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Mechanism of Action of Uncouplers of Oxidative Phosphorylation*

David F. Wilson,† H. P. Ting,‡ and Michele S. Koppelman

ABSTRACT: Four chemical types of uncouplers of oxidative phosphorylation in rat liver mitochondria have been studied with respect to the pH dependence of their uncoupling activity.

These uncouplers include salicylanilides, carbonyl cyanides, halogenated benzimidazoles, and thiophenols. The uncoupling activity in each case is pH dependent and, when the p*K*'s of the dissociable groups and solubilities in aqueous media are considered, this pH dependence is consistent with the uncouplers acting as acid or base catalysts of a reaction occurring in the nonaqueous region of the mitochondrial membrane. The carbonyl cyanide derivatives and halogenated benzimidazoles appear to be active as acids (protonated form) while the salicylanilides and halogenated thiophenol appear to be active as bases (anionic form). The measured pH dependences of the uncoupling activities do not support hypotheses in which the uncouplers act as proton carriers or as transported anions.

The compounds which uncouple the energy conservation reactions from the oxidation–reduction reactions of mitochondria (as opposed to those which activate ion transport, such as valinomycin, etc.) were first thought to act by chemical mechanisms (Loomis and Lipman, 1948; Slater, 1953; Lardy and Wellman, 1953; Chance and Williams, 1956). In recent years, however, the proposed mechanisms have been expanded to include additional chemical mechanisms (Hemker, 1964a,b; Weinbach and Garbus, 1969; Wang, 1967), proton conduction mechanisms (Mitchell, 1961, 1966), and transported anion mechanisms (van Dam and Slater, 1967).

The uncoupler molecule, in general, is characterized by having a chemical group from which a proton is dissociable with a p*K* between 3 and 9 (DeDeken, 1955; Parker, 1958). In addition a solubility in organic solvents seems to be important (DeDeken, 1955; Hemker, 1964a,b).

In the present work we have attempted to determine the role of the dissociable proton in uncoupling. For this purpose

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† To whom to address correspondence.
‡ Present address: Department of Biochemistry, Michigan State University, East Lansing, Mich.

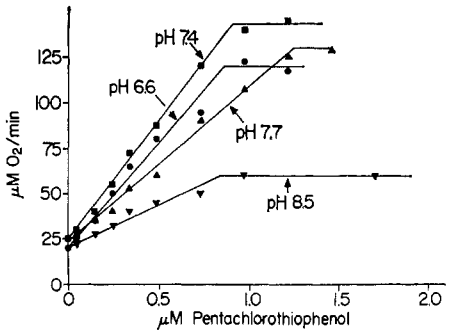

FIGURE 1: The effect of P-Cl-TP on the rate of succinate oxidation by rat liver mitochondria. The rat liver mitochondria were suspended at 0.3 μM cytochrome *a* in a KCl medium with the buffer system given in the Methods section and the pH indicated in the figure. The substrate was 8 mM succinate in the presence of 3 μM rotenone.

we have selected a number of very active uncouplers and measured the pH dependence of the uncoupling activity in rat liver mitochondria. In addition, we have measured the pH dependence of the uncouplers' ability to conduct protons through lipid bilayers (Skulachev *et al.*, 1967; Hopfer *et al.*, 1968, 1970; Liberman *et al.*, 1968; Markin *et al.*, 1969; Ting *et al.*, 1970), their p*K*'s in aqueous media, and their solubility in aqueous media.

From the data presented in this paper, one can conclude that the pH dependence of the uncoupling activity is consistent with a general acid–base catalysis in which both the protonated and anionic forms are active. The data are inconsistent with uncoupling mechanisms which require the presence of both the anionic and protonated species (Mitchell, 1961, 1966; van Dam and Slater, 1968). This latter conclusion is supported by the observation that the pH profiles for the uncoupling activity are completely different from the pH profiles for the ability to decrease the electrical resistance of lipid bilayers.

