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PHOTO-INDUCED BINDING OF 2,2′,4,4′,5,5′-HEXACHLOROBIPHENYL TO CULTURED HUMAN CELLS

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Abstract—The polychlorinated biphenyl congener 2,2′,4,4′,5,5′-hexachlorobiphenyl can be photoactivated by brief high-intensity ultraviolet irradiation. Photoactivated intermediates are bound to neighboring biological macromolecules.

Properties and stability of hexachlorobiphenyl photobinding were examined with bovine serum albumin, a protein known to strongly bind lipophilic compounds. Photobinding to cultured human Chang liver cells was a function of ligand and cell protein concentration as well as of irradiation time. Binding increased with incubation time, in support of the time course of uptake previously measured in the same system by alternative methods. Separation of cell proteins by gel electrophoresis showed that the distribution pattern of photobinding changed at different rates for different proteins. Photobinding to major cell lipid groups and to individual phospholipids likewise reflected uptake of the compound. Notably, photobinding to phosphatidyl choline was elevated relative to phosphatidyl ethanolamine.

Thus, the presented method is suitable to follow up transport and intracellular equilibrium distribution of photoactivatable ligands. As a particular advantage, artefactual redistribution of persistent lipophilic compounds during cell fractionation can be avoided.

Accumulation and persistent storage of polychlorinated biphenyls (PCB)† in animal tissues have been recognized as major environmental problems. An important factor in estimating the risk of exposure is the pharmacokinetics of PCB deposition in lipid-rich tissue cells. In particular, the slow rate of uptake, the metabolic persistence and the relatively high accumulation levels have made the PCB isomer 2,2′,4,4′,5,5′-hexachlorobiphenyl a valuable tool in studying uptake and intracellular distribution of higher chlorinated biphenyls in tissue cells and cell cultures [1–6].

We have recently investigated binding and uptake of the model compound (U-^{14}C)2,2′,4,4′,5,5′-hexachlorobiphenyl (6-CB) by cultured human Chang liver cells. After initial binding to the cell plasma membrane, temperature-dependent endocytic transport of this PCB congener was shown to play a major role in cellular uptake [7]. As the compound is concentrated in the lipid phase of the cell plasma membrane, transport by membrane endocytosis was quantitatively comparable with uptake by passive diffusion [7]. Despite these investigations, little information is yet available on the ensuing intracellular distribution routes following uptake.

Reversible non-covalent interaction of small organic ligands with biological macromolecules can be stabilized by photoaffinity labeling. For example, the photo-induced formation of stabile covalent bonds between photoactivatable hormones and their receptor proteins has greatly facilitated isolation and biochemical characterization of receptor–ligand complexes [8, 9]. In general, the photolabile ligand is an azido- or diazirino-derivative of the parent compound [8]; ideally, however, the small ligand itself should be photoactivatable.

We adapted this labeling technique onto time-dependent transport processes of small unmodified organic chemicals in cell culture. As a prerequisite, the ligand of choice must be photoactivatable within a reasonably short time. In the present paper, we show that the PCB congener 2,2′,4,4′,5,5′-hexachlorobiphenyl is photoactivatable by brief exposure to high-intensity ultraviolet radiation. Reactive excited intermediates of the photolyzed compound bind instantaneously to neighboring molecules such as protein and lipid of monolayer cells. These newly introduced covalent bonds stabilize the original weak reversible interaction of the ligand with cell macromolecules. As a particular advantage, the problem of redistribution of the lipophilic compound during subcellular fractionation [10] can be overcome.

EXPERIMENTAL SECTION

Chemicals and materials. Chemicals and materials were obtained from the following suppliers (mostly in the F.R.G.): (U-^{14}C)2,2′,4,4′,5,5′-hexachloro-

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† Abbreviations used: 6-CB, (U-^{14}C)2,2′,4,4′,5,5′-hexachlorobiphenyl; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; PCB, polychlorinated biphenyls.

biphenyl (6-CB): New England Nuclear (Dreieich, 0.30 GBq/mmol = 8.2 mCi/mmol) and Pathfinder Laboratories (St. Louis, MO; 0.34 GBq = 9.4 mCi/mmol). 2,2',4,4',5,5'-hexachlorobiphenyl: Promochem (Wesel). Lipid standards, the SH-reagents, *p*-aminobenzoic acid, bovine serum albumin (essentially fatty acid free) and the marker protein kit for gel electrophoresis: Sigma (Deisenhofen). Silica Gel 60 thin layer plates and the reagent 2-tert-butyl-4-methoxy phenol: Merck (Darmstadt). Reagents for polyacrylamide gel electrophoresis: LKB (Gräfelfing). Plastic ware and cell culture media: Biochrom (Berlin) and Greiner (Nürtingen).

