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MITOCHONDRIAL TARGETS OF DRUG TOXICITY

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■ **Abstract** Mitochondria have long been recognized as the generators of energy for the cell. Like any other power source, however, mitochondria are highly vulnerable to inhibition or uncoupling of the energy harnessing process and run a high risk for catastrophic damage to the cell. The exquisite structural and functional characteristics of mitochondria provide a number of primary targets for xenobiotic-induced bioenergetic failure. They also provide opportunities for selective delivery of drugs to the mitochondrion. In light of the large number of natural, commercial, pharmaceutical, and environmental chemicals that manifest their toxicity by interfering with mitochondrial bioenergetics, it is important to understand the underlying mechanisms. The significance is further underscored by the recent identification of bioenergetic control points for cell replication and differentiation and the realization that mitochondria play a determinant role in cell signaling and apoptotic modes of cell death.

INTRODUCTION

At the dawn of history, our single-celled glycolytic ancestors welcomed a new inhabitant, an oxygen-utilizing primitive proteobacteria. These newcomers would bring final relief to the eukaryotic community from the ever-increasing energy demands of ongoing evolution. Eventually, eukaryotic hosts completely integrated their new inhabitants, turning them into specialized energy-producing organelles, which we refer to as mitochondria. Mitochondria are present in almost all types of modern eukaryotic cells, their chief (but not their only) function being energy production for the benefit of the host cell.

In the long run, however, infection with mitochondria brought with it additional vulnerabilities. As with any power source, malfunctioning mitochondria present a hazard to the host cells. Insufficient energy production is an obvious but not the only consequence of mitochondrial poisoning; a great deal of harm at the cellular level arises from mitochondria-catalyzed side reactions, such as exothermic oxygen combustion and free radical emission. There is also an emerging

appreciation for the important role that mitochondria play in metabolic cell signaling pathways and in the regulation of cell morphology, mobility, multiplication, and apoptosis. Accordingly, pharmacologists and toxicologists have recently addressed mitochondria as an important intracellular target in the manifestation of a variety of both beneficial and adverse biological activities.

What are mitochondria, how do they produce energy, and what features render them vulnerable to chemical-induced malfunction? This treatise addresses these questions, with a focus on better understanding the critical characteristics that determine the biological activities of mitochondrial poisons.

Energy Production in Mitochondria: An Overview

Mitochondria are intracellular organelles, varying in both shape and size. They may be spherical or elongated, or even branched, and the number may vary from 6–12 small discrete organelles per rat thymus lymphocyte, to a massive and dynamically fluctuating network composed of an indefinable number of single interconnected mitochondria in a typical human fibroblast. Despite the wide variability in number and morphology, all mitochondria share several fundamental properties regardless of the cell type.

All mitochondria are bound by two lipid bilayer membranes. The outer membrane is permeable to ions and solutes up to 14 kDa. It is rich in cholesterol and contains embedded or attached enzymes that interface the mitochondrion with the rest of the cellular metabolic network. The inner membrane encloses a water-containing compartment, the so-called matrix, where mitochondrial DNA and various soluble enzymes, such as those of the tricarboxylic acid cycle and the β-oxidation pathway, are located. This membrane is not freely permeable to ions and metabolites, but instead contains special membrane proteins that transport selected metabolites across the membrane. This feature, the protein-mediated and -regulated permeability of the inner membrane, is of vital importance for the morphological and functional integrity of the mitochondrion: It is also the most common target for mitochondrial toxicants. Many foreign chemicals damage mitochondria either by increasing the permeability of the inner membrane or by inhibiting transport proteins embedded within it. The lipid composition of the inner membrane is unique in that it contains large amounts of cardiolipin and virtually no cholesterol. The presence of cardiolipin represents a second important feature: Many drugs (e.g. adriamycin-like anthraquinones) have a very high affinity for cardiolipin and thereby preferentially bind to and concentrate in the inner mitochondrial membrane.

The inner membrane also contains many different proteins that participate in various metabolic activities, including the production of energy. It also contains a mobile electron carrier, ubiquinone, dissolved in the lipid phase of the membrane. The primary form of energy generated in mitochondria is the so-called electrochemical proton gradient ($\Delta\tilde{\mu}_H+$) that is produced by three respiratory chain complexes, which are sophisticated protein ensembles composed of varying

numbers of polypeptide subunits. The electrochemical proton gradient furnishes the energy required to produce ATP and to support other activities of the mitochondria, such as the electrophoretic or protonophoric transport of ions, metabolic substrates, and proteins destined for the mitochondrial matrix. Several reviews on energy production and transformation in mitochondria are available [1; for a textbook devoted entirely to bioenergetics, see also Skulachev (36)]. Here, we briefly summarize the major features of mitochondrial energy production. This summary provides the basis for understanding the selective actions of mitochondria-targeted toxicants.

The mitochondrial respiratory chain catalyzes the oxidation of various substrates by oxygen. Substrates dissolved in the aqueous matrix space are oxidized by their specific dehydrogenases, which use the released energy to reduce NAD^+ or the ubiquinone of the inner membrane. Reduced NADH and ubiquinol are then oxidized by the respiratory chain complexes. NADH is oxidized by complex I (NADH:ubiquinone reductase) and the released energy is used to reduce a ubiquinone molecule and to generate $\Delta\tilde{\mu}_H+$. The majority of substrates are oxidized by this route, meaning that this is the main entry point for channeling electrons toward the final electron acceptor, molecular oxygen. Because of this, inhibition of NADH:ubiquinone electron flow blocks most of the oxidative metabolic reactions conducted by mitochondria. The second entry point of the respiratory chain is complex III (ubiquinol:cytochrome *c* reductase), which is also known as the bc_1-complex. This complex oxidizes the reduced ubiquinol. It also generates $\Delta\tilde{\mu}_H+$ and reduces the third member of the respiratory chain, cytochrome *c*. The latter is a mobile protein attached to the cytosolic (intermembrane space) side of the inner mitochondrial membrane. It serves as an electron carrier between complex III and complex IV, which is the terminal cytochrome *c* oxidase. Complex IV directly reduces molecular oxygen to water and generates $\Delta\tilde{\mu}_H+$. It is obvious that inhibition of complex III or complex IV completely blocks all of the energy production in mitochondria. In situations of partial inhibition of complex III or cytochrome oxidase, the electrons supplied by substrates may be diverted to the bulk production of toxic reactive oxygen species by the respiratory chain.

The $\Delta\tilde{\mu}_H+$ is generated by means of electrogenic pumping of protons from the mitochondrial matrix to the cytosol (intermembrane space), which is catalyzed by the membrane-spanning respiratory chain complexes. Proton pumping is ultimately coupled to electron flow so that there is no respiration without proton pumping and vice versa. The generated $\Delta\tilde{\mu}_H+$ consists of two components, the electrical membrane potential and the pH gradient across the inner mitochondrial membrane. The electrical potential represents the major component of $\Delta\tilde{\mu}_H+$. However, it is interchangeable with the pH gradient in such a way that, to some degree, a decrease in membrane potential alone results in an increase in the pH gradient with no changes in $\Delta\tilde{\mu}_H+$. Similarly, dissipation of the pH gradient results in a slight hyperpolarization of the membrane. The inner mitochondrial membrane is anisotropic, the matrix side being negatively charged and slightly alkaline; membrane potential is typically in the range of -180 to -220 mV with

a ΔpH of 0.4–0.6 U. This charge and pH anisotropy represents the third important feature of mitochondria that contributes to their vulnerability. Because of this feature, mitochondria can accumulate large amounts of positively charged lipophilic compounds and some acids. In the case of a positively charged compound such as 1-methyl-4-phenylpyridium ion or ethidium bromide, the concentration in the mitochondrial matrix can exceed that of the cytosol by several orders of magnitude, providing a strong basis for selective poisoning of mitochondria.

The overall rate of electron transport in the respiratory chain of mitochondria is regulated by the amplitude of $\Delta\tilde{\mu}_H+$, a phenomenon known as respiratory control. When $\Delta\tilde{\mu}_H+$ is high, respiration rate is low. Conversely, a decrease in $\Delta\tilde{\mu}_H+$ causes an immediate stimulation of oxygen consumption. Under physiological conditions, the decrease in $\Delta\tilde{\mu}_H+$ is due to the metabolic work performed in mitochondria, so the rate of respiration is tightly coupled to the rate of metabolism. It is obvious that the permeability of the inner mitochondrial membrane to protons and other charged species must be exquisitely low and that the dissipation of $\Delta\tilde{\mu}_H+$ must be mechanistically coupled with the performance of work. This coupling occurs by means of enzymes of the inner membrane that are capable of converting the energy stored in the form of $\Delta\tilde{\mu}_H+$ into the desirable kind of work (e.g. protein transport or ATP synthesis). A nonspecific increase in permeability of the inner membrane or a decrease in the degree of coupling of the protonomotive and phosphorylative complexes will dissipate $\Delta\tilde{\mu}_H+$ nonproductively in the form of heat emission. Such a malfunction in mitochondrial bioenergetics instantaneously transforms the mitochondrion from an essential powerhouse of the cell into a molecular furnace, efficiently wasting the metabolic energy of substrates. This is the most common mechanism of mitochondrial poisoning; literally hundreds of different toxicants damage mitochondria by increasing the permeability of the inner membrane to protons and other ions. In the following sections, we consider the major groups of mitochondria-specific toxicants, with particular attention to their mechanisms of action.

