The Influence of Chlorosubstituent Sites of Hexachlorobiphenyl on the Respiration of Rat Liver Mitochondria

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The actions of three hexachlorobiphenyls (HCBs) 2,3,4,2′,3′,4′-, 2,3,4,3′,4′,5′- and 3,4,5,3′,4′,5′-HCBs, on the respiration of rat liver mitochondria with succinate as the substrate were compared, and the effect of chloro-substitution sites in HCB on the respiration was examined. 2,3,4,2′,3′,4′-HCB strongly inhibited both state 3 and 2,4-dinitrophenol (DNP)-stimulated respiration with 50% inhibition dose of 52 and 54 μM for state 3 and DNP-stimulated respiration, respectively. The inhibitory action of 2,3,4,3′,4′,5′-HCB on both respiration was approximately half as potent as that of 2,3,4,2′,3′,4′-HCB. On the other hand, 3,4,5,3′,4′,5′-HCB did not inhibit any respiration at all. These results indicate that both inside (*ortho*) and outside (*meta* or *para*) positions in each phenyl ring of the biphenyl molecule should be replaced with chlorines for HCB to be an effective inhibitor.

Either the actual position of chloro-substituent or steric conformation caused by its substitution or both can be considered as factors affecting the inhibition. On the basis of the conformational energy, calculated by AM1 (Austin model 1) method, with increases in chlorine number in *ortho* position, HCB molecule became angulated. Furthermore, calculated probability of the conformation distribution for HCB indicated that the probability of nonplanarity was higher for effective HCB than for less effective HCB. These structural features suggest the significance of steric conformation as well as chloro-substituent sites in determining the inhibitory ability of HCB.

The extent of inhibition of state 3 respiration by effective HCBs was similar to that of DNP-stimulated respiration, indicating that the inhibition of state 3 respiration due to effective HCBs is mainly due to the interference with the electron transport chain in succinate oxidase. The examination with 2,3,4,2′,3′,4′-HCB revealed that the inhibition sites in succinate oxidase were succinate dehydrogenase and cytochrome bc_1 complex.

Keywords hexachlorobiphenyl; respiration; mitochondria; molecular orbital calculation

Introduction

Polychlorinated biphenyls (PCBs), whose biological and toxic effects have been extensively studied in animal models in the last two decades, are widely distributed environmental pollutants.[1,2] PCBs are large lipophilic molecules with very low water solubility and high octanol–water partition coefficient.[3] Once absorbed in living organisms, therefore, they tend to accumulate in the lipid biophase of living cells such as biomembranes, and can remain there for quite a long time.[4] Particularly, highly chlorinated PCBs can persist there for longer periods of time than less chlorinated ones without being metabolized to any great extent.[5]

It has been recognized that the biological effects of PCBs differ according to the number and the position of chlorines in the biphenyl ring.[6,7] The structure-activity relationships of PCBs have been extensively studied in the field of induction of drug metabolizing enzyme system. That is, individual PCBs are separated into two groups: those which induce cytochrome P-448 (3-methylcholanthrene-type induction), and those which induce cytochrome P-450 (phenobarbital-type induction).[8,9] PCBs that induce cytochrome P-448 contain chlorines in the *meta* and *para* positions of both phenyl rings, but not in the *ortho* position (3,4,3′,4′-tetra, 3,4,5,3′,4′-penta, and 3,4,5,3′,4′,5′-hexachlorobiphenyl). From these data, it has been suggested that the optimum conformation for PCBs to show 3-methylcholanthrene (3-MC)-type induction is a planar rectangle 3 × 10 Å with chlorines in at least three of the four corners.[10] Most PCBs which contain chlorines in the *ortho* position are phenobarbital (PB)-type inducer.[8] These compounds possess nonplanar structure because of the steric effect of chlorines in the *ortho* position.[11] Although the structural requirements for PCBs to show PB-type induction are not clear, it is noted that at least a nonplanar structure is needed.

