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Amiodarone–liposome interaction: a multinuclear NMR and X-ray diffraction study

Gordon L. Jendrasiak [1], Thomas J. McIntosh [2], Anthony Ribeiro [3] and R. Stephen Porter [4]

[1] *Department of Radiation Oncology, East Carolina University, Greenville, NC,* [2] *Department of Cell Biology, Duke University Medical Center, Durham, NC,* [3] *Nuclear Magnetic Resonance Center, Department of Radiology, Duke University Medical Center, Durham, NC, and* [4] *Likoff Cardiovascular Institute, Hahnemann University, Philadelphia, PA (U.S.A.)*

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Amiodarone, a potent antiarrhythmic drug, is widely used in cardiology. Its electrophysiological effects, as well as many of its side effects, seem to involve lipids. We report here a multinuclear NMR and X-ray diffraction study of amiodarone in egg phosphatidylcholine liposomes and lipid multilayers. In proton NMR experiments, amiodarone alters the signal from the lipid trimethyl ammonium group for pH values ranging from 3.2 to 8.4; cholesterol does not cause this alteration. The addition of SCN^- changes both the proton and phosphorus NMR spectra of liposomes containing amiodarone. For both proton and carbon NMR, amiodarone modifies the signal from the lipid methylene groups, but to a far lesser extent than does cholesterol. Incorporation of amiodarone in EPC bilayers also modifies the low-angle X-ray diffraction patterns, decreasing the lamellar repeat period at low water contents, but swelling the fluid spaces between bilayers at high water contents. Electron density profiles and modeling studies using the X-ray data indicate that amiodarone decreases the bilayer thickness and adds electron density at the interfacial region of the bilayer. Our analysis of the NMR and X-ray data indicates that the iodine atoms of amiodarone are located near the hydrocarbon / water interface and that the tertiary amine of amiodarone is in the headgroup region of the bilayer.

Introduction

Amiodarone is a widely used and very effective cardiac antiarrhythmic drug [1,2]. Unfortunately, it has a number of side effects while its mechanism of antiarrhythmic action is still largely unknown. Because of its chemical structure amiodarone might be expected to interact strongly with membrane lipids; the solubility of amiodarone in water is only about 0.72 mg/ml and its partition coefficient with respect to liposomes is in the order of 10^6. Recently, a number of reports have appeared on the physical-chemical properties of this drug [3,4] and these properties may well have relevance to the cardiac action of the drug. In particular, it has been suggested by some that amiodarone is 'buried deep' in the hydrocarbon core of liposomes, much like cholesterol [5] and, therefore, the drug–lipid interactions are mainly hydrophobic. Some X-ray diffraction [6] and fluorescent probe studies [7] seem to agree with this interpretation.

In this work, we report the results of multinuclear magnetic resonance and X-ray diffraction studies on unilamellar liposomes and lipid multilayers containing amiodarone or its derivatives and compare these results with liposomes containing cholesterol. These liposomes, prior to amiodarone incorporation, can be neutral, negatively or positively charged [8]. We also address the problem of pH of the liposomal dispersions. Even though our amiodarone concentration is relatively high, nevertheless, our results can provide information about the interaction of amiodarone with phospholipids present in cardiac cell membranes, and should have some applicability to both the cardiac and side effect mechanisms of amiodarone. A preliminary report of this work has appeared [9].

Methods

All phospholipids were obtained from Avanti Polar Lipids, Inc., Birmingham, AL, and were used without further purification. The lipids used were egg phosphatidylcholine (EPC), egg phosphatidic acid (PA), stearylamine (SA), and cholesterol (CHOL). Lipid pur-

Correspondence: G.L. Jendrasiak, Department of Radiation Oncology, East Carolina University, Greenville, NC 27858-4354, U.S.A.

ity was checked by chromatography. Tests indicate that trace impurities have no effect on our results. Potassium thiocyanate (ACS certified) was obtained from Fisher Scientific Co. and was the highest purity available. D_2O (> 99% pure) was used for all of our multinuclear NMR experiments. Amiodarone and desethylamiodarone, in their HCl forms, were generously provided by Wyeth Laboratories, Inc. while desoxyamiodarone was a gift from Dr. Thomas Kennedy, Duke University Medical School.

The lipids and drug were mixed in the appropriate molar ratios in chloroform and thoroughly dried by a steam of dry nitrogen and then vacuum dried for several hours. The lipids were then dispersed in D_2O and sonicated in a sonicator bath, Laboratory Supplies, Inc., Hicksville, NY. The sonication was done in a bath of cool water (20°C) and generally only a few minutes sonication time was necessary to achieve optical clarity, i.e., an optical density of 0.4 at 546 nm. During this time period of sonication the sample temperature rose only a few centigrade degrees. During the sonication procedure, the sample was sealed under nitrogen. The concentration of egg phosphatidylcholine (PC) used was 0.029 M and the PC/phosphatidic acid (PA) molar ratio was 7:1. The PC/drug molar ratio was 3:2. Thin-layer chromatography was performed before and after sonication and indicated no increase in lipid degradation products.

Size measurement of the liposomes were performed with a Nicomp Model 370 Submicron Particle Size Analyzer. The measurements indicated that the majority of particles were 1000 Å or less in diameter. Freeze-fracture electron microscopy verified these size measurements.

^{1}H- and ^{31}P-NMR data were obtained on 0.5-ml liposome preparations in D_2O in 5 mm NMR tubes. The ^{1}H-NMR spectra were recorded on a General Electric GN-500 spectrometer, at the Duke NMR Center, operating at 500.12 MHz. A large number of ^{1}H-NMR spectra were also obtained on a 400 MHz instrument, as well as a 200 MHz unit. The ^{1}H 90° pulse for liposome preparations in the 5 mm ^{1}H probe was 10.5 μs. The ^{1}H-NMR spectra were recorded using a spectral window of $\pm$1501 Hz with the transmitter frequency adjusted to focus on the 0–6 ppm region of the ^{1}H-NMR scale. These NMR spectra were digitized into 32 768 computer points to yield a digital resolution of 0.183 Hz/point. Standard one-pulse ^{1}H-NMR experiments used an 8 μs pulse (68° tip angle), an acquisition period of 5.46 s and a delay of 1 s. ^{1}H T_1 measurements were carried out with the 180°-τ-90° inversion recovery sequence which features a composite 180° pulse to compensate for field homogeneity imperfections and off-resonance effects. The T_1 values were measured using multiple (at least twelve) delay values and a recovery time of 6 s. The longest T_1 value measured for the liposome signal was 0.85 s (CH_3 resonance of phospholipid fatty acyl chain at 0.91 ppm). The above conditions therefore correspond to fully relaxed ^{1}H-NMR signals for the liposomal systems.

^{31}P-NMR spectra were obtained with a 10 mm broad-band NMR probe operating at 202.45 MHz. The ^{31}P-NMR shift scale was calibrated with a 85% H_3PO_4 sample set to 0.00 ppm. The phospholipid ^{31}P-NMR spectra were recorded using a spectral window of $\pm$2232 Hz with the transmitter frequency adjusted to focus on the +11 to −11 ppm region of the ^{31}P-NMR scale. These NMR spectra were digitized into 32 768 computer points to yield a digital resolution of 0.272 Hz/pt. Standard one-pulse ^{31}P experiments used a 15 μs pulse, an acquisition period of 3.68 s and a delay of 1 s.

^{13}C-NMR spectra were obtained on the Bruker WH-400 at the South Carolina NSF NMR facility using a multinuclear broadband probe operating at 100.61 MHz. These spectra were acquired using a total spectral window of 20 012 Hz with the transmitter frequency adjusted to detect the 1.82 ppm to 200.7 ppm region of the ^{13}C-NMR scale. These NMR spectra were digitized into 32 768 points yielding an effective digital resolution of 1.22 Hz/pt. The one-pulse ^{13}C experiments used a 16.5 μs pulse, an acquisition period of 0.819 s and a delay of 0.1 s.

