unrecognized mechanism by which thyroid hormone might affect any of several important physiological processes.

ACKNOWLEDGMENTS

We thank Dr. J. E. Rall for his interest and advice during the course of this work. We also thank Dr. J. Robbins and Dr. J. Wolff for their critical reading of the manuscript.

Registry No. T_3, 6893-02-3; protease inhibitor, 37205-61-1; contrapsin, 80700-40-9.

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Interaction of Europium(III) with Phospholipid Vesicles As Monitored by Laser-Excited Europium(III) Luminescence†

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Received January 9, 1986; Revised Manuscript Received April 17, 1986

ABSTRACT: The technique of laser-excited Eu(III) luminescence was applied to monitor Eu(III) binding to a variety of phospholipids. Eu(III) excitation spectra were similar with and without the presence of neutral phospholipids, while acidic phospholipids changed the spectrum in a concentration-dependent manner. Eu(III) appears to bind to the phosphate moiety with at least a 2:1 phospholipid:metal ion stoichiometry. Analysis of luminescence lifetimes reveals that only one or two waters of hydration are removed from Eu(III) by addition of neutral phospholipids, whereas acidic phospholipids and inorganic phosphate strip off all but one or two waters. Implications with regard to fusion and use of lanthanides as probes in membrane preparations are discussed.

The interaction of metals with phospholipids has received much attention in view of the effects of metals in physiologically important membrane processes such as membrane fusion and transport. In particular, Ca(II) is proposed to mediate vesicle fusion in a number of systems, although some other divalent ions can mediate such fusion to a limited extent (Papahadjopoulos & Vail, 1978; Zimmerburg et al., 1980; Liao & Prestegard, 1980; Duzgunes et al., 1981; Ekerdt & Papahadjopoulos, 1982; Ohki, 1981; Silvius & Gange, 1984). The details of divalent cation interaction with phospholipids are therefore of interest. The luminescent lanthanide probes

† This work was supported in part by the Department of Energy, Contract EV10329, and the National Institutes of Health, Grant RO1 HL30677.

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0006-2960/86/0425-5834$01.50/0

Eu(III) and Tb(III) have proven useful as analogues for the spectrally silent Ca(II) ion. Their utility has been greatly increased by the development of laser-induced excitation, largely by Horrocks and co-workers (Horrocks & Sudnick, 1979; Horrocks et al., 1977; Sudnick, 1979; Horrocks et al., 1980). We present here a detailed characterization of Eu(III) interaction with a variety of phospholipids, examining the effects on luminescence lifetimes and excitation spectra. Some of our results are similar to those of Saris (1983).

Materials and Methods

Diphytanoylphosphatidylcholine (DPPhoCho), phosphatidylserine (PhoSer), and dioleoylphosphatidylethanolamine (PhoEt) were obtained from Avanti Polar Lipids (Birmingham, AL). PhoEt (bacterial origin), phosphatidylinositol (PhoIn), and phosphatidic acid (PhoAc) were from Supelco (Bellefonte, PA). Diphosphatidylglycerol (DiPhoGly, cardiolipin) was obtained from both the above sources; no differences were noted. $EuCl_3$ was purchased from Alfa-Ventron (Danvers, MA); D_2O (99.9%) was from Sigma Chemical Co. All other chemicals were reagent grade.

Solutions of $EuCl_3$ were calibrated by passing a small volume through a column of Aminex 50-X2 cation-exchange resin (Bio-Rad, H^+ form), eluting with water, and performing a pH titration on the eluant to determine H^+ ions released. Phospholipids were stored in $CHCl_3$ at −80 °C. Vesicles were usually made by evaporating the solvent under N_2 in test tube, adding a small volume of 2 mM piperazine hydrochloride, pH 6.8, and sonicating for 10–20 min in an 80-kHz bath sonicator (Laboratory Supplies Co., Hicksville, NY) under N_2 at room temperature. Vesicles prepared in such a manner have been previously characterized by Papahadjopoulos et al. (1967), by Huang (1969), and by us (Shamoo & Brenza, 1980) as being mostly small, unilamellar vesicles. The vesicles suspensions were faintly opalescent but scattered laser light considerably. Lipid concentrations are given in the figure captions. Final phospholipid concentrations were determined by the total phosphate assay of Bartlett (1959). $EuCl_3$–vesicle mixtures were sonicated briefly before luminescence measurements to ensure homogeneity.

