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Effects of phenylamide herbicides on the physical properties of phosphatidylcholine membranes

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A number of phenylamide herbicides are observed to uncouple electron transport in isolated chloroplasts and mitochondria and alter the H^+ permeability of artificial liposomes. Several of these phenylamides were incorporated into phosphatidylcholine multilamellar and small unilamellar vesicles to measure their effects on the physical properties of membranes. X-ray diffraction analysis of the multilamellar vesicles revealed that the herbicides partitioned into the hydrocarbon chain region of the bilayer, but caused only minimal perturbations on hydrocarbon chain packing. ^{31}P-NMR spectroscopy of these multilamellar vesicles showed both a broadening and lowering of the phase transition temperature of the bilayer lipids upon addition of the herbicides. ^{13}C-NMR spectroscopy of small, unilamellar phosphatidylcholine vesicles was performed to measure the effects of the phenylamides on the chemical shifts and the spin-lattice relaxation times of the individual phosphatidylcholine carbon atoms. None of the added compounds had any measurable effect on the ^{13}C-NMR chemical shifts of the phosphatidylcholine. However, the herbicides significantly modified spin-lattice relaxation times of certain of the lipid carbon atoms. These results generally indicate that the herbicides orient in the lipid bilayers such that the hydrocarbon chains of the phenylamides associate with the hydrocarbon chains of the lipid, whereas the phenyl moiety resides in the polar region of the bilayer.

Introduction

Many phenylamide herbicides (phenylureas, acylanilides, and *N*-phenylcarbamates) have been shown to interfere with photosynthetic electron transport by binding to a chloroplast thylakoid membrane protein associated with photosystem II [1]. However, some phenylamide herbicides have also been shown to interfere with reactions of both chloroplast and mitochondrial ATP synthesis and mitochondrial electron transport that do not involve binding at photosystem II [2,3]. These latter herbicides inhibit ATP synthesis by acting as uncouplers. Support for this characterization was provided by studies which showed that the herbicides stimulate electron transport in mitochondria and chloroplasts and increase K^+ permeability in chloroplasts [1,3]. Further, these herbicides have been found to increase H^+ permeability and inhibit other activities associated with phosphatidylcholine liposomes in an order equivalent to

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Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine.

their uncoupling ability in chloroplast and mitochondrial membranes [1,3–6]. This selectivity in a bilayer lacking any protein component serves to strengthen the concept that these particular phenylamides are acting generally to perturb membrane organization and not at specific protein receptor sites [3].

The effects of phenylamide herbicides on membranes are of interest for two reasons. First, relatively subtle structural alterations of the phenylamides dramatically alter the observed effects of the compound on membrane function. For example, micromolar concentrations of isopropyl-3,4-dichlorocarbanilate were observed to increase the K^+ permeability of chloroplast membranes, whereas 6-fold greater concentrations of the 2,3-dichlorinated isomer were required to produce the same effect [3]. A similar order of selectivity has been observed in the ability of these compounds to promote H^+ permeability across the bilayers of PC-containing liposomes [3–6]. Second, the mechanistic basis for the uncoupling effect of phenylamides is not readily apparent; their structures do not suggest a mechanism of action similar to either carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (protonophore) or gramicidin (ion channel). It has been suggested that the effects of the phenylamides on membrane ion permeability can be explained in terms of a general, nonspecific effect on membrane bilayer organization [2,3]. This suggestion was based on the observation that many of the phenylamide herbicides cause changes in the 'fluidity' of the mung bean inner mitochondrial membrane [2] and alter the ion permeability of liposomes membranes in which no possible proteinaceous sites of action are present [3–6].

In this report, X-ray diffraction, ^{31}P-NMR, and ^{13}C-NMR were used to measure the effects of a series of phenylamide herbicides and herbicide derivatives on the structure, physical organization and internal motional dynamics of phosphatidylcholine membranes in an attempt to identify a physical basis for herbicide effects on membrane properties.

Materials and Methods

Lipid vesicles. Lipid/phenylamide vesicles were prepared by co-dissolving egg PC (more than 95% pure) or DPPC (Avanti Polar Lipids) in chloroform with the appropriate amount of phenylamide and then evaporating the solvent under a stream of nitrogen. The preparation was further degassed under vacuum to remove any residual chloroform. Triply distilled water was then added to the dried preparation, and the system was purged with nitrogen and vortexed above the lipid phase transition temperature to generate large multilamellar vesicles [7]. For the X-ray diffraction experiments, water was added to lipid-phenylamide preparations to make the system 70% water by weight. For the ^{31}P-NMR experiments, 50 mg of DPPC and 0.5 ml ^2H_2O were used with 0.09 mole fraction (herbicide/lipid) of phenylamide.

