Biophysical Chemistry, 33 (1989) 265–275
Elsevier

BIOCHE 01361

Physical characterization of and ionophore-mediated europium(III) transport through unilamellar phosphatidylcholine vesicles

A laser-induced europium(III) luminescence spectroscopy study

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Received 14 November 1988
Revised manuscript received 14 March 1989
Accepted 16 March 1989

Europium(III) luminescence; Laser spectroscopy; Phospholipid bilayer vesicle; Ion transport; Mobile carrier mechanism; Phosphatidylcholine

A continuation of the study of phospholipid bilayer vesicles as model membrane systems by laser-induced europium(III) luminescence spectroscopy is presented here (B.M. Cader and W. DeW. Horrocks, Jr, Biophys. Chem. 32 (1988) 97). This spectroscopic technique was used to characterize further the physical properties of small and large vesicles composed of dipalmitoylphosphatidylcholine and egg phosphatidylcholine, respectively. Unilamellar preparations were confirmed and internal aqueous volumes were calculated. The calcium-binding carboxylic ionophores, lasalocid A and A23187, were incorporated into the lipid bilayers of these vesicles for the purpose of modeling the mobile carrier mechanism of ion transport across cell membranes. Spectroscopic data implicate the presence of 1:1 and 1:2 europium(III)/lasalocid A complexes within the hydrophobic region, both capable of efficient transport and containing no water molecules in the inner sphere of europium(III). First-order rate constants for lasalocid A-mediated europium(III) transport were determined at 37 and 62° C (0.018 and 0.11 min^{-1}, respectively) using EGTA as a 'flag' to bind and detect the post-transported metal ion.

1. Introduction

A number of hypotheses have resulted from efforts to elucidate the mechanism(s) by which metal ions are transported across biological membranes [2,3]. One of the most popular theories, the mobile carrier mechanism, involves the presence of organic-soluble molecules that bind aquated cations on one side of the membrane, diffuse through the central hydrophobic region of the membrane, and dissociate on the other side, releasing the ions into another aqueous medium. These molecules, known as ionophores, have the ability to function as efficient mobile carriers within biomolecular lipid or artificial membranes and possess the following characteristics: (1) a polar interior with sufficient ligating atoms (e.g., ether, alcohol, carbonyl, and carboxyl oxygens; nitrogens of substituted amines) to replace some or all of the solvent molecules in the primary solvation sphere of the cation; (2) a nonpolar exterior which 'masks' the cation and allows it to be solubilized in a hydrophobic medium; (3) a high degree of cation selectivity; (4) binding constants large enough to bind cations effectively, yet small enough to release them efficiently; (5) complexation-decomplexation kinetics and diffusion rates favorable enough to allow high transport turnover numbers (> 500 s^{-1} across biological membranes).

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Ionophores fall into two classes; the first, naturally occurring antibiotics, is further divided into neutral ionophores (which contain no dissociable protons) and carboxylic ionophores (which contain a terminal carboxylic acid functionality that deprotonates upon complexation). The other category consists of synthetic ionophores such as the cyclic crown ethers and cryptands. This study focuses on lasalocid A (X-537A), as well as A23187 (calcimycin), the calcium-binding, carboxylic ionophores isolated from bacteria [4].

The effectiveness of Eu(III) as a substitutional probe for the spectroscopically and magnetically silent Ca(II) ion has been detailed in our earlier publication involving the characterization of Eu(III) interactions with the head group regions of phospholipid bilayer vesicles [1]. The laser-induced Eu(III) ion luminescence technique used in that study has been further exploited here to investigate metal ion transport phenomena across model biological membranes, viz., unilamellar phosphatidylcholine vesicles.

The ability of Eu(III) to luminesce in solution at room temperature and its possession of nondegenerate ground ($^7F_0$) and excited ($^5D_0$) electronic states, separated by an energy of approx. 17250 cm^{-1} (~ 580 nm), have allowed the development of convenient and useful spectroscopic methods. The nondegeneracy of the $^7F_0 \rightarrow {}^5D_0$ transition precludes any ligand field splitting and, barring coincidental superimposition of two or more bands, results in excitation spectra containing a single band for each unique Eu(III) environment. In addition, a relationship exists between the frequency of the $^7F_0 \rightarrow {}^5D_0$ transition and the total electrostatic charge of the ligands coordinated to Eu(III) [5], as well as between the number of water molecules in the primary coordination sphere of Eu(III) and the difference in $^5D_0$ excited-state lifetimes in H_2O and ^2H_2O [6].

Numerous investigations of phospholipid bilayer vesicles as model systems for the mobile carrier theory of ion transport have been reported in the literature. Most of these involve the application of ^1H- or ^{31}P-NMR to determine ionophore-mediated transport rates of paramagnetic lanthanide (Ln(III)) ions such as Pr(III) and Eu (III) [7,8]. These experiments are generally based on chemical shift or coupling constant measurements of phospholipid head group nuclei (*N*-methyl protons or phosphorus), using the paramagnetic species to differentiate between the inner and outer head group regions. The data accumulated were used to derive information regarding transport rates and stoichiometry. Discrepancies exist concerning the stoichiometry of the transporting complexes in Ln(III)/lasalocid A and Ln(III)/A23187 systems; both 1:1 and 1:2 metal : ligand species have been implicated [8–10].

