Biochemical Pharmacology, Vol. 29, pp. 3311–3317.
Pergamon Press Ltd. 1980. Printed in Great Britain.

0006-2952/80/1215-3311 $02.00/0

EFFECTS OF POLYCHLORINATED BIPHENYLS ON BIOLOGICAL MEMBRANES

PHYSICAL TOXICITY AND MOLAR VOLUME RELATIONSHIPS

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(*Received* 12 *April* 1980; *accepted* 7 *July* 1980)

Abstract—Perturbation of biological membranes by a series of purified polychlorinated biphenyls (PCBs) was studied, with the objective of distinguishing nonspecific physical toxicities from specific chemical interactions. The molar volume parameter 36,000 $V_x - E_B$, used in the past to estimate physical toxicities and partition coefficients, was calculated for a series of PCBs. The cytotoxicity of various PCBs and other lipophilic compounds to mouse spleen lymphocytes *in vitro* was investigated, and LD_{50} values were determined, using a ^{51}Cr-release assay to measure cell viability. All PCBs tested showed similar toxicities with LD_{50} values in the range of 2.33×10^{-6} to 3.55×10^{-5} M. PCBs were also capable of inhibiting the lymphocyte plasma membrane enzyme 5′-nucleotidase *in vitro*, with K_i values falling between 3.45 and 6.40×10^{-5} M. When both log LD_{50} and log K_i values were plotted against molar volume for the various compounds tested, the curves obtained followed the form predicted for a pure physical toxicity effect, with an initial linear region of slope 1 and a plateau region for compounds of large molar volume. Molar volume correlations were used to estimate the volume of lipid biophase in the systems studied and the toxic concentration of chemical in the membrane. Inhibition of plasma membrane enzymes such as 5′-nucleotidase and ATPase by PCBs is thus a nonspecific physical toxicity effect based on their lipid solubility, and there appears to be no specific chemical interaction between these molecules and membrane components.

Halogenated hydrocarbon pollutants such as polychlorinated and polybrominated biphenyls have become increasingly widespread in the environment in recent years, causing concern over their possible adverse effects on living organisms. These molecules have much in common with other persistent pollutants in that they are large hydrophobic molecules with very low water solubilities and correspondingly high octanol–water partition coefficients [1]. They thus tend to concentrate in the lipid biophase of living cells and can persist for long periods of time without being metabolized to any great extent. Although PCBs† do have specific biochemical effects, e.g. induction of drug-metabolizing enzyme systems [2] and stimulation of cytochromes P-448 and P-450 [3], we would expect their physical properties as lipophilic molecules to play a significant role in their overall toxicity.

It has been recognized for some time that the toxicities of many compounds depend on their physical properties rather than on their chemical natures [4, 5]. Many chemicals are equitoxic when present in the same concentration in the lipid biophase of a living organism [6], and this toxicity has been related to the molar volume [7] and partition coefficient [8] of the compound. It is now recognized that physical toxicity—toxicity caused primarily by the physical properties of the chemical such as size and hydrophobicity—occurs when the toxic substance reaches a certain concentration or volume fraction in the lipid biophase. For example, a membrane-soluble drug produces anesthesia (a physical toxicity effect) when the volume occupied by the drug in the membrane biophase reaches approximately 0.03 to 0.06 molal [9]. Additional contributions to overall toxicity may be the result of specific chemical interactions between the compound and biological molecules contained in the biophase, that is, chemical toxicity.

Most studies on PCBs and related chemicals have concentrated on the metabolic and biochemical perturbations they induce, and there has been little attempt to separate out the physical toxicity component of their overall action. Cell membranes are a "first target" for lipophilic compounds and we would expect PCBs to affect both overall membrane integrity and the functioning of membrane-bound enzymes. Recent reports show that PCBs [10] and other polyhalogenated hydrocarbons [11] can inhibit plasma membrane ATPases, but no attempt was made to distinguish chemical from purely physical effects. We have made use of molar volume correlations to distinguish nonspecific physical effects from specific chemical toxicity and, while this study deals with the physical toxicity of PCBs, similar results can be expected for other large hydrophobic molecules.

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†Abbreviations: PCB, polychlorinated biphenyl; HBSS, Hanks' balanced salt solution; DMSO, dimethylsulfoxide; and HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid.

