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IONOPHORE-CATALYZED CATION TRANSPORT BETWEEN PHOSPHOLIPID INVERTED MICELLES MANIFEST IN DNMR *

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Studies of hyperfine shifts of lipid ^{31}P resonances due to hydrated phospholipid inverted micelles in benzene are presented. Systems with distinct resonances from micelles containing no paramagnetic ions, and from micelles containing a single praseodymium(III) or a single europium(III) ion (and three nitrate counterions) have been generated. The addition of an ionophoric antibiotic from *Streptomyces lasaliensis*, lasalocid-A (X537A), causes both resonances to broaden and, with further additions, coalesce and eventually resharpen as a single line. Dilution of only the ionophore reverses these spectral changes. This is interpreted as a manifestation of dynamic NMR (DNMR, exchange broadening); i.e., that the ionophore catalyzes the equilibrium exchange of metal ions from micelle to micelle to the point where it becomes fast on the NMR time scale. This exchange is inhibited by protons or other competitive metal ions. We have simulated the spectra with a total lineshape analysis program and have thus extracted the average preexchange lifetimes for various concentrations of the antibiotic. We find a reasonably good first-order dependence on lasalocid-A concentration in each of several different experiments. This is in contrast to the higher order concentration dependences often observed by others using different techniques employing bilayer membranes. We favor a diffusional carrier mechanism involving surface aggregates of lasalocid-A for our process. This leads to the implication that a higher order concentration dependence found for a bilayer system arises from a different mechanism. The ionophore valinomycin does not catalyze rapid exchange in our system.

1. Introduction

Aggregates of amphiphilic molecules in nonpolar solvents are called inverted micelles [1–3]. Often these have been used to solubilize water and/or other polar molecules or ions as microemulsions [1,4,5]. In recent years, such systems have found increasing popularity for any number of imaginative applications including, for example, solar energy conversion and storage [6]. When the amphiphiles are naturally occurring lipid molecules, such systems can take on model biological implications. Thus, phospholipid inverted micelles have been postulated as the structures of equilibrium 'lipidic particles' observed in bilayer membranes [7–11] and as transient species occurring during the fusion of two bilayer membranes [7,12–14]. Lipid inverted micelles could be present in the annular and microlens regions [15–17] of the black lipid membranes (BLM) often used for transport studies, and could conceivably be involved in some ion and solute transbilayer transport in such systems [18]. Inverted micelles of phospholipids [19,20], detergent amphiphiles [19] and bile salt anions [19,21] have been postulated as diffusional carriers for transbilayer transport of metal cations and water-soluble proteins. Hydrated phospholipid inverted micelles are used as precursors in the formation of large bilayer vesicles by the reverse-phase evaporation technique [22]. Finally, solutions of protein-lipid complexes in nonpolar solvents have recently been used in methods for the reconstitution of membrane proteins in model membranes [23,24].

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We have recently reported studies of the solubilization and exchange of paramagnetic lanthanide ions, Ln^{3+}, in hydrated phospholipid inverted micelles in benzene, by ^{31}P and ^{1}H nuclear magnetic resonance (NMR) techniques [1]. In this paper, we extend these studies and show that an ionophoric molecule, the polyether antibiotic lasalocid-A, can catalyze the exchange of Ln^{3+} between the micelles to the point where it becomes fast on the NMR time scale. The analysis of the lineshapes so generated can provide a measure of the transport rate and thus, in a sense, this represents a dynamic NMR version of the Pressman cell transport experiment [25,26].

2. Experimental

2.1. Materials

Chemicals were from the same sources as described in our earlier paper [1]. Samples of X-537A and its sodium salt were generous gifts from Dr. J. Berger of the Hoffman-La Roche Co., Nutley, NJ. Valinomycin was obtained from the Sigma Chemical Co.

2.2. Preparation of hydrated inverted micelles

The method used to prepare hydrated inverted micelles was the same as that described in our earlier paper [1]. The addition of X-537A to the micelle solutions was accomplished in the following manner. A small measured amount (μl) of a stock solution of either HX or NaX in benzene (36.05 and 36.7 mM, respectively) was injected (micropipette) into the NMR sample tube which already contained the desired micelle solution. The resultant solution was mixed thoroughly by shaking. Valinomycin was simply added to the solution as the crystalline solid.

2.3. NMR measurements

$^{31}P\{^{1}H\}$ NMR measurements were accomplished in the same manner as described in ref. [1]. For the total lineshape analyses, the spectra were digitized (up to 24000 points/spectrum) and stored on magnetic tape (Varian 620-L computer).

2.4. Computing

The total lineshape analyses were accomplished using a simple uncoupled two-site exchange program, based on the classical Bloch equations [27], originally written by H.S. Gutowsky, modified by T.L. Brown, their students, and ourselves, and previously described by us [28]. The program was originally written in Fortran and was translated into the Basic language so that the search could be processed with a Hewlett-Packard calculator (model No. 9830A). The results were plotted with the associated plotter. The program requires for input: the experimental spectrum in digitized form, the value of the separation of the two resonances in the absence of exchange ($\Delta\nu_{\infty}$) in Hz, the populations of the two sites, and the linewidths ('effective' T_2 values) of the two resonances in the absence of exchange. The program will vary τ (an average, reduced lifetime of the DPL-Pr^{3+} hyperfine interaction) until it achieves the best fitting to the experimental spectrum as judged by a nonlinear least-square analysis.

3. Results

Fig. 1a depicts a $^{31}P\{^{1}H\}$ NMR spectrum of a dispersion of hydrated dipalmitoyl lecithin (DPL) inverted micelles in benzene. Some of these micelles contain one Pr^{3+} each (and three NO_3^- counterions) in their aqueous cores, some contain one La^{3+}, and some contain no lanthanide, Ln^{3+}. We have discussed such spectra at length in an earlier paper [1]. The sharp resonance arises from the phosphorus nuclei in the head groups of lipid molecules in those micelles which contain no Ln^{3+} and those micelles which contain one La^{3+}. The broader resonance downfield represents lipids in those micelles containing one Pr^{3+}. The temperature is 52°C, 10 degrees above the thermal phase transition temperature for DPL. In general, the average phospholipid aggregation number, $\bar{n}$, in such solutions is about 100, although this value appears to depend strongly on a number of factors, not the least of which is the molar ratio of water to phospholipid [1]. The solution of fig. 1a was prepared with a ratio appropriate to produce