Another field where the structure–activity relationship of PCBs has been studied is porphyrin synthesis;[11,12] the structure–activity relationships of PCBs in the rest of the fields, however, are poorly understood. We have studied the interaction of PCBs with biomembranes, especially with mitochondrial membranes.[13–15] It has been revealed that some PCBs inhibit the electron transport chain and collapse the membrane potential of mitochondria.[16,17] In a previous paper,[18] we reported the effects of symmetrical hexachlorobiphenyl (HCB) isomers on succinate oxidizing enzyme of rat liver mitochondria. We showed that HCBs which contain chlorines in both inside (*ortho*, *ortho*′) and outside (*meta*, *para*; *meta*′, *para*′) positions of the biphenyl ring are effective inhibitors of this enzyme, and that HCB which lacks chlorines in innermost positions (3,4,5,3′,4′,5′-HCB) does not inhibit at all. Therefore, it has been suggested that chlorine substitution to *ortho* position of the biphenyl ring is necessary for HCB to be an effective inhibitor of succinate oxidizing enzyme (succinate oxidase). However, it remains to be elucidated whether both *ortho*, *ortho*′ positions in the biphenyl ring must be replaced with chlorines for this to be true. For this purpose, it is desirable to use unsymmetrical HCB which contains an *ortho* chlorination pattern in one ring, and a *non-ortho* chlorination pattern in the other ring.

In the present study, as a representative compound of this type of HCB, we synthesized 2,3,4,3′,4′,5′-HCB which

contains an *ortho* chlorination pattern (2,3,4-chlorination) in one ring, and a *non-ortho* chlorination (3,4,5-chlorination) in the other ring. We examined its effect on succinate oxidase, and compared its potency with that of corresponding symmetrical isomers (*i.e.*, 2,3,4,2′,3′,4′-, and 3,4,5,3′,4′,5′-HCB). The results show that these HCBs inhibit succinate oxidase in the order 2,3,4,2′,3′,4′->2,3,4,3′,4′,5′-≫3,4,5,3′,4′,5′-HCB, suggesting that the structural requirement for HCB to be an effective inhibitor is that the *ortho* position in both phenyl rings be occupied by chlorines. Moreover, to evaluate the steric factor of the HCBs that may affect the inhibition, the structures of these isomers were optimized by molecular orbital calculations.

Materials and Methods

Synthesis of HCBs 2,3,4,2′,3′,4′-, 2,3,4,3′,4′,5′- and 3,4,5,3′,4′,5′-HCBs were synthesized by the Cadgan modification of the Gomberg–Bachmann coupling reaction[19] using the appropriate chlorinated aniline in excess chlorinated benzene, as described by Püttmann *et al.*[20] All HCBs were purified by alumina or florisil column chromatography or flash chromatography and by recrystallization from methanol. The structure of 2,3,4,2′,3′,4′-(mp 151—152 °C), 2,3,4,3′,4′,5′- (mp 159 °C) and 3,4,5,3′,4′,5′-HCB (mp 201 °C) were confirmed by ^{1}H-NMR and mass spectrometry. The purity as determined by gas chromatography was in all cases greater than 98%. The stock solutions of these HCBs were prepared in dimethylformamide.

Biochemicals Adenosine 5′-diphosphate (ADP), antimycin A, bovine serum albumin, and ferricytochrome c were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4-Dinitrophenol (DNP), phenazine methosulfate (PMS), sodium azide, and *N,N,N′,N′*-tetramethyl-*p*-phenylendiamine (TMPD) were obtained from Nakarai Chemicals (Kyoto, Japan). All other reagents were of the highest purity commercially available.

Isolation of Mitochondria Mitochondria were isolated from the liver of adult male Wistar rats in a medium containing 0.25 M sucrose, 5 mM Tris–HCl (pH 7.4), and 0.1 mM EDTA, as described by Mayers and Slater.[21] Protein concentration was determined by the biuret method using bovine serum albumin as a standard.[22]

Measurements of Mitochondorial Respiration 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 reaction medium consisted of 0.2 M sucrose, 20 mM KCl, 3 mM $MgCl_2$, and 5 mM potassium phosphate (pH 7.4). Five mM succinate was used as the substrate, and mitochondrial concentration was 1 mg/ml. When present, ADP was 150 μM, and DNP was 25 μM.

