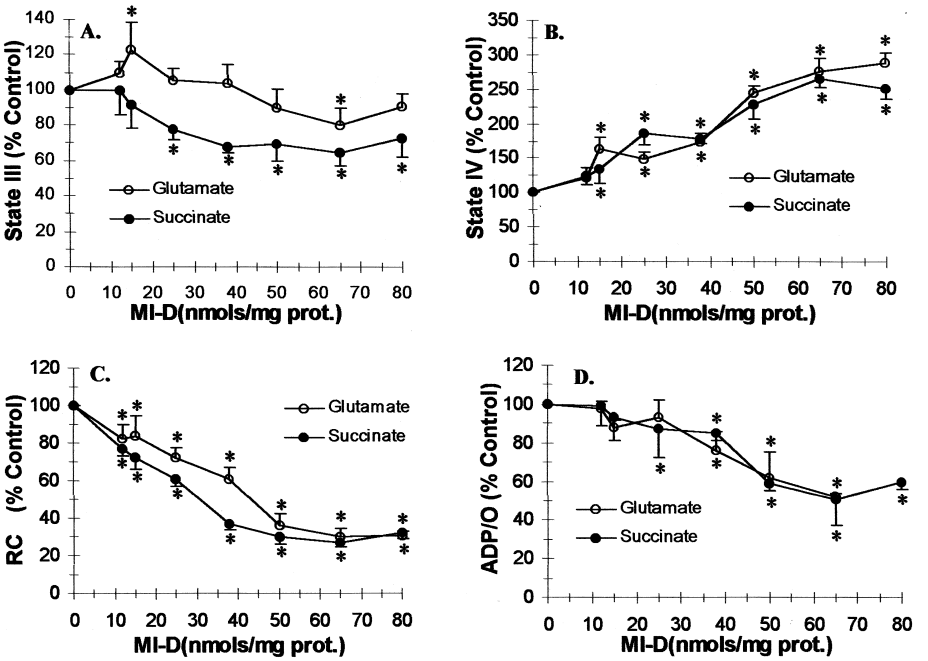

Fig. 2. Effects of MI-D on mitochondrial respiration with sodium glutamate (○) or sodium succinate (●) as substrate. Conditions for oxygen uptake measurements are described in Section 2. A: State III, mitochondrial oxygen uptake in the presence of ADP and substrate. 100% activity corresponds to 34.6 ± 4.2 and 38.0 ± 4.0 nmol of oxygen consumed per min per mg of mitochondrial protein for glutamate and succinate, respectively. B: State IV, mitochondrial oxygen uptake after exhaustion of ADP. 100% activity corresponds to 7.0 ± 1.1 and 8.0 ± 1.3 nmol of oxygen consumed per min per mg of protein for glutamate and succinate, respectively. C: RCC, respiratory control coefficient. 100% corresponds to 4.6 ± .0.2 and 4.6 ± 0.6 for glutamate and succinate, respectively. D: ADP/O, ADP concentration/oxygen consumption. 100% corresponds to values of 2.8 ± 0.2 and 1.8 ± .0.2 for glutamate and succinate, respectively. Each value represents the mean ± S.D. of six different experiments. *Significantly different from control ($P < 0.05$).

on the respiratory parameters of functionally intact mitochondria. In these experiments, glutamate and succinate were the oxidizable substrates and the analyzed parameters were: (i) the rates of oxygen consumption in states III (Fig. 2A) and IV (Fig. 2B) of mitochondrial respiration; (ii) the respiratory control coefficient (RC) (Fig. 2C); and (iii) the ADP/O ratio (Fig. 2D). In Fig. 2A it can be observed that the effect of MI-D on preparations of mitochondria respiring in the presence of substrates and ADP (state III) was discrete for both substrates, besides not being identical. When glutamate was used, MI-D at 15.0 nmol/mg protein promoted a slight but statistically significant increase in the respiratory rate, but with a decrease (22%) when 65.0 nmol MI-D/mg protein was used. When succinate was the substrate, the effect of MI-D on state III was inhibitory (30%), up to 25.0 nmol/mg protein. In contrast, as can be seen in Fig. 2B, the rate of oxygen consumption after ADP exhaustion (state IV) was strongly increased reaching 80.0 nmol/mg protein, a stimulus of 190% and 152% during oxidation of glutamate and succinate, respectively (Fig. 2B). The values for RC and ADP/O ratios were lower (Fig. 2C and D), indicating that MI-D could, in addition to an effect on components of respiratory chain, give rise to an accentuated uncoupler effect.