Enveloped within the mitochondrial membranes are a number of soluble enzyme activities related to the generation of reducing equivalents for the membrane-embedded electron transport chain. Among these soluble enzyme activities are the fatty acid β-oxidation and tricarboxylic acid oxidation pathways. Although there are many examples of chemicals that interfere with specific enzymatic steps within these pathways that lead to bioenergetic deficits within the cell, the focus of this article is the activity and turnover of membrane-associated bioenergetic functions, such as electron transport and respiration.

BIOENERGETIC POISONS

There are two means by which chemicals can affect mitochondrial bioenergetics, either by interfering with the generation of $\Delta\tilde{\mu}_H{}^+$ or by causing its dissipation. Acute poisoning with inhibitors of electron transporting complexes cause symp-

toms such as muscle weakness, easy fatigability, hypotension, headache, facial flushing, nausea, confusion, and aggravation of latent myocardial angina. This inability to utilize oxygen is manifested as a cytotoxic hypoxia wherein the chemicals cause a metabolic acidosis and hyperpnea, despite the normal pA_{O2} and absence of cyanosis. Inhibitors of the supply of reducing substrates for the respiratory chain, such as the fluoroacetates and acetamides, cause a remarkably similar metabolic syndrome that is difficult to distinguish from inhibitors of the electron transport chain.

Contrast this to poisons that dissipate $\Delta\tilde{\mu}_H+$, which likewise cause ATP deficits and metabolic acidosis and hyperpnea, but also induce excessive oxygen consumption, as reflected by the lower pA_{O2} and cyanosis. The free energy of substrate oxidation is liberated as heat causing fever in individuals poisoned by such agents. Examples of this latter group of chemicals include agents that increase membrane permeability to individual ions, such as channel-forming proteins, ionophores, uncouplers of oxidative phosphorylation, and inducers of the permeability transition of the mitochondrial membrane. The next several paragraphs summarize the characteristics of each of these classes of chemicals.

Inhibitors of Respiratory Chain

Inhibitors of Complex I Mitochondrial NADH:ubiquinone oxidoreductase (complex I; EC 1.6.5.3) is the first in the series of membrane-associated proton pumps of the mitochondrial respiratory chain (Figure 1*A*). The details of the path of electron transfer through components of complex I, the exact stoichiometry of proton pumping, and the mechanism of proton pumping are not clear. However, significant progress is being made and several excellent recent reviews are available (3–5). This enzyme is the most vulnerable of the respiratory chain complexes to chemical-induced malfunction. More than 60 different types of natural and synthetic compounds are known to inhibit mitochondrial complex I activity. Characteristics of the various inhibitors of this complex, the mechanisms of inhibition, and structure-activity relationships for major classes of potent inhibitors of complex I are all reviewed in a recent series of papers (5–8). The list of inhibitors of complex I includes pesticides; neuroleptics and natural neurotoxins; antihistaminic, antianginal, and antiseptic drugs; rodenticides; phenolic pollutants; fluorescent dyes; and myxobacterial and other antibiotics. The large number and structural diversity of inhibitors of complex I is in excellent agreement with the summation by Degli Esposti (5) that a ‘‘potent . . . inhibitor of complex I has a modular similarity with ubiquinone, with a cyclic ‘head’ . . . and a hydrophobic ‘tail’.’’

All of the inhibitors can be classified into one of three categories based on their specificity toward complex I. The first group includes compounds that inhibit at the level of the NADH-flavin interaction, such as rhein. Such compounds are not specific to complex I because they also affect a variety of other dehydrogenases. The second category is represented by quinole antagonists, which are inhib-

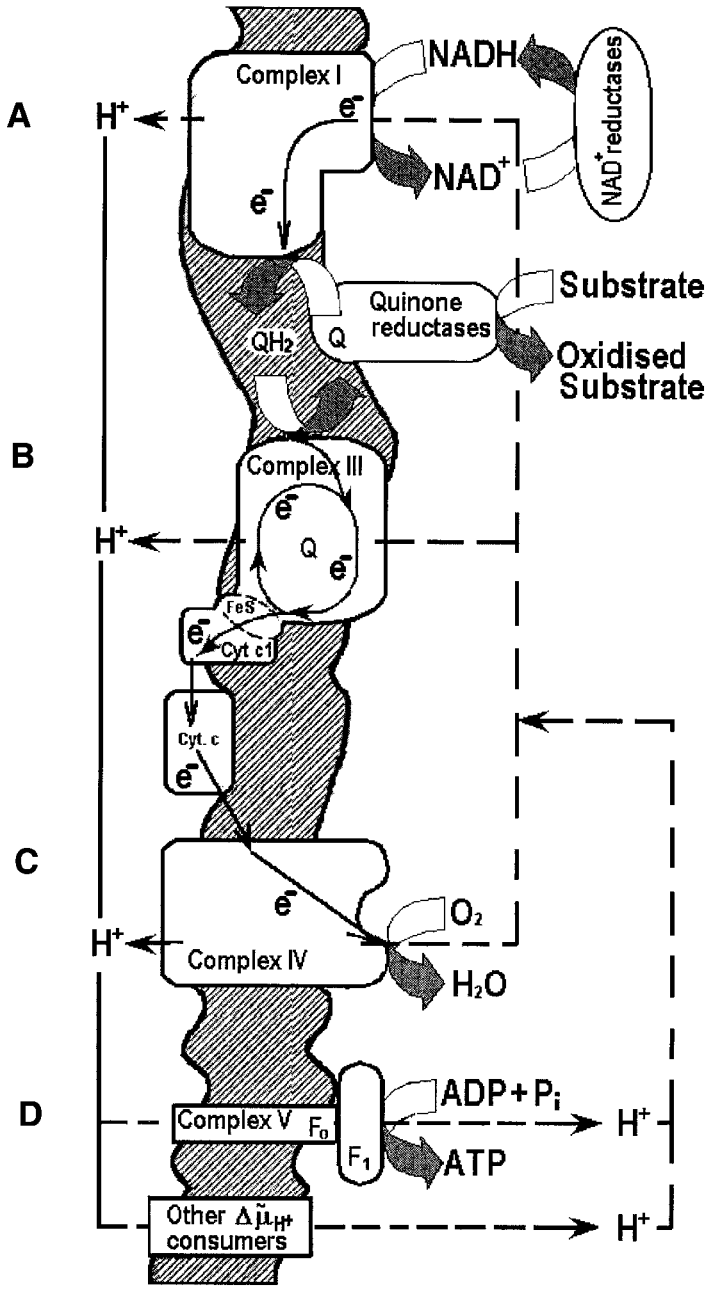

Figure 1 See next page.

itory for both complex I and the bc_1-complex. Examples of this group include myxothiazol and the quinolone aurachins produced by *Pseudomonas aeruginosa* and *Stigmatella aurantiaca.* These compounds interfere with quinol binding and some are twice as potent as rotenone in inhibiting mammalian mitochondrial NADH:ubiquinone oxidoreductase (5). The third group of inhibitors consists of compounds that seem to be specific and potent inhibitors of complex I, acting at concentrations low enough to have no effect on other respiratory chain complexes. Among this group is the classical inhibitor rotenone, which is a member of the rotenoid family of naturally occurring isoflavonoids produced by Leguminosae

Figure 1 Mitochondrial respiratory chain. (*A*) Mitochondrial NADH:ubiquinone oxidoreductase (complex I; EC 1.6.5.3) catalyzes the oxidation of NADH generated by NAD-linked dehydrogenases within the mitochondrial matrix. The enzyme reduces ubiquinone to the ubiquinol and generates $\Delta\tilde{\mu}_H+$ across the inner membrane of the mitochondria. Complex I is the largest and most sophisticated enzyme of the respiratory chain. It is composed of several polypeptide subunits encoded both by mitochondrial and nuclear DNA, and it contains FMN and several FeS centers. (*B*) Complex III (bc_1-complex, ubiquinol:cytochrome *c* oxidoreductase, EC 1.10.2.2) oxidizes ubiquinol, reduces cytochrome *c,* and generates $\Delta\tilde{\mu}_H+$. Mammalian complex III is composed of 11 subunits, including cytochrome *b,* having both a low and a high potential heme group (b_L and b_H), Rieske iron-sulfur protein containing a single Fe_2S_2 cluster, cytochrome *c*1 with a covalently bound heme *c,* and eight polypeptide subunits whose functions are unclear. Complex III subunits are encoded both by nuclear and mitochondrial DNA (12, 13). The bc_1-complex works through a Q-cycle mechanism. It contains two separate ubiquinone-binding sites, a quinol-oxidizing site Q_o, and a quinone-reducing site Q_i. Reduced ubiquinol dissolved in the inner mitochondrial membrane is oxidized in a bifurcated reaction. The first electron is transferred via the Rieske protein and cytochrome *c*1 to cytochrome *c.* This leaves an ubisemiquinone at center Q_o, which is unstable and quickly donates the remaining electron to heme b_L and b_H and to a ubiquinone or a stable ubisemiquinone anion bound in the Q_i site. The oxidation of one molecule of ubiquinol to ubiquinone yields two reduced molecules of cytochrome *c,* two protons are consumed on the negative (matrix) side of the membrane, and four protons are released on the positive (cytoplasmic) side. (*C*) Cytochrome c oxidase (complex IV; EC 1.9.3.1) is a heme/copper terminal oxidase that uses cytochrome *c* as electron donor. It catalyzes the four-electron reduction of O_2 to water, coupled to the generation of $\Delta\tilde{\mu}_H+$ across the inner mitochondrial membrane in which it is embedded. The mammalian enzyme contains 13 polypeptide subunits, two iron centers, heme *a* and heme a_3, and two copper centers, Cu_A and Cu_B. Subunit I of COX contains the heme *a* and the oxygen-binding site, which is composed of the heme a_3 and Cu_B. The binuclear Cu_A center is contained within subunit II of the complex. (*D*) H^+-ATP synthetase (complex V, EC 3.6.1.34) uses the $\Delta\tilde{\mu}_H+$ to produce ATP from ADP and phosphate. The enzyme consists of several polypeptide subunits that are encoded both in the cell nucleus and in mitochondria. The part of the enzyme that is located in the mitochondrial matrix forms soluble ATPase or so-called F_1-ATPase; if separated from the rest of enzyme, it can hydrolyze ATP. F_1-ATPase is connected by the stalk to the membrane-buried part, which is termed F_0. This part participates in proton conduction. For more details, two well-written reviews are recommended (87, 88).

