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# EFFECT OF 4-CHLOROBIPHENYL ON SUBSTRATE TRANSPORT AND PHOSPHOLIPID METABOLISM IN MOUSE L5178Y LYMPHOMA CELLS

JUDSON W. SPALDING, ELIZABETH FORD and DIANE LANE

Environmental Toxicology Branch, National Institute of Environmental Health Sciences, Research Triangle Park, N.C. 27709, U.S.A.

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**Abstract**—The toxicity of 4-chlorobiphenyl, a constituent of Aroclor 1221, was studied in mouse L5178Y cells, *in vitro*. 4-Chlorobiphenyl had a varied effect on the uptake of small precursor molecules. Uptake of [$^3$H]L-leucine, [$^3$H]L-serine, [$^3$H]uridine and [$^3$H]thymidine was inhibited, while that of [$^3$H]inositol was stimulated. There was no significant effect on either [$^{14}$C]ethanolamine or [$^{14}$C]choline uptake. However 4-chlorobiphenyl significantly inhibited the incorporation of [$^{14}$C]ethanolamine into phosphatidylethanolamine and caused a 2- to 3-fold stimulation in the incorporation of [$^{14}$C]choline into phosphatidylcholine. This effect on phosphatidylcholine metabolism depended on the adsorption and continued presence of 4-chlorobiphenyl on the cell plasma membrane. The stimulation of [$^{14}$C]choline incorporation was reversed when treated cells were placed in fresh growth medium under conditions where 95 per cent of the 4-chlorobiphenyl was desorbed from the cell surface. The effect of 4-chlorobiphenyl on substrate uptake and phospholipid metabolism appears to depend upon the interaction of the agent with the cell membrane surface.

The widespread occurrence of organochlorine compounds and their persistence in the environment have raised concern with respect to their detrimental biological activity in man as well as in a wide range of animal and plant species. The toxicity of polychlorinated biphenyl mixtures (PCBs) to several tissue culture cell lines has been recently reported [1–3]. When toxicity of these commercial mixtures (Aroclors) was compared on an equal weight basis in Chinese hamster cells, it was observed that the toxicity increased as the average percentage of chlorine in the Aroclor mixture decreased [3]. In toxicity studies with chicks and fish, the toxicity of the Aroclors was also found to be inversely related to the percentage of chlorine [4, 5]. Other investigators have reported that several different Aroclor mixtures inhibited both $Mg^{2+}$ ATPase and $Na^+$-$K^+$ ATPase in fish preparations [6] and disrupted osmoregulation in marine fish [7]. These results imply that some component(s) of PCBs are active in affecting membrane transport mechanisms.

Previous investigation in this laboratory on 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) toxicity in mouse L5178Y lymphoma cells suggested that DDT was expressing its toxic effect at the plasma membrane level [8]. Furthermore, the toxic effect did not depend on any detectable change in the molecular structure of the parent compound. Subsequently, DDT was shown to cause a perturbation in phospholipid metabolism [9]. The availability of specific low molecular weight compounds of the PCB mixture Aroclor 1221 provided an opportunity to study the effects of this class of organochlorine compounds on transport and cell metabolism.

Several constituents of Aroclor 1221 were compared for toxicity in mouse L5178Y lymphoma cells, and 4-chlorobiphenyl (4-Cl-BP) proved to be the most toxic of the compounds studied. The effects of 4-Cl-BP on the incorporation of selected precursors of macromolecular synthesis have been investigated with the objective of attempting to characterize the mode of action by which the toxicity is being expressed.

## MATERIALS AND METHODS

*Cell culture and exposure protocols.* Mouse L5178Y lymphoma cells were grown in suspension as shake cultures in a model G25 Brunswick Gyrotory incubator-shaker at 37°. The complete growth medium was composed of Fisher's medium, pH 7.0, supplemented with 10% horse serum, 50 units of penicillin/ml, 50 mg streptomycin/liter, 2.2 g $NaHCO_3$/liter and 0.1% pluronic F-68 (Wyandotte Chemical Corp.). Cell numbers were determined with a model B Coulter counter. Cells were determined to be uncontaminated by mycoplasmas.*

Exposure of cells to chlorinated biphenyls or to mixtures of polychlorinated biphenyls (e.g. Aroclor 1221) was performed by adding dimethylsulfoxide (DMSO) solutions of the biphenyls to cell suspensions ($1.30 \pm 0.10 \times 10^5$ cells/ml) which had been subcultured in fresh medium. Corresponding amounts of dimethylsulfoxide were added to controls. Inhibition of cell replication was determined after 24–26 hr of incubation at 37°. The degree of toxicity was

* Both lysed cells and spent culture medium (cells removed) were tested for the presence of mycoplasmas by culturing aliquots on mycoplasma agar base (BBL 11456, BioQuest) and mycoplasma broth base (BBL 11457) supplemented with mycoplasma enrichment (BBL 11865). Incubations were both aerobic and anaerobic at 35–37°. The results were negative for mycoplasmas.

expressed as per cent inhibition relative to the control or as an $ID_{25}$ or $ID_{50}$ concentration (μg/ml).