Cell culture. Chang liver cells (American Type Culture Collection, No. CCL 13) were obtained through Gibco-Europe (Karlsruhe). Monolayer stock cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with glutamine (2 mM), $NaHCO_3$ (45 mM) and 8% of fetal calf serum (FCS) in an atmosphere of 95% air/5% CO_2 at 37° in a humidified incubator.

Photolysis of 2,2',4,4',5,5'-hexachlorobiphenyl. 0.4 μmoles (150 μg) of the compound was dissolved in 20 ml of ethanol and filled into the cylindrical lumen (2 mm width) between the inner, water-cooled quartz glass jacket and the outer, concentric glass support of a custom-made double-walled beaker. A Philips HPK 125-W/L lamp was placed in the centre to irradiate the solution with uniform intensity. After various periods of time, samples were taken to record the UV absorption spectra (Beckman DU-7 spectrophotometer). In a separate experiment, photolysis products were identified by GLC/MS analysis after 30 sec of irradiation (Dr Clausen, Institut für Ökologische Chemie der GSF). To this, samples of the concentrated solution were injected onto a DB 5 column of a Hewlett Packard HP 5995 GC/MS system that was run with a temperature program from 180 to 300°.

6-CB photobinding to bovine serum albumin (BSA). Fifteen millilitres of 0.5% BSA were equilibrated in phosphate-buffered saline (PBS) with 3.3×10^6 cpm = 11 μM of (U-^{14}C)2,2',4,4',5,5'-hexachlorobiphenyl (6-CB) at 37° overnight. Samples (2.8 ml) were filled into plastic culture dishes (6 cm dia.), and, eventually, the reagents shown in Table 2 were added. The open dishes were placed under the radiation source at a distance of 2.5 cm and irradiated for 20 sec. The whole unfiltered output of the mercury lamp was used to maximize intensity at minimal irradiation time. Following irradiation, the protein was precipitated with 0.8 ml of 25% ethanolic TCA. The precipitates were washed free from residual non-covalently bound material with 4 × 1.5 ml of the corresponding water/ethanol/TCA reagent mixture, until constant specific radioactivity was achieved. The final precipitates were solubilized in 0.5 ml of 0.5 N NaOH. Aliquots were then used for protein determination [11] and radioactivity counting by liquid scintillation. Alternatively, samples were subjected to HPLC and photobinding to the eluted BSA fractions was measured. A Beckman HPLC unit was equipped with a TSK-3000 column; flow rate: 0.9 ml/min at 0.52 kpsi; photometric detection at 229 nm; retention time of BSA: 11.1 min. "Dark control" samples (no irradiation) were treated alike to remove reversibly bound compounds.

Incubation and 6-CB photobinding to monolayer cells. Stock solutions were prepared by diluting the commercial 6-CB samples with a small volume of dimethylsulfoxide. Aliquots of the stock were pipetted into cell culture medium (DMEM + 8% FCS; "6-CB medium") to the final concentration required; the solvent concentration never exceeded 0.1%. This 6-CB medium was equilibrated overnight at 37° in a humidified CO_2-incubator. Uniform dissolution of 6-CB was checked by counting radioactivity of samples.

On the day preceding the experiment, cells had been plated in plastic culture dishes (6 cm diameter) at a density of 1×10^6 cells/dish. For the experiments, the culture medium of the cells was replaced with 2.5 ml of the above 6-CB medium, and the cells were incubated as long as indicated. If 10 cm dia. dishes were used, the experimental conditions were appropriately adjusted. Cells of 4–6 culture dishes were pooled for each single measurement.

Incubation was stopped by diluting the culture medium with 5 ml of chilled PBS. The cell monolayer was then irradiated in the open culture dishes as described above. Light exposure caused the temperature of the diluted incubation medium to slightly rise from 15° to 20°. The medium was then aspirated and the cell monolayer was washed successively with chilled solutions of 5 ml of PBS, 1 ml of FCS (to exchange loosely adsorbed photolysis products, cf. Ref. 7), and again 5 ml of PBS. The cells were scraped off the dishes in 1 ml of PBS and pelleted by low speed centrifugation. Protein was precipitated with 25% ethanolic TCA, and the precipitates were repeatedly extracted with 4 × 1 ml of the same reagent to remove reversibly bound 6-CB photolysis products. "Dark control" samples were not irradiated, but were processed alike.

