ENERGY CONSERVATION AND UNCOUPLING IN MITOCHONDRIA

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Energy conservation and uncoupling in mitochondria are examined in the light of three important new findings: (a) Studies with the photoaffinity-labeling uncoupler 2-azido-4-nitrophenol have shown that mitochondria contain a specific uncoupler binding site (apparently a polypeptide of $M_r = 30,000 \pm 10\%$). (b) This site fractionates into an enzyme complex (complex V), which is capable of oligomycin- and uncoupler-sensitive ATP-Pi exchange. It is absent from electron transfer complexes I, III, and IV, which represent segments of the respiratory chain containing coupling sites 1, 2, and 3, respectively. (c) Trinitrophenol is a membrane-impermeable uncoupler (uncouples submitochondrial particles, but not mitochondria) and a poor protonophore. There is an excellent correlation between the uncoupling potencies and the affinities of uncouplers for the mitochondrial uncoupler-binding site. There is no correlation between uncoupling potency and protonophoric activity of uncouplers when a membrane-permeable uncoupler is compared with a membrane-impermeable one.

INTRODUCTION

Uncoupling of oxidative phosphorylation has been extensively studied and various theories for the mechanism of this process have been proposed. These theories include the classical uncoupler-induced hydrolysis of a high-energy intermediate (1), conformation change and altered function of coupling proteins upon interaction with uncouplers (2), energy-linked transport of the uncoupler anion into, and passive diffusion of the protonated species out of the mitochondrion (3), acid or base catalyzed hydrolysis by uncouplers of an intermediate reaction of oxidative phosphorylation in a nonaqueous membrane region (4), increase of membrane conductance and collapse of transmembrane proton gradient by uncouplers acting as protonophores (5), and variations of the above (6–12).

Recent studies in our laboratory (13–16) have revealed several important features of uncoupling and uncoupler interaction with mitochondria, which have considerable bearing not only on the characteristics of the uncoupling process but also on the mechanism of oxidative phosphorylation. The present paper offers a brief review of these new developments. The characteristics of uncoupler interaction with mitochondria are as follows.

1. MITOCHONDRIAL UNCOUPLER-BINDING SITE

An uncoupler, which is capable of photoaffinity labeling, was designed and synthesized in radioactive form by Dr. W. G. Hanstein in our laboratory (13, 15). This

uncoupler, 2-azido-4-nitrophenol (NPA), is a structural analog of the classical uncoupler 2, 4-dinitrophenol (DNP). It is also two to three times more potent than DNP as an uncoupler. Studies on the equilibrium binding of tritiated NPA to mitochondria and submitochondrial particles have revealed that mitochondria contain a specific uncoupler-binding site. The concentration of this site in mitochondria is about 0.6 nmole/mg protein at pH 7.0 and 3°C. Under these conditions, the dissociation constant (K_D) of NPA is $6 \pm 3 \mu M$. The binding of NPA to this site is noncooperative, and uniform to the extent determined (90% saturation of the site). In addition, thermodynamic studies have indicated that NPA binding does not involve a major conformation change in mitochondria. The equilibrium binding of NPA to the specific uncoupler-binding site in mitochondria or submitochondrial particles is competitively inhibited by DNP,[1] TNP, PCP, Cl-CCP, S-13, dicoumarol, and azide. Addition of only 0.4 nmole of S-13 per mg of mitochondrial protein raises the apparent K_D of NPA by more than 2.5-fold. Extraction of more than 80% of mitochondrial lipids by aqueous acetone did not change the extent of NPA binding, and photoaffinity labeling of mitochondria and particles depleted of F_1 (ATPase) with triated NPA showed that the uncoupler binds predominantly to a polypeptide of $M_r = 30,000 \pm 10\%$.

