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## THE INTERACTION OF ATEBRIN WITH PHOSPHOLIPID VESICLES

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### SUMMARY

The interaction of atebtrin with phosphatidylcholine and phosphatidylcholine–phosphatidic acid vesicles has been followed by equilibrium dialysis, and by photometric, fluorimetric and NMR techniques. The presence of negative charges in the phospholipids enhances the binding of atebtrin. The absorbance and NMR spectral changes and fluorescence quenching occurring with phosphatidic acid are attributed to dimerization of the dye interacting electrostatically with negative groups.

The dissociation constant of the binding of the dye to phosphatidylcholine vesicles was 1.4 mM; those of binding to the negative sites of phosphatidic acid were approx. 150 and 3  $\mu$ M.

The dye is probably located at the interphase with the acridine ring interacting with the anionic groups of phosphatidic acid and the tail freely floating in the aqueous phase. The results are discussed also in view of the use of atebtrin as a probe of the energized state in natural membranes and of the suggestion that atebtrin may be used as a transmembrane pH indicator in liposomes or natural membranes.

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### INTRODUCTION

The use of fluorescent probes in order to follow structural changes accompanying the activity of soluble and membrane-bound enzymes is expanding rapidly, and has already provided a variety of information as to the probe environment, namely viscosity, polarity, charge, etc. In general, this approach requires that (a) the probe behaviour be preliminarily interpreted in model systems and (b) the assumption be made that the same interpretation applies in the more complex natural systems.

The acridines have been extensively used to analyze the structure of synthetic and biological biopolymers. More recently it has been reported that metabolic transitions in energy transducing membrane, such as chloroplasts, chromatophores and sub-mitochondrial particles, are accompanied by marked changes in the fluorescence of atebtrin. Two types of interpretation have been proposed. First, that the fluorescence

quenching in the energized state be correlated with a transmembrane or an intramembrane pH gradient [1–3], and, therefore, that atebrin could be used as a pH indicator. Second that the fluorescence quenching be correlated with the extent of the dye–dye interaction [4] and that atebrin could be used as an indicator of the anionic sites present on the membrane. In support of the first view, Deamer et al. [5] reported a correlation between the fluorescence of various amines and the ratio between the internal and external pH of phospholipid vesicles. The behaviour of atebrin was not an ideal one but this did not invalidate the conclusion that atebrin equilibrates across the membrane and that the  $\Delta\text{pH}$  is responsible for most of fluorescence changes.

Since the conclusion of Deamer et al. depends largely on the assumption that the amines are located in the aqueous phase, the present study has been devoted to the problem of the localization of atebrin in the presence of phospholipid vesicles. It will be shown that atebrin interacts strongly with acidic phospholipids and that the dye is most probably localized with the acridine ring near the polar head of the phospholipid while the tail moves freely in the aqueous phase. The data are not consistent with the use of atebrin as a proton gradient-sensitive dye in model or natural systems.

## METHODS

To prepare the phospholipid dispersion, lyophilized phosphatidylcholine or phosphatidylcholine–phosphatidic acid mixtures were suspended in a buffered medium and sonicated under a nitrogen atmosphere in a vessel maintained at 2 °C, until the solution was clear. The suspension was centrifuged at  $25\,000\times g$  for 20 min. In NMR experiments the supernatant was passed on a Sepharose 4B column at 4 °C, and only the fractions corresponding to the second peak were used [6]. Phosphorous determinations were made with the method of Ames and Dubin [7]. DL- $\alpha$ -Lecithin, phosphatidic acid and atebrin were provided by Sigma and used without further purification.

Fluorescence measurements were carried out with the spectrofluorimeter Hitachi Perkin–Elmer Model MPF-2A at room temperature. For the determination of fluorescence polarization the corrected formula of Shinitzky et al. [8] was used in order to eliminate the scattering contribution of the phospholipids.

Absorbance measurements were recorded with a split-beam spectrophotometer constructed in the workshop of the Johnson Foundation. In order to eliminate any interference from the emitted light, the spectra were recorded in the presence of a Wratten filter No. 34.

NMR spectra were recorded at 270 MHz using a modification of a Bruker spectrometer in conjunction with a superconducting magnet made by Oxford Instruments Ltd. High resolution  $T_1$  measurements were made using a  $180^\circ$ – $90^\circ$  pulse sequence, for several values of delay time, by Fourier transformation of the resulting free induction decay.  $T_1$  values were obtained by fitting the data to:

$$\ln(I_\infty - I_t) = \ln(2I_\infty) - t/T_1$$

where  $I_t$  and  $I_\infty$  are the peak heights at time  $t$  and  $\infty$  (no  $180^\circ$  pulse), respectively.

