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Electrical Conductivity, Transfer of Hydrogen Ions in Lipid Bilayer Membranes and Uncoupling Effect Induced by Pentachlorobenzenethiol (Pentachlorothiophenol)

Pavel Smejtek, A. Ranjith Jayaweera and Kwan Hsu
Environmental Science Program, Department of Physics, Portland State University, Portland, Oregon 97207

Summary. Pentachlorobenzenethiol (PCBT) has been considered an anomalous uncoupler. It was reported as active in mitochondria, but not effective in inducing electrical conductivity in lipid bilayer membranes. We have overcome the experimental difficulties associated with accurate determination of the induced conductivity. The main contributing factors to the difficulties, we discovered, are the photolability and the low solubility of the compound in aqueous medium. We have conclusively demonstrated that PCBT does induce conductivity in lipid bilayers and compared this conductance with its uncoupling activity reported by other investigators in the literature. We present the results of steady-state current-voltage measurements: conductance dependence on applied voltage for various values of pH, buffer strength and PCBT concentration, as well as the dependence of the conductance on pH, buffer strength and PCBT concentration in the limit of zero applied voltage. We have also compared the above results with those obtained previously with pentachlorophenol. Our experimental results on PCBT-induced membrane conductance suggest that PCBT belongs to class II uncouplers and that "disulfide dimer" of PCBT is membrane inactive. Thus the replacement of oxygen in molecular structure of pentachlorophenol (R-OH) by sulfur (R-SH) does not change the protonophoretic activity of the compound. The conductivity of a membrane is due to PCBT-induced hydrogen ion transfer and it was found to be limited by the kinetics of reactions coupled to transmembrane charge transfer. The kinetic limitations became prominent at higher PCBT concentrations and at both low and high pH. Our findings support the existence of correlation between the uncoupling effect and the magnitude of membrane electrical conductance associated with the protonophoretic effect because (1) the pH dependence of PCBT-induced membrane conductance was found to be similar to the pH dependence of its uncoupling activity in rat liver mitochondria, and (2) PCBT, which induced greater membrane conductivity than PCP, was also found to be a more effective uncoupler.

Key Words pentachlorobenzenethiol · pentachlorothiophenol · uncouplers · membrane conductivity · lipid bilayers

Introduction

Chloro- and nitro-substituted phenols, salicylanilides, benzimidazoles and carbonyl cyanide hydrazones are small molecules classified as uncouplers that ionize with the release of a hydrogen ion, typically within the pH range 3 to 9, and disrupt the energy conversion process in cells. Specifically, in their presence, the free energy of oxidation of substrates is dissipated as heat [23]. There are two dominating and competing hypotheses attempting to explain the membrane action of uncouplers. According to the chemiosmotic models [17, 18], biological activity of uncouplers is intimately associated with their ability to transfer hydrogen ions across energy-transducing membranes. Weak acid uncouplers induce electrical conductivity in lipid bilayer membranes due to their protonophoretic action [5, 24]. An alternative hypothesis assumes specific interaction between the uncoupler molecule and the protein component of electron transport chain [35]. The latter concept of uncoupling has been strongly supported by the results of binding studies with 2-azido-4-nitrophenol (NPA) [6–8], 2-nitro-4-azidocarbonylcyanide phenyl hydrazone (N_3CCP) [10] and their ^3H analogs [8, 11]. The results of binding studies indicate the existence of high affinity binding sites in the inner mitochondrial membrane and it is argued that this binding site or sites mediate the uncoupling action [10, 11].

The question of the significance of specific binding to components of electron transport chain remains unresolved. The opponents of the specific binding hypothesis point out that binding of very potent uncouplers, such as 3,4-di-tert-butyl-4-hydroxybenzilidenmalonitrile (SF6847) to mitochondria does not parallel its uncoupling activity [30]. Other uncouplers, such as 5-chloro-3-tert-butyl-2′-chloro-4′-nitro-salicyl anilide (S13) were found to bind more strongly to mitochondria than to uncoupler SF6847, but were less effective as uncouplers. Models based on the assumption of specific interaction between uncouplers and proteins imply binding of at least one uncoupler molecule

per respiratory chain. Terada [30] found that under the conditions of maximum uncoupling activity, the ratio of bound SF6847 molecules to the number of electron transport chains in rat liver mitochondria was about 1/3 or 1/4. This experimental finding contradicts the models of uncoupling action based on specific binding, which require a stoichiometric binding ratio of 1:1.