In acute toxicity studies, 4-Cl-BP was preincubated at 37° overnight (16 hr) in complete growth medium. Subsequently, cells taken from cultures in exponential growth were then subcultured (at $5.25 \pm 0.25 \times 10^5$ cells/ml) in the above biphenyl preincubation medium. At this cell concentration, the uptake of all labeled substrates was linear for 90–120 min; therefore, 60 min was selected for the incubation time.

*Determination of radioactivity in whole cells and acid-insoluble fractions.* For the determination of labeled precursor uptake into whole cells, three replicate 10-ml volumes of cell suspension (approximately $5.0 \times 10^6$ cells) were collected at appropriate time intervals and were immediately placed in an ice bath. The cells were sedimented at 200 *g* in an IEC model PR-2 refrigerated centrifuge for 7 min and were then washed twice in 5 ml of Earle's balanced salt solution (EBSS).

In order to prepare the acid-insoluble fractions, a second set of three replicate 10-ml volumes of cell culture was also cooled in an ice bath. All subsequent steps were carried out at 4°. The cells were sedimented and washed once with 5 ml EBSS. The cell pellet was suspended in 1.0 ml saline and mixed with 1.0 ml of 1 N perchloric acid. The mixture was allowed to sit for 20 min to facilitate complete precipitation. The precipitate was sedimented at 400 *g* for 7 min, washed twice with 2.0-ml volumes of 0.5 N perchloric acid, and once with 2.0 ml of 0.5 N trichloroacetic acid.

When incorporation of [$^3$H]L-serine was being determined, another set of acid-insoluble fractions was prepared and the lipid fraction extracted in order to distinguish between the [$^3$H]L-serine that was incorporated into phospholipid from that incorporated into protein. The lipid fractions were removed by washing the acid-insoluble residues successively with 2.0-ml volumes of ethanol–water (80:20, v/v), ethanol–diethylether (1:1, v/v) and finally with diethylether.

All cell pellet residues were solubilized in 0.2 ml of 88% formic acid at 37°, and added with 1.0 ml water to 12 ml scintillator solution.

*Phospholipid isolation.* Cell suspensions containing $100–150 \times 10^6$ cells were sedimented and the cell pellet was washed with Earle's balanced salt solution, suspended in 2.0 ml saline, and homogenized by sonication. The phospholipid fraction was isolated according to the procedure of Folch *et al.* [10] and recovered quantitatively with $CHCl_3$. Aliquots were taken for analysis of radioactivity, phosphate and for thin-layer chromatography (t.l.c.).

*Thin-layer chromatography of phospholipids and phosphate determination.* The phosphatides were chromatographed on Silica gel H, magnesium acetate-impregnated t.l.c. plates (Analtech) in a two-dimensional system according to the method of Rouser *et al.* [11].

The phospholipids were located by exposure to iodine vapor. The four major phospholipid components, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol and phosphatidylserine, were identified by comparing relative mobilities with those of standards obtained from Supelco, Inc.

Phospholipid spots from replicate plates were encircled and recovered. Phosphate analysis was performed according to the method of Bartlett [12]. Either one or the other of two procedures was employed to determine the specific activity of [$^{14}$C]methyl-choline or [$^{14}$C]1-2-ethanolamine incorporated into their corresponding phospholipid fraction. In the first procedure, replicate phospholipid-containing Silica gel spots were recovered and assayed directly for radioactivity. Another set of corresponding Silica gel spots was placed in tubes and digested directly with 62% perchloric acid prior to phosphate analysis. In the second procedure, replicate gel spots were extracted three times with 2.0-ml volumes of chloroform methanol acetic acid water (12:6:1:1, v/v). The pooled extracts were taken to dryness under $N_2$ and the residue recovered in 1.0 ml chloroform–methanol (2:1, v/v; 0.1 ml was removed for determination of radioactivity. The remaining volume was taken to dryness for phosphate analysis. In both procedures, appropriate-sized blank areas of Silica gel were treated in a similar manner to serve as controls.

*Adsorption of [$^{14}$C]4-chlorobiphenyl to L5178Y cells.* [$^{14}$C]4-Cl-BP was diluted with carrier 4-Cl-BP and added to complete medium so that the specific activities (dis./min/ml) would be identical at two concentrations, 20 μg and 40 μg/ml. After overnight preincubation, the medium was filtered through a 0.20 μm Nalgene filter. The specific activities of the medium were lower after filtration, and the concentrations of [$^{14}$C]4-Cl-BP were recalculated. The final concentrations of [$^{14}$C]4-Cl-BP, after the addition of cells, were 18 μg/ml (sp. act. 1700 dis./min/μg) and 33 μg/ml (sp. act. 1670 dis./min/μg). Cells were resuspended in the medium at two concentration levels, $0.500 \times 10^6$ cells/ml and $1.0 \times 10^6$ cells/ml at each concentration of [$^{14}$C]4-Cl-BP.