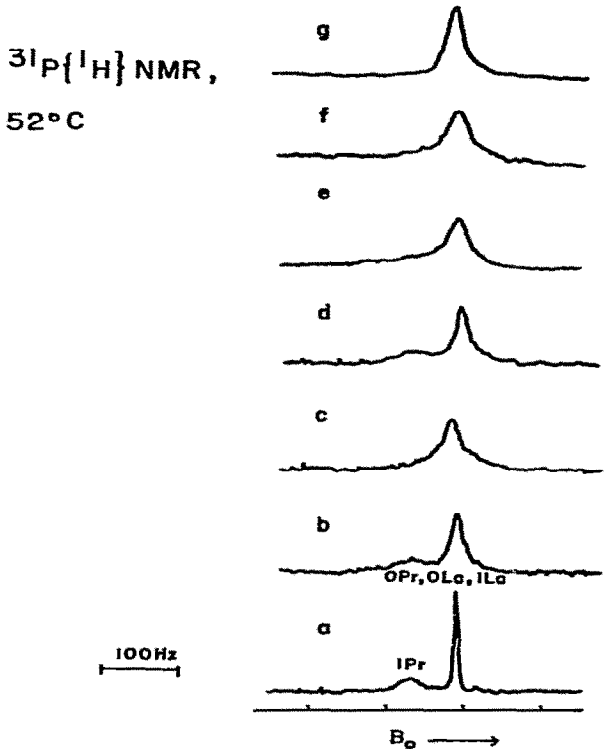


Fig. 1. $^{31}P\{^1H\}$ NMR spectra of DPL/water dispersions in benzene at 52°C. (a) Unsonicated dispersion of inverted micelles with total (stoichiometric) concentrations of 0.31 mM Pr^{3+}, 0.31 mM La^{3+}, 70.23 mM DPL and 1110 mM D_2O. These concentrations are based on the total solution volume. (b) Solution of a made 0.91 mM in . (c) Solution of b made 1.82 mM in NaX. (d) Mixture of c with an equal volume of a solution identical to that of a: [NaX] is 0.91 mM. (e) Solution of d made 1.82 mM in NaX. (f) Solution of e made 2.73 mM in NaX. (g) Solution of f made 5.45 mM in NaX.

spherical hydrated inverted micelles with $n = 117$ *. We have shown that a measurement of $\bar{n}$ can be estimated from the ratio of the spectral peak intensities, assuming that $n \neq f(L)$, where L is the lanthanide occupation number, and that the Ln^{3+} is distributed among the micelles randomly (Poisson) [1]. For the spectrum in fig. 1a (intensity ratio in table 1), such an estimate yields a value of 116 for $\bar{n}$. This agreement is certainly fortuitous, for we have found that, in general, either or both of the above assumptions are not strictly valid [1].

Since two distinct resonances are observed in fig. 1a, it is clear that the exchange of phospholipid molecules between micelles not containing a Pr^{3+} and those with a Pr^{3+} is slow on the NMR time scale. Although the exchange of the Pr^{3+} between binding sites on the phosphates of the various lipids within a micelle is fast on the NMR time scale, it is clear that Pr^{3+} exchange between micelles also must be slow on the NMR time scale.

Lasalocid-A (X-537A) is an antibiotic molecule, soluble in nonpolar solvents, which is capable of extracting metal ions into such solvents and also of transporting them across phospholipid bilayer membranes [30]. Fig. 1b shows the spectrum of the solution of fig. 1a after it is made 0.91 mM in the sodium salt of the anion of lasalocid-A, NaX. It is clear that both resonances have become noticeably broadened. If the NaX concentration is doubled to 1.82 mM, the two lines coalesce into a single broad resonance. Since lasalocid-A is known to act as an ionophore for Pr^{3+} [31–33], one suspects that NaX is able to transport Pr^{3+} among the inverted micelles and that its presence is catalyzing the equilibrium Pr^{3+} exchange between micelles to the point where it becomes fast on the NMR time scale. Presumably the exchange of the diamagnetic La^{3+} is also catalyzed but these ions are 'magnetically invisible' here.

If the broadening in fig. 1b and c is due to an increased exchange rate (dynamic NMR, DNMR [27]), it should be reversible. Fig. 1d shows the result of diluting the concentration of NaX (only) back to 0.91 mM. (This is accomplished by mixing equal volumes of the solutions of fig. 1a and c.) The spectral changes are indeed reversed; two resonances reappear. The spectrum shown in fig. 1d is almost identical to that of fig. 1b. If the concentration of NaX is redoubled again to 1.82 mM, the two lines recoalesce (fig. 1e). As the NaX concentration is increased further (2.73 mM in fig. 1f, 5.45 mM in fig. 1g) the single line sharpens. All

* The calculation of the expected value of n from the water/lipid molar ratio (after Fendler and co-workers [4]) involves two major assumptions; a value for the amphiphile headgroup area, and that all water added is incorporated into the micellar inner cores. We assumed 60 $Å^2$ for the headgroup area [29], and used benzene which was presaturated with water [1].

of this is consistent with the DNMR phenomenon. An alternative interpretation of the lineshape changes might be that the ionophore is simply sequestering the Pr^{3+} into the benzene phase. This would give rise to a different lineshape change, however, and is ruled out by the success in fitting the observed spectra with the DNMR equation (vide infra).

Fig. 2 offers additional evidence that the DNMR effect is caused by the transport ionophoric property of lasalocid-A. Fig. 2a depicts the spectrum of a solution very similar to that of fig. 1a. Fig. 2b shows the spectrum when that solution is made 7.29 mM in NaX. Complete coalescence and almost complete resharpening are observed. Fig. 2c depicts the spectrum when an aliquot of the solution of fig. 2a is made 8.23 mM in HX, the protonated form of lasalocid-A. The DNMR broadening and coalescence have reached nowhere near the stage of that of fig. 2b, implying that the rate of exchange is significantly less. It has been observed in a number of studies [31,34–37] that at low aqueous phase pH values (< 3.7), the ion transport induced by lasalocid-A is significantly decreased, indicating H^+ inhibition. This seems to be also manifest in our results. Fig. 2d–f illustrates the same effect except for a three-site NMR exchange situation. The diamagnetic La^{3+} of figs. 1 and 2a–c has been replaced by paramagnetic Eu^{3+}. These are also partitioned inside the hydrated