Binding data were obtained by equilibrium dialysis. The experiments were

conducted as follows: the dialysis tube was filled with 0.5 ml of solution with a certain amount of atebtrin and various amount of phospholipids, and placed in 1.5 ml of external medium, containing the same dye concentration, and devoid of phospholipids. After equilibration in a cold room for 48 h, the external medium was analyzed for atebtrin by means of a Hitachi Perkin-Elmer spectrophotometer Model 124.

## RESULTS

The structure of atebtrin is seen in Fig. 1. It consists of an acridine ring, where the nitrogen has a  $pK_a$  around 7.7, and a tail which contains, at the end, a tertiary amine whose  $pK_a$  is around 10.3. The spectral characteristics were reported by Massari et al. [4].

### (A) Phosphatidylcholine vesicles

The absorbance spectra transitions are shown in Fig. 1 following the addition of an increasing amount of phosphatidylcholine vesicles. The higher the amount of phosphatidylcholine, the higher the ratio of the two maxima, at 425 and 445 nm. This behaviour is typical of acid-base transitions [4]. No isosbestic point could be observed, probably because of the modifications, induced by the dye, of the scattering properties of the phospholipid vesicles. If the shift of Fig. 1 were due to an acid-base transition, there are two possibilities: either the dye moves across the membrane into a very alkaline space, or the dye binds to phosphatidylcholine only in the mono-protonated form. The first alternative is very improbable, because the pH during sonication was constant at 7.1, and the medium was buffered with 10 mM Hepes. If, on the other hand, the dye binds to phosphatidylcholine, it is possible to analyze the fluorescence of atebtrin according to the following treatment. Assuming that all the fluorescence changes are due to dye-phospholipid interactions, we write:

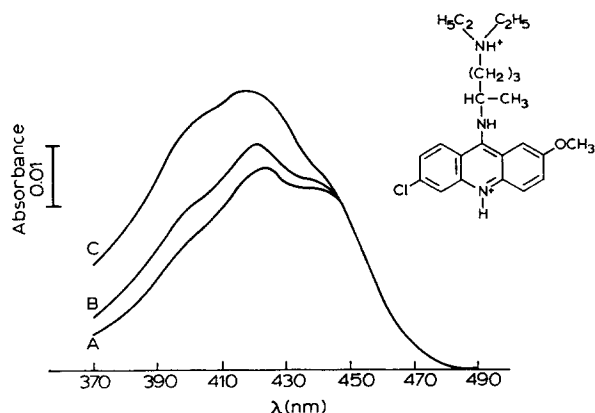
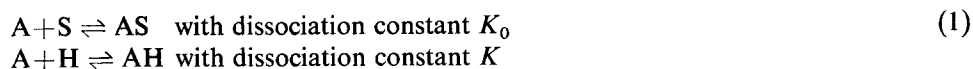


Fig. 1. Absorbance spectra of atebtrin with phosphatidylcholine vesicles. The medium contained 5 mM Tris-Cl pH 7.1 and 10  $\mu$ M atebtrin. Volume 1 ml. Spectrum A, no phosphatidylcholine; spectrum B, 1.2  $\mu$ moles  $P_1$ /ml; spectrum C: 5.8  $\mu$ moles  $P_1$ /ml.

where A and AH are the mono and diprotonated forms of atebrin and S is the binding site. Since the concentration of the site (phosphatidylcholine concentration) is much greater than that of the dye we have:

$$K_0 = \frac{[A][S_0]}{[AS]} \quad (2)$$

where  $[S_0]$  is the total site concentration. The total dye concentration  $[A_0]$  is given by:

$$[A_0] = [A] + [AH] + [AS] \quad (3)$$

In the absence of the inner filter effect (the dye absorbance was always less than 0.1), the fluorescence  $F$  is:

$$F = 2.3I_0\phi w\epsilon c l \quad (4)$$

where  $I_0$  is the intensity of the incident light,  $\phi$  is the quantum yield of atebrin,  $w$  is an instrumental constant,  $\epsilon$  is the molar extinction coefficient of the dye,  $c$  is the dye molar concentration, and  $l$  the optical path. The total emitted light may be expressed as:

$$F_0 = \phi'_A [A] + \phi'_{AH} [AH] + \phi'_{AS} [AS]$$

where  $\phi'_x = 2.3I_0\phi_x w\epsilon_{xx} l$ . Solving the system of equations, we find:

$$F_0 = \frac{\phi'_A K K_0 + \phi'_{AH} K_0 [H] + \phi'_{AS} K [S_0]}{K_0 [H] + K [S_0] + K K_0} [A_0] \quad (5)$$

The total fluorescence is proportional to the dye concentration, at constant  $[S_0]$  and