Exciting light (0.15 mJ/pulse) was obtained from a Molectron DL-12 laser (using Rhodamine 6G) pumped by a Molectron UV-12 nitrogen laser. The line width at 580 nm was approximately 0.01 nm. The sample housing, collection optics, and associated electronics were built by Photochemical Research Associates (London, Ontario, Canada). A cooled broad-band phototube (Hamamatsu) was used in photon-counting mode. Lifetime data were collected on a Tracor Northern TN-1750 multichannel scaler and transferred to a MINC-23 computer (Digital Equipment Corp.) for storage and analysis. Typically ($1–2 \times 10^5$ counts were taken for a lifetime analysis. Excitation spectra were usually taken at a scan rate of 0.006 nm/s, with a pulse rate of 10 pps, using single-photon counting. In later measurements a D/A interface was available; each data point (0.1–0.2 nm apart) was the sum of photon for 16 or 32 pulses. Luminescence from an EDTA–Eu(III) complex exhibited sharp excitation peaks at 579.63 and 580.12 nm, in agreement with the data of Sudnick (1979). Lifetimes for simple exponential decays were fit by linear regression using the logs of the amplitudes.

Results

Excitation Spectra. The nondegenerate $^7F_0 \rightarrow {}^5D_0$ transition is especially useful for excitation since there is no ligand field splitting. Sudnick (1979) has shown the excitation peak wavenumber for a given Eu(III)–ligand complex to be roughly proportional to the charge of the overall complex. The wavenumber is seen to increase about 7.5 cm^{-1} for a 1-unit increase in positive charge.

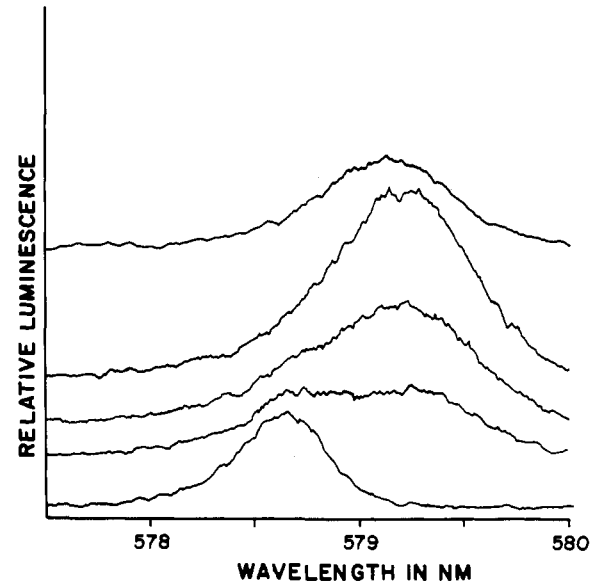

FIGURE 1: Excitation spectra for titration of Eu(III) with PhoAc (mixed with DPPhoCho; see text). The bottom spectrum is 0.10 mM Eu(III) alone, followed by PhoAc:Eu(III) mole ratios of 0.5:1, 1:1, 2:1, and 4:1 (top). Spectra are vertically displaced for clarity.

PhoCho and PhoEt changed the excitation spectrum very little when added to Eu(III) sample with sonication. The peak is not shifted even at a 10:1 mole ratio. Any peak height change could be ascribed to slightly increased turbidity (data not shown). This is evidence that these netural phospholipids bind Eu(III) only weakly, in agreement with Hanse et al. (1977). One should note that these phospholipids were oxidized very little if at all. This contrasts with air-oxidized egg lecithin or such preparation as asolectin, both of which induce shifts in excitation spectra and large increases in luminescence lifetimes (data not shown). These were not studied further since preparations with such poorly defined composition were not well suited to the present study.

In our hands, PhoAc will not form closed vesicles easily by itself, so we formed vesicles of PhoAc/DPPhoCho mixtures at various mole ratios. Figure 1 shows the excitation spectra obtained when 100 μM $EuCl_3$ was titrated with vesicles of this mixture in a 3:1 DPPhoCho:PhoAc mole ratio while $EuCl_3$ concentrations were kept constant. We see clearly that the aquo peak at 578.65 nm is replaced by a broad peak at 579.15 nm. The peak height increases up to a 2:1 PhoAc:Eu(III) mole ratio and is constant thereafter. This saturation is independent of the DPPhoCho:PhoAc mole ratio; up to 10:1 DPPhoCho:PhoAc was used. Thus, PhoAc appears to form a 2:1 complex with Eu(III). The peak position 15 cm^{-1} down from the aquo species peak suggests a change in charge of about 2, supporting this conclusion.