The NMR techniques mentioned above are useful, but limited in that they do not involve direct examination of the transporting species. The direct observation of metal ions and high sensitivity of laser-induced Eu(III) luminescence make it a potentially valuable tool for determining the stoichiometry of these species, as well as to observe and quantitate rate processes. An additional application of the luminescence technique reported herein is the determination of the average encapsulated aqueous volume within a system of phospholipid bilayer vesicles.

2. Materials and methods

The chemicals used in this study were employed without further purification and are listed with their sources as follows. L-α-Dipalmitoylphosphatidylcholine (DPPC, synthetic, ~ 99 + %), L-α-phosphatidylcholine (EPC, type V-E from frozen egg yolk, ~ 99%), EGTA (97–98%), octylglucoside (crystalline), Triton X-100 (octyl phenoxy polyethoxyethanol), Sepharose 4B and Sephadex G-50–80 were obtained from Sigma. $EuCl_3 \cdot 6H_2O$ (99.9%) and lasalocid A (Las, sodium salt, 99 + %) were purchased from Aldrich. A23187 (free acid) was obtained from Calbiochem-Behring Corp.

Small unilamellar vesicles (SUVs) were ultrasonically prepared as described previously [1], using 20 mg DPPC dispersed in 2 ml buffer. The buffer system consisted of 10 mM piperazine (Sigma, laboratory grade), 150 mM KCl (Fisher, ACS grade), in either ^1H_2O or ^2H_2O at pH 6.0. For those cases in which Eu(III) was encapsulated in the SUVs, 20 μM or 1 mM $EuCl_3$ was added to

the buffer prior to ultrasonication. Subsequently, external $EuCl_3$ was removed from the vesicle sample using the Sephadex minicolumn technique of Fry et al. [11]. A 1-ml aliquot was used for each spectroscopic study.

Larger vesicles were prepared by the detergent dialysis method of Philippot et al. [12], using 15 mg EPC and 60 mg octylglucoside in an identical buffer system to that described above, with or without 1 mM $EuCl_3$. When necessary, the vesicle samples were passed through a Sepharose 4B column equilibrated with buffer at 4°C, to remove external $EuCl_3$. For each of the internal volume determinations and the quantitative rate experiments, 1 ml of the vesicle preparation was diluted to 2 ml with buffer prior to spectroscopic analysis.

For those studies requiring the presence of Eu(III) in the external aqueous medium only, appropriate microliter additions to the vesicle samples were made from a 1 mM $EuCl_3$/buffer stock solution. Ionophore additions were made from 1 or 10 mM ionophore/methanol stock solutions. All volumetric measurements were performed with Eppendorf digital pipets (±1%).

Phosphorus determinations were accomplished with the $Mg(NO_3)_2$ digestion method of Ames and Dubin [13] with the inclusion of an additional digestion step involving 60 μl of 72% perchloric acid. The samples were incubated for 30 min at 60°C; absorbances were measured at 625 nm.

The pulsed Nd-YAG-pumped tunable dye laser described previously was used to obtain Eu(III) $^7F_0 \rightarrow {}^5D_0$ excitation spectral and lifetime data [1,14]. Vesicle scattering information was obtained from a Cary 210 spectrophotometer. All spectroscopic studies were performed by using a Teflon-capped quartz cuvette with a 1 cm path length.

The Gaussian/Lorentzian spectral peak deconvolution and excited-state lifetime exponential decay analyses were performed using computer programs developed in this laboratory, which are based on the nonlinear regression method of Marquardt. Complete discussions of these programs have been previously documented [1,15].

The quantitative ionophore-mediated transport data were collected as follows and analyzed assuming first-order kinetics. The dye laser was tuned to 580.1 nm, allowing only the excitation of $EuEGTA^-$. Data collection was initiated prior to the addition of EGTA and continued as EGTA, ionophore, and detergent were added at the appropriate times during the course of the experiment. This procedure resulted in a plot of intensity at 580.1 nm vs. time, in which each point represented the signal intensity averaged over a set period of time. Typically, the gate box was set at 40 pulses per channel and, given a laser pulsing frequency of 10 Hz, this corresponded to 4 s per channel. The intensity in every twenty-fifth channel (this number varied with the length of the experiment), beginning with the addition of the ionophore (time zero), was corrected for signal present before time zero. This value, denoted by I_t, was used to determine the rate constant from eq. 2 (vide infra). In some cases, the data contained within a specific number of channels were averaged and used for the logarithmic plot. The intensity at $t = \infty$, I_∞, is defined as the intensity beyond the point at which transport had ceased, i.e., the leveling off of the $EuEGTA^-$ signal.

The intensity of the $EuEGTA^-$ signal at any given time, I_t, is related to the rate constant, k, through eq. 1:

$$I_t = I_\infty - I_\infty e^{-kt} \tag{1}$$

Rearranging eq. 1 and taking the natural logarithm gives a linear expression (eq. 2)

$$\ln\left(\frac{I_\infty - I_t}{I_\infty}\right) = -kt \tag{2}$$

and enables k to be determined from a plot of $\ln\{(I_\infty - I_t)/I_\infty\}$ vs t.

3. Results and discussion

3.1. Determination of average internal volume of phospholipid bilayer vesicles

The physical characterization of a model system is an essential precursor to the performance of actual model studies. A number of experiments

have been carried out on phospholipid bilayer vesicles which not only reveal information regarding their physical properties, but also demonstrate the utility of the laser-induced Eu(III) luminescence technique for the study of these systems. Vesicle formation, integrity, and temperature dependence of Eu(III) binding to vesicles have been discussed in a recent article [1]; this paper presents a method for the determination of the average internal volume (and, concomitantly, the average diameter) for a sample of phospholipid bilayer vesicles prepared by detergent dialysis. In addition, a means is presented to quantitate the average concentration of Eu(III) encapsulated in a vesicle sample and therefore, the initial concentration of Eu(III) in a given transport experiment.