The above results indicated, therefore, that mitochondria contain a specific binding site for the uncouplers mentioned above. This site is located in the inner membrane, appears to be associated with protein, and occurs at a concentration comparable to the concentration of components which participate in oxidative phosphorylation (cyt. $c_1 \simeq$ 0.3, cyt. $a \simeq 0.7$, $F_1 \simeq 0.27$ nmole/mg protein) (17−19). The uniform binding of NPA up to 90% saturation of the site suggests that the uncoupler-binding site is probably the same for the three energy coupling sites. This agrees with the relative concentration of F_1 with respect to the individual electron carriers, which again indicates one ATP synthesizing system per three coupling sites. It also agrees with the fact that site-specific uncouplers are not known.[2]

2. RELATIONSHIP OF THE UNCOUPLER-BINDING SITE TO THE OXIDATIVE PHOSPHORYLATION ENZYMES

The machinery for oxidative phosphorylation in beef heart mitochondria appears to be composed of five enzyme complexes (16, 20). These five segments can be isolated in high yield from the same batch of mitochondria by a general procedure, which employs deoxycholate and cholate for differential solubilization and ammonium acetate and ammonium sulfate for fractionation and isolation of the fragments (Fig. 1). Enzyme

[1] Abbreviations: DNP, 2, 4-dinitrophenol; TNP, 2, 4, 6-trinitrophenol; PCP, pentachlorophenol; Cl-CCP, carbonylcyanide m-chlorophenylhydrazone; S-13, 5-chloro-3-t-butyl-2'-chloro-4'-nitrosalicylanilide; K_D, dissociation constant; cyt., cytochrome; DCCD, dicyclohexylcarbodiimide; $\phi_{1/2}$, uncoupler concentration required for 50% uncoupling; U, uncoupler molecular.

[2] Guanidinium and alkyguanidinium ions have been suggested to be specific inhibitors of site I phosphorylation (25). However, the effect of these ions on the NADH-ubiquinone reductase segment of the respiratory chain is complex. It involves structural changes, inhibition of ubiquinone reductase activity, and activation of NADH dehydrogenase (20, 26, 27).
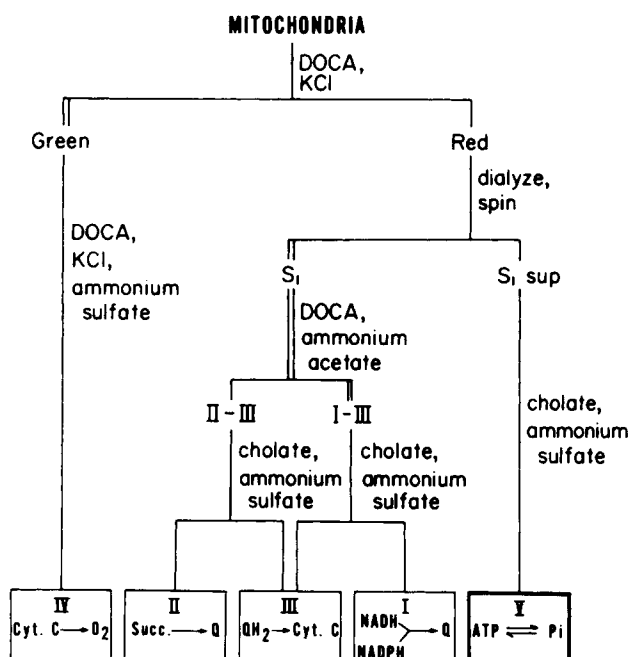
MITOCHONDRIA

Fig. 1. Scheme showing the fractionation of bovine heart mitochondria into enzyme complexes I, II, III, IV, and V with the use of deoxycholate (DOCA), cholate, ammonium acetate, and ammonium sulfate. From Hatefi et al. (20).

complexes I—IV are segments of the respiratory chain. Complex V appears to contain the mitochondrial enzymes concerned with energy conservation and transfer. In the isolated state, complex V exhibits high ATP-Pi exchange activity (> 200 nmoles/min X mg protein at 30°C). This activity is inhibited by uncouplers, oligomycin, venturicidin, DCCD, arsenate, azide, triethyl tin sulfate, adenylyl imidodiphosphate, and valinomycin plus K^+ (16, 20). It is highly significant that the mitochondrial uncoupler-binding site is concentrated (two- to threefold as compared to mitochondria) in complex V, and appears to be absent from complexes, I, III, and IV, which in mitochondria contain the three coupling sites (Table I). The uncoupler-binding site of complex V exhibits the same characteristic as in mitochondria in that the equilibrium binding of NPA to this site is competitively inhibited by other uncouplers.