For comparison, we examined the Eu(III)-complexing behavior of α-glycerophosphate, which is essentially PhoAc without the hydrocarbon chains. The Eu–α-glycerophosphate complex shows a symmetric excitation peak at 579.18 nm, with a 0.5-nm full-width at half-maximum [slightly narrower than that of PhoAc–Eu(III)]. This is with a 2:1 mole ratio of α-glycerophosphate to $EuCl_3$, at pH 6.8.

PhoSer causes a similar shift, to about 579.2 nm, but its titration behavior is more complex than that of PhoAc. Figure 2 shows excitation spectra of several mole ratios of PhoSer to Eu(III). The peak height stops increasing at a 1:1 Pho-

Table I: Summary of Eu(III) Luminescence Lifetime Measurements

ligand	mole ratios of ligand:Eu(II) used	emission wavelength used for lifetime (nm)	single-exp or multiexp decay	av no. of coordinated H_2O's
α-glycerophosphate	2:1	579.2	single	2.0
PO_4^{2-}	1:1, 5:1	579.05	single	1.7
egg PhoCho, DPPhoCho	4:1	578.7	single	8
PhoEt	4:1, 5:1	578.65	single	7
PhoAc[a]	2:1, 4:1	579.3	single	1.7
PhoSer	2:1, 4:1	579.1	some multiexp char	1.6
PhoIn	1:1	579.5	single	1.8
DiPhoGly	1:1	579.1	multiexp	1.2–1.5[b]

[a] Mixed vesicles, using DPPhoCho as a "carrier". Results were identical for both 3:1 and 9:1 mole ratios of DPPhoCho:PhoAc. [b] Extimated with longer lived components.

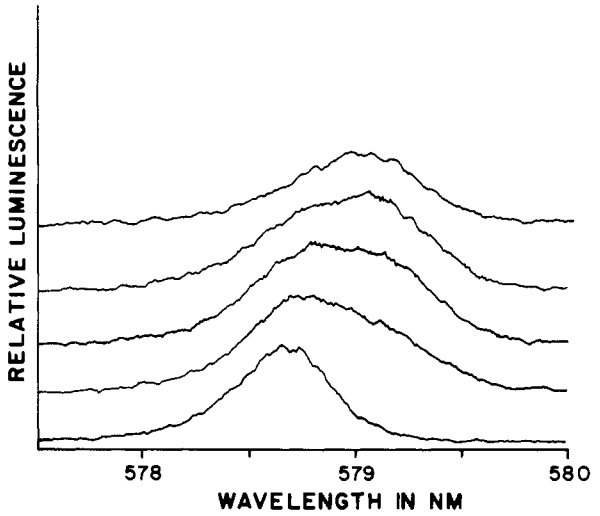

FIGURE 2: Excitation spectra for titration of Eu(III) with PhoSer. The bottom spectrum is 0.10 mM Eu(III) alone, followed by PhoSer:Eu(III) mole ratios of 0.5:1, 1:1, 2:1, and 4:1. Spectra are vertically displaced for clarity.

Ser:Eu(III) ratio and decreases when the ratio is above 3:1. This behavior occurs reproducibly and also is observed in mixed PhoSer:DPPhoCho vesicles, the peak height being dependent only on the mole ratio of PhoSer to Eu(III). The shape of the spectrum is similar to that reported earlier by Rhee et al. (1982) for a Folch fraction III–PhoEt mixture. PhoSer is a major constituent of Folch fraction III.

Titration of $EuCl_3$ with DiPhoGly exhibits similar behavior: the peak for the complex is at 579.05 nm, and the height of this peak decreases for lipid phosphate to Eu(III) mole ratios above 3. The behavior of the excitation peaks during titration of $EuCl_3$ solutions with phospholipids is summarized in Figure 3. While the PhoAc–Eu(III) peak levels off at a lipid phosphate to Eu(III) mole ratio of about 2, the behavior of other acid phosphate–Eu(III) complexes is not so simple, showing a decrease in peak height for mole ratios above 3. This drop is not due simply to increasing turbidity, as the optical density due to turbidity was always below 0.1 in these preparations, and the same behavior was observed when a microcuvette (path length 0.25 cm) was used to hold the sample.