For the ^{13}C-NMR experiments, 0.4 g of egg PC and 2.0 ml ^2H_2O were used with 0.17 mole fraction phenylamide. After suspension of the lipid-phenylamide was completed, the multilamellar vesicles were placed in a 10 ml test tube under a nitrogen atmosphere and sonicated to clarity in a sonicator bath (1–6 h). The resultant small unilamellar vesicles were transferred to a 10 mm NMR tube under a nitrogen atmosphere, and ^{13}C-NMR spectroscopy was performed within 24 h.

X-ray diffraction analysis. Diffraction patterns from the lipid-phenylamide suspensions were recorded using a pinhole collimation camera as previously described [7]. All patterns were recorded on Kodak No-screen X-ray film, and exposure times were on the order of 5–7 h.

NMR spectroscopy. NMR spectra were obtained on a JEOL FX-90Q Fourier transform NMR spectrometer equipped with a multi-band probe. All spectra were recorded in the presence of broad band proton decoupling.

^{31}P-NMR spectra were obtained at 36.2 MHz using multilamellar DPPC vesicles in 5 mm NMR tubes. The probe was allowed to equilibrate at the appropriate temperature for approx. 10 min prior to the beginning of data accumulation. A 45° pulse angle, a 1 s pulse delay, and a 5 kHz sweep width were used in all spectra. Spectra were obtained by averaging 1000 transients and employing a 30 Hz line-broadening sensitivity enhancement factor.

^{13}C-NMR spectra were obtained at 22.5 MHz using sonicated small unilamellar egg phosphatidylcholine vesicles in 10 mm NMR tubes. The ^{13}C

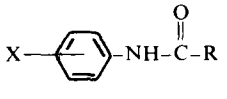
spin-lattice relaxation times (T_1) were measured using the fast inversion-recovery technique of Canet et al. [8]. This technique uses a (180°-τ-90°-PD) pulse sequence in which the pulse delay (PD) is set at about twice the value of the slowest relaxation time. A plot of ln $((S_\infty - S_\tau)/S_\infty)$ vs. τ yields a line with slope equal to $-1/T_1$ where τ is the time delay between 180° and 90° pulses, S_τ is the signal intensity at pulse interval τ and S_∞ is the signal intensity obtained when τ is greater than 5 times the longest T_1 value. In these experiments, temperature was 36°C, pulse delay was set at 5 s, eight values of τ were used, 500 transients were accumulated for each τ value, and a 2.3 Hz line broadening sensitivity enhancement factor was employed. The plots of $\ln((S_\infty - S_\tau)/S_\infty)$ vs. τ were fitted with a linear regression and all resultant lines had regression coefficients of 0.95 or better. The chemical shift of the terminal methyl carbon of the phosphatidylcholine was defined as 14.40 ppm relative to tetramethylsilane and was used as an internal standard. In most cases, the T_1 measurements were not replicated for any given phenylamide/liposome preparation. However, in the cases where replicate experiments were performed, the T_1 values were reproducible within 10%.

Herbicides and derivatives. Table I gives the trivial names or designations and abbreviations of the compounds used in this study. The compounds were available as crystalline material, and the identities of the compounds were confirmed by ^{13}C-NMR. All molar ratios of phenylamide to lipid used in these studies were within the range of concentrations used in mitochondrial inhibition studies [2]. For example, a molar fraction of phenylamide of 0.23 is obtained for a solution that contains 0.2 mg mitochondrial protein/ml and 10 μM phenylamide. This is based upon a value of 0.22 μmoles of phospholipid per mg protein in intact rat liver mitochondria [9]. The use of mole fractions is relevant to biological activity because the large values associated with the octanol/water partition coefficients of many of the phenylamides [10] can result in appreciable partitioning of these compounds into the lipid phase of the bilayer in the presence of lipid-containing membranes.

With the phenylamides, increasing the length of the alkyl side chain leads to increased inhibitor activity [1]. Further, dichlorination in the 3 and 4 ring positions is more inhibitory than monochlorination in either position. Chlorination in the *ortho* position (2,3-DCIPC) has been associated with decreased inhibitory activity. DCMU was utilized as a control compound in these experiments because other than its effects on photosystem II, it has only marginal effects on reactions mediated by mitochondria, chloroplasts and phosphatidylcholine liposomes.

