Proc. Nat. Acad. Sci. USA
Vol. 71, No. 2, pp. 288–292, February 1974

Trinitrophenol: A Membrane-Impermeable Uncoupler of Oxidative Phosphorylation

(mitochondria/anisotropic uncoupling/uncoupler-binding sites/transmembrane proton gradient)

W. G. HANSTEIN AND Y. HATEFI

Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California 92037

Communicated by Britton Chance, October 3, 1973

ABSTRACT **Picrate (trinitrophenol) is a unique uncoupler of oxidative phosphorylation. Unlike the commonly used uncouplers (e.g., 2,4-dinitrophenol, pentachlorophenol, *m*-chlorocarbonylcyanide phenylhydrazone, and 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide), picrate seems to penetrate the mitochondrial inner membrane very slowly. Consequently, it is ineffective when added to intact mitochondria or to mitochondria depleted of their outer membranes. In contrast, when added to phosphorylating submitochondrial particles prepared by sonication in which the inner membrane orientation is inside-out, picrate binds to the uncoupler-binding sites and uncouples oxidative phosphorylation. This unique property of picrate has made it possible to compare the potencies of picrate and dinitrophenol for (*a*) uncoupling and (*b*) increasing the proton permeability of submitochondrial particle vesicles. At 50% uncoupling concentration, dinitrophenol increased the proton permeability of submitochondrial particle vesicles by 9- to 12-fold. In contrast, at 100% uncoupling concentrations or higher, picrate augmented the proton permeability of the particles by only about 3-fold. These results indicate that facilitation of transmembrane proton equilibration does not determine the degree of uncoupling, and lead to the corollary conclusion that the magnitude of transmembrane proton gradient need not be the quantitative driving force for ATP synthesis.**

Study of energy uncoupling in mitochondria can furnish crucial information regarding the mechanism of oxidative phosphorylation. Equilibrium binding studies with a new, radioactive uncoupler, 2-azido-4-nitrophenol (NPA), have shown that mitochondrial inner membranes contain 0.56 ± 0.13 nmol/mg of protein of a uniformly reacting uncoupler-binding site, which is shared by various uncouplers and which appears to be functionally involved in the act of uncoupling (1).

The present communication is concerned with the unique property of picrate (trinitrophenol) as an uncoupler. This compound, which is a structural analogue of 2,4-dinitrophenol (DNP) and NPA, has afforded the important information that the mitochondrial inner membranes are anisotropic with respect to binding and the uncoupling activity of picrate. Thus, when introduced to mitochondria and inner-membrane preparations (digitonin particles), picrate does not readily penetrate the inner membranes and does not uncouple oxidative phosphorylation. In contrast, when added to submitochondrial particles (ETP_H), which are inside-out in orientation with respect to the medium, then picrate binds to the uncoupler-binding sites described above and uncouples oxidative phosphorylation. In view of these results, the effects of picrate and DNP were studied on the proton permeability of ETP_H. It was found that the potencies of picrate and DNP for increasing the proton permeability of ETP_H vesicles could not be correlated with their strength as uncouplers. These results are not only of interest with regard to the mechanism and site of uncoupling, but have important implications on the current theories of oxidative phosphorylation.

Abbreviations: AP-DPN, 3-acetylpyridine DPN; NPA, 2-azido-4-nitrophenol; DNP, 2,4-dinitrophenol; TNP, 2,4,6-trinitrophenol (picrate); S-13, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide; log P, logarithm of the octanol–water partition coefficient (20); $\phi_{1/2}$, concentration of uncoupler necessary to uncouple oxidative phosphorylation by 50%; RCR, respiratory control ratio (2); ETP_H, phosphorylating submitochondrial particles prepared by sonication; $[NPA]_b$ and $[NPA]_f$, respectively, concentration of bound and free NPA.