Titration of Eu(III) by phosphatidylinositol is still more difficult to cleanly characterize. A broad excitation peak due to the PhoIn–Eu(III) complex appears at 579.4 nm but drops at lipid phosphate to Eu(III) mole ratios above 1:1 and virtually disappears at ratios above 2:1 (data not shown).

As shown by Horrocks and Sudnick (1979) one can obtain an estimate of the number of Eu(III)-coordinated water molecules in a complex by measuring luminescence lifetimes of the complexed Eu(III). Following Horrocks et al. (1977),

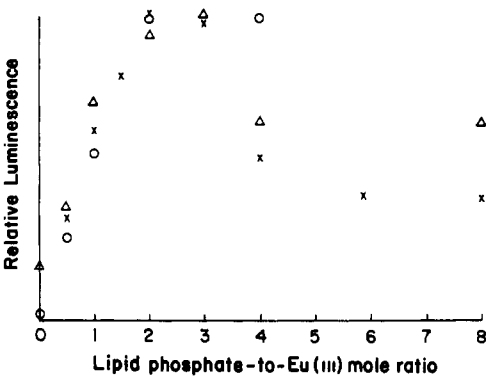

FIGURE 3: Plot of luminescence intensity at excitation peaks of phosphate–Eu(III) complexes, as a function of lipid phosphate:Eu(III) mole ratio. $EuCl_3$ concentration in all cases is 0.1 μM Eu(III), in 2 mM piperazine hydrochloride at pH 6.8. Lipids and excitation peak wavelengths: (⊙) PhoAc, 579.1 nm; (×) PhoSer, 579.2 nm; (▲) DiPhoGly, 579.0 nm.

we perform the lifetime measurements on samples containing different mole ratios of D_2O:H_2O, generally in the range 0:1–9:1. Plotting the reciprocal lifetimes against mole fraction of H_2O, we find the best fit and extrapolate linearly to obtain $\tau_{D_2O}^{-1}$ and $\tau_{H_2O}^{-1}$, the reciprocal lifetimes in pure D_2O and pure H_2O.

The number q of D_2O-exchangeable waters coordinated to Eu(III) is estimated by the equation (Horrocks et al., 1977)

$$q = (1.05 \text{ ms})(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1})$$

Representative data are presented in Figure 4, and the results are summarized in Table I. In general, lifetimes were not strongly dependent on mole ratio of ligand to Eu(III) for ratios above 1:1. The wavelength used for lifetime measurements does not always correspond to a peak in intensity; for PhoIn the measurement was done at a longer wavelength to eliminate any aquo ion overlap. Phospholipid preparations were similar to those for the excitation scans.

Examination of the lifetimes confirms that the neutral lipids PhoCho and PhoEt do not strongly complex Eu(III). The ion binds weakly, with one or possibly two H_2O's being removed. Acidic phospholipids, in contrast, show extensive coordination to the ion, stripping all but one to two H_2O's. Certain other phosphate compounds show similar behavior, stripping all but about two H_2O's upon complexing Eu(III). We take this as further evidence that Eu(III) binds to the phosphate moiety of the phospholipids.

The luminescence decays for Eu(III) complexed to α-glycerophosphate, PhoAc, and PhoIn are single exponential in nature. Those observed with PhoSer or DiPhoGly are not as well-defined; two or more exponential components contribute to the luminescence. This effect is most pronounced with