Materials and Methods

Measurement with Black Lipid Membranes. The black lipid membranes were prepared as previously described (Ting *et al.*, 1970) from a chloroform–methanol (2:1, v/v) extract of ox brain white matter with 20 mg of additional cholesterol and 75 mg of α-tocopherol added per ml of lipid solution. The aqueous phases consisted of 0.1 M KCl buffered with 10 mM MOPS or Tris as required by the pH. The temperature for all experiments was maintained at 25°. The electrical circuit for electrical resistance measurements was that of Mueller *et al.* (1964) later employed by Tien and Diana (1967). The potential across the membrane was measured with a London Radiometer pH-mV meter using a pair of calomel electrodes. The method of calculating the membrane conductance and the uncoupler dependence of the membrane conductance were the same as previously reported (Ting *et al.*, 1970).

Measurements of Uncoupling Activity in Rat Liver Mitochondria. The rat liver mitochondria were prepared in a medium containing 0.22 M mannitol, 0.07 M sucrose, and 200 μM EDTA. Mitochondrial respiration was measured polarographically in an assay medium containing 0.12 M KCl, and buffered with 30 mM MOPS or Tris at the indicated pH. Buffer specificity was tested by using alternating buffers at pH values between the two p*K* values, *i.e.*, buffers of pH 7.6, 7.8, and 8.0 were prepared with MOPS while buffers of pH 7.7, 7.9, and 8.1 were prepared with Tris. There was no detectable difference in the uncoupling activity using the two different buffers. The concentration of cytochrome *a* in the mitochondrial assay medium was 0.22–0.3 μM unless otherwise specified. Rotenone was added to a final concentration of 3 μM, and the substrate was 8 mM succinate. The reaction temperature was 24°. The cytochrome concentrations were assayed as previously reported (Wilson, 1969).

The FCCP was the generous gift of Dr. P. G. Heytler (Central Research Department, Experimental Station, du Pont de Nemours and Co., Wilmington, Del.). The 5-chloro-3-*tert*-butyl-2′-chloro-4′-nitrosalicylanilide (S-13) and 5-chloro-3-(*p*-chlorophenyl)-4′-chlorosalicylanilide (S-6) were gifts of Dr Phillip Hamm (Agricultural Division of Monsanto Chemical Co., St. Louis, Mo.). TTFB was the gift of Dr. Brian Beechey (Shell Grundlagen-forschung G.m.b.H., Schloss Birlinghaven, 52 Siegburg, Postfach 88, West Germany). Br-N-CCP and benzothiazole-CCP were synthesized and crystallized by D. F. Wilson by the method given by Heytler and Prichard (1962). P-Cl-TP was obtained from Aldrich Chemical Co., purified and crystallized by sublimation.

The ox brain lipid extract was generously provided by Drs. P. Mueller and D. O. Rudin (Eastern Pennsylvania Psychiatric Institution, Philadelphia, Pa.), and all other reagents were the same as previously used (Wilson, 1969).

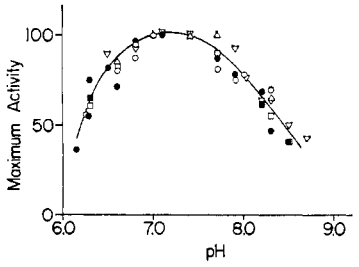

FIGURE 2: The pH dependence of the maximum rate of succinate oxidation by rat liver mitochondria. The rat liver mitochondria were suspended as given in the Methods section and titrated with uncoupler to achieve maximum respiration rate as shown in Figure 1. The measured maxima were normalized to 100 at pH 7.4 (data from 12 different mitochondrial preparations) and the rates plotted on the ordinate. The pH of the titration is plotted on the abscissa. The individual plotted points are for: (○) FCCP, (△) TTFB, (▽) S-13, (■) Br-N-CCP, (●) benzothiazole-CCP, and (□) P-Cl-TP.

Results

Respiratory Control as a Measure of Uncoupling Activity. When rat liver mitochondria in state 4 (with excess substrate but without ADP or P_i) are titrated with an uncoupler such as P-Cl-TP, there is a linear relationship between the increase in respiration rate and the concentration of uncoupler added (Figure 1). Both the slope of the titration curve and the maximum respiration rate are pH dependent.

pH Dependence of the Maximum Rate of Oxidation of Suc-

[1] Abbreviations used are: MOPS, morpholinopropanesulfonate; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; TTFB, tetrachlorotrifluoromethylbenzimidazole; Br-N-CCP, 4-bromonaphthylhydrazone of carbonyl cyanide; benzothiazole-CCP, *p*-(6-methyl-2-benzothiazyl)phenylhydrazone of carbonyl cyanide; P-Cl-TP, pentachlorothiophenol.

