INTERACTION OF TETRACHLOROBIPHENYLS WITH ISOLATED RAT LIVER MITOCHONDRIA

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A comparative study of the effects of tetrachlorobiphenyls (TCBs) on the succinate-supported respirations of rat liver mitochondria was made, and some differences in effects caused by the different chlorine positions of the biphenyl rings were clarified. The inhibitory actions of 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs on both state 3 and uncoupler-stimulated respirations were potent, while those induced by 2,6,2',6'-, and 3,4,3',4'-TCBs were weak. 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-TCBs stimulated state 4 respiration, but 3,4,3',4'-TCB had very little effect on this respiration. The latent adenosine triphosphatase activity was stimulated by 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs, but 2,6,2',6'-, and 3,4,3',4'-TCBs had no effects. The relationship between these effects and chemical structure of TCBs is discussed.

Keywords — tetrachlorobiphenyl; respiration; ATPase activity; uncoupling action; rat liver mitochondria

INTRODUCTION

Polychlorinated biphenyls (PCBs) are toxic industrial chemicals that have been used in various products and industrial processes, and are widely distributed in the environment.¹⁾ On the toxicity of PCBs on mitochondria, Stotz and Greichus reported alterations in the shape of liver mitochondria from the white pelican by *in vivo* treatment with PCBs.²⁾ That is, mitochondria from the PCB-treated white pelican were rounded and swollen instead of long and slender as in untreated animals, which is similar to that produced by 2,4-dinitrophenol (protonophoric uncoupler),³⁾ suggesting uncoupling of oxidative phosphorylation. Jonsson *et al.* also reported by morphological studies that mitochondrial changes compatible with necrosis were found in the livers of rats fed with PCBs.⁴⁾ We have shown by biochemical studies that Kanechlor-400 (a commercial PCB mixture)

inhibits state 3 respiration (active respiration in the presence of adenosine 5'-diphosphate (ADP)) of rat liver mitochondria; state 3 respiration with succinate as the substrate is more sensitive to inhibition than that observed with α -ketoglutarate-malate, and that Kanechlor-400 stimulates state 4 respiration (resting respiration after the expenditure of ADP in the assay system); state 4 respiration in the presence of α -ketoglutarate-malate is more intensely stimulated than by succinate.^{5,6)}

The present study was undertaken to compare the effects of several tetrachlorobiphenyl (TCB) isomers, whose action on other enzymatic systems such as hepatic microsomal enzymes have been well characterized,⁷⁻⁹⁾ on the succinate-supported respirations of rat liver mitochondria, and some differences in effects caused by the different chlorine positions in TCBs were clarified.

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MATERIALS AND METHODS

2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, 2,6,2',6'-, and 3,4,3',4'-tetrachlorobiphenyls (TCBs) were synthesized by the Ullman condensation of the corresponding dichloriodobenzenes.¹⁰⁾ The purities of these isomers were found to be more than 99% by gas liquid chromatography. The stock solutions of these isomers except 3,4,3',4'-TCB were prepared in ethanol, and that of 3,4,3',4'-TCB was prepared in dimethylformamide. Adenosine 5'-diphosphate (ADP), adenosine 5'-triphosphate (ATP), oligomycin, and bovine serum albumin were purchased from Sigma Chemical Co (St. Louis, MO). Other chemicals were commercial products of reagent grade.

Liver mitochondria were isolated from male Wistar rats (200–300 g) in 0.25 M sucrose, 5 mM Tris-HCl (pH 7.4), and 0.1 mM ethylenediaminetetraacetic acid (EDTA) by the method of Hogeboom.¹¹⁾ The last washing was carried out in an EDTA-free medium. Mitochondrial protein was assayed by the biuret method,¹²⁾ using bovine serum albumin as a standard.

Respiration rates were measured polarographically with a Clark-type oxygen electrode in a 2 ml water-thermostated glass reaction cell maintained at 25 °C. The respiratory buffer consisted of 0.2 M sucrose, 20 mM KCl, 3 mM MgCl₂, and 5 mM potassium phosphate (pH 7.4). 5 mM Succinate was used as the respiratory substrate, and mitochondrial concentration was 1 mg/ml. When present, ADP was 150 μM, and 2,4-dinitrophenol (DNP) was 25 μM.