These results show that the mitochondrial uncoupler-binding site is not spread throughout the membrane. Rather, the uncoupler-binding site fractionates into an enzyme complex, which is essentially devoid of respiratory chain components and which appears to be concerned with energy capture and transformation. The absence of the specific uncoupler-binding site of mitochondria from the electron transfer complexes also suggests that uncoupler-induced release of "respiratory control" in vesicles made from the electron transfer complexes is not related to uncoupling of oxidative phosphorylation. These vesicles are proton-impermeable, contain anisotropically oriented enzymes, and

TABLE I.   NPA-Binding Capacity of Complexes
I, III, IV, and V

Complex	Nmoles NPA bound/mg protein
I	$\leqslant$ 0.05
III	< 0.01
IV	< 0.01
V	0.81

The NPA binding experiments were carried
out at pH 8.0 and 4°C according to reference
13. Under these conditions (i.e., at pH 8.0)
the NPA binding capacity of whole
mitochondria was 0.35 nmole/mg protein.
From Hatefi et al. (16).

catalyze reactions which involve protons. It is possible, therefore, that proton require-
ment or accumulation on the inside of the vesicles would inhibit electron transfer. Un-
couplers acting as protonophores would relieve this inhibition by increasing transmem-
brane proton conductance.

3. RELATIONSHIP OF UNCOUPLER BINDING TO UNCOUPLING

An important question which cannot be satisfactorily answered at this stage of our
knowledge is whether the uncoupler-binding site is concerned with the act of uncoupling.
However, the data of Table II suggest that uncoupler-binding and uncoupling are closely
related. This table offers for several water-soluble uncouplers a comparison between K_D
(dissociation constant), which is an expression of uncoupler-binding affinity, and $\phi_{1/2}$
(uncoupler concentration required for 50% uncoupling), which is a measure of uncoupler
potency. The values shown in Table II are not directly comparable, because the K_D values
were determined at 3°C, and the $\phi_{1/2}$ values at 30°C. However, the K_D values shown in
column 3 were obtained from sets of data on the competitive inhibition of NPA binding
by each uncoupler under conditions that enthalpy change for NPA binding $\triangle H = -8 \pm 1$
kcal/mole. Using van't Hoff's equation, it can be calculated that the K_D of NPA at 30°C
would be three to four times the value given in Table II. Assuming a similar temperature
correction factor for other uncouplers, it may be concluded, therefore, that (a) K_D and
$\phi_{1/2}$ are of comparable magnitude ($K_D \approx 3 \phi_{1/2}$) for each uncoupler, and (b) $K_D/\phi_{1/2}$
is essentially the same for several uncouplers, even though their binding affinities and un-
coupling potencies might vary by as much as three orders of magnitude (compare NPA
and PCP with azide, Table II). These results suggest, therefore, (a) that uncoupler binding
and uncoupling are related phenomena, and (b) that in the case of water-soluble uncouplers,
binding affinity of an uncoupler is an indication of its uncoupling potency. Others
(10, 21–23) have correlated the uncoupling potencies of a number of uncouplers with
their ability to increase lipid bilayer conductance, which is in agreement with the
chemiosmotic concept of uncoupling. However, as will be seen below, membrane perme-

TABLE II.   Comparison of Binding Affinities and Uncoupling Potencies

Uncoupler	K_D	$K_{D,i}$	$\phi_{1/2}$
	μM	μM	μM
NPA	4–8		5–10
DNP	13–20	15–25	15–20
TNP		33	40–90
PCP		6–12	4–8
Azide		$3–4 \times 10^3$	$3–4 \times 10^3$

$K_{D,i}$: dissociation constant determined as in Fig. 4 from the competitive inhibition by each uncoupler of NPA binding to mitochondria or submitochondrial particles. From Hatefi and Hanstein (15).

ability and protonophoric potency do not hold as requirements for the uncoupler trinitrophenol (TNP), whereas binding to the uncoupler-binding site appears to be a requirement for TNP and the relationship between the magnitudes of K_D and $\phi_{1/2}$ (see above) also holds for this membrane-impermeable uncoupler.