Enzyme Assays Succinate dehydrogenase activity was assayed polarographically at 25 °C using PMS as an electron acceptor in the respiratory medium (2 ml) containing 2 μg/mg protein of antimycin A, 2 mM NaN_3, 5 mM succinate, and 1 mg/ml of mitochondria. Three min after exposure of mitochondria to the test compound, respiration was initiated by the addition of 0.5 mM PMS.[23] Succinate–cytochrome c reductase activity was assayed by the method of King[24] in the respiratory medium (2.5 ml) containing 2 mM NaN_3, 0.1 mM ferricytochrome c, and 1 mg/ml of mitochondria at 25 °C. Mitochondria were allowed to interact with test compound for 3 min, then the reaction was initiated by the addition of 5 mM succinate. Cytochrome c oxidase activity was assayed polarographically at 25 °C in the respiratory medium (2 ml) containing 25 μM DNP and 1 mg/ml of mitochondria. Mitochondria were allowed to interact with test compound for 3 min, then the reaction was initiated by the addition of 5 mM ascorbate/0.1 mM TMPD.

In all experiments, the control contained the same volume of solvent, and the final concentration of solvent was less than 0.7% (v/v). The concentration of solvent did not affect the activities assayed.

Molecular Orbital Calculations Molecular orbital calculations were performed using the semiempirical Austin model 1 (AM1)[25] method *via* the program MOPAC[26] on a Fujitsu Facom M-760 computer at the University of Tokushima. The conformational energy of HCB isomers was calculated as a function of the interplanar angle (θ) between phenyl rings (torsion angle, at every 30° starting from 0° up to 180°). The probability of the conformation of each isomer at 25 °C was calculated using Boltzmann's distribution equation

$$P(\theta)=\frac{\exp(-\Delta E(\theta)/RT)}{\sum_{0^\circ}^{180^\circ}\exp(-\Delta E(\theta)/RT)}$$

where R and T are gas constant and absolute temperature, respectively, and ΔE is the energy difference between each conformation and the lowest energy conformation, assuming that the probability follows the Boltzmann distribution.

Results

The effect of HCBs on state 3 respiration with succinate as the substrate are shown in Fig. 1. Of the three isomers studied, 2,3,4,2′,3′,4′-HCB showed the strongest inhibition, with 83% inhibition at 200 μM. Fifty percent inhibition dose (ID_{50}) was 52 μM. The unsymmetrical HCB, 2,3,4,3′,4′,5′-HCB, showed about half as much inhibition as 2,3,4,2′,3′,4′-HCB, with 51% inhibition at 200 μM. In contrast to these HCBs, 3,4,5,3′,4′,5′-HCB barely showed any inhibition, with only 6% at 200 μM. Two way analysis of variance and *t*-test ($p<0.05$) revealed that the order of inhibitory potency on state 3 respiration was 2,3,4,2′,3′,4′->2,3,4,3′,4′,5′-≫3,4,5,3′,4′,5′-HCB.

Figure 2 shows the effects of HCBs on the oxygen consumption 3 to 4 min after the addition of HCBs during state 4 respiration with succinate as the substrate.

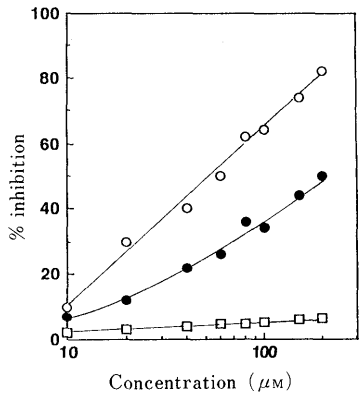

Fig. 1. Comparison of the Inhibitory Effects of HCBs on State 3 Respiration of Rat Liver Mitochondria with Succinate as the Substrate

Mitochondria (1 mg/ml) were allowed to interact with HCB for 3 min, then state 3 respiration was initiated by the addition of 150 μM ADP. Control rate was 130.3 ± 3.8 natoms oxygen/min/mg protein. Each point is the mean of 3 separate experiments. Symbols: 2,3,4,2′,3′,4′-, ○; 2,3,4,3′,4′,5′-, ●; 3,4,5,3′,4′,5′-HCB, □.

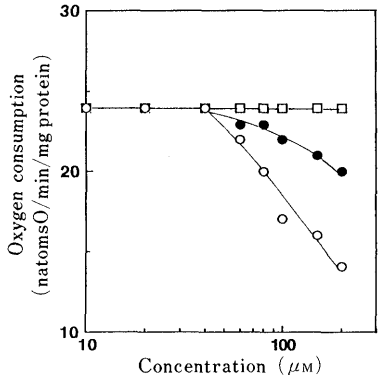

Fig. 2. Effects of HCBs on State 4 Respiration of Rat Liver Mitochondria with Succinate as the Substrate

Shown are the rates of oxygen consumption 3 to 4 min after the addition of HCB during state 4 respiration. Mitochondrial protein was 1 mg/ml. Each point is the mean of 3 separate experiments. Symbols same as Fig. 1.