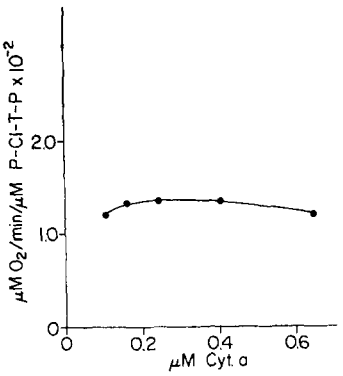

FIGURE 3: The protein concentration dependence of the uncoupling activity of P-Cl-TP. The mitochondria were suspended and titrated as given in the legend of Figure 1 except that the cytochrome *a* concentration (protein concentration) was varied as given on the abscissa. The ordinate is the slope of the measured titration curve (see Figure 1).

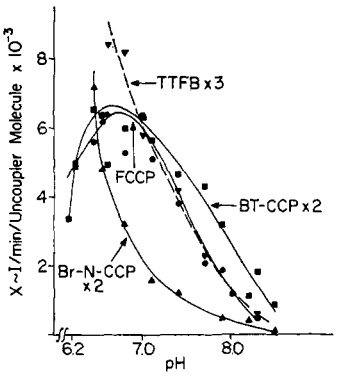

FIGURE 4: The pH dependence of the uncoupling activity of the carbonyl cyanide derivatives and TTFB. The rat liver mitochondria were titrated (as shown in Figure 1) with the designated uncoupler; the slope of the titration curve was plotted on the ordinate and the pH of the determination plotted on the abscissa. In order to fit all of the uncouplers to a single ordinate scale the measured values were multiplied by the factors indicated in the figure. As indicated in the figure, the measured numbers have been multiplied by 10^{-3} to give the ordinate scale numbers, *i.e.*, at pH 7.0 approximately 6 $\times 10^3$ high-energy intermediates are discharged per min per FCCP molecule.

cinate by Rat Liver Mitochondria. The maximum respiration rate is a function of the respiratory chain itself and is essentially independent of the uncoupler used to attain the maximum (Figure 2). A broad maximum in the rate is observed at pH 7.2–7.4 with decreasing activity on both the acid and alkaline sides of the pH maximum. The activity is decreased to 50% of the maximum by pH 6.2 on the acid side and pH 8.4 on the alkaline side. There is no significant difference in the pH profiles for any of the six uncouplers shown.

pH Dependence of the Uncoupling Activity of Several Uncouplers. The slope of the titration curve is independent of mitochondrial concentration (Figure 3) and represents a pseudo-first-order rate constant that may be expressed in the units of "high-energy" intermediates (X~I) per uncoupler molecule (Wilson, 1969) where each high-energy intermediate is assumed to be energetically equivalent to the terminal phosphate bond of ATP. It is an accurate measure of the rate at which the respiration controlling form of conserved energy may be dissipated by the designated uncoupler.

The pH profile for the slope of the titration curve is the resultant of two potentially pH-dependent terms. First, an intrinsic pH dependence arising from a pH dependence in the reaction

$$C\sim I + U^* \longrightarrow C + I + U^* \qquad (1)$$

in which C~I represents the inhibited respiratory carrier and U* represents an active uncoupler—*i.e.*, one that is in the active form and is in the physically optimum phase or location. The second source of a pH dependence comes from the necessity of correcting for the uncoupler not in the active form or location

$$U \rightleftharpoons U^* \qquad (2)$$

The pH dependence of eq 2 is most important in determining the properties of the uncoupler molecules required for their uncoupling activity. Mitochondria in aqueous suspension can be approximated as a two-phase system in which the phosphorylation enzymes in the mitochondrial membrane are in a nonaqueous phase representing less than 0.3% of the total assay volume. At the same experimental concentration a compound that partitions preferentially into the nonaqueous phase will have a much higher effective concentration in the mitochondrial membrane than will a compound that partitions preferentially into the aqueous phase.