MATERIALS AND METHODS

Heavy beef-heart mitochondria and various types of submitochondrial particles were prepared according to published procedures (3–6). Rat-liver mitochondria and digitonin-treated particles were prepared according to ref. 7 and 8, respectively. Protein was estimated by the biuret method (9). Oxygen uptake was measured polarographically, and P_i esterification was estimated either according to Estabrook (2) from the extent of state-3 respiration after ADP addition (Figs. 1 and 4), or with $^{32}P_i$ in the presence of hexokinase and glucose, as described in the legend for Fig. 2. The experiments of Figs. 1 to 4 were conducted at 30°. Uncoupler-binding studies (Figs. 5 to 8) were conducted as before (1). Valinomycin was obtained from Calbiochem; 3-acetylpyridine DPN (AP-DPN) from P-L Biochemicals; DNP from East-

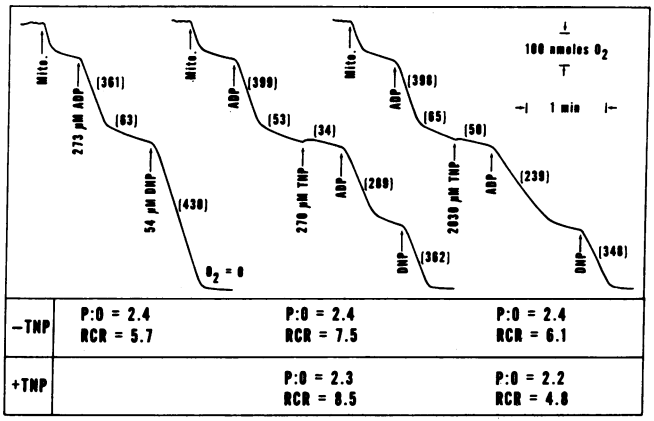

FIG. 1. Effect of picrate on oxidative phosphorylation activity of intact mitochondria. Conditions: 20 mM DL-3-hydroxybutyrate, 10 mM potassium phosphate, pH 7.4, 5 mM $MgCl_2$, 20 mM KCl, 0.25 M mannitol, and 1 mg of heavy beef-heart mitochondria protein per ml. Oxygen uptake rates shown in *parentheses* are expressed as nanogram atoms of oxygen per min per mg of protein at 30°.

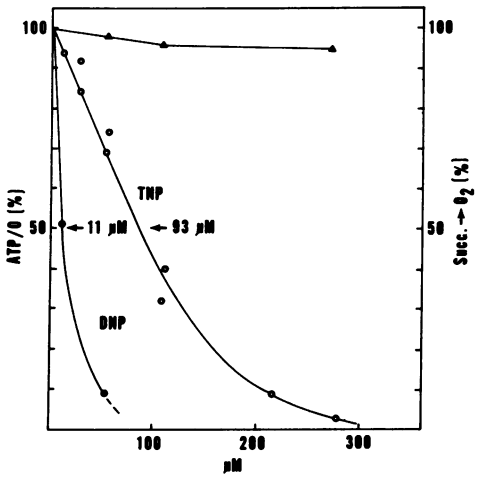

FIG. 2. Effects of picrate and DNP on the oxidative phosphorylation activity of ETP_H. Conditions: The reaction mixture (1.8 ml of 0.25 M sucrose) contained 0.3 mg of hexokinase, type V, 3 μmol of ATP, 60 μmol of glucose, 25 mM Tris-SO_4, pH 7.5, 1–2 mg of ETP_H (5), 2.5 mM succinate, and various amounts of uncoupler. The reaction was started by addition of 2.5 μmol of potassium phosphate, containing 100 to 200 cpm of $^{32}P_i$/nmol of phosphate, and was stopped by addition of 0.2 ml of 35% perchloric acid. Aliquots (0.3–0.5 ml) were analyzed for esterified [^{32}P]phosphate, as described by Pullman (10). Phosphate controls were exactly the same as the reaction mixture described above, except that perchloric acid was added before radioactive phosphate. They were analyzed for both total inorganic phosphate and $^{32}P_i$. Oxidative phosphorylation results (esterified phosphate/oxygen uptake) are expressed as ATP/O ratio. The *left-hand ordinate* refers to traces with *open* and *filled circles* and the *right-hand ordinate* refers to the trace with *triangles*. *Succ.* = succinate.