Adenosine 5'-triphosphatase (ATPase) activity was determined in a reaction mixture (final volume 1.5 ml) containing 0.15 M KCl, 3 mM MgCl₂, 5 mM Tris-HCl (pH 7.4), and 1.5 mg of mitochondria; when present, DNP was 25 μM, and oligomycin was 1.5 μg. The reaction was initiated by the addition of ATP at a final concentration of 5 mM. After incubation for 10 min at 25 °C, 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.¹³⁾

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

RESULTS

The effects of TCBs on state 3 respiration with succinate as the substrate are shown in Fig. 1. 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs inhibited strongly state 3 respiration. The concentrations of these agents that gave 50% inhibition (*I*₅₀) are shown in Table I. There was no significant dif-

FIG. 1. Effects of TCBs on State 3 Respiration of Rat Liver Mitochondria with Succinate as the Substrate

Oxygen uptake was measured polarographically with a Clark-type oxygen electrode at 25 °C. The respiratory buffer consisted of 0.2 M sucrose, 20 mM KCl, 3 mM MgCl₂, 5 mM potassium phosphate (pH 7.4), and 5 mM succinate as the substrate. Mitochondria (1 mg/ml) were interacted with TCB for 3 min, then state 3 respiration was initiated by the addition of 150 μM ADP. Control rate was 132.5 ± 4.8 natoms O/min/mg protein. Symbols are as follows: 2,3,2',3'-, ○; 2,4,2',4'-, ▲; 2,5,2',5'-, □; 2,6,2',6'-, ●; 3,4,3',4'-TCB, △. Each point is a mean of 3 separate experiments.

TABLE I. Concentrations that Gave 50% Inhibition (I_{50}) of State 3 and DNP-Stimulated Respirations of Rat Liver Mitochondria with Succinate as the Substrate

Compound	State 3 I_{50} (μM)	DNP-stimulated I_{50} (μM)
2,3,2',3'-TCB	43.8 ± 4.7	47.5 ± 2.8
2,4,2',4'-TCB	45.7 ± 4.6	50.8 ± 3.9
2,5,2',5'-TCB	50.5 ± 2.2	51.6 ± 4.3

The values of I_{50} are calculated from linear regression equations correlating activity with logarithm of TCB concentration with the same data used for Figs. 1 and 4. Each value is a mean $\pm$ S.D. of 3 independent estimations.

ference among these I_{50} values ($p < 0.05$), which indicates that treatment with 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs resulted in similar rates of inhibition. On the other hand, the inhibitory actions of 2,6,2',6'-, and 3,4,3',4'-TCBs were relatively weak with only 22, and 27% inhibitions even at 200 μM , respectively.

Figure 2 shows the effects of TCBs on the oxygen consumption 3 to 4 min after the addition of TCBs during state 4 respiration with succinate as the substrate. As the concentration of 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs was increased, oxygen consumption was stimulated, reaching a peak where maximum rate was observed (more than 2-fold increases at 80 μM), after which further increases in concentration repressed the respiration. However, 2,6,2',6'-TCB stimulated state 4 respiration in a concentration-dependent manner (more than 6-fold increase at 200 μM). 3,4,3',4'-TCB did not stimulate state 4 respiration.

Figure 3 shows the effects of TCBs on the ATPase activity of mitochondria. 2,3,2',3'-TCB caused a substantial activation of the activity; in the presence of oligomycin, however, the stimulatory action of this agent was completely abolished. 2,3,2',3'-TCB produced marked stimulation of the ATPase activity also when added in the presence of 2,4-dinitrophenol (DNP). Identical results were obtained for 2,4,2',4'-, and 2,5,2',5'-TCBs (therefore, data were omitted). On the other hand, 2,6,2',6'-TCB had no effect on the ATPase activity of both in the absence

and presence of DNP. The result by 3,4,3',4'-TCB was similar to that of 2,6,2',6'-TCB (data not shown).

The effects of TCBs on DNP-stimulated respiration with succinate are shown in Fig. 4. Inhi-

FIG. 2. Effects of TCBs on State 4 Respiration of Rat Liver Mitochondria with Succinate as the Substrate

Shown are the rates of oxygen consumption 3 to 4 min after the addition of TCBs during state 4 respiration. Each point is a mean of 3 separate experiments. Symbols are as follows: 2,3,2',3'-TCB, $\circ$; 2,4,2',4'-TCB, $\blacktriangle$; 2,5,2',5'-TCB, $\square$; 2,6,2',6'-TCB, $\bullet$; 3,4,3',4'-TCB, $\triangle$. At 20, and 40 μM , symbol of 2,3,2',3'-TCB ($\circ$) represents those of 2,4,2',4'-, 2,5,2',5'-, 2,6,2',6'-, and 3,4,3',4'-TCBs. At 60 μM , symbol ($\circ$) also represents those of 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-TCBs.