The chemiosmotic models of uncoupler action do not require the assumption of specific interaction between the uncoupler and the energy-transducing proteins. The dissipation of hydrogen ion free energy can be achieved by passive transfer of protons across the lipid matrix of energy-transducing membranes. Thus the existence of a correlation between the electrical conductivity induced in lipid bilayer membranes by uncouplers and their uncoupling activity is consistent with the chemiosmotic models. Such a correlation has been reported in some [1, 14, 16, 24] but disputed in other studies [31, 36]. The issues of protonophoretic action of uncouplers in biological and artificial membranes have been discussed in great depth in a review by McLaughlin and Dilger [16].

The existence of some compounds that would uncouple oxidative phosphorylation but fail to exhibit membrane electrical conductivity due to the protonophoretic effect can be regarded as a strong argument against the concept of the protonophoretic mode of uncoupling. Several compounds meeting the above criterium, i.e. failing to induce electrical conductivity in lipid bilayer membranes, have been reported [6, 31, 36]. One of them was thiosalicylic acid; another one was pentachlorobenzenethiol, also called pentachlorothiophenol. The controversial action of thiosalicylate, specifically the absence of induced electrical conductance in lipid bilayer membranes, was resolved by McLaughlin and Dilger [16]. They demonstrated that it was necessary to use highly purified lipids to determine the conductivity with much reliability.

We compare the electrical conductance characteristics of lecithin-cholesterol bilayers modified by the presence of pentachlorobenzenethiol (PCBT) and pentachlorophenol (PCP). The structures of both compounds are shown in Fig. 1. Both are known to be uncouplers of oxidative phosphorylation [32–34, 36]. PCP is a commonly used pesticide that has been extensively studied [13, 21, 22, 25, 26, 32–34]. We have found, for example, that the onset of toxic effect due to PCP measured by the rate of carbon assimilation by an alga, occurs in parallel with the decrease of membrane electrical resistance [9].

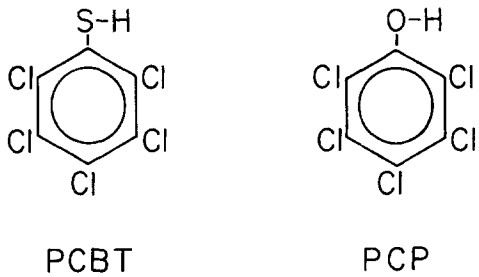

Fig. 1. Molecular structure of pentachlorobenzenethiol (PCBT) and pentachlorophenol (PCP)

The effect of PCBT on oxidative phosphorylation in rat liver mitochondria and on electrical conductivity of the lipid bilayer membranes have been studied by Wilson et al. [36]. These authors also measured conductance of lipid bilayer membranes in the presence of PCBT at several values of pH and obtained no significant change of membrane conductance around neutral pH, with the exception of an approximately threefold increases at pH 4. The absence of a correlation between uncoupling activity in mitochondria and membrane electrical conductivity was used as an argument against the presence of protonophoretic mode of action of PCBT.

In the paper we address the question of the absence of a correlation between the uncoupler activity and the induced membrane conductivity. We demonstrate, contrary to a previous report [36], that the induced membrane conductance was about 10^3-fold above the background, and furthermore, that the pH dependence of PCBT membrane conductance resembles closely the pH dependence of PCBT uncoupling activity.

Materials and Methods

Purification of Pentachlorobenzenethiol

Pentachlorobenzenethiol (PCBT) was obtained from Dupont (RPA No. 6), and purified according to the method of Lucas and Peach [15]. The PCBT of technical grade was extracted three times with aqueous caustic soda, and the filtrate was acidified with HCl to obtain thiol precipitate, which was filtered off, washed and vacuum dried. The thiol was further purified by decolorizing it with activated charcoal in toluene solution instead of benzene. The filtrate was allowed to stand for crystallization. The crystals were dissolved in ammoniacal aqueous solution containing 50% ethanol to remove any disulfide formed in the previous steps, and the filtrate was further acidified to give purer crystals. The crystals were vacuum dried, and their melting point verified (m.p. 241.5 to 242 °C). The purified crystals were further purified by sublimation at 100 °C in vacuum.