2. PROPERTIES OF MITOCHONDRIA CONTAINING COVALENTLY-BOUND UNCOUPLER

Covalent binding of NPA to mitochondria under photolabeling conditions occurs at the same site as identified by equilibrium binding (13, 15). Our preliminary studies have indicated that mitochondria in which the uncoupler-binding sites are partially occupied by covalently bound NPA show a decrease in coupled (state 3) respiration rate with little change in state 4 rate or P/O value. Addition of free uncoupler does not increase the respiration rate to the level of control mitochondria. These results are in agreement with the findings of Wang and his colleagues (27) on the effect of the uncoupler 2, 4-dinitro-5-(bromoacetoxyethoxy) phenol (DNBP). According to Wang et al., this alkyl bromide analog of DNP uncouples oxidative phosphorylation when added to rat liver mitochondria. Then after several minutes of incubation, DNBP inhibits respiration as it covalently binds to thiol groups in the vicinity of the uncoupler-binding site. Covalent binding diminishes only the rate of respiration, not the P/O ratio. In contrast, 2, 4-dinitro-5-(acetoxyethoxy) phenol (DNAP), which is incapable of covalent binding, only uncouples, and the uncoupled rate of respiration does not become inhibited upon further incubation of mitochondria with DNAP.

The results with mitochondria containing covalently bound NPA or DNBP suggest that their respective uncoupler-binding sites participate in oxidative phosphorylation, and that the species containing covalently bound uncoupler become inhibited and incapable of recycling. Therefore, the rate of respiration drops as if some of the mitochondria were removed from action, but P/O ratio does not diminish because the population of mitochondria not containing covalently bound uncoupler can function normally.

5. EFFECT OF DIFFERENT REDOX AND ENERGIZED STATES OF MITOCHONDRIA ON UNCOUPLER BINDING

Equilibrium binding studies with NPA under various conditions of respiration and phosphorylation have shown that the specific binding of NPA to mitochondria is not affected by the presence or absence of substrate, ATP, valinomycin + K^+, or gramicidin $D + K^+$. This is also true for sufficient quantities of antimycin A or cyanide to inhibit electron transport completely; rutamycin, DCCD, or triethyl tin to inhibit ATP synthesis completely; or arsenate to uncouple completely (13, 15). Thus, contrary to the conclusion of others from kinetic data (7), uncouplers do not appear to have a greater affinity for an energized intermediate. In addition, various conformational or energized states of the membranes neither modify the binding affinity nor alter the effective concentration of the uncoupler binding site. Further, the ineffectiveness of antimycin A and cyanide indicates that inhibition of electron transfer at or near the coupling sites in the respiratory chain does not alter the uncoupler-binding site. This agrees with the absence of electron carriers, and with the presence of the uncoupler-binding site, in complex V. The ineffectiveness of rutamycin, DCCD, and triethyl tin indicates that the uncoupler-binding site is distinct from the inhibition sites of these reagents, and is unaffected by any structural modification which might result from the binding of these compounds. The ineffectiveness of arsenate is in agreement with the earlier conclusions of others that the sites of uncoupler action and arsenate uncoupling are different in mitochondria. Kinetically, uncouplers appear to act between the level of electron transport and the site of oligomycin inhibition, whereas arsenate seems to uncouple between the oligomycin inhibition site and the site at which ADP is phosphorylated.

6. TRINITROPHENOL, A MEMBRANE-IMPERMEABLE UNCOUPLER

As stated above, trinitrophenol (picrate, TNP) is a membrane-impermeable uncoupler (14, 15). It does not penetrate the mitochondrial membrane, as evidenced from its inability to interact with the specific uncoupler-binding site, and does not uncouple oxidative phosphorylation in mitochondria or digitonin particles (mitochondria depleted of outer membrane). However, when added to sonicated submitochondrial vesicles, which have an inside-out orientation of the inner membrane, or to complex V, then TNP interacts with the uncoupler binding site and uncouples. Table III gives the $\phi_{1/2}$ of TNP for uncoupling of oxidative phosphorylation (lowering of P/O ratio), energy-linked reverse electron transfer from succinate to NAD (driven by ATP), energy-linked transhydrogenation from NADH to NADP (driven by succinate oxidation), and release of rutamycin-induced respiratory control in "EDTA particles" (15). Since TNP cannot penetrate the mitochondrial membrane, the above results suggest that the uncoupler-binding site is located at or near the matrix side of the inner membrane, which, in inverted submitochondrial vesicles, is accessible to the medium. This agrees with the location of F_1 on the inner surface of the inner membrane and the fact that both F_1 and the uncoupler-binding site are components of complex V. The inability of TNP to penetrate the mitochondrial membrane does not appear to be related to the lipid solubility of picric acid. The octanol-water partition coefficient of undissociated TNP is only 1.5 times less