The experimentally measured pH dependence of the uncoupling activities of several uncouplers is shown in Figures 4 and 5. In order to fit all the uncouplers to a single ordinate scale the measured values were multiplied by the factor indicated in the figures. The profile for the carbonyl cyanide derivatives is maximum at acid pH and decreases sharply with increasing pH. The actual pH region of the most rapid decrease in uncoupling activity is different for each derivative. TTFB has a pH dependence very similar to that of the carbonyl cyanide derivatives. Both types of uncouplers differ sharply in pH dependence from the salicylanilides. The latter show a clear maximum in uncoupling activity at about 7.9. P-Cl-TP has the least pH dependence of the uncouplers used in this study with very little pH dependence on the acid side

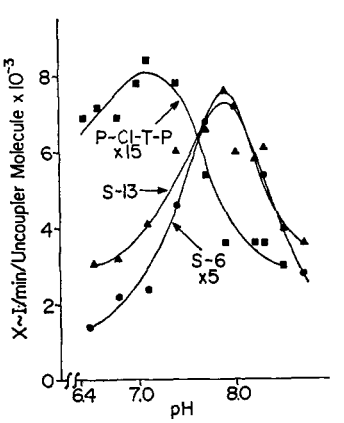

FIGURE 5: The pH dependence of the uncoupling activity of the salicylanilide derivatives and P-Cl-TP. The rat liver mitochondria were titrated (as shown in Figure 1) with the designated uncoupler. The slope of the titration curve is plotted on the ordinate and the pH of the assay medium used is plotted on the abscissa. In order to fit all of the uncouplers to a single ordinate scale the measured values were multiplied by the factors indicated.

TABLE I: Some Properties of the Uncouplers in Aqueous Solution.[a]

Uncoupler	pK	Solubility (μM) Acid Form	Solubility (μM) At the Indicated pH
Br-*N*-CCP	5.4	0.4	100 (pH 7.85)
FCCP	6.2	1	5 (pH 9.6)
Benzothiazole-CCP	7.3	0.06	0.5 (pH 9.4)
S-13	6.4	<0.1	<0.1 (pH 8.95)
S-6	6.3	<0.1	<0.1 (pH 9.6)
TTFB	5.3	20	600 (pH 8.5)
P-Cl-TP	4.3	<0.1	<0.1 (pH 8.5)

[a] The pK values were determined by optically measuring the spectral shift accompanying protonation as a function of pH. The solvents used were water, 10% ethanol in water (v/v), and 20% *N*,*N*-dimethylformamide in water (v/v) buffered with 10 mM MOPS or acetate. The solubilities were measured approximately 2 pH units to the acid side of the pK or at the indicated pH on the alkaline side of the pK.

of pH 7.1. Its activity decreases to about 40% of maximal activity by pH 8.5.

Some Physical Properties of the Uncouplers. The pK for the acid groups of the uncouplers in aqueous solution have been determined by optical methods. Where the uncouplers are not soluble in concentrations that give sufficient absorbance for titrations, the measurements were made in 10% ethanol or 20% *N*,*N*-dimethylformamide. Control experiments showed that neither the 10% ethanol nor the 20% dimethylformamide produces a measurable shift in the pK of Br-N-CCP or bromthymol blue from that obtained in an aqueous medium, and the values thus determined for the salicylanilides and carbonyl cyanide derivatives are assumed to be correct. The pK's of the uncouplers in aqueous solution (Table I) range from 4.3 for P-Cl-TP to 7.3 for benzothiazole-CCP.

A most interesting difference in the uncouplers is their relative solubilities in the aqueous mitochondrial suspending medium at the pH values used to measure uncoupling activity. As shown in Table I the salicylanilides and P-Cl-TP are quite insoluble in the aqueous phase throughout the relevant pH range and aqueous solubility need not be considered in interpretations. By contrast the TTFB and carbonyl cyanide derivatives are relatively soluble in their anionic forms as evidenced by the increased solubility of these uncouplers at alkaline pH values. The decrease in uncoupling activity of the latter uncouplers at more alkaline pH values could be the result either of the increased solubility of the uncouplers in the aqueous phase, or of a requirement for the protonated form for uncoupling or both factors simultaneously.