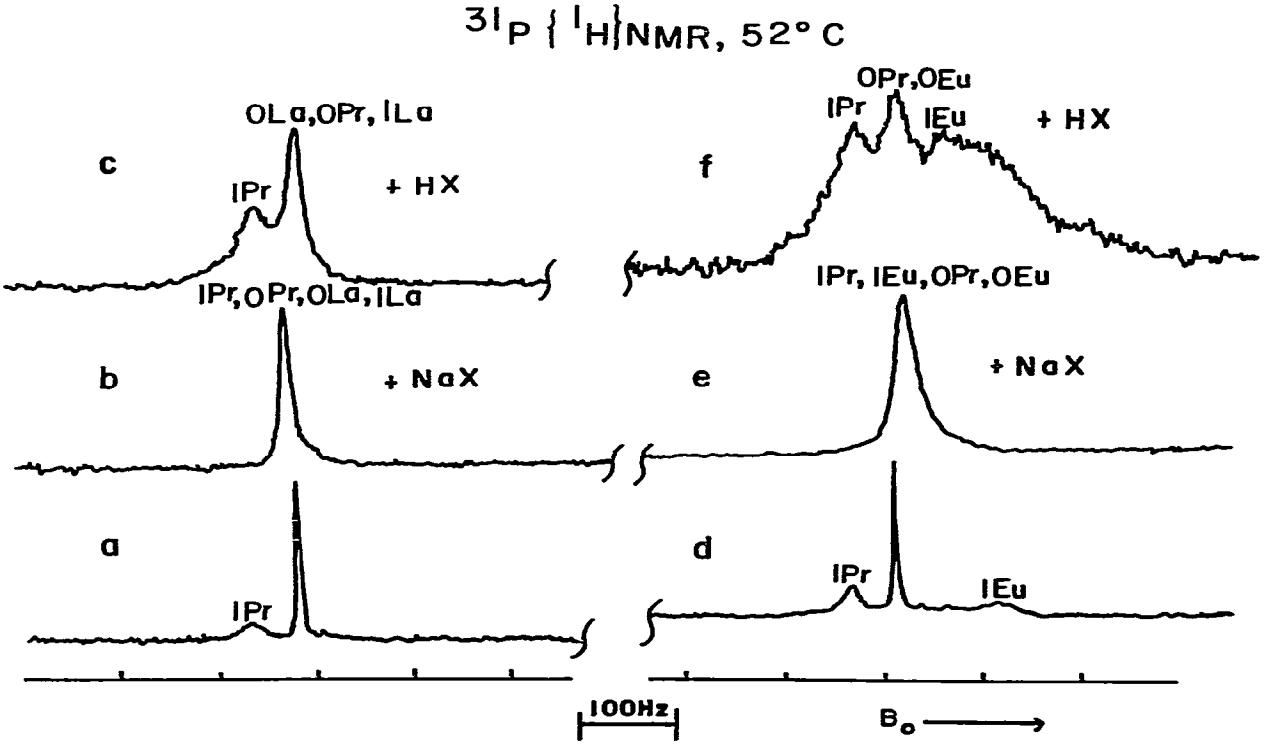


Fig. 2. $^{31}P\{^1H\}$ NMR spectra of DPL/water dispersions in benzene at 52°C. (a) Unsonicated dispersion of inverted micelles with total (stoichiometric) concentrations of 0.35 mM La^{3+}, 0.35 mM Pr^{3+}, 1111 mM D_2O and 55.64 mM DPL. The concentrations are based on the total solution volume. (b) Solution of a made 7.29 mM in NaX. (c) Solution of a made 8.23 mM in HX. (d) The same as a except La^{3+} replaced by 0.35 mM Eu^{3+}. (e) Solution of d made 9.34 mM in NaX. (f) Solution of d made 9.69 mM in HX.

inverted micelles but give rise to an upfield hyperfine shift. Thus, there are three kinds of observable micelles in the solution; those with one Pr^{3+}, those with one Eu^{3+}, and those with no Ln^{3+}. The probability of micelles having two Ln^{3+} of any kind is very small at these concentrations [1].

In order to gather data for total lineshape analyses under several different conditions, we conducted further experiments. Two of these are shown in figs. 3 and 4. The solutions of fig. 3 are similar to those of fig. 1 in that NaX is used as the exchange catalyst, but different in that no La^{3+} is present. The solutions of fig. 4 are similar to those of fig. 3 except that HX is used as the exchange catalyst. Details of species concentrations are given in the figure captions. The results of the spectral simulations using the uncoupled two-site exchange computer program described in section 2 are shown in figs. 5–7. The input parameters and the numerical results are listed in table 1.

The simulations are seen to be quite good, in general, especially considering the problems commonly encountered in a rigorous lineshape analysis of spectra obtained by the FT method after moderately long accumulations. An inspection of the comparisons between experimental and calculated spectra in figs. 5–7 might lead to the conclusion that the phase angles of the experimental spectra were such that pure absorption curves were

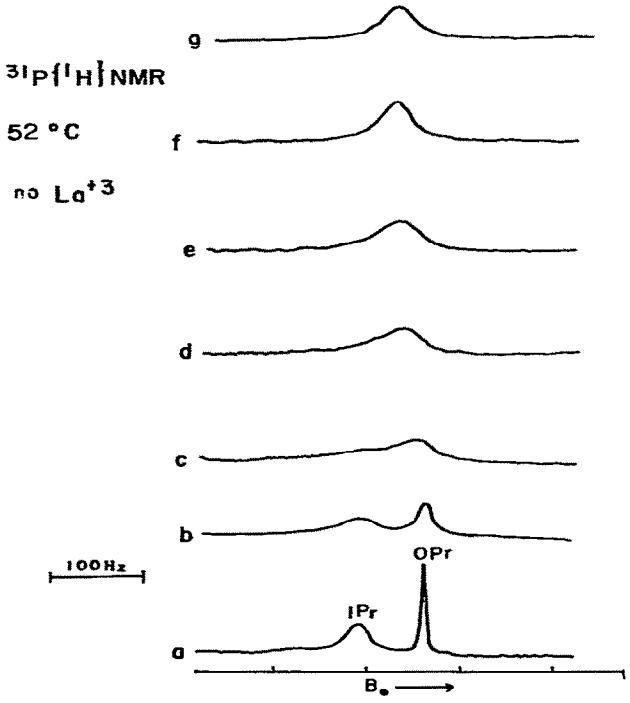


Fig. 3. $^{31}P\{^1H\}$ NMR spectra of sonicated DPL/water dispersions in benzene at 52°C. (a) Dispersion of inverted micelles with total (stoichiometric) concentrations of 0.49 mM Pr^{3+}, 1103 mM D_2O and 77.2 mM DPL. These concentrations are based on the total solution volume. (b) Solution of a made 0.71 mM in NaX. (c) Solution of b made 1.39 mM in NaX. (d) Solution of c made 2.04 mM in NaX. (e) Solution of d made 2.67 mM in NaX. (f) Solution of e made 4.43 mM in NaX. (g) Solution of f made 6.00 mM in NaX.