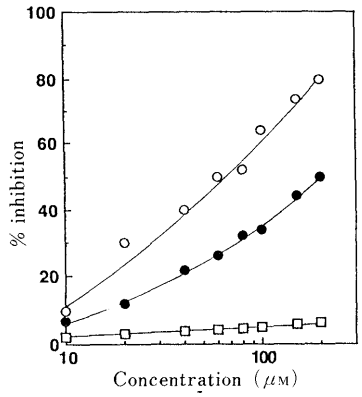

Fig. 3. Comparison of the Effects of HCBs on DNP-Stimulated Respiration of Rat Liver Mitochondria with Succinate as the Substrate

Mitochondria (1 mg/ml) were allowed to interact with HCB for 3 min, then DNP-stimulated respiration was initiated by the addition of 25 μM DNP. Control rate was 151.3±3.7 natoms oxygen/min/mg protein. Each point is the mean of 3 separate experiments. Symbols same as Fig. 1.

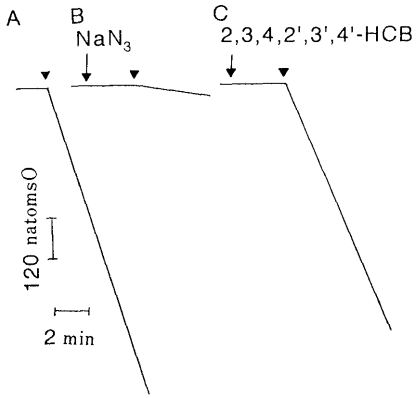

Fig. 4. Effects of 2,3,4,2',3',4'-HCB on Cytochrome c Oxidase of Rat Liver Mitochondria

Cytochrome c oxidase activity was assayed polarographically with a Clark-type oxygen electrode. Mitochondria (1 mg/ml) were allowed to interact with the test compound for 3 min, then the reaction was initiated by the addition of 5 mM ascorbate/0.1 mM TMPD (▼). (A) Control, (B) Effect of 2 mM NaN_3, (C) Effect of 100 μM 2,3,4,2',3',4'-HCB.

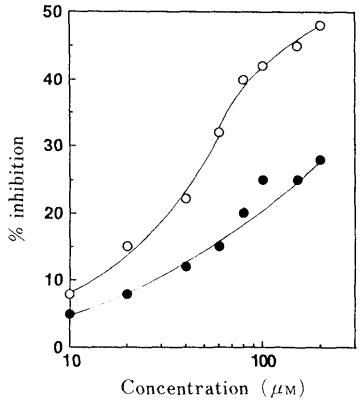

Fig. 5. Effects of 2,3,4,2',3',4'- and 2,3,4,3',4',5'-HCBs on Succinate Dehydrogenase of Rat Liver Mitochondria

Each point is the mean of 3 separate experiments. Symbols: 2,3,4,2',3',4'-, ○; 2,3,4,3',4',5'-HCB, ●.

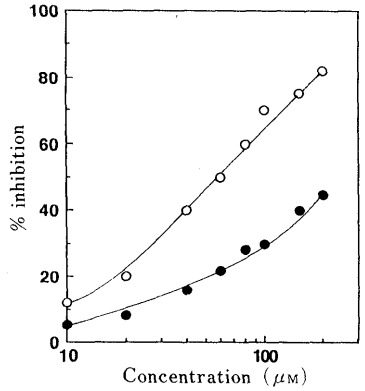

Fig. 6. Effects of 2,3,4,2',3',4'- and 2,3,4,3',4',5'-HCBs on Succinate–Cytochrome c Reductase of Rat Liver Mitochondria

Each point is the mean of 3 separate experiments. Symbols same as Fig. 5.

Mitochondria oxidizing succinate in state 4 usually respire at a rate of 23 natoms oxygen/min/mg protein. When the effects of HCBs on state 4 respiration were tested, none of the HCBs stimulated the respiration. 2,3,4,2',3',4'-HCB inhibited state 4 respiration at higher concentrations. The inhibitory action of 2,3,4,3',4',5'-HCB was weak compared to 2,3,4,2',3',4'-HCB, while 3,4,5,3',4',5'-HCB did not inhibit the respiration at all.

