*A SUGGESTED MECHANISM OF UNCOUPLING OF RESPIRATORY-CHAIN PHOSPHORYLATION**

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A general property of uncouplers of respiratory-chain phosphorylation is that they inhibit respiration if added in concentrations greater than necessary for maximal uncoupling.[1-3] Hülsmann[1] suggested that, whereas uncoupling is due to release from an inhibited form[4] of the hypothetical coupling factors of the energy-conserving reactions,[5] inhibition by excess uncoupler is due to binding of one or more of these factors by the uncoupler. Hemker[2, 3] provided further experimental evidence in favor of this view that the uncoupling and inhibitory activities of uncouplers are not unrelated phenomena.

More recently, however, Wenner[6] demonstrated that the inhibitory effect of uncouplers can be partly overcome by higher substrate concentration. Wilson and Merz[7] extended this observation to show that the inhibition of succinate oxidation is kinetically of the competitive type between substrate and uncoupler. These results suggested an inhibition at the level of the primary dehydrogenation rather than in the energy-conserving reactions or in the respiratory chain proper. This was borne out by the demonstration that the respiratory carriers are completely oxidized in the uncoupler-inhibited state.[8]

Independent evidence for the idea that substrate availability may be the factor limiting respiration in the presence of high concentrations of uncoupler came from the experiments of Harris, van Dam, and Pressman[9, 10] on substrate accumulation. It was shown that all substrates tested are actively concentrated by intact mitochondria (see also ref. 11), and that this accumulation is inhibited by high concentrations of uncouplers in a manner similar to the inhibition of substrate utilization.

Harris, Höfer, and Pressman[12] have also proposed that the inhibitory effects of high uncoupler concentrations are related to the inhibition of energy-dependent substrate transport.

It should also be noted that the inhibitory effect of uncouplers is observable already at low concentrations, when not masked by the stimulatory effect of uncouplers on respiration measured in the absence of adenosine 5′-diphosphate (ADP). Thus, uncouplers in a concentration equal to that required for maximal stimulation of respiration in the absence of ADP inhibit in the presence of ADP.[8]

Since a typical uncoupler of respiratory-chain phosphorylation is a weak acid, and since the inhibition by uncouplers of substrate oxidation occurs only with anionic substrates,[8] it appears reasonable to relate the inhibitory effect to a competition between uncoupler anion and substrate anion for entry into the mitochondria. Indeed, the possibility must be seriously considered that many other phenomena such as the release by uncouplers of inhibition of respiration induced by Amytal,[13] 2-heptyl-4-hydroxyquinoline-N-oxide,[14] or azide[15, 16] may be simply explained on the basis of a competition between the anions of uncoupler and inhibitor for entry into the mitochondrion.

Investigations by Chappell and Haarhoff,[17] de Haan and Tager,[18] and Meijer and Tager[19] have revealed the presence of a number of specific "permeases" for substrate anions in the mitochondrial membrane. To reconcile these findings with the relatively nonspecific mutual inhibition of substrate and uncoupler anions observed, we propose a system similar to that suggested for the bacterial membrane,[20, 21] viz. that the molecules selected by a number of specific permeases are transported through the membrane by a common carrier.

We propose that the affinity of the energy-linked nonspecific anion carrier for uncoupler anions explains not only the inhibitory effects at high concentrations of uncouplers but also the uncoupling activity at lower concentrations. A diagram to illustrate this hypothesis is given in Figure 1. Under normal conditions, a

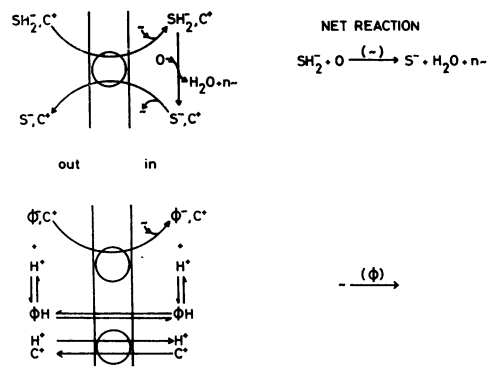


FIG. 1.—Proposed energy-linked carrier for substrate anions and mechanism of uncoupling.