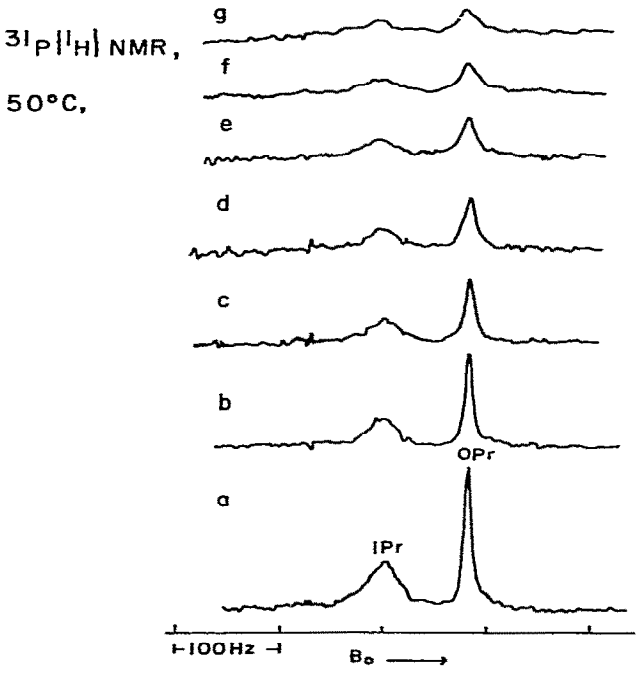


Fig. 4. $^{31}P\{^1H\}$ NMR spectra of sonicated DPL/water dispersions in benzene at 50°C. (a) Conditions are the same as those of fig. 3a. (b) Solution of a made 1.22 mM in HX. (c) Solution of b made 2.45 mM in HX. (d) Solution of c made 4.06 mM in HX. (e) Solution of d made 5.51 mM in HX. (f) Solution of e made 7.63 mM in HX. (g) Solution of f made 9.44 mM in HX.

Table 1

The effects of increasing concentration of NaX or HX on the rate constants of praseodymium(III) exchange (obtained from computer simulations) in hydrated inverted DPL micelle solutions in benzene [a]

The input parameters were as follows:

	$\Delta\nu_\infty$ (Hz)	$p(0)$ *	$T_{2,0}$ (s) **	$T_{2,1}$ (s) **
Data from fig. 1a	60	0.66	0.072	0.004
A replicate analysis of data from an aliquot of the solution of fig. 1	60	0.66	0.072	0.004
Data from fig. 3a	72	0.42	0.04	0.01
Data from fig. 4a	82	0.46	0.045	0.013

* $p(0)$ is the relative population of lipid in micelles with no Pr^{3+} (i.e., $p(0)+p(1)=1$).

** $T_{2,0}$ and $T_{2,1}$ are equal to $1/\pi(\Delta H_{1/2})_0$ and $1/\pi(\Delta H_{1/2})_1$, respectively, where $\Delta H_{1/2}$ is the full width of the resonance at half-height, in Hz (no lasalocid-A present).

Spectrum	[NaX] (mM)	[HX] (mM)	τ (ms)	τ_1^{-1} (s^{-1})
Fig. 1 [b]				
d	0.91		17.70	37.18
e	1.82		8.84	74.6
f	2.73		5.50	120
g	5.45		2.78	236
A replicate analysis of data from an aliquot of the solution of fig. 1 [b,c]				
a	0.60		25.40	25.90
b	1.40		9.13	72.1
c	2.06		6.46	102
d	2.69		4.66	141
e	3.90		4.12	160
f	5.01		3.29	200
Fig. 3				
b	0.71		8.55	49.1
c	1.39		3.97	106
d	2.04		2.52	167
e	2.67		1.57	265
f	4.43		0.92	450
g	6.00		[e]	[e]
Fig. 4				
b		1.22	[d]	[d]
c		2.45	[d]	[d]
d		4.06	20.90	22.05
e		5.51	15.80	29.17
f		7.63	11.70	39.39
g		9.44	9.50	48.5

[a] The experimental conditions are listed in the figure captions.

[b] The conditions are such that La^{3+} is present in some of the inverted micelles.

[c] It is not possible to determine τ accurately, since the data are in the fast exchange limit.

[d] The same as c above except that the data are in the slow exchange limit.

[e] The figures are not shown in the text.

not obtained. In general, the calculated curves are slightly lower than the experimental curves on the downfield sides and slightly higher on the upfield sides. We investigated this to some extent by varying the phase angles (the digitized accumulated free-induction decays were stored on magnetic

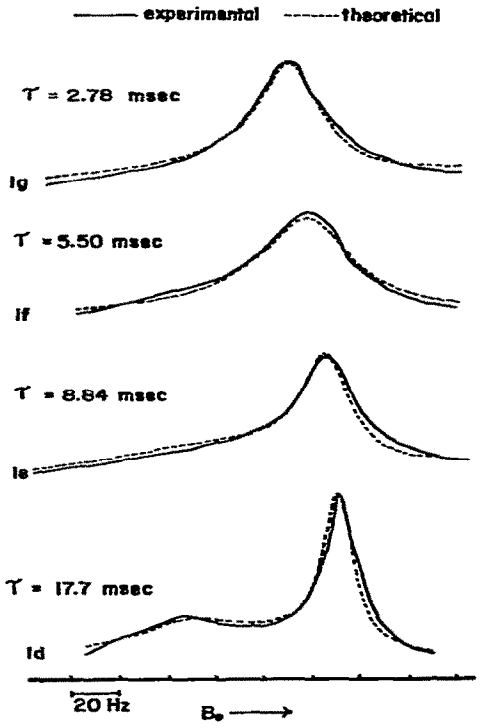
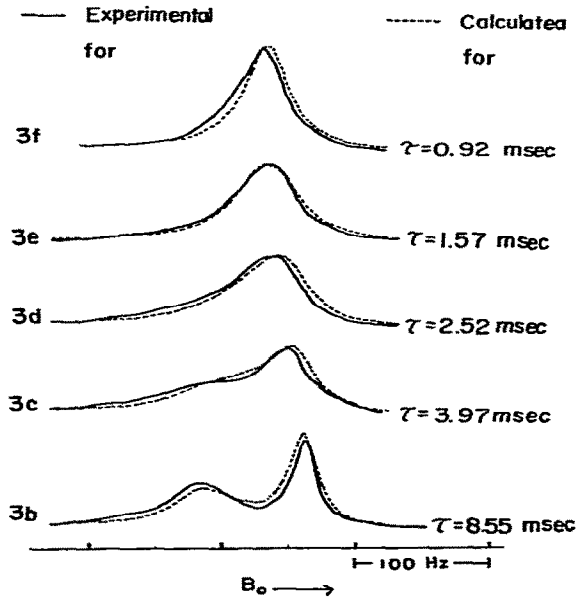


Fig. 5. Computer simulations for the spectra of fig. 1d–g. The solid lines represent experimental spectra while the dashed lines are calculated spectra. The value of τ corresponding to each calculated spectrum is given.