PCBT is very light sensitive. The ultraviolet spectra of PCBT decane solution left in the laboratory and exposed to room light, indicate that PCBT slowly oxidizes into bipenta-

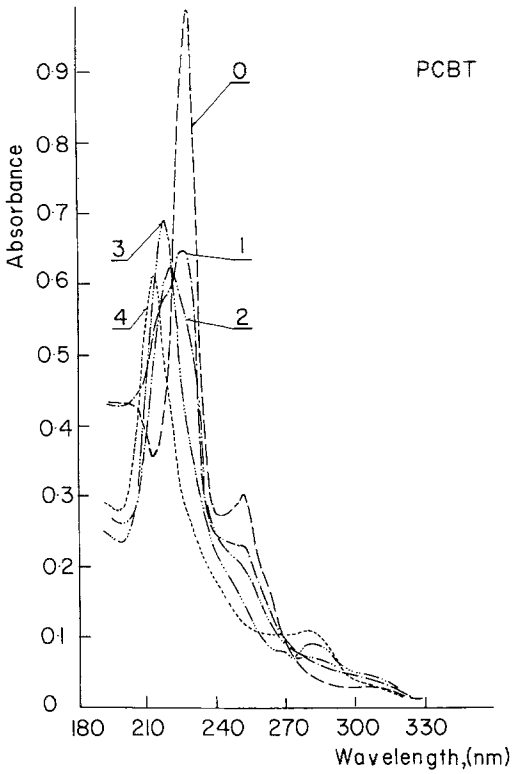

Fig. 2. UV spectra of 20 μM of PCBT in decane continuously exposed to room light in a course of 29 days. 0 – freshly prepared solution; 1 – after 21 hr; 2 – after 29 hr; 3 – after 67 hr; 4 – after 650 hr. The last spectrum corresponds to that of synthesized "disulfide dimer" of PCBT

chlorophenyl disulfide, $(C_6Cl_5)_2S_2$, the "disulfide dimer" of PCBT. Figure 2 gives the UV spectra of 20 μM PCBT in decane, taken at various times during a period of 29 days. It shows that in the first 11 hr, the maximum absorption peak at 227 nm decreased continuously from an absorbance of 0.98 to 0.80, indicating a half-life of about 34 hr. This peak then slowly shifted toward lower wavelengths with decreased absorbance until it reached a lowest value of 0.62 at 217 nm after 29 hr. The transformation continued. The absorbance of the maximum peak decreased until the absorbance reached the minimum value of 0.60 at an even lower wavelength at 211 nm. The last spectrum, which was taken after 29 days, corresponds to that of a "disulfide dimer" of PCBT, which we have synthesized.

Preparation of "Disulfide Dimer" of PCBT

Bipentachlorophenyl disulfide was synthesized from the purified PCBT by the method of Tadros and Saad [29]. Phosphorus pentachloride (0.37 g) was added to a solution of PCBT in carbon tetrachloride (0.5 g/10 ml); the mixture was heated on a water bath for 10 min and left overnight. Carbon tetrachloride was removed and the residue was treated with water, filtered off, washed with a few ml of 3% NaOH, and then with water. The disulfide, which was crystallized from acetic acid after the carbon tetrachloride treatment, according to Tadros and Saad, had a melting point of 225 °C, while the disulfide which was crystallized from benzene was reported to have a melting point of 234 to 235 °C.

Membrane Experiments

Optically black lipid membranes were formed on a 2-mm diameter hole in a wall of TFE (tetrafluoroethylene resin) cell using the brush method. The membrane-forming solution contained egg lecithin, cholesterol and a membrane modifier (PCBT or PCP) dissolved in *n*-decane. The lipid content was approximately 10 mg/ml. All solutions containing PCBT were kept in the dark, sealed under nitrogen and were spectroscopically monitored for PCBT decay. The incorporation of PCBT into the membrane-forming solution is essential, because if PCBT were present only in the aqueous medium, the membrane conductance would increase very slowly and never reach a stationary level. Due to the low solubility of PCBT in water (about 6.5 μM), and to avoid any artifact associated with the presence of PCBT suspensions in the aqueous medium, all experiments were performed with PCBT incorporated in the membrane-forming solution. The aqueous medium contained only electrolytes: 0.5 M KCl, and phosphate/citrate/borate buffer at concentrations 0.02:0.02:0.005 M, except in the one case noted. All measurements were done at room temperature, 21 to 23 °C. At a high buffer concentration the stirring effect was unimportant.

