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COMPARATIVE TOXICITY OF 4-CHLOROBIPHENYL AND ITS METABOLITE 4-CHLORO-4′-BIPHENYLOL IN ISOLATED RAT LIVER MITOCHONDRIA

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Abstract—4-Chlorobiphenyl (4-CB) is converted by the microsomal cytochrome P-450 system to its hydroxylated metabolite 4-chloro-4′-biphenylol (4′-OH-4-CB). A study of the effects of 4-CB and 4′-OH-4-CB on the energy-linked functions of rat liver mitochondria was carried out. 4′-OH-4-CB was more effective than 4-CB in causing the inhibition of state 3 respiration of mitochondria with both succinate and glutamate/malate. As a substrate specificity, with glutamate/malate the inhibition by each compound (ID_{50}, 30 μM for 4′-OH-4-CB, 76 μM for 4.CB) was more significant than that with succinate (ID_{50}, 200 μM for 4′-OH-4-CB, never reached 50% for 4-CB). From the effects on DNP-stimulated respiration, it was indicated that the electron transport from both glutamate/malate and succinate to oxygen was more sensitively inhibited by 4′-OH-4-CB than by 4-CB, with the same substrate specificity as for state 3 respiration (i.e. the inhibition by both compounds was greater with glutamate/malate than with succinate). Since there existed a good coincidence in the inhibition between state 3 and DNP-stimulated respiration with both substrates, the inhibition of state 3 respiration by both compounds was due to the inhibition of the electron transport. With succinate, the uncoupling of oxidative phosphorylation by both compounds was observed, the extent of which was greater with 4′-OH-4-CB than with 4-CB, although the uncoupling by higher concentrations of 4′-OH-4-CB was masked because of the increased inhibition in respiration. With glutamate/malate, the uncoupling action of 4-CB was largely, while that of 4′-OH-4-CB was completely, masked by progressive respiratory inhibition. 4′-OH-4-CB was more effective than 4-CB in causing stimulation of latent ATPase in mitochondria. These results indicate that both 4-CB and 4′-OH-4-CB impair mitochondrial energy-transducing functions, but 4′-OH-4-CB is more effective than 4-CB in damaging these functions. Thus, the product of the metabolism is more biologically active than the parent compound. The impairment of energy-linked mitochondrial reactions by the metabolite as well as of the parent compound may be an important factor in the toxicity of 4-CB.

Polychlorinated biphenyls (PCBs) are persistent organochlorine chemicals ubiquitously distributed as environmental pollutants [1]. PCBs taken up in mammals are metabolized primarily by the hepatic microsomal mixed function oxidase system. In general, the less chlorinated PCBs are more readily metabolized than the highly chlorinated ones [2]. PCBs are complex mixtures of many of the 209 possible isomers and congeners, and the evaluation of the effects of PCBs on human health is dependent on a knowledge of the properties of both individual isomers and their metabolites. The extent to which the metabolism affects PCB toxicity has been only partially elucidated. Some hydroxylated metabolites of PCBs exhibit greater toxicity than the parent compounds. For example, the monohydroxylated metabolite of 2,4,3′,4′-tetrachlorobiphenyl has a significantly lower LD_{50} in mice than the parent compound [3].

4-Chlorobiphenyl (4-CB) has been shown to be hepatotoxic, irrespective of its short stay in the body compared to highly chlorinated PCBs [4, 5]. Hepatocytes from rats treated with 4-CB (50 mg/kg/day) for 3 days had a proliferation of smooth endoplasmic reticulum, alteration of the rough endoplasmic reticulum, and increased numbers of lipid droplets and microbodies [5]. 4-CB also caused necrotic foci, centrolobular necrosis and proliferation of biliary ductules in hepatocytes of rats treated with 100 mg/kg/day for 7 days [5]. 4-CB is metabolized by rat liver microsomes to yield a major metabolite, 4-chloro-4′-biphenylol (4′-OH-4-CB) [6].

Mitochondria are of importance during the development of cellular damage, because they possess the energy-transducing functions. Owing to its high yield, mitochondrial oxidative phosphorylation is responsible for supplying over 95% of the total ATP requirement in eukaryotic cells [8].

In a previous paper, we reported the effects of 4′-OH-4-CB on several functions of mitochondria [9]. 4′-OH-4-CB uncoupled oxidative phosphorylation with succinate as the respiratory substrate, but did not with glutamate/malate. 4′-OH-4-CB severely inhibited the NADH-CoQ span of the electron transport chain [9]. The present study was undertaken to compare the effects of parent compound, 4-CB and its metabolite, 4′-OH-4-CB on mitochondrial functions, and the extent to which the metabolism affects the toxicity to mitochondria has been elucidated.

MATERIALS AND METHODS

Chemicals. 4-Chlorobiphenyl (4-CB) and 4-chloro-4′-biphenylol (4′-OH-4-CB) were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan) and

Ultra Scientific (Hope, RI), respectively. The stock solutions of these compounds were prepared in ethanol. Adenosine 5′-diphosphate (ADP), adenosine 5′-triphosphate (ATP), bovine serum albumin, oligomycin, phenazine methosulfate (PMS), and valinomycin were obtained from Sigma Chemicals Co. (St Louis, MO). 2,4-Dinitrophenol (DNP) and tetraphenylphosphonium chloride (TPP^+) were from Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents were of the highest purity commercially available.

Isolation of mitochondria. Mitochondria were isolated by the method of Hogeboom [10] from the livers of adult male Wistar albino rats weighing 200–300 g in a medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), and 0.1 mM EDTA. EDTA was excluded in a final wash and resuspension. Protein concentration was determined by the biuret reaction using bovine serum albumin as a standard [11].

Measurements of mitochondrial respiration. The respiration of mitochondria was measured polarographically using a Clark-type oxygen electrode in a closed 2 ml glass vessel mixed with a magnetic stirring bar at 25°, with temperature regulated by a thermostatically controlled bath. The respiratory buffer used to measure mitochondrial respiration consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 5 mM potassium phosphate (pH 7.4). The respiratory substrates were 5 mM succinate, 5 mM glutamate/5 mM malate, and 5 mM ascorbate/0.1 mM *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine (TMPD). To determine the effects on state 3 and DNP-stimulated respiration, test compound was added to the reaction vessel shortly after the addition of excess ADP (1.25 mM) and 20 μM DNP, respectively. Effects on state 4 respiration were determined by adding test compound to the reaction vessel during state 4 respiration.

Assay of ATPase activity. ATPase activity was determined in a reaction mixture (final volume 2.0 ml) containing 0.15 M KCl, 3 mM $MgCl_2$, 5 mM Tris–HCl (pH 7.4), and 2.0 mg of mitochondria; when present, DNP was 20 μM, and oligomycin was 2.0 μg. The reaction was initiated by the addition of ATP at a final concentration of 5 mM. After interaction for 10 min at 25°, the reaction was terminated by adding 0.5 ml of 40% trichloroacetic acid. Inorganic phosphate released by the hydrolysis of ATP was determined by the method of Takahashi [12].

Assay of succinate dehydrogenase activity. Succinate dehydrogenase activity was assayed polarographically at 25° using PMS as an electron aceptor in the respiratory buffer (2 ml) containing 2.0 μg/mg protein of antimycin A, 2 mM NaN_3, 5 mM succinate, and 1 mg/ml of mitochondria. Both shortly and 5 min after exposure of mitochondria to test compound, respiration was initiated by the addition of 0.5 mM PMS [13]. Under the blockades of complex III and IV with antimycin A and NaN_3, respectively, succinate dehydrogenase reduced by succinate is reoxidized by PMS. Reoxidation of the reduced PMS is accomplished by oxygen, thus allowing the specific measurement of succinate dehydrogenase activity.