plants. In isolated beef heart or liver mitochondria, the median inhibitory concentration (IC_{50}) for rotenone is 0.07 nmol/mg of protein with a K_i of 4 nM. The most powerful and specific inhibitor is rolliniastatin-1, which belongs to the family of acetogenins produced by Annonaceae plants. For complex I, it has an IC_{50} of 0.03 nmol/mg of protein with a K_i of 0.3 nM (5).

Although complex I is present in all eukaryotic organisms possessing mitochondria and in many bacteria, the structure of this enzyme and its sensitivity toward inhibitors is vastly different in different species. In general, insect and fish mitochondria are the most sensitive to complex I inhibition whereas plant and fungi mitochondria are fairly resistant. In mammalians, neuronal mitochondria tend to be most sensitive to inhibitors of complex I.

Inhibitors of Complex III Complex III (bc_1-complex, ubiquinol:cytochrome *c* oxidoreductase, EC 1.10.2.2) is the second membrane-spanning, proton-translocating complex of the mitochondrial electron transport chain. The mechanism of proton pumping is not clear, however the overall reaction sequence in this segment of the respiratory chain is well understood (9–11) (Figure 1*B*). The minimal sufficient structure of the bc_1-complex consists of only three subunits, as in bacteria *Paracoccus denitrificans,* and there is considerable variability in the structure of complex III in mitochondria from different species. The sensitivity of various species to a particular inhibitor of the bc_1 complex also varies greatly (12–16), which allows relatively safe practical application of some of these compounds as fungicides and as antimalarial, antiprotozoan, and anticancer drugs (17–24).

The major inhibitors of the bc_1-complex have been reviewed by von Jagow & Link (25), who classified them in four groups according to the site of action and the part of electron transfer within the bc_1-complex that is blocked by a particular inhibitor. Group I includes compounds of natural origin, such as myxothiazol, strobilurines, and oudemansins. These quinol antagonists contain a β-methoxyacrylate group that resembles part of the structure of ubiquinone. As a result, they block ubiquinol oxidation at center Q_o. Myxothiazol, which is produced by the myxobacterium *Myxococcus fulvus,* is the most tightly binding and potent inhibitor. In beef heart mitochondria, 0.58 molecule of myxothiazol per bc_1-complex produces 50% inhibition of respiration (26).

Group II inhibitors also resemble ubiquinone in that they contain a 6-hydroxyquinone fragment as a common structural element. They block electron transfer between the Rieske Fe_2S_2 center and cytochrome *c*1, thereby inhibiting the reduction of cytochrome b_L. This group includes undecylhydroxydioxobenzothiazole (UHDBT), undecylhydroxynaphtoquinone (UHNQ), and similar compounds (25).

Group III includes inhibitors acting at center Q_i. The antibiotics antimycin A, funiculosin, and quinolones such as heptylhydroxyquinoline-N oxide (HHQNO) inhibit electron transfer from heme b_H to a quinone or semiquinone molecule bound at center Q_i. The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) may

also belong to this class of inhibitors of complex III (27). The most frequently used inhibitor of this group is antimycin, which is produced by various *Streptomyces* species and has the highest affinity for the bc_1-complex among all the other inhibitors ($K_d = 3.2 \times 10^{-11}$ M). The structural factors required for inhibition were studied with synthetic antimycin analogues (28). Mitochondria are not the only targets of antimycin in a cell, however. Antimycin also inhibits peroxisomal β-oxidation by inhibiting acyl-coenzyme A oxidase (29) and interferes with thyroid hormone transport in the cell nucleus (30). However, all such effects require much higher concentrations of antimycin than are necessary to completely inhibit mitochondrial respiration.

In mitochondria, inhibition of the respiratory chain by group III inhibitors is not the only harmful effect. The generation of superoxide and hydrogen peroxide (reactive oxygen species) contributes to both the cellular and tissue toxicity of antimycin, funiculoside, and HHQNO (31–33). The mechanism of reactive oxygen species generation consists of one-electron reduction of oxygen by a semiquinone, which is normally formed at center Q_o but which is rapidly oxidized by b_L:b_H (see above). In the presence of group III inhibitors, the transfer of electrons from b_H to center Q_i is blocked, which increases the probability of one electron reduction of molecular oxygen by the ubisemiquinone at center Q_o (34).

Two more classes of complex III inhibitors of toxicological importance should be considered, namely substituted phenols, including those with uncoupling capability, and metal cations. Many phenolic uncouplers partially inhibit complex III. This can be easily observed in experiments with isolated mitochondria; a curve of the dependence of respiration rate on uncoupler concentration is typically bell-shaped both with NADH-dependent substrates and with succinate (in the former case because of the inhibition of both complex I and III). The structure-activity relationships of inhibition of the bc_1-complex by phenolic uncouplers and by a series of substituted nitrophenols have been studied (35); however, no simple conclusions were made.

Zinc ions are also known to inhibit mitochondrial electron transport at complex III (36, 37). Zn^{2+} (5 μM; $K_i = 10^{-7}$ at pH 7.0) induces practically complete inhibition of activity of the bc_1-complex isolated from beef heart mitochondria; zinc ions bind reversibly and with high affinity to a single site that Link & von Jagow (37) suggested is part of the proton channel at center Q_o. With isolated mitochondria, 2 μM of Zn^{2+} inhibits succinate:O_2 activity by 40%, whereas more than 400 μM is required to achieve 90% inhibition. Among 20 other di- and trivalent metal cations tested, Hg^{2+}, Ag^{+}, Cu^{2+}, and Cd^{2+} were all found to be inhibitory of complex III, but less effective (37).

Inhibitors of center Q_i such as antimycin, funiculosin, and HQNO (hydroxyquinoline-N-oxide) are specific for the bc_1 complex, whereas naturally occurring center Q_o inhibitors, which possess ubiquinone-like structure, are less specific and inhibit complex I as well (38, 39). It should be noted, however, that the concentrations at which Q_o center inhibitors affect complex I activity are substantially higher than is necessary to completely block the bc_1 complex (39).

Inhibitors of Complex IV, Cytochrome c Oxidase Complex IV, cytochrome *c* oxidase (COX) (EC 1.9.3.1) is a heme/copper terminal oxidase that uses cytochrome *c* as electron donor (Figure 1*C*). According to the classification by Nicholls & Chance (40), COX inhibitors fall into four categories: (*a*) heme-binding inhibitors that are noncompetitive with both O_2 and cytochrome *c* (e.g. azide, cyanide, and sulfide), (*b*) inhibitors competitive with oxygen, such as carbon monoxide (CO) and nitric oxide (NO), (*c*) inhibitors competitive with cytochrome *c* (polycations); and (*d*) noncompetitive inhibitors not affecting the heme groups, such as phosphate ions and alkaline pH.

Noncompetitive Heme-Binding Inhibitors Cyanide and azide are the oldest known and the most frequently used inhibitors of COX. Both react with heme a_3 noncompetitively with oxygen [for a review, see Nicholls & Chance (40)]. Contrary to the wide-spread belief, cyanide is neither a specific nor a selective inhibitor of COX. It inhibits other heme-containing enzymes (e.g. peroxidases, cytochrome *c*) with the same or even greater potency. The pattern of inhibition of mitochondrial respiration by azide is unique in that state 3 respiration is much more sensitive to inhibition ($IC_{50} \sim 60$ μM) than state 4 respiration ($\geq$300 μM azide), and the inhibition of state 3 can be released by various protonophoric uncouplers (41). These features can be explained by the well-known inhibitory effect of azide on ATP synthase and uncoupling of oxidative phosphorylation.

Hydrogen sulfide (H_2S) is a naturally occurring toxic compound [the toxicity of sulfide has been reviewed (42)]. Inhalation of high concentrations (50–400 ppm) of gaseous H_2S inhibits COX activity in mitochondria of rat lungs both in vivo and ex vivo (43). Brain cytochrome *c* oxidase activity was reported to be particularly sensitive to inhibition (IC_{50} for $H_2S = 0.13$ μM) (44). Like cyanide, sulfide is not a specific inhibitor of COX; it inhibits other hemoproteins as well (e.g. superoxide dismutase, glutathione peroxidase, glutathione reductase, and catalase (45)).