The steady-state current-voltage characteristics were measured after the current stabilized, which was monitored by periodic application of 25-mV pulses. The area of the hole was used for the computation of conductance. The escape of PCBT from the membrane and membrane torus into the aqueous phase did not present a serious problem, because the efflux is very small due to the very low solubility of PCBT in water. The concentration of PCBT in the membrane is maintained by its diffusion from the torus, which acts as a PCBT reservoir. Only in the case of PCP the membrane current gradually decreased with time at high pH. Under such circumstances, the data were taken as soon as an apparent equilibrium was reached. The data points represent an average obtained on at least 4 membranes.

Results

pH Dependence of PCBT-Induced Membrane Conductance

Figure 3 illustrates the steady-state membrane conductance as a function of pH. Several features of the experimental results are significant. First, the high level of membrane conductance was only obtainable with PCBT incorporated in the membrane-forming solution; the zero-voltage conductance was found to be 3 orders of magnitude higher than the background conductance. Second, the bell-shaped pattern of the conductance indicates that both the neutral and the ionized species participate in the formation of membrane-permeable ions. Third, the induced conductance was found to be rather insensitive to the cholesterol content of the membrane; the set of data points obtained with 0.2 mole fraction of cholesterol does not differ significantly from that obtained with 0.8 mole fraction. It is interesting to see that the pH dependence of the PCBT-induced conductance is similar to the pH dependence of the uncoupling activity of PCBT

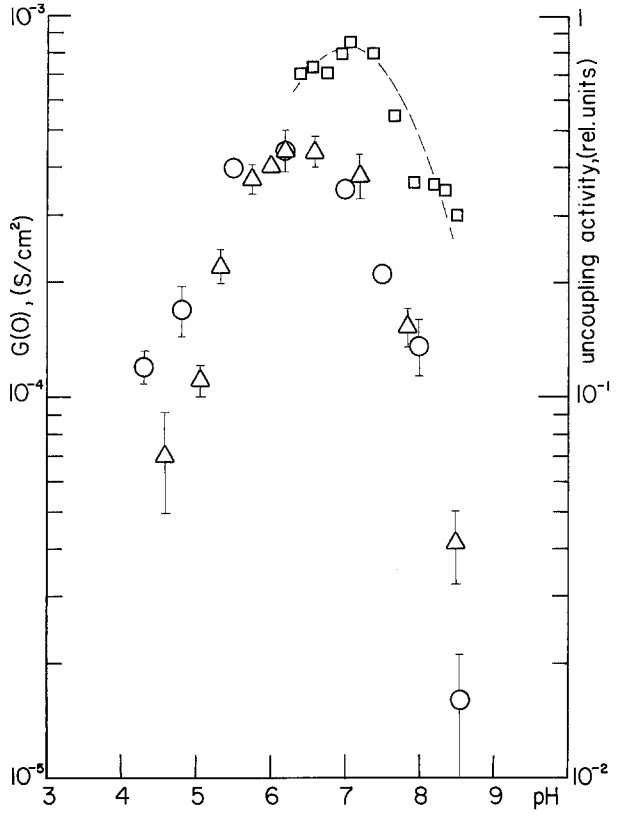

Fig. 3. Comparison of PCBT-induced conductance in lecithin-cholesterol membranes with the uncoupling activity of PCBT in rat liver mitochondria. Circles – membranes with cholesterol mole fraction = 0.2, and $C_{PCBT} = 10$ mM. Triangles – membranes with cholesterol mole fraction 0.8, and $C_{PCBT} = 10$ mM. Uncoupling activity is shown by squares; data obtained from reference [36]