TABLE III.   Uncoupling of Energy-Linked Functions
of SMP by Trinitrophenol

Function	$\phi_{1/2}$ (μM)
Ox. Phos.	93
Succinate $\xrightarrow{\ e\ }$ DPN	41
DPNH $\longrightarrow$ TPN	59
Release of oligomycin-induced resp. control (EDTA particles)	58

From Hatefi and Hanstein (15).

than that of DNP, whereas rough estimates have indicated that its rate of diffusion into the mitochondrial membrane is 1,000 times less than that of DNP. We feel that the difference in their penetrability into the mitochondrial hydrophobic membrane might be related, however, to the concentration of undissociated DNP (pK_a = 4.0) and TNP (pK_a = 0.8) (28) at the neutral pH of enzymatic experiments.

While TNP uncouples various energy-linked functions in submitochondrial particles, it has very little effect on the proton conductivity of these vesicles (14, 15). As shown in Fig. 2, DNP has a pronounced effect on facilitation of proton conductance across the membranes of submitochondrial vesicles, while TNP is a poor protonophore. More importantly, the data of Fig. 2 show that there is no correlation between the uncoupling and the protonophoric potencies of DNP and TNP. At $\phi_{1/2}$ concentration of DNP (15—20 μM), the rate of proton conductance of the particles is increased 12—15-fold, whereas at $\phi_{1/2}$ concentration of TNP (50—90 μM), transmembrane proton conductance is facilitated only 2—3-fold. Even at several times $\phi_{1/2}$ concentration of TNP (200—500 μM), there is not more than a 3—4-fold increase in the proton permeability of the membranes. A four-fold increase of proton conductivity can be achieved with less than 5 μM DNP, a concentration which has only a marginal (~20%) uncoupling effect.

Skulachev and his colleagues (11, 12) have assigned TNP and tetraphenylboron (TBP) to a special class of uncouplers which uncouple submitochondrial particles, but not mitochondria, by the following mechanism. They postulate that TNP⁻ and TBP⁻ are pulled into the submitochondrial vesicles because the membrane electric potential is positive inside. This energy-linked transfer is considered to result in collapse of the membrane potential and uncoupling. The inability of TNP⁻ and TBP⁻ to uncouple mitochondria is explained by the argument that in mitochondria membrane potential is opposite that of submitochondrial vesicles, i.e., negative inside. This interesting hypothesis does not agree, however, with our results. As stated above, TNP competitively inhibits NPA binding to the uncoupler-binding site of submitochondrial particles (Figs. 3 and 4). These experiments were performed in the presence of such concentrations of NPA (up to 40 μM) which are sufficient for total uncoupling. Under these conditions, membrane potential and transmembrane proton gradient would be collapsed. Therefore, TNP could not enter the membranes by the Skulachev mechanism to interfere with NPA for interaction with the uncoupler-binding site. However, as stated above, such a competition between TNP and NPA for binding to the specific uncoupler-binding site did take place in submitochondrial particles, but not in mitochondria under the same conditions.
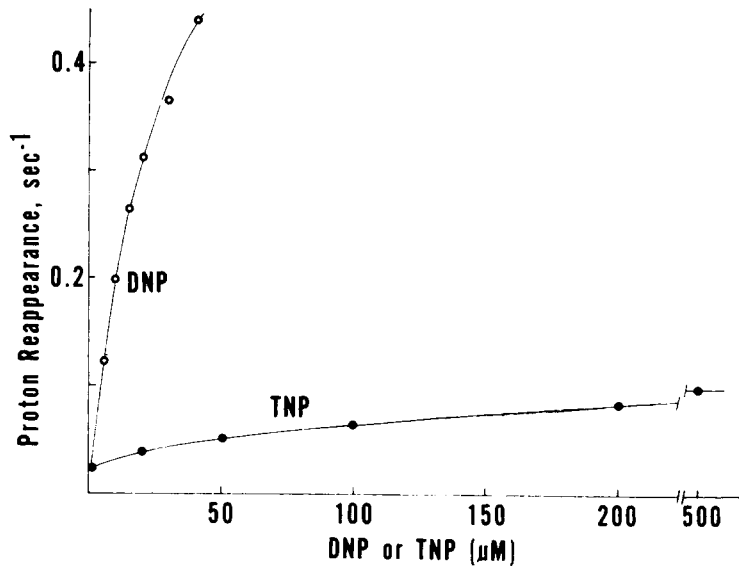
Fig. 2. Effects of DNP and TNP on the proton permeability of submitochondrial vesicles .
Ordinate: pseudo-first-order rate constants of proton reappearance from the vesicles into the
medium after uptake induced by a substrate pulse. Conditions: the reation mixture at 25° C contained
1 mg of submitochondrial particle protein per ml, 150 mM KCl, 3.3 mM glycylglycine, 5 mM MgCl$_2$,
20 μg/ml of rutamycin, and 1 μg/ml of valinomycin. The pH gradient was generated at pH 7.1 – 7.2
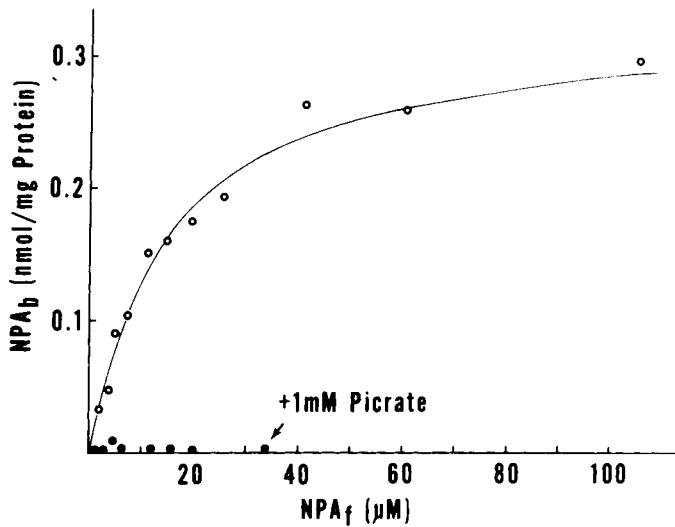by a pulse of 20 μM NADH. From Hanstein and Hatefi (14).

