Biochemical Pharmacology, Vol. 29, pp. 2471–2475.

0006-2952/80/0915-2471 $02.00/0

USE OF THE FLUORESCENT PROBE 1-ANILINO-8-NAPHTHALENE SULFONATE TO MONITOR THE INTERACTIONS OF CHLOROPHENOLS WITH PHOSPHOLIPID MEMBRANES (LIPOSOMES)

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(*Received* 15 *December* 1979; *accepted* 19 *February* 1980)

Abstract—The fluorescence intensity (F_{480}), at pH 8, of 1-anilino-8-naphthalene sulfonate (ANS) bound to phospholipid membranes (liposomes) was decreased in the presence of mono-, di- and pentachlorophenols (pesticides and chemical intermediates that are toxic to animals). No shifts in emission spectra occurred, and no decreases in ANS fluorescence intensity were observed, in the presence of chlorophenols if liposomes were absent. An exception was 2,6-dichlorophenol which, at pH 8, had no effect on membrane-bound ANS-F_{480}. Using liposomes prepared from dimyristoyl lecithin, a 50 per cent decrease in membrane-bound ANS-F_{480} was produced by 0.046 mM pentachlorophenol and 0.2–0.5 mM dichlorophenols (excluding the 2,6-derivative). These results are consistent with published toxicological data on polychlorinated phenols that show an order of toxicity: pentachlorophenol > 2,4-dichlorophenol > 2,6-dichlorophenol.

The widespread use of chlorinated phenols in industry and agriculture [1, 2] is a current cause of concern due to their increasing contamination of the environment. PCP* in particular is an extensively used biocide [2] and was reported to be the cause of several cases of accidental infant poisonings [3]. PCP has also been found, along with a metabolite, in the urine of exposed workers [4, 5].

The toxicities of chlorinated phenols have been measured by whole animal toxicological studies, and LD_{50} values have shown that there is a gradation of response [6]. One of the difficulties posed by the whole animal studies is the inability to monitor the effects of low exposures that do not result in overt symptoms. An alternative approach is to use a model system that mimics cellular processes, e.g. membrane mediated phenomena.

The use of a model membrane system for the study of chlorinated phenols appeared feasible due to the known membrane active properties of the derivatives. They uncouple oxidative phosphorylation [7, 8], alter the microsomal electron transport system [9], and inhibit amino acid transport across cell membranes [10, 11]. PCP itself induces electrical conductivity in model membranes [12]. Furthermore, the membrane activities of other biologically active substances, e.g. drugs and pesticides, have been monitored by model membranes (liposomes) [13–15].

The alteration in the fluorescence intensity of membrane-bound ANS that is produced by the herbicide dicryl [15] suggested that this particular system may be able to monitor the interaction(s) of the chlorinated phenols with membranes. The present study was undertaken to test this possibility. The data show that liposome-bound ANS can be used to monitor the binding of chlorophenols to membranes and suggest that the number and positions of the chlorine atoms may play a role in the relative toxicities of these substances.†

MATERIALS AND METHODS

o- and *m*-Chlorophenol, and 2,4- and 2,5-dichlorophenol were purchased from the Eastman–Kodak Co. (Rochester, NY). 2,3-, 2,6-, 3,4- and 3,5-Dichlorophenol, pentachlorophenol and pentachlorobenzene were products of the Aldrich Chemical Co. (Milwaukee, WI). *p*-Chlorophenol was purchased from the Fisher Scientific Co. (Fairlawn, NJ). ANS was purchased from the Alfred Bader Chemical Co. (Milwaukee, WI) and recrystallized as the Mg^{2+}-salt [16]. Phenol was purchased from the Mallinckrodt Chemical Co. (New York, NY). Stock solutions of the above compounds were prepared in 95% ethanol (EtOH) and stored in the dark at 4°. Lecithins (egg yolk and dimyristoyl) and dicetylphosphate were products of the Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade. Deionized glass distilled water was used for all experiments.

Liposomes were prepared from dried phospholipid by the method of Huang and Thompson [17]. Sonication (Heat Systems Sonifier, model W185) was carried out for 1 hr at 16° for EL and 37° for DML. These temperatures are above the transition temperatures for the respective phospholipids [18]. The

* Abbreviations: PCP, pentachlorophenol; ANS, 1-anilino-8-napthalene sulfonate; FCCP, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone; EL, egg lecithin; and DML, dimyristoylecithin.