In order to clarify the effects of MI-D on state III respiration, assays were carried out using broken mitochondria. Under this experimental condition, an evaluation could be made of electron transport throughout the respiratory chain, independent of the membrane barrier, phosphorylation activity or other processes dependent on $\Delta\Psi$; in addition, a determination could be performed of the site of respiratory chain inhibition. The presence of the drug in the system decreased

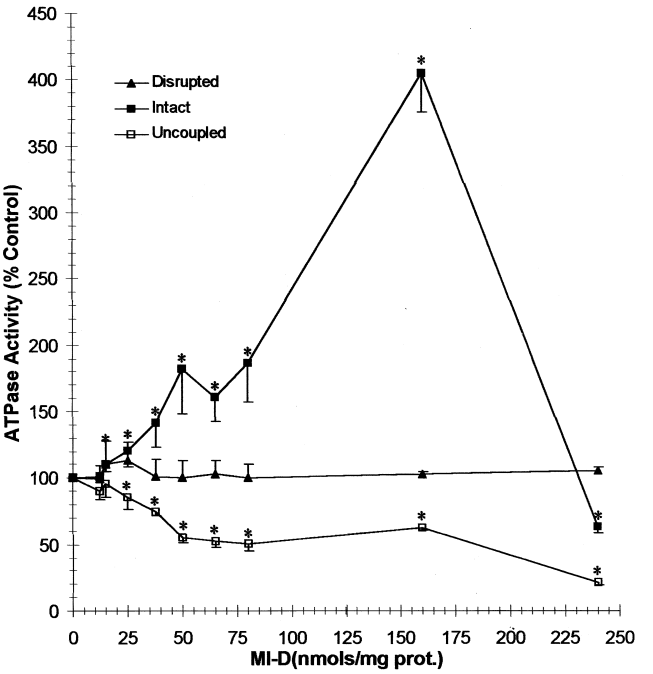

Fig. 4. Effects of MI-D on ATPase. (▲) Disrupted mitochondria. 100% activity is that of 700.0 ± 112.0 nmol of Pi released per min per mg of mitochondrial protein. (■) Intact mitochondria. 100% activity is that of 20.0 ± 1.1 nmol of Pi released per min per mg of mitochondrial protein. (□) FCCP-uncoupled mitochondria. 100% activity is that of 91.3 ± 5.6 nmol of Pi released per min per mg of mitochondrial protein. The assay conditions are described in Section 2. The data are the means ± S.D. of four different experiments. *Significantly different from control ($P < 0.05$).

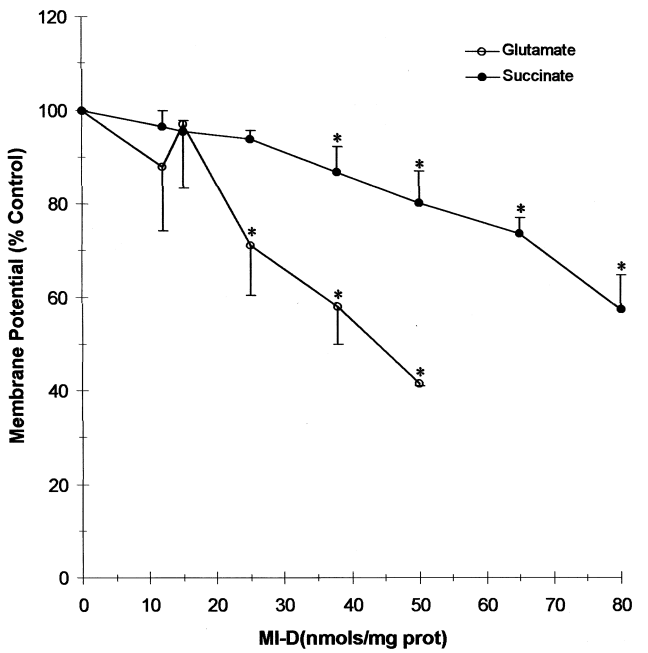

Fig. 3. Effect of MI-D on $\Delta\psi$. Rat liver mitochondria (1.5 mg) was incubated under the standard incubation conditions, as described in Section 2 and energized with sodium glutamate (○) or sodium succinate (●). Each value represents the mean ± S.D. of four different experiments. *Values significantly different relative to absence of MI-D ($P < 0.05$). 100% corresponds to 170 ± 10.9 mV of transmembrane electrical potential.

the activities of NADH and succinate oxidase by 32% and 55%, respectively. The segments of the respiratory chain, namely NADH-cytochrome *c* reductase, succinate-cytochrome *c* reductase, as well as succinate dehydrogenase were inhibited by 20%. No effect was observed on NADH dehydrogenase and cytochrome oxidase, suggesting that the site of MI-D inhibition is further on than complex I and between complexes II and III. These results explain those pointed out by Fig. 2A, in terms of a decrease of oxygen consumption at state III. Small differences in inhibition values could signify particular properties of the mitochondrial preparations used, especially considering that in broken mitochondria the disrupted membrane barrier could allow direct interaction of MI-D with the enzymatic complexes.