Photobinding to cell proteins. Following irradiation the monolayer cells were washed with PBS and scraped off in 0.8 ml of water. Protein and lipids were separated by extraction with 2 × 3 ml of chloroform/methanol (final ratio of chloroform/methanol/water = 1/2/0.8; Ref. 12). The organic phase was saved for lipid analysis as described below. The precipitated cell protein was solubilized in electrophoresis buffer [13] and subjected to discontinuous SDS-polyacrylamide gel electrophoresis according to the method of Laemmli [13]. Approximately 800 μg of protein were layered onto cylindrical gels (stacking gel: 3.5% acrylamide; resolving gel: 7.5%), with subsequent overnight electrophoresis. The gels were fixed, stained with Coomassie Brilliant Blue, destained and sliced. Each 2 mm slice was then decolorized and digested with 30% H_2O_2 at 50° overnight in a closed scintillation vial prior to counting.

Photobinding to cell lipids. The lipids were extracted from the above mixed organic phase with chloroform [12]. After concentration under a stream of nitrogen, lipid groups were separated by one-dimensional TLC in the solvent system *n*-hexane/diethylether/acetic acid/water = 80/20/4/2. Phospholipids were separated by two-dimensional TLC in the systems chloroform/methanol/aqueous

ammonia = 65/35/5 (first dimension) and chloroform/acetone/methanol/acetic acid/water = 10/8/2/2/1 (second dimension; Ref. 14). Lipid spots were made visible by iodine vapour. Standards were used to identify phospholipid specimens and to ascertain identical R_f-values of parent and photolabeled phospholipids. Phospholipids were quantitated by digestion with 70% perchloric acid and subsequent phosphate determination using the Boehringer kit 124 974. Lipid spots were scraped off the plates directly into the scintillation vials for counting radioactivity.

RESULTS

Photolysis of 2,2',4,4',5,5'-*hexachlorobiphenyl*

Exposure of the PCB congener 2,2',4,4',5,5'-hexachlorobiphenyl to sunlight [15] or, under laboratory conditions, to low-energy ultraviolet radiation (280–400 nm) has been shown to result in gradual decomposition of the molecule [16–18]. The rate of photolysis can be enormously accelerated if an ethanolic solution of the compound is exposed to the whole ultraviolet output of an unfiltered high-intensity mercury lamp. As shown in Fig. 1, irradiation of 10 sec is sufficiently short to change the UV absorption spectrum; the peak increase at 255 nm is essentially completed after 30 sec of exposure. A log plot of the peak change over irradiation time yielded a curvilinear function of multiple photolytic reactions (data not shown). The approximate composition of photolysis products was calculated from GLC/MS analysis and is listed in Table 1. At least 21 different chlorinated biphenyl photolysis products can be identified in the reaction mixture. The parent compound is approximately 80% degraded after 20 sec of photolysis.

Table 1. Photolysis products of 2,2',4,4',5,5'-hexachlorobiphenyl

Compounds identified	Approximate percentage
Unchanged hexachlorobiphenyl	21
Pentachlorobiphenyls	16
Tetrachlorobiphenyls	20
Trichlorobiphenyls	13
Dichlorobiphenyls	19
Chlorinated biphenyl-O-ethylether	11

After irradiation of the compound (20 μM in ethanol) photolysis products were identified by GLC/MS analysis and their approximate percentage calculated.

6-*CB photobinding to bovine serum albumin*

The basic features of photobinding to biological macromolecules were examined with soluble BSA as a model protein. After equilibration with 6-CB and irradiation, the protein/ligand complex was isolated by two different techniques. The results are compiled in Table 2. In the absence of irradiation, the reversible hydrophobic association of 6-CB with BSA is broken by protein precipitation and repeated extraction with ethanolic TCA; the combined treatment with acid and organic solvent removes more than 97% of reversibly bound substances (expt 1, −irradiation). In contrast, the photo-generated bonds are stabile under the conditions of repeated acid-solvent extraction (expt 1, +irradiation). The covalent protein/ligand complex can also be isolated by HPLC. In this case, the percentage of "dark control" binding is slightly higher than with the precipitation method, but does still not exceed 10% of photobinding (expt 2). In the literature, thiol or

