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A Protein Binding Radio-Assay Method for Measuring PCB (Hexachlorobiphenyl) Incorporation in Culture Cells

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Measurement of polychlorinated biphenyl (PCB) incorporation into cells has been approximated by several methods in various cell types. MURAKAMI & FUKAMI (1978) used monolayer cultures of human embryonic lung diploid cells to assess ^{14}C hexachlorobiphenyl uptake, KEIL et al. (1971) examined the uptake of Aroclor 1242 in the marine diatom Cylindrotheca closterium, by extracting PCB with acetone and analyzing it by gas chromatography. SPALDING et al. (1976) employed a cellular suspension of L 5178 Y mouse lymphoma cells containing ^{14}C-tetrachlorobiphenyl in Fisher media. The results of these varied experiments on PCB uptake in cells are important for elucidating the possible mechanism of PCB toxicity.

Methods described in this paper employ protein binding of ^{14}C-PCB as a means of preventing the cross contamination of the precipitated cellular pellet with unbound free ^{14}C-PCB. The binding of organic compounds to serum proteins is not an uncommon phenomena. MOSS & HATHWAY (1964) showed strong evidence for the binding of dieldrin and isobentan to the soluble proteins in circulating blood. PCBs have been found associated with red blood cells, serum albumin and lipoproteins (MATTHEWS 1977) and the lipoproteins reportedly contained proportionally the greatest amount of PCB. HESSE et al. (1978) reported the irreversible binding of 2,2'-^{14}C dichlorobiphenyl and 2,2',4,4',5,5'-^{14}C hexachlorobiphenyl to both microsomes and albumin. Less than 10% of ^{14}C-PCB was reported to be covalently bound to liver macromolecules (SHIMADA 1978). In light of this evidence the binding of ^{14}C-PCB to albumin (bovine serum albumin) was investigated in this study as a mechanism for separating free ^{14}C-PCB from that bound to the cell. In this way we hope to demonstrate a more precise method of estimating the properties of PCB incorporation into and unto the cell and also to determine the extent of ^{14}C-PCB sedimentation during centrifugation compared to the sedimentation of cells.

METHODS AND MATERIALS

Gravitational Force on ^{14}C-PCB Precipitation. Approximately $2x10^6$ dpm's of 2,2',4,4',5,5'-^{14}C hexachlorobiphenyl (specific activity 24 mCi/mM, California Bionuclear was evaporated under nitrogen and resuspended in 5 uL of 95% ethanol and then diluted with 10 mL of physiological saline (0.9% NaCl) solution. This solution was centrifuged for 20 min at 10,000 x g at 4°C. Aliquots of 0.1 mL were taken before and after centrifugation and

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each aliquot was reacted with 3 mL of Hydrofluor (National Diagnostics) and counted on a liquid scintillation counter.

A second ^{14}C-PCB solution was prepared to a concentration of 14,000 dpm/3 mL and sonified for 15 s and centrifuged at 1000 x g for 10 min. A third solution was prepared exactly as described above except that it was not sonified and thus served as a control. For comparative purposes, a 0.1 mL aliquot was counted from each solution before and after centrifugation.

A fourth solution of ^{14}C-PCB was sonified for at least 15 s and incubated for 2 h at 37°C in a shaking water bath, and then centrifuged for 10 min at 1000 x g. An aliquot of 0.1 mL was taken after sonification, incubation and centrifugation and then counted.

^{14}C-PCB Binding to Bovine Serum Albumin (BSA). BSA fraction V (Sigma) solutions in concentrations of 0,0.1, 0.5, 1 and 2% were prepared in 0.9% NaCl. Following incubation with 300,000 dpm of ^{14}C-PCB for 2 h at 37°C in a shaking water bath, the samples were centrifuged for 1.0 h at 10,000 x g. The total of each solution was 3 mL per sample and a 0.5 mL aliquot was taken after centrifugation and counted.

Uptake of ^{14}C-PCB in Hepatocytes. Female Sprague-Dawley rats (210-250 g) were killed by cervical dislocation and one g of liver tissues was immediately removed and suspended in a 1 mg/mL trypsin solution (Sigma) of Dulbeccos modified Eagle media (Gibco) using a procedure after SAYERS et al. (1971). The mixture was then incubated at 37°C in a shaking water bath for 20 min after which it was filtered through a layer of sterile gauze and subsequently centrifuged at 1000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 5 mL of media containing 5 mg of trypsin inhibitor from lima bean type II-L (Sigma). The cells were counted in a Levy counting chamber and then diluted with media to a final concentration of 10^6cells/1.5 mL.

Approximately 40,000 dpm of ^{14}C-PCB in 0.5 mL of 0.9% NaCl solution was sonified and added to each cell culture. The cell cultures, which contained 10^6 cells plus ^{14}C-PCB were incubated at 37°C in a shaking water bath for either 2 or 20 h.