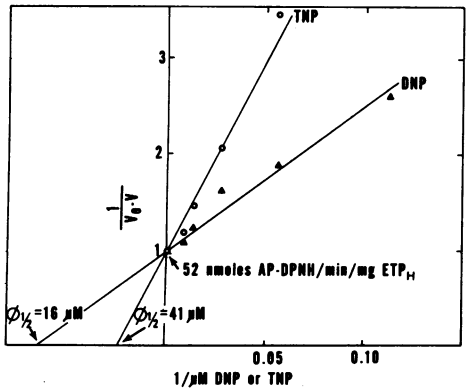

FIG. 3. Effects of picrate and DNP on ATP-dependent reverse electron transfer from succinate to AP-DPN as catalyzed by ETP_H. Conditions: ATP-driven reverse electron transfer from succinate to 3-acetylpyridine-DPN (AP-DPN) was measured at 363 – 400 nm in an Aminco-Chance Dual Wavelength Spectrophotometer. The reaction mixture contained 0.26 mg/ml of ETP_H (5), 250 mM sucrose, 50 mM Tris-SO_4, pH 7.5, 5.5 mM $MgCl_2$, 1.8 mM NaCN, 7.3 μM AP-DPN, 3.6 mM succinate, and various amounts of uncoupler as indicated. The reaction was started by the addition of 1.8 mM ATP. V_0 and V, rates of AP-DPN reduction in the absence and presence, respectively, of uncouplers as indicated.

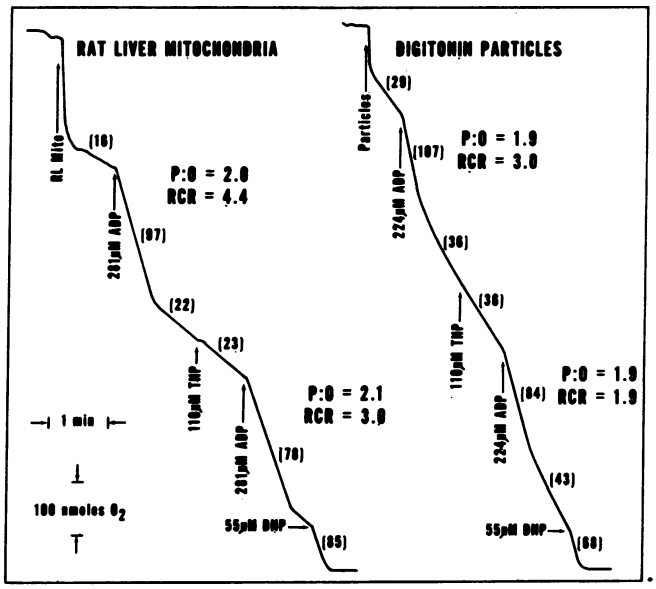

FIG. 4. Effects of picrate and DNP on the oxidative phosphorylation activity of digitonin-treated rat-liver mitochondria. Conditions were the same as in Fig. 1, except that mitochondria and digitonin particles were used at 2.13 and 2.72 mg/ml, respectively.

man Kodak; picrate from J. T. Baker; hexokinase (type V) from Sigma; and $^{32}P_i$ as phosphoric acid in 0.02 N HCl, carrier-free, from New England Nuclear Corp. Rutamycin was a gift from Eli Lilly and Co.

RESULTS

Anisotropy of the Mitochondrial Inner Membrane with Respect to the Uncoupling Activity of Picrate. The effect of picrate on the oxidative phosphorylation activity of intact mitochondria is shown in Fig. 1. The left-hand trace is a control, which demonstrates the effects of ADP and DNP on the respiratory activity of mitochondria in the presence of 3-hydroxybutyrate as substrate. The oxygen consumption rates in state 3, state 4, and after DNP addition are shown in parentheses, and P:O and RCR values are given underneath each polarographic trace. The middle and the right-hand traces show the

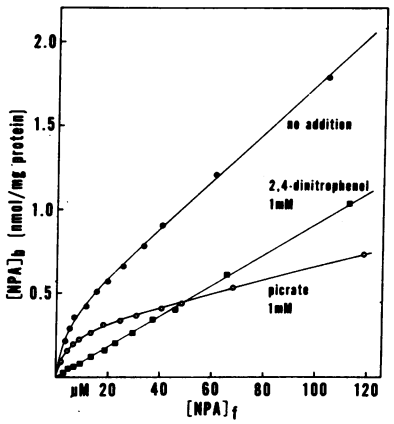

FIG. 5. Effects of picrate and DNP on the total binding of NPA to mitochondria.