MATERIALS AND METHODS

Adenosine-5′-monophosphate (Type II) and *p*-nitrophenyl phosphate were purchased from the Sigma Chemical Co., St. Louis, MO. Medium RPMI 1640 buffered with 25 mM HEPES and heat-inactivated fetal bovine serum were obtained from Gibco Canada, Burlington, Ontario. Chlorooctane and chlorodecane were purchased from the Aldrich Chemical Co., Milwaukee, WI; 1-hexanol, 1-octanol, 1-nonanol, 1 decanol and *trans*-retinol were purchased from the Sigma Chemical Co.; and 1-dodecanol and 1-tetradecanol were purchased from Applied Science Inc., State College, PA.

Polychlorinated biphenyls. All polychlorinated biphenyls were obtained from Dr. S. H. Safe, Department of Chemistry, University of Guelph, and were of high purity as determined by gas chromatographic analysis.

Radioactive compounds. Sodium [^{51}Cr]chromate (sp. act. 200 mCi/mg Cr) and [2-^{3}H]adenosine-5′-monophosphate (sp. act. 15.8 Ci/mmole) were obtained from the Amersham/Searle Corp., Oakville, Ontario.

Lymphocyte separation. Individual spleens from 6 to 8-week-old male Swiss–Webster white mice were gently homogenized by hand, using a Teflon-glass homogenizer, in 10 ml of cold HBSS. Cell debris was allowed to settle for 5 min and then the supernatant fraction containing the cells was removed. The pellet was washed with a further 10 ml of HBSS and the combined supernatant fractions were centrifuged at 280 *g* for 10 min at 4°. The cell pellet was cleared of intact erythrocytes according to the method of Boyle [12]. The resulting pellet was resuspended in 10 ml of cold HBSS and passed through a short, prewashed column of non-absorbent cotton to remove red blood cells and debris. The column was then washed with a further 4 ml of HBSS and the combined effluent was centrifuged at 280 *g* for 10 min at 4°. The cells were resuspended in medium RPMI 1640 buffered with 25 mM HEPES and counted in a hemocytometer. Cell viability was estimated by trypan blue exclusion and was always over 90 per cent. All lymphocyte suspensions were used within a few hours of preparation.

Determination of LD_{50} *by* ^{51}Cr-*release assay.* Cell viability after PCB treatment was measured quantitatively by adapting a ^{51}Cr-release assay designed for measuring target cell death in cytotoxicity assays [13].

Lymphocytes (2.5×10^8) were suspended in 15 ml medium RPMI 1640–25 mM HEPES that was supplemented with 5% heat-inactivated fetal bovine serum. The cell suspension was incubated with 250 μCi of sterile sodium [^{51}Cr]chromate for 1 hr at 37°. The suspension was centrifuged at 280 *g* for 10 min at 0°, and the cells were washed twice with 10 ml RPMI 1640–25 mM HEPES–5% fetal bovine serum and once with 10 ml RPMI 1640–25 mM HEPES to remove excess chromate. The cells were resuspended in RPMI 1640–25 mM HEPES at a concentration of 5×10^6/ml.

Aliquots (1.0 ml) of the labeled cell suspension in 1.5 ml microcentrifuge tubes were incubated with a series of concentrations of PCBs or other test compounds for 1 hr at 37°. PCBs and test compounds were added as 10 μl aliquots in DMSO; appropriate controls were treated with DMSO only. The incubation was terminated by centrifugation on a microcentrifuge for 30 sec, and 200 μl of the supernatant fraction was removed for counting of the released radiochromium in a Nuclear Chicago 4233 Auto Gamma counter. Total chromium release was measured in control tubes by freezing and thawing three times; spontaneous release was usually about 15 per cent of total release. Per cent viability of the lymphocytes was calculated and LD_{50} values were defined as the concentration of a compound which reduced lymphocyte viability by 50 per cent.

Assay for 5′-nucleotidase. Spleen lymphocytes from individual mice were suspended in medium RPMI 1640–25 mM HEPES at a concentration of 5.71×10^7/ml. Aliquots (175 μl) of this suspension (10^7 cells) in 600 μl microcentrifuge tubes were incubated with a series of concentrations of test compound for 30 min at 37°. All test compounds were added as 5 μl aliquots in DMSO. Control tubes were treated with DMSO only.