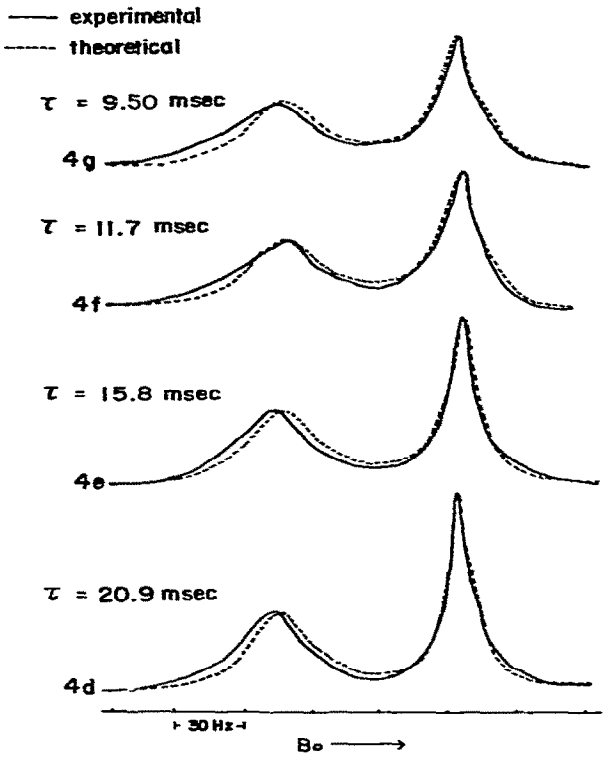


Fig. 7. Computer simulations for the spectra of fig. 4d–g. The solid lines are experimental spectra while the dashed lines are calculated spectra. The τ value corresponding to each calculated spectrum is given.

tape). We conclude that the more likely explanation for this small systematic discrepancy is the fact that we are ignoring the small, but detectable, amount of two Pr^{3+} micelles in our computer simulations. These always give rise to a small broad resonance downfield of the one Pr^{3+} micellar resonance [1]. The effect is small and does not affect the major conclusions we draw from these simulations (vide infra).

Fig. 6. Computer simulations for the spectra of fig. 3b–f. The solid lines represent the experimental spectra while the dashed lines represent calculated spectra. The value of τ corresponding to each calculated spectrum is given.

4. Discussion

It seems clear from the results described above that we see here a DNMR manifestation of a physical transport process: the lasalocid-A-facilitated transport of Pr^{3+} between hydrated phospholipid inverted micelles in benzene. In the course of our preparation of this paper, a similar DNMR effect, for the unfacilitated transport of acetic acid across vesicular bilayer membranes, has been reported [38]. Less closely related NMR relaxation studies of the dynamics of physical transport have been reported in the past [38–43].

The average lifetime, τ, varied as a parameter in our computer fittings, is a reduced lifetime for those of the two sites. The symbol τ_0

$$\tau^{-1} = \tau_0^{-1} + \tau_1^{-1} \tag{1a}$$

$$\tau = \frac{\tau_0 \cdot \tau_1}{\tau_0 + \tau_1} \tag{1b}$$

represents the average length of time a phosphorus nucleus spends in a micelle with no Pr^{3+} while τ_1 represents the average length of time in a micelle with one Pr^{3+}. Since we have attributed the observed exchange to Pr^{3+} transport, τ_0 and τ_1 also represent the average lifetimes for micelles to be without and with a Pr^{3+}, respectively. If the exchange is in equilibrium (evidence for this is presented in ref. [1]), then eq. (2) is valid

$$\frac{p(0)}{p(1)} = \frac{\tau_0}{\tau_1} \tag{2}$$

($p(0)$ and $p(1)$ are the fractional populations of the two sites) and τ_0 or τ_1 can be expressed in terms of τ*. For example:

$$\tau_1 = \left[1 + (p(1)/p(0))\right]\tau \tag{3}$$

The quantity τ_1 can be crudely related to the flow quantities of macroscopic membrane transport experiments. Consider scheme 1 which depicts a micelle containing one Pr^{3+} and a micelle without a Pr^{3+}. For 'Fickian' diffusion of a solute into and out of a spherical space, the rate of concentration change is given by eq. (4) [44]:

$$\frac{dC_1}{dt} = \frac{AP}{V}(C_0 - C_1) \tag{4}$$

where, C_1 and C_0 are the concentrations of the solute inside and outside the space, respectively, A and V are the average values of the surface area and volume of the space, respectively, and P is the permeability coefficient. In order to find the average lifetime of a Pr^{3+} in a micelle, we set C_0 equal to zero and rearrange eq. (4):