Replicate 10-ml volumes of cell suspension were taken at 0 time and after 1, 2 and 4 hr of incubation. Adsorption of [$^{14}$C]4-Cl-BP was measured by determining the radioactivity associated with the cell pellet after sedimentation at 200 *g* for 10 min. Ten-ml volumes of control medium (no cells) were taken at zero-time and at 4 hr. The amount of radioactivity sedimenting from the control medium did not exceed 100 dis./min/10 ml at either concentration of [$^{14}$C]4-Cl-BP.

After initial sedimentation, [$^{14}$C]4-Cl-BP was desorbed from the cells by careful washing in 10 ml of complete medium. One wash was sufficient to remove 95 per cent of the radioactivity associated with the cell pellet. A second wash reduced the radioactivity to background levels. The incubation, sedimentation and washing procedures were carried out at 37°. The cell pellets were solubilized in 0.2 ml of 88% formic acid and assayed for radioactivity as previously described.

*Chemicals and radioisotopes.* The commercial polychlorinated biphenyl mixtures, Aroclors 1221, 1242, 1248, 1254 and 1260, were obtained from Monsanto. Biphenyl was obtained from Eastman Kodak Co.; 2-chlorobiphenyl and 3-chlorobiphenyl were obtained from K & K Laboratories Inc.; 4-chlorobiphenyl and 4,4′-dichlorobiphenyl were obtained from Aldrich Chemical Co.

[$^3$H]5-Uridine (20 Ci/m-mole) was purchased from Schwarz BioResearch. [$^{14}$C]1,2-Choline (0.25 mCi 5.6 mg), [$^{14}$C]methyl-choline (1 mCi/3.37 mg), [$^3$H] methyl-thymidine (5 mCi/0.26 mg), [$^{14}$C]1,2-ethanolamine HCl (1 mCi/48.7 mg), [$^3$H]4,5-L-leucine (5 mCi/0.021 mg), [$^3$H]2-myoinositol (5 mCi/0.26 mg), and [$^3$H]L-serine (1 mCi/0.047 mg) were obtained from New England Nuclear. [$^{14}$C]4-Chlorobiphenyl (0.91 mCi/188 mg, ring-labeled) was a gift from Dr. E. Oswald.

*Radioactivity measurements.* The liquid scintillation solution contained 1330 ml of Triton X-100, 2670 ml toluene, 22 g of 2,5-diphenyloxazole (PPO) and 400 mg of 1,4-bis[2(5-phenyloxazolyl)]benzene (POPOP). Radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer (model 3380-544).

## RESULTS

*Toxicity studies.* The sensitivity of L5178Y cells to polychlorinated biphenyl mixtures was first investigated with the commercial preparation, Aroclor 1221. The $ID_{50}$ concentration was 11.6 ± 0.8 μg/ml; this concentration is equivalent to $6 \times 10^{-5}$ M when based on a molecular weight of 194 which is assumed to be appropriate for Aroclor 1221. Five commercial Aroclor mixtures were then compared for toxicity at a $5 \times 10^{-5}$ M concentration. All of the Aroclor mixtures inhibited cell growth to nearly the same degree, from 27 to 36 per cent (Table 1). No single Aroclor mixture was significantly more toxic than another.

Five major chemical constituents of Aroclor 1221 were obtained in pure form (see Materials and Methods). These compounds, which comprised 80 per cent of Aroclor 1221, were compared for toxicity on the basis of equivalent molarity, at $5 \times 10^{-5}$ M (Table 1). 4-Chlorobiphenyl (4-Cl-BP) proved to be four times as toxic as 2-chlorobiphenyl, the next most toxic component of Aroclor 1221. Biphenyl, 3-chlorobiphenyl and 4,4'-dichlorobiphenyl showed little or no significant toxicity.

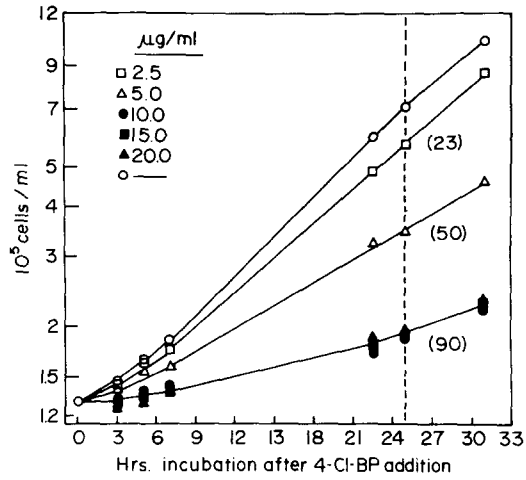


Fig. 1. Typical growth curve of L5178Y cells in the presence of varying concentrations of 4-Cl-BP. The per cent inhibition of cell growth at 25 hr is indicated by the figures enclosed in parentheses.