Measurements of membrane potential. Membrane potential across the mitochondrial membranes was monitored at 25° with a TPP^+ electrode constructed according to Kamo *et al.* [14]. The reaction medium was the same one as used for the measurement of respiration rate plus 5 μM TPP^+ in a final volume of 2.5 ml. The calibration of the electrode was performed by multiple additions of a known amount of TPP^+ after each set of experiments.

Swelling of mitochondria. Swelling was monitored spectrophotometrically at 520 nm on a Shimadzu UV-300 dual-wavelength spectrophotometer in a double beam mode at 25° in a volume of 2.5 ml [15]. Swelling was conducted in the presence of respiratory substrate (5 mM succinate) in the same medium as used for respiration rate, and initiated by the addition of either 4-CB or 4′-OH-4-CB as indicated in the figure.

Measurements of H^+ movement across the mitochondrial membranes. H^+ movements across the mitochondrial membranes were monitored by a glass electrode at 25° in a volume of 2.5 ml. The reaction medium consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, 1 mM succinate, and 1 mM Tris–HCl (pH 7.2). The electrode was calibrated by multiple additions of a known amount of HCl after each set of experiment.

Measurements of K^+ release. The measurements of efflux of endogenous K^+ was carried out with a K^+ electrode (Radiometer, model F2132K) connected to a pH meter at 25° in a volume of 2.5 ml. The incubation medium consisted of 0.2 M sucrose, 3 mM $MgCl_2$, 5 mM Tris–HCl (pH 7.4). The electrode was calibrated by multiple additions of a known amount of KCl after each set of experiment.

In all experiments, the control contained the same volume of solvent (ethanol), and the final concentration of solvent was less than 1% (v/v); the concentration of solvent did not affect the activities assayed.

RESULTS

Effects on respiration rates

When isolated rat liver mitochondria were exposed to either parent compound 4-CB or its metabolite 4′-OH-4-CB with succinate as the respiratory substrate, polarographic traces as shown in Fig. 1 were obtained. This was presented in order to clarify the differences in time course of effect between 4-CB and 4′-OH-4-CB. Figures 1A and B show the effects on state 3 respiration. Upon the addition of 1.25 mM ADP, the respiration rate was stimulated from 18.6 (state 4) to 96.5 (state 3) natoms oxygen/min/mg protein, and the stimulated-respiration was sustained until the reaction medium became anaerobic. One minute after the addition of ADP, various concentrations of 4-CB and 4′-OH-4-CB were added. State 3 respiration was linearly inhibited along the time course by 100 μM 4-CB, where the respiration rate was decreased to 60.6 natoms oxygen/min/mg protein (37.3% inhibition) as shown in Fig. 1A, trace a. 4-CB below 100 μM also showed a linear inhibition pattern (data not shown). However, it is important to note that 4-CB exhibited a more complicated effect above 200 μM. That is, 4-CB immediately after addition caused a marked inhibition of state 3

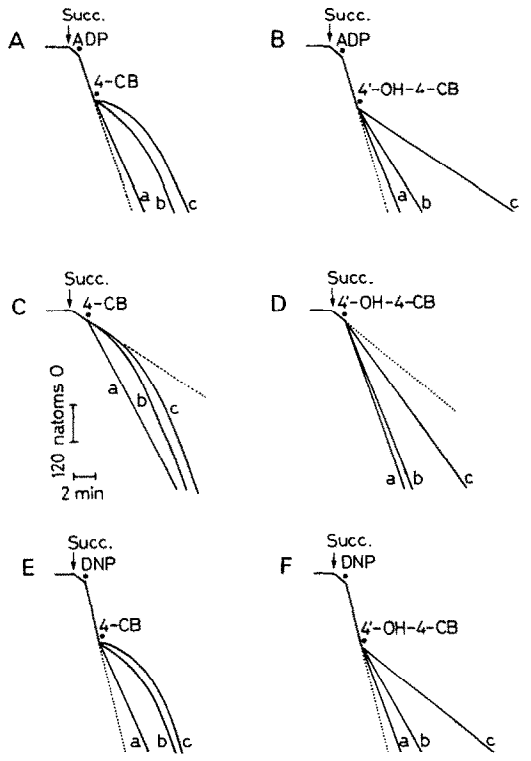

Fig. 1. Representative polarographic traces depicting the time course of effects of 4-CB and 4'-OH-4-CB on state 3 (A, B), state 4 (C, D), and DNP-stimulated (E, F) respirations of mitochondria. Rat liver mitochondria (1 mg/ml) were incubated in the respiratory buffer consisting of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 5 mM potassium phosphate (pH 7.4). The respiratory substrate was 5 mM succinate. State 3 and DNP-stimulated respirations were initiated by adding 1.25 mM ADP, and 20 μM DNP, respectively. In all traces, a, b and c represent 100, 200 and 300 μM of each compound, respectively. Temp. 25°, vol. 2 ml.

respiration to the level of state 4. The inhibition persisted for at least 5 min, then surprisingly the inhibited-respiration was relieved gradually, and finally restored to almost identical level obtained by 100 μM 4-CB (Fig. 1A, traces b and c). Thus, the effect of more than 200 μM 4-CB on state 3 respiration with succinate was biphasic; an initial significant inhibitory and subsequent less inhibitory phase. 4'-OH-4-CB, however, inhibited the respiration only in a linear and concentration-dependent manner as shown in Fig. 1B (below 100 μM traces not shown).

Figures 1C and D show the effects on state 4 respiration. When added to mitochondria energized with succinate, 4-CB stimulated the respiration instantaneously at 100 μM, where the respiration rate was increased from 18.6 (basal) to 59.8 natoms oxygen/min/mg protein (3.2-fold increase), and the stimulated-respiration continued constantly to anaerobiosis (Fig. 1C, trace a). 4-CB below 100 μM also caused an instantaneous stimulation similar to 100 μM (traces not shown). Moreover, it is worthy of note that after 5–6 min lag period, 4-CB, above 200 μM, at length caused a stimulation of the respiration; the extent of which was almost identical to that attained by 100 μM 4-CB (Fig. 1C, traces b and c). On the other hand, 4'-OH-4-CB did not show this phenomenon; it caused an instantaneous and time-linear stimulation of the respiration at all concentration ranges examined (Fig. 1D), although the rate of stimulation reduced when 4'-OH-4-CB concentration became more than 200 μM.

Figures 1E and F show the effect on DNP-stimulated respiration. Control rat liver mitochondria had DNP-stimulated respiration rate of 108.6 natoms oxygen/min/mg protein. 4-CB inhibited the respiration in a time-linear and concentration-dependent manner up to 100 μM, where the respiration rate was reduced to 59.1 natoms oxygen/min/mg protein (45.6% reduction, Fig. 1E, trace a). As observed in the case of state 3 respiration, the effect of more than 200 μM 4-CB on DNP-stimulated respiration was biphasic consisting of a significant inhibition soon after the addition of 4-CB and a subsequent release of the inhibited-respiration 5–6 min after the addition of the compound (Fig. 1E, traces b and c). On the other hand, 4'-OH-4-CB showed both time-linear and concentration-dependent inhibition at all concentration ranges tested (Fig. 1F), which was also consistent with the inhibition pattern observed in state 3 respiration (Fig. 1B).