TABLE I

COMMON NAMES AND DESIGNATIONS FOR PHENYLAMIDE DERIVATIVES STUDIED



X	R	Name or designation
3,4-Cl_2	$-CH_2CH_3$	Propanil
3,4-Cl_2	$-(CH_2)_3CH_3$	DCPA
3,4-Cl_2	$-CH(CH_3)CH_2CH_2CH_3$	Karsil
3,4-Cl_2	$-OCH(CH_3)_2$	3,4-DCIPC
2,3-Cl_2	$-OCH(CH_3)_2$	2,3-DCIPC
H	$-OCH(CH_3)_2$	IPC (Propham)
3-Cl_2	$-OCH(CH_3)_2$	CIPC (Chlorpropham)
3-Cl_2	$-(CH_2)_5CH_3$	CHPC
3-Cl_2	$-O(CH_2)_7CH_3$	CCPC
3,4-Cl_2	$-N(CH_3)_2$	DCMU (Diuron)

Results and Discussion

X-ray diffraction

All lipid suspensions, with mole fractions of phenylamide ranging from 0.0 to 0.33, gave diffraction patterns with lamellar low-angle reflections and wide-angle reflections typical of bilayer organization. For liquid-crystalline state lipid (egg PC at 20°C), the addition of up to 0.33 mole fraction 2,3-DCIPC, 3,4-DCIPC, CHPC or DCMU did not change the lamellar repeat period of 63 ± 1 Å. The broad, wide-angle reflection centered at 4.6 Å was also unmodified by these phenylamides. For gel-state lipid (DPPC at 20°C), 0.25 or 0.33 mole fraction of these same phenylamides modified both the low-angle and wide-angle diffraction patterns. Typical diffraction patterns for hydrated DPPC and DPPC with 0.25 mole fraction CHPC are shown in Fig. 1. The lamellar repeat period was increased from the value of 63 Å for hydrated