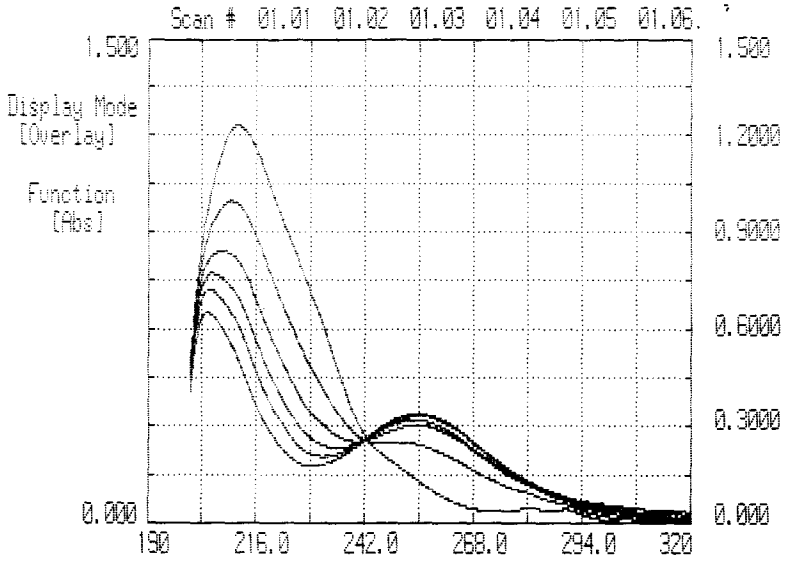

Fig. 1. Ultraviolet absorption spectra of 2,2',4,4',5,5'-hexachlorobiphenyl irradiation. The compound (20 μM in ethanol) was irradiated as described in the Experimental Section. UV spectra of samples were recorded after 0 (top spectrum at 212 nm), 10, 20, 30, 50 and 90 sec of irradiation.

Table 2. 6-CB photobinding to bovine serum albumin

Experiment	Additions	Method of separation	Irradiation	ng 6-CB bound mg BSA protein
1	—	TCA precipitation	—	3
	—	TCA precipitation	+	130
2	—	HPLC	–	5
	—	HPLC	+	75
	10 mM p-ABA*	HPLC	+	35
	1 mM BMPS†	HPLC	+	155

The covalent protein–ligand complex was purified to constant specific radioactivity by either TCA precipitation in aqueous ethanol (expt. 1) or by HPLC (expt. 2). The radical scavengers *p*-aminobenzoic acid and 2-tert-butyl-4-methoxy phenol, respectively, were added prior to photoactivation. The yield of photobinding in 3 independent experiments was: expt 1: 10.5%; expts 2: 9.0% and 9.7%.

* *p*-Aminobenzoic acid.

† 2-Tert-butyl-4-methoxy phenol.

amine compounds have been described to trap long-lived chemical radicals which might escape into solution prior to reacting with their original binding site [8, 19]. In our experiments, even a 10^3-fold molar excess of the scavenger *p*-aminobenzoic acid did not inhibit 6-CB photobinding by more than 60% (expt 2). The radical stabilizing scavenger 2-tert-butyl-4-methoxy phenol even doubled the yield of 6-CB photobinding (expt 2).

Thus, photolytically generated reactive 6-CB intermediates, presumably of radical nature, form stable covalent bonds to neighboring biological macromolecules.

Photobinding to monolayer cells

Prior to subsequent experiments, the effect of irradiation on monolayer cell viability was checked. The Trypan Blue exclusion test [20] indicated (Fig. 2) that plasma membrane integrity is maintained up to 30–40 sec of irradiation; thereafter, cell viability is rapidly declining. In consequence, the irradiation time of subsequent experiments will routinely be 20 sec. In the presence of *p*-aminobenzoic acid (15 mM), monolayer cells tolerate irradiation up to at least 60 sec (Fig. 2).

As shown in Fig. 3, binding linearly increases in the low 6-CB concentration range, but with the slope declining above 20 μM (panel A). Since uptake is not yet saturated at these concentrations [7], the declining slope very likely mirrors the onset of self quenching by 6-CB photolysis products. Binding also increases with duration of photolysis (panel B) and with cell number (dependence on cell protein not shown). Reversibly bound photoproducts are practically eliminated by the work-up procedure: "dark

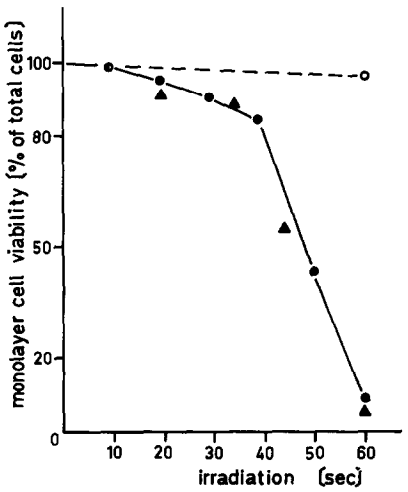

Fig. 2. Effect of irradiation on cell viability. Viability of monolayer cells was followed by the Trypan Blue exclusion test [20]: ●—●, percentage of stained cells; ○– – –○, same but in the presence of 15 mM *p*-aminobenzoic acid; ▲—▲, data of a second independent experiment.