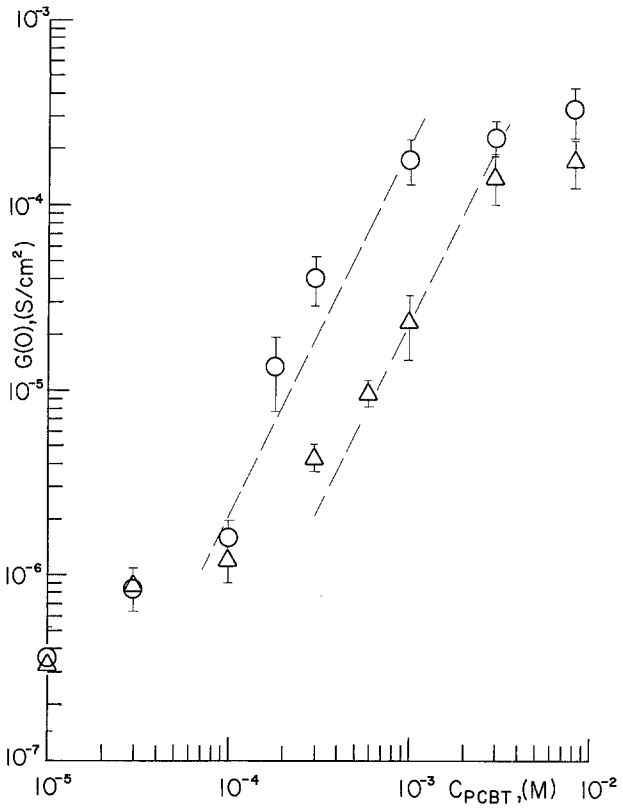

Fig. 4. Dependence of membrane conductance on PCBT concentration. Circles – aqueous phase pH = 7; triangles – aqueous phase pH = 5.5. The broken lines indicate a quadratic dependence of conductance on the concentration

in rat liver mitrochondria (indicated by squares in Fig. 3), as reported by Wilson et al. [36]. These points were taken from Fig. 5 in the above-mentioned reference. The points were given as the slopes of the titration curves of rat liver mitochondria with PCBT at the designated pH values.

Dependence of Membrane Conductance on PCBT Concentration

The data in Fig. 4 establish that above 3×10^{-5} M of PCBT in the membrane-forming solution and pH in the vicinity of the conductance maximum, the membrane conductance increases with the PCBT concentration, with a slope greater than unity. This suggests that probably two PCBT molecules participate in the formation of membrane-permeable ion. The tendency toward the saturation of conductance above 3×10^{-3} M of PCBT in the membrane-forming solution can be associated with the distribution of PCBT between the membrane torus and the bilayer membrane.

Effect of Buffering Capacity of the Aqueous Solution

The membrane conductance measurements indicate that the rate of transfer of electric charge across the membrane induced by PCBT is strongly dependent upon the buffering capacity of the aqueous medium surrounding the membrane. The magnitude of the effect is shown in Fig. 5. In spite of the high concentration of electrolyte, $C_{KCl} = 0.5$ M, the aqueous solution with low concentration of buffer cannot maintain a high level of membrane conductance. In addition, at low buffer concentration, the bell-shaped pattern of the dependence of membrane conductance on the pH (as seen in Fig. 3) disappears. Slow transient currents, indicative of the development of diffusion polarization regime [19] were observed when the voltage bias across the membrane was changed. Such electrical response supports the hypothesis that the charge transfer across the membrane is accompanied by the diffusion of protons in the immediate vicinity of the membrane [3, 20, 25].

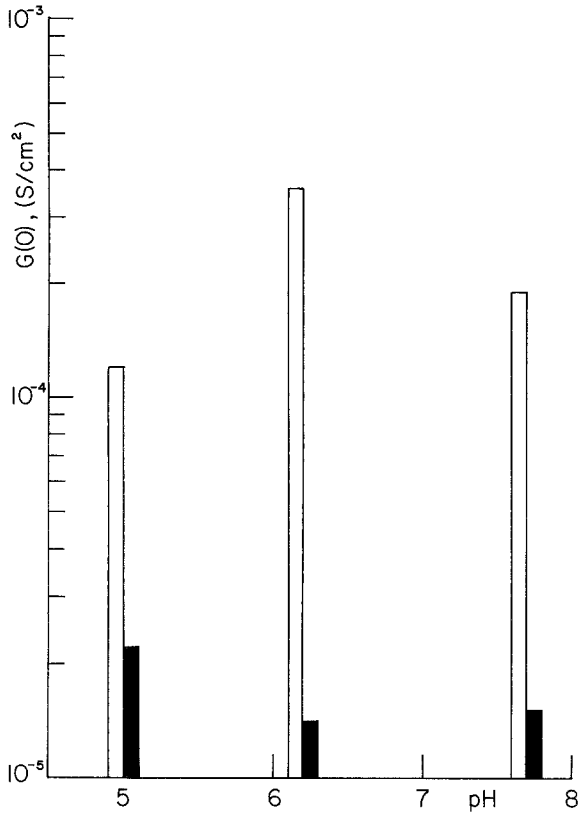

Fig. 5. Effect of buffering capacity of the aqueous phase on the conductance of electrolyte/membrane/electrolyte system; $C_{PCBT} = 10$ mM, cholesterol mole fraction = 0.8; open bars indicate the conductance in well-buffered solution, with the concentrations of the buffer components phosphate/citrate/borate = 0.02:0.02:0.005 M; closed bars indicate the conductance in weakly buffered solution, with the concentration of the buffer components = 2×10^{-5}:2×10^{-5}:5×10^{-6} M