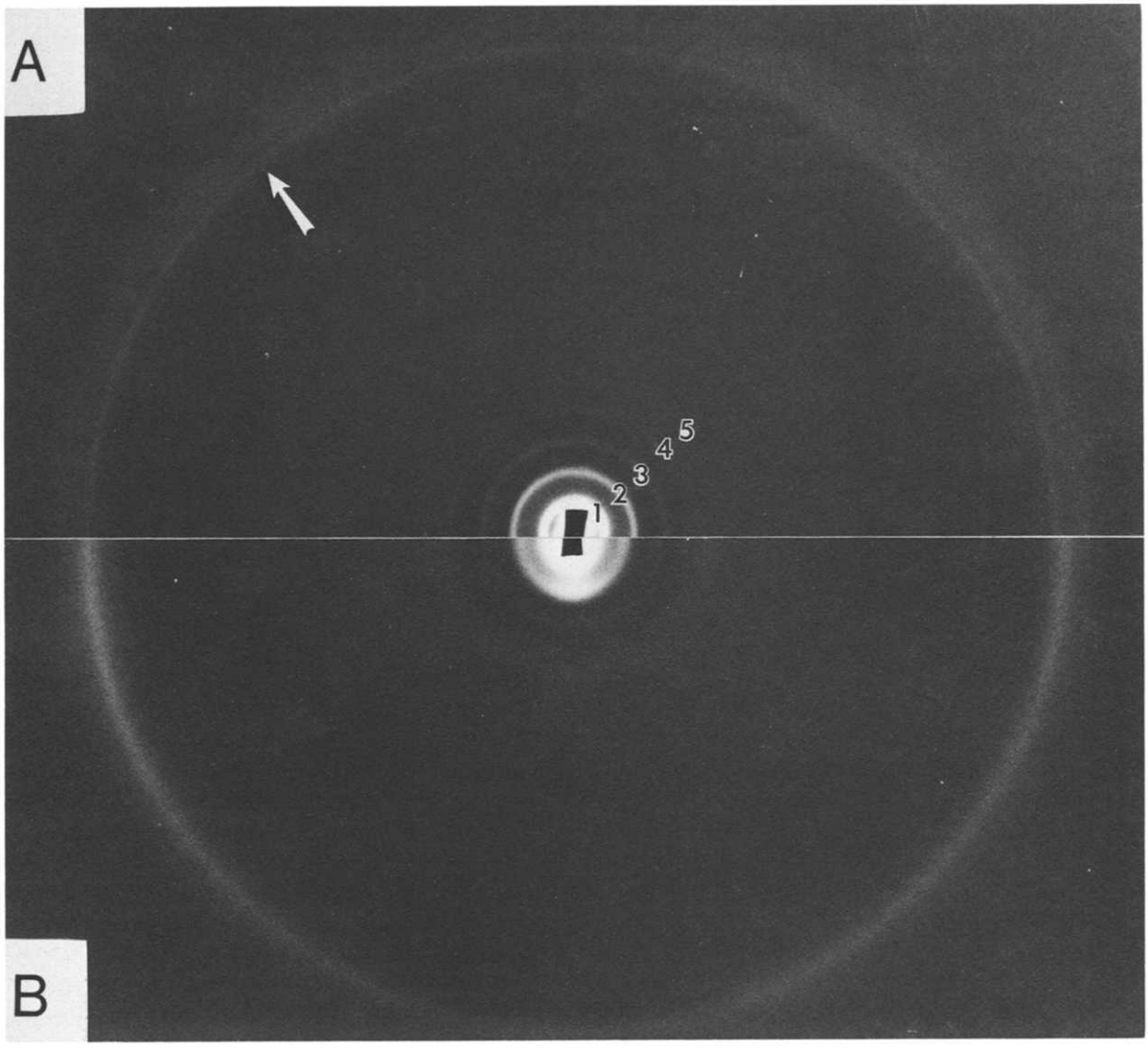


Fig. 1. (A) X-ray diffraction pattern from DPPC multilamellar vesicles at 20°C. The first five orders of a lamellar repeat are labelled, and the arrow indicates the sharp, wide-angle reflection at 4.2 Å with a surrounding band at 4.1 Å. (B) Pattern from DPPC vesicles at 20°C containing 0.25 mole fraction CHPC. The lamellar repeat period is 73 Å, and a sharp 4.15 Å wide-angle reflection is also observed.

DPPC to between 69 Å and 73 Å with phenylamide present. In addition, each of these phenylamides converted the characteristic double wide-angle pattern of gel state DPPC (a sharp 4.2 Å reflection and a broad 4.1 Å band; see arrow in Fig. 1A) to a single sharp 4.15 Å reflection. This change in the wide-angle pattern is a direct indication that these phenylamides modify the gel-state hydrocarbon chain packing and eliminate hydrocarbon chain tilt [11]. The elimination of chain tilting fully accounts for the increase of 6 to 9 Å in the lamellar spacing for the gel state [11,12]. Both liposome-active (3,4-DCIPC, CHPC) and liposome-inactive (2,3-DCIPC, DCMU) phenylamides gave identical results in these X-ray studies.

The fact that the diffraction patterns of liquid-crystalline bilayers are unmodified by the presence of phenylamides implies that phenylamides either do not enter the bilayer or partition into the bilayer without changing the bilayer thickness.

However, the changes in both the wide-angle and low-angle regions of the diffraction patterns of gel-state DPPC (Fig. 1) show that the phenylamides do partition into the gel-state bilayer, and the reduction in transition temperature as observed by ^{31}P-NMR (see below) indicates that the solubility of the phenylamides is greater in the liquid-crystalline than in the gel phase. The X-ray data indicate that the phenylamides do not increase the thickness of the bilayer in the liquid-crystalline state and increase the thickness of the gel-state bilayer only by an amount consistent with the elimination of hydrocarbon chain tilt. This suggests that for both liquid-crystalline and gel-state bilayers, the phenylamides position between adjacent lipid hydrocarbon chains, rather than between the opposing monolayers of the bilayer. Long chain alkanes [11–13] and fatty acids [14], which have also been found to localize between adjacent lipid acyl chains, produce modifications in gel and liquid state diffraction patterns similar to those observed with the phenylamides.

^{31}P-NMR spectroscopy

^{31}P-NMR spectra of DPPC multilamellar vesicles were recorded in the presence of phenylamides at 50°C, above the lipid phase transition temperature. the compounds looked at included propanil, 3,4-DCIPC, 2,3-DCIPC, and DCMU. In all cases, the observed NMR lineshapes were characteristic of a bilayer organization of DPPC in a liquid-crystalline state [15]. The ^{31}P-NMR lineshapes of other phospholipid organizations, such as a hexagonal phase or an isotropic phase, are markedly different than the lineshape observed with phospholipid bilayers [16,17]. This observation supports the results from the X-ray diffraction studies (Fig. 1) which showed that the presence of phenylamides did not alter the bilayer organization of aqueous DPPC dispersions.

Below the lipid phase transition temperature, the ^{31}P-NMR linewidth of DPPC multilamellar vesicles increases from about 40 ppm to 70 ppm (Fig. 2). This increase in linewidth has been interpreted in terms of a general decrease in lateral mobility of the phospholipid molecules as the bilayer undergoes a transition from the liquid-crystal to the gel state [17,18]. The incorporation of propanil into DPPC bilayers lowered the phase transition temperature, and increased the temperature range of the phase transition (Fig. 2). The reduction in phase transition temperature results from the incorporation of propanil into the bilayer and indicates a preferential solubility of the herbicide in the liquid-crystalline versus the gel phase [19,20]. The general increase in the temperature range over which the ^{31}P linewidth broadening takes place, as shown by the solid line in the presence of propanil (Fig. 2), was representative of all DPPC/phenylamide preparations tested. No marked differences were observed between previously identified liposome-active and -inactive compounds. In other trials, egg PC was used instead of DPPC, and the ^{31}P-NMR spectra of these vesicles at room temperature were not affected by incorporation of a number of different phenylamides (data not shown). Because the liquid crystal-gel transition temperature for egg PC is −15 to −7°C [21], the lack of effect of phenylamides on the ^{31}P-NMR spectrum is analogous to the results obtained for DPPC above 41°C.