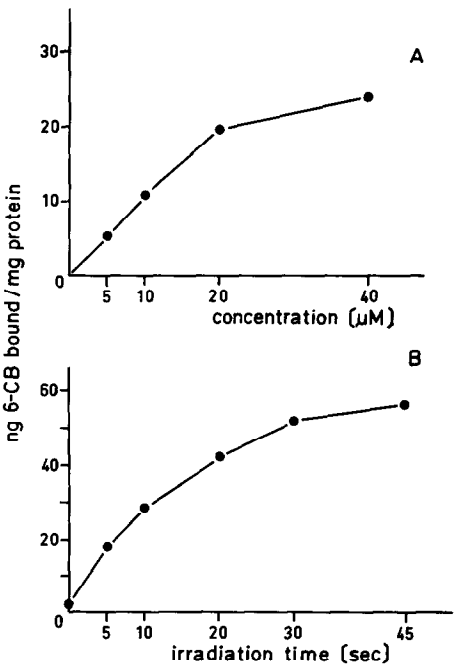

Fig. 3. 6-CB photobinding to monolayer cells. Photobinding is plotted as a function of 6-CB concentration (panel A; irradiation for 20 sec) and as a function of irradiation time (panel B; 6-CB concentration 20 μM; irradiation time "zero" = "dark control" binding). Number of experiments: panel A: N = 1; panel B: N = 2.

Table 3. Conditions of 6-CB photobinding to monolayer cells

Line	Additions	Concentration of added reagent	Irradiation	Photobinding (% of control)	
1	—	—	—	4 ± 1	(N = 4)
2	—	—	+	100	
3	Dithiothreitol	2 mM	+	115	
4	Glutathione	18 mM	+	104	
5	*p*-Aminobenzoic acid	12 mM	+	2 ± 0.5	(N = 3)
6	*p*-Aminobenzoic acid, then change to basal medium	12 mM —	+ +	37;44	(N = 2)
7	2-Tert-butyl-4-methoxy phenol	1 mM	+	90	
8	Pre-irradiated 6-CB	20 μM	+	45	

Monolayer cells were incubated at 37° for 2 hr with 6-CB (10–20 μM, lines 1–7) or with the pre-irradiated compound (line 8). The reagents listed were added prior to irradiation. Photobinding is given as % of control, that is of irradiation-induced 6-CB binding in the absence of any addition (line 2). The data are compiled from N different experiments.

control" (no irradiation) binding ranged at 5–10% of photobinding (cf. panel B at irradiation time "zero").

A possible source of error could arise from additional binding of photolytic intermediates, generated from the parent compound in the aqueous incubation medium. Moreover, 6-CB photointermediates could have escaped from their original interaction sites into the medium prior to photolytic fixation, diffusing to and binding at remote target sites. To get an idea whether such side reactions could significantly add to the observed binding, the influence of some scavengers known from photoaffinity labeling experiments [8, 19] was measured. As shown in Table 3, the SH-reagents dithiotreitol and glutathione are without effect (lines 3 + 4). The presence of *p*-aminobenzoic acid abolishes binding (line 5). If this compound was present during the initial irradiation but was then removed with the medium (as was all of the dissolved 6-CB), a second irradiation of the cells yields still 37% of control binding (line 6). This amount of photobinding is tantamount to 6-CB association with the cell monolayer. The monolayer-associated fraction of 6-CB was protected from photolysis by the shield effect of *p*-aminobenzoic acid in the overlaying aqueous medium (line 6 + 5). The radical stabilizer 2-tert-butyl-4-methoxy phenol does not affect 6-CB photobinding to monolayer cells (line 7). Incubation of cells with a 6-CB preparation that had been pre-irradiated for 45 sec and subsequent irradiation still yields 45% of control binding (line 8). Thus, binding is mainly the result of photoactivation of the 6-CB parent molecule; some primary photointermediates, for example such as listed in Table 1, may also be photoactivated and could contribute to overall binding.