$$-\frac{dC_1/C_1}{dt} = \frac{AP}{V} \tag{5}$$

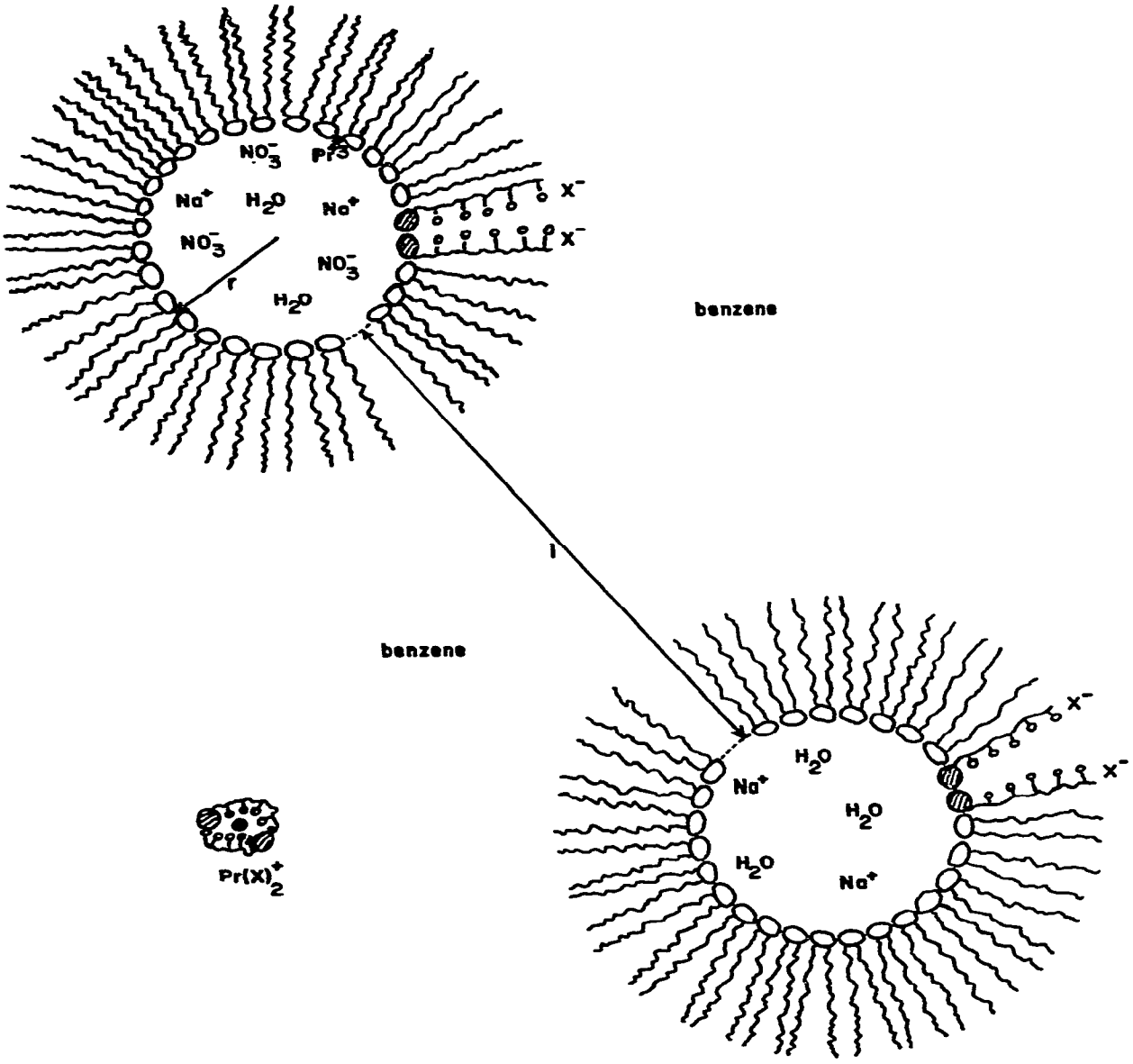
However, the left left-hand side of eq. (5) is the reciprocal average lifetime of a kinetic process [45]. Thus, we arrive at eq. (6),

$$\tau_1^{-1} = \frac{AP}{V} = \frac{3P}{r} \tag{6}$$

where r is the average radius of the aqueous core of the micelle (scheme 1). Strictly speaking, the right-hand side of eq. (6) should be multiplied by an electrostatic factor due to the fact that the diffusing particle in our case, Pr^{3+}, is charged [44]. However, we are mostly interested in trends in τ_1^{-1} and the constant electrostatic factor can be ignored for the present.

The major trend we can ascertain, of course, is the lasalocid-A molecularity of the transport process: since we effect change in τ_1^{-1} by changing the concentration of lasalocid-A (figs. 5–7). The molecularity of lasalocid-A-facilitated transport has been the subject of considerable investigation. Thus, it has been reported that the conductance of BLM due to Li(I) [46], Ca(II) [36,37,46,47], and Pr(III) [31] increases with the square of the lasalocid-A concentration. The tracer permeability coefficient of a BLM to radioactive $^{45}Ca^{2+}$ has been reported to increase with the square of the lasalocid-A concentration [48] as has the rate of transport of Mn^{2+} into sonicated phospholipid bilayer vesicles measured by an NMR technique [34,35,49]. This general result has been considered consistent with the fact that bis mononuclear complexes of divalent metal cations and mono dinuclear complexes of monovalent metal cations seem to be

* Eq. (3) was used to calculate the minimum value of τ_1 from $\tau_{coalescence}$ in ref. [1]. Note that there we erroneously multiplied the right-hand side by $\bar{n}$ in one instance (p. 31).



Scheme 1. Schematic representation (drawn to scale) of a micelle containing one Pr^{3+} and a micelle without Pr^{3+}

required for lasalocid-A sequestration of metal cations into bulk nonpolar phases [30] and thus strong support for a diffusional carrier mechanism employing such complexes. Two lasalocid-A molecules are required to complete a hydrophobic covering for the transported cation (s).

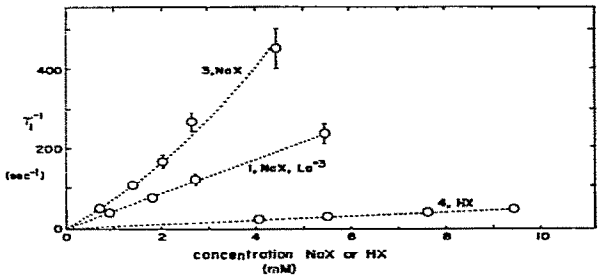
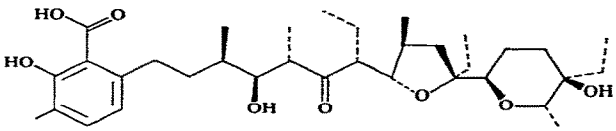


Fig. 8. Plots of (τ_1^{-1}) versus the concentration of lasalocid-A for the data of figs. 1, 3 and 4. (See table 1.) The dotted lines are not fittings but merely intended to guide the eye. The sizes of the error bars arise from the considerations of fitting accuracy discussed in the text.

Hence, it is interesting to see that when we plot τ_1^{-1} versus the concentration of lasalocid-A (fig. 8), we find reasonably good first-order dependences. (The fact that these lines extrapolate to the origin is considered strong support for the DNMR interpretation and is thus evidence against the sequestration interpretation of the observed lineshape changes.) From plots of $\ln(\tau_1^{-1})$ versus ln([lasalocid-A]) (not shown), we obtain slopes of 1.04 ± 0.10 (data of fig. 1), 0.95 ± 0.10 (replicate data of fig. 1, table 1), 1.20 ± 0.10 (data of fig. 3), and 0.93 ± 0.10 (data of fig. 4). Therefore, the permeability coefficient for Pr^{3+} transport in our system depends linearly on the lasalocid-A concentration. This may not be in such strong contrast to the results reported above as it first appears.