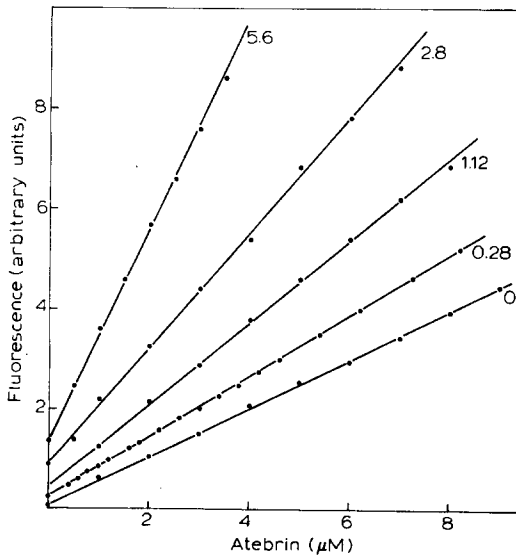


Fig. 2. Fluorescence changes at various atebrin concentrations. The medium contained 5 mM Tris-Cl and 5 mM HEPES pH 7. The numbers indicate the  $\mu$ moles of phosphatidylcholine  $P_i$ /ml during each titration. Emission wavelength 500 nm, excitation wavelength 420 nm. Volume 2.5 ml.

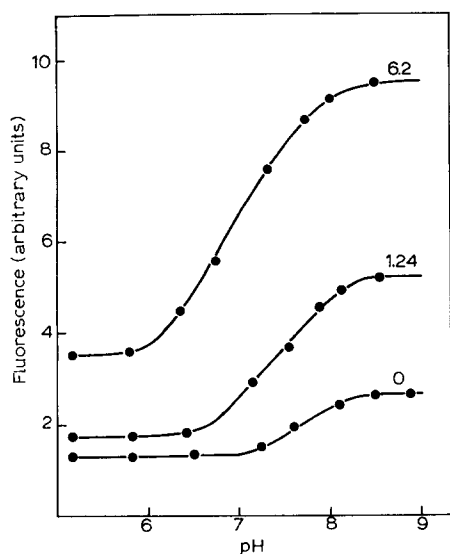


Fig. 3. Fluorimetric titrations at various pH values. The medium was the same as in Fig. 2. Volume 1 ml.  $10 \mu\text{M}$  atebrin. The numbers indicate the  $\mu\text{moles } P_1/\text{ml}$  of phosphatidylcholine during each titration.

pH. This is shown in Fig. 2. Fig. 2 also shows that the atebrin fluorescence increases by increasing the phospholipid concentrations, at constant  $[A_0]$  and pH. The increase in fluorescence may be due either to the immobilization of the dye or to a less hydrophilic environment, because both phenomena result in an enhancement of fluorescence [4]. As to the pH dependence, Eqn 5 predicts, at constant  $[A_0]$  and  $[S_0]$ , a titration curve where the  $[H]$  value at  $F_0/2$  is:

$$[H] = K \frac{[S_0] + K_0}{K_0} \quad (6)$$

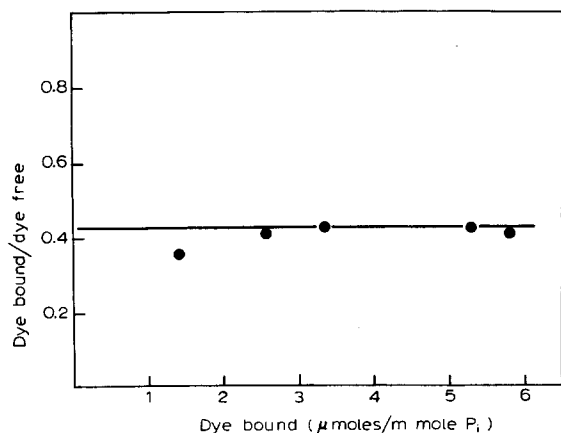


Fig. 4. Scatchard plot for atebrin binding to phosphatidylcholine vesicles. The medium contained 5 mM Tris-Cl, 5 mM HEPES pH 7.2.  $20 \mu\text{M}$  atebrin. Dye bound/dye free is measured in  $1/\text{m mole } P_1$ .

Eqn 6 predicts that with increasing  $[S_0]$  a  $pK_a$  shift toward the acid occurs. From this shift it is possible to calculate the dissociation constant between atebtrin and site. Fig. 3 agrees with these predictions: the  $pK_a$  changed from a value of 7.7 in the absence of phosphatidylcholine to a value of 7.4 in the presence of 1.24  $\mu\text{moles/ml}$  of  $P_i$  and to 7.0 in the presence of 6.2  $\mu\text{moles/ml}$  of  $P_i$ . The dissociation constant  $K_0$ , calculated on the basis of these values, was 1.4 mM.