To separate the ^{14}C bound to the cells from free ^{14}C-PCB, BSA was used. One mL of 2% BSA was added to each suspension after incubations of 2 and 20 h and before centrifugation. The suspensions were vigorously vortex mixed for 30 s and the incubations were continued in a shaking water bath at 37°C for an additional 30 min. The suspensions were then centrifuged at 1000 x g for 10 min. A blank, without cells was included in each assay and it contained 40,000 dpm ^{14}C-PCB in Dulbeccos MEM. A 0.5 mL aliquot of the supernatant from each tube was combined with 3 mL of Hydrofluor and counted. The pellet was resuspended in 0.8 mL of distilled water and a 0.5 mL aliquot of the resuspended pellet was then combined with 3 mL of Hydrofluor and counted.

In a second aspect of this experiment, hepatocyte concentrations in 1 mL of culture media were diluted to $0.25x10^6$, $0.5x10^6$, $1.0x10^6$ and $2.0x10^6$ while the ^{14}C-PCB added was held constant at 40,000 dpm. The uptake of ^{14}C-PCB was measured following a 2 h incubation as described above.

Effect of BSA Affinity for ^{14}C-PCB and Its Effect on Uptake in Hepatocytes. Approximately 10^6 isolated hepatocytes were incubated with ^{14}C-PCB for 2 h, according to the previously outlined procedures. However, after incubation, three different concentrations of BSA were added to the culture media. One mL aliquots of 1.2 and 5 g/100 mL of BSA in 0.9% NaCl was added to the media. The amount ^{14}C-PCB bound to the cells was measured as previously described.

All data were analyzed using either a one way analysis of variance (ANOVA) with Duncans Multi-Range Test for separating the differences between means or the students t test. Unless otherwise stated ($p < 0.05$).

RESULTS

Centrifugation greatly decreased ($p < 0.05$) the concentration of ^{14}C-PCB remaining in the supernatant (Table 1). There was also a relationship between the g force and the amount of ^{14}C-PCB lost from the supernatant. As the force of gravity increases, there is a decrease in the amount of ^{14}C-PCB remaining in the supernatant. At 10,000 x g for 20 min, 4.4% of the ^{14}C-PCB remained in the supernatant; while at 1,000 x g for 10 min, 30% remained in the supernatant. Sonification reduced the loss of ^{14}C-PCB from the 0.9% NaCl solution. In an unsonified solution only 30% of the ^{14}C-PCB remained in the supernatant, while over 75% of the ^{14}C-PCB remained in the supernatant of a sonified solution (Table 1).

Albumin (BSA) prevented the precipitation of ^{14}C-PCB from the 0.9% NaCl solution during centrifugation (Table 2). Even at the lowest concentration, BSA (0.1 g/100 mL) prevented the precipitation of ^{14}C-PCB since 81% remained in the supernatant after centrifugation. At the highest concentration (2 g/100 mL) over 99% of the PCB remained in the solution.

Increasing the incubation time had no effect upon the cellular uptake of ^{14}C-PCB between 2 h (35 ± 7 picomole) and 20 h (30 ± 7 picomole) (Table 3). There was no further increase ($p > 0.05$) in the ^{14}C-PCB uptake in hepatocytes as the concentration of BSA increased from 1 to 5 g/100 mL. The total amount of ^{14}C-PCB found to hepatocytes was below (42 picomole) 10% of the initial concentration which is relatively low (Table 3).

The cell concentration not surprisingly determined the total amount of ^{14}C-PCB uptake. As the cell concentration increased there was an increase ($p < 0.05$) in the amount of ^{14}C-PCB (Table 4). The total molecular amount of PCB uptake increased ($p<0.05$) as the cell concentration increased from $1x10^6$ to $2x10^6$ cells/mL, although it was not directly proportional. At the highest cell concentration there was a considerable amount of quenching upon the ^{14}C-PCB dpm, almost 27% of the pellet dpm values.

TABLE 1: Effect of Sonification, Incubation Time and Centrifugational g Force on 2,2',4,4',5,5'-PCB Concentration in 0.9% NaCl Solution

Centrifugation	Initial[a] dpm	Final[a] dpm	Percent[b] remaining
20 min at 10,000 g	1,940,956 ±176,494	83,778[d] ±27,598	4.4
10 min at 1,000 g not sonified	44,638 ±3,514	13,602[d] ±1,088	30.5
10 min at 1,000 g and sonified	15,750 ±2,405	11,905[d] ±1,811	75.6
10 min at 1,000 g sonified & incubated for 2 h at 37°C	620,714 ±24,655	291,505[c,d] ±14,903	46.9

a. Each value is the mean ± the standard error of the mean (n=7). b. Percent of the dpm remaining in the supernatant. c. This dpm represents the count after centrifugation and there was no loss after incubation. d. All of the final dpm were significantly different from the initial dpm ($p<0.05$).