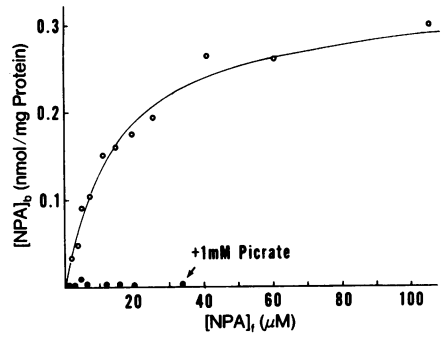

FIG. 6. Binding of NPA to ETP_H in the absence (O) and presence (●) of picrate.

effects of 0.27 mM and 2.0 mM picrate (TNP) on the state-4 respiration rate of mitochondria. In each case the mitochondria were taken first through a state 3 to 4 transition by addition of a limited amount of ADP, and the P:O values were determined. At this point picrate was added, state-4 respiration rate in the presence of picrate was recorded, and then ADP was added to induce a second state 3–state 4 cycle. This allowed determination of P:O and RCR values in the presence of picrate. Finally, DNP was added to demonstrate its effect as compared to picrate. It is seen that picrate did not augment the inhibited state-4 rate, and did not prevent the induction of state 3 upon subsequent addition of ADP. The state-3 rates in the presence of large amounts of picrate were slightly inhibited, but once again respiratory control prevailed, which was released upon addition of the uncoupler, DNP. These results, and the P:O and RCR values in the presence of picrate, clearly demonstrate that in mitochondria picrate does not act as an uncoupler (see also ref. 11).

In contrast, Figs. 2 and 3 show that picrate is an effective uncoupler of oxidative phosphorylation and ATP-dependent reverse electron transfer from succinate to AP-DPN when sonicated submitochondrial particles (ETP_H) are used as

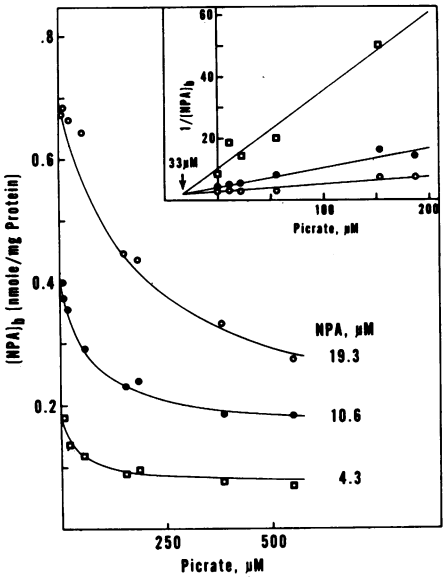

FIG. 7. Inhibition by picrate of NPA binding to ETP_H.

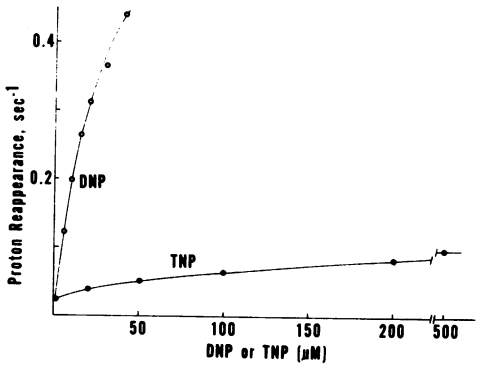

FIG. 8. Effects of picrate and DNP on the proton permeability of ETP_H vesicles. Conditions: The experiments were conducted at 25°, using a Brinkman EA 120 combination electrode, an Orion model 801 pH meter, and a Honeywell Electronic 19 recorder. The reaction mixture contained 1 mg/ml of ETP_H (5), 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 substrate pulse consisting of 20 μM DPNH.

catalyst.* As will be seen below, the crucial difference in the response of mitochondria and ETP_H particles to picrate is that ETP_H particles are inside-out (13–16). In the case of mitochondria, the natural orientation of the inner membrane poses a barrier for the approach of picrate to the site of action of uncouplers, whereas with inside-out ETP_H particles these sites are accessible to picrate in the medium.†