Data in Fig. 2 show the effects of varying the concentration of test compound on the mitochondrial respiration with succinate. As shown in Fig. 1, the duration of the mitochondrial incubation period with 4-CB significantly influences the magnitude of both inhibition (state 3, DNP-stimulated) and stimulation (state 4) of the respiration; achieving a near-definite level of respiration when mitochondria were incubated with 4-CB for 5–6 min, whereas the time course effect due to 4'-OH-4-CB was simply constant. Therefore, the rates of oxygen consumption 5–6 min after the addition of the test compound are plotted against both 4-CB and 4'-OH-4-CB concentrations in order to compare the effects of the two compounds. The effects on state 3 respiration are shown in Fig. 2A. Exposure of mitochondria to 20 μM 4-CB caused no detectable inhibition of the respiration. 4-CB began to inhibit the respiration at 40 μM. The inhibition became maximum at 100 μM, retaining plateau with further increases in 4-CB concentration (never gave 50% inhibition even at 300 μM). 4'-OH-4-CB, up to 100 μM, exhibited a similar rate of inhibition to that of 4-CB. However, in contrast to 4-CB, the inhibition by 4'-OH-4-CB proceeded significantly when 4'-OH-4-CB concentration was further raised, with 50% inhibition dose (ID_{50}) of 200 μM. Effects on state 4 respiration are shown in Fig. 2B. 4-CB initiated threshold stimulation of the respiration at 60 μM. As the 4-CB concentration was increased, oxygen consumption was further accelerated, reaching a plateau around 200 μM (71.8 natoms oxygen/min/mg protein). 4'-OH-4-CB up to 80 μM showed the same rate of stimulation as 4-CB. Moreover, oxygen consumption was further accelerated with further increases in concentration, reaching a peak at 150 μM (98.7 natoms oxygen/min/mg protein), after which

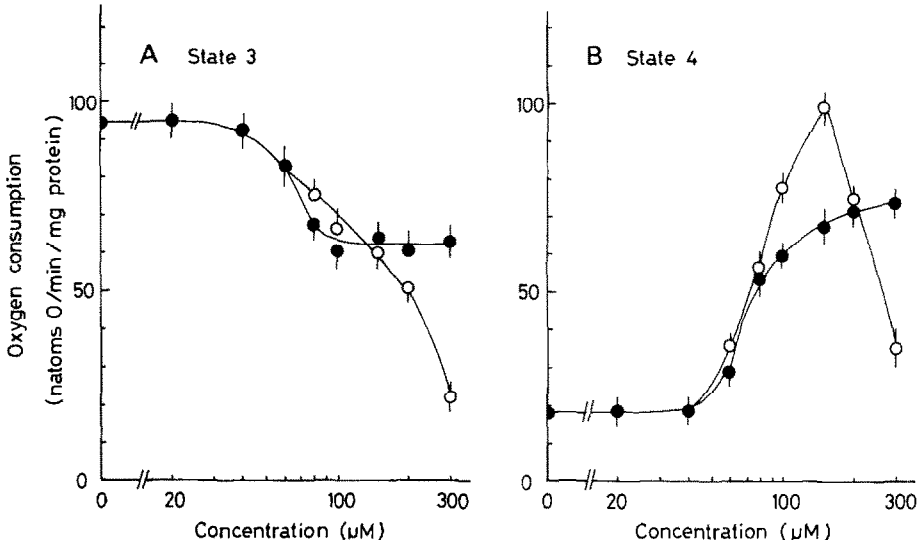

Fig. 2. Effects of 4-CB and 4'-OH-4-CB on the respiration of mitochondria with succinate as the substrate. Rat liver mitochondria (1 mg/ml) were incubated as described in Fig. 1. Shown are the rates of oxygen consumption 5–6 min after the addition of either 4-CB (●) or 4'-OH-4-CB (○) during state 3 (A) and state 4 (B) respirations of mitochondria with 5 mM succinate. Each point is a mean ± SD of 3 separate experiments. Temp. 25°, vol. 2 ml.

further increases in concentration resulted in a concentration-dependent inhibition of the stimulated-respiration.

Figure 3A shows the oxygen consumption rates 1–2 min after the addition of test compound during state 3 respiration with glutamate/malate. Inhibition of the respiration occurred with both 4-CB and 4'-OH-4-CB, but was more significant after 4'-OH-4-CB exposure, as exhibited by the ID_{50} of 76 μM for 4-CB and 30 μM for 4'-OH-4-CB. In contrast to succinate as the substrate, with glutamate/malate, 4-CB inhibited state 3 respiration in a time- and concentration-dependent manner. With glutamate/malate, 4'-OH-4-CB also showed a time- and concentration-dependent inhibition similar to that with succinate. The oxygen consumption rates 1–2 min after the addition of the test compound during state 4 respiration with glutamate/malate are shown in Fig. 3B. The stimulation of state 4 respiration by 4-CB was observed at 80–100 μM ranges. It was immediate with no time lag. Moreover, the stimulated respiration gradually declined with the passage of time, and was no longer seen after 8 min. On the other hand, 4'-OH-4-CB did not stimulate state 4 respiration throughout the concentration ranges studied.

To determine whether the electron transport chain in mitochondria was affected, the effects of 4-CB

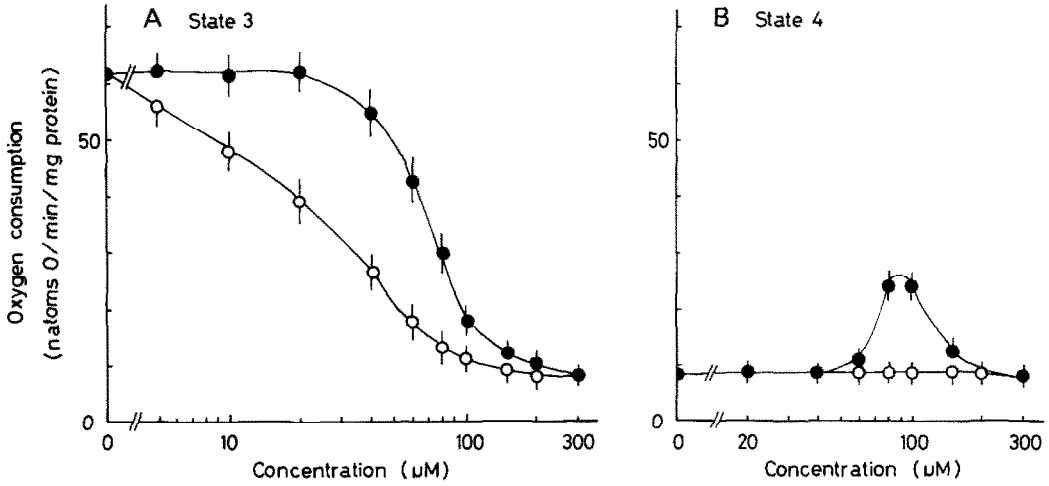

Fig. 3. Effects of 4-CB and 4'-OH-4-CB on the respiration of mitochondria with glutamate/malate as the respiratory substrate. Shown are the rates of oxygen consumption 1–2 min after the addition of either 4-CB (●) or 4'-OH-4-CB (○) during state 3 (A) and state 4 (B) respirations of rat liver mitochondria with 5 mM glutamate/5 mM malate as the substrate. Mitochondrial concentration was 1 mg/ml. Each point is a mean ± SD of 3 separate experiments. Temp. 25°, vol. 2 ml.