After the 30-min incubation, 20 μl of radiolabeled substrate solution was added. Final substrate concentrations in a total volume of 200 μl were 1 mM 5′-AMP (0.2 μCi [2-^{3}H]-5′-AMP per tube), 5 mM Mg^{2+} and 5 mM *p*-nitrophenylphosphate. *p*-Nitrophenylphosphate is added to minimize the effects of nonspecific phosphatases present on the cell surface [14]. The cells were incubated for a further 30 min at 37° after which the reaction was stopped by addition of 150 μl of 0.15 M $ZnSO_4$. $Ba(OH)_2$ (150 μl; 0.15 M) was added to precipitate protein and unreacted 5′-AMP, while [^{3}H]adenosine released remained in the supernatant fraction. The tubes were then centrifuged in a microcentrifuge at 8500 *g* for 1 min. Aliquots (300 μl) of supernatant fraction were removed, added to 5 ml of Anderson's Scintillator [15], and counted in a Beckman LS 7000 Liquid Scintillation counter.

Controls for 100 per cent enzyme activity were treated with DMSO only. Controls for 0 per cent enzyme activity were cells to which $ZnSO_4$ solution was added at the same time as radiolabeled substrate, followed by a 30-min incubation at 37° and addition of $Ba(OH)_2$ solution. Enzyme activity was expressed as per cent control and the concentration of a compound which produced half-maximal inhibition was defined as the K_i for that compound.

Under the conditions described above, the hydrolysis of 5′-AMP by intact lymphocytes was linear with cell concentration in the range of 0.3 to 1.6×10^7 cells per assay tube.

Calculation of the molar volume parameter. Calculation of 36,000 V_X–E_B for a typical PCB is shown below, using the data given in Table 1.

2,2′,4,5′-tetrachlorobiphenyl $C_{12}H_6Cl_4$ (23 bonds)

The characteristic volume V_x is found as follows:

12 C atoms with atomic characteristic volume 1.635×10^{-5} each $= 1.962 \times 10^{-4}$

6 H atoms with atomic characteristic volume 8.71×10^{-6} each $= 5.226 \times 10^{-5}$

4 Cl atoms with atomic characteristic volume 2.095×10^{-5} each $= 8.38 \times 10^{-5}$

Total $= 3.323 \times 10^{-4}$

Substract 23 bonds with characteristic volume 6.56×10^{-6} each $= -1.509 \times 10^{-4}$

Characteristic volume of PCB,

$$V_x(\text{m}^3, \text{mole}^{-1}) = 1.814 \times 10^{-4}$$

$$36{,}000\ V_x = 6.53$$

The interaction term E_B is found as follows:

4 Cl groups of $E_B - 0.35$ each $= -1.40$

The molar volume parameter

$$36{,}000\ V_x - E_B = 7.93$$

RESULTS AND DISCUSSION

In this study we have looked at membrane perturbation by PCBs and other lipophilic molecules at two different levels. First of all, we have looked at the cytotoxic effects of these chemicals on intact cells under conditions where cell death is apparently due to disruption of the integrity of the plasma membrane. Second, inhibition of the plasma membrane enzyme 5'-nucleotidase by these compounds has been studied. In this case, enzyme inhibition may be due to perturbation of the form and fluidity of the lipid bilayer matrix, or perhaps to direct interaction between the toxicant and the enzyme protein. Purified PCB isomers and other highly hydrophobic molecules were chosen to cover a wide range of molecular size. All experiments were designed to look at short-term effects, of the order of 1 hr, so that metabolism of PCBs should play no significant role in the results obtained. The basic question we wanted to answer was whether the toxic effects of PCBs can be predicted from their physical properties, or if there is evidence of specific interactions between PCBs and membrane components.

Molar volume relationships. McGowan [7] first realized the importance of molar volume as a physical property which correlated very well with the anesthetic potency and toxicity of many chemicals. For interaction of a toxic compound with a system containing both an aqueous and a nonaqueous phase (the lipid biophase) we can describe the relationship between toxicity and molar volume as follows:

$$C_t = A + B \times 10^{-(36{,}000\ V_x - E_B)} \qquad (1)$$

where C_t is the toxic concentration required in the aqueous phase to produce a certain manifestation of toxicity (defined by the experimenter), $36{,}000\ V_x - E_B$ is the molar volume parameter, and A and B are constants for the system. B gives the toxic concentration of the chemical in the lipid biophase, and the value of A/B equals the ratio of nonaqueous phase volume to aqueous phase volume for the system. (For further details on the derivation and use of equation 1, see Refs. 16 and 17.) A plot of $-\log C_t$ vs $36{,}000\ V_x - E_B$ should give a curve consisting of two parts: (a) a straight line portion of slope 1 at low molar volume; the intercept of this line when $36{,}000\ V_x - E_B$ is zero equals $-\log B$, and (b) a horizontal plateau at high molar volumes, where the value of $-\log C_t$ equals $-\log A$.