In a typical internal volume determination, phospholipid bilayer vesicles were produced by detergent dialysis of egg phosphatidylcholine in a 1.2 mM $EuCl_3$ medium. With Eu(III) incorporated in the internal aqueous volume and all external Eu(III) removed, detergent (Triton X-100) was added to resolubilize the vesicle sample and free the entrapped Eu(III) in the presence of excess EGTA. The intensity of the $EuEGTA^- {}^7F_0 \rightarrow {}^5D_0$ excitation signal at 580.10 nm was measured and compared with a linear calibration plot of intensity vs. concentration of known $EuEGTA^-$ solutions; the total concentration of Eu(III) in the vesicle sample was determined from this line to be 3.8 μM for a 2.0 ml aliquot. Since the internal Eu(III) concentration, prior to solubilization, was identical to that of the medium in which the vesicles were formed (1.2 mM), the fraction encapsulated by the lipid is simply the dilution factor, 3.8 μM/1200 μM = 0.0032 or 6.4 μl of the 2.0 ml total volume.

This information is used to calculate the average diameter and the internal aqueous volume of an average vesicle in a particular sample. An assay of the above sample for phosphorus determined the total amount of lipid to be 7.0×10^{-7} mol per 2.0 ml sample. The validity of the Eu(III) luminescence and phosphorus assay results can be evaluated from the following formulation. Eq. 3 expresses the relationship between the total internal volume, V_{TI}, determined by Eu(III) luminescence to be 6.4 μl and the radius of the internal aqueous volume of a single vesicle, r_I, in a homogeneous sample containing N_V vesicles:

$$V_{TI} = \tfrac{4}{3}\pi r_I^3 N_V \tag{3}$$

The total volume of the lipid (PC) bilayer in the sample, V_B, is calculated according to eq. 4:

$$V_B = 1250(\text{mol PC} \cdot N_A) = \tfrac{4}{3}\pi\left(r_T^3 - r_I^3\right)N_V, \tag{4}$$

where N_A is Avogadro's number, r_T the total vesicle radius, and 1250 the volume of an average PC molecule in Å^3 [16]. In addition, the literature value of approx. 40 Å for the width of a PC bilayer relates r_T and r_I according to eq. 5 [16]:

$$r_T = r_I + 40 \tag{5}$$

Combination of eqs. 3–5 to eliminate the unknown quantity, N_V, and subsequent rearrangement yield eq. 6, which contains a single unknown, r_T.

$$\left(\frac{V_B + V_{TI}}{V_{TI}}\right)^{1/3} = \frac{r_T}{r_T - 40} \tag{6}$$

Solving for r_T requires knowledge of V_B and V_{TI}. As previously mentioned, V_{TI} was determined experimentally by Eu(III) luminescence; a total internal aqueous volume of 6.4 μl or 6.4×10^{21} Å^3 was obtained for a typical experiment. V_B was calculated (eq. 4) from the amount of phospholipid in the vesicle sample (7.0×10^{-7} mol), which was determined by a phosphorus assay, yielding a total bilayer volume of 5.3×10^{20} Å^3. With this information in hand, r_T may be calculated from eq. 6; $r_T = 1530$ Å. This corresponds to an average diameter of approx. 3000 Å, which is in good agreement with the previously reported electron micrographs [1], as well as with the results reported by the developers of the detergent dialysis method of vesicle preparation [12] *.

* One can simplify this expression even further by assuming, as suggested by a referee, that the vesicle size is large compared to the width of the lipid bilayer and therefore, $r_T = r_I$. V_B can now be described as $(dV_{TI}/dr_I)\,\Delta r N_V$ which is equal to $4\pi r_I^2 \Delta r N_V$, where $\Delta r = 40$ Å. By dividing this form of V_B into that given above for V_{TI} and rearranging, one obtains $r = 3\Delta r V_{TI}/V_B$. Substitution of the appropriate values yields $r = 1455$ Å, which is in good agreement with that reported above.

It is important that the vesicle preparations be unilamellar, as this feature is necessary for the quantitative rate experiments discussed in section 3.5.2. The good agreement between the luminescence and electron microscopy data is evidence for the unilamellar character (single bilayer) of the vesicles. The presence of concentric bilayers within each vesicle (i.e., multilamellar) would result in the prediction of a smaller average radius, since the amount of Eu(III) incorporated in the internal aqueous volume would be lower and V_{TI} would be smaller. Further evidence for unilamellar vesicles is presented in section 3.5.2.