The effect of HCBs on DNP-stimulated respiration with succinate is shown in Fig. 3. 2,3,4,2',3',4'-HCB strongly inhibited the respiration by 80% at 200 μM. The ID_{50} was 54 μM. 2,3,4,3',4',5'-HCB showed about half as much inhibition as 2,3,4,2',3',4'-HCB, with 46% at 200 μM. Generally speaking, the inhibition of DNP-stimulated respiration is interpreted to be inhibition of the electron transport chain of mitochondria. Therefore, these HCBs were found to inhibit the electron transport chain of mitochondria (succinate oxidase). However, 3,4,5,3',4',5'-HCB had almost no effect on the respiration, indicating no effect on succinate oxidase. Two way analysis of variance and t-test ($p<0.05$) revealed that the order of inhibitory potency on DNP-stimulated respiration was 2,3,4,2',3',4'->2,3,4,3',4',5'-≫3,4,5,3',4',5'-HCB. Thus, there was a good agreement with the manner of inhibition between DNP-stimulated respiration and state 3 respiration (Fig. 1).

Figure 4 shows the effects of 2,3,4,2',3',4'-HCB on cytochrome c oxidase, the final segment of the electron transport chain. Mitochondria oxidizing ascorbate/TMPD in the presence of DNP respire at a rate of 72 natoms oxygen/min/mg protein (Fig. 4A). The effects of NaN_3, the specific inhibitor of cytochrome c oxidase, are also shown for comparison (Fig. 4B). Sodium azide completely inhibited the respiration. In contrast to NaN_3, as shown in Fig. 4C, 2,3,4,2',3',4'-HCB, even at 200 μM, did not affect the respiration, indicating no effect on cytochrome c oxidase. 2,3,4,3',4',5'-HCB also did not inhibit cytochrome c oxidase (data not shown). Therefore, from the facts shown in Figs. 3 and 4, it is apparent that these HCBs inhibit the components of the electron transport chain located on the substrate side of cytochrome c in succinate oxidase.

To determine the precise interacting sites of 2,3,4,2',3',4'- and 2,3,4,3',4',5'-HCBs in the electron transport chain, the effects of these compounds on succinate dehydrogenase and succinate–cytochrome c reductase were examined. Figure 5 shows their effects on succinate dehydrogenase. Both initially inhibited succinate dehydrogenase at 10 μM, and the inhibition increased in a concentration-dependent manner with 48% and 27% inhibition at 200 μM for 2,3,4,2',3',4'- and

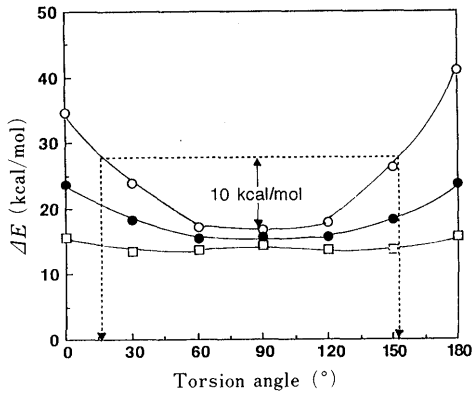

Fig. 7. Conformational Energy of HCBs as a Function of Torsion Angle

The conformational energy of each HCB was calculated as a function of torsion angle at every 30°. The dotted line shows the energy level associated with the biological interaction (10 kcal/mol). Symbols same as Fig. 1.

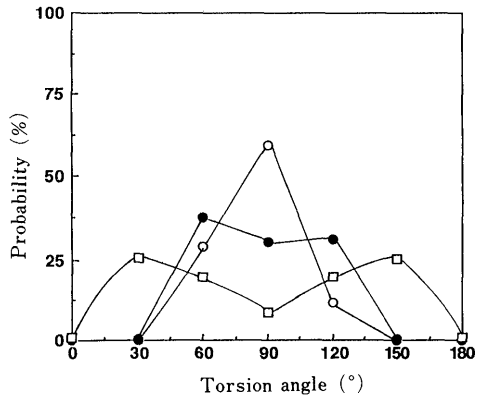

Fig. 8. Probability of HCB Conformation as a Function of Torsion Angle

The probability of HCB conformation was calculated as a function of torsion angle at every 30° under the assumption that the probability follows the Boltzmann distribution. Symbols same as Fig. 1.

