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ACCELERATION OF PROTON EXCHANGE BETWEEN OCTANOL AND WATER BY 2,4-DINITROPHENOL DETERMINED BY ^{1}H-NMR SPECTROMETRY

A NEW MODEL FOR PROTON TRANSFER IN A HYDROPHOBIC ENVIRONMENT

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The rate of proton transfer between the octanol -OH group and water dissolved in octanol after partition equilibrium was determined by ^{1}H-NMR spectrometry. The rate was found to depend on the pH of the aqueous phase, being minimal at about pH 11. The uncoupler of oxidative phosphorylation 2,4-dinitrophenol at about 10^{-3} M accelerated proton transfer several-fold. Its effect was shown to depend on the concentration of the neutral form of 2,4-dinitrophenol in the octanol phase, irrespective of the pH of the aqueous phase. This effect is suggested to be based on the catalytic action of the phenolic -OH group in 2,4-dinitrophenol. The importance of this effect in the uncoupling action of 2,4-dinitrophenol is discussed.

Introduction

The partitioning of bioactive compounds between a water-immiscible organic solvent and water has been used as a simple model system for evaluation of their transfer into biomembranes [1–3]. Furthermore, the hydrophobicity of these compounds, represented by their partition coefficients, is known to be well correlated with their biological activities [4–6]. In these studies, octanol has been used most often as an organic phase [7]. Thus, studies on the mechanism of partioning of bioactive compounds provide clues to understanding the mechanisms of action of bioactive compounds, especially of those which act on biomembranes.

Uncouplers of oxidative phosphorylation are typical compounds that act on the membrane. This class of compounds uncouples the link between the reactions of phosphorylation and oxidoreduction in mitochondria and other energy-synthesizing membranes, interacting nonspecifically with these membranes [8,9]. A wide variety of compounds are known to be uncouplers. Most of them, such as 2,4-dinitrophenol, are hydrophobic and are weak acids. The role of uncouplers as carriers of protons across the proton-impermeable mitochondrial membrane (protonophores) is especially noteworthy [10–15].

We recently reported that the transfer of K^+ from aqueous solution to a water-immiscible organic phase mediated by the K^+ ionophore valinomycin was greatly accelerated by the presence of uncouplers, such as SF6847, FCCP and 2,4-dinitrophenol, due to formation of a ternary valinomycin-K^+-uncoupler anion complex [16]. The unique dynamic structure of uncouplers, self-regulating their electronic properties, was sug-

Abbreviations: SF6847, 3,5-di(*tert*-butyl)-4-hydroxybenzylidenemalononitrile; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

gested to be responsible for this preferential formation of ternary complexes between various kinds of anions [17,18]. Furthermore, it was found that the uncoupler 2,4-dinitrophenol itself has the ability to act as a carrier for some cations, such as K^+ and Na^+, besides H^+ in a two-phase partition system between octanol and water at pH 7 [19].

In the present study, as an extension of these studies, we measured the ^{1}H-NMR spectrum of octanol after partition equilibrium at various pH values, and found that there was a pH-dependent proton exchange between the octanol -OH and water that had been transferred to octanol. We also found that the presence of 2,4-dinitrophenol in the octanol phase accelerated the proton exchange reaction. Since octanol is regarded as representing biomembranes very well in hydrophobic properties [7], the effect of partitioned 2,4-dinitrophenol on the microenvironment of water dissolved in octanol should be useful in understanding the mechanism of action of compounds including uncouplers acting on membranes.

Materials and Methods

2,4-Dinitrophenol from Wako Pure Chemicals Co., Osaka (Japan), was used without further purification, since it appeared pure on thin-layer chromatography. 1-Octanol from Nakarai Chemicals Co., Kyoto (Japan), was distilled three times before use. *n*-Butyltrimethylammonium hydroxide was prepared as described before [19]. Other reagents used were commercial products and were used without further purification.