That the difference in the response of mitochondria and ETP_H to the uncoupling effect of picrate is due to the sidedness of the inner membrane, and not a consequence of the screening of picrate by the outer membrane, is shown in the experiments of Fig. 4. In these experiments the effect of picrate was tested on mitochondria depleted of their outer membranes by treatment with digitonin. The experiments are similar to those described in Fig. 1. The left-hand trace shows the effect of picrate on intact rat-liver mitochondria, and the right-hand trace shows its effect on a digitonin-treated sample of the same mitochondrial preparation. It is seen that 110 μM picrate (similar results were obtained with 830 μM picrate) abolished neither the P:O ratio nor the respiratory control property of such particles that lack their outer membrane, but have a natural orientation of the inner membrane. In connection with these results, the following points were also ascertained: (*a*) that the respiratory control observed with digitonin-treated particles in the presence of

* We have also tested the ability of picrate in (*a*) abolishing the "respiratory control" achieved by addition of rutamycin to "EDTA particles" (see ref. 6), and (*b*) inhibiting respiration-supported transhydrogenation from DPNH to TPN as catalyzed by ETP_H particles treated with rotenone and rutamycin (12). In both tests, picrate was shown to act as an uncoupler at concentrations similar to those shown in Figs. 2 and 3. In the case of "EDTA particles," it was also found by Mcntal *et al.* (17) that 51 μM picrate plus 9.5 mM KCl released the oligomycin-inhibited DPNH oxidation when added in the presence of nigericin or nigericin plus valinomycin.

† For the anisotropic response of mitochondrial inner membranes toward accumulation of large "penetrating" ions such as phenyldicarbaundecaborane, see ref. 15.

picrate was indeed associated with ATP formation ([^{32}P]-ATP:O values were essentially the same as the ADP:O values determined from Fig. 4), (*b*) that digitonin treatment had indeed resulted in removal of the outer membranes (electron microscopic data), and (*c*) that digitonin-treated beef-heart mitochondria were also capable of oxidative phosphorylation and respiratory control in the presence of such amounts of picrate, which completely uncoupled the ETP_H particles.

Anisotropy of the Mitochondrial Inner Membrane with Respect to Binding of Picrate. With the use of a new uncoupler, 2-azido-4-nitrophenol (NPA), we have shown elsewhere (1) that mitochondria contain a finite number of uncoupler-binding sites. These sites are common for NPA, DNP, pentachlorophenol, *m*-chlorocarbonylcyanide phenylhydrazone, azide, and S-13, are located in the inner membrane, and appear to participate in the act of uncoupling. The binding of NPA to mitochondria under equilibrium conditions occurs in two distinct phases or sites. There is a high-affinity site, which is saturated at 0.56 ± 0.13 nmol of NPA per mg of protein and is competed for by the uncouplers mentioned above. There is also a low-affinity site(s), which takes up NPA as a linear function of NPA concentration in the medium, is not saturable even up to 600 μM NPA, and is not competed for by other uncouplers. Thus, the high-affinity phase appears to represent the uncoupler-binding sites of mitochondria.

When NPA-binding experiments were performed in the presence of picrate, it was shown, in agreement with the results of Figs. 1 to 4, that picrate competed with NPA for the uncoupler-binding sites in inside-out ETP_H particles, but not in intact mitochondria. Fig. 5 shows the binding pattern of NPA to mitochondria. It is seen that NPA binding is initially hyperbolic (high affinity) and subsequently linear (low affinity). Dinitrophenol, which uncouples mitochondria, competes for, and at high concentration completely abolishes, the high-affinity phase of NPA binding, but has little effect on the linear, low-affinity phase. In contrast, picrate, which does not uncouple mitochondria, has an appreciable effect on the low-affinity phase of NPA binding, but very little effect on the high-affinity phase. A double-reciprocal plot of the specific binding data derived from Fig. 5 showed that the dissociation constant of NPA was, indeed, unaffected by picrate. These results indicate that in mitochondria the uncoupler-binding sites either are not accessible to picrate or do not bind it.

However, the experiments of Figs. 6 and 7 with inside-out ETP_H particles clearly indicate that picrate binds to the uncoupler-binding sites and that such binding is competitive with respect to NPA concentration. It is seen in Fig. 6 that the high-affinity phase of NPA binding in ETP_H is completely abolished by the same picrate concentration (1 mM) that had no effect in mitochondria. Fig. 7 shows the competitive inhibition results, a Dixon plot of which in the inset gives a dissociation constant of 33 μM for picrate.