Formic acid (HCOOH) is a natural by-product of metabolism of methanol, methyl ethers, esters, and amides. Its toxicity is due primarily to inhibition of mitochondrial respiration resulting in histotoxic hypoxia [for a review, see Liesivuori & Savolainen (46)]. Formate inhibits COX with a K_i depending on the degree of oxidase reduction and varying from 30 mM (100% reduction) to 1 mM (100% oxidation) at pH 7.4. The apparent affinity increases with acidification. The formic acid (HCOOH) molecule binds to ferric heme iron of cytochrome a_3, thus preventing its reduction by cytochrome *c*. In isolated mitochondria, formate also inhibits succinate-–cytochrome *c* reductase activity in a reaction competitive with succinate.

Hydroxylamine (NH_2OH), a naturally occurring product of cellular metabolism, exerts various toxic effects [some of the biological activities of hydroxylamine are reviewed elsewhere (47)]. At millimolar concentrations, hydroxylamine inhibits mitochondrial respiration with an azide–like pattern, state 3 being par-

ticularly sensitive. Hydroxylamine can form complexes with copper and iron, and it also can generate NO, a potent COX inhibitor.

O2 Competitive Inhibitors Inhibition of COX by CO or NO is competitive toward oxygen. Binding of CO to cytochrome oxidase is reversible; dissociation of the complex is strongly promoted by light. There are several reports indicating the significance of CO inhibition of COX in vivo (48, 49). CO–induced inhibition of COX could also be a mechanism of toxicity of some xenobiotics, such as dichloromethane (methylene chloride), which liberates CO as a product of its metabolism by cytochrome P4502E1 (50).

NO is a naturally occurring metabolite that exerts a number of important biological activities. It has long been known that NO binds reversibly to COX. However, the inhibition of mitochondrial respiration by NO was shown only recently (see 51 and references therein). This inhibition shows competition with oxygen, the K_i being lower at low oxygen concentrations. Although the exact mechanism of inhibition is not clear, it probably involves the reaction of NO with oxidized Cu_B of the COX binuclear center, leading to the reduction of this metal center and formation of nitrite.

Cytochrome c Competitive Inhibitors Polycations represent a different type of inhibition of COX activity that does not involve direct binding to heme. High-molecular-weight polylysine, histones, cationic lysosomal proteins, protamine, and some other high-molecular-weight synthetic and natural polycations are known to inhibit mitochondrial respiration as well as isolated COX activity (52). The inhibition is competitive toward cytochrome *c*. These compounds bind to anionic sites of mitochondrial membranes and COX with high affinity, thus decreasing the mobility of the cytochrome and/or preventing the proper orientation of the cytochrome *c* molecule toward its reduction and oxidation sites. A polycationic type of inhibition was also suggested for the inhibitory effect of adriamycin–Fe^{3+} complexes, where COX was competitively inhibited by the complex with a K_i of 12 μM, whereas free adriamycin was without effect (53).

Other Inhibitors Local anesthetics such as dibucaine, lidocaine, and tetracaine are widely used in clinical practice. In vitro, these compounds inhibit mitochondrial respiration at the level of COX. Because of the correlation between hydrophobicity and inhibitory potency, it is proposed that this activity reflects the nonspecific interaction of anesthetics with the lipid phase in which COX is embedded (54). Others (55), however, have shown that dibucaine inhibits the oxidase activity by interacting with cytochrome *a* and its associated copper. However, significant inhibition is observed only at millimolar concentrations of anesthetics.

Hydrophobic metal chelators inhibit COX activity as well, most probably by chelating a copper atom of the binuclear center. The inhibition occurs at relatively

low concentrations; bathocuproine at 14 μM inhibits oxidase activity by 75% both in mitochondria and in solubilized enzyme (56). However, the physiological significance of this class of inhibitors is not clear.

Psychosine (galactosylsphingosine), a cytotoxic lipid that is accumulated in brain cells of some animals and humans with Krabbe disease, is a powerful inhibitor of complex IV. In vitro, it produces 50% inhibition at concentrations of 0.1 μg/mg of mitochondria (57).

Recently, it was found that 4-hydroxynonenal, a major product of lipid peroxidation, can inhibit COX activity. This establishes a potential direct link between oxidative stress and inhibition of respiration, which may represent a primary mechanism of oxidative stress–induced damage to mitochondria (58).

Inhibitors of ATP-Synthetase

H^+-ATP synthetase (complex V, EC 3.6.1.34) uses the $\Delta\tilde{\mu}_H+$ to synthesize ATP from ADP and phosphate (Figure 1*D*). It is the major source of ATP in aerobic cells [for recent reviews, see Junge et al (59), Boyer (60)]. The enzyme is reversible. Under some conditions it can work as an ATPase to hydrolyze ATP and generate $\Delta\tilde{\mu}_H+$. Evolutionarily, the mitochondrial ATP synthetase is a well-conserved protein, and it is not surprising that many of its known inhibitors are of natural, mostly fungal, origin. These antibiotics were intensively searched for, isolated, and studied because of their selective and potent toxicity against other fungi. Examples of mycotoxins possessing significant ATPase inhibiting activity are the aurovertins A-E, leucinostatins A and B, venturicidin and ossamycin, efrapeptin, and the classic inhibitors of mitochondrial ATPase, oligomycins A-D. Consumption of rice contaminated with the mycotoxin citreoviridin, which also inhibits mitochondrial ATPase, causes symptoms of acute cardiac beri-beri (convulsions, vomiting, ascending paralysis, and respiratory arrest) in humans and experimental animals. Injection of oligomycin to rats causes a marked inhibition of oxygen consumption and severe lactic acidemia with no change in arterial pO_2 (61). The mechanisms of inhibition of ATPase by the mycotoxins involve binding the F^1 subunit or to the F^0 subunit of the enzyme to block proton conduction. With isolated mitochondria, the range of IC_{50} values for ATPase inhibition for all mycotoxins is 0.1–5.0 nmol/mg of protein (62–64). The acute 50% lethal dose (LD_{50}) values (intraperitoneally, and subcutaneously) are on the order of 1–10 mg/kg in rats and mice (64–67).

Although mycotoxins are the most powerful inhibitors of mitochondrial ATP synthetase, many other compounds and classes of compounds share this same activity. Examples include naturally occurring flavonoids (68), a commonly used beta-adrenergic receptor antagonist propranolol (69), local anesthetics (70), the herbicide paraquat (71), several pyrethroid insecticides (72) and possibly DDT and parathion (73, 74), diethylstilbestrol (75), several cationic dyes (76), and organotin compounds (64, 77).

Uncouplers of Oxidative Phosphorylation

A wide variety of compounds indispensable to our everyday activities are uncouplers of mitochondrial oxidative phosphorylation. The most abundant are compounds used as drugs or pesticides. Uncoupling activity is considered to be a common characteristic of antiinflammatory agents with an ionizable group (78); nonsteroidal antiinflammatory drugs (diclofenac, aspirin, nimesulide, meloxicam, piroxicam, and indomethacin) exert an uncoupling effect both in isolated mitochondria and in perfused rat liver at concentrations that correspond to the pharmacological doses employed in antipyretic and antiinflammatory treatments (79, 80). Antipsychotic and antidepressant drugs (81, 82), some of the antitumor drugs (83, 84), a number of plasticizers, lipid-lowering drugs and other peroxisome proliferators (85–88), antimycotics (89, 90), drugs used to treat trypanosomiasis and leishmaniasis (91), numerous antihelmintics (92–94), antispermatogenic drugs (95), agents that are implicated in causing Reye's syndrome (96), and various herbicides and insecticides (27, 97–99) are all reported to uncouple isolated mitochondria.

In the mitochondria-related literature, the term uncoupler is traditionally suffixed with the words ''of oxidative phosphorylation,'' which emphasizes the impact of these compounds on mitochondrial ATP production. Uncouplers are compounds that decrease the efficiency of ATP production. Unfortunately, such a definition implicitly blurs the fundamental fact that all of the other known energy-dependent metabolic functions of mitochondria are equally affected by all of the known uncouplers of oxidative phosphorylation (perhaps, with the only exception being the so-called decouplers, which are not considered here due to the highly debatable nature of the subject). In this treatise, the term uncoupling is taken to mean any energy-dissipating process competing for energy with routine mitochondrial functions, thus inducing a metabolically futile wasting of energy. This then explains the hyperpyresis that is characteristic of intoxication with mitochondrial uncouplers. Under such a definition, any xenobiotic-induced enhancement of any energy-consuming mitochondrial function (such as ion, metabolite, or protein transport across the inner membrane) would also be considered uncoupling.

The following sections review some of the properties of representative uncouplers of different chemical classes that we believe to be most important to the fields of pharmacology and toxicology on the basis of abundance and use characteristics (Figures 2–5). Critical reviews are cited where available, which can be consulted for more detailed information on a particular class of uncouplers. Some additional types of uncouplers and uncoupling mechanisms are listed in Figure 2.