Partitioning of 2,4-dinitrophenol between octanol and water was carried out at 25°C as reported previously [19]. The concentration of 2,4-dinitrophenol in the octanol phase was determined spectrophotometrically in a Shimadzu spectrophotometer, model UV 180, or model UV 300.

^{1}H-NMR spectra were recorded at 60 MHz with a Varian EM 360 NMR spectrometer at room temperature (25°C).

Results

Proton exchange between water and octanol

Fig. 1 shows the ^{1}H-NMR spectrum of octanol equilibrated with water (0.05 M phosphate buffer, pH 7.0). The signals were assigned as shown in the figure. The chemical shifts of these peaks from tetramethylsilane were as follows: CH_3, 1.0; CH_2, 1.4; αCH_2, 3.6 and OH, 4.9 ppm. It is noteworthy that the signals of the protons of the octanol -OH and water coalesce to give a single resonance.

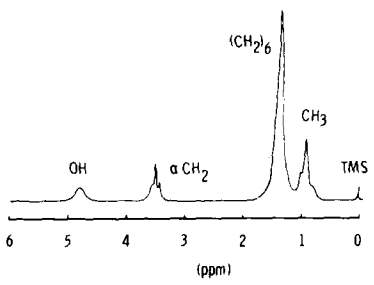

Fig. 1. ^{1}H-NMR spectrum of octanol after partition equilibrium with water at pH 7. Octanol was equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, before measurement. Tetramethylsilane (TMS) was added as a standard.

The effect of the pH of the aqueous phase on the signals of the octanol -OH and water dissolved in the octanol phase was examined. For this purpose, octanol was equilibrated with water at various pH values, and after equilibrium was attained, ^{1}H-NMR spectra of the octanol phase were recorded. As shown in Fig. 2, the signals of the octanol -OH and water in the octanol phase change with the pH of the aqueous phase. At pH 2.1, the signals of the octanol -OH and water give a very sharp single resonance at 4.9 ppm. With increase in pH, this signal becomes broader (pH 3.4 and 6.5) and then separates into two peaks at 4.4 and 5.2 ppm (pH 9.3, 10.8 and 13.5). These two peaks

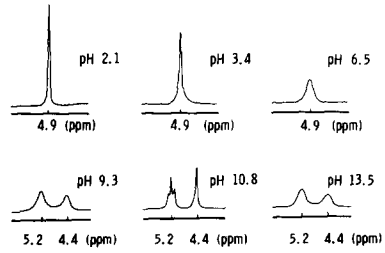

Fig. 2. Dependence of the ^{1}H-NMR signals of octanol -OH and water on pH. The spectra of the octanol phase were measured after partition equilibrium with water at various pH values. The pH values in the figure are those of the aqueous phase.

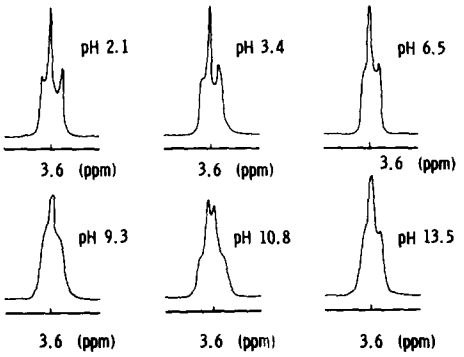

Fig. 3. Change in the pattern of octanol αCH_2- signals in the ^{1}H-NMR spectrum with pH. Experimental conditions were as for Fig. 2.

are assigned as the signals of water and octanol -OH, respectively, from the NMR spectra of pure water and octanol.

The dependence of the pattern of signals of octanol -OH and water on the pH of the aqueous phase indicates that there is a proton exchange between octanol -OH and water dissolved in octanol [20]. From Fig. 2 it can be seen that the rate of proton exchange is very great at pH 2.1, and becomes smaller with increase in pH, reaching a minimum at pH 10.8, where these two signals are essentially the same as those of pure water and octanol. Then the rate is increased again at pH 13.5. As shown in Fig. 3, the signal of αCH_2 protons also changes with pH, being affected by the proton exchange between octanol -OH and water: The signal of αCH_2 is a triplet at low pH values due to the rapid exchange of spin resonance between octanol -OH and water. However, the signal is split into a quadruplet at pH 10.8, indicating that spin-spin coupling between αCH_2 and -OH becomes apparent at the lowest rate of proton exchange. The triplet signal of the -OH proton of octanol at this pH also indicates that the spin resonance of the octanol -OH is coupled with that of αCH_2.