bition of uncoupler (in this case, DNP)-stimulated respiration reflects the inhibition of the electron transport chain. Therefore, these experiments were conducted to determine if TCBs affected the electron transport chain of mitochondria. Concentration-dependent inhibitions were observed with 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs. The amounts required for 50% inhibition (I_{50}) are shown in Table I; there was no significant difference among these TCBs ($p < 0.05$). However, inhibitory actions of 2,6,2',6'-, and 3,4,3',4'-TCBs were weak. These results suggest that 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs are potent inhibitors of the electron transport chain of mitochondria, whereas 2,6,2',6'-, and 3,4,3',4'-TCBs are weak inhibitors.

FIG. 3. Effects of TCBs on the ATPase Activity of Rat Liver Mitochondria

The reaction medium consisted of 0.15 M KCl, 3 mM $MgCl_2$, 5 mM Tris-HCl (pH 7.4), 5 mM ATP, and without ($\circ$, $\triangle$) or with ($\bullet$, $\blacktriangle$) 25 μ M DNP. The reaction was initiated by adding 1 mg/ml of mitochondria. After incubation for 10 min at 25°C, inorganic phosphate released by the hydrolysis of ATP was determined. Symbols are as follows: 2,3,2',3'-, $\circ$, $\bullet$; 2,6,2',6'-TCB, $\triangle$, $\blacktriangle$. Each point is a mean of 3 separate experiments.

This is in good agreement with the manner of inhibition on state 3 respiration. Inhibition of state 3 respiration is caused by the inhibition of either the ATPase system or the electron transport chain. Since it has turned out that TCBs do not act as ATPase inhibitors (Fig. 3) such as oligomycin which suppresses the stimulatory effect exerted on the ATPase activity by uncoupler, the inhibition on state 3 respiration caused by TCBs is attributed to interference with the electron transport chain.

Representative polarographic traces depicting the effect of 2,6,2',6'-TCB on state 4 respiration with succinate are shown in Fig. 5A. The stimulation of state 4 respiration began at 60 μ M, and became marked with increasing concentration of the agent. A lag period was observed, the length of which was dependent on the agent concentration before stimulation became obvious.

FIG. 4. Effects of TCBs on DNP-Stimulated Respiration of Rat Liver Mitochondria with Succinate as the Substrate

Mitochondria (1 mg/ml) were interacted with TCB for 3 min, then DNP-stimulated respiration was initiated by the addition of 25 μ M DNP. Control rate was 156.3 ± 5.4 natoms O/min/mg protein. Symbols are as follows: 2,3,2',3'-, $\circ$; 2,4,2',4'-, $\blacktriangle$; 2,5,2',5'-, $\square$; 2,6,2',6'-, $\bullet$; 3,4,3',4'-TCB, $\triangle$. Each point is a mean of 3 separate experiments.

This was in contrast to DNP which showed an instantaneous stimulation. 2,6,2',6'-TCB released the oligomycin-inhibited state 3 respiration (Fig. 5B); identical results were observed for 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs.

DISCUSSION

The results presented here clearly indicate that marked differences exist in the effects of TCBs on respiratory and energy-linked functions of rat liver mitochondria. 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs showed potent inhibitory actions on both state 3 and DNP-stimulated respirations, while those induced by 2,6,2',6'-, and 3,4,3',4'-TCBs were weak. The fact that the extent of inhibition due to same TCB is similar in both state 3 and DNP-stimulated respiration (Figs. 1 and 4) indicates that the inhibitory

action of TCBs on state 3 respiration is mainly caused by interference with the electron transport chain. It is clear that 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs, which are potent inhibitors of these respirations, have a common structure with chlorine atoms equally attached to both inside (*ortho*, *ortho'*) and outside (*meta*, *para*; *meta'*, *para'*) positions of the biphenyl ring, and that TCBs of which all chlorine atoms are localized in either inside (2,6,2',6'-TCB) or outside (3,4,3',4'-TCB) positions of the biphenyl ring are poor inhibitors. As far as TCBs are concerned, the former-type of compounds (*i.e.*, 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-TCBs) may be optimum configurations to interact with enzymes which constitute the electron transport chain when compared to the latter-type compounds (*i.e.*, 2,6,2',6'-, 3,4,3',4'-TCBs). In fact, the former-type of compounds inhibited succinate dehydrogenase, and the CoQ-cytochrome *c* region of the electron transport chain, while the latter-type had very little effect (data not shown).