Attempts to determine the average diameter of ultrasonically prepared vesicles (SUVs) resulted in values over an order of magnitude too high (~ 14000 Å from Eu(III) luminescence; ~ 250 Å from electron micrographs [1]). The $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of SUVs sonicated in the presence of Eu(III) contains a single peak centered at 579.25 nm, even after complete removal of the external metal ion. The Eu(III) environment corresponding to this signal, discussed in detail in the previous manuscript [1], is located within the phosphatidylcholine head group. All Eu(III) contained in the outer monolayer (ultrasonically prepared vesicles contain no more than one bilayer due to the high radius curvature), as well as in the external aqueous space, is effectively removed by adding EGTA to the external aqueous region and passing the sample through a Sephadex minicolumn. This $^7F_0 \rightarrow {}^5D_0$ band is intense enough to obliterate the relatively less intense signal which may be due to solvated Eu(III) encapsulated in the internal aqueous space. The entrapment of a fraction of Eu(III) in the inner monolayer has been attributed to the rapid rate of vesicle formation by this method of preparation. Indeed, this phenomenon would significantly increase the observed encapsulated Eu(III) concentration (and therefore, V_{TI}) as measured by luminescence, causing an excessively high radius to be calculated. Using the r_T measured from the electron micrographs (~ 125 Å), and substituting into eq. 6, a value of 8.3×10^{-5} ml for V_{TI} was obtained (from phosphorus assay, $V_B = 1.8 \times 10^{20}$ Å^3 per 1.0 ml aliquot). This is only approx. 1% of the total internal volume determined from the Eu(III) luminescence experiment, implying that very little Eu(III) was actually contained within the aqueous cavity (from Eu(III) luminescence, $V_{TI} = 0.011$ ml per 1.0 ml aliquot). In addition, the proposed location of the excess Eu(III) is not unreasonable since these data correspond to only one Eu(III) ion for every 20 phospholipid molecules in a vesicle.

3.2. *The effects of methanol on aqueous vesicle solutions*

The insolubility of ionophores in water, the medium in which phospholipid bilayer vesicles are formed, precludes the use of aqueous ionophore stock solutions in any of the ionophore/vesicle studies discussed in this article. For this reason, methanol was chosen as the most reasonable solvent for ionophore stock preparation, since the ionophores are soluble in it and it is highly miscible with water. A number of experiments were performed to determine the effects of methanol on the components of a vesicle/metal ion transport system and, in particular, to evaluate the aqueous environment as seen by the Eu(III), and the integrity of the vesicles.

A plot of the excited-state lifetime of a 1 mM Eu(III) solution (10 mM piperazine, 150 mM KCl, pH 6.0) as a function of percent methanol (by volume) is shown in fig. 1. The lifetimes plotted were derived from the observed single-exponential decay curves and represent weighted averages of the Eu(III)/H_2O/CH_3OH species present. It ap-

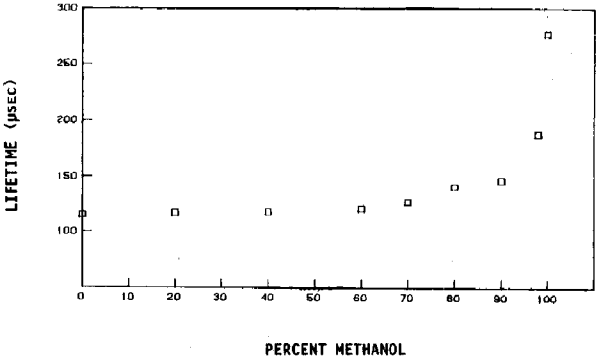

Fig. 1. Plot of excited-state lifetime (λ_{ex} = 578.95 nm) of 1 mM Eu(III) vs. percent methanol in water (λ_{max} (nm) = 578.90 (H_2O); 579.00 (CH_3OH)).

pears that methanol does not compete effectively with water as a ligand for Eu(III) until it reaches approx. 70% by volume. Indeed, the higher affinity of Eu(III) for water over methanol is reflected in the fact that H_2O-bound species are major contributors to the overall lifetime at 90, and even 98% methanol.

Methanol additions to phospholipid bilayer vesicles at temperatures greater than that of the gel → liquid-crystalline phase transition (T_c) do not affect vesicle stability. Additions below T_c, on the other hand, result in a marked increase in turbidity of the vesicle sample. Upon addition of 200 μl MeOH to 0.5 ml of a DPPC vesicle preparation (~ 5 mg lipid) in the presence of Eu(III) at 78° C, the sample maintained its relatively clear, opalescent character for a period of time exceeding that of an experiment; lowering the temperature below T_c caused an immediate clouding of the sample.

To minimize the effects of methanol, all ionophore/vesicle experiments were carried out using microliter additions of ionophore stock solutions to yield a total methanol content no greater than 15%.

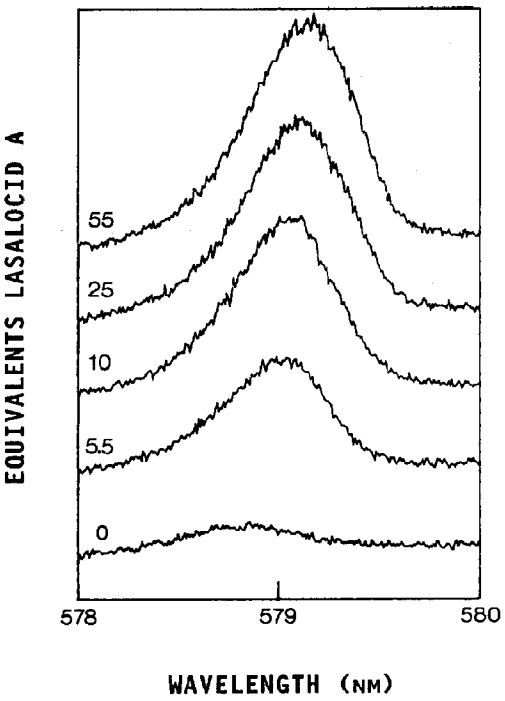

Fig. 2. $^7F_0 \rightarrow {}^5D_0$ excitation spectra of DPPC vesicles with external Eu(III) vs. equivalents lasalocid A at 70° C (all intensities are relative). DPPC/Las = 0, 150, 80, 30, 15.