The molar volume parameter, $36{,}000\ V_x - E_B$, is composed of two parts. V_x, the characteristic volume, is an estimate of the molar volume of the chemical at absolute zero and is calculated by summing individual contributions for each atom in the molecule and subtracting a contribution for each bond (see Table 1). E_B is a term describing the interaction between the compound and the nonaqueous phase and is a constant for a given functional group. In physical toxicity it has been found that E_B for compounds with a carbonyl ester, aliphatic hydroxyl, or aliphatic ether is 1.2 [18] and for a halogen E_B is -0.35 [19]. (Reference 16 should be consulted for more information on the calculation of molar volume parameters).

Cytotoxic effects of PCBs on spleen lymphocytes. When intact mouse spleen lymphocytes are incubated with sodium [^{51}Cr]chromate, some of the radioactivity is taken up by the cell. If the cell is treated with toxic agents the plasma membrane is disrupted and radiolabel is released into the surrounding medium. The extent of ^{51}Cr-release provides a good quantitative indication of cell viability. The concentration of chemical required to produce 50 per cent cell death (LD_{50}) was determined for a series of PCB isomers and other lipophilic compounds (see Table 2). A plot of $-\log LD_{50}$ vs $36{,}000\ V_X - E_B$ as shown in Fig. 1, follows the form predicted by equation 1 for a physical toxicity effect. The initial part of the curve is a straight line of slope 1, with a plateau region being reached for all compounds with molar volume larger than that of dodecanol. All the PCB isomers are of relatively large molar volume and thus should fall in the plateau region if they have purely physical toxicity. Figure 1 shows that this is indeed the case, with all PCBs from monochloro- to octachloro-isomers falling on

Table 1. Calculation of the molar volume parameter

Atom	$36{,}000\ V_x - E_B$ Atomic characteristic volume V_x $m^3\ mole^{-1}$	Functional group	E_B
Carbon	1.635×10^{-5}	−Halogen	−0.35
Hydrogen	8.71×10^{-6}	−OH	1.2
Oxygen	1.243×10^{-5}		
Chlorine	2.095×10^{-5}		
Bond between atoms (single, double or triple)	-6.56×10^{-6}		

Table 2. Molar volumes and biological activities*

Compound	(36,000 $V_x - E_B$)	$-\log LD_{50}$	$-\log K_i$
(1) Butanol	1.43	1.08	1.12
(2) Hexanol	2.45	2.25	2.24
(3) Octanol	3.46	3.19	3.24
(4) Nonanol	3.97	3.55	3.61
(5) Decanol	4.55	4.08	3.94
(6) Dodecanol	5.48	4.72	4.16
(7) Tetradecanol	6.50	4.79	
(8) *trans*-Retinol	8.38	4.99	4.12
(9) Chlorooctane	5.24	4.49	
(10) Chlorodecane	6.25	4.75	4.15
(11) 4-PCB	5.56	4.45	4.19
(12) 2,2'-PCB	6.35	4.83	4.38
(13) 2,4,5-PCB	7.14	4.91	4.41
(14) 2,2',4,5'-PCB	7.93	5.20	4.46
(15) 2,2',3,4,5'-PCB	8.72	5.14	4.30
(16) 2,2',3,4,4',5-PCB	9.51	5.36	4.34
(17) 2,2',4,4',5,6'-PCB	9.51	5.63	4.23
(18) 2,2',3,4,4'5,6'-PCB	10.31	4.98	4.20
(19) 2,2',3,4,4'5,6,6'-PCB	11.09	4.56	4.26

* Values for 36,000 $V_x - E_B$ were calculated as described in the text, using the information given in Table 1.

or near the predicted plateau line. Although several PCBs, notably the tetra-, penta- and hexachloro-isomers, appear to be more toxic than expected on a physical basis, these variations are not dramatic enough to be classified as chemical toxicity. Specific chemical toxicity would be expected to produce points significantly above the plateau region.