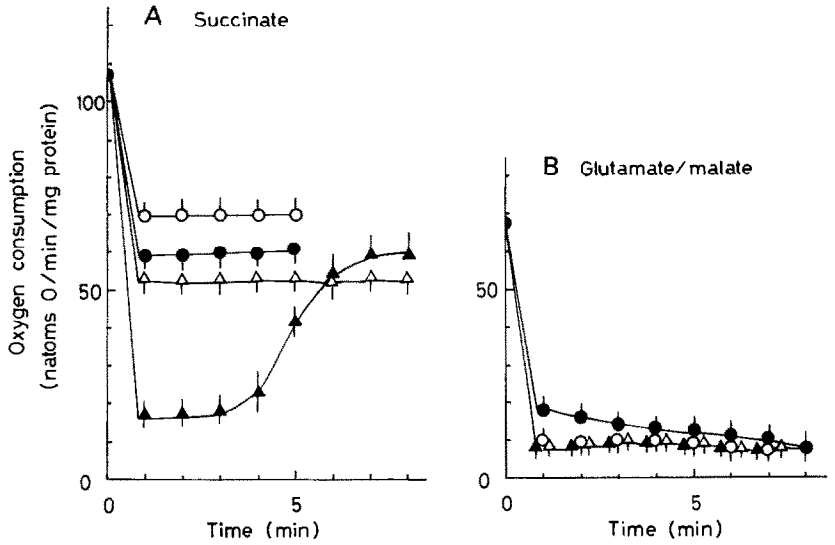

Fig. 4. Time course of effects of 4-CB and 4′-OH-4-CB on DNP-stimulated respiration of mitochondria. Rat liver mitochondria (1 mg/ml) were incubated with either 5 mM succinate (A) or 5 mM glutamate/5 mM malate (B), and DNP-stimulated respiration was initiated by the addition of 20 μM DNP. In both A and B, either 4-CB or 4′-OH-4-CB was added during DNP-stimulated respiration, and rates of oxygen consumption were plotted against time. Each point is a mean ± SD of 3 separate experiments. Symbols are as follows: 4-CB, 100 μM ●, 200 μM ▲; 4′-OH-4-CB, 100 μM ○, 200 μM △. Temp. 25°, vol. 2 ml.

and 4′-OH-4-CB on DNP-stimulated respiration (uncoupled respiration) were examined (Figs 4 and 5). Figure 4 shows the time course of inhibition of DNP-stimulated respiration caused by selected concentrations (100 and 200 μM) of the test compounds. When succinate was used as the substrate (Fig. 4A), the inhibition of the respiration by 100 μM of both 4-CB and 4′-OH-4-CB was constant with respect to time. However, when a comparison was made on 200 μM, a significant difference was observed in inhibition pattern between the two compounds. By 3 min after exposure of mitocondria to 200 μM 4-CB, DNP-stimulated respiration was inhibited significantly to the state 4 level. The inhibited-respiration was gradually relieved with the passage of time, and by 6 min the respiration recovered to the level attained by 100 μM 4-CB. 4-CB also showed a similar pattern at 300 μM (data

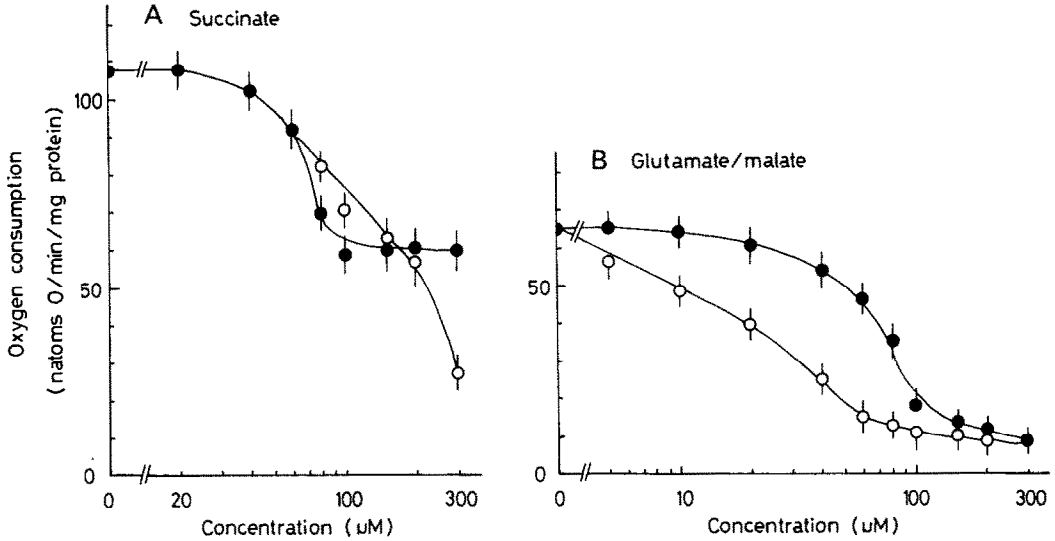

Fig. 5. Effects of 4-CB and 4′-OH-4-CB on DNP-stimulated respiration of mitochondria. Rat liver mitochondria (1 ml/ml) were incubated with either 5 mM succinate (A) or 5 mM glutamate/5 mM malate (B), and DNP-stimulated respiration was initiated by adding 20 μM DNP. Shown are the rates of oxygen consumption 5–6 min (A) to 1–2 min (B) after the addition of either 4-CB (●) or 4′-OH-4-CB (○) during DNP-stimulated respiration. Each point is a mean ± SD of 3 separate experiments. Temp. 25°, vol. 2 ml.

not shown). On the other hand, the inhibition by 200 μM 4′-OH-4-CB was similarly linear to that seen for 100 μM 4′-OH-4-CB; 300 μM of 4′-OH-4-CB also linearly inhibited DNP-stimulated respiration (data not shown). Regarding the time behavior, these findings suggest that with succinate 4-CB impairs the electron transport chain linearly at relatively low concentrations (below 100 μM), but biphasically (an initial significant and subsequent less progressive impairment) at higher concentrations, while 4′-OH-4-CB linearly inhibits the electron transport chain throughout all concentration ranges. With glutamate/malate (Fig. 4B), the inhibition of DNP-stimulated respiration by each compound was time-dependent at both 100 and 200 μM, suggesting that the flow of electrons through the electron transport chain is time-dependently inhibited by both 4-CB and 4′-OH-4-CB.

The rates of oxygen consumption of DNP-stimulated respiration are given as a function of the test compound concentration in Fig. 5. With succinate, the oxygen concentration rates 5–6 min after the addition of the test compound were plotted (Fig. 5A). Both 4-CB and 4′-OH-4-CB began to inhibit DNP-stimulated respiration at 40 μM. Furthermore, the inhibition by 4-CB plateaued at 100 μM, while that caused by 4′-OH-4-CB was concentration-dependent with an ID_{50} of 210 μM. Thus, 4′-OH-4-CB at higher concentrations was more potent than 4-CB in causing inhibition of DNP-stimulated respiration (i.e. the ability to inhibit electron flow from succinate to oxygen). When glutamate/malate was the substrate (Fig. 5B), 4′-OH-4-CB was more effective than 4-CB in inhibiting the respiration as seen in ID_{50} of 27 μM for 4′-OH-4-CB and 74 μM for 4-CB. This indicates that the electron flow from glutamate/malate to oxygen is more sensitively inhibited by 4′-OH-4-CB than by 4-CB.

Figure 6 shows the effects of 4-CB and 4′-OH-4-CB on state 4 respiration with abcorbate/TMPD as the substrate. The effect of DNP is also shown for comparison. DNP stimulated state 4 respiration for 41.8 (basal) to 72.1 natoms oxygen/min/mg protein (Fig. 6A). The same rate of stimulation occurred after addition of any of the three concentrations (100, 200 and 300 μM) of 4-CB (Figs 6B, C and D), the extent of which was the same as that achieved by DNP. Moreover, the stimulation by 4-CB was instantaneous even more than 200 μM (Figs 6C and D). This was in contrast to succinate as the substrate, with which there was observed a lag period before the stimulation by 4-CB became obvious (Fig. 1C). This suggests that the lag time seen with succinate is caused by the inhibition of electron transport chain located before cytochrome *c* oxidase. 4′-OH-4-CB also stimulated state 4 respiration instantaneously (Figs 6E, F and G); however, rate of stimulation was reduced at higher 4′-OH-4-CB concentrations (Figs 6F and G).