X-ray diffraction patterns were recorded from both oriented lipid multilayers and unoriented lipid dispersions. The oriented multilayers were formed by placing a small drop of chloroform solution of the appropriate lipid or lipid/amiodarone mixture on a flat strip of aluminum foil and evaporating the chloroform under a gentle stream of nitrogen. The aluminum foil substrate was given a convex curvature by bending it around a Pasteur pipet, as described previously [10,11]. The specimen was then mounted in a controlled humidity chamber on a single-mirror line-focussed X-ray camera, so that the X-ray beam was oriented at a grazing angle relative to the lipid multilayers. Relative humidity was controlled in the chamber by means of a cup of a saturated salt solution [12]. To speed equilibration, a gentle stream of nitrogen gas was passed through a flask of the saturated salt solution and then through the chamber [10,11]. Unoriented dispersions were formed by the following procedure. The appropriate lipid or lipid/amiodarone mixtures were rotary evaporated from chloroform and a measured volume of doubly distilled water was added. The suspensions were incubated under nitrogen for several hours with periodic vortexing, sealed in quartz glass X-ray capillary tubes, and mounted in a point collimation X-ray camera.

As described previously [10,11], for both oriented multilayers and unoriented dispersions X-ray patterns were recorded on stacks of three or four sheets of Kodak DEF 5 X-ray film. Films were processed by standard techniques and densitometered with a Joyce-

Loebl Model MKIIIC microdensitometer. For the unoriented suspensions the densitometer trace was taken in a radial direction from the center of film, whereas for the oriented multilayers the trace was taken through the center of each reflection. After background subtraction, integrated intensities, $I(h)$, were obtained for each order h by measuring the area under each diffraction peak. For unoriented patterns the structure amplitude $F(h)$ was set equal to $[h^2I(h)]^{1/2}$ [13]. For the oriented patterns, taken with a line-focussed camera, there was no detectable arcing of the reflections, which were of uniform height. In this case, the intensities were corrected by a single factor of h due to the cylindrical curvature of the multilayers so that $F(h) = [hI(h)]^{1/2}$ [13,14]. One-dimensional electron density profiles, $\rho(x)$ were calculated by

$$\rho(x) = (2/d)\sum \exp[i\phi(h)] \cdot F(h) \cos(2\pi hx/d) \quad (1)$$

where x is the distance from the center of the bilayer, d is the lamellar repeat period, and (h) is the phase angle, either 0 or for each order h in these centrosymmetric lipid multilayers. All profiles were calculated at the same resolution, $d/2h_{max} \cong 6$ Å. Published phase angles were used for EPC multilayers [15,16], whereas for multilayers containing amiodarone phase angles were obtained from a sampling theorem analysis, according to the procedures of McIntosh et al. [17]. For each lipid system, structure amplitudes were normalized by the method of Blaurock [18].

Continuous transforms were calculated from the structure amplitudes and each possible phase combination for each data set by use of the sampling theorem [19] as described previously [16]. For transforms of EPC and EPC/amiodarone bilayers, the values of the structure factors at the origin of reciprocal space were calculated by the formalism of King and Worthington [20]. The phase combination was selected which gave the best agreement among the possible transforms.

Results

Fig. 1 shows 500 MHz proton spectra for PC-PA and PC-PA-CHOL liposomes. The molar ratio of PC/PA is

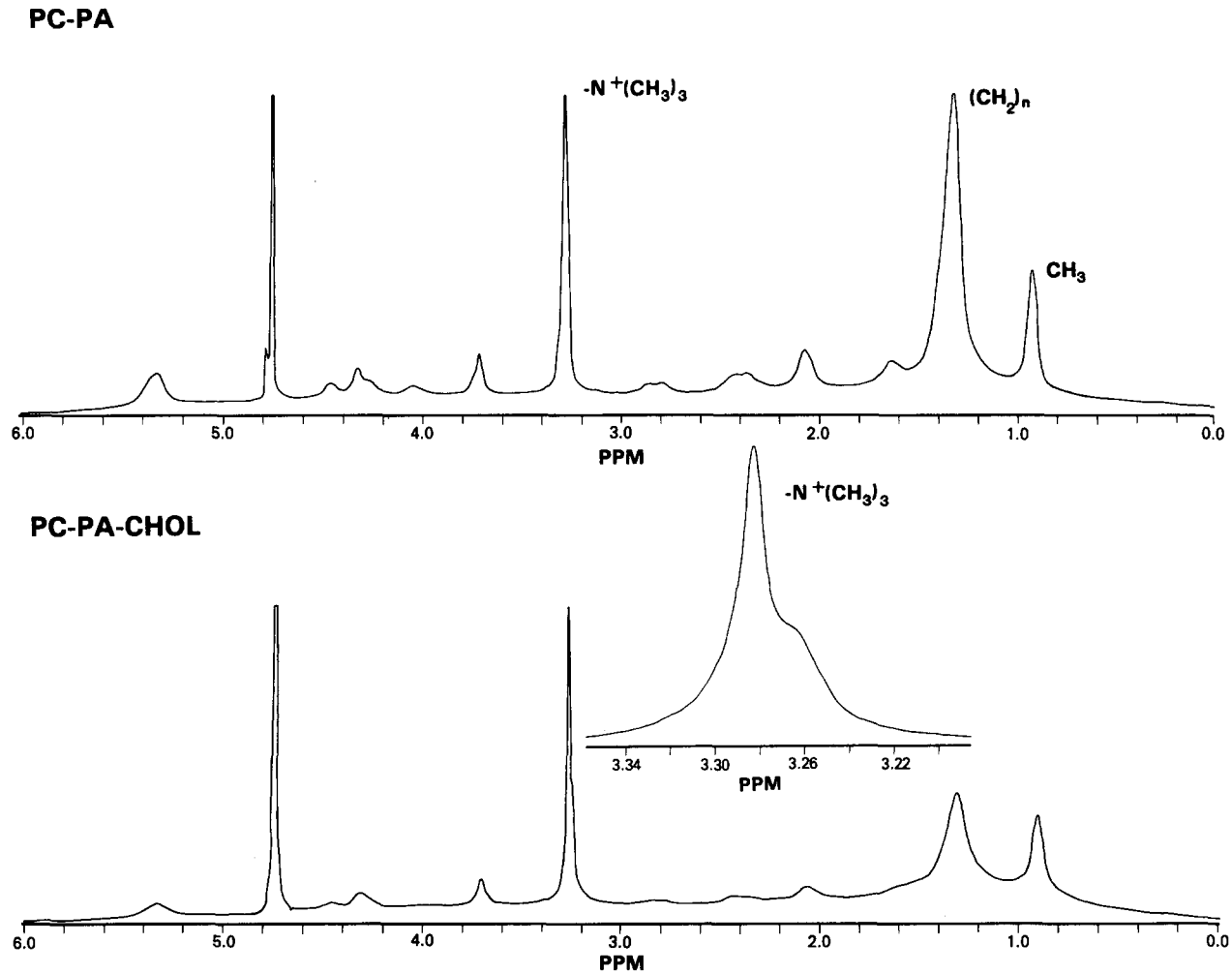

Fig. 1. 500 MHz proton NMR spectra of PC-PA (top) and PC-PA-CHOL (bottom) liposomes. $T = 29\,^{\circ}$C. See text for liposome preparation. Note insert of N^+ $(CH_3)_3$ signal on expanded scale for PC-PA-CHOL.