The $^{45}Ca^{2+}$ permeability data of Kafka and Holz [48] appear to us to be better fitted by a cubic dependence on lasalocid-A concentration than a quadratic dependence. Fluorescence studies of a process attributed to Ca^{2+} transport in sonicated phospholipid vesicles indicate that the dependence on lasalocid-A increases from near first order at low Ca^{2+} concentrations to greater than third order at higher Ca^{2+} concentrations [50]. Degani et al. [49] report that, at lasalocid-A/vesicle ratios less than 0.5, Mn^{2+} transport is dominated by a first-order pathway. Hunt [32] has found that the rate of transport of Pr^{3+} into sonicated phospholipid bilayer vesicles, as measured by an NMR technique, depends on the lasalocid-A concentration with an exponent of 1.28. (The only one of our data sets which shows significant deviation from linearity is that of fig. 3 (see fig. 8). This set shows a dependence with an exponent of 1.2 (vide supra), very similar to that of Hunt [32]. The conductance, due to Ca^{2+}, in a BLM depends on the square of the lasalocid-A concentration only over a limited concentration range, being of lower order at both lower and higher concentrations [37]. The same is true for the conductance, due to Pr^{3+}, at higher concentrations [31]. In any case, it appears that almost all transport mediated by lasalocid-A is electrically silent [25,48] and thus the molecularity determined by conductance studies may be that of a minor pathway.

The lifetime of an inverted micelle between collisions can be estimated from the Smoluchowski equation to be about 10^2 ns under our conditions. Thus, the pretransport lifetime of a Pr^{3+}, of the order of milliseconds (longer in the absence of ionophores [1]), indicates that micelle collisions, of themselves, are most ineffective in inducing transport even though they probably do so in the absence of ionophores [1]. Also, we have found that the average lifetime of Pr^{3+} bound to the salicylate end of lasalocid-A, in methanol, is of the order of microseconds.



Lasalocid-A

at 30°C [30,52]. Thus, the Pr^{3+}-lasalocid-A interaction is probably also broken and reformed many times for each transport event. The first-order dependence on lasalocid-A concentration observed here argues against some sort of mechanism requiring the collision of lasalocid-A-containing micelles (vide infra). Therefore, it seems most reasonable that a diffusional carrier mechanism is operative in this system. By this, we mean a mechanism in which lasalocid-A accompanies Pr^{3+} in transit from one micelle to another, even if this occurs at the time of collision between the two micelles. One can calculate that the average dis-

tance between the centers of adjacent inverted micelles is about 15.7 nm (see scheme 1). This means that the average distance from one aqueous core to an adjacent one is about 10.9 nm (the average value of r for the concentrations of fig. 1 is 2.4 nm, vide supra), or more than twice the thickness of the hydrocarbon region of a normal phospholipid bilayer membrane in the liquid crystalline state. Therefore, one would expect the necessity of at least bis complex formation in order to render the Pr^{3+} hydrophobic. The expectation of even tris complex formation, to yield a neutral species, would not be unprecedented [30]. However, if the addition of a second (or third) ionophoric ligand were the rate-limiting step (as postulated by Haynes et al. [50]), higher order dependence on lasalocid-A would be manifest. Thus, how can we interpret a first-order concentration dependence? It seems to us that aggregation of lasalocid-A molecules at the aqueous/hydrocarbon interface, independent of metal ion complexation or transport, (scheme 1), is the most reasonable hypothesis. Strong equilibrium surface aggregation would produce a first-order dependence on lasalocid-A concentration for transport irrespective of the molecularity of the aggregation [51].

There is independent evidence that lasalocid-A molecules do reside on the surfaces of monolayer vesicles, with their salicylate fluorophores in the aqueous phase [50,53]. However, we are not aware of any results in the literature which shed light on the state of aggregation of lasalocid-A bound to phospholipid membranes. The fluorescence studies of Haynes and co-workers [50] indicate that the bound ionophore exists in more than one form. It is certainly quite reasonable that lasalocid-A would aggregate in order to prevent exposure of its hydrophilic portions to the lipid hydrocarbon chains. Lasalocid-A is known to be a flexible molecule, able to adopt a number of difference conformations in response to its environment [30,54,55]. Model building indicates that it can even take on a reasonably linear conformation, nearly equal to the length of a phospholipid hydrocarbon chain, with all of the oxygen atoms on one side [33]. Aggregation of such structures, with hydrophilic sides together, finds precedent in the postulated aggregates of the polypeptide antibiotic alamethicin [56] and the antiobiotics nystatin and amphotericin B, although the latter also require sterol molecules [57,58].

The inverted micelle system investigated here might be capable of yielding even more information on diffusional carrier-mediated transport. An expression for the permeability coefficient, P, can be derived for a diffusional model [59,60]. This is shown in eq. (7),

$$\frac{1}{P} = \frac{l}{D \cdot K} + \frac{2\lambda}{D_{\text{inter}}} \tag{7}$$

where the quantity l represents the average distance between aqueous cores which the diffusing particle must traverse and λ the thickness of the interface (see scheme 1), D and D_{inter} are the diffusion coefficients for the diffusing particle (the complex, in the carrier model) in the region between the aqueous cores and across the interface, respectively, and K represents the partition coefficient for the particle into the region between the aqueous cores. The second term, arising from the two interfaces which must be crossed in the transport event, has often been dropped [38,61]. When this is done the value of l includes the 2λ and the D_{inter} factor becomes implicitly incorporated into D. If the transported species spends a significant fraction of time in the interfacial region (this might be quite realistic for the current case), one might wish to include a partition factor in the denominator of the second term of eq. (7). Combinding eqs. (7) and (6) and multiplying the right-hand side by the afore-mentioned electrostatic factor [44], we arrive at eq. (8),

$$\tau_1 = \frac{r}{3}\left\{\frac{l}{D \cdot K} + \frac{2\lambda}{D_{\text{inter}}}\right\} \times \frac{RT(1-\exp(-zEF/RT))}{zEF} \tag{8}$$

where z is the charge on the diffusing particle, E the electrical potential drop across the space between the aqueous cores, F the Faraday constant and R and T have their usual meanings. The inverted micelle system may allow a degree of control over some of the parameters in eq. (8). As discussed above and in ref. [1], the magnitude of r can be controlled over a certain range by varying

the water-to-phospholipid (PL) molar ratio. If the transport event is completely independent of micellar collisions the value of l can be controlled, independently of r, by adjusting the mole fraction of nonpolar solvent while keeping $[H_2O]/[PL]$ constant. Since a number of different nonpolar solvents, with some variation in polarity, can support the formation of inverted micelles [1], one can vary the product $D \cdot K$ over a restricted range. The use of different kinds of lipid molecules should offer some limited control over λ/D_{inter}. Finally, of course, one can control the temperature. Temperature variations would affect the magnitudes of D, K and D_{inter}. One could obtain the activation parameters (ΔH^* and ΔS^*) for the transport process *.