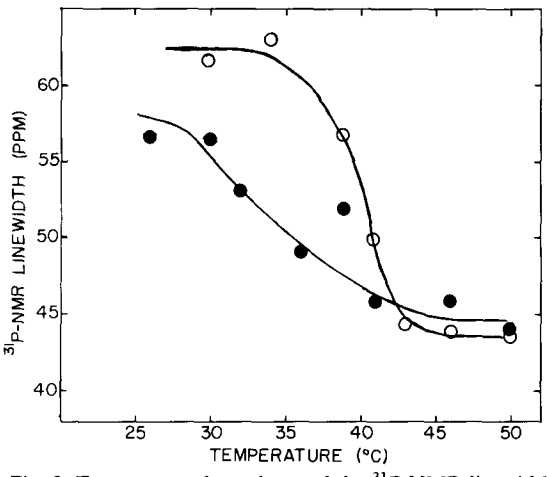


Fig. 2. Temperature-dependence of the ^{31}P-NMR linewidth of DPPC multilamellar vesicles in the absence (○———○) and presence (●———●) of 0.09 mole fraction propanil. The linewidth was taken as the chemical shift difference between the points at half height on either end of the spectral line.

^{13}C-NMR spectroscopy

In order to obtain a more detailed understanding of the nature of the phenylamide/PC interactions, ^{13}C-NMR spectroscopy of sonicated egg PC

TABLE II

^{13}C-NMR CHEMICAL SHIFTS OF EGG PHOSPHATIDYLCHOLINE LIPOSOMES AT 36°C IN THE PRESENCE OF 0.17 MOLE FRACTION PHENYLAMIDE

General formula: 3,4-Cl_2-Ø-N(H)C(O)-R.

PC carbon	Chemical shift (ppm)				
	control [a]	R			
		$-CH_2CH_3$	CH_3 | $-CHCH_2CH_2CH_3$	$-OCH(CH_3)_2$	$-N(CH_3)_2$
$-N^+(CH_3)_3$	54.65	54.60	54.65	54.54	54.65
$-N^+(CH_2)$	66.57	66.46	66.52	66.50	66.52
CH_2OP	60.18	60.14	59.96	60.07	60.07
C-1	174.21	174.21	174.16	174.16	174.16
C-2	34.61	34.61	34.66	36.58	-
$-(CH_2)_n-$	30.38	30.11	30.38	30.27	30.27
C-8 + C-11 oleic; C-8 + C-14 linoleic	27.73	27.73	27.67	27.62	27.89
C-9 linoleic; C-10 oleic	130.17	130.12	129.95	129.90	130.17
C-10, C-12 linoleic	128.44	128.38	128.82	128.33	128.49
C-11 linoleic	26.10	26.10	26.10	26.05	26.16
$-\underline{C}H_2CH_2CH_3$	32.55	32.55	32.55	32.06	32.49
$-\underline{C}H_2CH_3$	23.18	23.18	23.18	23.17	22.23
$-CH_3$ [a,b]	14.40	14.40	14.40	14.40	14.40

[a] Resonance assignments were taken from Cushley and Forrest [26].

[b] Chemical shifts are referenced to the terminal CH_3 peak assigned a value of 14.40 ppm relative to tetramethylsilane.

liposomes was performed in the presence of the phenylamides. The alkyl side chains and ring substituents of the phenylamides were systematically altered in these studies to determine if specific interactions between phosphatidylcholine and phenylamides existed.Table II shows the chemical shifts of the egg PC carbons in control liposomes and in liposomes containing various phenylamides. The chemical shift data were all characterized by a lack of effect of added phenylamide. This result was somewhat surprising because intermolecular ring current effects of the phenyl ring on the phenylamides might have been expected to have some minimal effect on the ^{13}C chemical shifts of egg PC.

^{13}C-NMR signals corresponding to the phenylamide were not observed in any of the spectra. Several factors could account for this. The amount of phenylamide present in the liposome might have been too small to give observable signals. This possibility is unlikely because smaller amounts of phenylamide in C^2HCl_3 gave strong signals with similar spectrometer settings. A second factor could be that the phenylamide resonances were obscured by lipid resonances. This is relevant for $-CH_2-$ and $-CH_3$ groups of the phenylamide alkyl chains because these signals in organic solvents are observed in the 14–35 ppm region of the spectrum. However, the chemical shifts of the phenyl-carbons and the -CH= groups should appear in clear regions of the spectrum. A third factor that could account for the absence of phenylamide signals in the spectra is the possibility that the spin-lattice relaxation times (T_1) of the phenylamide carbons are slow relative to the pulsing conditions so that saturation of the phenylamide signals occurred. This possibility cannot be entirely excluded for the phenyl carbons, which can have long (greater than 10 s) relaxation times [22], although as noted previously strong signals from the phenylamides were obtained in organic solvents under similar pulsing conditions. Also, this ex-