Thus, the binding experiments with mitochondria (Fig. 5) parallel the oxidative phosphorylation experiments involving inner membranes in their natural orientation (Figs. 1 and 4). In the former experiments, picrate did not bind to the uncoupler-binding sites, and in the latter it did not uncouple ATP synthesis. Similarly the binding experiments with EPT_H (Figs. 6 and 7) parallel the oxidative phosphorylation and ATP-dependent reverse electron transfer experiments (Figs. 2 and 3), which involved inside-out particles. In the former experiments picrate reacted with the uncoupler-binding sites, and in the latter it uncoupled.

Effect of Picrate on the Proton Permeability of Mitochondria and ETP_H. The chemiosmotic theory of oxidative phosphorylation (13) proposes that the electron transport-induced anisotropic deposition of protons across the mitochondrial inner membrane is the driving force for energy coupling. Thus, uncoupling is visualized as facilitation of transmembrane proton equilibration by uncouplers (13).

We have studied the relative effects of picrate and DNP on facilitation of transmembrane proton movement in intact mitochondria and ETP_H particles. The experiments with mitochondria were conducted according to Skulachev (18), and those with ETP_H were performed as follows. In a medium containing particles, valinomycin, rutamycin, KCl, and picrate or DNP as indicated, a transmembrane proton gradient was induced with a substrate (DPNH) pulse (see ref. 19). Then the collapse of the gradient was monitored as a function of time. In intact mitochondria treated with succinate, valinomycin, and KCl, the addition of 100 μM DNP caused a rapid alkalinization of the medium, whereas 200 μM picrate was completely ineffective. In ETP_H, picrate had a slight effect on the decay of the proton gradient (Fig. 8). However, there was no correlation between the potencies of picrate for uncoupling and for facilitation of proton diffusion. When DNP was used at half uncoupling concentration (11–16 μM, see Figs. 2 and 3), the rate of proton equilibration was increased 9- to 12-fold as compared to the control (Fig. 8). By contrast, when picrate was used at half uncoupling concentration (40–90 μM, see Figs. 2 and 3), the rate of proton efflux was increased only 2- to 3-fold. It might also be seen that even at 500 μM picrate (i.e., well above the concentration needed for complete uncoupling), the rate of proton efflux was increased only 4-fold. This facilitated rate is achieved by about 5 μM DNP, which has only a slight (about 20%) uncoupling effect.

DISCUSSION

It is clear from the data presented here and elsewhere (1) that the mitochondrial inner membranes contain a finite number of sites for the binding and the energy-dissipating action of uncouplers. These sites appear to be accessible from either side of the inner membrane to most uncouplers, but not to picrate. The inability of picrate to interact with and uncouple mitochondria and digitonin-treated particles appears to be referable to its slow diffusion rate into the inner membrane. Rough estimates have indicated that the rate constant for the diffusion of picrate into the mitochondrial inner membrane is about three orders of magnitude smaller than that of DNP. This difference does not appear to be due to the lipid solubilities of the undissociated DNP and TNP, because the octanol–water partition coefficient (P) of DNP is only 1.5 times that of TNP (DNP, log P = 1.5; TNP, log P = 1.34; refs. 20 and 21). However, the difference in their pK_a values (20) suggests a possible explanation, since the undissociated acids are most likely the more diffusible species. Above pH 6, the concentration of undissociated DNP is three orders of magnitude greater than that of TNP.

Experiments with inside-out ETP_H particles have shown, on the other hand, that picrate is an effective uncoupler ($\phi_1/_2$ = 40–90 μM) and binds to the same uncoupler-binding

sites shared by NPA, DNP, *m*-chlorocarbonylcyanide phenylhydrazone, pentachlorophenol, azide, and S-13. In addition, as was found for NPA and DNP, the ratio of the dissociation constant of picrate (33 μM) to its $\phi_{1/2}$ is close to unity. This result suggests that the degree of saturation of the binding sites (at least in the case of water-soluble uncouplers such as NPA, DNP, and picrate) is related to the degree of uncoupling.