† A preliminary account of this work was presented in abstract form to the FASEB Meetings, Anaheim, CA, April 1980.

sonicated material was spun for 1 hr at the sonication temperature and the supernatant fraction used within 24 hr. Liposome preparations were maintained above the transition temperatures to minimize aggregation to multilamellar structures [19, 20]. Phospholipid concentration was based on total phosphate analysis [21] in which Elon was the reducing agent.

Fluorescence measurements were carried out on a Farrand Mark I spectrofluorometer equipped with a Heath Servo Recorder, model EU 2026, and a filter to cut off light below 400 nm. The slit widths were 5 nm for excitation and 10 nm for emission. The excitation wavelength was set at 380 nm and the emission wavelength at 480 nm. The data have been expressed as relative fluorescence intensity (F_{480}).

Measurements were carried out in 10^{-2} M Tris·HCl (pH 8) + 10^{-1} M KCl at 23° (EL liposomes) or 40° (DML liposomes) unless otherwise stated. Samples were incubated for at least 15 min at the appropriate temperature in a Lauda K-4/R circulating water bath. The content of each tube was transferred to a cuvette that was kept in a thermostated jacket through which water at the desired temperature was circulated. In this way, neither the ANS nor the chlorophenols were exposed to the high intensity ultraviolet light that might have partially destroyed the aromatic moieties [22], and no significant temperature changes occurred during the transfer to the fluorometer cuvette.

Ultraviolet absorption spectra were recorded on a Carey 14 spectrophotometer.

RESULTS

Effect of pentachlorophenol. The emission spectrum of ANS in the presence of EL liposomes is shown in Fig. 1. When liposomes were omitted, the fluorescence intensity was negligible, but a broad peak around 515 nm was observed. Hence, in Fig. 1 we are observing ANS associated with liposomes. After PCP was added, the emission spectrum of ANS bound to liposomes exhibited a decrease in intensity but no change in the emission peak. Furthermore, the order of addition of ANS and PCP did not alter the results. The decreased fluorescence intensity of ANS in the presence of PCP required the presence of membranes. In their absence, the ANS emission spectrum was unaltered by the presence of PCP in aqueous buffer or 95% EtOH.

The effect of PCP concentration on the fluorescence intensity of ANS bound to EL liposomes is shown in Fig. 2. Under the conditions of the experiment, 0.037 mM PCP produced a 50 per cent decrease in F_{480}. When DML liposomes were used, 0.046 mM PCP produced a 50 per cent decrease.

The contributions of the chlorine atoms and the hydroxyl group of PCP to the change in F_{480} were investigated by using pentachlorobenzene and phenol in place of PCP. Pentachlorobenzene (0.034 mM) resulted in a 28 per cent decrease in F_{480}, whereas PCP at the same concentration caused a decrease of 39 per cent. Higher concentrations of the former were not used because of its limited solubility. Phenol had no effect up to a concentration of 1 mM.