3.3. Proof of ionophore incorporation into phospholipid bilayers

Ionophore incorporation into the hydrophobic lipid bilayer of phospholipid vesicles is demonstrated at temperatures above T_c as follows. For DPPC vesicles in their fluid, liquid-crystalline state (> 41° C), lasalocid A (Las) intercalates into the bilayers in the presence of Eu(III) in the internal and external aqueous regions as demonstrated by the $^7F_0 \rightarrow {}^5D_0$ excitation spectra (fig. 2). As successive aliquots of a Las stock in methanol were added, a significant shift from the ionophore-free spectrum occurred, along with an increase in intensity of the $^7F_0 \rightarrow {}^5D_0$ excitation peak maximum. Since ionophores do not bind metal ions in water [4], the lipid bilayer is the only medium in which a Eu(III)-ionophore complex can exist.

Further evidence for this phenomenon resulted from exploitation of the nonradiative deexcitation pathway provided by the ionophore A23187, described elsewhere in detail [4]. Addition of this ionophore to a sample of Eu(III)/DPPC vesicles at a temperature greater than T_c resulted in complete quenching of Eu(III) luminescence upon excitation of the $^7F_0 \rightarrow {}^5D_0$ transition, in accord with the findings in pure methanol [17]. This observation is consistent with Eu(III)-ionophore complexation being limited to the lipid bilayer.

The degree of light scattering (apparent absorbancy at 400 nm) of DPPC vesicles was measured over the course of a typical Las titration at 70° C (see section 3.4), in an attempt to detect a precipitated species in the aqueous medium. No change was observed upon addition of increasing amounts of Las (and methanol), consistent with quantitative ionophore incorporation into the bilayer.

3.4. Direct examination of Eu(III)/ionophore complexes within a phospholipid bilayer by Eu(III) luminescence

Owing to the lack of an $^7F_0 \rightarrow {}^5D_0$ excitation spectrum for A23187-bound Eu(III) species, this