Because of its relatively high toxicity, 4-Cl-BP was selected for further investigation. The $ID_{50}$ of 4-Cl-BP was found to be 4.6 ± 0.6 μg/ml. Concentrations of 10 μg/ml consistently inhibited cell growth 80–90 per cent of control when toxicity was compared at intervals equivalent to two or more generation times (Fig. 1). There was no greater inhibitory effect at 15 or 20 μg/ml, when toxicity was compared at similar time intervals.

*Effect of 4-chlorbiphenyl on protein, RNA and DNA synthesis.* The effect of 4-Cl-BP on protein, RNA and DNA biosynthetic pathways was investigated by measuring the uptake and subsequent incorporation of appropriate radioisotopically labeled precursors into whole cells and acid-insoluble fractions of L5178Y

Table 1. Toxicity of chlorobiphenyl compounds in mouse L5178Y lymphoma cells

| Compound | No. of chlorines* | Mol. wt† | μg/ml at $5 \times 10^{-5}$ M | % Inhibition |
|---|---|---|---|---|
| Aroclor-1221 | 1 | 194 | 9.7 | 36 |
| 1242 | 3 | 258 | 12.9 | 27 |
| 1248 | 4 | 292 | 14.6 | 32 |
| 1254 | 5 | 327 | 16.3 | 33 |
| 1260 | 6 | 361 | 18.1 | 35 |

| Compound | %Composition 1221‡ | Mol. wt | μg/ml at $5 \times 10^{-5}$ M | % Inhibition |
|---|---|---|---|---|
| Biphenyl | 22 | 154 | 7.7 | 8 |
| 2-Chlorobiphenyl | 28 | 188 | 9.4 | 17 |
| 3-Chlorobiphenyl | 3.2 | 188 | 10.2§ | 3 |
| 4-Chlorobiphenyl | 22.6 | 188 | 9.4 | 70 |
| 4-4'-Dichlorobiphenyl | 3.7 | 223 | 11.1 | 10 |

* Average number of chlorine atoms substituted on biphenyl based on the per cent of chlorine in the mixture.

† Average molecular weight of the Aroclor mixture based on the average number of chlorine substitutions on biphenyl.

‡ The figures for the per cent composition of the listed constituents of Aroclor 1221 were kindly provided by Dr. Phillip Albro, Analytical Chemistry Branch, NIEHS.

§ 3-Chlorobiphenyl is in liquid form; it was added at $5.4 \times 10^{-5}$ M concentration.

Table 2. Effect of 4-chlorobiphenyl on [$^3$H]L-leucine, [$^3$H]uridine and [$^3$H]thymidine incorporation into whole cells and acid-insoluble fractions of mouse L5178Y lymphoma cells

| | Whole cells | | Acid-insoluble | |
|---|---|---|---|---|
| 4-Cl-BP (μg/ml) | Radioactivity (dis./min/$10^5$ cells) | % Inhibition | Radioactivity (dis./min/$10^5$ cells) | % Inhibition |
| [$^3$H]L-leucine* | | | | |
| | 4,390 | | 1,800 | |
| 20 | 3,800 | 13 | 1,530 | 15 |
| 40 | 3,030 | 31 | 1,200 | 33 |
| [$^3$H]uridine* | | | | |
| | 3,430 | | 1,230 | |
| 20 | 2,710 | 21 | 1,070 | 13 |
| 40 | 2,300 | 33 | 880 | 29 |
| [$^3$H]thymidine* | | | | |
| | 13,650 | | 9,950 | |
| 20 | 8,850 | 35 | 5,750 | 42 |
| 40 | 6,250 | 54 | 3,430 | 66 |

* Sixty-min incubation.

cells. [$^3$H]5-Uridine and [$^3$H]methyl-thymidine supplemented with unlabeled carrier ($1 \times 10^{-5}$ M) were used as precursors for RNA and DNA biosynthesis respectively. [$^3$H]4,5-L-leucine was the precursor for protein synthesis. Acute toxicity of 4-Cl-BP was studied at 20 and 40 μg/ml, concentrations which could be expected to inhibit cell growth 90 per cent or more (Fig. 1). The toxicity at these concentrations was reversible, since the cell viability of washed cells (measured by cloning efficiency) was 96 and 86 per cent, respectively, after the 60-min incubation period in the presence of the compound.

The uptake of all three precursors into whole cells and their subsequent incorporation into the acid-insoluble fractions was inhibited at both concentrations of 4-Cl-BP (Table 2). The inhibition of [$^3$H]methyl-thymidine uptake and incorporation was nearly twice that of [$^3$H]5-uridine and [$^3$H]4,5-L-leucine. In all instances, there was a high correlation between the degree of inhibition of precursor uptake into the whole cell and the subsequent degree of inhibition of precursor incorporation into the acid-insoluble fraction. These results indicate that 4-Cl-BP does not directly inhibit either protein, RNA or DNA synthesis. Rather, the primary effect of 4-Cl-BP appears to be on the uptake of these small precursor molecules across the cell membrane.