Fig. 3 shows that MI-D impairs the development of $\Delta\Psi$ by mitochondria energized with succinate or glutamate, the concentration of the drug for total potential collapse being different for each substrate. While 65.0 nmol/mg protein was sufficient to abolish $\Delta\Psi$ completely when glutamate was the substrate, with succinate a decrease of 40% was observed at 80.0 nmol/mg protein. The different magnitude effects could be results of the mechanism by which each substrate had access into the mitochondria; glutamate depended on the ΔpH across the inner mitochondrial membrane, while succinate depended on the dicarboxylate carrier [23]. The effects promoted by MI-D on $\Delta\Psi$ must be related with its uncoupling property, since its effect as inhibitor of electron transport was not sufficient to explain the drastic collapse of the transmembrane potential observed.

In order to better visualize the uncoupler action of MI-D, the effect of this xenobiotic was evaluated on ATPase activity; the results are shown in Fig. 4. In these experiments the enzyme was assayed in preparations consisting of disrupted, intact and FCCP-uncoupled mitochondria. This last condition was included considering the reports that excess or simultaneous combinations of uncoupler agents might result in submaximal rates of mitochondrial parameters as a decrease of ATP hydrolysis [24] and/or oxygen consumption [25]. No effect on ATPase activity was detected when MI-D was evaluated using disrupted mitochondria preparations, excluding a direct interference of this drug on the enzyme. In contrast, as would be expected for an uncoupler agent, MI-D promoted a strong stimulus on the hydrolytic activity of ATPase, assayed in intact mitochondria. This stimulus was dependent on MI-D concentration and reached 300% at 160.0 nmol/mg protein. A further increase in drug concentration, up to 240.0 nmol/mg protein, was accompanied by an accentuated decrease of ATP hydrolysis. This has also been described for compounds such as 2,4-dinitrophenol (DNP) [26] and FCCP [27], when used in concentrations higher than those necessary for uncoupling mitochondria. The effect of MI-D, as evaluated on ATPase of FCCP-treated mitochondria, was inhibitory (Fig. 4). In addition, MI-D was able to remove the inhibition caused by oligomicin (data not shown). These results characterize and include MI-D as a new uncoupler agent, although of lower potency than FCCP, and suggest that the effects of this xenobiotic depend on its interaction with membrane constituents. In this context it has been shown that both membrane lipids and proteins change simultaneously during the uncoupling event [28]. Specific interactions with membrane polypeptides, similar to those described for analogues of the classical uncouplers DNP and FCCP [29,30] and also suggested from studies with recoupling agents [31], could be involved during the interaction of MI-D with the membrane. In order to deal with this possibility, experiments of fluorescence polarization developed in our laboratory with dimyristoylphosphatidylcholine (DMPC) liposomes (data not shown), have pointed to a distribution of MI-D molecules in both phospholipid bilayer region and phospholipid/water interface, as described for uncoupler agents [32].

The actions of MI-D on mitochondria are complex with a diverse spectrum of responses being manifested. While MI-D satisfies most, if not all of the criteria for an uncoupler agent, at higher concentrations it can act as electron transport inhibitor. An inhibition of state III, particularly when succinate was the substrate, became evident within the stimulatory concentration range of state IV. Compounds with such properties have been classified as inhibitory uncouplers [33]. The effects of MI-D on the mitochondria were in close relationship with its structural properties, the bulky hydrophobic groups, in addition to the presence of an acid-dissociable group, justifying all the presently observed results.

In a recent review Skulachev [34] pointed that in some instances an uncoupling action could explain the therapeutic effect of a drug. This seems true for the antitumor action of carbonylcyanide-*m*-chlorophenylhydrazone (CCCP) [35] and other anticancer drugs [36]. In this respect, the results presented herein could be involved in the antitumor activity of MI-D described by Grynberg et al. [10].

Acknowledgements: This investigation was supported by the Brazilian Research Council (CNPq and CAPES). The authors thank Dr. Aguinaldo José Nascimento for suggestions for the statistical analysis, Dr. Maria Lúcia W. Klüppel for critical reading and Dr. Philip Albert James Gorin for the language corrections.