A Comparison of the Uncouplers with Respect to Their Ability to Uncouple Mitochondrial Respiration and Their Ability to Lower the Electrical Resistance of Lipid Bilayers. The ability of compounds with a dissociable proton to conduct protons through a lipid bilayer depends on the presence of both the protonated and anionic forms. Several theoretical treatments are available which describe the pH dependence of this property (Markin *et al.*, 1969; Lea and Croghan, 1969). The principal characteristic of the proton conductance is the sharp maximum in the pH dependence which occurs at, or somewhat alkaline of, the pK of the dissociable group (Markin *et al.*, 1969). In Figures 6, 7, and 8 the pH dependence of the ability of the selected uncouplers to increase the conductance of lipid bilayers is compared with the pH dependence of their uncoupling activity. For direct comparison the uncoupling activity is presented as a calculated value in the units of protons per min per uncoupler molecule per cm^2 of membrane surface. In the case of uncoupling activity the calculation assumes 2 H$^+$ penetrating the membrane per high-energy intermediate and 40 m^2 of membrane per g of protein (Mitchell, 1961, 1966). Such calculations are intended only for comparison of orders of magnitude.

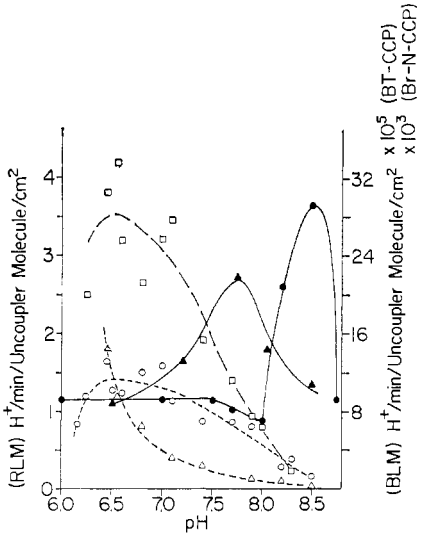

FIGURE 6: A comparison of the pH dependence of the ability of the carbonyl cyanide derivatives to uncouple oxidative phosphorylation and to increase the conductance of lipid bilayers. The uncoupling activity is plotted as a calculated value of protons per sec per cm^2 of membrane as shown on the ordinate on the left, while the increase in conductance of the lipid bilayer (BLM) is plotted against the ordinate on the right. The uncoupling activities are given with open symbols and the conductance activity given as solid symbols. (Δ, ▲) Br-N-CCP, (○, ●) BT-CCP, and (□) FCCP.

The experimental points for the mitochondrial data are more scattered on this type of plot. The reason lies in the assumption of the calculation of a dependence of activity on membrane area (protein concentration). Experimentally (Figure 3) no such dependence is observed, and corrections for the different protein concentrations used in the assays introduce a corresponding error in the relative values obtained from the experiments.

It is evident that the uncouplers do not have a direct correlation between their uncoupling activity and ability to increase the conductance of lipid bilayers (see also Ting *et al.*, 1970). More important, however, is the observation that the pH profiles are different. As expected, the pH dependence of the conductance of the lipid bilayer has a maximum at a pH value somewhat greater than the pK of the uncoupler in aqueous media. The pH profile for the effect of TTFB on the conductance of lipid bilayers not presented here has been shown by Markin *et al.* (1969) to be maximal between pH 5 and 6 and to decrease approximately 10-fold for each pH unit to either the acid or the base side of the maximum.

The pH profiles in Figure 6 show that the carbonyl cyanide derivatives have maximal uncoupling activity at pH values

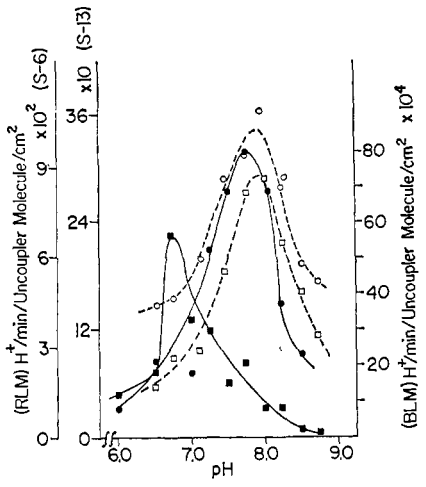

FIGURE 7: A comparison of the pH dependence of the ability of the salicylanilide derivatives to uncouple oxidative phosphorylation and their ability to increase the conductance of lipid bilayers. The experimental conditions were the same as for Figure 6. The uncoupling activities are given as open symbols and the activities for increasing the conductance of lipid bilayers are given as solid symbols. (○, ●) S-13 and (□, ■) S-6.

more acid than 6.7, while maximum increase of the conductance in lipid bilayers occurs at values greater than pH 7.5. The salicylanilides (Figure 7) have nearly the same pH profile for each activity in the case of S-13, but S-6 has a maximum in uncoupling activity at pH 7.9, a value which is more alkaline than the pH 6.6 maximum for the bilayer conductance changes. P-Cl-TP has very little effect on the conductance of the lipid bilayer at neutral pH, but has a much more pronounced effect at pH 4. The conductance changes measured at near-neutral pH for P-Cl-TP are so small that there must be some doubt as to their experimental significance.