The curve shown in Fig. 1 corresponds to a form of equation 1 where $A = 1.62 \times 10^{-5}$ and $B = 2.24$,

i.e. $LD_{50} = 1.62 \times 10^{-5} + 2.24 \times 10^{-(36{,}000\ V_x - E_B)}$

The toxic concentration of chemical in the lipid biophase is thus 2.24 M, and the ratio of nonaqueous to aqueous phase volume A/B is 7.2×10^{-6}. Thus 1 liter of cell suspension (5×10^6/ml) contains 7.2 μl of lipid biophase. This is close to estimates of the plasma membrane volume of spleen lymphocytes based on a diameter of 7 μm and a membrane thickness of 8 nm.

Inhibition of 5'-nucleotidase by PCBs. To look at membrane perturbation in more detail, we studied the ability of PCBs and other compounds to interfere with the functioning of a plasma membrane enzyme. Previous work has shown that chlorinated hydrocarbons, dichloro/diphenyl/trichloroethane (DDT), and mixtures of PCBs can inhibit both Mg^{2+} and Na^+, K^+-ATPases *in vivo* [20–22]. La Rocca and Carlson [10] have demonstrated ATPase inhibition *in vitro* using a variety of purified PCB isomers and

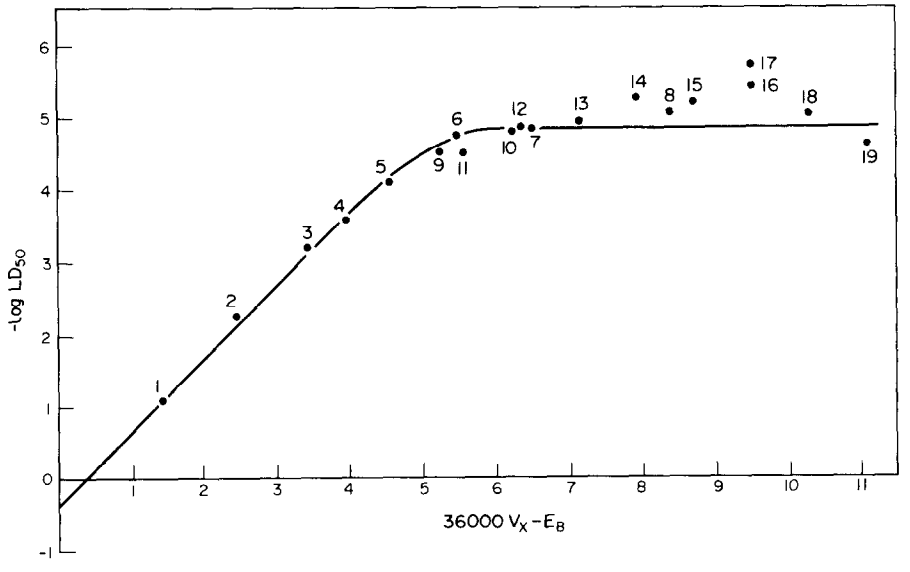

Fig. 1. Molar volume and cytotoxicity for PCBs and other lipophilic compounds. Numbers refer to specific compounds as listed in Table 2. The line drawn follows the form described by equation 1 in the text.