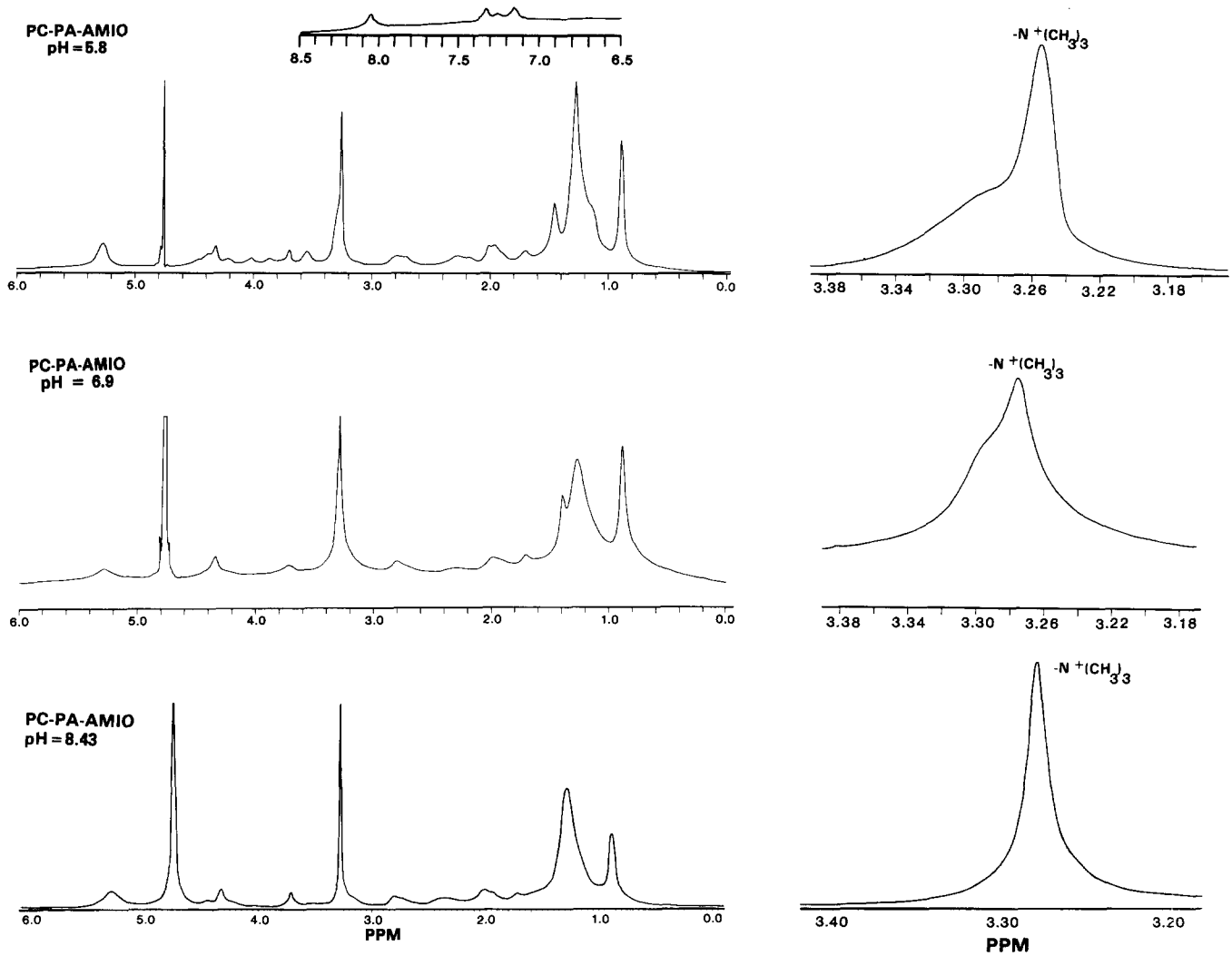

Fig. 2. 500 MHz proton NMR spectra of PC-PA-AMIO at pH = 5.8, pH = 6.9 and pH = 8.4. The PC/PA molar ratio is 7:1 and the PC/AMIO molar ratio is 3:2. At the right are shown the expanded scale spectra for the $N^+(CH_3)_3$ group signals at each pH. The inset shows the signal from the AMIO aromatic ring protons.

7:1 and that for PC/CHOL is 3:2. The two spectra are essentially unchanged over the pH range of 3 to 8.5. Note that cholesterol decreases the height of the $(CH_2)_n$ signal at 1.3 ppm as has been shown by others [21]. This is due to hydrophobic interactions between cholesterol and the phospholipid hydrocarbon chains [22,23]. The insert shows the $N^+(CH_3)_3$ signal for PC-PA-CHOL on an expanded scale. This headgroup signal splitting is similar to that observed for PC liposomes [24,25] where we have ascribed the downfield narrower peak to $N^+(CH_3)_3$ groups 'exterior' to the liposomes whereas the upfield broader peak is from $N^+(CH_3)_3$ groups interior to the liposomes [24].

Fig. 2 shows PC-PA-AMIO proton spectra at pH values of 5.8, 6.9 and 8.4, respectively. The PC/PA molar ratio is again 7:1 and the PC/AMIO molar ratio is 3:2, the same as the PC/CHOL molar ratio. The PC-PA-AMIO proton spectrum is essentially unchanged at pH values as low as 3.2. The spectrum at pH 5.8 shows several differences from the cholesterol case (Fig. 1): First, the $(CH_2)_n$ peak height (1.3 ppm), relative to the $N^+(CH_3)_3$ peak height, is significantly greater than was the case for cholesterol, suggesting that amiodarone does not order the hydrocarbon interior of the bilayer to the extent that cholesterol does. Second, there is an additional peak near 1.5 ppm, which we have tentatively assigned to the CH_3 moieties of the ethyl groups attached to the amiodarone nitrogen atom. We have made this assignment by studying the NMR patterns of amiodarone and its derivatives in deuterated chloroform, Fig. 3. As can be seen, the terminal methyl groups on the ethyl moieties of amoidarone are located at this chemical shift position; comparison with desethylamiodarone spectra indicate that the 1.5 ppm peak for PC-PA-AMIO can be assigned to these groups. Also note the shoulder, on the $(CH_2)_n$ peak, at about 1.2 ppm. We have not yet identified this shoulder although it presumably arises from amiodarone. The inset

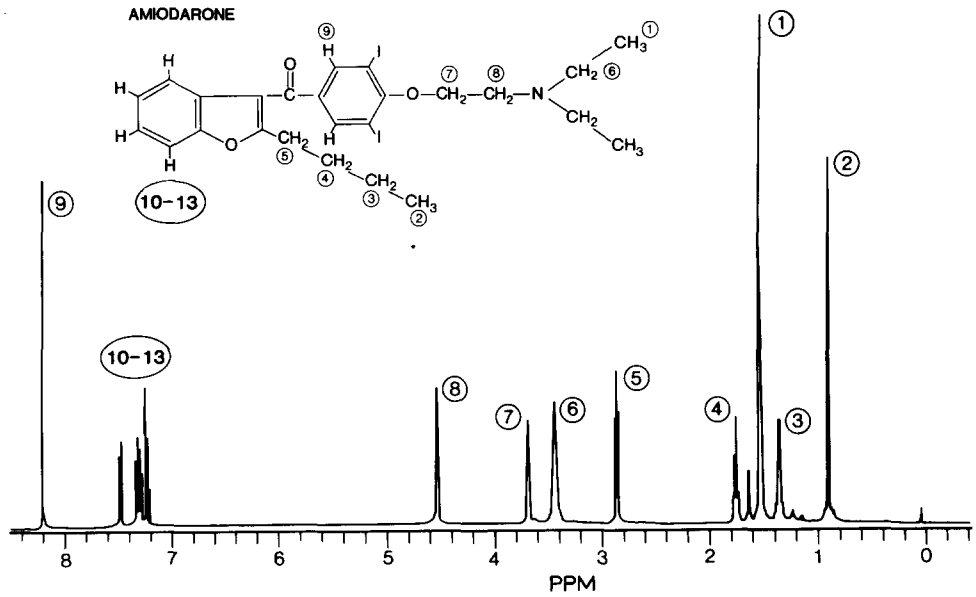

Fig. 3. Proton spectrum of amiodarone in $CDCl_3$ at the same molar concentration as was used for the liposomes shown in Fig. 2.

shows the signals between 7 and 8.5 ppm, from the ring protons of amiodarone.

Although the AMIO in $CDCl_3$ spectrum shows a peak, peak 6, in the neighborhood of the PC headgroup peak, peak area measurements and experiments with desethylamiodarone convince us that the downfield peak of the split headgroup pair in PC-PA-AMIO does not arise from this group, but rather from the PC, $N^+(CH_3)_3$ group.