The Scatchard plot for the atebtrin binding to phosphatidylcholine vesicles obtained by equilibrium dialysis is reported in Fig. 4. The dissociation constant can be calculated on the basis of the number of polar heads which can bind the dye, by supposing that only external phospholipids interact with the dye. This requires the calculation of the ratio between the external and total surface. For the smaller vesicles of the second peak obtained after elution in Sepharose 4B, the ratio is 0.73 [9]. In our preparation, which contains also bigger vesicles, the ratio could be about 0.6. With this value the dissociation constant  $K_0$  is 1.4 mM, the same as that obtained with fluorimetric titration.

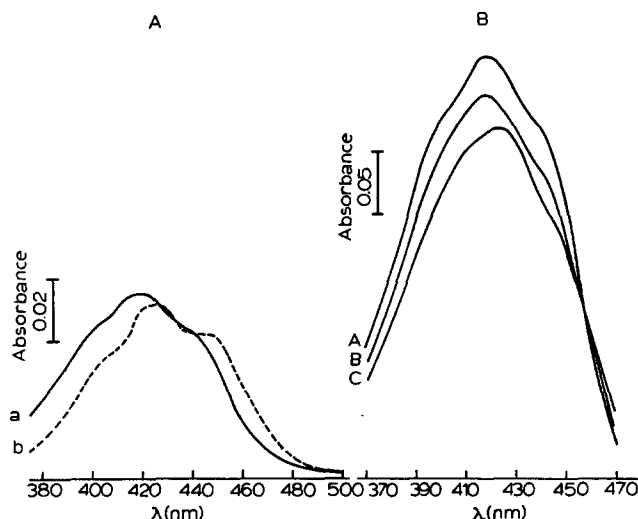


Fig. 5. Absorbance spectra of atebtrin with phosphatidylcholine-phosphatidic acid vesicles. The medium was that of Fig. 2. (A) 10  $\mu\text{M}$  atebtrin pH 8. Spectrum a, without phospholipids; spectrum b, 1.3  $\mu\text{moles } P_i/\text{ml}$ . (B) 100  $\mu\text{M}$  atebtrin pH 8. Spectrum A, without phospholipids; spectrum B, 0.174  $\mu\text{mole } P_i/\text{ml}$ ; spectrum C, 0.7  $\mu\text{mole } P_i/\text{ml}$ . Total volume: 1 ml.

#### (B) Phosphatidylcholine-phosphatidic acid vesicles

Figs 5A and 5B show the absorbance spectra of atebtrin with phosphatidylcholine-phosphatidic acid vesicles in the ratio 3 : 1. In Fig. 5A the ratio dye/site was low; in this case the transition was clearly base-acid, due to stronger binding of the diprotonated form of the dye. In Fig. 5B the ratio dye/site was high. In this case the scattering change due to atebtrin was negligible because the amount of phospholipids was low and the phosphatidylcholine-phosphatidic acid vesicles were clearer than the phosphatidylcholine vesicles. There was an isosbestic point between 450 and 460 nm, coinciding with that of the transition monomer-aggregate [4]. The spectra also in-