Effects on ATPase

Figure 7 shows the effects of 4-CB and 4′-OH-4-CB on the rates of coupled and DNP-stimulated ATP hydrolysis. Both 4-CB and 4′-OH-4-CB began to stimulate ATPase at 60 μM. The stimulation of ATPase by 4-CB never exceeded the level obtained by DNP. However, 4′-OH-4-CB stimulated ATPase to a larger extent than did DNP. Both 4-CB and 4′-OH-4-CB could not stimulate ATPase in the presence of oligomycin. Moreover, even DNP-stimulated ATPase activity was further stimulated by both compounds; the extent of stimulation was higher with 4′-OH-4-CB than with 4-CB.

Effects on succinate dehydrogenase

The effects of 4-CB and 4′-OH-4-CB on succinate dehydrogenase are shown in Fig. 8. 4-CB, shortly after the addition into suspension of mitochondria respiring with succinate, initially inhibited succinate

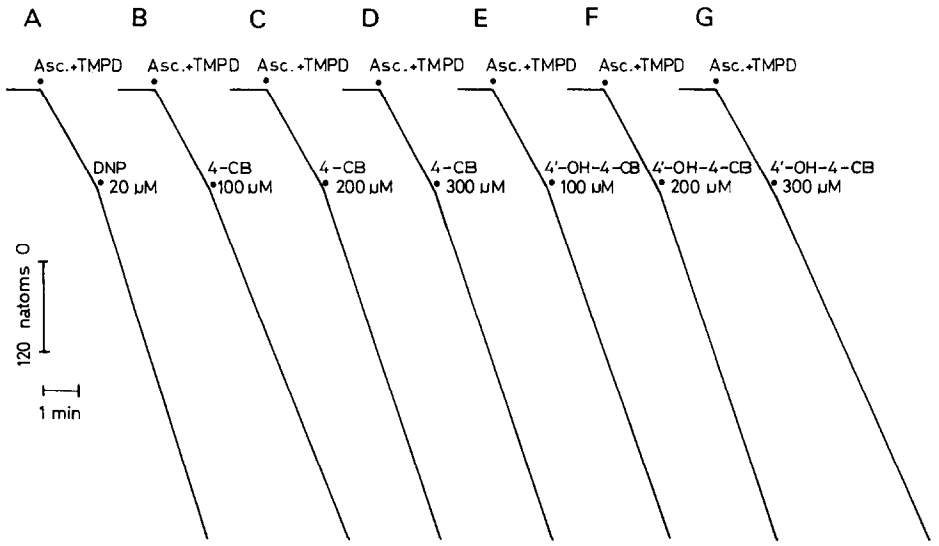

Fig. 6. Representative polarographic traces depicting the effects of 4-CB and 4′-OH-4-CB on state 4 respiration of mitochondria with ascorbate/TMPD as the substrate. Rat liver mitochondria (1 mg/ml) were incubated with 5 mM ascorbate/0.1 mM TMPD as the substrate. Test compounds were added at the indicated point. Temp. 25°, vol. 2 ml.

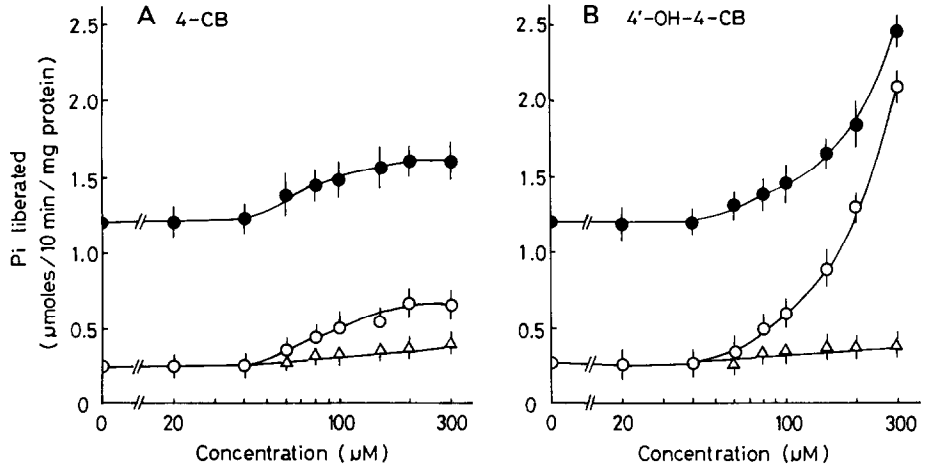

Fig. 7. Effects of 4-CB and 4'-OH-4-CB on ATPase activity of mitochondria. Rat liver mitochondria (1 mg/ml) were incubated in a reaction medium consisting of 0.15 M KCl, 3 mM $MgCl_2$, 5 mM Tris–HCl (pH 7.4), when present, DNP was 20 μM, and oligomycin was 2 μg. The reaction was initiated by adding 5 mM ATP. After incubation for 10 min, inorganic phosphate released by the hydrolysis of ATP was determined. In both A and B, symbols are as follows: −DNP, −oligomycin ○; +DNP, −oligomycin ●; −DNP, +oligomycin △. Each point is a mean ± SD of 3 separate experiments. Temp. 25°, vol. 2 ml.

dehydrogenase at 100 μM, and the inhibition proceeded in a concentration-dependent manner with 56% inhibition at 300 μM. Surprisingly, longer (5–6 min) exposure of mitochondria to 4-CB completely attenuated the inhibited activity of the enzyme that had been seen immediately after exposure (Fig. 8A). Thus, the inhibition of succinate dehydrogenase by 4-CB was reversible. On the other hand, both shortly after and 5–6 min after exposure of mitochondria to 4'-OH-4-CB, the compound exhibited a similar extent of inhibition with 31% inhibition at 300 μM, indicating that the inhibition of succinate dehydrogenase by 4'-OH-4-CB is not influenced by the duration of mitochondrial incubation period with 4'-OH-4-CB (independent of time).

Effects on membrane potential

The changes in the membrane potential across the mitochondrial membranes during the action of 4-CB and 4'-OH-4-CB are shown in Fig. 9. The permeant cation TPP^+ was taken up from the reaction medium into the inner side of mitochondria when they are energized with succinate (upward deflection in the figure), indicating the formation of membrane potential. The addition of 4-CB and 4'-OH-4-CB induced concentration-dependent release of incor-

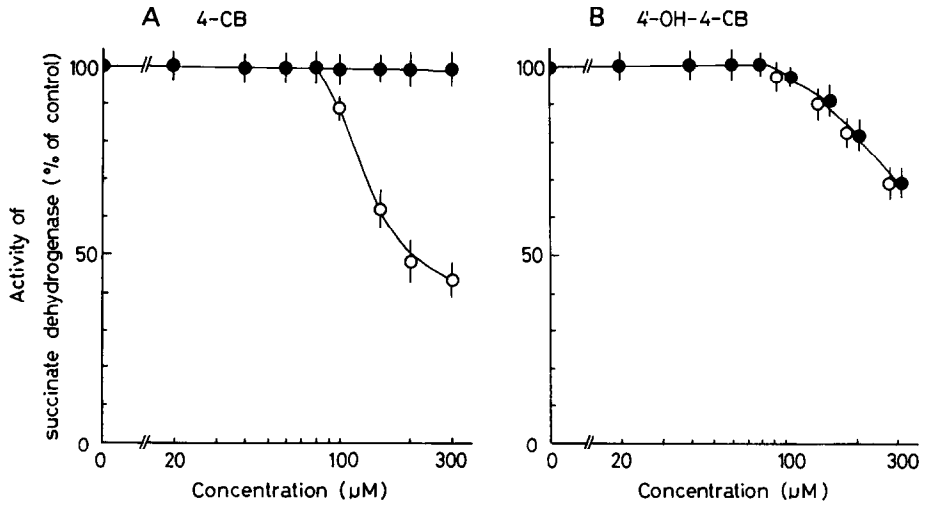

Fig. 8. Effects of 4-CB and 4'-OH-4-CB on succinate dehydrogenase of mitochondria. Succinate dehydrogenase activity was assayed polarographically with a Clark-type oxygen electrode. Shortly (○) and 5 min (●) after exposure of mitochondria (1 mg/ml) with various concentrations of test compound, reaction was initiated by adding 0.5 mM PMS. Control activity was 159.3 ± 4.8 natoms oxygen/min/mg protein. Each point is a mean ± SD of 3 separate experiments. Temp. 25°, vol. 2 ml.