A Comparison of the Effect of PCBT and PCP on Membrane Conductivity

In order to provide some insight into the relationship between the molecular structure and the effect of the compound on membrane conductivity, we performed similar experiments with PCP, by incorporating PCP in the membrane-forming solution. The results obtained with PCP in the membrane-forming solution were found not to be different from those with PCP in the bathing medium, as reported earlier [25]. For given concentrations of uncoupler the conductance of PCP membranes was always found to be smaller, typically by about one order of magnitude, than those treated with PCBT. Furthermore, the voltage dependences of membrane conductance of PCP and PCBT containing membranes were distinctly different. This can be concluded from Fig. 6*a* and 6*b*, which show the normalized membrane conductance, $G(V)/G(O)$, as a function of applied voltage. For PCP-treated membranes, the membrane conductance increases with the applied voltage, and within the experimental error the normalized conductance is pH independent. In contrast, for PCBT-treated membranes the normalized membrane conductance either remains close to unity or decreases with the applied voltage. For comparison we have shown in Fig. 6 by broken line, the ratio of sinh(eV/2kT)/(eV/2kT), which represents the case of transmembrane charge transport solely limited by electrodiffusion across the membrane potential energy barrier.[1] The fact that the experimental $G(V)/G(O)$ data are considerably below the broken curve indicates that the charge transport across the membrane is limited by other processes coupled to the charge translocation across the membrane [12, 28]. This conclusion is supported by our observation of fast transient currents (time constant 10^{-5} to 10^{-4} sec) in response to change of the applied voltage. The magnitude and time dependence of the transients have been found to be a function of the pH, applied voltage, and membrane modifier concentration.[2] The above steady-state data clearly indicate that the PCBT-induced charge transport is significantly more limited by chemical kinetics than that induced by PCP. Data in Fig. 7 show how the kinetic limitations develop with the increasing concentration of PCBT in membranes. Furthermore the PCBT-induced conductance exhibits an interesting pattern of the dependence of $G(V)/G(O)$ on the pH: the kinetic limitations of transport (manifested by the smaller slope of $G(V)/G(O)$ versus V), are more pronounced at both the low and high ends of the pH range (*compare* Fig. 6*b* and 3).

Discussion and Conclusions

In this work we have experimentally demonstrated the existence of disputed membrane conductivity induced by PCBT. Its significance rests in removing another compound from the list of those used in the past as an argument against the existence of correlation between the phenomenon of uncoupling of oxidative phosphorylation and induced membrane conductivity.

Our major findings show that PCBT induces electrical conductivity in lipid bilayers as other

[1] The implied assumptions are that the profile of ion potential energy barrier is not distorted by the applied electric field, space charge, and that the applied electric potential difference is fully effective in driving transmembrane transport.

[2] The scattering of current relaxation data due to the large variation of relaxation parameters from different membranes did not make it possible to arrive at a meaningful kinetic analysis of PCBT-induced charge transport.