2,3,4,3′,4′,5′-HCB respectively, indicating that the inhibitory reaction for succinate oxidase (Fig. 3) due to these compounds occurs partially in succinate dehydrogenase. In addition, the inhibitory potency of the latter was about half as strong as that of the former.

Figure 6 shows the effects of 2,3,4,2′,3′,4′- and 2,3,4,3′,4′,5′-HCB on succinate–cytochrome c reductase. The inhibition by 2,3,4,2′,3′,4′-HCB (ID_{50},57 μM) was about twice as strong as that by 2,3,4,3′,4′,5′-HCB. Moreover, the inhibitory actions of these compounds were far greater than those on succinate dehydrogenase, indicating that these compounds also inhibit the region between succinate dehydrogenase and cytochrome c (cytochrome bc_1 complex).

The conformational energy of these HCB isomers calculated, using AM1, as a function of their torsion angle occurring between the two phenyl rings is shown in Fig. 7. In these calculations, the torsion angle of the coplanar conformation is referred to as $\theta=0°$, and that of the conformation in which two phenyl rings are perpendicular as $\theta=90°$. 2,3,4,2′,3′,4′-HCB showed the highest energy at 0° (180°), and the energy lowered as the torsion angle approached 90°. The energy difference between 0° (180°) and 90° was 18 (24) kcal/mol, indicating the existence of a rotational barrier between the different states of the molecule. 2,3,4,3′,4′,5′-HCB showed the highest energy at 0° (180°), but this value was less than that of 2,3,4,2′,3′,4′-HCB. The energy levels of the compound at 60°, 90°, and 120° were equal. The energy difference between the highest and the lowest states was 8 kcal/mol, indicating the existence of a lower rotational energy barrier than that seen with 2,3,4,2′,3′,4′-HCB. 3,4,5,3′,4′,5′-HCB also showed the highest energy at 0° (180°), but this value was far less than that at corresponding states of 2,3,4,2′,3′,4′- and 2,3,4,3′,4′,5′-HCB. The energy level was the lowest at 30° and 150°. The energy difference between the highest and the lowest was only 2 kcal/mol, indicating that the rotational barrier is very small and that the compound rotates freely around $C_{(1)}$–$C_{(1')}$ axis. Figure 7 also shows the possible range of the torsion angle of each isomer (*i.e.*, the angle giving the energy level below the dotted line in the figure; $20°<\theta<150°$, $0°<\theta<180°$, $0°<\theta<180°$ for 2,3,4,2′,3′,4′-, 2,3,4,3′,4′,5′- and 3,4,5,3′,4′,5′-HCB, respectively), under the assumption that the energy associated with the biological interaction is 10 kcal/mol.

Table I. Relationship between Chlorine Number at *ortho* Position of HCB and Parameters Associated with Molecular Orbital Calculations

Chlorine number at *ortho* position	Energy difference[a] (kcal/mol)	Possible range of the torsion angle (°)	Probability at the conformation at 90°	Torsion angle giving the maximum probability (°)
0	2	0—180	8.4	30
1	8	0—180	30.5	60
2	24	20—150	59.5	90

a) Energy difference between the highest and the lowest conformation.

Figure 8 shows the probability of isomer conformation at each torsion angle. 2,3,4,2′,3′,4′-HCB had a maximum probability (59%) at 90°, followed by 29% (60°) and 11% (120°). The probability at the rest of the angle was 0%. Therefore, the compound existed essentially at 90°. The probability of 2,3,4,3′,4′,5′-HCB at each angle was 37% (60°), 30% (90°), 31% (120°) and 1% (30° and 150°), indicating that 2,3,4,3′,4′,5′-HCB existed equally between 60° and 120°. 3,4,5,3′,4′,5′-HCB had a maximum probability (25%) at 30° and 150°, followed by 20% (60° and 120°), 8% (90°), and <1% (0° and 180°). This indicates that 3,4,5,3′,4′,5′-HCB existed equally over a wide range ($0°<\theta<180°$).

Table I summarizes the characteristics of each isomer obtained from molecular orbital calculations. As the number of chlorine atoms in *ortho* position increased from zero to two, the energy at 0° or 180° increased. Furthermore, the energy difference between the highest and the lowest state also increased, resulting in the sharpening of the slope of parabola in Fig. 7, which narrowed the possible range of the torsion angle.