The spectrum at pH 6.9 is different in two respects from the one taken at pH 5.8: first the $(CH_2)_n$ peak height, relative to the $N^+(CH_3)_3$ peak height is less. Second, the peak at about 1.5 ppm, putatively arising from amiodarone, has shifted upfield. The spectrum at pH 8.4 for the PC-PA-AMIO system is clearly different from the spectra at lower pH values. In particular, although the appearance of the $(CH_2)_n$ peak, relative to the $N^+(CH_3)_3$ peak has not changed significantly from that for the pH = 6.9 case, the peak at about 1.5 ppm is no longer observed. This 1.5 ppm peak may have shifted further upfield so as to be unresolvable from the $(CH_2)_n$ peak. Also the (CH_3) peak height at 0.9 ppm is decreased relative to the $(CH_2)_n$ peak height.

Fig. 2 also shows the expanded $N^+(CH_3)_3$ group proton spectra for PC-PA-AMIO. Note that at pH = 5.8, the headgroup proton peak is split, but in the reverse manner from that obtained with PC-PA-CHOL, i.e., the taller, narrower peak is upfield whereas the shorter, broader peak is downfield. At a pH value of 6.9, this splitting is less pronounced but, nevertheless, clearly present. At a pH value of 8.4, the splitting is not observable and a single peak is seen. The PC-PA-AMIO and PC-PA-CHOL spectra are clearly different over the pH range of 5.8 to 8.4. The results shown at pH = 5.8 are essentially unchanged at pH values as low as 3.2.

Fig. 4 shows the ^{13}C spectra for PC-PA, PC-PA-CHOL and PC-PA-AMIO liposomes. The presence of cholesterol has a rather dramatic effect on the $(CH_2)_n$ signal located between 30 and 35 ppm, which is consistent with that obtained for protons (Fig. 1). Thus, cholesterol which is known to extend deeply into the hydrocarbon chain region of the liposomes [22,23] decreases the $(CH_2)_n$ peak height relative to the $N^+(CH_3)_3$ peak height, both for ^1H and ^{13}C. The ^{13}C spectrum of the PC-PA-AMIO system is at acidic pH, i.e., 5.8. Amiodarone has much less of an effect on the $(CH_2)_n$ signal between 30 and 35 ppm, than does cholesterol. This result is quite consistent with the proton results.

The addition of SCN^- clearly increases the splitting of the proton headgroup signal for PC-PA-CHOL (Fig. 5, inset). This comes about by the shifting of the downfield peak, further downfield, by somewhat more than 0.02 ppm whereas the upfield peak maintains its original chemical shift value. The spectra shown in the figures were obtained by setting the water signal at 4.750, however, since SCN^- shifts the water signal, we have calculated the chemical shift changes relative to the terminal CH_3 group; we have found this procedure gives the same results as using TSP as an internal standard. This splitting is consistent with the downfield peak arising from $N^+(CH_3)_3$ groups exterior to the liposome since we have found that SCN^- penetrates into liposomes but takes many hours to equilibrate, at which time the splitting disappears. This increased splitting by chaotropic anions was studied by us in some detail for PC liposomes [24]. Using computer decon-

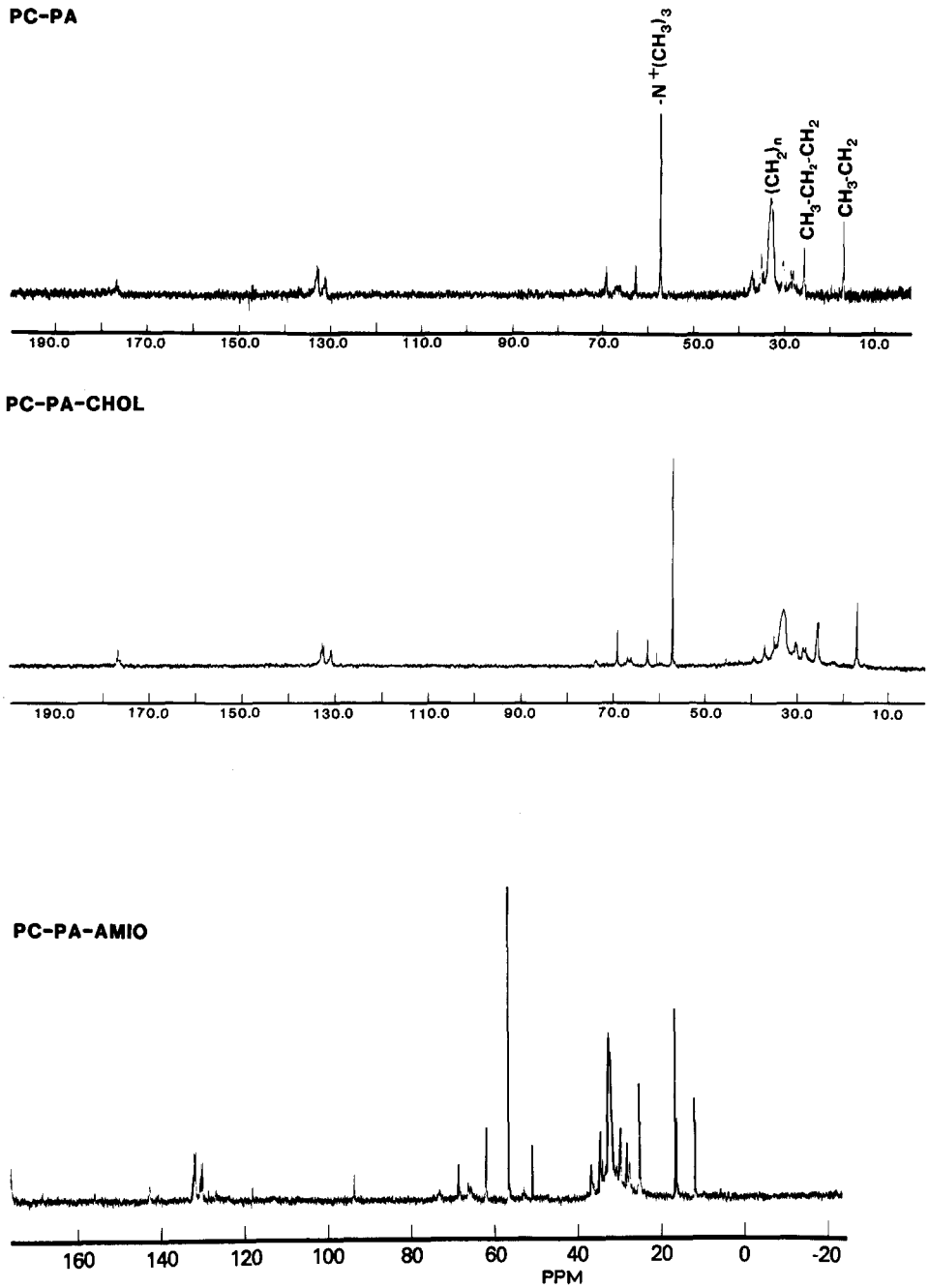

Fig. 4. ^{13}C-NMR spectra of the PC-PA, PC-PA-CHOL and PC-PA-AMIO liposome systems. Liposome preparation same as that for Figs. 1 and 2.

volution techniques, we find that the ratio of the areas of the two headgroup peaks is very close to one; for unilamellar vesicles, this would mean that approximately equal numbers of headgroups exist on both the outside and on the inside of the liposome.

When Mn^{2+} is added to the PC-PA-CHOL system, before or after SCN^- addition, only the downfield peak is significantly broadened, implying that this peak arises from headgroups in the outer monolayer of the liposomes. SCN^- has little, if any, effect on the NMR signals from the hydrocarbon chain protons. This same behavior was found for PC liposomes [24].