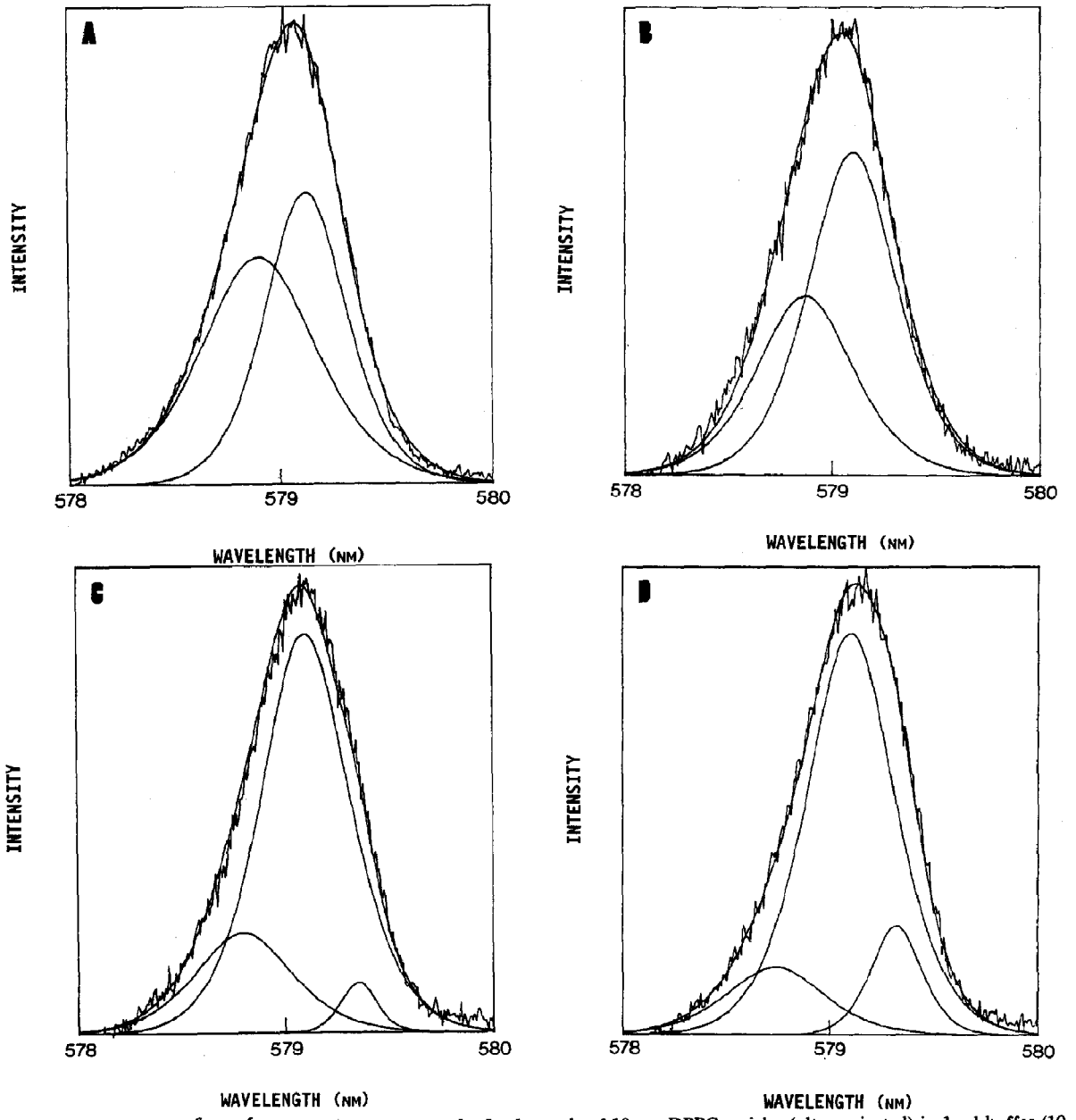

Fig. 3. Curve resolved $^7F_0 \rightarrow {}^5D_0$ excitation spectrum of a 1 ml sample of 10 mg DPPC vesicles (ultrasonicated) in 1 ml buffer (10 mM piperazine, 150 mM KCl, pH 6.0) with 20 μM $EuCl_3$ and (A) 5.5, (B) 10, (C) 25, (D) 55 equiv. Las. $T = 73°C$; λ_{max} (nm) and full-width and half-maximum (nm, cm^{-1}): 578.75, 0.67, 20 (free Eu^{3+}); 579.10, 0.51, 15 (EuLas); 579.35, 0.24, 7 ($Eu(Las)_2$).

section deals only with the characterization of Eu(III)/lasalocid A complexes in the lipid bilayer. Eu(III) luminescence reveals the presence of two types of metal ion/ionophore complexes during the titration of Las into a solution of Eu(III)-containing DPPC vesicles at a temperature above T_c. Gaussian/Lorentzian curve resolution analyses of the individual spectra discussed in section 3.3

implicate the initial formation of a 1:1 Eu(III):Las species with the coordination of a second ionophore ligand occurring at ligand-to-metal molar ratios greater than 10 (fig. 3A–D), with a lipid concentration of ~10 mM. Similar spectra (not shown) were obtained for the larger vesicles prepared with egg PC by detergent dialysis. Stoichiometries have been assigned based on the relative peak positions of 579.10 nm (1:1) and 579.35 nm (1:2); the red shift is consistent with a higher degree of complexation by a negatively charged species, as predicted by the $^7F_0 \rightarrow {}^5D_0$ transition energy/ligand charge correlation [5]. The presence of a third peak at 578.75 nm (in systems containing excess Eu(III) in the external aqueous region) is attributed to ionophore-free Eu(III); more specifically, the 'superficial' Eu(III) species described in our earlier paper [1], which actually represents the rapid equilibrium between the free hydrated Eu(III) ion and the phosphate-bound ion. The wavelength maxima for the ionophore complexes in the lipid bilayers are in excellent agreement with findings for the corresponding methanol system, with one exception [17]. Unlike the methanol case, no more than two Las molecules appear to be able to bind Eu(III) within the lipid bilayer; in fact, a relatively large excess of the ionophore is necessary to produce the bis complex. The probable cause of this discrepancy is that the lipid bilayer environment is considerably more hydrophobic and the initial sequestration by the ionophore is therefore more complete, which inhibits the coordination of additional ligands.

Eu(III) excited-state lifetime data were obtained for the mono(Las) species formed in DPPC vesicles prepared by ultrasonication in the presence of 20 μM $EuCl_3$. Studies in ^1H_2O and ^2H_2O systems were performed with the intention of determining the number of water molecules, if any, coordinated to the complex during Eu(III) transport through the lipid bilayer. The relationship between $^5D_0$ excited-state lifetimes and the number of O-H oscillators in the primary coordination sphere of Eu(III) has been previously discussed [1]. The lifetime values, derived from double-exponential decay curves, are listed in table 1.

In the absence of ionophore, the lifetimes generated correspond to the head-group-bound 'sequestered' and 'superficial' Eu(III) species referred to in section 3.3 and described in detail elsewhere (330, 350 and 180, 1400 μs, respectively, in ^1H_2O and ^2H_2O) [1]. Upon addition of Las at 73°C, no apparent change in values for the ^1H_2O system was observed, whereas the deuterated system showed significant alterations. Most notable is the absence of the 1400 μs species in ^2H_2O (which represents the highly solvated superficial species), indicating that the 180 μs lifetime observed in ^1H_2O with lasalocid A is not due to the 'superficial' species. A reasonable interpretation assigns this lifetime (as well as the 190 μs lifetime in ^2H_2O) to the mono(Las) complex, while the 330 and 450 μs values are attributed to a Eu(III) species containing no ionophore, similar, if not identical, to the 'sequestered' species. In addition, the 180 μs lifetime is consistent with that found in protonated methanol for the 1:1 Eu(III)/Las complex. Further comparison of these data to those obtained in methanol (for which a significant isotope effect was observed) [17] reveals a minimal change in lifetime of the mono(Las) complex due to lack of an isotope effect. These results imply that there are no water molecules contained within the inner coordination sphere of Eu(III) in the 1:1 transporting complex.

Table 1

Eu(III) excited-state lifetimes (in μs) of Eu(III)/DPPC vesicle systems in ^1H_2O and ^2H_2O at 73°C, with and without lasalocid A (λ_{ex} = 579.0 nm)

	^1H_2O	^2H_2O
No Las	330 [a]; 180 [b]	350 [a]; 1400 [b]
7 equiv. Las	330 [a]; 180 [c]	450 [a]; 190 [c]

[a] Sequestered (or similar) species.
[b] Superficial species.
[c] 1:1 Eu/Las species.