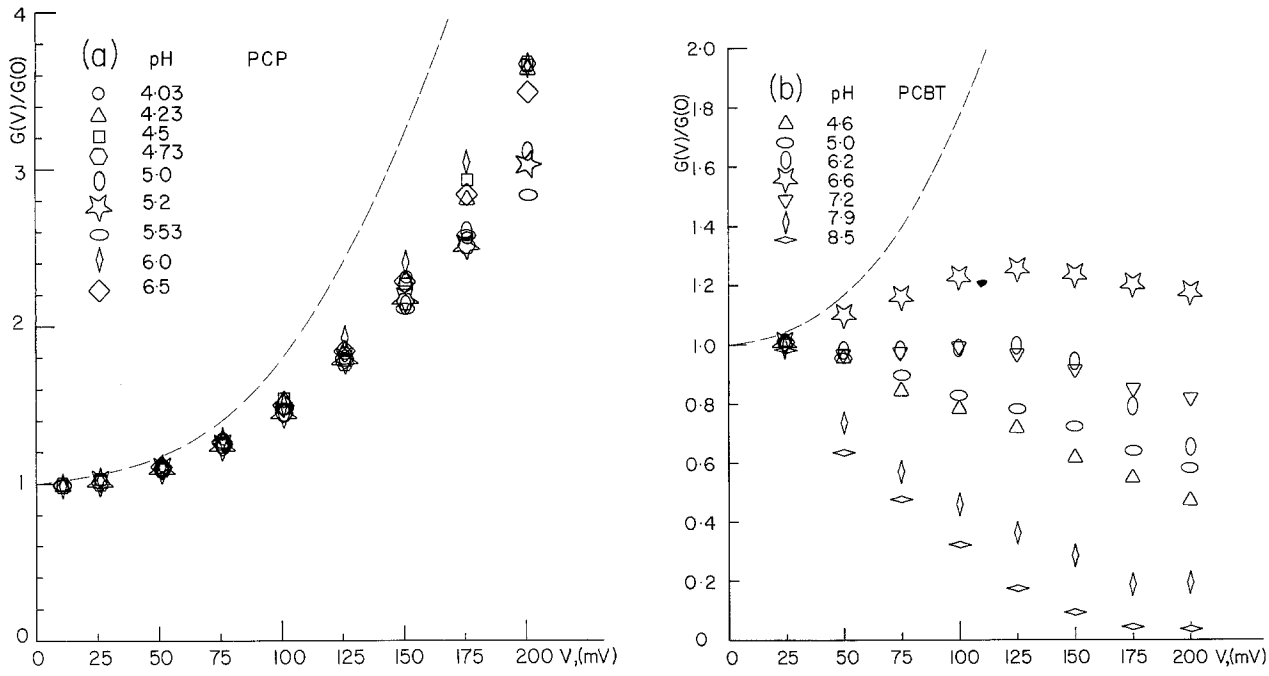

Fig. 6. *a.* Voltage dependence of normalized membrane conductance of PCP-treated membranes; $C_{PCP}=10$ mM in the membrane-forming solution, cholesterol mole fraction = 0.8. The broken curve corresponds to the normalized conductance for transmembrane transport limited by a central membrane barrier. *b.* Voltage dependence of normalized conductance of PCBT-treated membranes, indicating strong kinetic limitations of membrane conductance; $C_{PCBT}=10$ mM in the membrane-forming solution, cholesterol mole fraction = 0.8. The broken curve corresponds to the normalized membrane conductance for transmembrane transport limited by a central membrane barrier

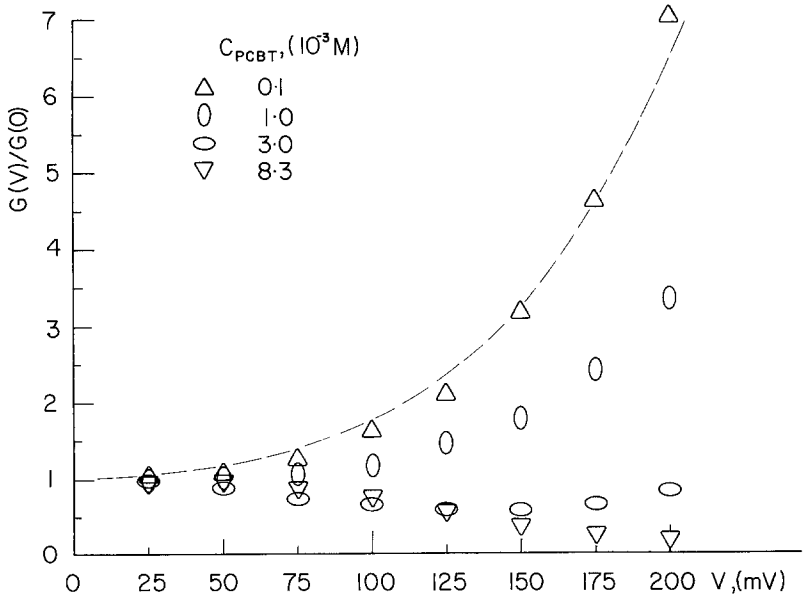

Fig. 7. Voltage dependence of normalized conductance of PCBT-treated membranes, indicating the development of kinetic limitations of transmembrane transport with increasing concentration of PCBT; pH = 5.5, cholesterol mole fraction = 0.2. The broken curve corresponds to the normalized membrane conductance for transmembrane transport limited by a central membrane barrier