Lipophilic Weak Acids The majority of compounds possessing protonophoric activity are lipophilic weak acids (Figure 2) with a pK_a in the range of 5–7. Generally, structural requirements for uncoupling activity include the presence of

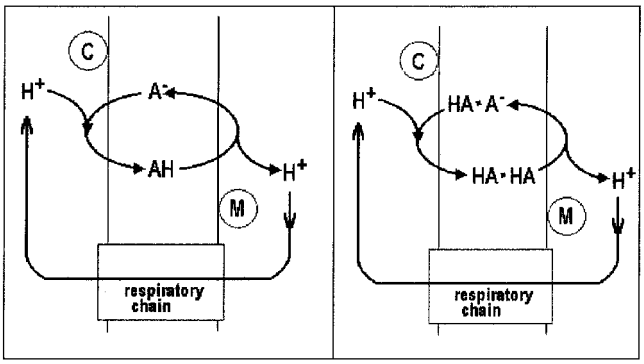

Figure 2 Lipophilic weak acids: proton shuttling. All the known uncouplers of this type can selectively increase the permeability of natural and artificial lipid membranes to protons. Numerous studies with artificial bilayer membranes reveal two major mechanisms of proton translocation. According to the first model, a molecule of a lipophilic weak acid (HA) penetrates the lipid core of the membrane both in the protonated H form and in the ionized A^- form. This model describes the proton shuttling mechanism of potent uncouplers such as FCCP, CCCP, S-13, and SF6847. In mitochondria, an undissociated uncoupler molecule dissolves in the lipid of the inner mitochondrial membrane, crosses it, and releases the proton into the mitochondrial matrix (M), which is slightly more alkaline than the external medium. This discharges the ΔpH; in turn, the ionized negatively charged molecule diffuses across the membrane, down the gradient of electric field, and discharges the membrane potential. At the cytoplasmic side of the membrane (C), it can again bind a proton and complete the cycle. The second model of proton shuttling differs in that the charged molecule of an uncoupler crosses the membrane as a HA_2^- dimer, a complex of the protonated (HA) and anionic (A^-) forms of the weak acid. This mechanism describes the proton translocation by uncouplers similar to 2,4-dinitrophenol and substituted benzimidazoles. Both mechanisms result in the net transport of protons catalyzed by cyclic movement of an uncoupler molecule, which dissipates $\Delta\tilde{\mu}_H+$ [for a review, see McLaughlin & Dilger (101)].

an acid-dissociable group, bulky lipophilic groups, and a strong electron-withdrawing moiety [for reviews, see Terada (100), McLaughlin & Dilger (101)]. These properties determine the important features of proton-transporting lipophilic weak acids that affect their uncoupling efficiency, such as the solubility in lipid membranes, the stability of the ionized form in the membrane, and the ability to release and bind a proton. The most representative uncouplers of this class are substituted phenols, trifluoromethylbenzimidazoles, salicylanilides, and carbonyl cyanide phenylhydrazones.

2,4-Dinitrophenol and Other Substituted Phenols Substituted phenols are the best represented and studied class of mitochondrial poisons. Some of these compounds, such as 2,4-dinitrophenol (DNP) have a long and curious history. There were times when DNP was considered to be a miracle drug, a new hope in an

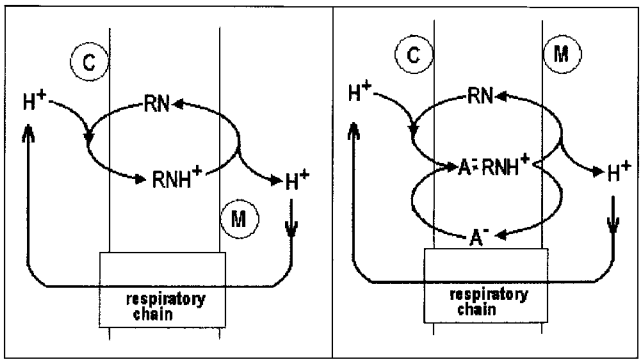

Figure 3 Local anesthetics: lipophilic ion pairs. Uncoupling by a mechanism involving an electrophoretic H^+ uniport does not necessarily require the compound to be a protonophore. A concerted action of some compounds exerting no protonophoric activity can significantly increase membrane permeability to protons. Many pharmacologically active amines with local anesthetic properties are known to exert various effects on mitochondria, including uncoupling (see 102 and references therein). It was shown that uncoupler-like activity of amine local anesthetics could be fully explained by their ability to form lipophilic ion pairs with certain anions. Electrophoretic H^+ uniport and uncoupling results from transmembrane cycling of neutral amine, charged anion, and neutral ion pair (102, 103). More lipid-soluble local anesthetics, such as bupivacaine, can uncouple mitochondria even in the absence of pair–forming lipophilic anions (103). General anesthetics: changes in fluidity of the inner membrane. Millimolar concentrations of the general anesthetics chloroform and halothane inhibit ATP synthesis in rat liver mitochondria, stimulate mitochondrial ATPase activity, and reduce the respiratory control and ADP:O ratio. The same concentrations of halothane and chloroform increase the fluidity of the inner mitochondrial membrane (104). An increase in membrane fluidity potentiates the intrinsic proton permeability of lipid bilayer, the so-called proton leak. In addition, it may affect the functioning of proteins embedded in the membrane, such as ATP synthetase and proton pumps, decreasing the degree of coupling between electron transport and proton pumping, thus decreasing overall efficiency of energy conservation.

everlasting fight of humankind against obesity. These hopes died in the 1930s along with those unlucky patients who had received the ''miracle drug'' (cf 119). However, the wide-spread use of DNP and compounds such as the phenolic herbicides and insecticides has sustained the interest in this mode of toxicity. In man, the syndrome of DNP poisoning ''consists of lassitude, malaise, headache, increased perspiration, thirst, and dyspnea which may progress to hyperpyrexia, profound weight loss, respiratory failure, and death'' (120). In mice, the LD_{50} for DNP is 141 μmol/kg (121). The uncoupling efficiency of substituted phenols (expressed as the concentration inducing 50% uncoupling) varies from 30–100 μM for relatively inefficient uncouplers like dicoumarol and DNP, to 5–10 nM for SF6847 [2,6-di-tert-butyl-4-(2',2'-dicyanovinyl)phenol]. The latter compound is the most powerful uncoupler known, and the ''turnover number'' of the SF6847

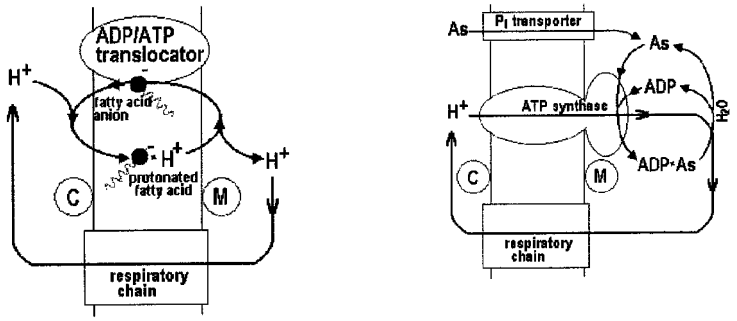

Figure 4 Long-chain free fatty acids: protein–mediated uncoupling. The phenomenology of long-chain free fatty acids–induced uncoupling resembles that of classical protonophores such as 2,4-dinitrophenol. The protonophoric action requires that fatty acids (FAs) cross the membrane in both the protonated and the anionic forms. Protonated long-chain FAs are sufficiently hydrophobic and easily penetrate lipid membranes. However, in a deprotonated form they cannot cross the hydrophobic barrier because of the strong negative charge. The mechanism of apparent protonophoric action of FA remained a mystery for about 40 years [for a review, see Wojtczak & Schonfeld (105)]. Recently, it was shown that ATP/ADP translocase, an integral protein of the inner mitochondrial membrane, facilitates FA anion transport across the hydrophobic core of the inner membrane, thus allowing net H^+ cycling (106). It has also been suggested that the aspartate/glutamate antiporter, which is another membrane protein of the same family, participates in FAs–induced uncoupling (107). Uncouplers acting at the level of ATP synthetase. It has long been known that arsenate uncouples oxidative phosphorylation and releases state 4 respiration of isolated mitochondria, the effects being completely inhibited by oligomycin (108). The sensitivity to this highly specific (109) antibiotic and the well–known ability of arsenate to participate in phosphorylation reactions points to the involvement of ATP synthase in the mechanism of arsenate's action. It was shown that arsenate uncouples oxidative phosphorylation by a mechanism involving intramitochondrial synthesis of ADP-arsenate, followed by its rapid nonenzymatic hydrolysis (110). The formation of ADP-arsenate in an ATP synthetase–catalyzed reaction at the expense of the protonmotive force and its rapid hydrolysis establishes a futile energy-dissipating cycle in the matrix of a mitochondrion. This cycle abolishes energy conservation and turns normally functioning ATP synthetase into an energy-burning furnace. Strictly speaking, it is not correct to term this kind of energy-dissipating mechanism as uncoupling. The oxidation of substrates is well coupled with phosphorylation, but the product of the phosphorylation is unstable and cannot serve to conserve energy. However, we use the term uncoupling to emphasize the futile nature of this pathway.

molecule in mitochondrial membranes is close to the theoretical maximum according to Brownian motion of the uncoupler molecule (122). Structure-activity studies with various substituted phenols (123–125) confirmed the protonophoric mechanism of their action in mitochondria and revealed important correlations between the uncoupling activity and physicochemical properties such as hydro-