substrate anion (SH_2^-) is transported into the mitochondrion at the cost of energy. In order to conserve electrical neutrality a cation is transported with the substrate anion. Indeed, it is possible that the anion carrier is in reality a cation carrier, and that the anions are drawn in with the cations. Oxidation of the substrate by the respiratory chain generates a number of high-energy bonds and the product (S^-). It is further proposed that release of this product (together with cation) through the transporter system to the outside regenerates completely or nearly completely the energy utilized to bring the substrate inside. Thus, the net effect would be an exchange of product for substrate anion, and energy would be required only to initiate substrate utilization. In the steady state, there is little or no net consumption of energy. According to this hypothesis, an uncoupler would be transported in its anionic form (ϕ^-) exactly like a substrate anion. Once it is inside, the uncoupler (being a weak acid) can take up a proton. The undissociated and uncharged molecule can leave the mitochondrion by simply diffusing through the membrane. Since the membrane contains much lipid, the observed relation between lipid solubility and uncoupling activity of a series of nitrophenols[2] finds a ready explanation. Once the uncharged uncoupler molecule is outside it dissociates again into a proton and the anion. Since, in our experience, there is no net formation of H^+ outside the mitochondrion when substrates are oxidized in the presence of uncoupler, we propose that the cation and proton re-equilibrate by means of an

exchange-diffusion system similar to that proposed by Mitchell.[22] The exchange-diffusion system might be either a C^+/H^+ antiport, as envisaged in Figure 1, or an anion/H^+ symport.[22] Alternatively, one might imagine that H^+ accompanies the substrate or uncoupler anions. In this case, no exchange-diffusion system would be necessary.

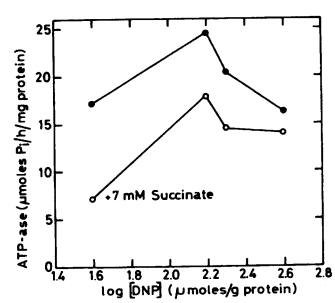
Other variants of the proposed hypothesis are possible. The essential feature is a cyclic transport of the uncoupler, inwardly energy-consuming and outwardly spontaneous. The inward transport system utilizes an anion-carrier system whose physiological function is the transport of substrate anions. Our proposed hypothesis is analogous to that proposed by Pressman[12] to explain uncoupling by valinomycin in the presence of K^+: namely, a valinomycin-induced cyclic transport of K^+.

The proposed hypothesis explains why uncoupler alone is sufficient to equilibrate protons between the mitochondria and the surrounding medium, when protons are liberated to the surrounding medium after an oxygen-pulse experiment, but both valinomycin and uncoupler are needed to equilibrate the protons after the addition of HCl.[22] In the first case, cations taken up during the oxygen pulse[23] are available as counter ion to the H^+ transported into the mitochondria under the influence of uncoupler. In the second case, valinomycin is required to transport K^+ outside the mitochondria to counteract the uncoupler-induced uptake of H^+.

The hypothesis that we propose is quite different from that of Mitchell and Moyle[22] who have shown that uncouplers, by acting as proton conductors, may under certain circumstances bring about the equilibration of H^+ inside and outside the mitochondrial membrane. However, according to Mitchell's view, the primary act of energy conservation is the setting up of a pH differential (however, cf. ref. 12). According to our view, energy is primarily conserved by a chemical mechanism, and this energy is needed to drive anion uptake. The energy-linked uptake of uncoupler anion would, in any case, prevent any equilibration of H^+ by the proton-conducting properties of the uncoupler, and we invoke an exchange between H^+ and cation to bring about this equilibration.