Discussion

Uncoupler activity is dependent on pH for each uncoupler tested but differs from one chemical group to another. P-Cl-TP (Figure 8) is particularly useful in interpreting the results because its pK is very acid (4.3), and yet its anionic form is expected to be quite soluble in nonaqueous media from structural considerations (delocalized charge, etc.). Experimentally, the formation of the lipid soluble anion is strongly supported by the observation that this uncoupler greatly increases the proton conduction of lipid bilayers at pH values near 4 (Figure 8). It is reasonable to conclude that the pK of P-Cl-TP is not shifted by the 4 pH units required to make the concentration of the protonated form essentially constant from pH 6.5 to 8. The uncoupler is quantitatively associated with the mitochondrial membrane because solubility in the aqueous phase is insignificant throughout the range used to measure uncoupling activity. These considerations support the assumption that the small pH dependence in the uncoupling activity of pentachlorothiophenol arises not from the uncoupler but from the nature of the mitochondrial membrane and its relevant constituents (intrinsic pH dependence).

If we assume that the pH dependence of the uncoupling activity of the P-Cl-TP is the intrinsic pH dependence (eq 1) and correct the pH profiles of all the other uncouplers for this intrinsic pH dependence, the remaining pH dependence is the result of eq 2 and relates directly to the transition of the inactive forms of each uncoupler. In Figure 9, this correction has been applied to each of the other uncouplers. The curves drawn to the experimental points are theoretical curves for acid–base dissociations (Henderson–Hasselbalch equation). It is apparent that the theoretical curves fit the data points remarkably well. The theoretical curves are drawn by selecting a pK and a maximum uncoupler activity. The latter is a theoretical maximum activity at ideal pH and may be regarded as analogous to the maximal velocity of enzyme reactions. It is designated U_{max}. In the case of the FCCP, benzothiazole-CCP, and the salicylanilides, the pK and U_{max} are more easily selected than for the other uncouplers.

TABLE II: Some Properties of the Uncouplers as Determined by Their Uncoupling Activity.

Uncoupler	pK Aqueous	pK Uncoupling	ΔpK	U_{max} × 10^{-3}
Br-*N*-CCP	5.4	≈6.5	+1.1	≈5
FCCP	6.2	7.5	+1.3	7.5
BT-CCP	7.3	8.2	+0.9	3.5
S-13	6.4	7.6	+1.2	15
S-6	6.3	7.6	+1.3	3
P-Cl-TP	4.3			0.5
TTFB	5.3	≈7.1	+1.8	4

[a] The pK values for the aqueous phase are the same as in Table I, and the pK and U_{max} values are the parameters used to fit the theoretical curves to the data in Figure 9A,B.

In Table II the selected pK and U_{max} are presented in addition to the pK of the uncouplers in aqueous media. The pK as measured by uncoupling activity is more alkaline than that in aqueous solution by 0.9–1.8 pH units. This is good evidence that the active uncouplers are in a hydrophobic region of the membrane where the anionic form is less stabilized by interaction with water. The change in pK is equivalent to a change of 600–1200 cal in the free energy of ionization. The U_{max} values are approximate and vary from 0.5 × 10^3 to 15 × 10^3

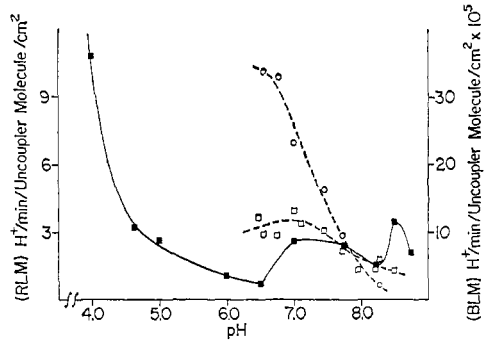

FIGURE 8: A comparison of the pH dependence of the ability of P-Cl-TP and TTFB to uncouple oxidative phosphorylation and to increase the conductance of lipid bilayers. The experimental conditions are the same as given in the legend of Figure 6. The uncoupling activities are given as open symbols, and their ability to increase the conductance of lipid bilayers is given as solid symbols. (□, ■) P-Cl-TP and (○) TTFB.