Stimulation of state 4 respiration is generally explained by uncoupling action. Judging from this, 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-TCBs possess uncoupling actions. The uncoupling actions of 2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs, however, are masked at higher concentrations because of the increased inhibition of the electron transport chain. 2,6,2',6'-TCB showed a potent uncoupling action, since this agent did not inhibit electron transfer. 3,4,3',4'-TCB did not show an uncoupling action. 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-TCBs possess considerably hindered structure because of the steric hindrance of chlorines in *ortho*, *ortho'* position, while 3,4,3',4'-TCB is a coplanar structure because of the increased double-bond character of the C₍₁₎-C_(1') bond due to chlorine atoms in *para*, *para'* positions.¹⁴⁾ According to the chemiosmotic theory,¹⁵⁾ the dissipation of membrane potential is generally regarded as decisive for exhibition of uncoupling action. With a protonophoric uncoupler, dissipation of membrane potential is performed by shuttling protons across the mem-

FIG. 5. Polarographic Traces That Depict the Effects of 2,6,2',6'-TCB on Respiration of Rat Liver Mitochondria

Trace A, stimulation of state 4 respiration by various concentrations of 2,6,2',6'-TCB: (a) none, (b) 60, (c) 80, (d) 100, (e) 150, (f) 200 μ M. ADP, 150 μ M. Trace B, release of oligomycin-inhibited state 3 respiration by 100 μ M 2,6,2',6'-TCB. ADP, 1 mM; oligomycin (Olig), 2 μ g/mg protein. In both traces A and B, succinate (Succ), 5 mM, rat liver mitochondria (RLM), 1 mg/ml. A dotted line indicates DNP (25 μ M)-induced respiration.

brane with an acid-dissociable group within the molecule. However, TCBs do not possess an acid-dissociable group. 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-TCBs, therefore, dissipate membrane potential through a different mechanism from that of a protonophoric uncoupler. We showed that Kanechlor-400 (a mixture of PCBs) dissipated membrane potential by increasing membrane permeability to protons or other ions.¹⁶⁾ Therefore, a most probable candidate for these TCBs may be also an increased membrane permeability to ions. When 2,3,2',3'-, 2,4,2',4'-, 2,5,2',5'-, and 2,6,2',6'-TCBs are incorporated into the lipid phase of the mitochondrial membranes, these TCBs may perturb the lipid phase because of their hindered structure, and increase membrane permeability to ions, thereby dissipating membrane potential, which leads to uncoupling. In contrast, the membrane damage due to 3,4,3',4'-TCB is not enough to cause the permeability increase because of its coplanar structure.

Recent publications have indicated that increase of intracellular Ca^{2+} is a potential mechanism of cell injury following contact with toxic agents.^{17,18)} It was suggested that the accumulation of Ca^{2+} is the feature which converts initially non-lethal into irreversible cell injury by hepatotoxic agents.¹⁹⁾ The intracellular free Ca^{2+} concentration in hepatocytes is controlled by the active transport of this ion across the mitochondrial, endoplasmic reticular, and plasma membranes. Mitochondria, which can accumulate large amounts of Ca^{2+} by an energy-dependent pathway, play a major role in Ca^{2+} sequestration.^{20,21)} Addition of an uncoupler such as DNP abolishes this ability of mitochondria. It may be, similarly, that TCBs possessing uncoupling action also disrupt intracellular Ca^{2+} homeostasis, causing cell injury.

2,3,2',3'-, 2,4,2',4'-, and 2,5,2',5'-TCBs activate the latent ATPase, and stimulate state 4 respiration. These phenomena are similar to those of classical protonophoric uncouplers. In case of 2,6,2',6'-TCB, however, the activation of the latent ATPase is not seen; only a stimulation of

state 4 respiration is observed. This is in contrast to protonophoric uncoupler. There are some compounds of which the effects on energy-transducing membranes are similar in some, but not all, respects to those of protonophoric uncouplers. Fatty acids such as oleic acid stimulate state 4 respiration without significant activation of the latent ATPase.²²⁾