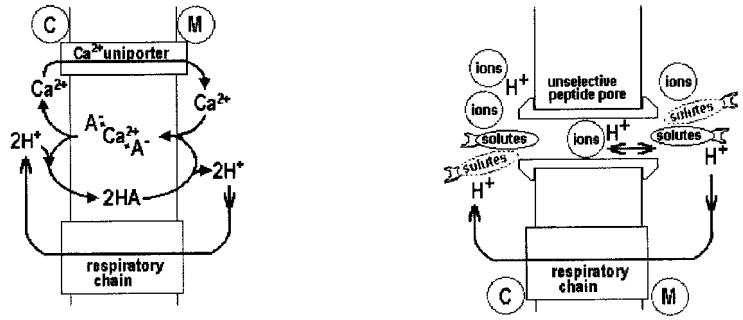

Figure 5 A23187-mediated uncoupling: Ca^{2+} cycling and other mechanisms. The mechanism consists of A23187-mediated ΔpH–dissipating release of cations from the organelle followed by reuptake of Ca^{2+}, which is mediated by an electrogenic Ca^{2+} uniporter of the inner mitochondrial membrane. The uptake of Ca^{2+} by the uniporter decreases the membrane potential, thus completing the energy-dissipating cycle (111). Another mitochondria-specific mechanism of A23187-mediated uncoupling is related to the ability of this ionophore to bind and transport Mg^{2+}. It was reported that A23187 can severely deplete mitochondria of endogenous Mg^{2+}. In the presence of physiological concentrations of K^+, the Mg^{2+} depletion activates a latent K^+ uniport pathway in the mitochondrial membrane. This, in turn, induces electrogenic uptake of potassium ions, which is accompanied by membrane depolarization and stimulation of respiration (112, 113). The protein conferring the K^+ uniport activity was not identified; however, the participation of Ca^{2+} uniporter was proposed (114). The Me^+ uniport pathway activated in mitochondria by Mg^{2+} depletion can be a more universal mechanism of divalent ionophore–induced uncoupling. Another *Streptomyces* antibiotic, oleficin, was shown to uncouple liver mitochondria, the features being similar to the divalent cationophoric mechanism of A23187–induced K^+ uniport (115). A23187–mediated uncoupling has been demonstrated in isolated mitochondria and may also occur in situ, in tissue, and in isolated cells. However, taking into account the high concentrations of Ca^{2+} in biological fluids and the high activity of the mitochondrial Ca^{2+} uniporter, it is probable that exposure to A23187 specifically uncouples mitochondria in situ even without interacting directly with organelles. Flooding of cellular cytosol with Ca^{2+} would stimulate continuous electrogenic uptake of the cation into mitochondria, which will compete with other energy-dependent processes and induce osmotic swelling and damage of organelles. Membrane-active peptides. Various short peptides (especially of fungal origin) are known to increase the conductance of lipid bilayer membranes. Such peptides are usually amphipathic, 15–20 amino acids long, and enriched in α-aminoisobutyric acid. They form channels of various sizes in bilayer lipid membranes, thus inducing high permeability to normally impermeable ions and solutes. Alamethicin is the most widely studied peptide of this class; it forms voltage-gated pores and exerts numerous biological activities (116, 117). Structural requirements for uncoupling activity were studied with synthetic derivatives of alamethicin A. It was shown that a minimum peptide chain length of 13 residues is necessary for uncoupling activity. Peptide esters were more potent than the corresponding acid forms, and in general the structural requirements for uncoupling activity were similar to those for ionophoretic activity in liposomes (118).

phobicity, acidity, and the stability of ionized intermediate in the lipid phase of a membrane [for a review, see Terada (122)].

Trifluoromethylbenzimidazoles 2-Trifluoromethylbenzimidazoles (TFBs) were introduced in the early 1960s as a new class of potent herbicidal and insecticidal compounds. The high toxicity of these compounds to animals was immediately evident, and much effort has been aimed at the synthesis of derivatives in an attempt to obtain an ''acceptably safe and yet active herbicide'' (126). In mitochondria, many of these compounds act similar to DNP (Figure 2); however, their efficiency is significantly higher. The most active uncoupler of this class is 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), which produces 50% uncoupling of oxidative phosphorylation (measured as ATP synthesis) at 8 x 10^{-8} M in isolated liver mitochondria (127) (compared with 8×10^{-6} M for DNP under the same experimental conditions). Similar to other acidic uncouplers, the efficiency of TFB derivatives increases with the acidity of the dissociable -NH- group (128, 129).

The toxicity of a number of substituted TFBs in mice, houseflies, and honeybees was compared with their uncoupling efficiency in isolated mitochondria (130). In this study, the LD_{50} in mice for a number of TFBs and other uncouplers, such as salicylanilide, dinitrophenol, and phenylhydrazone type, was plotted against their uncoupling activity in isolated mouse liver and brain mitochondria. A reasonably good correlation was obtained; generally, the toxicity increased with the increase in uncoupling efficiency. In the same study, it was also found that with some of the TFB derivatives, brain mitochondria were three to five times more sensitive than liver mitochondria. The principal manifestations of toxicity of injected TFBs were dyspnea, occasional salivation, weakness, and death in an extended position with immediate rigor mortis (126, 130), which is typical for other mitochondrial uncouplers as well.

Salicylanilides Salicylanilide (2-hydroxy-N-phenylbenzamide) derivatives have been shown to possess bacteriostatic, fungicidal, and molluscicidal activity; some of the members of this class are widely used as anticestodal, antitrematodal, and antihelmintic drugs. Many of these chemicals are protonophoric uncouplers of mitochondrial oxidative phosphorylation (127, 128). The most efficient uncoupler of this class is S-13, 2',-5-dichloro-4'-nitro-3-tert-butyl-salicylanilide, which induces complete uncoupling at only 0.2 molecule per cytochrome oxidase heme *aa*3 (which is about 42 pmol of uncoupler per mg of mitochondrial protein). The mechanism of the uncoupling action is similar to that of other A^- protonophores (129). Structure-activity relationships with 28 derivatives substituted at both the salicylic acid moiety and the aniline moiety revealed that both hydrophobicity and electron-withdrawing power were necessary for uncoupling activity (130).

Carbonyl Cyanide Phenylhydrazones Carbonyl cyanide *p*-trifluromethoxyphenyl-hydrazone (FCCP) and carbonyl cyanide *meta*-chlorophenylhydrazone

(CCCP) are probably the most frequently used uncouplers in experimental biology. In mice, FCCP and CCCP induce rapid rigor mortis at death after intraperitoneal injection (131, 132), with LD_{50}s of 32 and 40 μmol/kg, respectively. These compounds were introduced in the early 1960s by Heytler & Prichard (133, 134) and since that time have remained among the most powerful commercially available protonophoric uncouplers. The uncoupling activity of these phenylhydrazones correlates well with their protonophoric activity (135), and the requirements for maximal uncoupling efficiency are a lipophilicity (octanol/buffer partition coefficient) above 2×10^3 and a pKa (in diluted aqueous solution) between 4.5 and 5.5 (136).

The protonophoric activity, however, may not be the only mechanism by which these compounds interact with mitochondria. FCCP can form stable complexes with other mitochondria-active compounds, such as lipophilic amine local anesthetics and K^+-valinomycin. FCCP, CCCP, and other carbonyl cyanide phenylhydrazone ring-substituted analogs readily react with mitochondrial thiols and aminothiols (cysteine, glutathione), yielding corresponding N-(substituted phenyl)-N'-(alkylthiodicyano)-methylhydrazine derivatives. The reactivity of carbonyl cyanide phenylhydrazone with thiols is comparable to the reactivity of phenyl isothiocyanate and N-ethylmaleimide (134, 137), well-known SH-group modifiers. Thiols are known to protect and reverse the FCCP uncoupling effects in mitochondria (138) in vitro.

Other Types of Uncouplers

Protein–Mediated Uncoupling by Free Fatty Acids Endogenous and exogenous long-chain free fatty acids (FFA) (Figure 4) have long been known to be efficient uncouplers of mitochondrial oxidative phosphorylation both in situ (e.g. in perfused liver) and in vitro (105). Studies over the past decade reveal that adenine nucleotide translocase, an integral protein of the inner mitochondrial membrane, is involved in the uncoupling action of long chain FFAs (106). Adenine nucleotide translocase-–mediated uncoupling of mitochondria represents an important mechanism of FFA toxicity because many pathologies are associated with the accumulation of free long-chain FFA in the affected tissues. It should be expected that an increase in FFA would preferentially affect mitochondrial energetics, wasting energy at first and inhibiting respiration at higher concentrations. In mitochondria-rich tissue, the effect of excessive FFA accumulation can be devastating, strongly increasing local temperature and oxygen consumption at the expense of oxidizable substrates [e.g. as is the case in malignant muscle hyperthermia (139)]. Other fatty acid–like compounds, such as perfluorodecanoic acid, sulfuramide, and methyl-substituted hexadecanedioic acid, uncouple mitochondria apparently by the same mechanism (88, 140, 141).

Ionophores Ionophores are compounds of various chemical structures that are capable of transporting small ions across a lipid membrane. There are hundreds of such compounds of both natural and synthetic origin. Physical and chemical properties of various ionophores, mechanisms of ion transport, and their numerous biological activities have been thoroughly reviewed (e.g. see 142, 143). Here, we mention only the most general properties of ionophores that render them efficient uncouplers of mitochondrial oxidative phosphorylation.