Fig. 3. Specific binding of NPA to submitochondrial particles in the absence (open circles) and
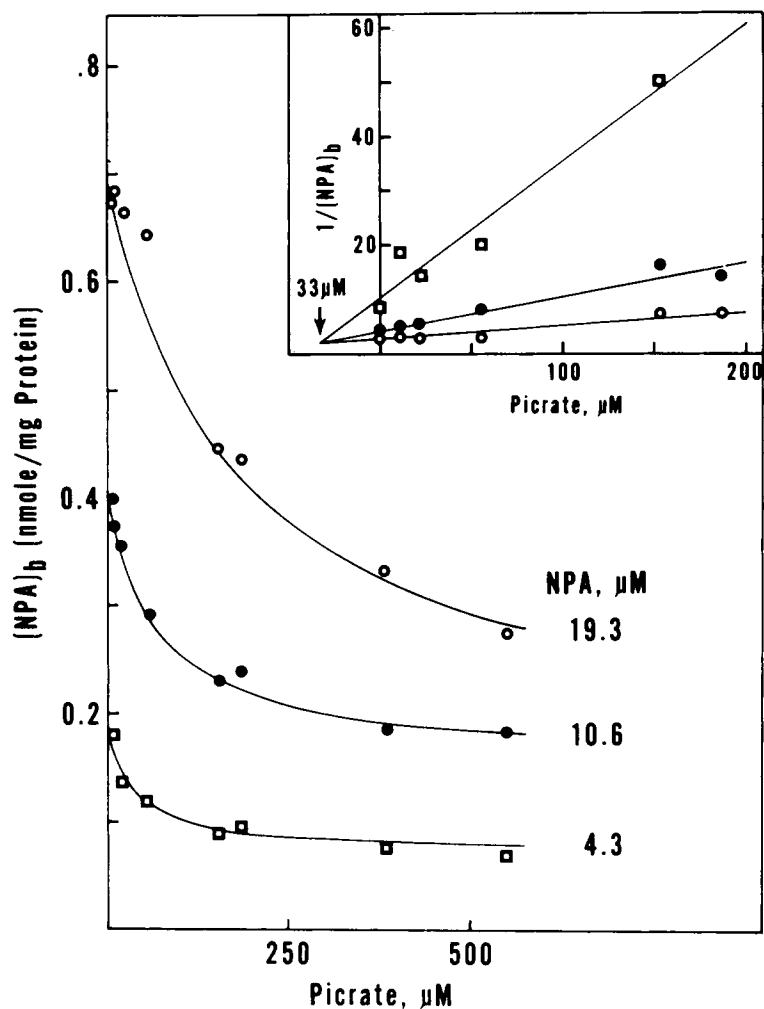presence (filled circles) of 1 mM TNP. From Hanstein and Hatefi (13).