Time course of uptake

6-CB photobinding to Chang liver cells increases over time and approaches an equilibrium level at 90–120 min of incubation (Fig. 4). This profile closely mirrors the time course of uptake as measured in previous "conventional" experiments [7], the only

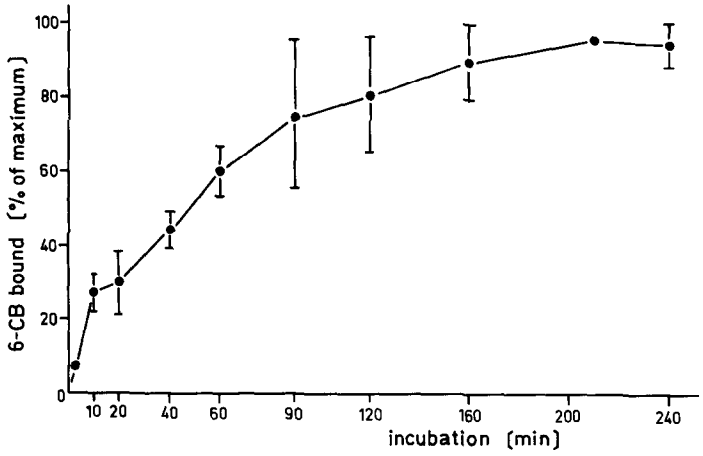

Fig. 4. Time course of 6-CB photobinding to monolayer cells. After incubation with 6-CB, monolayer cells were irradiated at the indicated times. Photobinding to cell protein is plotted vs incubation time. The mean ± SD of 4 independent experiments is shown, with 100% of maximum binding denoting 27, 55, 60 and 200 ng 6-CB bound/mg protein, respectively.

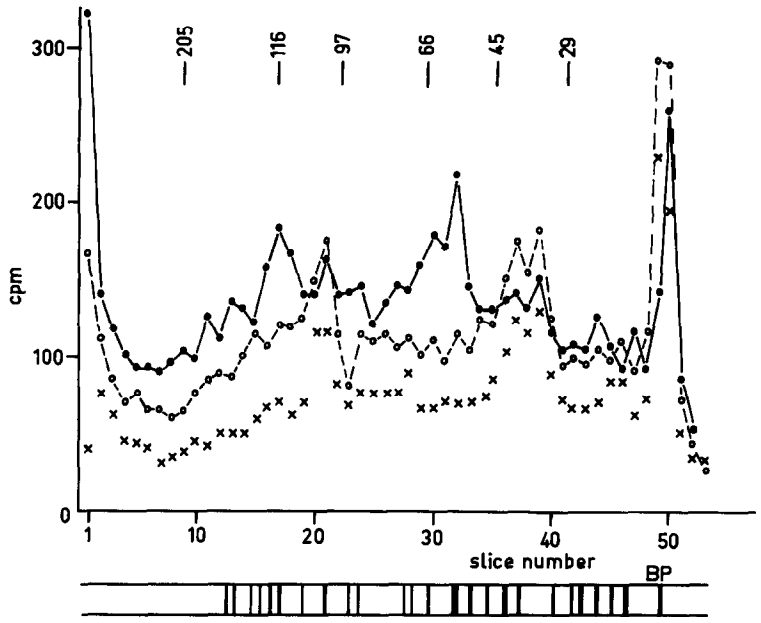

Fig. 5. 6-CB photobinding to cell protein. After incubation of monolayer cells and irradiation cell protein was isolated and subjected to SDS–polyacrylamide gel electrophoresis. The distribution of protein-bound radioactivity in consecutive 2 mm gel slices is shown. Figures on top indicate molecular weight (kDa) and position of marker proteins. Coomassie Brilliant Blue staining pattern is drawn underneath the figure. BP = Bromophenol dye front. Incubation was at 37° for 10 min (× × × ×), 45 min (○– – –○), and 150 min (●—●), respectively. "Dark control" binding at equilibrium did not exceed 6% of photobinding (not shown). Three experiments were performed.

difference being the earlier onset of uptake equilibrium. At low temperature (4°), 6-CB photobinding to monolayer cells stands at approximately 30% of binding at 37° (data not shown), in accordance with the previous experiments [7].

The profile of radioactivity distribution among separated cell proteins should likewise illustrate the time-dependent progressive invasion of cells by 6-CB. Following incubation and irradiation, the cell protein was subjected to discontinuous SDS–polyacrylamide gel electrophoresis. The profile of the protein-bound radioactivity distribution in the sliced gel rods is shown in Fig. 5. After brief incubation for 10 min, the incorporated radioactivity is rather low, and a few regions only of the gel are labeled. After the prolonged time periods of 45 and 150 min, however, the total protein-bound radioactivity as well as the number of labeled proteins are increased. The electrophoretic profiles of 6-CB photobinding to cell proteins thus reflect the time-dependent migration of the compound into the cells.