The proton exchange between octanol and water can be expressed by Eqn. 1:

$$CH_3(CH_2)_7OH^* + HOH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CH_3(CH_2)_7OH + HOH^* \quad (1)$$

where k_1 and k_{-1} denote the rate of proton transfer from octanol to water, and that from water to octanol, respectively.

The rate constant k_1 can be determined quantitatively by line-shape analysis of the ^{1}H-NMR spectra of octanol -OH and water by the following equations [20]. The band shape $v(\nu)$ of the signal of the NMR spectrum in the exchange system is given by Eqn. 2:

$$v(\nu) = -C_0\{P[1+\pi\tau(P_wW_{oct}+P_{oct}W_w)]+QR\}/(P^2+R^2) \quad (2)$$

The following relations also hold:

$$\delta\nu = \nu_{oct} - \nu_w \quad (3)$$

$$\Delta\nu = 0.5(\nu_{oct}+\nu_w) - \nu \quad (4)$$

$$P = \pi^2\tau\left[W_{oct}W_w - 4(\Delta\nu)^2 + (\delta\nu)^2\right] + \pi(P_{oct}W_{oct} + P_wW_w) \quad (5)$$

$$Q = \tau[2\pi\Delta\nu - \pi\delta\nu(P_{oct} - P_w)] \quad (6)$$

$$R = 2\pi\Delta\nu[1+\pi\tau(W_{oct}+W_w)] + \pi^2\delta\nu\tau(W_{oct}-W_w) + \pi\delta\nu(P_{oct}-P_w) \quad (7)$$

$$\tau = P_{oct}/k_{-1} = P_w/k_1 \quad (8)$$

where C_0 is a constant, ν and W the chemical shifts (Hz) and linewidths, respectively, of octanol -OH (subscript oct) and water (subscript w) in the absence of exchange, and P_{oct} and P_w the molar fractions of protons of octanol -OH and water, respectively. The values of ν_{oct}, ν_w, W_{oct}, W_w, P_{oct} and P_w can be obtained from the observed spectra. Using these equations, we can calculate the simulated spectra chosing an appropriate k_1 value. The

TABLE I

LIFETIME OF PROTONS IN OCTANOL OH (τ_{oct}) IN THE PROTON EXCHANGE REACTION BETWEEN OCTANOL -OH AND WATER

pH	τ_{oct} (s)
2.1	$1.2\cdot10^{-3}$
3.4	$1.7\cdot10^{-3}$
6.5	$7.3\cdot10^{-3}$
9.3	$4.8\cdot10^{-2}$
10.8	$8.8\cdot10^{-2}$
13.5	$3.7\cdot10^{-2}$

lifetime of a proton in octanol τ_{oct} can be determined by Eqn. 9:

$$\tau_{oct} = 1/k_1 \quad (9)$$

The lifetimes τ_{oct} at various pH values thus calculated are summarized in Table I. The value changes with pH, but was of the order of 10^{-3}–10^{-2} s. It is interesting that the proton exchange between octanol -OH and octanol-dissolved water proceeds more rapidly than that in a homogeneous mixture of water and ethanol: values for the lifetime of a proton in ethanol are reported to be in the range of 10^{-3}–1 s, depending on the pH [21].

Fig. 4 shows the dependence of log k_1 on the pH of the aqueous phase. As can be seen, log k_1 decreases linearly with increase in pH, to a minimum value at about pH 11, and then increases again. It is noteworthy that k_1 at extremely low pH is more than 100-fold that at pH 11. The composition of the ion species in the squeous phase has no influence on the exchange rate.