Fig. 5 shows the PC-PA-AMIO spectra at pH values of 5.8, 6.9, and 8.4, respectively, but after the addition of 0.2 M SCN^- for the two lower pH values and 0.1 M SCN^- at pH = 8.4. At the right are shown the expanded spectra of the $N^+(CH_3)_3$ groups. At pH = 5.8, the SCN^- causes the upfield headgroup peak to move downfield, effectively merging the two peaks. The downfield shift of the upfield headgroup peak is about 0.06 ppm for 0.2 M SCN^- addition; this is a greater shift than was obtained for the cholesterol-containing liposomes and indicates that, in contradistinction to the PC-PA-CHOL case, the upfield peak arises from

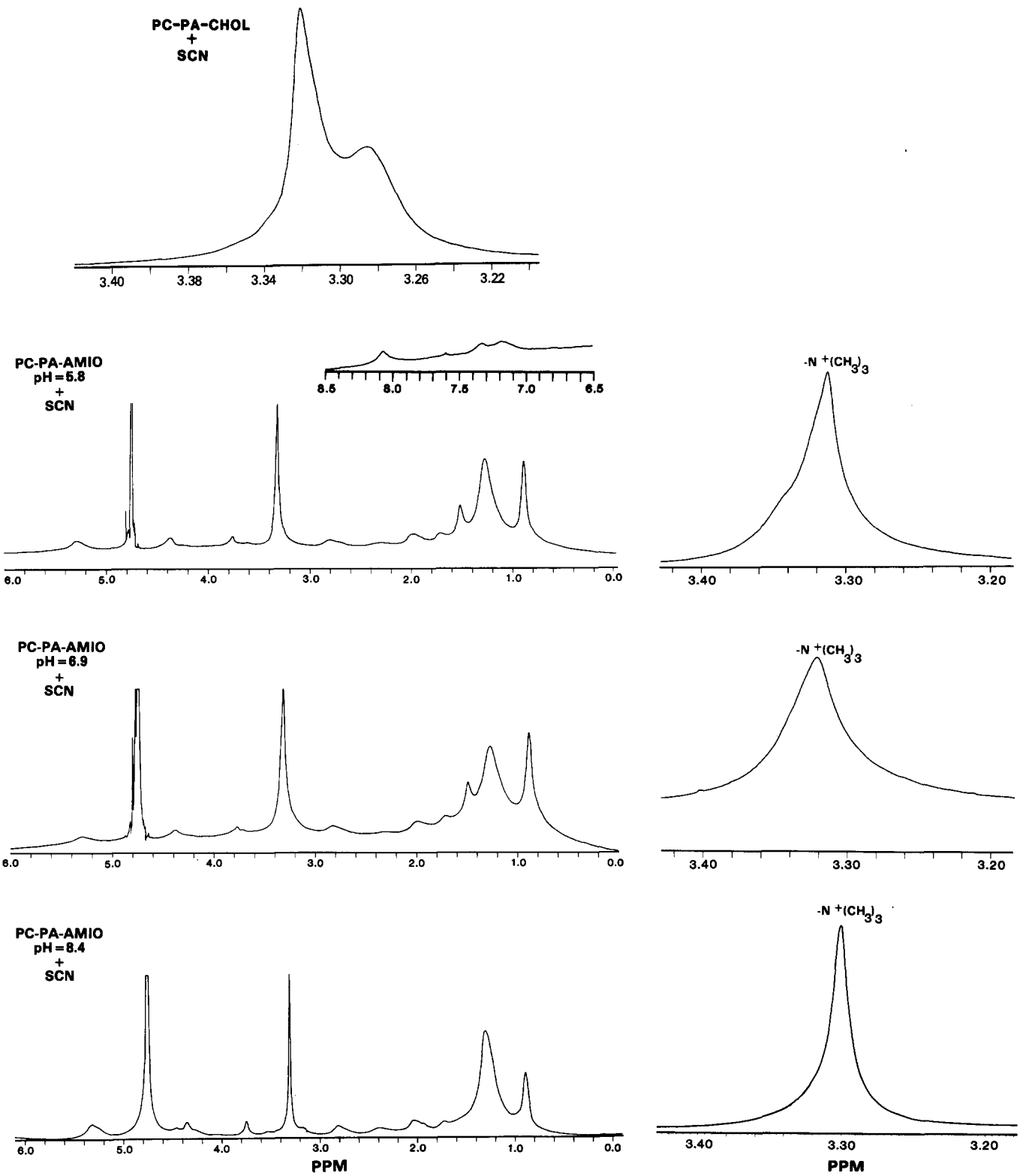

Fig. 5. Effect of the pseudohalide, SCN^-, on the PC-PA-AMIO liposomal systems shown in Fig. 2. Note the insets for the PC-PA-CHOL headgroup and the PC-PA-AMIO, AMIO aromatic ring protons.

$N^+(CH_3)_3$ groups in the outer monolayer of the liposome. The ratio of the area of the upfield peak to the downfield peak, obtained by computer deconvolution, is 1.4, and would suggest that there are a greater number of PC molecules in the outside monolayer of the liposome than interior to the liposome. This ratio was

obtained prior to SCN^- addition, since SCN^- caused the two peaks to merge to a single peak. At a pH value of 6.9, the behavior of the head-group peak upon SCN^- addition is quite similar to the lower pH behavior, although the actual downfield shift is about 0.04 ppm. At pH = 8.4, the entire single headgroup peak shifts downfield about 0.02 ppm, upon SCN^- addition.

SCN^- has a noticeable effect on the peak located at 1.5 ppm, the peak we ascribed to the CH_3 groups of amiodarone. At pH = 5.8, this peak shifts upfield some 0.06 ppm upon SCN^- addition whereas at pH = 6.9 this shift is 0.09 ppm. At pH = 8.4, this peak is not observable and, no anion effect can be measured.

The amiodarone ring proton signals (inset) Fig. 5, located between 7 and 8.5 ppm, are noticeably affected by the SCN^- at acidic pH values. We have not studied the amiodarone ring proton signals for SCN^- addition at pH = 8.4. For the PC-PA-CHOL system, SCN^- does not modify signals from other parts of the lipid suggesting that the SCN^- headgroup interaction does not affect the cholesterol orientation since cholesterol is situated mainly in the hydrocarbon region.

The addition of Mn^{2+} to the PC-PA-AMIO dispersion, at pH = 5.8 in even greater concentration than was used for the PC-PA-CHOL case results in no obvious effects on the $N^+(CH_3)_3$ signal. If the same amount of Mn^{2+} is added after the 0.2 M SCN^- addition, however, the single $N^+(CH_3)_3$ peak is now broadened. This behavior is again clearly different than that obtained with PC-PA-CHOL.

The SCN^- effect on the $(CH_2)_n$ and (CH_3) signal intensities for the PC-PA-AMIO system depends on the pH; the signal intensities decrease significantly upon anion addition at pH 5.8, decrease only slightly if at all at pH 6.9, but are unchanged at pH 8.4. These effects do not occur for the PC-PA-CHOL liposomes. At pH, 5.8, the upfield shoulder on the $(CH_2)_n$ peak is no longer observable after SCN^- addition.

The proton NMR results are not dependent on the magnetic field strength; we have obtained similar results at both 200 MHz and 400 MHz proton resonance frequencies.

We have also obtained proton spectra of two other amiodarone-containing liposome systems: a PC-AMIO system (PC/AMIO molar ratio 3:2) and a PC-SA-AMIO system (PC/SA molar ratio 7:1) at pH 5.8. The spectra are, in their essential features, quite similar to those for the PC-PA-AMIO system. At these pH values, the PA provides a negative charge to the liposome, whereas the SA provides a positive charge; the PC, of course, provides no net charge. We have also used liposomes containing cardiolipin as a provider of negative charge; again, the results are quite similar to those obtained with PA. The charge on the bilayer lipids thus has no major effect on the NMR spectra of the amiodarone-containing liposomes.