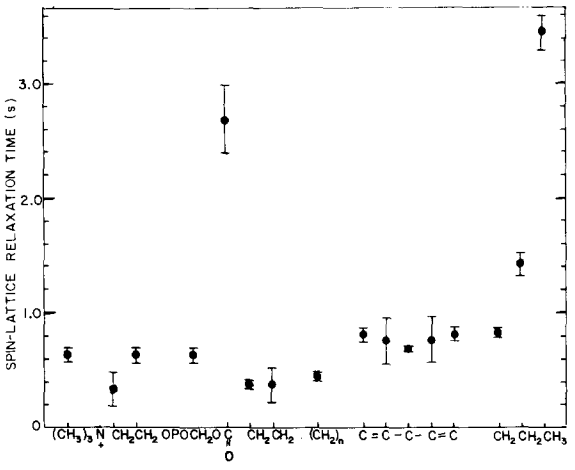


Fig. 3. ^{13}C-NMR spin-lattice relaxation times of the individual carbon atoms in egg phosphatidylcholine unilamellar vesicles (liposomes). NMR spectra were recorded at room temperature and T_1 values calculated as described in Materials and Methods.

planation would not apply to the phenylamide -CH= carbons, because these groups are not expected to have long T_1 values. A fourth possibility to explain the absence of phenylamide signals is that the phenylamide associates with the membrane in a rigid lattice environment resulting in dipolar broadening of the phenylamide signals. This explanation has been invoked to explain the absence of cholesterol signals in cholesterol-egg PC liposome ^{13}C-NMR spectra [23,24], and it would best appear to account for the lack of signals in the present studies.

In the absence of effects of phenylamides on the PC ^{13}C chemical shifts, attempts were made to look at phenylamide-lipid interactions by measuring the effects of added phenylamides on the spin-lattice relaxation times of the various PC carbon atoms. Fig. 3 presents a plot of T_1 values of the PC carbon atoms in egg PC liposomes in the absence of added herbicide. the T_1 values of the lipid carbon atoms directly reflect the relative motion of the nuclei [25]. The T_1 values increase from a low value of 0.31 s for the relatively rigid C-2 carbon near the glycerol moiety to a high value of 3.45 s for the mobile, terminal methyl carbon, at the center of the bilayer. These data are similar to results in other ^{13}C-NMR reports [23–28], and the trend of increasing mobility going from the surface to the interior of the bilayer is consistent with the picture of the physical state of the bilayer derived from other magnetic resonance studies [25]. The T_1 values were reproducible within $\pm 10\%$ using different liposome preparations, and the constancy of the chemical shift data (Table II) and the T_1 values for the terminal methyl carbon (Figs. 3 and 4, Tables III and IV) provided evidence that differences were not caused by variability in liposome preparation, egg PC, or instrument performance.

It was anticipated that those regions of the phospholipid bilayer most affected by incorporation of the phenylamides would show the largest effect on their ^{13}C relaxation times. Such an approach has been used previously to look at the incorporation of vitamin E, phytol, and phytanic acid into PC vesicles [26,27]. Spin-lattice relaxation times are particularly sensitive in those regions of the bilayer where a general destabilization occurs, leading to clear increases in measured T_1 values [26,27]. The present study had two goals: (1) to provide some insights into how the phenylamides are oriented within the bilayer and (2) to see whether biologically active phenylamides would show some common effects on relaxation times not seen with inactive compounds. Again, previous studies indicated that PC-containing liposomes showed the same specificity toward a number of phenylamides as isolated chloroplast and mitochondrial membranes [3–6].

The effects of three chlorinated carbanilates on the PC T_1 values are given in Fig. 4. These carbanilates differed only in their alkyl chain length. Bilayers that contained CIPC and CHPC showed reductions in the T_1 values of the CH_2OP group of the choline moiety, whereas bilayers that contained CCPC showed an increase in the T_1 value for this carbon. CIPC-containing bilayers showed T_1 values similar to control bilayers for the remaining carbon resonances. The CHPC-containing bilayers also showed T_1 values similar to control bilayers although the average T_1 value for the middle of the hydrocarbon chain (i.e. excluding the last three carbons) was 10% higher than in the control bilayers, suggesting some interaction between the alkyl chain of CHPC and the PC hydrocarbon chain. CCPC-containing bilayers were observed to have significantly higher T_1 values (average around 30%) for each nucleus of the PC hydrocarbon chain relative to control preparations.