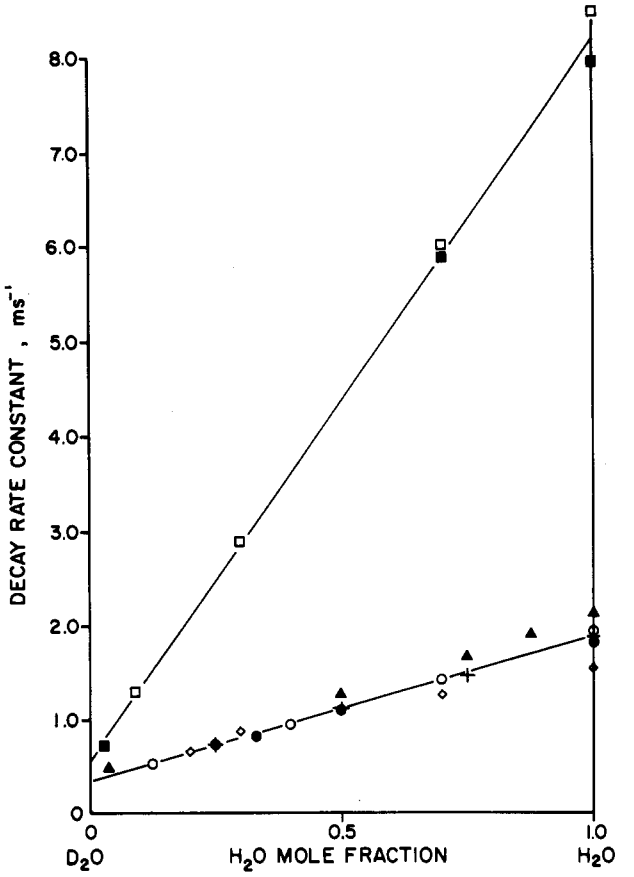

FIGURE 4: Eu(III) luminescence decay constants in varying mole fractions of H_2O to D_2O: α-glycerophosphate:Eu(III), 2:1 (▲); PhoAc:Eu(III), 4:1 (●); PhoIn:Eu(III), 1:1 (○); PhoSer:Eu(III), 2:1 (+); DiPhoGly:Eu(III), 1:1 (◊) (longer lived components); egg PhoCho:Eu(III), 4:1 (□); DPPhoCho:Eu(III), 2:1 (■).

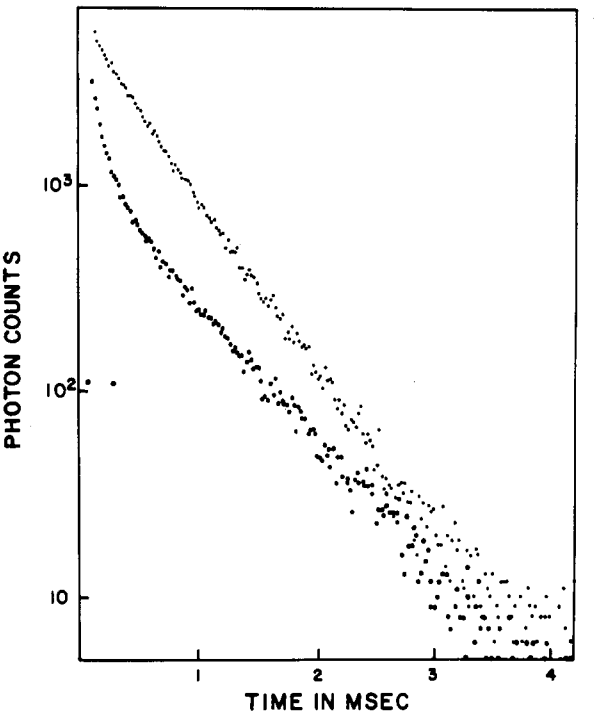

FIGURE 5: Representative luminescence decay data in H_2O and 2 mM piperazine, pH 6.8. The small dots are for 25 μM $EuCl_3$ and 100 μM PhoAc in a PhoAc/DPPhoCho mixture, 4:1 mol/mol. The larger dots are for 50 μM DiPhoGly. The monoexponential decay of the former contrasts with the multicomponent decay of the DiPhoGly:Eu(III) complex.

DiPhoGly. Figure 5 compares the single-exponential decay of PhoAc–Eu(III) with the multiexponential nature of DiPhoGly–Eu(III) luminescence decays.

Discussion

When Eu(III) is complexed by acidic phospholipids and certain other phosphate compounds, the excitation peak shifts from 578.65 to about 579.1 nm, varying ±0.1 nm from this depending on the complexing species. At the same time, all but one or two waters of hydration are removed from Eu(III), indicating extensive coordination of the lanthanide ion, almost certainly by phosphate oxygens. The wavelength shift indicates a change in the effective charge around the ion from 3+ to between 1+ and 2+.