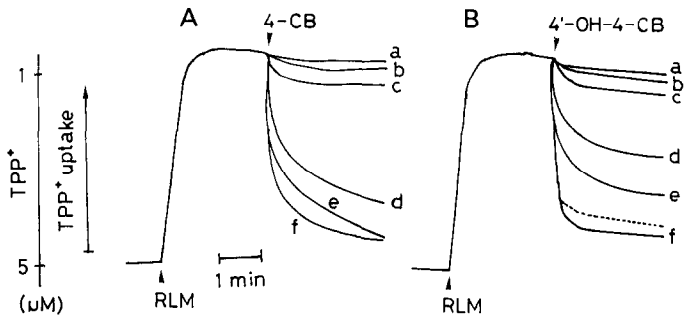

Fig. 9. Effects of 4-CB and 4'-OH-4-CB on the movement of TPP^+ across the mitochondrial membranes. Rat liver mitochondria (RLM, 1 mg/ml) were incubated in the respiratory buffer containing 5 mM succinate and 5 μM TPP^+. In both A (4-CB) and B (4'-OH-4-CB): a, none; b, 40; c, 60; d, 80; e, 100; f, 200 μM of test compound. In B, dotted line indicates 20 μM DNP. Temp. 25°, vol. 2.5 ml.

porated TPP^+ into the incubation medium (downward deflection in the figure, dissipation of membrane potential). Below 60 μM of 4-CB and 4'-OH-4-CB that had no stimulatory effect on state 4 respiration with succinate (Fig. 2B), the dissipation of membrane potential due to these compounds was also small (Fig. 9, traces a–c in both A and B). Above this concentration, although the potency to dissipate it was slightly larger with 4-CB than with 4'-OH-4-CB, both compounds rapidly dissipated the membrane potential (Fig. 9, traces d–f in both A and B), which was accompanied by the acceleration of state 4 respiration (Fig. 2B).

Effects on the volume of mitochondria

Figure 10 shows the effects of 4-CB and 4'-OH-4-CB on the volume of mitochondria energized with succinate, monitored as the changes in the optical absorbance at 520 nm. 4-CB at 60 μM began to cause a decrease in the absorbance (Fig. 10A). The

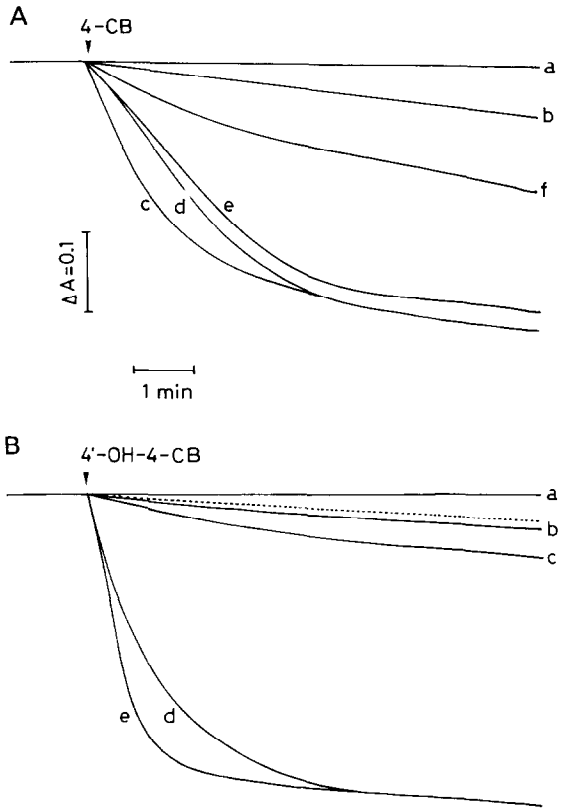

Fig. 10. Swelling of mitochondria induced by either 4-CB or 4'-OH-4-CB. Swelling was monitored at 520 nm in the respiratory buffer containing 5 mM succinate. Concentration of rat liver mitochondria was 1 mg/ml. In A: a, none; b, 60; c, 80; d, 100; e, 200; f, 300 μM of 4-CB. In B: a, none; b, 80; c, 100; d, 200; e, 300 μM of 4'-OH-4-CB, dotted line indicates 20 μM DNP. Temp. 25°, vol. 2.5 ml.

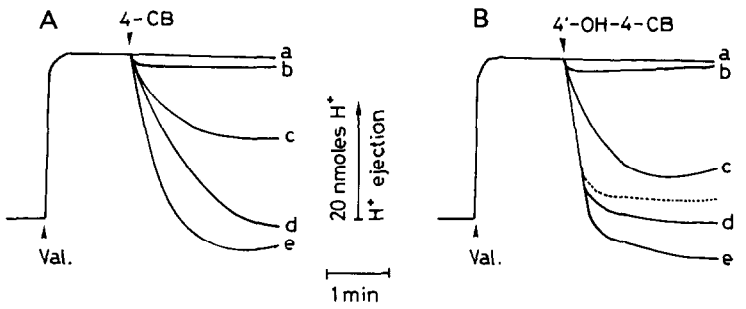

Fig. 11. Effects of 4-CB and 4'-OH-4-CB on H^+ ejected by the addition of valinomycin to energized mitochondria. Valinomycin (Val, 40 nM) was added to a suspension of rat liver mitochondria (1 mg/ml) in 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 1 mM Tris–HCl (pH 7.2). Mitochondria were energized by the addition of 1 mM succinate. The movement of H^+ was monitored with a combined glass electrode. In both A (4-CB) and B (4'-OH-4-CB): a, none; b, 60; c, 80; d, 100; e, 200 μM of test compound. In B, dotted line indicates 20 μM DNP. Temp. 25°, vol. 2.5 ml.

decrease can be interpreted as due to swelling of the mitochondria [15]. With increases in 4-CB concentration, the rate of swelling became greater, reaching a peak at 80 μM, after which further increases in concentration resulted in a concentration-dependent repression of the swelling. The concentration (60 μM) at which 4-CB began to induce swelling corresponded well to that of initiation of the stimulation of state 4 respiration by 4-CB (Fig. 2B). On the other hand, the swelling by 4'-OH-4-CB was not large at concentrations lower than 100 μM. However, 4'-OH-4-CB induce large swelling when the concentration was further raised.

Effects on the movement of H^+ *and* K^+ *across the mitochondrial membranes*

Since both 4-CB and 4'-OH-4-CB caused the dissipation of membrane potential, in order to correlate the dissipation of membrane potential with the increase in ion-permeability across the mitochondrial inner membranes, the ability of these compounds to cause changes in either H^+ or K^+ movements across the mitochondrial inner membranes was tested. Figure 11 shows the effects of 4-CB and 4'-OH-4-CB on the movement of H^+ across the mitochondrial membranes. Since the first report by Henderson *et al.* [16], the effects of uncouplers on the H^+ ejection from energized mitochondria by the addition of valinomycin plus potassium have been used as the model system to monitor the protonophoric activity of uncouplers [17]. As shown in the figure, the addition of valinomycin to mitochondrial suspension caused H^+ ejection (upward deflection in the figure). After H^+ ejection reached steady-state, the addition of uncoupler such as DNP caused H^+ influx (downward deflection in Fig. 11B, dotted line). This H^+ influx induced by uncouplers is taken as evidence of the increase of H^+ conductance of the inner mitochondrial membranes. When the effects of 4-CB and 4'-OH-4-CB on the valinomycin-ejected H^+ were examined, both 4-CB and 4'-OH-4-CB, above 60 μM, caused concentration-dependent H^+ influx, indicating that 4-CB and 4'-OH-4-CB increase H^+ conductivity of the inner mitochondrial membranes.