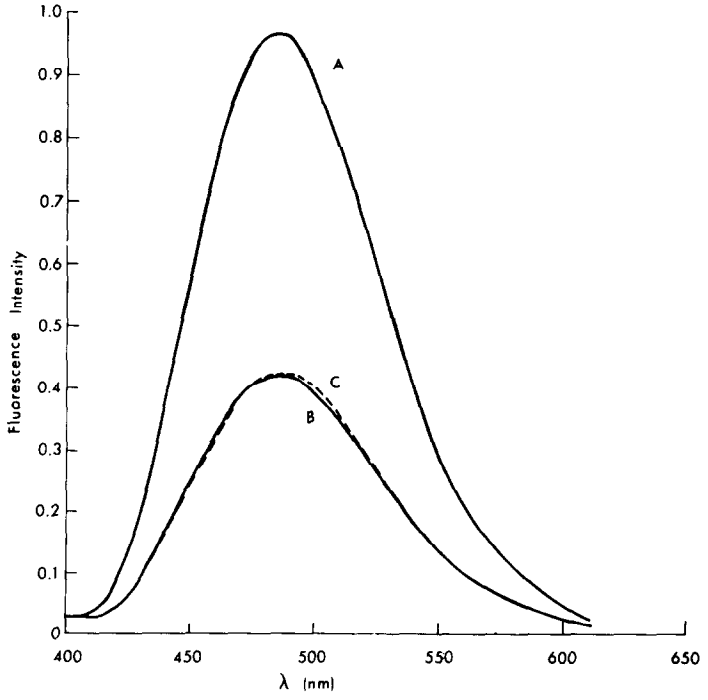

Fig. 1. Effect of PCP on the fluorescence intensity of ANS bound to EL liposomes. [ANS] = 7.5 μM; [PCP] = 0.25 mM; [EL] = 0.33 mM + 4 mole% dicetylphosphate. Curve A: ANS + EL liposomes. Curve B: PCP added to EL liposomes preincubated with ANS. Curve C: ANS added to EL liposomes preincubated with PCP.

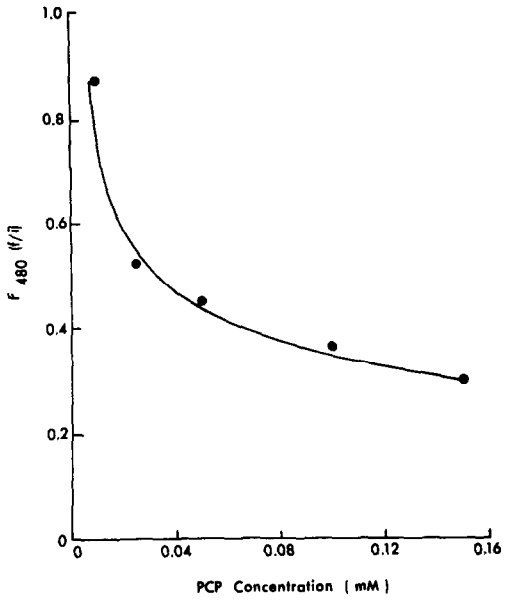

Fig. 2. Effect of PCP concentration on the decrease in F_{480} of ANS bound to EL liposomes. [ANS] = 16 μM; [EL] = 0.092 mM. F_{480} (f/i): F_{480} in the presence of PCP/F_{480} in the absence of PCP.

Effect of mono- and dichlorophenols. To gain some insight into the contribution of the individual chlorine atoms, the effects of dichlorophenol isomers on the fluorescence intensity of DML liposome-bound ANS were studied. The data in Fig. 3 and Table 1 show that, whereas 2,3-, 2,4-, 2,5-, 3,4- and 3,5-dichlorophenol caused a decrease in F_{480}, the 2,6-analogue did not. Spectral shifts did not accompany intensity changes.

The absence of an effect by 2,6-dichlorophenol was also observed by ultraviolet spectroscopy. Whereas the presence of DML liposomes caused a change in the u.v. spectrum of 2,4-dichlorophenol, no such change was observed for 2,6-dichlorophenol (Fig. 4).

o-, *m*- and *p*-Chlorophenol (1 mM) also caused a dimunition in the fluorescence intensity of ANS bound to DML liposomes. The decreases were 29, 49 and 43 per cent with *o*-, *m*- and *p*-chlorophenol, respectively.

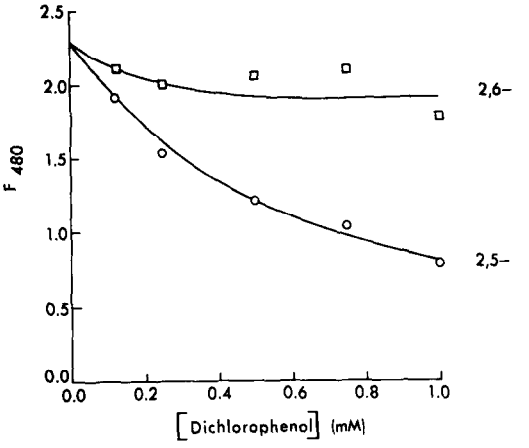

Fig. 3. Effect of 2,5- and 2,6-dichlorophenol concentrations on the F_{480} of ANS bound to DML liposomes. [ANS] = 4.8 μM; [DML] = 0.14 mM + 4 mole% dicetylphosphate. In the absence of dichlorophenol, F_{480} was 2.3.

Double reciprocal plots were constructed to determine if the chlorophenols and ANS were interacting in a competitive manner with the liposomes [23]. The data of Fig. 5 suggest that under the conditions of the experiment the decreases in F_{480} brought about by PCP and 2,4-dichlorophenol were due to competition with ANS for binding sites. Under the conditions specified in Fig. 5, an apparent fluorescence dissociation constant for ANS was estimated at 41 μM, assuming that the interactions were a result of binding to specific sites [24].