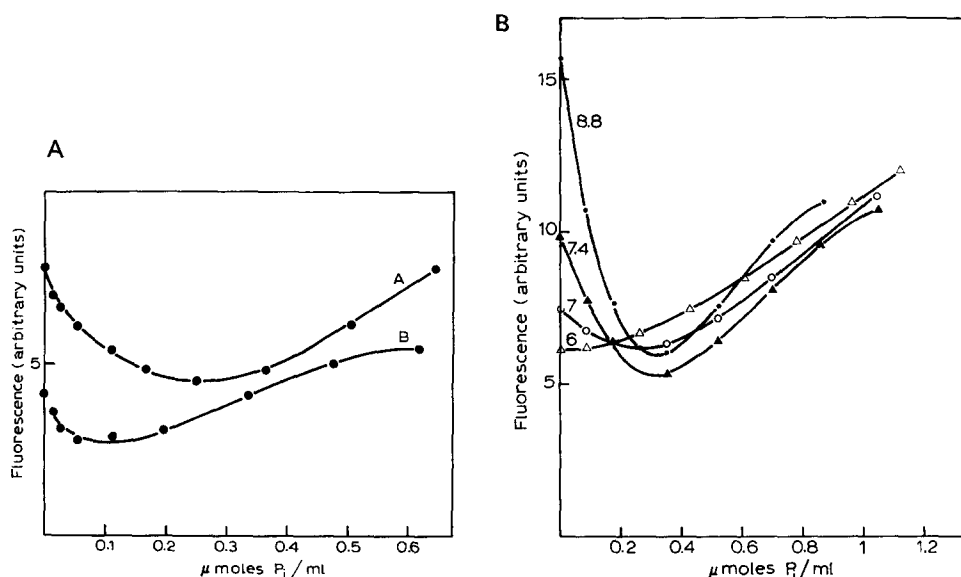


Fig. 6. Fluorescence changes at various phosphatidylcholine-phosphatidic acid concentrations. The medium was the same as in Fig. 2. Volume 1 ml. (A) Curve A, 50  $\mu\text{M}$  atebtrin; curve B, 30  $\mu\text{M}$  atebtrin. pH 6.85. (B) 40  $\mu\text{M}$  atebtrin. The pH values are reported in the figure.

licated a monomer-aggregate transition. The conclusion is that the diprotonated form of atebtrin binds tightly to phosphatidic acid: at a high dye/site ratio, the dye molecules, which bind to nearby sites, can interact, whereas at a low dye/site ratio binding without aggregation predominates.

If this conclusion is correct, we expect quenching and enhancement of fluorescence at high and low dye/site ratios, respectively. In Fig. 6A, by increasing the  $P_i$  concentration, there was first a large quenching, followed by a fluorescence increase. At a higher dye concentration the quenching was greater and the minimum of fluorescence shifted toward higher values of  $P_i$  concentration. This is in accordance with the aggregation model discussed above, because by increasing the dye concentration a greater amount of phospholipid is required in order to have the maximum of quenching.

Dye-dye interactions leading to fluorescence quenching may be due to a dye binding either to a certain amount of phosphatidic acid molecules whose distance permits dye-dye interactions or to the two charges of the same phosphatidic acid molecule. The first group of phosphatidic acid molecules has a  $pK_a$  around 3–3.8; the second around 8–8.6, depending on the preparation. The titration curve for mixed phosphatidylcholine-phosphatidic acid systems were quite similar to those of phosphatidic acid alone [10]. Fig. 6B shows the dependence of fluorescence intensity on the pH values. In alkaline media, where the second group of phosphatidic acid molecules is charged, the quenching is much greater than in the acid media, where phosphatidic acid carries only one charge. This may be an indication that the second group of phosphatidic acid plays an important role. Nevertheless even at very low pH values (pH 4.3, not shown in the figure) there was a small effect on the fluorescence at low  $P_i$  concentration. This may be an indication that the distribution of phospho-

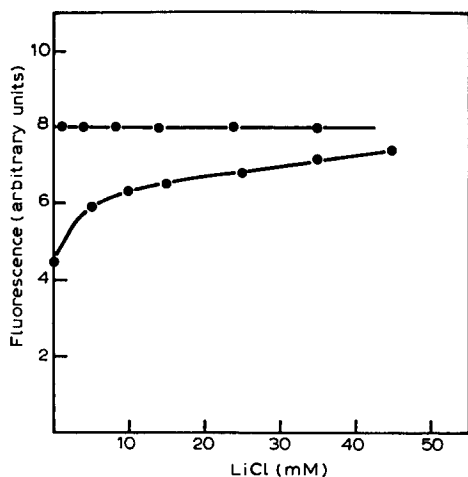


Fig. 7. Salt effect on atebtrin fluorescence. The medium was that of Fig. 2;  $40 \mu\text{M}$  atebtrin, 1 ml volume. Upper curve,  $1.7 \mu\text{moles P}_i/\text{ml}$ , pH 5.6; lower curve,  $0.43 \mu\text{mole P}_i/\text{ml}$ , pH 8.4.

tidic acid molecules is also involved in the dye-dye interaction phenomena.

Fig. 6B also shows that the pH dependence of fluorescence intensity was very complex and dependent on the dye/site ratio. In fact, by varying the pH, four overlapping phenomena occur: a) acid-base titration of the dye, b) free-bound transition of the dye, c) acid-base titration of the second site of phosphatidic acid and d) quenching of fluorescence of the dye due to aggregation. In particular we found, at a certain value of the ratio dye/site, the same dependence of the fluorescence intensity on the pH of Deamer et al. [5] that is a maximum of fluorescence at a certain pH value.