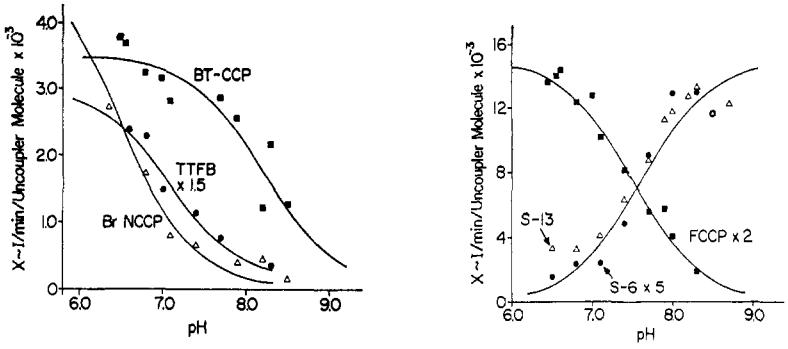

FIGURE 9: The pH dependence of the uncoupling activity of the uncouplers after correction for the intrinsic pH dependence. The uncoupling activity of each of the uncouplers is corrected by the factor required to make the uncoupler activity of P-CL-TP independent of pH. The experimental points are plotted after the appropriate correction while the drawn lines are theoretical curves drawn according to the Henderson–Hasselbalch equation for the dissociation of an acid or base. The curves for all except TTFB are constructed assuming 100% activity for either the acid or base form and 0% activity for the other form. In the case of TTFB the base form is assumed to have 4% of the activity (per unit concentration) of the acid form.

high-energy intermediates per minute per uncoupler molecule for these uncouplers.

The uncoupling activities of the carbonyl cyanide derivatives and TTFB are maximal at acid pH's and decrease as the pH becomes more alkaline. This pH dependence could arise from either of two sources: first, the anionic form is more soluble in the aqueous phase, and thus the uncoupler is effectively diluted by many fold as the pH becomes more alkaline and the uncoupler disperses into the aqueous phase; second, it is possible that the anionic form of the uncoupler is less active than the protonated form. The pH profile for the salicylanilides makes the first possibility most likely because these uncouplers are active in the alkaline form but not in the acid form. The P-Cl-TP as well must be active in the anionic form in view of its pK of 4.3 and essential lack of pH dependence in uncoupling activity. It must be concluded that uncouplers can act as either an acid or base. The minimum hypothesis is that a single catalytic site is involved and the uncouplers are acting as general acid–base catalysts. The relative catalytic activities of the acid and base forms of a compound are usually different even in general acid–base catalysis. The present experiments would detect activity by both forms if they had a combined factor due to activity and phase partition coefficient which totaled less than 10. The theoretical curve for TTFB (Figure 9B) is calculated assuming an activity of the anionic form of 4% of that of the protonated form. Some evidence is present in the deviation from the theoretical curves for activity of the anionic form of Br-N-CCP and the protonated form of S-13. These deviations are very small and more detailed experiments would be necessary to unequivocally establish their existence.

The proposed mechanisms of uncoupler action which require the presence of both anionic and protonated forms, such as the chemiosmotic hypothesis (Mitchell, *1961*, *1966*), predict a sharp maximum in the pH profile. The present data are inconsistent with such mechanisms which will not be discussed here (for discussion, see Skulachev *et al.*, 1967, Hopfer *et al.*, 1968, 1970, Liberman *et al.*, 1968, and Markin *et al.*, 1969). The data are completely consistent with the concept of uncouplers as acids or bases which catalyze the hydrolysis of a high-energy chemical intermediate present in the "hydrophobic" region of the membrane. This concept is also consistent with the apparent stoichiometry observed in the effect of uncouplers in releasing azide inhibition (Wilson, 1969) and hydroxylamine inhibition (Wilson and Brooks, 1970).

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