3.5. *The transport experiment*

The design of an optimal model ion-transport system for study by laser-induced Eu(III) luminescence involves detection of the post-transport species. Since this method of analysis focuses on the metal ion itself and since the metal ion is present during the transport process in both the

internal and external aqueous media (as well as being sequestered by the ionophore within the lipid bilayer), the design must allow for the discrimination of Eu(III) in its various environments. The system used to determine the average internal volume, in which Eu(III) is contained in the internal aqueous space with excess EGTA in the external volume, was chosen for the transport studies based on the following considerations. Firstly, EGTA in the external region serves as an excellent 'sink' for Eu(III) due to its high affinity ($K_b \approx 10^{17}$ M^{-1}) [18] and the $^7F_0 \rightarrow {}^5D_0$ excitation profile of the $EuEGTA^-$ complex ($\lambda_{max} = 580.1$ nm) does not overlap with excitation bands produced by any other Eu(III) species present during the course of a transport experiment. In addition, it has been previously shown that phospholipid bilayer vesicles with Eu(III) encapsulated in their internal aqueous cavities remain intact over a time period which exceeds the duration of a typical transport experiment [1]. This ensures that any Eu(III) detected externally is due to legitimate transport through the bilayer and not to leakage resulting from vesicle degradation.

It is generally agreed upon that the rate-limiting step in the Ln(III)/ionophore transport process is the binding of a metal ion to the ionophore at the membrane interface, as opposed to diffusion of the complex through the hydrophobic region [9]. If this is the case, the data produced in our experimental system should legitimately reflect the rate-limiting step, despite Eu(III) binding to EGTA in the external medium. The rate data presented herein were analyzed assuming first-order kinetics, as described in section 2.

3.5.1. Qualitative observations – The effect of varying Eu(III) / lasalocid A ratios

Initial transport experiments were conducted to evaluate the effectiveness of Eu(III) luminescence in qualitatively observing Eu(III) transport in phosphatidylcholine bilayer vesicles. The systems examined consisted of DPPC vesicles ultrasonically prepared in the presence of Eu(III). The greater part of the external Eu(III) was removed by passing the vesicle sample through a Sephadex mini-column, the remainder being tied up by the addition of excess EGTA to the external aqueous region.

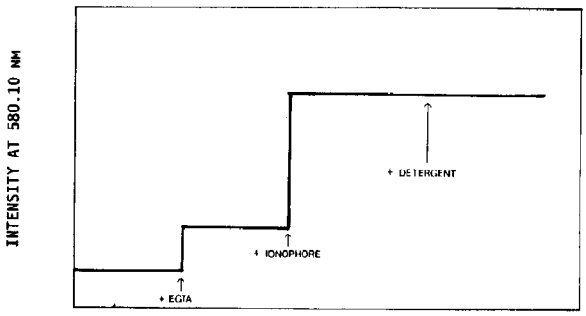

Fig. 4. Eu(III) transport experiment with excess lasalocid A within ultrasonicated DPPC vesicles ($EuEGTA^-$ signal monitored at 580.10 nm).

It was discovered that in the presence of a large excess of ionophore (either Las or A23187) over metal ion, Eu(III) is transported (only at a temperature greater than T_c) [19] from the internal to external aqueous region at a rate too high to measure (the formation of $EuEGTA^-$ was monitored at 580.10 nm). At this point, detergent (Triton X-100) was added to the vesicle sample for the purpose of solubilizing the lipid and allowing any remaining encapsulated Eu(III) to bind to EGTA. No increase in $EuEGTA^-$ signal was observed, indicating the complete transport of Eu(III) to the external aqueous region (fig. 4).

Analysis of an identical vesicle system to that described above, with the exception that only 2.5 equivalents of lasalocid A were added per Eu(III), yielded a nonlinear logarithmic plot. This biphasic curve is consistent with competitive binding, most likely with a sequestered Eu(III) site in the head group region, similar, if not identical, to that described previously [1] (the $^7F_0 \rightarrow {}^5D_0$ spectral data for ultrasonically prepared vesicles indicated that Eu(III) was contained in the head group region prior to ionophore addition; see section 3.1). Vesicles of larger diameter (such as those produced by detergent dialysis) are better systems to study because of their lower radii of curvature (making them more similar to natural cell membranes than the smaller, ultrasonicated vesicles),

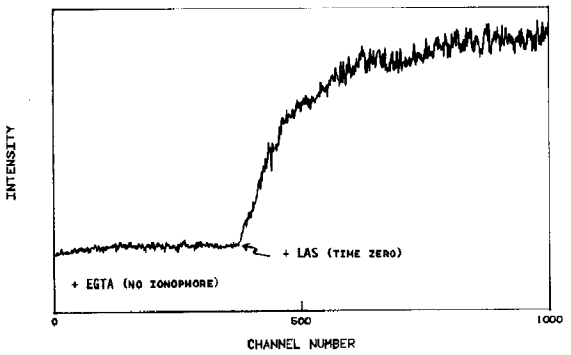

Fig. 5. Intensity at 580.10 nm as a function of time for Eu(III) transport through EPC vesicles at 62° C (40 laser pulses per channel; EPC/Las = 13).

greater capacity for solute encapsulation, and the absence of sequestered Eu(III) during formation.