*Effect of 4-chlorobiphenyl on phospholipid metabolism.* Results from investigation on DDT toxicity in this laboratory had suggested that DDT was expressing its toxic effect at the plasma membrane of L5178Y cells; subsequently, DDT was shown to cause a perturbation of phospholipid metabolism [8, 9]. L5178Y cells have been analyzed quantitatively for their phospholipid content and it was found that four phospholipid species comprised nearly 94 per cent of the total cell phospholipid. Phosphatidylcholine accounted for 54 per cent; phosphatidylethanolamine, 28 per cent; phosphatidylinositol, 7.5 per cent; and phosphatidylserine, 4.0 per cent. The remaining 6 per cent was not identified. These values are similar to those described for the phospholipid composition of mouse fibroblasts [13].

The results cited above suggested that 4-Cl-BP toxicity, like that of DDT, was being expressed at the plasma membrane level, and causing some aberration in phospholipid metabolism; therefore, the effect of 4-Cl-BP on the metabolism of four precursors of phospholipid biosynthesis was investigated.

Uptake and incorporation of [$^3$H]L-serine and [$^3$H]2-myoinositol into whole cells and the acid-insoluble fraction were compared at 4-Cl-BP concentrations of 20 and 40 μg/ml. [$^3$H]L-serine uptake and incorporation were inhibited to the same degree as

Table 3. Effect of 4-chlorobiphenyl on [$^{14}$C]1,2-ethanolamine incorporation into whole cells, acid-insoluble and phospholipid fractions of mouse L5178Y lymphoma cells*

| | Whole cells | | Acid-insoluble | | Phospholipid fraction | | Phosphatidylethanolamine | |
|---|---|---|---|---|---|---|---|---|
| 4-Cl-BP (μg/ml) | Radio-activity (dis./min/$10^6$ cells) | % Inhibition | Radio-activity (dis./min/$10^6$ cells) | % Inhibition | Radio-activity (dis./min/μg $PO_4$) | % Inhibition | Radio-activity (dis./min/μg $PO_4$) | % Inhibition |
| | 23,650 | | 17,120 | | 12,750 | | 30,890 | |
| 20 | 23,860 | (1)† | 12,740 | 26 | 8,540 | 33 | 21,850 | 29 |
| 40 | 20,330 | 14 | 5,430 | 68 | 4,170 | 67 | 11,760 | 62 |

* Sixty-min incubation.

† Indicates increase in uptake: essentially, no change.

Table 4. Effect of 4-chlorobiphenyl on [$^{14}$C]methyl-choline incorporation into whole cells, acid-insoluble and phospholipid fractions of mouse L5178Y lymphoma cells*

| | Whole cells | | Acid-insoluble | | Phospholipid fraction | | Phosphatidylcholine | |
|---|---|---|---|---|---|---|---|---|
| 4-Cl-BP ($\mu$g/ml) | Radioactivity (dis./min/cells) | % Control | Radioactivity (dis./min/$10^6$ cells) | % Control | Radioactivity (dis./min/$\mu$g $PO_4$) | % Control | Radioactivity (dis./min/$\mu$g $PO_4$) | % Control |
| | 5950 | | 170 | | 135 | | 220 | |
| 20 | 5850 | 98 | 410 | 241 | 300 | 222 | 485 | 220 |
| 40 | 5480 | 92 | 540 | 318 | 420 | 311 | 655 | 298 |

* Sixty-min incubation.

that observed for [$^3$H]L-leucine and [$^3$H]5-uridine (Table 2) at similar concentrations of 4-Cl-BP. In contrast, [$^3$H]2-myoinositol uptake and incorporation were stimulated 17–20 per cent; there was no significant difference in the degree of stimulation at either 4-Cl-BP concentration. The primary effect of 4-Cl-BP appeared to be on uptake of [$^3$H]L-serine and [$^3$H]2-myoinositol, and in both instances, the incorporation of labeled precursor into the acid-insoluble fraction reflected the degree of uptake by the cells.

Though 4-Cl-BP had relatively little effect on [$^{14}$C]1-2-ethanolamine uptake, there was a significant inhibition of incorporation into the acid-insoluble fractions at both concentrations (Table 3). In order to confirm that the inhibition of incorporation into the acid-insoluble fraction did reflect inhibition of phosphatidylethanolamine (PE) synthesis, the phospholipids were extracted from the cells, and the PE fractions isolated and recovered via t.l.c. The degree of inhibition reflected in the relative specific activities of PE compared well with the specific activities calculated on the basis of [$^{14}$C]1-2-ethanolamine incorporation into the acid-insoluble fraction (dis./min/$10^6$ cells) or into the total phospholipid fraction (dis./min/$\mu$g $PO_4$) (Table 3). Ninety-five per cent of the radioactivity in the total phospholipid extract was recovered as PE.