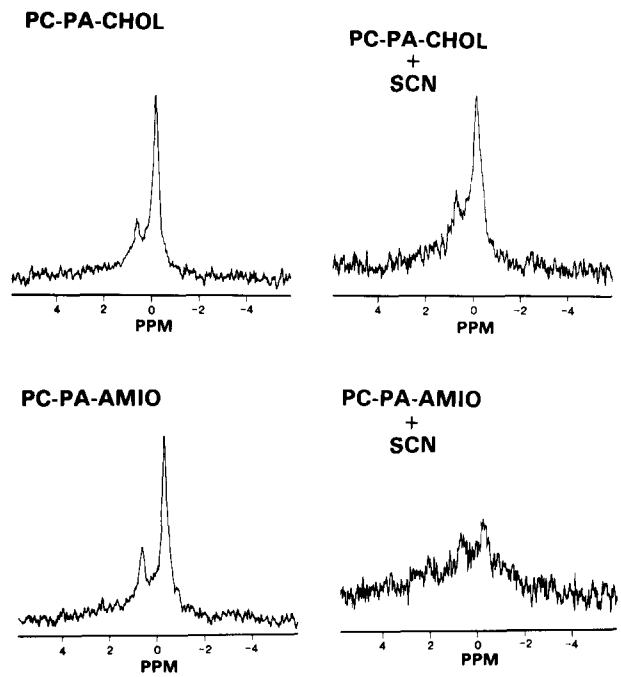

Fig. 6. ^{31}P-NMR spectra for PC-PA-AMIO, pH = 5.8 and PC-PA-CHOL. Two ^{31}P signals are seen corresponding to the 7:1 molar ratio of PC/PA. Note that for the cholesterol case, SCN^- has virtually no effect on the ^{31}P signals whereas for the amiodarone case, the ^{31}P signal is greatly broadened by SCN^-.

In Fig. 6, we show ^{31}P spectra at the acidic pH values, for both PC-PA-AMIO and PC-PA-CHOL. The spectra of these two systems are quite similar; upon the addition of 0.2 M SCN^-, however, the ^{31}P signal for the cholesterol containing liposomes is affected very little whereas that for the amiodarone-containing liposomes almost totally disappears into the baseline. The initial interaction of the anion is with the exterior of the liposome, and the observed spectral differences should represent responses to the anion interactions with the polar headgroups of the lipids and/or with the amiodarone in the outer monolayer of the liposome.

We have also measured the T_1 values for the headgroup proton signals as shown in Table I. Note that the upfield peak for amiodarone liposomes corresponds to outer headgroups while the reverse situation holds for cholesterol liposomes. In both systems, the outer-headgroup T_1 values are larger than those for the inner headgroups. Such larger T_1 values would be consistent with a more mobile, less tightly packed arrange-

TABLE I

T_1 values for the headgroup protons

	T_1	
	Upfield peak	Downfield peak
PC-PA-CHOL	0.470 ± 0.013 s	0.560 ± 0.005 s
PC-PA-AMIO	0.620 ± 0.013 s	0.540 ± 0.005 s

ment of headgroups. It can be seen that the T_1 values for the headgroups are very close for the AMIO and CHOL liposome systems. The T_1 measurements have been repeated for several samples and have an uncertainty of no more than 10%. The standard deviation shown refers to the deviation of the experimental points used in calculating T_1 from the inversion recovery fitting function.

Spectra (data not shown) for liposomes containing desethylamiodarone (DESAM) and desoxyamiodarone (DESOAM), a 'less toxic' form of the drug, are similar to those obtained with amiodarone. This might be expected since the two drugs differ only in the loss of one ethyl group from the nitrogen atom in the case of DESAM and the removal of an oxygen atom for the case of DESOAM. More detailed NMR studies on these systems are now in progress.

For unoriented 3:2 EPC/amiodarone dispersions the X-ray diffraction patterns consist of a broad wide-angle band centered at about 4.5 Å, and a low-angle pattern which depends on the water content. For 40% water, the pattern contains four orders of a 65 Å repeat period, whereas for 50% water the low-angle pattern contains a single broad band centered at about (34 Å)$^{-1}$.

For oriented multilayers, the X-ray patterns contain four or five reflections which index as orders of a lamellar repeat period. For 3:2 EPC/amiodarone multibilayers, the repeat period decreases monotonically with decreasing relative humidity, ranging from $d = 51.3$ Å at 98% relative humidity to $d = 44.2$ Å at 32% relative humidity. In comparison, for EPC bilayers under similar conditions the repeat period varies from $d = 53$ Å to $d = 49$ Å [26].

Structure factors for multilayers of EPC and 3:2 EPC amiodarone are shown in Fig. 7. The data for EPC are taken from Ref. 16. Note that the incorporation of amiodarone affects the magnitude of the structure factors. Similar results are obtained for 3:2 EPC/amiodarone bilayers containing either stearoylamine or phosphatidic acid at a mole ratio of 7:1 EPC/SA or EPC/PA. Electron density profiles for bilayers of EPC and 3:2 EPC/amiodarone at 93% relative humidity are shown in Fig. 8. The EPC profiles were calculated using data from McIntosh et al. [26]. In each profile the geometric center of the bilayer is at the origin. The high density peaks, centered at ±19 Å for EPC and at ±16 Å for 3:2 EPC/amiodarone, correspond to the lipid headgroups and the low density regions between the headgroup peaks correspond to the lipid hydrocarbon chains. The low density regions at both sides of each profile correspond to the fluid spaces between adjacent bilayers, which at 93% relative humidity are quite narrow [26]. Although the two profiles are similar in shape, there are some distinct differences between them. First, the distance between headgroups peaks across the bi-

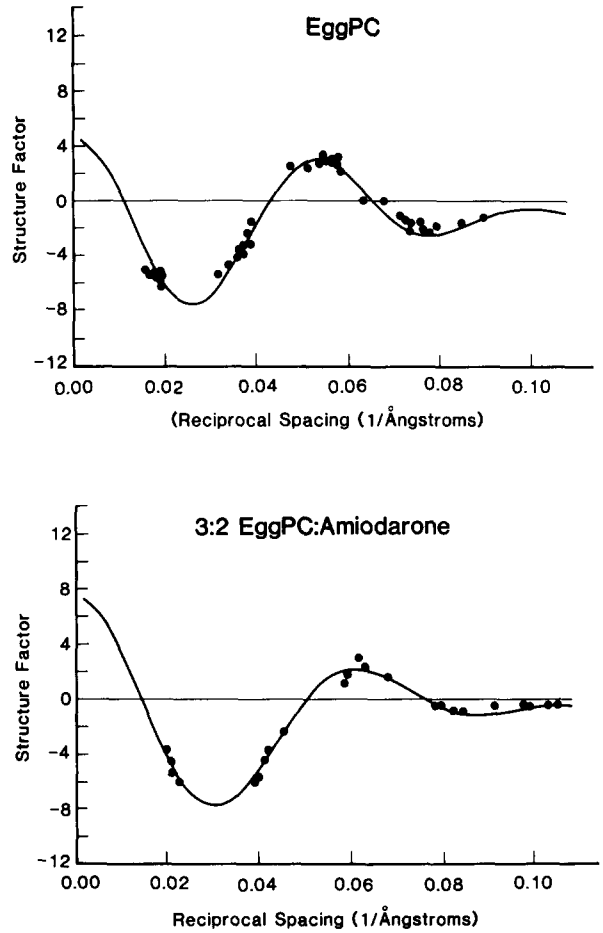

Fig. 7. Structure factors (●) for oriented multilayers of egg PC (top) and egg PC/AMIO, molar ratio 3:2, (bottom) from a series of relative humidity experiments. The solid lines are a continuous transform calculated from electron density strip models. See text for details.

layer is decreased by the incorporation of amiodarone. The distance between head group peaks is 37.8 ± 0.8 Å (mean ± 1 S.D., $N = 10$ experiments) for EPC [16] and 31.3 ± 0.4 Å ($N = 5$) for 3:2 EPC/amiodarone. Second, the shape of the profiles are slightly different. For example, in the case of EPC, the profile (Fig. 8) contains a low density trough centered at the origin, which corresponds to the localization of the lipid terminal methyls in the geometric center of the bilayer. Such a trough is less apparent in the profile of 3:2 EPC/amiodarone (Fig. 8). In addition, the incorporation of amiodarone appears to raise the average density of the bilayer with respect to the density of the fluid spaces between adjacent bilayers.