Effect of 2,4-dinitrophenol on the proton exchange

2,4-Dinitrophenol was added to the aqueous phase at final concentrations of 10^{-3}–10^{-1} M and partitioned into the octanol at pH 4, 7 and 13. After equilibrium had been attained, the ^{1}H-NMR spectrum of the octanol phase was recorded. Fig. 5 shows representative results on the effect of 2,4-dinitrophenol on the signals of octanol -OH and water. The concentration of 2,4-dinitrophenol shown in the figure is that in the octanol phase. It is apparent from Fig. 5 that at both pH 4 and 7, 2,4-dinitrophenol accelerates proton exchange between octanol and water, making the coalescent signal of protons sharper. This effect was greater at higher pH and at higher concentration of 2,4-dinitrophenol. However, at pH 13, 2,4-dinitrophenol had little effect.

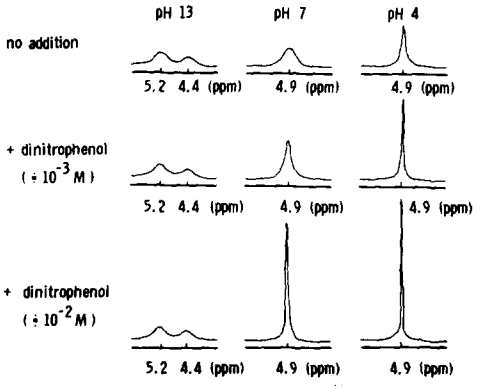

Fig. 5. Effect of 2,4-dinitrophenol on the signals of octanol -OH and water at various pH values. Experimental conditions were as for Fig. 2, except that 2,4-dinitrophenol was added to the aqueous phase before partition equilibrium. Concentrations of dinitrophenol shown in the figure are those in the octanol phase.

Fig. 6 shows the proton exchange rate k_1 as a function of the concentration of 2,4-dinitrophenol in the octanol phase after partition equilibrium. At pH 4, k_1 increased hyperbolically as the concentration of 2,4-dinitrophenol increased, being more than 4-times greater at 10^{-2} M than in the absence of 2,4-dinitrophenol. Increase in concentration of 2,4-dinitrophenol also accelerated proton exchange progressively at pH 7, but its effect was rather sigmoidal. In contrast to the results at this pH, it

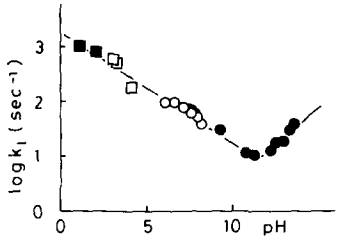

Fig. 4. pH dependence of the rate of the proton exchange reaction (k_1) between octanol -OH and water. Experimental conditions were as for Fig. 2. Various ions were added as buffer components in the aqueous phase: (■) HCl, (□) HCl and KH_2PO_4, (○) KH_2PO_4 and K_2HPO_4, (●) KOH.

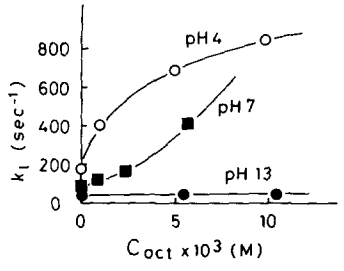

Fig. 6. Effect of 2,4-dinitrophenol on the rate of the proton exchange reaction (k_1) between octanol -OH and water. Experimental conditions were as for Fig. 5. C_{oct}, concentration of 2,4-dinitrophenol in the octanol phase after partition equilibrium.

scarcely accelerated the exchange reaction at pH 13.

Judging from its absorption spectrum in the octanol phase, 2,4-dinitrophenol is present almost entirely as the neutral form at pH 4, as the neutral and anionic forms at pH 7, and almost entirely as the anionic form at pH 13, as reported previously [19]. Probably in the octanol phase, the phenol anion is present as an ion-pair complex with cations, such as K^+, Na^+ and *n*-butyltrimethylammonium cation, added to the aqueous phase as counterions [19]. It was confirmed that these cations did not have any effect on the proton exchange rate.