Table 4. 6-CB photobinding to major cell lipid groups

Lipid species	Total photobinding to lipid (μg)	Total photobinding to lipid, but related to protein (μg/mg protein)
Phospholipid	2.8	0.4
Cholesterol	0.55	0.08
Free fatty acids	0.2	0.03
Triglyceride	0.35	0.05
Total cell lipid	3.9	0.56

After incubation with 6-CB (20 μM; 37°, 150 min) and irradiation, cell lipids were extracted [12] and separated by one-dimensional TLC. Lipid spots were scraped off the plate directly into scintillation vials for counting. For comparison, photobinding to cell protein was 0.07 μg/mg protein.

Photobinding to cell lipids

6-CB photobinding to cell lipids was measured after TLC separation of the lipid extract of monolayer cells. The major lipid fractions were resolved by one-dimensional TLC. As shown in Table 4, photobinding to all major lipid fractions is found. The ratio of radioactivity bound to the different separated lipid groups is close to their respective weight percentage [21]; there is no accumulated photobinding to individual lipids. Total binding (w/w) to lipids is eight times enriched over the binding to protein (Table 4; assuming a lipid:protein ratio = 0.5; 0.56:0.07 = 8). However, an approximately 5-fold enrichment of protein binding is calculated, if the molar ratio of cellular protein and lipid is taken into account (assuming a mean molecular weight of 60,000 for protein and of 750 for lipid, respectively; 60,000:750:8 × 0.5 = 5). This is strong evidence that hexachlorobiphenyl is largely associated with cell proteins.

Phospholipids were resolved by two-dimensional TLC. As shown in Fig. 6, 6-CB radioactivity is bound to all lipid specimens and increases over time of incubation. The fractional binding to individual phospholipids, calculated from total binding, is

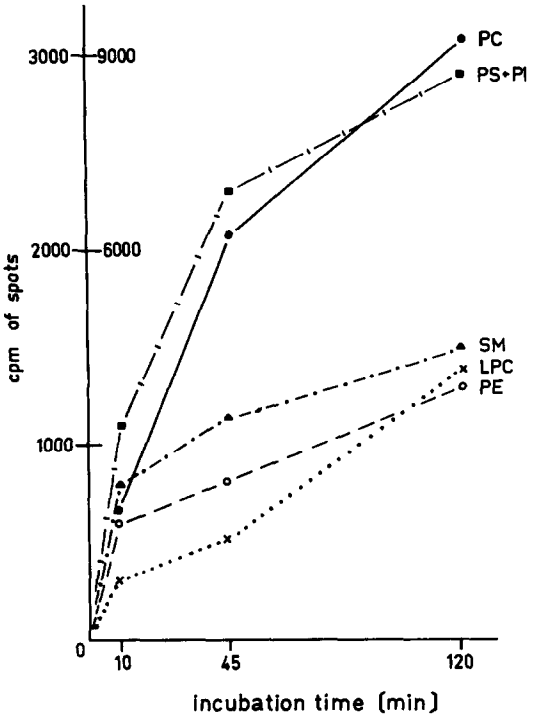

Fig. 6-CB photobinding to cell phospholipids. Phospholipids of the cell lipid extract were separated by two-dimensional TLC. Radioactivity of isolated phospholipid spots is plotted vs incubation time. The right-hand scale of the ordinate is for PC only. PC, phosphatidyl choline; PS + PI, phosphatidyl serine + phosphatidyl inisitol; SM, sphingomyelin; LPC, lyso-phosphatidyl choline; PE, phosphatidyl ethanolamine. The mean ± SD of 4 experiments (calculated as percentage of total phospholipid binding) is indicated in Table 5 for the 120 min incubation experiment. As an example, the cpm of photobinding to PC (120 min incubation) range from 6000–9000 cpm.

rather constant over all times of incubation (the figures of the 120 min incubation experiment are given in Table 5). This result can be expected provided that the rates of photobinding to individual phospholipids are similar. However, if 6-CB binding is related to the molar amount of individual phospholipids, binding to phosphatidyl choline is markedly elevated over binding to phosphatidyl ethanolamine (Table 5).

DISCUSSION

The suitability of the presented method depends on the generation of highly reactive photo-intermediates, such as chemical radicals, which are sufficiently reactive to ascertain instantaneous and indiscriminate binding of the excited molecules to neighboring biomolecules. Therefore, considerable effort was put into verifying the rapid photolysis of 6-CB and the covalent nature of the photo-induced 6-CB binding. The experiments show indeed that the persistent environmental chemical 2,2′,4,4′,5,5′-hexachlorobiphenyl can be photoactivated by brief high-intensity UV irradiation. After a few seconds of exposure, the parent compound is considerably dechlorinated while the biphenyl skeleton itself remains intact; the overall product composition strongly resembles the composite obtained after long-wavelength UV irradiation of PCB [15, 17, 18, 22, 23]. The photo-generated species are short-lived and are sufficiently reactive to attack adjacent biomolecules. The newly formed bonds are stable, they survive a variety of harsh treatments with organic solvents as well as the conditions of HPLC and of SDS gel electrophoresis.