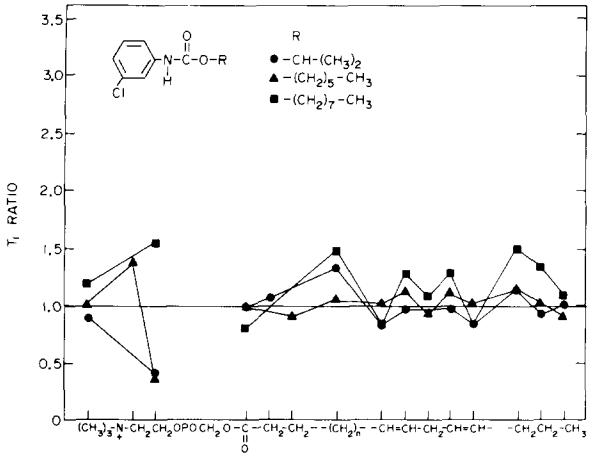


Fig. 4. The effects of ester chain length in 3-chlorocarbanilates on phosphatidylcholine T_1 values. ^{13}C-NMR spectroscopy of egg phosphatidylcholine liposomes prepared in the presence and absence of 0.17 mole fraction of the appropriate phenylamide was performed, and the spin-lattice relaxation times (T_1) of the phosphatidylcholine carbon atoms were determined using the fast inversion recovery pulse sequence as described by Canet et al. [8]. The T_1 ratio was calculated as the ratio of a given carbon atom's T_1 in the presence and absence of added phenylamide. Other details are as described in Materials and Methods.

TABLE III

EFFECT OF PHENYLAMIDE ALKYL CHAIN BRANCHING ON EGG PHOSPHATIDYLCHOLINE T_1 VALUES AT 36°C

Conditions were as in Fig. 4. General formula: 3,4-Cl_2-Ø-N(H)C(O)-R.

PC carbon	T_1 ratio	
	R = $-(CH_2)_3CH_3$	$-\underset{\displaystyle CH_3}{\underset{\displaystyle \vert}{CH}}(CH_2)_2CH_3$
$-N^+(CH_3)_3$	0.95	0.63
$-CH_2OP$	0.25	0.40
C-1	0.86	0.73
C-2	0.58	0.74
$-(CH_2)_n-$	1.02	1.23
$-\underline{C}H_2-CH=$	0.56	1.13
C-9 linoleic; C-10 oleic	0.70	0.95
C-10, C-12 linoleic	1.20	1.39
C-11 linoleic	1.12	1.46
$-\underline{C}H_2CH_2CH_3$	0.88	1.16
$-\underline{C}H_2CH_3$	1.09	1.09
$-CH_3$	0.94	0.92

Thus, the effect of lengthening the alkyl chain of the phenylamide was to increase the apparent depth of interaction of the phenylamide within the bilayer resulting in an increase in relative mobility of the PC hydrocarbon chains. The increased mobility of the hydrocarbon chains also followed the general order of effectiveness of these three compounds toward activities associated with biological membranes: CCPC $\geqslant$ CHPC > CIPC.

The effects on the PC T_1 values of two acylanilides with different alkyl chain structure are given in Table III. DCPA has a straight alkyl chain equal in length to the karsil alkyl chain (Table I), but the karsil alkyl chain contains a branched methyl group. Both anilides lowered the T_1 values of the choline CH_2OP and the first two carbons of the PC hydrocarbon chain. However, the two compounds differed in their effects on the remaining T_1 values; the DCPA-containing bilayers had T_1 values similar to control bilayers on average while the karsil-containing liposomes consistently showed higher T_1 values (average about 25%) for the PC hydrocarbon atoms down to the penultimate methylene. This comparison provides further evidence for the positioning of the phenylamide alkyl chain in the region of the PC hydrocarbon chains, and suggests that incorporation of a phenylamide containing a branched alkyl side chain results in a larger disruption of lipid hydrocarbon chain packing than does incorporation of a phenylamide containing a linear alkyl side chain. The marked effect of differing alkyl substituents (Fig. 4, Table III) on the PC hydrocarbon chain T_1 values, coupled with the generally observed decrease in T_1 values of the PC headgroup carbon atoms, allows some speculation on the orientation of the phenylamides within the bilayer. These results are consistent with the phenylamides being situated in the bilayer such that the phenyl moiety is located in the vicinity of the PC headgroup with the alkyl side chain of the phenylamide extending into the PC hydrocarbon region. The decreased T_1 values of the PC headgroup carbons indicate a more rigid lattice environment in the headgroup region when the herbicides are incorporated into the bilayer.

A compilation of PC T_1 values for bilayers containing three 3,4-dichlorophenylamides which

TABLE IV

EFFECT OF ALKYL GROUP CLASS OF PHENYLAMIDE ON EGG PHOSPHATIDYLCHOLINE T_1 VALUES AT 36°C

Conditions were as in Fig. 4. General formula: 3,4-Cl_2-Ø-N(H)C(O)-R.