Fig. 8 clearly shows the inhibition of the transport of Pr^{3+} by La^{3+} and H^+. We presume that both of these are manifestations of competitive inhibition although they probably differ in detail. La^{3+} certainly competes directly for the same binding site on lasalocid-A occupied by Pr^{3+}. H^+ more likely inhibits only the initial binding step, presumably at the salicylate end of the antibiotic molecule [30]. It would be of interest to accomplish a three-site exchange fitting of a series of spectra such as those in fig. 2d–f. This might shed additional light on the mechanistic details.

Fig. 9 depicts spectra of a series of solutions very similar to those of fig. 1 except that the ionophore valinomycin is added instead of lasalocid-A. Valinomycin is perhaps thought of as the archetypical example of a diffusional carrier [36]. As can be seen from fig. 9, valinomycin appears to cause little if any transport of Pr^{3+} on the NMR time scale. This could be interpreted to mean that valinomycin does not act as a carrier. Although seemingly heretical, there have been 'half-pore' mechanisms postulated for bilayer transport facilitated by valinomycin [62]. More likely, however, the negative result here simply means that valinomycin cannot transport Pr^{3+} rapidly. This ionophore is known to be rather K^+ specific [36]. One could say that if a molecule could not act as a diffusional carrier under any circumstances, it would not be able to catalyze metal ion exchange in the inverted micelle system to be fast on the NMR time scale.

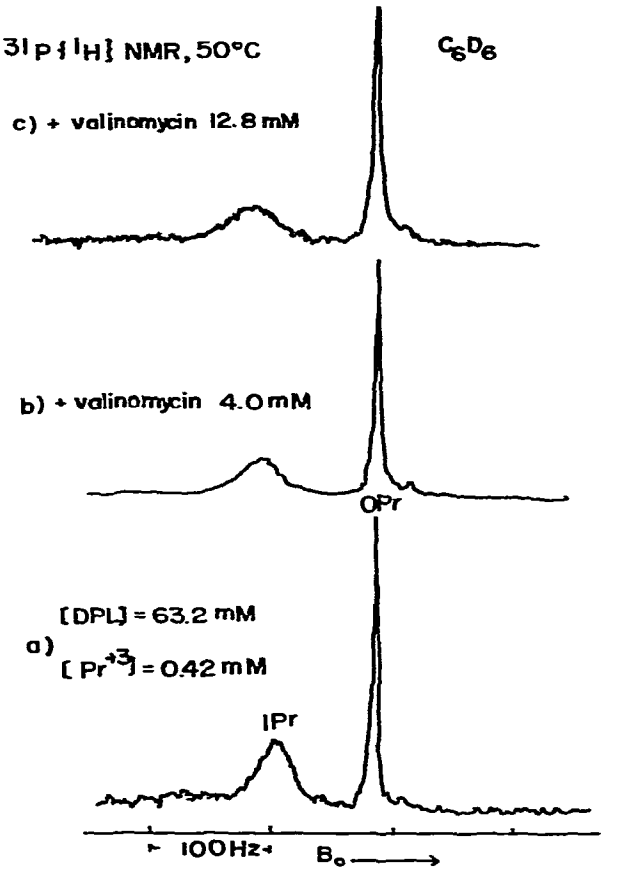


Fig. 9. $^{31}P\{^1H\}$ NMR spectra of sonicated DPL/water dispersions in benzene at 50°C. (a) Dispersion of inverted micelles with total (stoichiometric) concentrations of 0.42 mM $Pr(NO_3)_3$, 926 mM D_2O and 63.2 mM DPL. These concentrations are based on the total solution volume. (b) Solution of a made 4.0 mM in valinomycin. (c) Solution of a made 12.8 mM in valinomycin.

* Preliminary experiments with benzene/DPL inverted micellar solutions in our laboratories have indicated some kind of irreversible decomposition when the temperature is raised to 70°C (David Yarmush, personal communication). Experiments at lower temperatures with egg lecithin and other lipids with lower phase transition temperatures are certainly quite feasible.

5. Implications for lasalocid-A-facilitated transport across bilayers

It surface aggregation of lasalocid-A molecules is occurring in the inverted micelle system here, it would seem that such aggregation would be even more likely for lasalocid-A in the phospholipid bilayer milieu. Hence, bilayer lasalocid-A transport molecularities greater than unity might not necessarily imply the formation of a bis diffusional carrier complex (molecularities greater than two are a problem for this model in any case). Instead, such results might reflect the statistics of encounters involving one aggregate in each monolayer. If the aggregates were able to act as half pores, then such encounters would transiently yield whole pores which could affect the translocation of Pr^{3+}. Such a mechanism has precedents in those postulated for the antibiotics gramicidin A [63], nystatin [57] and amphotericin B [57], and for ion translocation mediated by lysolecithin [51]. Such a mechanism can be easily shown to give rise to higher molecularities (higher even than two) as the ionophore concentration in the membrane is lowered [51]. Degani et al. [49] have reported an activation energy of 9 kcal/mole for the lasalocid-A-mediated transport of Mn^{2+} across the bilayer of a sonicated DPL vesicle in the liquid crystalline state. This value is seemingly quite low for a carrier mechanism and is more in line with a pore mechanism [51].

Molecularities less than two, sometimes reported for lasalocid-A bilayer transport at low antibiotic concentrations (vide supra), where the occurrence of a transient pore simply becomes very improbable, could be explained by a switch to a carrier mechanism similar to that postulated here for transport in the inverted micelle system. It is very hard to determine the membrane lasalocid-A concentrations in most literature reports because the amount of ionophore actually partitioned from the aqueous phase into the membrane is not known. If we assume all lasalocid-A to be so partitioned, the concentrations studied by Degani et al. [34,35,49] range from 0.05 to 2.9 mole X-537A/1000 mole PL, while those of Hunt [32] range from 1.2 to 5.0 mole X-537A/1000 mole PL, in their respective studies with sonicated bilayer vesicles. Our concentration ratios cover a much larger range from 0 to 122 mole X-537A/1000 mole PL; of course for the very different inverted micelle system. Perhaps, by separating the monolayers, the inverted micelles have shown us to expect a molecularity of unity for each monolayer in the combined bilayer structure, at least at normal lasalocid-A concentrations

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