The titration results presented suggest that PhoAc complexes with Eu(III) in a 2:1 mole ratio. This is also consistant with the magnitude of the red-shift in the excitation peak, which indicates a loss of about two positive charges around the metal ion. Results for PhoSer and DiPhoGly are similar except that the peak height decreases when the ratio of phosphate groups to Eu(III) ions exceeds about 3:1. Close examination of Eu(III)–PhoSer excitation spectra shows that as this mole ratio is increased and the peak height goes down, the peak wavelength shortens (blue-shifts) by about 0.1 nm. This may indicate a net increase in the average positive charge seen by a Eu(III) ion. We speculate that this change in excitation spectra may result from multiple "bridging" of PhoSer head groups and Eu(III) ions at these increased mole ratios. For instance, formation of a long linear "string" of alternating metal ions and head groups would result in an average charge of 2+ on each metal ion. The average charge of an ion in a two-dimensional lattice structure (on the surface of a vesicle) would be between 1+ and 2+. Such an interpretation of the data is consistent with models of Ca(II)–membrane interactions in which Ca(II) bridges the phosphate moieties of acidic phospholipids in a membrane and in some instances triggers fusion (Zimmerburg et al., 1980; Liao & Prestegard, 1980; Duzgunes et al., 1981; Ohki et al., 1981; Ekerdt & Papahadjopoulos, 1982).

PhoAc titration of Eu(III) showed no decrease in excitation peak height above a 2:1 PhoAc:Eu(III) mole ratio. It is noteable that in mixed PhoCho/PhoAc or PhoCho/PhoSer vesicle systems similar to ours, Eu(III) at millimolar concentrations was necessary for significant fusion (Zimmerburg et al., 1980; Duzgunes et al., 1981; Ohki, 1981; Silvius & Gange, 1984). Our concentrations were necessarily far below this.

With several acidic phospholipids the excitation peak height decreases at lipid phosphate:metal ion mole ratios greater than 1.5:1. The quantum yield (as indicated by lifetime) changes very little above this ratio, so the peak height decrease is due to some other change in Eu(III) environment that is affecting the "hypersensitive" $^5D_0 \rightarrow {}^7F_2$ transition we are monitoring. We can currently offer no quantitative theoretical interpretation of this behavior. Further evidence for more than one physically distinct type of phospholipid–metal ion complex is seen in the luminescence decays of Eu(III)–PhoSer and Eu(III)-DiPhoGly. The multiexponential nature of the decay indicates the existence of multiple luminescent species. Each of these is at least metastable on a millisecond time scale, since if they interconverted rapidly on the time scale of luminescence a monoexponential decay would be observed.

We find that neutral phospholipids bind Eu(III) much more weakly since addition of these phospholipids has little effect on the Eu(III) excitation spectrum and removes an average of only one or two waters of hydration from the ion. This is

consistent with the data of an earlier report (Hanse et al., 1977) in which the binding constant of Eu(III) to PhoCho is in the millimolar range. The affinity of Eu(III) for acidic phospholipids is so strong that one cannot extract binding constants from our data, as the free Eu(III) concentration is too low to measure at a 1:1 mole ratio of phosphate to Eu(III). The use of Ca(II) as a competitor has not proven feasible in our preparation, either.

The measurements presented in this paper are of interest in interpreting luminescent lanthanide probe experiments with membrane proteins. Clearly, one must be aware of possible interference by lanthanide binding to acidic phospholipids and oxidized lipids in the membranes. One cannot, as some workers have done, assume this binding to be negligible.

The observation that Eu(III) complexing by neutral phospholipids is fairly weak has implications in membrane methodology, as well. In systems involving intrinsic membrane proteins, it is often undesirable to completely delipidate the preparation. Our work suggests that one may greatly reduce metal ion binding to phopholipids by replacing the acidic phospholipids by a neutral counterpart such as PhoCho. This laboratory is currently using this strategy effectively with sarcoplasmic reticulum preparations.

Registry No. DPPhoCho, 64626-70-6; PhOEt, 2462-63-7; Eu, 7440-53-1; PO_4^{2-}, 14265-44-2; α-glycerophosphate, 57-03-4.

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CORRECTION

Studies of 6-Fluoropyridoxal and 6-Fluoropyridoxamine 5′-Phosphates in Cytosolic Aspartate Aminotransferase, by Robert David Scott, Yen-Chung Chang, Donald J. Graves, and David E. Metzler*, Volume 24, Number 26, December 17, 1985, pages 7668–7681.

Page 7672. In Table III, the values of K_m for 2-oxoglutarate and aspartate are incorrectly cited as 0.82 mM and 6.6 mM, respectively. These values should read 0.040 mM and 1.93 mM, respectively. Also, the last sentence in the paragraph entitled Enzymatic Activity should be deleted.