*Incorporation of [$^{14}$C]methyl-choline.* The most striking effect of 4-Cl-BP was observed on [$^{14}$C]methyl-choline metabolism and the biosynthesis of phosphatidylcholine. There was no significant effect on choline uptake into whole cells (Table 4), but there was a 2- to 3-fold increase of incorporation into the acid-insoluble and phospholipid fractions. The increase of [$^{14}$C]methyl-choline incorporation into the acid-insoluble fraction increased with the 4-Cl-BP concentration. Nearly 100 per cent of the radioactivity in the acid-insoluble fraction could be accounted for in the total phospholipid extract and 95 per cent of this activity was recovered as phosphatidylcholine.

Since 4-Cl-BP did not appreciably affect choline uptake from the medium into the cell, it could be expected that the increased incorporation of [$^{14}$C]methyl-choline into phosphatidylcholine might be reflected in an increased incorporation of labeled precursor into one or several of the biosynthetic intermediates.

However, when the intermediates of phosphatidylcholine (PC) biosynthesis, phosphorylcholine, and cytidine diphosphate choline (CDP-choline) were isolated from the acid-soluble fraction and resolved by t.l.c. according to the method described by Plageman [14], there was no difference found in the specific activities of the recovered intermediates from control and treated cells. Ninety-five per cent of the acid-soluble radioactivity was recovered as phosphorylcholine. There were no quantitative differences in the relative amounts of total phosphatidylethanolamine or phosphatidylcholine recovered from control and treated cells. Thus, the change in the specific activities of these two phosphatides may reflect changes in their turnover rates in treated cells.

*Adsorption of [$^{14}$C]4-Cl-BP on L5178Y cells.* Since the evidence suggested that 4-Cl-BP toxicity was

Table 5. Adsorption of [$^{14}$C]4-chlorobiphenyl on the surface of mouse L5178Y lymphoma cells

| [$^{14}$C]4-Cl-BP ($\mu$g/ml) | Radioactivity* (dis./min/10 ml cell susp.) | Cell concn ($10^6$ cells/10 ml) | Radioactivity (dis./min/$10^6$ cells) |
|---|---|---|---|
| Zero-time incubation | | | |
| 18 | 4,200 | 5.4 | 780 |
| 18 | 8,800 | 10.3 | 850 |
| 33 | 8,370 | 5.2 | 1,610 |
| 33 | 16,860 | 10.2 | 1,650 |
| Two-hr incubation | | | |
| 18 | 4,330 | 5.4 | 800 |
| 18 | 8,450 | 10.6 | 800 |
| 33 | 8,840 | 5.4 | 1,640 |
| 33 | 17,400 | 10.7 | 1,630 |

* Amount of radioactivity adsorbed to cells present in 10 ml of cell suspension.

being expressed at the level of the plasma membrane, it became of interest to study the extent of the interaction of 4-Cl-BP with the membrane surface of the cells. L5178Y cells were incubated at intervals up to 4 hr at two concentrations of [$^{14}$C]4-Cl-BP (described in Materials and Methods). These concentrations were nearly the same as those which had been shown to stimulate [$^{14}$C]methyl-choline incorporation. The data for zero-time and the 2-hr incubation period are compared in Table 5. The results clearly show that nearly all of the [$^{14}$C]4-Cl-BP that will be adsorbed to the cells, absorbs immediately. The amount of radioactivity absorbed per $10^6$ cells remained constant during 1, 2 and 4 hr of incubation. At both concentrations of 4-Cl-BP, the degree of adsorption was proportional to the cell population density, and of the total [$^{14}$C]4-Cl-BP available in the culture medium, 1.5 and 3.0 per cent were adsorbed to the cells at the lower and higher cell densities respectively. It was noted with interest that, when the cell pellet was washed with complete medium, [$^{14}$C]4-Cl-BP was as easily desorbed as it was initially absorbed to the cells. Recent data (unpublished) indicate that the rate of desorption is significantly decreased when serum is omitted from the washing medium. This suggests that some serum component, e.g. serum protein, competes for the compound absorbed on the plasma membrane and that the 4-Cl-BP is not bound tightly to the cell surface.

The observation that 4-Cl-BP was so easily and nearly completely desorbed from treated cells when they were washed with fresh medium raised the following question. Did the 2- to 3-fold stimulation of choline incorporation into phosphatidylcholine by 4-Cl-BP occur only while the 4-Cl-BP was adsorbed on the cell membrane? In order to attempt to answer this question, the following experiment was performed.

Complete medium was preincubated with 20 and 40 $\mu$g 4-Cl-BP/ml in the usual manner. Exponentially growing cells were collected and suspended in replicate control and 4-Cl-BP-treated cultures. All cultures were incubated for 1 hr. In one set of cultures, [$^{14}$C]methyl-choline was added to demonstrate the usual effect of 4-Cl-BP on the uptake and incorporation of choline into whole cells and the acid-insoluble fraction respectively. At the end of this incubation period, the cells treated with 4-Cl-BP in the absence of [$^{14}$C]methyl-choline were centrifuged and resuspended in medium without 4-Cl-BP. Then the cells were either (a) added back to fresh medium containing the same concentration of 4-Cl-BP that they had been previously incubated in, or (b) added to fresh medium without 4-Cl-BP. [$^{14}$C]Methyl-choline was added to all cultures and they were incubated for an additional 60 min. Uptake of [$^{14}$C]methyl-choline into whole cells and further incorporation into the acid-insoluble fractions were measured in the usual manner and compared.