Figure 12 shows the release of endogenous K^+ from mitochondria treated with 4-CB and 4'-OH-4-CB. The experiment was carried out in order to examine whether these compounds also alter the membrane permeability to ions other than H^+. 4-CB did not release K^+ up to 40 μM. Above 60 μM, the K^+-release increased progressively with increasing 4-CB concentrations (Fig. 12A). On the other hand, the release of K^+ induced by 4'-OH-4-CB was slight up to 100 μM (Fig. 12B, traces a and b). Above these concentrations, 4'-OH-4-CB released K^+ progressively similar to 4-CB.

DISCUSSION

When a compound is administered to animals, the hydroxylation of the compound by microsomal cytochrome P-450 system sometimes produces a more toxic metabolite than the parent compound. For example, pentachlorophenol (PCP) is formed by the microsomal metabolism, when hexachlorobenzene (HCB, a fungicide) is fed to animals [18, 19].

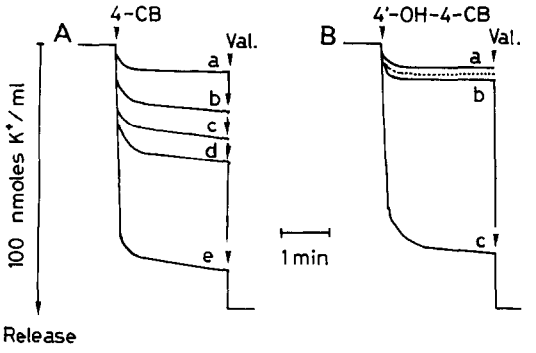

Fig. 12. Release of endogenous K^+ from mitochondria treated with either 4-CB or 4'-OH-4-CB. Rat liver mitochondria (1 mg/ml) were incubated at 25° with various concentrations of test compound in a 2.5 ml medium composed of 0.2 M sucrose, 3 mM $MgCl_2$, and 5 mM Tris–HCl (pH 7.4). In A: a, none; b, 60; c, 80; d, 100; e, 200 μM of 4-CB. In B: a, none; b, 100; c, 200 μM of 4'-OH-4-CB. Dotted lines indicates 20 μM DNP. At the end of each experiment, valinomycin (Val. 80 nM) was added to obtain the complete release of K^+.

The toxic events caused by HCB are attributed to endogenously formed PCP which inhibits ATP synthesis by uncoupling of oxidative phosphorylation [19]. Similarly, when 4-CB is administered to animals, 4-CB is metabolized to produce a phenolic compound, 4′-OH-4-CB [6]. The present study compares the effects of 4-CB and its metabolite 4′-OH-4-CB on the energy-transducing functions of rat liver mitochondria, and elucidates the influence of metabolism on the toxic events in mitochondria.

Regarding the effects on state 3 respiration (Figs 2A and 3A), the inhibition of the respiration by 4-CB with succinate never exceeded 50% even at 300 μM, while that with glutamate/malate was more intense with ID_{50} of 76 μM. On the other hand, 4′-OH-4-CB inhibited state 3 respiration with ID_{50} of 200 μM for succinate and 30 μM for glutamate/malate. These results indicate that as a substrate specificity, the inhibitory action of both 4-CB and 4′-OH-4-CB on state 3 respiration was more potent with NAD^+-linked substrate, glutamate/malate than with FAD-linked substrate, succinate. Moreover, when a comparison was made on the test compounds, 4′-OH-4-CB was more effective than 4-CB in causing inhibition of state 3 respiration with both substrates. Thus, the product of the metabolism of 4-CB more strongly impairs the ATP synthesis than the parent compound with both substrates. The inhibition of state 3 respiration by both compounds either with succinate (Fig. 2A) or with glutamate/malate (Fig. 3A) was found to occur in parallel to that of DNP-stimulated respiration with corresponding substrates (Fig. 5). From this fact, it is apparent that the inhibition of state 3 respiration by both 4-CB and 4′-OH-4-CB is mainly caused by the interference with the electron transport chain in mitochondria, and not by the inhibition of both ATPase (e.g. oligomycin-like) and adenine nucleotide translocator (atractyloside-like).

When succinate was used as the respiratory substrate, both 4-CB and 4′-OH-4-CB caused a stimulation of state 4 respiration. Maximum stimulation (5.3-fold increase) due to 4′-OH-4-CB (150 μM) was much greater than that (3.9-fold increase) caused by 300 μM 4-CB (Fig. 2B). As uncoupling of oxidative phosphorylation is manifested in the form of stimulation of state 4 respiration, the observed stimulation by both 4-CB and 4′-OH-4-CB is attributed to the uncoupling action. Thus, with succinate, the hydroxylation of the parent compound is shown to cause an increase in uncoupling ability. When substrate was changed to glutamate/malate, 4-CB only stimulated state 4 respiration within a narrow concentration range (80–100 μM), but 4′-OH-4-CB did not stimulate at all. Thus, with glutamate/malate, the hydroxylation of the parent compound completely eliminates (masks) the uncoupling ability of the parent compound (discussed later).

The electron transport from glutamate/malate to oxygen was more sensitively inhibited than that from succinate to oxygen by both 4-CB and 4′-OH-4-CB (Fig. 5). This suggests that either TCA cycle dehydrogenase or the NADH-CoQ span of the electron transport chain is sensitive to inhibition by both compounds at low concentrations where the inhibition of succinate oxidation is not apparent. In a previous paper, we have shown that the sensitive site of relatively low concentrations of 4′-OH-4-CB is located between the NADH-CoQ span of the electron transport chain, and not in TCA cycle dehydrogenase [9]. Using similar methods to 4′-OH-4-CB [9], it was shown that 4-CB did not inhibit the reduction of NAD^+ by glutamate/malate (data not shown), indicating that TCA cycle dehydrogenase is not affected by 4-CB. However, 4-CB inhibited the reduction of cytochrome *b* induced by the addition of glutamate/malate (data not shown). This indicates that 4-CB at relatively low concentrations interferes with the NADH-CoQ span of the electron transport chain similar to 4′-OH-4-CB. Moreover, the extent of inhibition of cytochrome *b* reduction by 4-CB was less than that by the same concentration of 4′-OH-4-CB (data not shown), indicating that in the ability to inhibit the NADH-CoQ span, metabolite is stronger than the parent compound.