$\text{Li}^+$  has been found to bind to phosphatidic acid more tightly than  $\text{Na}^+$  and

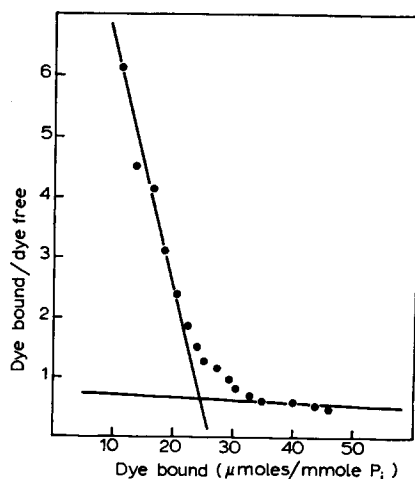


Fig. 8. Scatchard plot for atebtrin binding to phosphatidylcholine-phosphatidic acid vesicles. The medium contained 5 mM Tris-Cl, 5 mM HEPES, pH 6.2,  $60 \mu\text{M}$  atebtrin. Dye bound/dye free is measured in  $1/\text{m mole P}_i$ .



$K^+$ ; the stability constant for the complex of  $Li^+$  with the dibasic form of phosphatidic acid is 17 [11]. It is known also that monobasic and dibasic phosphate groups have a different ion binding capacity [12]. Fig. 7 shows the competitive inhibition induced by LiCl on the fluorescence changes at various concentrations of phosphatidylcholine–phosphatidic acid. At low pH there was no effect on the fluorescence intensity, whereas at high pH, from the maximum of quenching, there was a return of fluorescence. The same behaviour was obtained using  $NH_4Cl$  instead of LiCl. The experiment of Fig. 7 indicates that: (a)  $Li^+$  competes with atebirin for the dibasic form of phosphatidic acid; this causes a release of dye and a consequent decrease of quenching; (b)  $NH_4^+$  has the same effect of  $Li^+$ ; hence under these conditions  $NH_4^+$  acts as a monovalent cation which competes with the dye for the negative sites of phosphatidic acid.

The Scatchard plot for the binding of the dye to phosphatidylcholine–phosphatidic acid vesicles is shown in Fig. 8. It is apparent that the system consists of two different site species. The first are about 150  $\mu$ moles/mole  $P_i$  and have a dissociation constant of about 150  $\mu$ M. They probably are the negative polar heads of phospha-

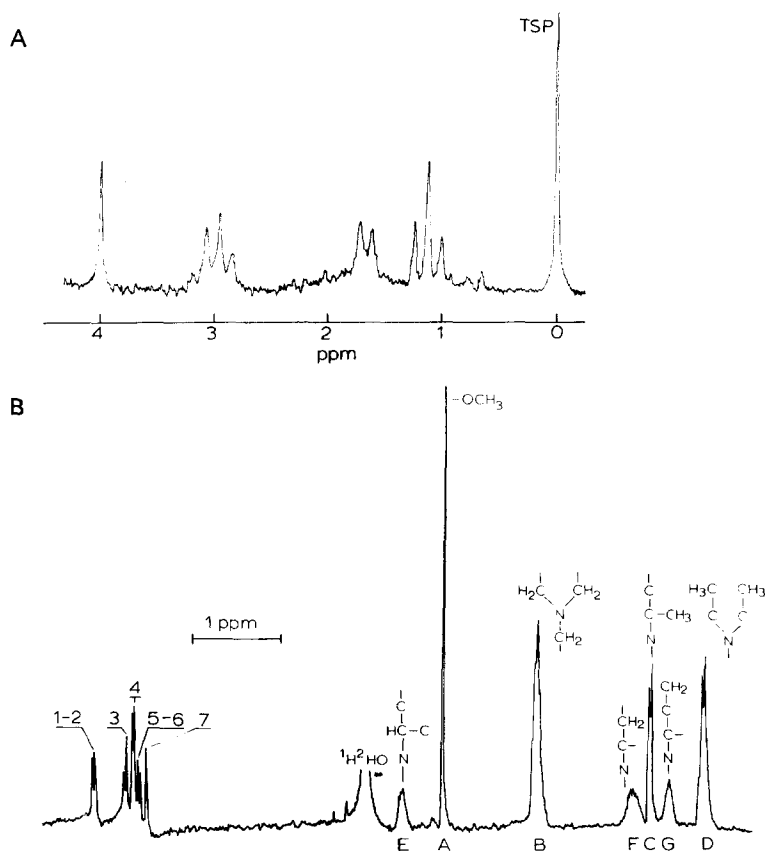


Fig. 9. NMR spectra of atebirin. (A) 60 MHz NMR spectrum of 0.1 M atebirin in  $^2H_2O$ , 37 °C. Reference standard TSP (2,2-dimethyl-2-silapentane-5-sulphonic acid) (B) 270 MHz NMR spectrum of 20 mM atebirin in  $^2H_2O$ , 20 °C, p<sup>2</sup>H 5.

tidic acid whose amount, in phosphatidylcholine–phosphatidic acid vesicles 3 : 1, is about 150  $\mu\text{moles}/\text{mmole } P_i$  taking into consideration only the external phosphatidic acid and a ratio of external and total surface of 0.6. The second are about 25  $\mu\text{moles}/\text{mmole } P_i$  and have a dissociation constant of about 3  $\mu\text{M}$ . This amount is about 15 times greater than the amount one would expect taking a  $pK_a$  value of 8.2 of the second phosphoric group of phosphatidic acid. The reasons why the amount is greater may be: i) the binding of atebtrin may modify the  $pK_a$  value, or ii) two phosphatidic acid molecules are near so that the binding of the dye to one molecule facilitates the binding to the other.