Thus, it may be concluded that (*a*) membrane diffusibility of uncouplers (neutral or charged species) is neither tantamount to nor a prerequisite for uncoupling, and (*b*) that uncoupling appears to result from the interaction of uncouplers with specific sites situated in the inner membrane and more accessible from the matrix side.

The relative potencies of DNP and picrate for uncoupling (Figs. 2 and 3) and for facilitation of transmembrane proton permeation (Fig. 8) clearly show that the latter effect cannot be a quantitative determinant of uncoupling. An obvious corollary of this conclusion is that the magnitude of a transmembrane proton gradient need not be a quantitative driving force for energy coupling. This is evident from Fig. 8, which in effect shows the following. In one instance (i.e., high picrate concentration), complete uncoupling occurs when membrane resistance to proton diffusion is little affected. In another instance (i.e., low DNP concentration), considerable phosphorylation (e.g., 50%) can take place when membrane proton permeability is increased by more than 10-fold.

We thank Dr. J. D. Feldman for electron microscopy of the mitochondrial preparations, and Miss L. Rademacher and Mr. C. Muñoz for expert technical assistance. This work was supported by grants to Y.H. of USPHS AM08126 and CA13609, and to W.G.H. of USPHS GM19734 and San Diego Heart Association Grant-in-Aid no. 108. W.G.H. is recipient of USPHS Research Career Development Award 5-K4-GM-38291.

1. Hanstein, W. G. & Hatefi, Y., *J. Biol. Chem.*, in press.
2. Estabrook, R. W. (1967) in *Methods in Enzymology*, eds. Estabrook, R. W. & Pullman, M. E. (Academic Press, New York), Vol. 10, pp. 41–47.
3. Hatefi, Y. & Lester, R. L. (1958) *Biochim. Biophys. Acta* **27**, 83–88.
4. Löw, H. & Vallin, I. (1963) *Biochim. Biophys. Acta* **69**, 361–374.
5. Hansen, M. & Smith, A. L. (1964) *Biochim. Biophys. Acta* **81**, 214–222.
6. Lee, C.-P. & Ernster, L. (1968) *Eur. J. Biochem.* **3**, 391–400.
7. Schnaitman, C. A. & Greenawalt, J. W. (1968) *J. Cell Biol.* **38**, 158–175.
8. Chan, T. L., Greenawalt, J. W. & Pedersen, P. L. (1970) *J. Cell Biol.* **45**, 291–305.
9. Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766.
10. Pullman, M. E. (1967) in *Methods in Enzymology*, eds. Estabrook, R. W. & Pullman, M. E. (Academic Press, New York), Vol. 10, pp. 57–60.
11. Weinbach, E. C. & Garbus, J. (1965) *J. Biol. Chem.* **240**, 1811–1819.
12. Lee, C.-P., Azzone, G. F. & Ernster, L. (1964) *Nature* **201**, 152–155.
13. Mitchell, P. (1966) *Biol. Rev.* **41**, 445–502
14. Fessenden-Raden, J. M. & Racker, E. (1971) in *Structure and Function of Biological Membranes*, ed. Rothfield, L. I. (Academic Press, New York), pp. 401–438.
15. Skulachev, V. P. (1971) *Cur. Top. Bioenerg.* **4**, 127–190.
16. Lee, C.-P. & Ernster, L. (1966) in *Regulation of Metabolic Processes in Mitochondria*, eds. Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C. (Elsevier, Amsterdam), BBA Library, Vol. 7, pp. 218–234.
17. Montal, M., Chance, B. & Lee, C.-P. (1970) *J. Membrane Biol.* **2**, 201–234.
18. Skulachev, V. P., Jasaitis, A. A., Navickaite, V. V., Yaguzhinsky, L. S., Liberman, E. A., Topali, V. P. & Zofina, L. M. (1969) *FEBS Symp.* **17**, 275–284.
19. Hinkle, P. C. & Horstman, L. L. (1971) *J. Biol. Chem.* **246**, 6024–6028.
20. Fujita, T. (1966) *J. Med. Chem.* **9**, 797–803.
21. Fujita, R., Iwasa, I. & Hansch, C. (1964) *J. Amer. Chem. Soc.* **86**, 5175–5180.