Discussion

The results of the previous study demonstrated several important characteristics of HCBs with respect to their ability to inhibit the electron transport chain of mitochondria (succinate oxidase).[18] Namely, the effective inhibitors (2,3,4,2′,3′,4′-, 2,3,5,2′,3′,5′-, 2,3,6,2′,3′,6′-, 2,4,5,2′,4′,5′- and 2,4,6,2′,4′,6′-HCBs) have a common structure: chlorine

atoms are present in both inside (*ortho*, *ortho'*) and outside (*meta*, *para*; *meta'*, *para'*) positions of the biphenyl molecule. HCB, in which all chlorine atoms are localized in outside positions (3,4,5,3′,4′,5′-HCB), is a poor inhibitor. Judging from this, it is speculated that the optimum condition for HCB to interact with succinate oxidase is that both inside and outside positions of the biphenyl molecule are occupied by chlorine atoms.

The present study was designed to investigate whether *ortho* positions of both phenyl rings in the biphenyl molecule must be replaced with chlorines in order for HCB to be the effective inhibitor of succinate oxidase. As shown by the results, for 2,3,4,2′,3′,4′-HCB, replacing inside and outside positions of each phenyl ring with chlorines strongly inhibited both state 3 and DNP- stimulated respirations. On the other hand, for the inhibitory actions of 2,3,4,3′,4′,5′-HCB, when inside and outside positions in one ring (2,3,4-chlorination pattern) and only outside positions in the other ring (3,4,5-chlorination pattern) were replaced with chlorines resulted in both respirations being about half as potent as those of 2,3,4,2′,3′,4′-HCB. Thus, chlorine substitution in the *ortho* position of only one phenyl ring is not sufficient for HCB to act as a strong inhibitor of succinate oxidase; instead, for HCB to exert full inhibitory ability it is necessary that *ortho* position of both phenyl rings be replaced with chlorines.

Furthermore, the fact that the extent of inhibition due to 2,3,4,2′,3′,4′- and 2,3,4,3′,4′,5′-HCB is similar in both state 3 and DNP-stimulated respiration (Figs. 1 and 3) indicates that the inhibitory action of these HCBs on state 3 respiration is not caused by the inhibition of ATPase but by the interference with the electron transport chain. From the facts shown in Figs 5 and 6, the inhibition sites of these HCBs in the electron transport chain were identified to be succinate dehydrogenase and cytochrome bc_1 complex.

The inhibition of the electron transport chain by these HCBs is closely related with the chlorine number substituted in *ortho* position. As summarized in Table I, the molecule becomes highly angulated and rigid with increases in chlorine number in *ortho* position. Therefore, when intercalated in the electron transport chain, especially at the inhibition sites, 2,3,4,2′,3′,4′-HCB (two phenyl rings are perpendicularly oriented) may perturb the smooth electron transfer between the assemblies because of this nonplanarity. As the rigidity of 2,3,4,3′,4′,5′-HCB, chlorinated at one *ortho* position and at *meta* and *para* positions of both phenyl rings, is not as large as that of 2,3,4,2′,3′,4′-HCB, the perturbation of the electron transfer by this chemical may not be as potent. On the other hand, 3,4,5,3′,4′,5′-HCB rotates freely along the axis, (*i.e.*, is flexible), so it is possible for the compound to take any conformation so as to cope with the condition of the enzyme assemblies. For this reason, the perturbation of the electron transfer by this chemical is thought to be very small.

Although the inhibition of the electron transport chain is closely associated with the steric factor of HCB discussed above, it is possible to consider another factor (*i.e.*, the actual position of chlorine substitution) rather than the steric factor itself. Namely, substitution of chlorine atoms in both the *ortho* and *meta* or *para* positions of each phenyl ring is more important than in only mono *ortho* or *non-ortho* positions for HCB to possess a molecular dimension or charge distribution (or an electronic environment)[27] suitable for inhibiting succinate oxidase; or, both factors may be equally important. According to the induced fit model proposed by Koshland,[28] the approach of substrate (in this case, succinate) induces a conformational change in the enzyme protein (succinate dehydrogenase) which causes amino acid residues on the enzyme to be aligned in the correct special orientation. Prior binding of 2,3,4,2′,3′,4′-HCB to the enzyme, because of its steric or substitution-site effect, may inhibit the conformational change of the enzyme when the substrate approaches.