TABLE 2: Binding of 2,2',4,4',5,5'-^{14}C Hexachlorobiphenyl to BSA in a Physiological Saline Solution[a]

BSA concentration (g/100 mL)	Percent ^{14}C-PCB[b] bound to BSA[c]
0	9 ± 1
0.1	81 ± 11[d]
0.5	98 ± 3[d]
1.0	97 ± 6[d]
2.0	99 ± 3[d]

a. Solution of 0.9% NaCl. b. Each value is the mean ± the standard error of the mean (n=7). c. Percent ^{14}C-PCB in supernatant after centrifugation at 10,000 g for 20 min. d. Each value is significantly different from the control ($p<0.05$).

TABLE 3: Effects of BSA on 2,2',4,4',5,5'-^{14}C Hexachlorobiphenyl Binding to Isolated Rat Hepatocytes

BSA concentration (g/100 mL)	Percent Uptake[a,b]	Uptake in picomoles
1.0	10.1 ± 1.1	42 ± 5
2.0	9.1 ± 0.9	38 ± 4
5.0	8.0 ± 1.0	33 ± 4

a. Each value is the mean ± the standard error of the mean (n=7).
b. There were no significant differences ($p<0.05$).

TABLE 4: Effect of Hepatocytes Concentration on the Uptake of 2,2',4,4',5,5'-^{14}C Hexachlorobiphenyl[a,b]

Number of Cells (10^6/mL)	Percent Uptake	Uptake in picomoles
0.25	4.2 ± 0.2	40 ± 1
0.5	4.9 ± 0.8	40 ± 5
1[d]	5.3 ± 0.2	48 ± 3
2[d]	9.1 ± 0.4	88 ± 3[c]

a. Free and bound ^{14}C-PCB were separated by using 2% BSA.
b. Each value is the mean ± the standard error of the mean (n=7).
c. Values for cell concentration of $1x10^6$ and $2x10^6$ are significantly different ($p<0.05$) from each other as well as the other values by one way ANOVA. d. At this concentration the cells exert a 27% quenching effect on the ^{14}C-PCB counting.

DISCUSSION

The factors of ^{14}C-PCB sedimentation, centrifugation and binding to glass and plastic cause quantitational artifacts during and after incubation of cells suspended in culture media. Therefore, it becomes important to elucidate the effect of these 3 factors on measuring the quantity of ^{14}C-PCB uptake in cells.

The focus of this study has been on the first two factors, that is, the effects of sedimentation and centrifugation on evaluating PCB uptake and incorporation into the cell. Our data strongly suggest that difficulties arise in quantifying PCB uptake when precautions are not taken to separate free PCB from PCB bound to the cell.

We have previously addressed the question of PCB binding to glassware and plasticware (PEPE & BYRNE 1980) and found that incubation of 2,2',4,4',5,5'-^{14}C hexachlorobiphenyl after 144 h caused adhesion-binding to the glassware equal to 24% and 46% to plasticware. The two h time course of the incubations in this study plus the blanks we employed both contributed to eliminating the errors of glassware adhesion-binding from our ^{14}C-PCB incorporation calculations.

Our data show that centrifugation is a major mechanism of removing PCB from aqueous solutions. It also shows that hexachlorobiphenyl and cells (hepatocytes) precipitate at similar g forces in the centrifuge. Therefore, separating free PCB from bound PCB must be accomplished by other means. Albumin and specifically BSA seems an adequate method for effectively separating free PCB from PCB bound to cells and thus eliminate the cross contamination of free PCB appearing in the cellular pellet.

The uptake of ^{14}C-PCB by rat hepatocytes was a relatively low percentage of the total ^{14}C-PCB added to the culture. Earlier studies have reported very high uptakes of PCB. Marine diatoms exposed to Aroclor 1242 reportedly incorporated almost 1100 times more PCB than remained in the supernatant media (KEIL 1971). The ciliate T. pyriforonia "incorporated" 60 times the media level after incubation (COLLEY 1972). In contrast, the uptake in human embryonic

lung cells in monolayer were very low compared to the total amount of ^{14}C-PCB added to the culture media (MURAKAMI 1978). Approximately 1.5-3% of 4-chloro ^{14}C-biphenyl was incorporated with mouse lymphoma cells (SPALDING et al. 1976). A probable explanation for these findings may be that the monolayers were removed from the media for PCB analysis without using centrifugation or filtration. What then appear as large differences between PCB uptake in unicellular organisms compared to mammal cells may be explained by the methodological means employed in separating the cells from their supernatant.

After a ^{14}C-PCB solution was incubated for 2 h at 37°C and then centrifuged for 20 min at 10,000 g, there was over a 95% loss of the ^{14}C-PCB from the supernatant (Table 1). This loss could be but should not be calculated as an uptake of ^{14}C-PCB by cells. To avoid this artifact we have found bovine serum albumin which was added to our cultures before centrifugation very effective in facilitating the separation of free PCB from bound.

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