The inhibition of the electron transport from succinate to oxygen by both 4-CB and 4′-OH-4-CB was also recognized at higher concentrations (Fig. 5). This indicates that both compounds at higher concentrations interfere with either succinate dehydrogenase or the electron transport chain common to both NAD^+- and FAD-linked substrate (the b–c_1 complex or cytochrome *c* oxidase). In DNP-stimulated respiration with ascorbate/TMPD, 4-CB did not cause any inhibition irrespective of time-course even at 300 μM, and 4′-OH-4-CB only caused a slight inhibition at higher concentrations (data not shown), indicating that cytochrome *c* oxidase is not affected by both compounds. Therefore, the main inhibitory sites of succinate oxidation by both compounds lie in the components before cytochrome *c* oxidase (i.e. succinate dehydrogenase or the b–c_1 complex). The effects of both compounds on succinate dehydrogenase was measured directly (Fig. 8), and that on the b–c_1 complex was examined indirectly as follows. 4-CB caused a considerable inhibition of succinate dehydrogenase immediately after interaction with mitochondria, but the observed inhibition was completely attenuated after 5–6 min (Fig. 8A), and even at this stage the inhibition of succinate oxidation by 4-CB was observed (Fig. 5A). Therefore, the 4-CB-induced inhibition of succinate oxidation 5–6 min after exposure is attributed to the inhibition of the b–c_1 complex. On the other hand, the inhibition of succinate dehydrogenase by 4′-OH-4-CB was constant with respect to time (Fig. 8B). This indicates that although the extent of inhibition by 4′-OH-4-CB is lower than that by 4-CB (immediately after exposure), the 4′-OH-4-CB-induced inhibition of succinate dehydrogenase is irreversible (while that caused by 4-CB is reversible). Furthermore, the extent of inhibition of succinate oxidation by 4′-OH-4-CB (Fig. 5A) was far greater than that of succinate dehydrogenase by 4′-OH-4-CB (Fig. 8B). Therefore, the inhibition by 4′-OH-4-CB of electron flow from succinate to oxygen also occurred at the b–c_1 complex of the electron transport chain as well as succinate dehydrogenase.

The reversible inhibition of succinate dehydrogenase by 4-CB affects the manner by which 4-CB interacts with mitochondria energized with succinate. Firstly, in both state 3 and DNP-stimu-

lated respiration, the significant inhibition of these respirations was observed soon after the exposure of mitochondria to more than 200 μM 4-CB (Fig. 1A, traces b and c; Fig. 1E, traces b and c). This inhibition is due to the initial inhibition of succinate dehydrogenase by 4-CB, since the inhibition of these respirations was gradually relieved as the inhibition of succinate dehydrogenase by 4-CB became weaker with the passage of time. Secondly, the stimulation by 4-CB of state 4 respiration with succinate is also the case. That is, when 4-CB was below 100 μM, state 4 respiration was linearly stimulated (Fig. 1C, trace a). However, a lag period was observed before the stimulation became obvious, when 4-CB concentration was further raised (Fig. 1C, traces b and c); the stimulation of state 4 respiration gradually appeared as the 4-CB-induced inhibition of succinate dehydrogenase decreased with the passage of time. Therefore, the lag period is thought to be due to the initial inhibition of succinate dehydrogenase. The mechanism by which 4-CB acts on succinate dehydrogenase reversibly is not clear at present, but there may be some relevance to the conformational changes of the enzyme complex due to 4-CB.

Both 4-CB and 4′-OH-4-CB activated the latest ATPase in mitochondria. The latter was more effective than the former in activating the ATPase both in the absence and presence of DNP. Thus, the metabolite is stronger than the parent compound in the ability to hydrolyse ATP.

According to the chemiosmotic theory [20], the dissipation of membrane potential is generally regarded as decisive for exhibition of uncoupling action. With a protonophoric uncoupler such as DNP, the dissipation of membrane potential is performed by shuttling protons across the membrane with an acid-dissociable group within the molecule. However, 4-CB does not possess an acid-dissociable group. 4-CB, therefore, dissipate membrane potential (Fig. 9A) through a different mechanism from that of a protonophoric uncoupler. We have shown that some tetrachlorobiphenyls dissipated membrane potential by causing nonspecific increases in membrane permeability to ions [21]. Therefore, a most probable candidate for 4-CB may be also an increased membrane permeability to ions. This is supported by evidence that 4-CB caused a large amplitude swelling of mitochondria (Fig. 10A), suggesting that 4-CB increases the membrane permeability to ions in a nonspecific manner. Additional evidence that 4-CB produces a nonspecific increase in the mitochondrial ion permeability comes from the experiments with H^+ and K^+ fluxes (Figs 11A and 12A). That is, 4-CB increased the mitochondrial ion permeability to not only H^+ (Fig. 11A) but also K^+ (Fig. 12A) at concentrations comparable to the dissipation of membrane potential. When 4-CB is incorporated into the lipid phase of the mitochondrial membranes, this compound may perturb the lipid phase, and increases membrane permeability to ions, thereby dissipating membrane potential, which leads to uncoupling. With glutamate/malate, the uncoupling action of 4-CB was masked at higher concentrations because of the increased inhibition of the electron transport chain as compared to that with succinate.

4′-OH-4-CB acquired an acid-dissociable group by microsomal metabolism. The uncoupling action of 4′-OH-4-CB may be due to the dissipation of membrane potential (Fig. 9B) through proton shuttling with the acid-dissociable group. This is evident from the fact that 4′-OH-4-CB, at relatively low concentrations, was uncoupled by increasing only H^+ conductance (Fig. 11B) without causing any appreciable K^+ flux (Fig. 12B, traces a and b). Another rationale for this is that 4′-OH-4-CB induced uncoupling without considerable swelling at relatively low concentrations (Fig. 10B, traces b and c), similar to a typical protonophoric uncoupler, DNP, which also uncoupled without causing large amplitude swelling (Fig. 10B, dotted line). However, 4′-OH-4-CB at higher concentrations is thought to dissipate membrane potential by causing nonspecific increases in membrane permeability to ions, since 4′-OH-4-CB induced K^+ flux at higher concentrations (Fig. 12B, trace c) as well as H^+ flux (Fig. 11B). This is also apparent from the fact that 4′-OH-4-CB caused a large amplitude swelling at higher concentrations (Fig. 10B, traces d and e), indicating nonspecific increases in membrane ion permeability. With succinate, the uncoupling action due to higher 4′-OH-4-CB concentrations was masked because of the increased inhibition of electron transport chain. Likewise, with glutamate/malate, the uncoupling action of 4′-OH-4-CB was totally masked through all concentration ranges because of the significant inhibition of the electron transport compared to that with succinate.

Significant swelling was evoked in the presence of less than 100 μM 4-CB, but the swelling induced by the corresponding concentrations of 4′-OH-4-CB (less than 100 μM) was small (Fig. 10). This suggests that 4-CB is greater than 4′-OH-4-CB in the ability to alter the permeability of mitochondrial membranes to ions. Thus, as far as the membrane permeability is concerned, the parent compound is more active than its metabolite, although the metabolite is more toxic than the parent compound in many other activities of mitochondria (i.e. electron transport or uncoupling). Regarding the effects of these compounds on membrane permeability, similar results were reported with human erythrocyte membranes; 4-CB was more hemolytic than 4′-OH-4-CB because of higher ability of 4-CB (compared to 4′-OH-4-CB) in altering the permeability of the erythrocyte membranes to ions [22]. However, it was also reported that in highly chlorinated biphenyls, reverse tendency was observed. Namely, hydroxychlorobiphenyls were more effective than the parent chlorobiphenyls in causing hemolysis [22].

In summary, both 4-CB and 4′-OH-4-CB interferes with the energy transducing functions of rat liver mitochondria. However, the product of the metabolism of 4-CB turns out to be more biologically active than the parent compound as follows. 4′-OH-4-CB is more effective than 4-CB in causing the inhibition of electron transport (hence the inhibition of ATP synthesis). This tendency is apparent irrespective of the species of substrate, but more evident with glutamate/malate than with succinate. Although 4-CB itself possesses the uncoupling action, the hydroxylation of 4-CB gives a pro-

tonophoric activity to 4-CB. This enhances the uncoupling action of the parent compound as evidenced by more elevated stimulation of state 4 respiration by 4′-OH-4-CB than by 4-CB with succinate (suitable substrate with which the uncoupling action of these compounds becomes obvious). Thus, either inactivation of electron transport or uncoupling action caused by the metabolite (4′-OH-4-CB) as well as the parent compound (4-CB) would produce an inactivation of mitochondrial ATP synthesis, leading to increased ineffectiveness of the energy metabolism of aerobic organism.

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