Ionophores can be divided into two general groups based on the mechanism of ion transport: a carrier type or a channel type. The latter is typical of short amphiphilic peptides that form channels of various sizes in lipid membranes (Figure 5). These channels can be selective toward small ions such as protons or K^+, depending on the particular peptide, the membrane structure, and the experimental conditions. Gramicidins (gramicidin A, D, and S) are the most studied antibiotics of this type and are powerful uncouplers of energy transduction in mitochondria. The short, linear gramicidins A and D assume helical conformation to form a channel whereas the cyclic gramicidin S forms a β-sheet structure, which disturbs lipid packing in the membrane. The resulting increase in membrane permeability toward protons and K^+ efficiently collapses $\Delta\tilde{\mu}_H+$ in mitochondria, due either to proton cycling or to electrophoretic K^+ transport (144, 145).

Carrier–type ionophores are able to form neutral or charged lipid-soluble complexes with an ion to facilitate its electrophoretic transport (uniport) or electroneutral exchange with protons (antiport) across a hydrophobic membrane. Depending on the chosen experimental conditions, these ionophores may or may not uncouple isolated mitochondria in vitro; Me^+/H^+ exchangers like nigericin can, by collapsing the ΔpH, actually hyperpolarize mitochondrial membranes. However, under in vivo conditions, alkali cations such as K^+ and penetrating anions such as inorganic P_i are present in high concentrations and mitochondrial membranes are energized to approximately 180 mV. This means that electrophoretic transport of a cation such as K^+ facilitated by valinomycin or a similar ionophore will proceed down the gradient of membrane potential, collapsing $\Delta\tilde{\mu}_H+$ and uncoupling the mitochondria. Moreover, because of the presence of P_i and the P_i/H^+ symporter in mitochondrial membranes, a significant amount of K^+ can be accumulated, which will induce osmotic swelling of the organelle and physical damage to the coupling membrane. This phenomenon is evidenced by the common experimental use of valinomycin to selectively eliminate mitochondria from cultured cells.

At high concentrations, even electroneutral Me^+/H^+ ionophores can uncouple mitochondria. For example, the K^+/H^+ exchanger nigericin at concentrations higher then 1 μM increases the conductance of black lipid membranes by forming a mobile dimer with both molecules of nigericin protonated and complexed with one K^+. If formed in the mitochondrial membrane, such a charge-transferring complex can indeed cause uncoupling due to electrophoretic cation transfer (146).

Ionophores that are selective to Ca^{2+} or Mg^{2+} can uncouple mitochondria by several different mechanisms. Some of these mechanisms are very specific

because they involve intrinsic mitochondrial proteins, as with the divalent cation ionophore A23187 (calcimycin). This carboxylic polyether antibiotic is produced by *Streptomyces chartreusensis.* It forms a lipid-soluble 2:1 ionophore:Me^{2+} complex and transports divalent cations across lipid membranes by means of electroneutral $Me^{2+}/2H^{+}$ exchange. The induction of such cation transport per se cannot uncouple mitochondria. However, by dissipating the calcium gradient, A23187 establishes a futile, energy–dissipating cyclic flux of Ca^{2+}, which is responsible for the uncoupling activity of this compound (Figure 5).

Some of carrier–type ionophores are far more efficient in mitochondrial membranes than in other cellular membranes. For example, the valinomycin-K^{+} complex turnover number is about 100 times higher in mitochondrial membranes than in erythrocytes. There is also evidence that valinomycin and nigericin increase the ionic conductivity of the inner mitochondrial membrane, but not that of the plasma membrane of intact lymphocytes (147) or yeast cells (148). What factors besides membrane potential may contribute to this membrane selectivity have yet to be identified.

Cationic Uncouplers Several cationic compounds uncouple mitochondria by increasing membrane permeability to ions (Figure 3). The uncoupling action of compounds such as cyanine dye tri-S-C_4(5), Cu^{2+}-(*o*-phenanthroline)$_2$ complex, and pentamidine requires the presence of inorganic phosphate and can be efficiently prevented by inhibiting the mitochondrial P_i:H^{+} symporter. The molecular mechanism of uncoupling action of these compounds is obscure; however, it most likely is that all such P_i-dependent compounds affect the physical integrity of mitochondrial membranes. Indeed, it was recently shown that tri-S-C_4(5) and Cu^{2+}-(*o*-phenanthroline)$_2$ uncouple by inducing the mitochondrial permeability transition pore (149).

Membrane-Active Peptides Membrane-active peptides (Figure 5) can form channels, which are more or less selective to alkaline cations and/or protons, or they can form large pores allowing permeation of high-molecular-weight ($>$100 Da) solutes. Pore-forming peptides such as alamethicin possess a high affinity toward mitochondrial membranes because insertion of the peptide into the membrane and/or pore formation is driven by the electrical potential. These peptides induce nonspecific permeability changes, which results in oncotic swelling and disruption of the charged organelles.

Many short amphipathic peptides (especially of fungal origin) are known to uncouple oxidative phosphorylation in mitochondria at submicromolar concentrations by rendering the inner mitochondrial membrane permeable to various solutes (118, 150, 151). However, membrane–active peptides can also uncouple mitochondria in a much more specific manner. Mastoparan, an amphipathic peptide from wasp bee venom, induces the opening of a Ca^{2+}-dependent cyclosporine A–sensitive mitochondrial permeability transition pore. At higher concentrations, mastoparan depolarizes the mitochondrial inner membrane by act-

ing on the lipid phase with no apparent involvement of the permeability transition pore (152).

Under certain circumstances, positively charged signal peptides, whose normal function is the targeting of newly synthesized proteins to mitochondria, can uncouple mitochondria (153, 154). The effect of the peptides on mitochondrial integrity was shown to be dependent on concentration. At low peptide/mitochondria ratios, signaling peptides induce a gradual lysis of the outer membrane and a release of enzymes from the intermembrane space. At higher peptide/mitochondria ratios, the permeability of the inner membrane increases, leading to complete uncoupling of respiration and dissipation of the membrane potential (155). Signal peptide–induced uncoupling is of great interest and importance for several reasons. These peptides are synthesized inside the cell and they are naturally and selectively targeted to the mitochondria by their very structure. In the case of malfunctioning of the protein import machinery, such peptides may accumulate within mitochondria, discharging the membrane potential and eliminating damaged organelles. Recently, signaling peptides have been explored as prototypes for creating new drugs selectively targeted to mitochondria within the cell (156).

Alternate Electron Acceptors Mitochondrial electron carriers are distributed randomly within the inner membrane, but functionally they are arranged according to their respective redox potential. ''Respiratory chain'' refers to the sequence of electron and proton-transferring reactions. The efficiency of conversion of chemical energy from the oxidation of substrates into a useful form of $\Delta\tilde{\mu}_{H}+$ depends on how precisely electrons follow their ''prescribed'' pathways through the ordered sequence of electron acceptors in the respiratory chain. In all known cases, any alternate pathway of electron transfer greatly diminishes the efficiency of energy conversion, speeding up the oxidation of substrates and diverting the excess energy to wasteful heat production (i.e. uncoupled oxidative phosphorylation). Technically, at least two kinds of alternate electron pathways can be distinguished. The first group are terminal electron acceptors, which are compounds capable of being reduced by an electron carrier of respiratory complex, thereby competing with the natural acceptor for this carrier. These compounds intercept electron flow and divert it toward their own reduction. In theory, these compounds can participate in so-called futile redox cycling, where a compound is reduced by the respiratory chain at the expense of energy derived from substrates and then oxidized back in a side reaction. Side reactions may include direct univalent reduction of oxygen, or reacting with protein thiols or glutathione. In either case, the net reaction is a stimulation of cyanide-insensitive respiration and consumption of both oxygen and reducing substrates with no net metabolism of the parent compound. As with all other uncouplers, this mitochondrial combustion leads to rapid dissipation of all transmembrane gradients, including the $\Delta\tilde{\mu}_{H}+$.

The second group of alternate electron acceptors are so-called electron shunts, which accept electrons and feed them back to the respiratory chain at some higher

redox potential. This allows electrons to bypass a portion of a respiratory complex or a whole segment of respiratory chain, excluding it from energy generation (see Figure 6). In these cases, the secondary electron acceptor that completes the redox cycle is a terminal complex of the mitochondrial electron transport chain, not molecular oxygen. Accordingly, such compounds do not generate oxygen free radicals or stimulate cyanide-insensitive respiration to the same extent as the redox cycling electron acceptors.