Effect of pH. To determine the relative importance of the neutral and ionized phenolic species, the effects of the phenols on membrane-bound ANS were observed at pH 4 as well as pH 8. The data of Fig. 6 show that at pH 4 the decrease in F_{480} caused by PCP was similar to that at pH 8.

Similar results were obtained with 3,4- and 3,5-dichlorophenol (Table 2). 2,3-, 2,4- and 2,5-Dichlorophenol, on the other hand, were slightly more effective at the lower pH. 2,6-Dichlorophenol, which at pH 8 exhibited a negligible effect on DML-bound ANS, became active at pH 4. Phenol, however, had little effect on membrane-bound ANS at either pH. In the absence of chlorophenols, the fluorescence intensity of DML-bound ANS was altered only slightly at pH 4.

Table 1. Effect of dichlorophenols on membrane-bound ANS-F_{480}*

Positions of chlorine atoms in dichlorophenol	$[Dichlorophenol]_{0.5}$† (mM)	LD_{50}-Rat, oral‡ [6] (mg/kg)
3,5	0.21	
2,4	0.21	580
3,4	0.23	
2,3	0.45	
2,5	0.51	
2,6	ND§	2940

* [DML] = 0.14 mM + 4 mole% dicetylphosphate, [ANS] = 4.8 μM.

† $[Dichlorophenol]_{0.5}$: concentration of dichlorophenol required for a 50 per cent decrease in fluorescence intensity of membrane-bound ANS.

‡ LD_{50} for PCP = 50 mg/kg [6]; $[PCP]_{0.5}$ = 0.05 mM using DML liposomes.

§ Not detected.

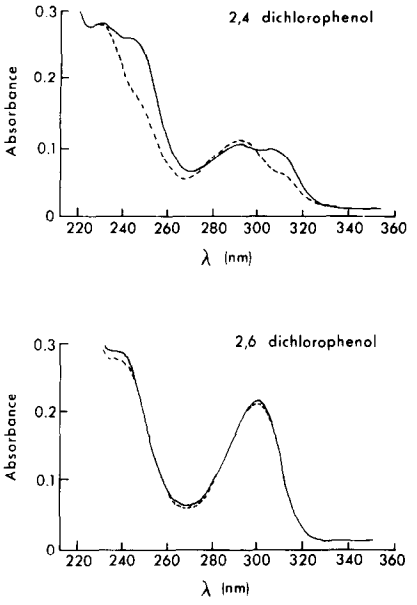

Fig. 4. Ultraviolet spectra of 2,4-dichlorophenol and 2,6-dichlorophenol in the presence or absence of DML liposomes. [DML] = 0.35 mM + 4 mole% dicetylphosphate; [dichlorophenol] = 0.1 mM. The samples were incubated for 15 min at 40° and the spectra were recorded at ambient temperature. Tandem cells (d = 0.439 cm) were used. The reference contained buffer. The sample cuvette contained dichlorophenol (with or without liposomes) in the back compartment and buffer or liposomes (respectively) in the front. Key: (———) dichlorophenol incubated without liposomes; and (– – – –) dichlorophenol incubated with liposomes.

DISCUSSION

The present study shows that liposome-bound ANS monitors the interaction of polychlorinated phenols with liposomes in a manner consistent with toxicological data. Monochlorophenols, which have LD_{50} values similar to 2,4-dichlorophenol [6], are less consistent than expected with the ANS–DML system (text and unpublished data). This apparent discrepancy may be a reflection of the closer resemblence of monochlorophenols to phenol which had a negligible effect on membrane-bound ANS-F_{480}.

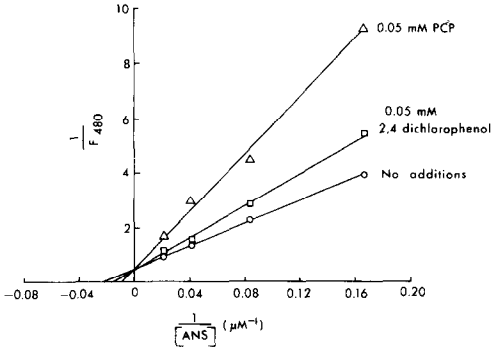

Fig. 5. Double reciprocal plot of F_{480} of ANS bound to DML liposomes. [DML] = 0.14 mM + 4 mole% dicetylphosphate. Key: (○) no additions; (□) 0.05 mM 2,4-dichlorophenol; and (△) 0.05 mM PCP.