Fig. 4. Inhibition by TNP of NPA binding to submitochondrial particles. Inset: Dixon plot showing competitive inhibition of NPA binding by TNP (K_D = 33 μM). From Hanstein and Hatefi (13).

CONCLUSIONS

The information accrued from the studies described above is summarized in Table IV. It is also depicted in the schemes shown in Figs. 5 and 6. The former scheme proposes that mitochondria contain a specific compenent, X, which can interact with uncouplers when the system is nonenergized (designated by X) or energized (designated by *X). In the absence of uncouplers, X participates in the process of oxidative phosphorylation. Noncovalent interaction of uncouplers (U) with *X results in energy dissipation. Consequently, noncovalently linked X. . .U is formed, which is in equilibrium with X + U, thus allowing both X and U to recycle and uncoupled respiration to proceed. In this
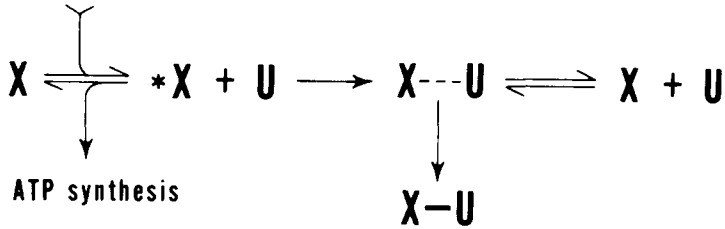
Electron transport

$$X \rightleftharpoons {}^{*}X + U \longrightarrow X\text{---}U \rightleftharpoons X + U$$

ATP synthesis   **X—U**

Fig. 5. Proposed scheme for the interaction of a component of mitochondria in nonenergized (X) and energized (*X) states with uncouplers (U) noncovalently (X . . . U) and covalently (X—U).
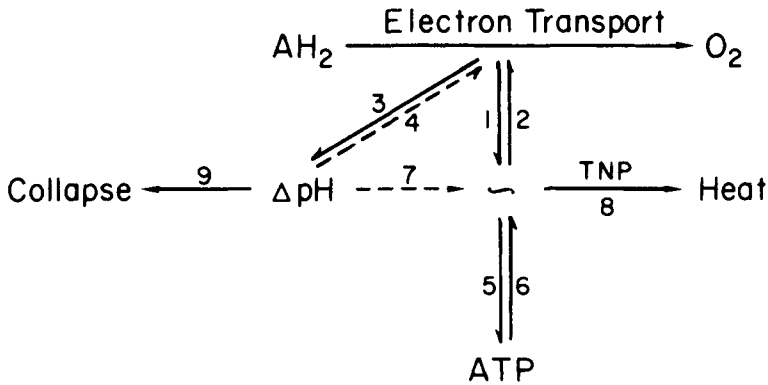

Fig. 6. Proposed scheme for synthesis of ATP and generation of transmembrane proton gradient (ΔpH), and for (a) uncoupling by the membrane-impermeable uncoupler, TNP (reaction 8), and (b) membrane perturbants and unspecific permeant ions (reaction 9). Reactions 4 and 7 shown by dashed arrows are assumed to be slower than the other reactions.

TABLE IV.   Characteristics of the Interaction of Uncouplers with Mitochondria

1. Mitochondria contain a specific uncoupler-binding site (UBS) common to various uncouplers. Uncoupler binding to this site is uniform and noncooperative.

2. UBS fractionates exclusively into complex V.

3. K_D and $\phi_{1/2}$ are of comparable magnitude. $K_D/\phi_{1/2}$ is the same for NPA, DNP, TNP, PCP, azide.

4. Covalent interaction of uncouplers with mitochondria appears to inhibit state 3 respiration. Inhibition is not relieved by addition of free uncoupler.

5. Uncouplers bind to UBS equally well when
   (a) membrane is energized, nonenergized, or deenergized;
   (b) electron transport is inhibited;
   (c) ATP—synthesizing system is inhibited.