In the first 60-min incubation period, the results were as expected. The rate of [$^{14}$C]methyl-choline incorporation into the acid-insoluble fraction was stimulated 2.5- and 3.5-fold (Table 6).

Cells which were treated with 4-Cl-BP for an additional 60-min interval, but now in the presence of [$^{14}$C]methyl-choline, also showed a stimulation of choline incorporation into the acid-insoluble fraction, though not to the same degree. At 20 $\mu$g 4-Cl-BP/ml, the stimulation was 175 per cent of control; and at 40 $\mu$g/ml, it was 230 per cent of control. In contrast, however, there was no continued stimulation of [$^{14}$C]methyl-choline incorporation into the acid-insoluble fraction of those cells from which 4-Cl-BP had been desorbed after a preincubation interval of 60 min in the presence of the compound: instead there was a decrease of incorporation which was inversely pro-

Table 6. Effect of preincubation with 4-Cl-BP on the incorporation of [$^{14}$C]methyl-choline into whole cells and acid-insoluble fraction of mouse L5178Y lymphoma cells

| | Whole cells | | Acid-insoluble fraction | |
|---|---|---|---|---|
| 4-Cl-BP ($\mu$g/ml) | Radioactivity (dis./min $10^6$ cells) | % Control | Radioactivity (dis./min $10^6$ cells) | % Control |
| I 60 min* | | | | |
| | 15,970 | | 250 | |
| 20 | 15,370 | 96 | 630 | 252 |
| 40 | 13,910 | 87 | 890 | 356 |
| IIA 60 min† | | | | |
| | 15,370 | | 340 | |
| 20 | 12,920 | 84 | 600 | 176 |
| 40 | 12,980 | 84 | 790 | 232 |
| IIB 60 min‡ | | | | |
| | 16,240 | | 380 | |
| (20) | 14,440 | 89 | 310 | 82 |
| (40) | 16,000 | 98 | 230 | 60 |

* I: first 60-min interval of [$^{14}$C]methyl-choline incorporation coinciding with the incubation of replicate cultures in 4-Cl-BP without labeled choline.

† IIA: second 60-min interval of [$^{14}$C]methyl-choline incorporation into 4-Cl-BP preincubated cells that were resuspended in medium containing the same corresponding concentrations of 4-Cl-BP.

‡ IIB: incorporation of [$^{14}$C]methyl-choline into cells preincubated in 4-Cl-BP, but resuspended in fresh medium without 4-Cl-BP. Preincubation concentrations of 4-Cl-BP are enclosed in parentheses.

portional to the concentration of 4-Cl-BP that the cells had previously been exposed to. Uptake of [$^{14}$C]methyl-choline into whole cells was slightly inhibited by 4-Cl-BP, but the degree of inhibition was variable and followed no consistent pattern of correlation with 4-Cl-BP concentration. These results do not explain how the adsorption of 4-Cl-BP on the cell membrane stimulates the incorporation of choline into phosphatidylcholine, but it is clear that the stimulation is dependent on the continued presence of 4-Cl-BP on the cell membrane surface.

## DISCUSSION

When the Aroclor mixtures were compared for toxicity at a $5 \times 10^{-5}$ M concentration, no significant difference in toxicity was observed. This observation differs from that reported earlier by Hoopingarner *et al.* [3]. These investigators found that, in Chinese Hamster cells, the various Aroclor mixtures were generally more toxic as the percentage of chlorine in the mixture decreased. However, in the latter studies, the Aroclor mixtures were compared on the basis of equal weight, e.g. μg/ml (ppm), rather than on the basis of equivalent molarity. If toxicity of Aroclor 1221 (average mol. wt, 194) is compared with that of Aroclor 1260 (average mol. wt, 361) on the basis of equal μg/ml (ppm), then Aroclor 1221 will be present in nearly twice the molar concentration of Aroclor 1260 (Table 1). This difference in molar concentration may account for the results of Hoopingarner *et al.* [3] cited above.

It is apparent that 4-Cl-BP is causing pertubations in at least two areas of cell metabolism: (1) in the transport of small precursor (substrate) molecules across the cell membrane, and (2) in phospholipid metabolism. Because of the limited number of substrates investigated, no generalization can be made with respect to the nature of the 4-Cl-BP effect on transport; however, one effect on transport was consistent. When 4-Cl-BP significantly inhibited the uptake of a specific precursor from the medium into whole cells (Table 2), the degree of uptake inhibition was reflected in a corresponding degree of inhibition of precursor incorporation into the acid-insoluble fraction and, by implication, in the inhibition of precursor incorporation into the terminal macromolecular species of the biosynthetic pathway being investigated.