conventional uncouplers. In addition its pH dependence resembles the pH dependence of PCBT uncoupling activity as reported for rat liver mitochondria by Wilson et al. [36]. The problem of correspondence of the mechanism of action of uncouplers in energy transducing membranes and lipid bilayers is of current interest. Benz and McLaughlin [2] have recently completed a detailed kinetic study of hydrogen ion transfer induced in membranes by uncoupler FCCP and made comparisons between FCCP action in lipid bilayers and mitochondrial membranes. They have shown that best agreement between the pH dependence of FCCP uncoupling activity in mitochondria and

membrane conductance due to FCCP-induced hydrogen ion transfer is achieved for bilayer membranes with dielectric characteristics resembling those of inner mitochondrial membrane.

The earlier failure in determining membrane electrical conductivity in the presence of PCBT [36] is due to several unfavorable properties of the compound. The most important one is its photolability (*see* Fig. 2). One also has to take special care to prepare true aqueous solutions rather than suspensions. The equilibration time of PCBT between the aqueous solution and the membrane is extremely long and thus only direct incorporation of PCBT into the membrane-forming solution made it possible to obtain meaningful results. Although we were unable to obtain reproducible results from our attempts to study kinetics, the steady-state data yielded some basic insight into the mechanism of PCBT-induced membrane conductivity. The following features of membrane conductivity indicate that PCBT acts in the membranes as conventional class II uncoupler. It means that the replacement of O-H group in pentachlorophenol by S-H does not impart to the PCBT molecule any unusual characteristics. The most relevant features of our experimental results are:

(a) the bell-shaped pH dependence of membrane conductance which was found insensitive to cholesterol content in the membrane;

(b) the "superlinear" dependence of membrane conductance on concentration of PCBT incorporated directly into the membrane; and that

(c) the movement of charge across the membrane is supported by the transport of hydrogen ions within the aqueous part of the membrane/water interfacial region.

The voltage dependence of normalized membrane conductance, $G(V)/G(O)$, provides information on membrane transport kinetics. It follows from the concept of carrier-mediated electrodiffusion that the drop of $G(V)/G(O)$ below that given by (2kT/eV)sinh(eV/2kT) is a measure of kinetic limitation [27]. For weak acid uncouplers such a limitation is usually observed at pH above the pK. This effect is associated with the unavailability of the neutral form, due to uncoupler ionization, to maintain the high rate of backflow of neutral uncoupler required to support the initial rate of charge translocation due to the transmembrane electric field [27]. This type of limitation was also present in PCBT-treated membranes; at high pH the slope of $G(V)/G(O)$ versus V decreased with increasing pH (Fig. 6*b*). In addition, we found that PCBT-induced hydrogen transfer became kinetically limited at low pH, at the value below that corresponding to conductance maximum. This effect, the drop of $G(V)/G(O)$ with decreasing pH, can be related to charge transfer limitation within the interfacial region. This is either due to an interfacial transfer of the ionized form of PCBT, slow rate of recombination of hydrogen ions with the ionized form of PCBT in case of heterogeneous surface reaction, or slow rate of formation of membrane-permeable dimers, HA_2. The kinetic limitation at low pH was also observed in relaxation studies with uncoupler TTFB by Dilger and McLaughlin [4]. These authors suggested that the effect was associated with reorientation of the ionized form of TTFB by the applied electric field. We would like to point out that molecular orbital calculation of electron density distribution in PCBT (R-SH) and PCP (R-OH) indicated reversal of net charge density on the R-portion of the molecules when oxygen is replaced by sulfur. This suggests the possibility of reversal of molecular electric dipole moment and different orientation of PCBT molecule, as compared to PCP, at the membrane/water interface.

The comparative studies with PCBT and PCP directly incorporated into the membrane have indicated that for a given uncoupler concentration the conductivity of membrane with PCBT and therefore the rate of PCBT-induced rate of hydrogen ion transfer was about one order of magnitude greater than that for PCP. Interestingly enough, Katre and Wilson [10] have also found PCBT to be a more effective uncoupler than PCP. The concentration of PCBT required for 50% uncoupling of pigeon heart mitochondria was found to be lower compared to that for PCP [10].

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