Experiments were done to measure the polarization values of the fluorescence of atebtrin in water and phosphatidylcholine–phosphatidic acid solutions. The polarization increased from 0.06 in water to 0.08 in vesicles at a ratio of  $\mu\text{moles dye}/\mu\text{moles } P_i = 1.2 \cdot 10^{-3}$ . This value which is very similar to that found in submitochondrial particles in passive conditions by Massari et al. [4] is direct evidence for the immobilization of the dye. The viscosity of the solution after the addition of phosphatidylcholine–phosphatidic acid vesicles did not vary appreciably.

### (C) NMR experiments

The position of the dye in the phospholipid membrane has been analyzed by means of NMR spectrometry.

In Figs 9A and 9B the spectra of atebtrin at 60 and 270 MHz are shown. The assignment of the various groups to the NMR lines, Fig. 9B, was obtained by: (a) taking the two spectra at different field strengths, (b) calculating the integrated areas under the peaks and (c) taking into account the relation between chemical shift and

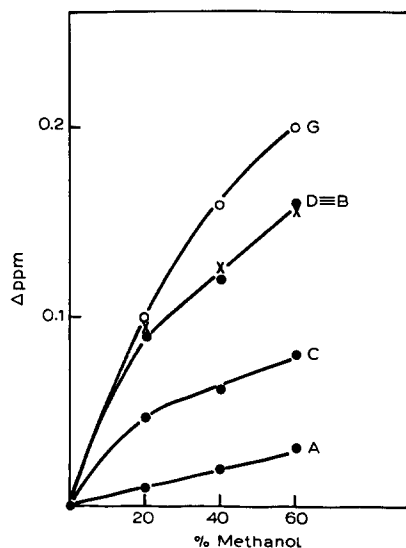


Fig. 10. Line shift at various methanol– $^2\text{H}_2\text{O}$  mixtures.  $\Delta\text{ppm}$  is the difference in chemical shift between a given percentage of methanol and pure water. It was calculated in a 270 MHz spectrometer taking as reference 2,2-dimethyl-2-silapentane-5-sulphonic acid. 10 mM atebtrin. The letters indicate the lines as in Fig. 9B.

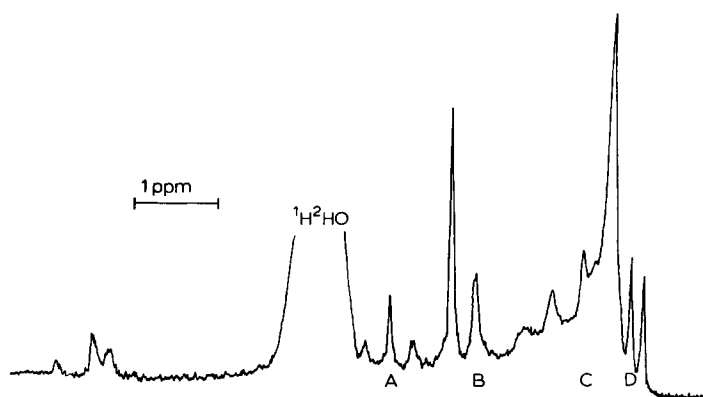


Fig. 11. NMR spectrum of atebirin with phosphatidylcholine-phosphatidic acid vesicles. 2 mM atebirin and 25  $\mu$ moles  $P_i$ /ml. The spectra were recorded at 270 MHz. The medium also contained 70 mM NaCl.

spin-spin coupling and the chemical structure of the molecule. The chemical shift of the tail protons of atebirin is dependent on the polarity of the medium. Fig. 10 shows that the lines shifted toward lower field by increasing the methanol concentration. The effect of aggregation on the NMR spectra was studied by increasing the dye concentration. The following phenomena were observed: (a) a large spectrum change of ring protons, (b) a large shift of the ring protons toward higher field and (c) a slight shift of tail protons toward lower field. The latter phenomenon is probably due to the enhanced hydrophobicity of the solution. In fact, by plotting the lines shift against the dye concentrations, there is coincidence between these shifts and that occurring at certain values of methanol-water mixture. The high field shift of the ring protons is peculiar to the aggregation of acridines [13].