To more quantitatively interpret the X-ray results, we performed modeling calculations in a manner similar to those done by McIntosh and Holloway [10]. The objectives were to determine the effects the incorporation of amiodarone has on the structure of EPC bilayers and to attempt to localize the amiodarone in the bilayer profile. In these calculations, uniform electron density strip

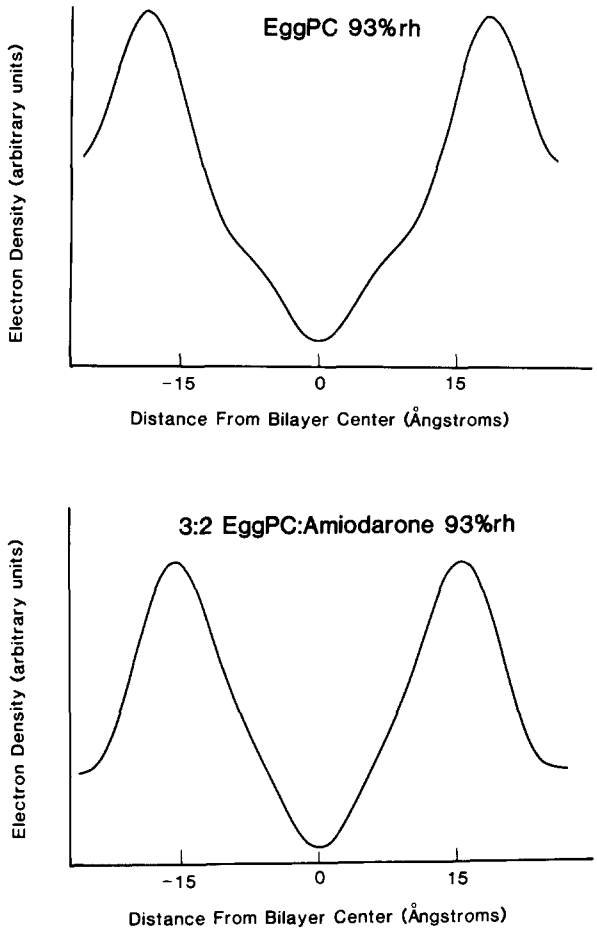

Fig. 8. Electron density profile of egg PC (top) and egg PC/AMIO molar ratio 3:2 (bottom), at 93% relative humidity.

functions [27] were used to model the lipid bilayer, and two Gaussian distributions per bilayer were used to represent the high electron density iodines in each amiodarone molecule. That is, it was assumed that the pair of iodines in each amiodarone molecule could be approximated by a Gaussian of height A, width w, and distances $+s$ and $-s$ from the bilayer center. Franks et al. [28] have used a similar procedure to localize brominated lipids in bilayers. The electron density of the iodine atoms in the bilayer is therefore approximated by

$$\rho_I(x) = A\exp\{-[(x+s)/w]^2\} + A\exp\{-[(x-s)/w]^2\} \quad (2)$$

whose Fourier transform is

$$F_I(X) = 2A(\pi w)^{1/2}\exp[-(X\pi w)^2]\cos(2\pi Xs) \quad (3)$$

The value of A was chosen so that the experimental and model transforms matched at the origin of reciprocal space. The initial procedure was to determine if the parameters w and s could be chosen so that when $F_I(X)$ was added to the continuous transform of EPC it would produce a transform which fit the 3:2 EPC/amiodarone structure factor data (Fig. 7). The model for the EPC data was the same as used by McIntosh and Holloway [10] for 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), a lipid which has a similar hydrocarbon chain composition to EPC. This model consists of a terminal methyl region 7 Å wide with an electron density of 0.25 $e/Å^3$, two methylene chain regions 11 Å wide with electron densities of 0.32 $e/Å^3$, and two head group regions 10 Å wide with electron densities of 0.44 $e/Å^3$. The electron density of the fluid space between bilayers was taken as that of water, 0.33 $e/Å^3$, and was subtracted from the electron density of each strip before the Fourier transform of the model was calculated [29]. The Fourier transform of this electron density strip model is shown as the solid line in Fig. 7. We took this transform and then used Eqn. 3 to estimate the effect of adding amiodarone to an unperturbed EPC bilayer. The parameters w and s were systematically varied in increments of 1 Å. We found that no values of the parameters w and s could be chosen which gave a close fit to the 3:2 EPC/amiodarone structure factors shown in Fig. 7. We then varied the bilayer width by changing the width of the methylene chain region of the strip model. When this was done, a satisfactory fit to the 3:2 EPC:amiodarone structure factors could be obtained (solid line Fig. 7 bottom) with a methylene chain width of 8 Å, $w = 6$ Å and $s = 14$ Å. These modeling calculations indicate that the incorporation of 40% mol amiodarone into EPC bilayers decreases the total bilayer thickness by about 4 Å, from 47 Å to 43 Å. The calculations also indicate that the iodine atoms of the amiodarone are located about 14 Å from the bilayer center, or near the water/hydrocarbon interface.

Discussion

Our NMR results show that amiodarone modifies the organization of bilayers in a pH-dependent manner, as discussed below. The NMR and X-ray data both indicate that at least part of the amiodarone molecule is located in the polar headgroup region of the bilayer.

It is evident that the $N^+(CH_3)_3$ NMR pattern for PC-PA-AMIO (Fig. 2) is qualitatively quite different than that for PC-PA-CHOL (Fig. 1) under the same conditions. The splitting of the headgroup signal peak for PC-PA – CHOL liposomes is very much like that observed by us [24] for PC as is the response of this signal to the pseudohalide SCN^-. We have identified the downfield peak as arising from $N^+(CH_3)_3$ groups exterior to the liposomes and, therefore, accessible to ions. For amiodarone, desethylamiodarone and desoxyamiodarone, we have the magnetically 'reverse' situation seen in Fig. 2. This type of 'reverse' splitting was previously found by us [30] for ANS, a molecule which is located near the headgroup region of the liposomes,

and which does not penetrate as deeply into the hydrocarbon region as does cholesterol [31]. For ANS we attributed the upfield position of the outside headgroup signal as being due to the ANS 'ring currents'; it seems reasonable to propose a similar mechanism for amiodarone. This would place the interaction between amiodarone and the lipid in the headgroup region rather than deep in the hydrocarbon interior, as suggested by others [5]. Recently, Erikkson [32] has observed NMR phospholipid headgroup signals, similar to those found for amiodarone, for a variety of amphiphilic drugs in interactions with phospholipids; his results would support our placement of amiodarone in the interfacial region of the liposome. We suggest that the alteration of the polar headgroup splitting in the amiodarone containing liposomes arises from an interaction between the iodine-containing ring of amiodarone and the headgroup.

Further support for this argument comes from an examination of the proton signals arising from the (CH_2) groups in the hydrocarbon chains of the phospholipid. Clearly, at least at neutral and acidic pH values, the amiodarone (Fig. 2) does not appear to interact with the hydrocarbon chains in the same manner as does cholesterol (Fig. 1). If we assume a pK value of 6.56 for amiodarone [33], then of course, the amiodarone would be positively charged at neutral and acidic pH values and might well be expected to interact with the phospholipid headgroups. At the more basic pH values, the amiodarone would be uncharged.

The differences in the proton spectra, as the pH value increases, are quite striking (Fig. 2) and likely arise because of this conversion of amiodarone from a protonated to a neutral species. The PC-PA-CHOL NMR pattern, on the other hand, is essentially unchanged over the entire pH range 3.2 to 8.4. For amiodarone-containing liposomes it can be seen that as the pH value passes through neutrality and becomes basic, the NMR hydrocarbon chain signal is altered. This would be consistent with the amiodarone interacting more in the hydrocarbon region of the lipid bilayers as amiodarone becomes neutral, although even near pH neutrality, the headgroup signal still clearly resembles that obtained at very acidic pH values. It has been found [34] that the headgroup splitting of PC disappears as the liposomes become larger. In the case of our PC-PA-AMIO liposomes, the solution at pH = 5.8 is quite translucent, consistent with a particle size, found by us, of about 0.1 μm or less in diameter, whereas at 6.9, the solution is noticeably more turbid. The increased turbidity would suggest larger particles consistent with the decreased headgroup signal splitting observed at pH = 6.9. At pH = 8.4, our solutions were very milky, suggesting even larger particles, thus, perhaps accounting for the absence of a $N^+(CH_3)_3$ NMR signal splitting. This turbidity is reversible with lowered pH. It may be that even at the higher pH values, the amiodarone still interacts with the phospholipid headgroup although perhaps not as strongly as at the lower pH values. At high pH the amiodarone may extend somewhat deeper into the hydrocarbon region of the liposomes, but probably not as deeply as cholesterol. It has been found [4], using differential scanning calorimetry and multilamellar vesicles formed from dipalmitoylphosphatidylcholine, that amiodarone ionization, as determined by the pH, modified the lipid transition temperature and the enthalpy of melting. The greatest modification of these two parameters was observed at lower pH values (pH = 4.3), i.e. for liposomes containing amiodarone in its ionized form. It is at these acidic pH values that we find the most noticeable interaction of the amiodarone with the phospholipid headgroup.