According to our hypothesis an uncoupler must be an acid with a sufficiently high pK so that both the dissociated and undissociated forms are present at neutral

FIG. 2.—Effect of succinate on the dinitrophenol-induced ATPase. Rat-liver mitochondria (3.8 mg protein) were incubated for 1 min at 25° in a reaction mixture (1.5 ml final vol) containing 75 mM KCl, 50 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl buffer, 3.0 mM $MgCl_2$, 3.0 mM ATP, and various concentrations of 2,4-dinitrophenol up to 1.0 mM. The final pH was 7.2. The reaction was stopped by addition of 1.5 ml 10% trichloroacetic acid, and the supernatant was assayed for inorganic phosphate (R. D. Veldsema-Currie, unpublished).



pH, and it must be sufficiently lipid-soluble to diffuse rapidly through the mitochondrial membrane. Both these properties are generally recognized. However, interpretation has been difficult since in some cases the anion, and in others the undissociated molecule, seemed to be the active uncoupling agent.[2, 3, 24] On the basis of the hypothesis developed here the active species will depend on whether

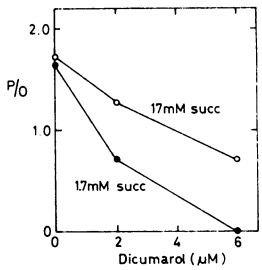


FIG. 3.—Effect of succinate concentration on the uncoupling by dicumarol of oxidative phosphorylation. The reaction mixture contained 50 mM Tris-HCl buffer, 50 mM KCl, 50 mM sucrose, 1 μg/ml rotenone, 1 mM EDTA, 1 mM ADP, 10 mM phosphate, and 2.0 mg protein per ml of rat-liver mitochondria. The final pH was 7.4. Oxygen uptake was measured at room temperature with the Clark electrode. The reaction was stopped with $HClO_4$ after 1.5 or 3 min, and ATP measured with hexokinase and glucose-6-phosphate dehydrogenase. The values are corrected for the small amount of ATP formed in the absence of succinate.

the transport of the anion or the diffusion of the uncharged molecule is the rate-limiting process in the uncoupling action.

The proposed mechanism of uncoupling by lipid-soluble weak acids is supported by the observation that substrate anions and malonate inhibit the dinitrophenol-induced adenosine triphosphatase (ATPase).[25-27] Inhibition by succinate is less at higher dinitrophenol concentrations (Fig. 2).

According to the proposed mechanism OH^- would also act as an uncoupler if it is transported by the energy-linked nonspecific anion carrier, since it is known that the mitochondrial inner membrane is permeable to water. Indeed, OH^- is a reversible uncoupler, as revealed by the effect of pH on the ATPase of liver mitochondria[28] and on the P:O ratio of heart mitochondria.[1]

As is also to be expected on the basis of the proposed mechanism, the uncoupling effects of either high pH or dicumarol are less at high than at low substrate concentrations (Figs. 3 and 4; cf. ref. 29).

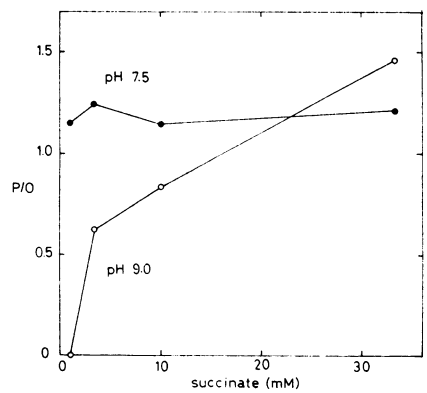


FIG. 4.—Effect of succinate concentration on uncoupling of oxidative phosphorylation by high pH. The reaction mixture and the experimental procedure were the same as in Fig. 3. Rat-liver mitochondria, 1.44 mg/ml. Reaction time, 2 or 3 min.

It is interesting that, whereas a typical uncoupler of mitochondrial oxidative phosphorylation is a weak acid, uncouplers of photosynthetic phosphorylation in chloroplasts are typically bases, such as amines, or are compounds which have both proton-accepting and proton-donating groups, such as trifluoromethoxycarbonyl cyanide phenylhydrazone. According to our hypothesis, chloroplast membranes would be expected to possess a cation carrier and not an anion carrier. Indeed, illuminated chloroplasts accumulate large amounts of H^+ in the absence of ADP and phosphate. The presence of an anion carrier in mitochondria and its absence in chloroplasts seems reasonable, since it is only the mitochondria that have the function of oxidizing substrate anions. According to this view, the differences between mitochondria and chloroplasts lie in the manner in which the primarily

conserved energy is utilized, rather than in the nature of the primary energy-conserving act. This is to be expected, since photosynthetic phosphorylation has many similarities to Site II respiratory-chain phosphorylation.

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