In Fig. 11 the spectrum of 2 mM dye with phosphatidylcholine-phosphatidic acid vesicles is reported. Addition of phospholipids induced the following changes. (a) A slight shift of ring protons toward higher field; this probably is due to the aggregation of the dye to the negative sites of phosphatidic acid. There was also a large spectral change of ring protons which took place at the same time. (b) Differential broadening of the lines: the ring protons were much more affected than the tail protons. This may be an indication that the ring is more immobilized than the tail of atebirin, if the broadening is a measure of proton mobility. An increase of the phospholipids concentration of 1 order of magnitude did not cause an appreciable chemical shift of ring protons (the dye/site ratio remained very high), whereas the linewidth increased several times. This excludes the possibility that the increase of linewidth was due to rapid exchange between two environments with different chemical shift.

To complement the linewidth measurements, we have also determined the high resolution  $T_1$  relaxation time of atebirin in the absence and in the presence of phosphatidylcholine-phosphatidic acid vesicles. The results are reported in Table I. The ring proton peaks were more affected, whereas the  $T_1$  of the tail protons remained unaltered. It seems, therefore, that the ring protons are more immobilized than the

TABLE I

$T_1$  measurements of atebtrin with and without vesicles at 270 MHz. The medium contained 10 mM atebtrin,  $p^2H$  5.5.  $T_{1f}$  is the  $T_1$  obtained without phospholipids.  $T_{1b}$  is the  $T_1$  with 25  $\mu$ moles  $P_1$ /ml and 50 mM NaCl.  $T_{1f}/T_{1b}$  is the ratio between the two  $T_1$  values. The numbers of ring protons and the letters of tail protons are that of Fig. 9B.

Lines	Ring protons				Tail protons		
	1-2	3-4	5-6	7	A	B	C
$T_{1f}$ (ms)	310-310	750-750	650-650	310	480	270	250
$T_{1b}$ (ms)	-	570		410	410	250	250
$T_{1f}/T_{1b}$	-	1.3		$\approx 1.4$	1.17	1.08	1

tail protons: probably because the ring nitrogen binds electrostatically to the phosphatidic acid-negative groups, whereas the tail of the dye is free to move in the outer solution.

## DISCUSSION

The following observations strongly suggest that when phospholipid vesicles are added to a solution of atebtrin, the dye binds the phospholipids and does not remain free in solution: (a) the fluorescence changes can be dealt with according to a model where the dye interacts with a fixed number of sites on the vesicles; (b) phosphatidylcholine vesicles induce an acid-base transition while phosphatidylcholine-phosphatidic acid vesicles induce a base-acid transition and the extent of transition depends on the phospholipid concentration. If the transition were due to the measurement of the pH of the inner osmotic space, following a translocation of the dye, the internal pH should be around 9 in the case of the phosphatidylcholine and around 5 in the case of the phosphatidylcholine-phosphatidic acid vesicles. This is unlikely considering the sonication conditions; (c) polarization measurements indicate a marked immobilization of the dye; (d) the fluorescence change depends on the relative concentrations of phospholipid and dye; there is quenching at a high dye/site ratio and enhancement at a low dye/site ratio: this is in accordance with a mechanism of interaction of dye molecules bound to near sites and immobilization of the dye bound to distant sites; (e) the fluorescence quenching is dependent on the dissociation of the second acidic group of phosphatidic acid; (f) the increase of ionic strength results in a marked inhibition of the interaction of the dye with the second group of phosphatidic acid. On the basis of these and NMR data it appears reasonable to conclude that the acridine ring of atebtrin interacts with the polar heads of the phospholipids. The observation that the tail protons mobility is not affected by the dye-phospholipid interaction suggests that atebtrin be localized almost at the interphase in order to permit the free movement of the amine group in water.

If atebtrin would be sensitive in some way to the intramembrane pH, this would require: (a) that the binding of atebtrin to phospholipids be negligible and (b) that one form of the dye be freely permeable across the membrane. The localization of atebtrin

near the polar heads of the phospholipids is in contrast with the former assumption. As to the latter assumption atebirin binds only with the externally located phospholipids. Indeed a good correspondence between equilibrium dialysis and fluorimetric data is obtained by assuming that the number of sites is given only by the outer layer of phosphatidylcholine molecules.

An interesting observation concerns the inhibitory effect of  $\text{NH}_4^+$  on the atebirin fluorescence. While Deamer et al. explained this phenomenon as reduction of the pH gradient following the  $\text{NH}_3$  equilibration across the membrane, it seems from our data that  $\text{NH}_4^+$  competes with the dye for the binding to the polar heads of phospholipids.

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