There are several examples of compounds that act as redox cycling alternate electron acceptors to uncouple mitochondrial oxidative phosphorylation. They include adriamycin, paraquat, and variously substituted naphthoquinones and N-nitrosoamines (157–159). The essence of their activity is the instability of the univalently reduced free radical intermediate, which under physiological conditions autooxidizes at the expense of reducing molecular oxygen to superoxide anion free radicals. Associated with this is a dramatic stimulation of oxygen

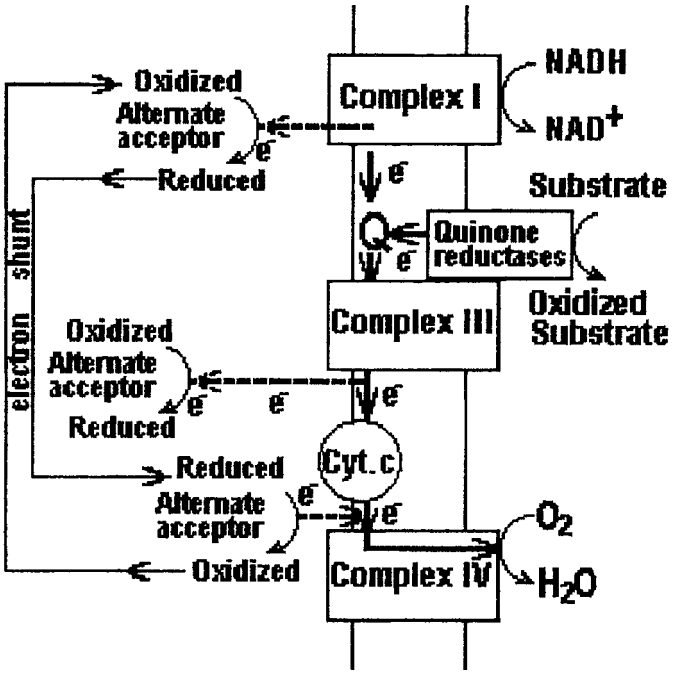

Figure 6 Interaction of alternate electron acceptors with mitochondrial respiratory chain. Alternate terminal electron acceptors are capable of being reduced by an electron carrier of the respiratory complex, thus competing with a natural acceptor of this carrier. These compounds intercept electron flow and divert it toward their own reduction. Electron shunts can be both reduced and oxidized by the respiratory chain, thus allowing electrons to bypass a portion of a respiratory complex or a whole segment of respiratory chain and excluding it from energy generation.

consumption and substrate oxidation, both of which are insensitive to inhibition by cyanide. Such chemicals stimulate the liberation of oxygen free radicals from isolated mitochondria, which most likely accounts for their observed cytotoxicity.

The antineoplastic agent doxorubicin (DXR) (adriamycin) represents a classic example of a compound that redox cycles on the mitochondrial electron transport chain [for a recent, thorough review, see Wallace (160)]. With a redox potential of approximately −320 mV, DXR is a good alternate electron acceptor for the mitochondrial respiratory chain. Incubation of DXR with cardiac mitochondria stimulates state 4 respiration and decreases the respiratory control ratio, as would be expected for an alternate electron acceptor. DXR accepts electrons exclusively from complex I of the mitochondrial respiratory chain to generate the univalently reduced semiquinone free radical intermediate (160, 161). This intermediate is highly unstable and rapidly autooxidizes to the parent quinone at the expense of reducing molecular oxygen to the superoxide anion free radical. Because the rate-limiting step in this redox cycle is the univalent reduction of the parent quinone, the overall reaction is controlled by the redox state of complex I; inhibitors of complexes III and IV, which increase the reduction state of complex I, stimulate the rate of DXR-induced oxygen consumption and free radical production (160, 161). The oxidation of mitochondrial glutathione (162), induction of the mitochondrial permeability transition (163), and cardioselective oxidation of mitochondrial DNA in vivo (164, 165) attest to the importance of mitochondrial redox cycling as a critical pathway in the mechanism of DXR-induced cardiotoxicity (160).

The aromatic amine 2-nitrosofluorene and its reduced hydroxylamine, N-hydroxy-aminofluorene, are metabolites of the carcinogen 2-acetylaminofluorene. 2-Nitrosofluorene is reduced at the level of complex I and complex III, and the reduced N-hydroxy-aminofluorene can be oxidized directly by oxygen to liberate oxygen free radicals, which appear to be central in mediating the pathogenic response (166, 167).

Naphthoquinones, both substituted and unsubstituted, represent another important class of alternate electron acceptors that redox cycle of the mitochondrial respiratory chain. Like DXR, naphthoquinones are reduced by complex I to their corresponding semiquinones, which autooxidize in aerobic solutions to the parent quinone and, in the process, generate superoxide anion free radicals from molecular oxygen. Structure-activity studies indicate that a univalent redox potential of −170 mV to +50 mV is the primary determinant of whether naphthoquinones are good alternate electron acceptors for the mitochondrial respiratory chain (168, 169). Furthermore, the rates of free radical generation correlate with the degree of mitochondrial dysfunction and the extent of cytotoxicity, implicating mitochondrial dysfunction and free radical generation as critical factors in the mechanism of cell killing by redox-active naphthoquinones (159, 170–174).

Not all naphthoquinones are pure terminal electron acceptors, however. Menadione (vitamin K_3, 2-methyl-1,4-naphthoquinone) is a classical alternate electron

acceptor that possesses a mixed chemical reactivity. It both redox cycles on the mitochondrial respiratory chain to generate oxygen free radicals and it is also a soft electrophile, which accounts for its arylation of critical cellular nucleophiles. Its cytotoxicity correlates with the depletion of ATP in hepatocytes (175, 176), which can be circumvented by providing glycolytic substrates (177). Like all redox-active naphthoquinones, menadione stimulates oxygen consumption in cell cultures (178). However, rather than reducing molecular oxygen, menadione serves as an electron shunt. It accepts electrons from complex I and feeds them back to complex IV, bypassing a portion of the electron transport chain (179). Some hepatotoxic fungal naphthoquinones, such as xanthomegnin and viomellein, act similarly to menadione (65). Another example of alternate electron shunts are the *p*-phenylenediamines (e.g. N,N,N',N'- tetramethyl-*p*-phenylenediamine), which are widely used industrial and household chemicals (180). A number of these compounds cause necrosis in cardiac and skeletal muscle tissues, with a concomitant decrease in respiratory control and ADP:O ratios (180).

Although terminal electron acceptors that redox cycle on the mitochondrial electron transport chain to generate oxygen free radicals are toxic and offer no benefit to the cell, electron shunts have been used therapeutically to combat certain bioenergetic disorders. The therapeutic rationale is that disorders associated with the inhibition or malfunction of complex III of the respiratory chain can be overcome by shuttling electrons from complex I to complex IV, bypassing the dysfunctional portion of the chain. The best example of this therapeutic strategy is the use of menadione to treat mitochondrial encephalomyopathies (181–183). This disorder is characterized by a functional deficiency in the bc_1 complex (complex III) and involves multiple organ systems, particularly those rich in mitochondria, such as nerve and muscle. Symptoms include ataxia and disorientation, muscle cramps and weakness, depressed phosphocreatine and ATP levels, and lactic acidosis. Administration of menadione to these individuals resolves many of the bioenergetic deficiencies (lactic acidosis and low phosphocreatine and ATP), with significant improvement in both neurological and muscular performance. This is an important demonstration that not all xenobiotics that interact with the mitochondrial respiratory chain are toxic and that it is possible to harness the energy of the electron transport chain to improve the bioenergetic well-being of the individual.

SIGNIFICANCE OF MITOCHONDRIAL-MEDIATED PATHOGENESIS

In this article, we review the various mechanisms by which xenobiotics (both therapeutics and toxicants) interact with the mitochondrial respiratory chain to alter the efficiency and/or capacity of oxidative phosphorylation. We also point out the major distinction in symptomology of poisoning by the two major classes of compounds that directly affect mitochondrial bioenergetics, inhibitors and

uncouplers of mitochondrial respiration. The significance of these mechanisms of cytotoxicity is underscored by the hundreds of commonly used products and byproducts that are known to interfere with the mitochondrial electron transport chain. However, there are many additional modes by which chemicals can be cytotoxic wherein the mitochondrial bioenergetic deficit is secondary to another critical target, and perhaps intermediary in the ultimate expression of toxicity (184).

Examples of secondary bioenergetic deficiencies include exposures to agents that interfere with mitochondrial biogenesis, gene expression, or protein synthesis, or compounds that inhibit essential membrane transporters. There are numerous examples of chemicals that inhibit each of these active processes, all of which cause a secondary mitochondrial bioenergetic deficit that is not easily distinguished from a primary mitochondrial dysfunction on the basis of clinical features. Although these modes of toxicity are not covered in this review, the reader is cautioned to be mindful of these potential pathways of mitochondrial dysfunction.

Finally, mitochondrial dysfunction is not always manifested as a classical bioenergetic failure; lactic acidosis, neurological impairment, and incoordination and muscle fatigue are the classic symptoms. In deed, there is a burgeoning of recent evidence demonstrating a primary role for mitochondria in the apoptogenic process, integral to activation of the caspase enzyme cascade (cf 185–189). It is widely accepted that mitochondria play a critical role in determining necrotic versus apoptotic cell death and that mitochondrial bioenergetics plays a defining role in regulating cell cycling and differentiation. For example, mitochondrial dysfunction may trigger a premature apoptotic cell death, thereby preventing the clonal expansion of bioenergetically compromised cells. Failure of this mitochondrially regulated program of controlled cell death may be a critical event in the pathogenesis of hyperplastic disorders, including carcinogenesis. This is an explosive area of current research and is only recently yielding promising opportunities for therapeutic interventions (24, 190–192). Armed with this understanding of the important features of mitochondrial bioenergetics, we are now on the verge of developing more selective pesticides and new therapeutic strategies for treating the debilitating metabolic disorders associated with genetic defects or exposures to the numerous agents that selectively interfere with normal mitochondrial bioenergetics.

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