Another striking difference between liposomes containing amiodarone and liposomes containing cholesterol is their NMR behavior in the presence of pseudohalides such as SCN^-. For liposomes composed of PC, PC-PA or PC-PA-CHOL, the proton headgroup splitting is significantly greater in the presence of this anion [24] because the downfield peak moves further downfield upon the addition of the anion. The downfield peak is broadened by the presence of Mn^{2+} and thus the anion and paramagnetic effects, considered together, strongly argue for the downfield peak being due to exterior $N^+(CH_3)_3$ groups [24]. For the PC-PA-AMIO liposomes, however, the upfield peak moves downfield upon anion addition, suggesting that it arises from the exterior headgroups. Mn^{2+} has little if any effect upon the headgroup signal prior to SCN^- addition, but does broaden the single peak existing after SCN^- addition. This behavior could well arise because of the repulsive nature of the protonated amiodarone as it 'protects' the headgroup against the paramagnetic cation. The SCN^-, however, may neutralize this charge to some extent, thus allowing the Mn^{2+} to affect the phospholipid headgroup. This would be consistent with the amiodarone being located in the headgroup region of the liposome.

The fact that the ^{31}P phospholipid signal from amiodarone-containing liposomes (Fig. 6) is affected by SCN^- is further evidence for the amiodarone being located near the phospholipid headgroups. If amiodarone were situated deep in the hydrocarbon interior of the lipid bilayer, it would be difficult to explain the dramatic effect on the ^{31}P signal of the anion for the liposomes containing amiodarone, vis-a-vis cholesterol containing liposomes. Still further support for this argument can be seen in Fig. 5 where the $(CH_2)_n$ and (CH_3) signals for the amiodarone preparations are affected by SCN^- whereas for the cholesterol liposomes, they are not. Our results thus suggest that the amiodarone may alter its position in the liposome headgroup region in

the presence of this anion. The amiodarone ring proton signals are also affected by the SCN (Fig. 5).

Equally interesting is the behavior of the proton signal arising from amiodarone itself: The peak at 1.5 ppm, which we ascribe to the ethyl group (CH_3) moieties of amiodarone, not only is clearly observable at neutral and acidic pH values (Fig. 2), but is also significantly downfield shifted by SCN^- (Fig. 5). Note that the downfield shift of this (CH_3) group is about the same as for the lipid $N^+(CH_3)_3$ signal. If the nitrogen of the amiodarone involved is localized in the polar headgroup region of the liposome, especially at acidic pH values, it is certainly reasonable that this (CH_3) group signal would be downfield shifted by SCN^- as are the lipid $N^+(CH_3)_3$ group signals. This is consistent with the (CH_3) group being located in the headgroup region.

There is one further aspect of our NMR results that bears discussion: Since the headgroup peaks exhibited by PC-PA-CHOL are reversed by the replacement of cholesterol by amiodarone, it may well be that much of the amiodarone is present in the outer monolayer of the liposome. Erikkson [32] has found such a condition for other amphiphilic drugs. The peak at 1.5 ppm, which arises from the (CH_3) group attached to the ethyl groups on the amiodarone nitrogen, is not split as might be expected if the amiodarone were distributed between the inner and outer monolayers. The outer monolayer does however, contain some 40% more PC molecules than does the inner bilayer as was found in our headgroup NMR area measurements, mentioned previously.

Both the electron density profiles (Fig. 8) and the modeling calculations (solid lines in Fig. 7) indicate that the incorporation of 40 mol% amiodarone into EPC bilayers decreases the bilayer width. The modeling calculation also localizes the high density iodine atoms of the amiodarone near the hydrocarbon/water interface. The decrease in headgroup peak separation in the electron density profiles (about 6 Å) is larger than the decrease in bilayer thickness indicated by the modeling analysis (4 Å). This 2 Å difference in the estimate for the change in bilayer thickness is probably caused by the localization of the iodine atoms located near the hydrocarbon/water interface which would tend to shift the centers of the high density peaks in the profile toward the center of the bilayer. It should be noted that the modeling calculations do not necessarily provide a unique fit to the X-ray structure factors (Fig. 7), for example, a second minor occupancy site for amiodarone can not be ruled out. However, the model obtained from the single site fit is consistent with all of the NMR and X-ray data.

The localization of the iodines of the amiodarone near the interfacial region is consistent with the NMR results (Figs. 2 and 3), which show that amiodarone modifies signals from both the head group and hydrocarbon regions. That is, with the iodines in the interfacial region, the hydrophobic portion of the amiodarone could extend into the hydrocarbon interior of the bilayer, and the charged tertiary amine portion of the amiodarone would be in the headgroup region. This location is consistent with the swelling behavior of 3 : 2 EPC/amiodarone liposomes. At 40% water, the lamellar repeat period is about 14 Å larger than observed at 98% relative humidity. The structure factors for the 40% water content specimen fall on the same transform as do the structure factors from the relative humidity experiments, indicating that the bilayer structure is approximately the same in oriented multilayers as in liposomes. At 50% water the discrete reflections are replaced by a broad band at about $(34\ Å)^{-1}$, which corresponds to the first peak in the continuous transform of the 3 : 2 EPC/amiodarone bilayer (Fig. 7). This implies that at 50% water content the fluid spaces between bilayers are large and irregular in width so that the continuous transform is observed, rather than discrete reflections. This type of swelling behavior is observed for bilayers containing a net charge on their surface, but not for uncharged bilayers in water [35,36]. For example, EPC bilayers in excess water swell to a maximum repeat period of about 63 Å [37,16].

Thus, the X-ray experiments on oriented multilayers and unoriented liposomes provide evidence that the iodine atoms of amiodarone are located near the hydrocarbon/water interface and the tertiary amine, at least at neutral pH, resides in the head group region of the bilayer. The extent to which the hydrophobic portion of the amiodarone extends into the bilayer cannot be determined from the X-ray experiments. The decrease in bilayer thickness observed upon the incorporation of 40% amiodarone into EPC bilayers can also be rationalized by the interfacial location of much of the amiodarone molecule. In this position amiodarone would increase the area per EPC molecule at the interface. To maintain a constant volume per EPC molecule, the thickness of the bilayer would have to decrease.

Previous X-ray diffraction experiments with gel phase dipalmitoylphosphatidylcholine bilayers containing less than 1 mol% amiodarone have placed the amiodarone molecule in the hydrocarbon region of the bilayer, near the bilayer center [6]. This result is not consistent with our NMR or X-ray diffraction data. Possible reasons for this discrepancy might be the difference in lipid phase and the amiodarone concentration used in the different studies; the charge on the phospholipid is not a reason for this discrepancy.

In conclusion our results apparently differ from those obtained by others [5–7] in that these workers place amiodarone deep in the hydrocarbon region of the lipid bilayers rather than near the polar headgroup as we find. Our amiodarone concentration is by experimental

necessity much higher than that used by these other workers; in this respect we do not claim that it is 'physiological'. Nevertheless, it is difficult to see, on thermodynamic grounds, why amiodarone would be situated deep in the hydrocarbon region at lower concentrations and near the headgroup at higher concentrations. Further work is necessary in order to reconcile our results with those of other workers, using other physical techniques.

The lipid model system described in the work here has been used in in vitro studies of the heart [38]. It has been found, using electrophysiological techniques, that the liposomal system is a very effective delivery system for amiodarone, at least as efficacious as the other systems presently used for such studies.

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