Some agents known from photoaffinity labeling experiments were included in some assays to check for the occurrence of radical intermediates and for possible side effects in the photoreaction. Of these, *p*-aminobenzoic acid decreased 6-CB photobinding, very likely as a result of radiation absorption. The sensitizing effect of 2-tert-butyl-4-methoxy phenol on 6-CB binding to albumin is indicative of the involvement of excited radical intermediates in the photoreaction.

Long-lived radicals, freely diffusing during their lifetime through the aqueous phase to distant binding sites, did not markedly contribute to 6-CB photobinding. The binding reaction upon 6-CB photoactivation is clearly much faster than the slow rate of cellular uptake [7]. In support of this conclusion are the clear time-dependence of uptake, the marked

Table 5. 6-CB photobinding to cell phospholipids

Phospholipid species	% of total phospholipid (N = 2)	% of total phospholipid radioactivity (N = 4)
Lyso-phosphatidyl choline	6	7 ± 2
Sphingomyelin	13	10 ± 4
Phosphatidyl serine + phosphatidyl inositol	20 ± 1	17 ± 3
Phosphatidyl choline	39 ± 1	56 ± 2
Phosphatidyl ethanolamine	22 ± 3	10 ± 1

After incubation with 6-CB (20–23 μM; 37°, 2 hr) and irradiation, phospholipids of the cell lipid extract were separated by two-dimensional TLC and quantitated as described in the Experimental Section. Photobinding to individual phospholipids is compared with the chemically determined content of the respective species. N = number of experiments.

time-dependent gel electrophoresis profile of photolabeled proteins, and the time-dependent profile of phospholipid-bound radioactivity after TLC. If photobinding by long-lived diffusing radicals had occurred, the radioactivity profile of the separated specimens, determined by different analytical methods, should have been much blurred throughout the individual fractions. Thus, the photoactivated parent compound serves as its own tracer of intracellular distribution. Even the break-down of endocytic transport at low temperature [7, 24] could be recorded.

It may be noted from the radioactivity distribution profile after gel electrophoresis that the protein staining pattern and the radioactivity distribution over the gels were not always in register. Aside from the obvious binding to most of the cell proteins, 6-CB binding to minor protein fractions is also evident suggesting intracellular 6-CB binding sites with rather high affinity. Relating the high molar binding ratio of 6-CB to protein and lipid, one can conclude that the lipophilic compound resides in a lipidic environment, closely associated with protein. For example, the compound may be buried in hydrophobic pockets of soluble proteins, or it could be enriched at the protein/lipid interphase in membranes, that is within the shell of membrane "boundary" lipids.

With respect to lipid binding, the PCB congener was associated with all major lipid groups. Noteworthy is that hexachlorobiphenyl binding to phosphatidyl choline was elevated, with the labeling of phosphatidyl ethanolamine concomitantly decreased. It may be speculated that 6-CB incorporation into biomembranes follows the asymmetric distribution of choline and ethanolamine phospholipids in the membrane bilayer halves [25]. Packing constraints, which are imposed on concave side, phosphatidyl ethanolamine-rich bilayer regions, could enforce an asymmetric distribution of the rather bulky hexachlorobiphenyl molecule in the bilayer halves.

Major advantages of the presented method and future applications may be outlined as follows. Weak hydrophobic interactions of a variety of persistent chemicals with biomolecules could be stabilized by photoactivation. As the parent compound is used, there are no time-consuming efforts required to synthesize photolabile derivatives with differing biochemical properties. The covalent nature of the protein–ligand binding makes it possible to apply rather rigorous purification methods in cell fractionation studies; artefacts due to redistribution of the compound are avoided. Uptake pathways that route small lipophilic compounds to destined intracellular positions can be explored, accumulation and storage sites could be detected, specific binding sites (for example in nuclear DNA) might be found. The biological significance rests mainly on the fact that mapping of the intracellular distribution as well as detection of high-affinity binding sites might greatly help in understanding the host of PCB effects in mammalian cells. Particularly, mechanisms of the assumed tumor-promoting activity of PCB might be re-examined in view of the then known intracellular localization of PCB.

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