The results are to be expected, because the inhibition of labeled substrate uptake will result in a decrease of the specific activity of the precursor in the acid-soluble pool, and subsequently, this in turn, will result in an apparent and similar degree of inhibition of precursor incorporation into the acid-insoluble fraction of the cells.

Plagemann [15] has reported that phenethyl alcohol causes a similar effect on protein, RNA, DNA and phosphatidylcholine synthesis in Novikoff hepatoma cells. He observed that the rapid and apparent inhibition of incorporation of isotopically labeled precursors into the acid-insoluble fraction was actually due to the failure of phenethyl-treated cells to take up the precursors from the medium and incorporate them into the acid-soluble cellular pools. More extensive studies [16] on uridine and choline transport and their subsequent phosphorylation revealed that the rate-limiting step in the conversion of uridine and choline into intracellular phosphorylated forms by whole cells was not that of the kinase activities, but rather of substrate entry into the cell. Thus, the major effect of phenethyl alcohol appeared to be on the permeation of these precursors.

The variation in the 4-Cl-BP effect on uptake of small precursor molecules reported here demonstrates that, in L5178Y cells, the substrates studied do not share a common mechanism of transport. Furthermore, there was no meaningful pattern to the relative degrees of uptake inhibition. At 40 μg/ml, 4-Cl-BP inhibited L-leucine, L-serine and uridine uptake 31–33 per cent and thymidine uptake by 54 per cent (Table 2). But uptake of choline; ethanolamine and inositol was not significantly affected. At 10 μg/ml (data not shown), 4-Cl-BP had no significant effect on the uptake of any of the substrates studied. It does not appear likely that inhibitory effect on precursor uptake represents the primary mechanism by which 4-Cl-BP toxicity is being expressed.

The effects of 4-Cl-BP on phosphatidylcholine and phosphatidylethanolamine metabolism appear to be independent of precursor transport, since uptake of the two substrates, [$^{14}$C]1,2-ethanolamine and [$^{14}$C]methyl-choline, into whole cells is only slightly affected compared to the significant inhibition of phosphatidylethanolamine synthesis or alternatively to the 2- to 3-fold increase in phosphatidylcholine synthesis. In the latter instance, there was no difference in the rate of choline incorporation into the phosphorylcholine pool of untreated and treated cells, and 95 per cent of the acid-soluble choline was recovered as phosphorylcholine. Only a trace of radioactivity was found in CDP-choline, indicating that the CDP-choline pool must be very small in L5178Y cells; a similar observation was made in Novikoff hepatoma cells [14]. It has been proposed that the rate-limiting reaction in the incorporation of choline into phosphatidylcholine is probably the synthesis of CDP-choline from phosphorylcholine and CTP [17].

Despite the 2- to 3-fold increase in the specific activity of PC which occurred in 4-Cl-BP-treated cells, no quantitative difference in the amount of PC recovered from control and treated cells could be detected. Since PC comprises 54 per cent of the total cell phospholipid, it could be expected that, if the increase in PC specific activity represented a significant increase in PC net synthesis, then that increase would be readily detectable by the analytical methods employed. Therefore, the stimulation of [$^{14}$C]methyl-choline incorporation into PC appears to represent an increase in the rate of PC turnover.

Phospholipid turnover has been previously observed and described in cultured mammalian cells and in rat tissues *in vivo*.

Gallaher *et al.* [18] found that the half-life of PC in cultured baby hamster kidney cells was 2.0 to 2.5 hr. Pasternak *et al.* [19, 20] described PC turnover rates in neoplastic mast cells, human lymphocytes, and in a variety of rat tissues *in vivo*. While the physiological significance is not understood, it does appear that PC turnover is characteristic of normal growing cells and tissues.

The stimulation of PC turnover in L5178Y cells by 4-Cl-BP suggests that the agent is affecting either the rate of PC biosynthesis or the rate of degradation. This effect is probably not a direct one. There is convincing evidence that the endoplasmic reticulum is the site of PC biosynthesis in both animal and plant tissues [21–23]. In the work presented here, it was observed that, while [$^{14}$C]4-Cl-BP was easily absorbed to the cells, there was no evidence that the labeled compound was taken up by the cells. In some way, not yet understood, the interaction of 4-Cl-BP with the cell membrane is able to influence the rate of PC turnover. A preliminary experiment has revealed that the stimulation of PC turnover occurs to the same extent in the mitochondrial, nuclear and microsomal fractions of these cells. Thus, PC turnover is not limited to the plasma membrane compartment of the cell.

It is not known at this time whether the stimulation of PC turnover is directly related to the mechanism of 4-Cl-BP toxicity. However, several other organochlorine compounds, e.g. DDT, chlordane and kelthane, which are also toxic to L5178Y cells [8], have a similar effect on PC turnover. Kelthane, the most toxic of the organochlorine compounds studied [8], also elicits the greatest stimulation of PC turnover. The mechanism by which these organochlorine compounds affect phospholipid metabolism is under further investigation.

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