From the integral of the signals of H_2O, octanol -OH and octanol αCH_2-, the amount of H_2O dissolved in octanol after partition equilibrium was calculated to be about 2.3 M, being constant over a pH range of the water phase of 1–13. The molar concentration of octanol in the octanol phase was 6.37 M. It was found that the partitioning of 2,4-dinitrophenol, either in the neutral molecular form or in the form of ion-pair complexes, did not significantly affect the water content of the octanol phase.

The findings that the exchange rate is great at acidic pH where 2,4-dinitrophenol is present almost entirely as the neutral form, and that it is small at alkaline pH where phenol is almost entirely in the anionic form suggest that the proton exchange is enhanced by the neutral form of 2,4-dinitrophenol present in octanol. Thus, we plotted the increase in the rate by phenol, Δk_1 (the difference between k_1 with and without 2,4-dinitrophenol), as a function of the concentration of the neutral form of phenol in the octanol phase after partition equilibrium.

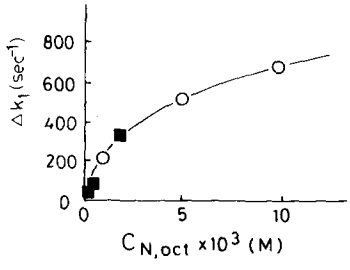

Fig. 7. Dependence of the rate of proton exchange between octanol -OH and water on the concentration of the neutral form of 2,4-dinitrophenol. Δk_1, difference between the rates of proton exchange with and without 2,4-dinitrophenol. $C_{N,oct}$, concentration of the neutral form of 2,4-dinitrophenol in the octanol phase after partition equilibrium at pH 4 (○) and pH 7 (■).

Fig. 7 shows the dependence of Δk_1 on the concentration of the neutral form of 2,4-dinitrophenol in octanol. This concentration was determined spectrophotometrically after partitioning. No value at pH 13 is shown in Fig. 7, since the amount of the neutral form at this pH is too small to be measured. It is apparent from Fig. 7 that the proton exchange increased with the concentration of the neutral form of 2,4-dinitrophenol, first almost linearly and then gradually, irrespective of the pH in the aqueous phase.

Discussion

In this study we found that after partition equilibrium H^+ exchange took place between octanol -OH and water dissolved in the octanol. We found that the rate of exchange depended on the pH of the aqueous phase: with increase in pH, it decreased to a minimum at about pH 11 and then increased again. Similar proton exchange has been observed with -OH protons in methanol and ethanol and with -NH protons in methylammonium chloride in water [22]. In these cases the proton transfer was shown to be catalyzed by H_3O^+ and OH^-, and thus the rate of transfer was minimum at pH 7, as reported for water and ethanol [21]. It is interesting to note that in the case of octanol -OH and water dissolved in octanol, the lowest exchange was at about pH 11, suggesting that the microenvironments around H_3O^+ and OH^- at this pH are similar to those in an aqueous solution at pH 7. The proton exchange in Eqn. 1 could be represented by Eqns. 10–13:

$$C_8H_{17}-OH + H_3O^+ \rightleftharpoons C_8H_{17}-O^+H_2 + H_2O \quad (10)$$

$$C_8H_{17}-OH + C_8H_{17}-O^+H_2 \rightleftharpoons C_8H_{17}-O^+H_2 + C_8H_{17}-OH \quad (11)$$

$$C_8H_{17}-OH + OH^- \rightleftharpoons C_8H_{17}-O^- + H_2O \quad (12)$$

$$C_8H_{17}-OH + C_8H_{17}-O^- \rightleftharpoons C_8H_{17}-O^- + C_8H_{17}-OH \quad (13)$$

The uncoupler of oxidative phosphorylation 2,4-dinitrophenol accelerated the proton exchange