PC Carbon	T_1 ratio		
	R = $-CH_2CH_3$	$-OCH(CH_3)_2$	$-N(CH_3)_2$
$-N^+(CH_3)_3$	1.19	0.89	1.29
$-CH_2OP$	0.95	1.00	0.46
C-1	1.02	0.80	0.70
C-2	0.77	1.19	3.55
$-(CH_2)_n-$	1.30	0.95	1.59
$-\underline{C}H_2-CH=$	0.87	0.81	1.35
C-9 linoleic; C-10 oleic	0.81	0.67	1.02
C-10, C-12 linoleic	0.89	1.37	1.21
C-11 linoleic	1.03	0.82	1.26
$-\underline{C}H_2CH_2CH_3$	1.02	1.11	1.34
$-\underline{C}H_2CH_3$	1.15	0.79	1.02
$-CH_3$	1.04	0.98	0.99

differ in the nature of the substituent bonded to the carbonyl atom is given in Table IV. Propanil has an alkyl $-CH_2CH_3$ group, DCIPC has an ester $-OCH(CH_3)_2$ group, and DCMU has an amide $-N(CH_3)_2$ group (Table I). The acylanilide propanil had minimal effect on any of the measured T_1 values. The T_1 values for DCIPC-containing bilayers were also similar to T_1 values of control bilayers. In contrast, the phenylurea, DCMU, had a marked effect on all of the T_1 values except for the terminal two carbons of the hydrocarbon chain. In general, the DCMU-containing bilayers showed T_1 values for the hydrocarbon chain atoms (excluding the C-1 and C-2 carbons and the terminal $-CH_2CH_3$ carbons) that were 30% greater than T_1 values in control bilayers. DCMU effects towards the middle of the bilayer were not expected due to the lack of an extended alkyl substituent. However, if DCMU caused a large disruption of lipid hydrocarbon chain packing around C-2, as indicated by the large increase in the T_1 value of C-2, it is possible that atoms deeper in the bilayer would experience greater motional freedom and, hence, increased T_1 values.

The results in Table IV can be put in the context with the effects of phenylamides on biological membranes. Previous studies on the interactions of phenylamides with chloroplast and mitochondrial membranes have shown that the phenylamide structure can be correlated with the effects of the phenylamide on membrane cation permeability, capacity for ATP synthesis, and 'fluidity' [2,3]. 3,4-DCIPC was observed to be active at concentrations of 10^{-5} M, whereas propanil required concentrations of 10^{-4} M, and DCMU was not active at concentrations up to 400 μM [3]. The nature of the substituent adjacent to the phenylamide carbonyl was judged to be important in determining membrane activity, because DCIPC, propanil, and DCMU were not equivalent as membrane-active compounds even though the phenyl ring substituent is the same for the three compounds.

The T_1 values of PC bilayers containing isopropylcarbanilates that differed in the chlorine substitution pattern on the phenyl ring were also measured (data not shown). Because the alkyl group adjacent to the carbonyl moiety (-OCH-$(CH_3)_2$) remained constant, any differences in PC bilayer T_1 values should reflect effects associated with substituents on the phenyl ring. In general, no systematic effect of chlorination pattern on the T_1 values was observed. It is clear from previous studies that the chlorine substitution pattern of the phenylamide is important in determining membrane activity, because 3,4-DCIPC has much greater activity than CIPC and 2,3-DCIPC was inactive [3] even though the isopropyl side chain is the same in all three compounds.

In the present study, it was observed that 3,4-DCIPC, CIPC, IPC, 2,3-DCIPC, and propanil did not significantly affect the T_1 values of the PC hydrocarbon atoms, whereas DCMU had a large effect on the PC hydrocarbon T_1 values. This result is somewhat surprising because DCMU shows the least activity of all these compounds with respect to its effects on ion permeability in chloroplast, mitochondrial, and liposome membranes [3]. This would indicate that the effects of phenylamides on hydrocarbon chain mobility as measured by PC ^{13}C relaxation times can serve as useful indicators of how these compounds interact with PC bilayers, but provide little insight into how they specifically alter membrane properties such as ion permeability. Possibly, phenylamide interactions in the polar region of the membrane

are more important than interactions with the hydrocarbon chain in determining phenylamide effects on ion-linked membrane activities.

Conclusions

In this report, the effects of phenylamide herbicides on the physical properties of phosphatidylcholine bilayers have been measured. From the X-ray diffraction patterns and the ^{31}P-NMR spectra of phenylamide/phosphatidylcholine preparations, it was concluded that the phenylamides partitioned into the hydrocarbon region of the bilayer causing disruption of lipid hydrocarbon chain packing below the lipid phase transition temperature. However, the phenylamides did not alter the basic bilayer organization of the phosphatidylcholine. The ^{13}C-NMR data support and extend the conclusions drawn from X-ray diffraction and ^{31}P-NMR regarding the positioning of the phenylamides in the bilayer. Phenylamide alkyl chain substituents were mainly responsible for observed effects on the mobility of the lipid hydrocarbon chains, while the phenylamide ring produced effects in the polar region of the lipid bilayer. Taken together, the data in this paper provide information regarding herbicide: lipid interactions within the membrane, but the results do not suggest an explanation for the differential effects of membrane-active and -inactive phenylamides.

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