6. Membrane permeability and protonophoric potency are not conditions for uncoupling, but interaction of uncoupler with UBS appears to be.

scheme, the effective concentration of U for uncoupling (e.g., $\phi_{1/2}$) would be a direct function of the rate of *X formation, which is in agreement with the finding of others regarding the direct relationship between uncoupler concentration needed for a given degree of uncoupling and the rate of phosphorylation (i.e., rate of coupled respiration X number of coupling sites involved) (29). The magnitude of $\phi_{1/2}$ would also be a function of the rate of energy dissipation (presumably a fast reaction) and X. . .U formation and the equilibrium between X. . .U and X + U. The latter is a measure of K_D, which, as seen in Table II, is directly related to $\phi_{1/2}$ for a number of water-soluble uncouplers. The finding that K_D and the saturation point of the uncoupler-binding site are independent of the redox, energized, or inhibited states of the membranes indicates that any conformation change which might accompany these modalities of the membrane does not modify the uncoupler-binding site. This fact and the presence of a specific uncoupler-binding site in mitochondria, F_1-deficient inner membrane particles, and electron carrier-deficient complex V should be taken into account in any hypothesis that might be formulated regarding the mechanism of action of uncouplers. Figure 5 also shows that covalent binding of U to X (X—U) could prevent X from recycling in the reactions of oxidative phosphorylation. This is consistent with the results of Wang et al. (24). with DNBP and our preliminary observations on mitochondria partially photolabeled with NPA. In both cases respiration was partially inhibited (presumably due to the population of mitochondria containing X—U), but P/O ratio did not drop (presumably because the unreacted population of mitochondria functioned normally in the absence of free uncoupler).

Figure 6 offers a simple explanation for the results obtained with the membrane-impermeable uncoupler, trinitrophenol. It proposes that as a result of electron transfer from substrate (AH_2) to oxygen, specific zones in the membrane are energized (reaction 1) (whether by conformation change, formation of a high-energy chemical intermediate, or some other mechanism remains to be seen), and concomitantly, protons resulting from the redox reactions of the respiratory chain are anisotropically deposited on one side of the membrane ($\triangle$pH) (reaction 3). The energized state of the membrane can lead to ATP synthesis (reaction 5), or conversely, energy from ATP hydrolysis can be utilized to energize the membrane (reaction 6), or drive reverse electron transfer (reactions 6 + 2). As shown by the dashed arrows (reactions 4 and 7), the energization of the membranes and reverse electron transfer driven by $\triangle$pH are assumed to be slow as compared to the other reactions shown.

This simple scheme incorporates a large number of known facts. Uncouplers with high protonophoric potency would uncouple either through reaction 8 by interacting with the membrane uncoupler-binding site, and/or through reaction 9 by collapsing the proton gradient and consequently dissipating the energy of the membrane through reactions 2 + 3.[3] Permeant ions, which do not interact with the uncoupler-binding site, and membrane perturbants (e.g., detergents) would also uncouple through reactions 9 + 3 + 2. Ion translocation would utilize energy through the same or a similar pathway. In contrast, membrane-impermeable uncouplers such as TNP would function almost exclusively through reaction 8. However, because TNP is a poor protonophore and reactions

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[3]The results of Table II suggest, however, that water-soluble uncouplers might function mainly by way of reaction 8.

4 and 7 are slow, it would be expected to have only a slight effect on facilitation of trans-membrane proton conductance and collapse of the pH gradient. It should be noted that Fig. 6 allows for ATP synthesis at the expense of $\triangle$pH (reactions 7 and 5). It only states that this process is slow as compared to ATP synthesis through reactions 1 and 5. The reason that the reversal of reaction 7 is not indicated on the scheme is not because there is evidence against it. It is rather because reactions 2 and 3 can account for energy utilization through $\triangle$pH. That both $\sim$ and $\triangle$pH are potential sources of energy does not necessarily pose a thermodynamic problem. Standard free energy change for oxidation of 1 mole of NADH by molecular oxygen is approximately twice that for the hydrolysis of 3 moles of ATP. Determination of the equilibria of the reactions shown in Fig. 6 under steady-state conditions (state 4) might show that the available energy can be accomodated in $\triangle$pH and the concentrations of ATP, ADP, and Pi.

ACKNOWLEDGMENTS

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