As for the uncoupling action of PCBs, we have made detailed reports on tetrachlorobiphenyls (TCBs).[16,17] Namely, nonplanar TCBs show strong uncoupling action, while planar 3,4,3′,4′-TCB does not.[17] Therefore, it is expected that nonplanar HCBs will also show an uncoupling action from the results of TCBs. Contrary to this expectation, all HCBs tested here, though highly angulated, did not stimulate state 4 respiration, suggesting that these HCBs do not have uncoupling action. Generally uncoupling is caused by the dissipation of membrane potential due to increases in membrane permeability to ions such as H^+.[29] Planar 3,4,3′,4′-TCB does not cause ion fluxes through mitochondrial membranes, so it does not show uncoupling action.[17] However, nonplanar TCBs increase the membrane permeability to ions, thereby causing this action.[17] We have already shown that Kanechlor-600, a mixture of HCBs, had weaker potency than Kanechlor-400, a mixture of TCBs, in its ability to cause membrane permeability changes.[15] Therefore, it can be speculated that PCBs possessing more than five chlorines, though highly angulated, have weak effects on the membrane permeability to ions. For this speculation, HCB used in this study may also be weaker than TCBs in its ability to increase membrane permeability to ions. Accordingly, these HCBs may not dissipate the membrane potential enough to stimulate state 4 respiration.

Besides 2,3,4,2′,3′,4′-HCB shown in this study, some nonplanar HCBs have been found to act as potent inhibitors of the electron transport chain of mitochondria.[18] This inhibition may finally result in a cessation of cellular adenocine 5′-triphosphate (ATP) generation. Because it is suggested that the inhibition of ATP generation is closely related to the induction of cell death in cytotoxicity induced by chemicals such as menadione,[30] this mechanism may also apply to nonplanar HCB-induced toxicity. Several studies concerning the mitochondrial dysfunction *in vivo* have been reported. Stotz and Greichus[31] reported alteration in the shape of liver mitochondria from the white pelican as a result of *in vivo* treatment with PCB. Mitochondria from the PCB-treated white pelican were round and swollen instead of long and slender as in the untreated animal; this change is similar to that produced by DNP,[32] a typical uncoupler, suggesting uncoupling of oxidative phosphorylation. Humans and animals exposed to PCBs exhibit morphologically abnormal mitochondria, although the authors do not state the physiological influence of this.[33] Ebner and Braselton[34] demonstrated that liver mitochondria from PCB-treated rats (300 mg/kg *p.o.* on 4 consecutive days) experienced a significant loss of body weight showed both suppression of state 3 respiration and increased membrane permeability to actively accumulated

or impermeable ions (*i.e.*, sign of uncoupling) compared to those from untreated controls. Moreover, these wasting rats had hyperuremia, hypoglycemia, stimulation of ureagenesis and suppression of gluconeogenesis after ammonium acetate was administered, and they exhibited decreased retention of assimilated nitrogen. From these data, they suggested that the increased mitochondrial membrane permeability to ions might predispose the PCB-treated rats toward urea formation rather than glucose synthesis and nitrogen retention, resulting in a significant loss of body weight. These findings point to a mitochondrial effect of treatment of PCBs. ID_{50} of 2,3,4,2′,3′,4′-HCB for state 3 respiration is relatively high. But PCBs tend to accumulate in lipid-rich membraneous fractions of the cell, and are resistant to metabolism.[35] Therefore, 2,3,4,2′,3′,4′-HCB may also be an effective inhibitor after a long *in vivo* exposure.

As 3,4,5,3′,4′,5′-HCB does not cause mitochondrial dysfunction, the compound may exert its toxic effect by another mechanism. This isomer shows several biochemical and toxic responses such as teratogenicity, porphyria, cleft palate, thymic atrophy, and immnosuppression.[36] These phenomena are considered to be mediated by the binding of the compound to Ah (aromatic hydrocarbon) receptor.[37] The planarity of the compound is essential for the binding to the receptor,[38] and because of the planarity, 3,4,5,3′,4′,5′-HCB binds to Ah receptor. The receptor/ligand complex thus formed translocates to the nucleus, and binds to the upstream region of structural genes to stimulate DNA transcriptions.[37] Subsequent events, however are not yet understood.

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