REFERENCES

- 1) R. W. Risebrough, P. Reiche, D. B. Peakall, S. G. Herman and M. N. Kirven: Polychlorinated biphenyls in the global ecosystem, *Nature* (London), **220**, 1098–1102 (1968).
- 2) I. J. Stotz and T. A. Greichus: The effects of a polychlorinated biphenyl (Arochlor 1254) on the white pelican, ultrastructure of hepatocytes, *Bull. Environ. Contam. Toxicol.*, **19**, 319–325 (1978).
- 3) M. George, R. J. Chenery and G. Krishna: The effects of ionophore A23187 and 2,4-dinitrophenol on the structure and function of cultured liver cells, *Toxicol. Appl. Pharmacol.*, **66**, 349–360 (1982).
- 4) H. T. Jr. Jonsson, E. M. Jr. Walker, W. B. Greene, M. D. Hughson and G. R. Henigar: Effects of prolonged exposure to dietary DDT and PCB on rat liver morphology, *Arch. Environ. Contam. Toxicol.*, **10**, 171–183 (1981).
- 5) Y. Nishihara: Effects of polychlorinated biphenyls (Kanechlor-400) on isolated rat liver mitochondria, *Arch. Environ. Contam. Toxicol.*, **12**, 517–522 (1983).
- 6) Y. Nishihara: Comparative study of the effects of biphenyl and Kanechlor-400 on the respiratory and energy linked activities of rat liver mitochondria, *Br. J. Ind. Med.*, **42**, 128–132 (1985).
- 7) A. Parkinson, R. Cockerline and S. Safe: Polychlorinated biphenyl isomers and congeners as inducers of both 3-methylcholanthrene- and phenobarbitone-type microsomal enzyme activity, *Chem.-Biol. Interact.*, **29**, 277–289 (1980).
- 8) A. Parkinson, L. Robertson, L. Safe and S. Safe: Polychlorinated biphenyls as inducers of hepatic microsomal enzymes; structure-activity rules, *Chem.-Biol. Interact.*, **30**, 271–285 (1980).
- 9) M. Ahotupa: Enhancement of epoxide-metabolizing enzyme activities by pure PCB isomers, *Biochem. Pharmacol.*, **30**, 1866–1869 (1981).
- 10) O. Hutzinger, S. Safe and V. Zitko: Polychlorinated biphenyls; synthesis of some individual chlorobiphenyls, *Bull. Environ. Contam. Toxicol.*, **6**, 209–219 (1971).
- 11) G. H. Hogeboom: Fractionation of cell component of animal tissues, *Methods Enzymol.*, **1**, 16–19 (1955).
- 12) A. G. Gornall, C. J. Bardawill and M. M. David: Determination of proteins by means of biuret reaction,

- J. Biol. Chem.*, **177**, 751–766 (1949).
- 13) T. Takahashi: Determination of true inorganic phosphate and creatine phosphokinase of swine spermatozoa, *J. Jpn. Biochem. Soc.*, **26**, 690–698 (1955).
 - 14) S. Kawanishi, T. Mizutani and S. Sano: Induction of porphyrin synthesis in chick embryo liver cell culture by synthetic polychlorobiphenyl isomers, *Biochim. Biophys. Acta.*, **540**, 83–92 (1978).
 - 15) P. Mitchell: “Chemiosmotic coupling in oxidative and photosynthetic phosphorylation,” Glynn Research Ltd., Bodmin (U.K.), 1966, pp. 135–156.
 - 16) Y. Nishihara: Uncoupling action of polychlorinated biphenyls (Kanechlor-400) on oxidative phosphorylation in rat liver mitochondria, *Arch. Environ. Contam. Toxicol.*, **13**, 225–229 (1984).
 - 17) F. A. Schanne, A. B. Kane, E. E. Young and J. L. Farber: Calcium dependence of toxic cell death; a final common pathway, *Science*, **206**, 700–702 (1979).
 - 18) A. B. Kane, E. E. Young, F. A. Schanne and J. L. Farber: Calcium dependence of phalloidin-induced liver cell death, *Proc. Natl. Acad. Sci. U.S.A.*, **77**, 1177–1180 (1980).
 - 19) J. L. Farber: The role of calcium in cell death, *Life Sci.*, **29**, 1289–1295 (1981).
 - 20) E. Murphy, K. Coll, T. L. Rich and J. R. Williamson: Hormonal effects on calcium homeostasis in isolated hepatocytes, *J. Biol. Chem.*, **255**, 6600–6608 (1980).
 - 21) S. K. Joseph, K. E. Coll, R. H. Cooper, J. S. Marks and J. R. Williamson: Mechanism underlying calcium homeostasis in isolated hepatocytes, *J. Biol. Chem.*, **258**, 731–741 (1983).
 - 22) I. Matsuoka and T. Nakamura: Reversible effects of fatty acids on respiration, oxidative phosphorylation, and heat production of rat liver mitochondria, *J. Biochem. (Tokyo)*, **86**, 675–681 (1979).