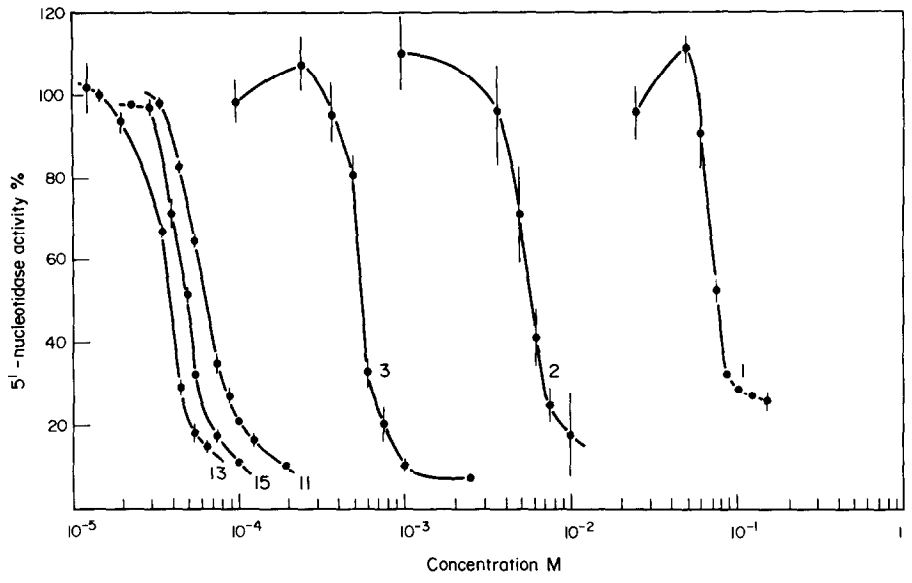

Fig. 2. Inhibition of lymphocyte 5'-nucleotidase by PCBs and other lipophilic compounds. Control 5'-nucleotidase activity varies considerably between individual spleens, with a measured specific activity of 28.8 ± 13.0 nmoles 5'-AMP hydrolyzed · hr^{-1} · 10^7 $cells^{-1}$ (mean ± S.D. for seventeen determinations). Each inhibition curve was measured using a single spleen, and points represent the mean ± range for duplicate tubes.

found a negative correlation between enzyme inhibition and PCB water solubility, that is a decrease in the water solubility of the PCBs caused an increase in ATPase inhibition.

5'-Nucleotidase (EC 3.1.3.5) catalyzes the hydrolysis of 5'-AMP to adenosine and phosphate. It is present in almost all mammalian plasma membranes and has been widely used as an enzyme marker during membrane purification. 5'-Nucleotidase is present exclusively on the plasma membrane in mouse spleen lymphocytes and is an ectoenzyme, that is, the active site faces the outside of the cell [14]. The enzyme is a glycoprotein and binds the mitogenic lectin concanavalin A [23]. It has been shown recently that adenosine (one of the products of 5'-nucleotidase action) is involved in adenylate cyclase regulation [24], thus suggesting a link between the two enzymes which may be important in lymphocyte transformation.

Figure 2 shows typical curves for the inhibition of 5'-nucleotidase by PCBs and other lipophilic chemicals. For some compounds, particularly the *n*-alkanols, there was a slight enzyme activation (up to 15 per cent over control) at low concentrations followed by a rapid decrease in activity in higher concentrations. Inhibition obtainable was generally in the 90–94 per cent range and did not decrease further at higher PCB concentrations. This is similar to the

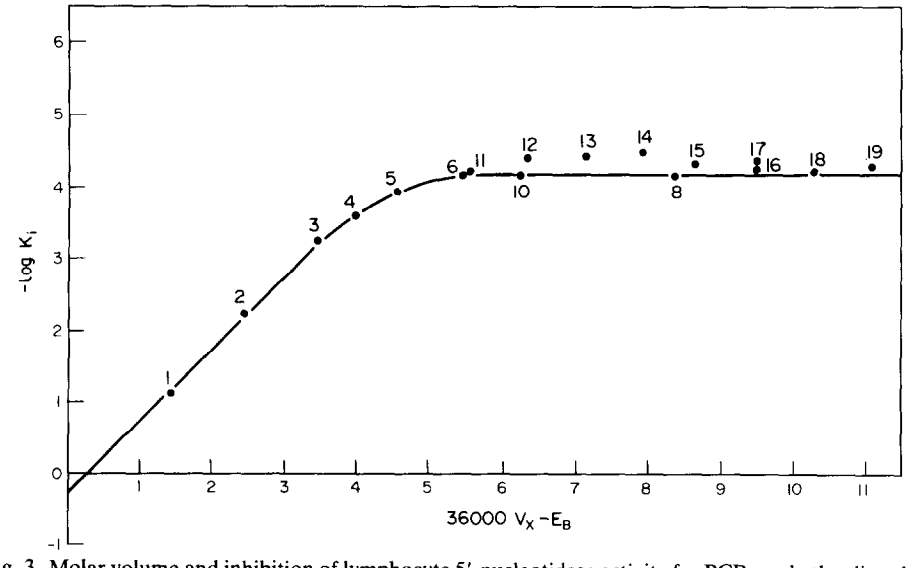

Fig. 3. Molar volume and inhibition of lymphocyte 5'-nucleotidase activity for PCBs and other lipophilic compounds. Numbers refer to specific compounds as listed in Table 2. The line drawn follows the form described by equation 1 in the text.

inhibition level of 90 per cent seen using con A as the inhibitor [23]. The K_i values for the various PCBs show much less variation than is seen for their corresponding LD_{50} values, and there seems to be very little correlation between the two, i.e. the more toxic isomers do not show a greater ability to inhibit 5′-nucleotidase.