3.5.2. Quantitative observations – The effect of temperature on transport rates

Large vesicles prepared by detergent dialysis of egg phosphatidylcholine were studied in an attempt to observe first-order kinetics of ionophore-mediated Eu(III) transport. A series of experiments was conducted under conditions where the 1 : 1 EuLas complex is the dominant metal ion-ionophore species present in the lipid bilayer (see section 3.4; $^7F_0 \rightarrow {}^5D_0$ spectrum contains a peak at 579.10 nm and no peak at 579.35 nm). A typical intensity vs. time plot is shown in fig. 5. All experimental runs produced linear logarithmic plots with excellent correlation coefficients (> -0.99; vide infra); an example is displayed in fig. 6.

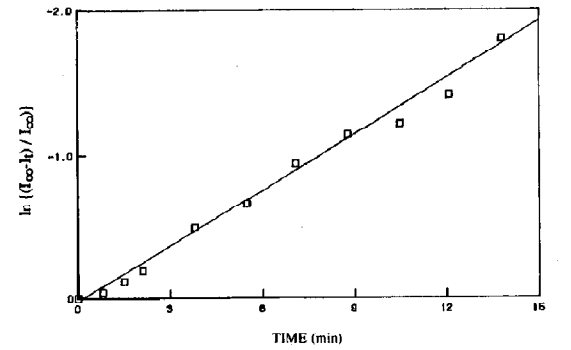

Fig. 6. Natural logarithm plot of intensity vs. time data for Eu(III) transport through EPC vesicles at 62° C; slope($-k$) = -0.13 min^{-1}, y-intercept = 0.035, correlation coefficient = -0.995 (11 points).

Table 2

Rate constants for lasalocid A-mediated transport of Eu(III) through large unilamellar phosphatidylcholine vesicles at 37 and 62° C ([Eu] = 1.9 μM; [Las] = 13 μM; [PC] = 0.17 mM)

Trial	k (min^{-1})	
	37° C	62° C
1	0.014	0.11
2	0.025	0.13
3	0.016	0.10

In order to demonstrate the utility of the laser-induced Eu(III) luminescence technique, the transport experiment was carried out at two temperatures: 37 and 62° C (both temperatures above the T_c for egg PC). The data are listed in table 2. The average rate constants were calculated to be 0.018 and 0.11 min^{-1} at 37 and 62° C, respectively.

Further evidence for the legitimacy of these experiments was obtained by addition of Triton X-100 to the vesicle sample after the EuEGTA$^-$ $^7F_0 \rightarrow {}^5D_0$ signal intensity leveled off. At this point, the sample turned from its opalescent appearance (typical of stable vesicles in aqueous media) to clear and colorless, indicative of complete lipid solubilization. In addition, the EuEGTA$^-$ intensity did not increase further, proving that Eu(III) had been completely removed from the internal aqueous region and transported to the external region by the ionophore. This result is also consistent with the presence only of unilamellar vesicles as opposed to the multilamellar variety. If the vesicles were composed of concentric bilayers, then the Eu(III) entrapped within the innermost aqueous region would not have been transported out by the ionophore (the ionophore would have been contained in the outer bilayer only). In such a case, a substantial increase in EuEGTA$^-$ signal intensity would have been observed upon lipid solubilization.

4. Conclusions

The utility of laser-induced Eu(III) luminescence spectroscopy in the study of small and large

phospholipid bilayer vesicles as model membrane systems has been demonstrated. Vesicles consisting of phosphatidylcholine, prepared by ultrasonication or detergent dialysis, are observed to be unilamellar. The average internal radius of the large vesicles prepared by detergent dialysis was determined from Eu(III) entrapment experiments and was found to be in excellent agreement with electron microscopy data. This method also revealed that significant quantities of Eu(III) were trapped within the bilayer region of the smaller vesicles during ultrasonication. This resulted in an excessively high apparent radius as compared to the corresponding electron micrographs.

The calcium-binding carboxylic ionophores, lasalocid A and A23187, were successfully incorporated into the phospholipid bilayers for the purpose of demonstrating, via Eu(III) luminescence, the mobile carrier mechanism theory of ion transport across cell membranes. Direct examination of ionophore-bound species in the bilayer indicated the presence of both 1:1 and 1:2 Eu(III)/Las complexes. Unlike the situation in methanol, higher order ionophore complexes do not appear to exist in the lipid bilayers. The mono and bis species contain no water molecules in the inner coordination sphere of Eu(III). The rate of ionophore-mediated transport of Eu(III) across the hydrophobic region was quantitatively observed by detecting the time-dependent appearance of Eu(III) in the outer aqueous region. At low ionophore-to-metal ion ratios (high lipid-to-ionophore ratios), conditions under which the 1:1 Eu(III)/Las complex prevailed in the lipid bilayer, competition between the ionophore and the phosphatidylcholine head group for Eu(III) precluded the evaluation of first-order rate constants in vesicle systems prepared by ultrasonication. Extremely large excess of ionophore (high concentration of 1:2 Eu(III)/Las in bilayer) resulted in transport events that were too rapid to measure by this technique. Transport experiments performed with vesicles prepared by detergent dialysis yielded linear first-order logarithmic data. Rate constants at 37 and 62°C were determined to be 0.018 and 0.11 min^{-1}, respectively.

In summary, the transport process was successfully monitored by laser-induced Eu(III) luminescence under conditions in which the 1:1 Eu(III)/Las species was the only ionophore complex present, and also under conditions where the 1:2 species prevailed. Both species appear able to transport Eu(III) across the lipid bilayer, the bis(Las) complex being the more efficient of the two.

Acknowledgements

The authors wish to thank Charles W. McNemar and Patrick J. Breen for the data analysis programs, Anjali Gharpure for assistance with the phosphorus assays, and the National Science Foundation for its support.

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