References

[1] Lima, E.O., Maia, R.F., Barbosa Filho, J.M. and Xavier Filho, L. (1986) Rev. Microbiol. 17, 132–136.
[2] Moustafa, M.A.A. and Eisa, H.M. (1991) Arch. Pharm. 325, 397–401.
[3] Corell, T., Pedersen, S.B., Lissau, B., Moilanen, E., Mëtsa-Ketelä, T., Kankaanranta, H., Vuorinen, P., Vapaatalo, H., Rydell, E., Andersson, R., Marcinkiewicz, E., Korbut, R. and Gryglewski, R.J. (1994) Pol. J. Pharmacol. 46, 553–566.
[4] Satyanarayana, K. and Rao, M.N.A. (1995) J. Pharm. Sci. 84, 263–266.
[5] Rehse, K., Ciborski, T. and Müller, B. (1995) Arch. Pharm. (Weinheim) 328, 125–126.
[6] Kier, L.B. and Roche, E.B. (1967) J. Pharm. Sci. 56, 149–169.
[7] Greco, C.V., Nyberg, W.H. and Cheng, C.C. (1962) J. Med. Pharm. Chem. 5, 861–865.
[8] Grynberg, N., Gomes, R., Shinzato, T., Echevarria, A. and Miller, J. (1992) Anticancer Res. 12, 1025–1028.
[9] Shinzato, T.O., Grynberg, N., Gomes, R.M., Echevarria, A. and Miller, J. (1989) Med. Sci. Res. 17, 865–866.
[10] Grynberg, N., Santos, A.C. and Echevarria, A. (1997) Anti-Cancer Drugs 8, 88–91.
[11] Voss, D.O., Campelo, A.P. and Bacila, M. (1961) Biochem. Biophys. Res. Commun. 4, 48–51.
[12] Voss, D.O., Cowles, J.C. and Bacila, M. (1963) Anal. Biochem. 6, 211–222.
[13] Chance, B. and Williams, G.R. (1955) J. Biol. Chem. 217, 383–393.
[14] Estabrook, R.W. (1967) in: Mitochondrial Respiratory Control and the Polarographic Measurement of ADP/O Ratios (Estabrook, R.W. and Pullman, M.E., Eds.) Methods in Enzymol., Vol. 10, pp. 41–47, Academic Press, New York, NY.
[15] Singer, T.P. (1974) in: Determination of the Activity of Succinate, NADH, Choline and α-Glycerophosphate Dehydrogenases (Glick, D., Ed.) Methods Biochem. Anal., Vol. 22, pp. 123–175, John Wiley, New York, NY.
[16] Somlo, M. (1965) Biochim. Biophys. Acta 97, 183–201.
[17] Mason, T.L., Poyton, R.O., Wharton, D.C. and Shatz, G. (1973) J. Biol. Chem. 248, 1346–1354.
[18] Pullman, M.E., Penefski, H.S. and Data, A. (1960) J. Biol. Chem. 235, 3322–3329.
[19] Sumner, J.B. (1944) Science 100, 413–414.
[20] Akerman, K.E.O. and Wikstrom, M.F.K. (1976) FEBS Lett. 68, 191–197.
[21] Rossi, E. and Azzone, G.F. (1969) Eur. J. Biochem. 7, 418–426.
[22] Lowry, O.H., Rosebrough, N.J., Farr, A.C. and Randall, R.J. (1951) J. Biol. Chem. 193, 262–275.
[23] Nicholls, D.G. and Ferguson, S.J. (1992) Bioenergetics 2, Academic Press, Harcourt Brace Jovanovich, London.
[24] Van Dam, K. and Kraayenhof, R. (1969) Biochim. Biophys. Acta 172, 189–197.
[25] Van Dam, K. (1967) Biochim. Biophys. Acta 131, 407–411.
[26] Slater, E.C. (1967) in: Application of Inhibitors and Uncouplers for a Study of Oxidative Phosphorylation (Estabrook, R.W. and Pullman, M.E., Eds.) Methods in Enzymology, Vol. 10, pp. 48–57, Academic Press, New York, NY.
[27] Bertina, R.M. and Slater, E.C. (1975) Biochim. Biophys. Acta 376, 492–504.
[28] Zimmer, G. (1977) Arch. Biochem. Biophys. 181, 26–32.
[29] Hanstein, W.G. (1976) Biochim. Biophys. Acta 456, 129–148.
[30] Katre, N.V. and Wilson, D.F. (1978) Arch. Biochem. Biophys. 191, 647–656.

[31] Starkov, A.A., Bloch, D.A., Chernyak, B.V., Dedukhova, V.I., Mansurova, S.E., Severina, I.I., Simonyan, R.A., Vygodina, T.V. and Skulachev, V.P. (1997) Biochim. Biophys. Acta 1318, 159–172.
[32] Terada, H. (1981) Biochim. Biophys. Acta 639, 225–242.
[33] Moreland, D.E. (1994) in: Effects of Toxicants on Oxidative Phosphorylation and Photophosphorylation (Hodgson, E. and Levi, P.E., Eds.) Introduction to Biochemical Toxicology, pp. 345–366, Appleton and Lange, Norwalk, CT.
[34] Skulachev, V.P. (1998) Biochim. Biophys. Acta 1363, 100–124.
[35] Newell, K.J. and Tannock, I.F. (1989) Cancer Res. 49, 4477–4482.
[36] Keller, B.J., Marsman, D.S., Popp, J.A. and Thurman, R.G. (1992) Biochim. Biophys. Acta 1102, 237–244.