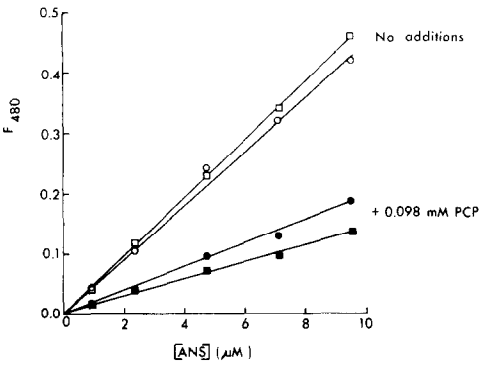

Fig. 6. Effect of pH and PCP on the F_{480} of ANS bound to DML liposomes. [DML] = 0.23 mM + 4 mole% dicetylphosphate. Key: (□, ■) pH 4; and (○, ●) pH 8. pH 4: 10^{-2} M acetate (Na^+) + 10^{-1} M KCl. pH 8: 10^{-2} M Tris·HCl + 10^{-1} M KCl.

The relative inactivity of phenol and 2,6-dichlorophenol, at pH 8, may be due to: (1) a high proportion of the unionized phenolic species; (2) an orientation effect imposed by two ortho chlorine atoms that precludes a favorable interaction with the membranes; or (3) a mechanism of binding that is different from the other chlorophenols. The first is probably not solely operative because at pH 4, where all chlorophenols are protonated [12, 25, 26], the dichloro- and pentachloro-phenols caused a reduction in the liposome-bound ANS-F_{480}. Furthermore, at pH 8 the monochlorophenols with pK_a values greater than 8 [25, 26] caused a similar decrease. In the case of phenol, the lack of effect at the lower pH suggests that, if binding does occur, its mechanism is different from that for the chlorinated species.

The association of ANS with the liposomes could be due to binding or partitioning or a combination. Binding was probably operative because the lines in the double reciprocal plot intersected the negative x-axis [24]. Haigh *et al.* [24] have shown that when partitioning occurs different curves are generated. The double reciprocal plot (Fig. 5) is consistent with a reversible binding process [23], which was also

Table 2. Effect of pH on the F_{480} of membrane*-bound ANS† in the presence of chlorophenols

Positions of chlorine atoms in dichlorophenol	F_{480} (f/i)‡	
	pH 8§	pH 4‖
3,5 (0.25 mM)	0.51	0.56
3,4 (0.25 mM)	0.55	0.52
2,4 (0.25 mM)	0.61	0.45
2,3 (0.25 mM)	0.71	0.52
2,5 (0.25 mM)	0.74	0.64
2,6 (1.0 mM)	0.91	0.54
(Phenol-1.0 mM)	0.84	0.90

* [DML] = 0.35 mM + 4 mole% dicetylphosphate.
† [ANS] = 4.8 μM.
‡ F_{480} (f/i): F_{480} in the presence of dichlorophenol/F_{480} in the absence of dichlorophenol.
§ 10^{-2} M Tris·HCl + 10^{-1} M KCl.
‖ 10^{-2} M Acetate + 10^{-1} M KCl.

suggested by the coincidence of the emission scans regardless of the order of addition of ANS and PCP.

The lack of spectral shifts accompanying the decrease in fluorescence intensity in the presence of chlorophenols indicates that the environment of the remaining ANS did not change [27]. Similar results were obtained with warfarin and microsome-bound ANS [28]. It is interesting to note that FCCP—like PCP and dichlorophenol, an uncoupler of oxidative phosphorylation [7, 8]—caused an efflux of ANS from energized electron transport particles [29].

This study has shown that a non-covalently bound model membrane probe can monitor the interactions of biologically active substances such as chlorophenols. The method is not subject to the uncertainties found in whole animal studies that arise from extended time periods, species differences, and small test populations. Model membrane systems similar to the one described here are amenable to experimental control and hence provide a means for reliably analyzing the effect of putative toxic substances on biological systems. Furthermore, some insight may be gained into the manner in which compounds such as the chlorophenols interact with cellular components, e.g. membranes.

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