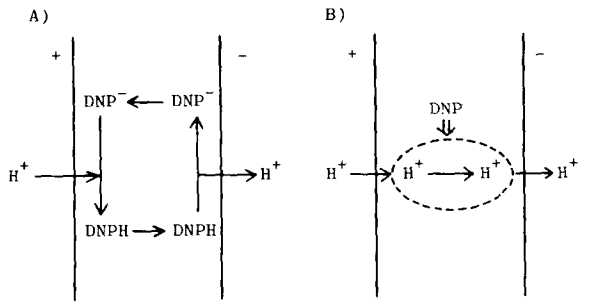

Fig. 8. Possible mechanisms of proton translocation across proton-impermeable membranes mediated by 2,4-dinitrophenol. (A) Shuttle-type mechanism. DNPH and DNP^- are the neutral and anionic forms of 2,4-dinitrophenol, respectively. (B) Proton-transfer model catalyzed by 2,4-dinitrophenol (DNP).

between octanol -OH and water to different extents depending on the pH and on its concentration. One explanation of the mechanism of the effect of nitrophenol in accelerating proton exchange is that its transfer from the aqueous to the octanol phase is accompanied by that of H_2O and that the H_3O^+ or OH^- thus transferred is responsible for the acceleration. However, this mechanism can be ruled out, since the water content of the octanol phase after partitioning of 2,4-dinitrophenol did not differ from that before partitioning.

In view of the fact that 2,4-dinitrophenol at about 10^{-3} M accelerated proton transfer several-fold, this compound could play a role as a catalyst like H_3O^+ and OH^-. In this respect, it is noteworthy that the concentrations of octanol and water are 6.37 and 2.5 M, respectively. The effect of nitrophenol depended solely on the concentration of its neutral form, irrespective of the pH of the aqueous phase, as shown in Fig. 7; although the anionic form exchanges protons with its neutral form, it has no effect on the rate of proton transfer between octanol -OH and water.

Generally, the action of uncouplers in energy-transducing membranes, such as mitochondrial membranes, is thought to be due to their protonophoric activities. The simplest shuttle-type mechanism is shown in Fig. 8A. In this case uncoupler anion (in this case DNP^-) absorbed at the membrane/solution interface picks up H^+ to form the neutral form (DNPH). It then crosses the membrane and at the other side of the membrane, it releases H^+ to become the anionic form. The uncoupler anion then moves back electrophoretically to the positively charged original membrane surface. As a result of this cycle, the uncoupler molecule transfers H^+ across H^+-impermeable membranes.

However, it is still controversial whether all actions of uncouplers are due solely to this shuttle-type mechanism [9]. The results of the present study suggest that 2,4-dinitrophenol in the membrane acts as a catalyst of movement of protons across the mitochondrial membrane by accelerating proton transfer between H_2O and certain membrane components such as proteins and phospholipids, as shown in Fig. 8B. Eventually, 2,4-dinitrophenol makes the membrane leaky to protons. Such an effect could induce uncoupling. This possibility seems worth examining.

We recently found that nonsteroidal anti-inflammatory drugs, such as indomethacin and phenylbutazone, accelerated the proton exchange reaction between octanol and water, and that their effects on proton exchange correlated well with their anti-inflammatory potencies and inhibitory actions on prostaglandin synthesis [23]. At present, it is not clear why there are such correlations between biological activities in vivo and effects in a simple model system. However, the lifetimes, τ $(= 1/k_1)$, of the proton-transfer reactions accelerated by 2,4-dinitrophenol and anti-inflammatory drugs were of the order of less than 10^{-2} s, and those of biologically important reactions are also of the same order of magnitude. Thus, it is reasonable to regard the nature of the effects of bioactive compounds on such a short-lived reaction in this model system as essentially the same as that of their biological effects in vivo. Hence, the effects of bioactive compounds on the proton-transfer reaction in this simple model system could be efficient indices for evaluating their effects in vivo and their mechanisms of action. We are now examining this possibility.

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