Figure 3 shows a plot of $-\log K_i$ vs 36,000 $V_X - E_B$ for PCBs and other lipophilic chemicals. Again we obtain a typical physical toxicity curve (equation 1) with an initial linear portion of slope 1, and a plateau region for all compounds of larger molar volume than decanol. The parameters obtained from this curve are $A = 6.92 \times 10^{-5}$, $B = 1.78$, and $A/B = 3.89 \times 10^{-5}$. Thus, the toxic concentration of chemical in the biophase is 1.78 M and 1 liter of cell suspension contains 38.9 μl of lipid biophase. This value for the volume of nonaqueous phase is larger than that obtained from the cytotoxicity curve, reflecting the higher cell concentration used in the enzyme assay. All the PCBs tested fall on or slightly above the plateau line with no evidence of chemical toxicity. Inhibition of 5′-nucleotidase by PCBs is thus a simple physical toxicity effect based on their lipid solubility, and there appears to be no specific chemical interaction between these molecules and the enzyme protein.

All membrane-bound enzymes will be inhibited by lipophilic chemicals (although their individual susceptibilities may vary somewhat), and a similar physical toxicity curve could be constructed for any one of them. In light of the results described above, the PCBs would be expected to produce nonspecific inhibition of all such enzymes at concentrations of $\sim 10^{-4}$ to 10^{-5} M. La Rocca and Carlson [10] used PCB concentrations of 10^{-5} M to obtain ATPase inhibition in the range 36–95 per cent, depending on the isomer under study. The inhibition of ATPase at these PCB concentrations is almost certainly due to physical toxicity effects and is unlikely to represent a specific mechanism for PCB toxicity since many other membrane enzymes will also be inhibited under these conditions.

The values of the constant B (from equation 1) for Fig. 1 and 3 and 2.24 and 1.78 respectively. Since B represents the toxic concentration in the lipid biophase, it therefore requires a 2.24 M concentration of the chemical in the cell membrane to cause cell death and a 1.78 M concentration to inhibit 5′-nucleotidase. Assuming for the sake of simplicity that the cell membrane is a simple bilayer composed only of phospholipid, this would translate into phospholipid–solute ratios of approximately 0.48 and 0.60 respectively. Thus, the cell membrane must be very highly perturbed, to the point where the number of lipophilic solute molecules greatly exceeds the number of phospholipid molecules, before 5′-nucleotidase is inhibited. This is in sharp contrast to the molal drug concentrations required to produce anesthesia, which are in the range 0.03 to 0.06, that is, a phospholipid:drug ratio of 22–44.

Mechanism of 5′-nucleotidase inhibition by PCBs. Anesthetics and other lipid-soluble compounds such as benzyl alcohol are lipid bilayer "fluidizers" causing membrane expansion. Some compounds, particularly steroids such as androstane, have the opposite effect, producing a more rigid and ordered membrane [25, 26]. Some lipophilic compounds seem to partition only into the bulk lipid bilayer [26, 27], while others also partition into the tightly bound layer of boundary lipid surrounding each membrane protein molecule [28]. Some drugs may aggregate preferentially around membrane protein at the hydrophobic lipid–protein interface [29], displacing boundary lipids important in maintaining enzyme conformation, and perhaps altering protein structure directly. Such binding of a lipophilic compound to a membrane protein need not imply chemical specificity. Some membrane enzymes require a very narrow range of fluidity in which to operate and may be perturbed indirectly via a change in bulk bilayer fluidity. Others are more sensitive to perturbations occurring at the lipid–protein interface and can thus be inhibited directly by a lipophilic compound.

Based on the results reported in this study it is not possible to differentiate between the possible mechanisms by which PCBs inhibit membrane-bound enzymes.

Acknowledgements—This work was supported by a grant from the National Science and Engineering Research Council. We would like to thank Dr. S. H. Safe for his gift of polychlorinated biphenyls, and Dr. J. C. McGowan for his advice and encouragement.

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