Determination of Membrane Potential and Cell Volume by ^{19}F NMR Using Trifluoroacetate and Trifluoroacetamide Probes

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ABSTRACT: The distribution of ionic species between intra- and extracellular compartments forms one basis for the determination of cell membrane potential. It is shown that fluorine-19 NMR studies of erythrocytes in the presence of trifluoroacetate, a stable, relatively nontoxic anion with $pK = -0.3$, provide a sensitive probe of membrane potential. Since such measurements are based on ion concentrations, the parallel use of the neutral analogue trifluoroacetamide to provide information on intra/extracellular volume ratios was also explored. In both cases, separate ^{19}F resonances corresponding to intra- and extracellular ions were observed, with the intracellular resonance shifted downfield by ~0.2 ppm and the intracellular peak typically somewhat broader than the extracellular resonance. Studies with the band 3 anion-exchange inhibitor 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) indicate that both transmembrane diffusion and flux involving the band 3 anion exchanger contribute to the observed transport of the trifluoroacetate anion. Intra/extracellular volume ratios determined on the basis of trifluoroacetamide intensity ratios were in good agreement with determinations based on measured hematocrits. On the basis of the high sensitivity of ^{19}F NMR and the capability of monitoring volume changes simultaneously, the time resolution for these measurements can approach the lifetime of intracellular trifluoroacetate ions and hence be limited by the trifluoroacetate flux rate.

The application of in vivo NMR[1] spectroscopy to the study of cell metabolism has been greatly extended in recent years as a consequence of the introduction of fluorinated indicators for cell parameters of interest. The combination of high intrinsic sensitivity of the ^{19}F nucleus for NMR detection, its 100% isotopic abundance, and the essential absence of endogenous ^{19}F NMR signals from cell systems makes ^{19}F NMR studies of fluorinated indicators an attractive strategy for in vivo studies. The use of fluorinated, NMR-active indicators for cellular pH (Deutsch et al., 1982; Taylor & Deutsch, 1983; Deutsch & Taylor, 1987a,b; Joseph et al., 1987), cytosolic calcium ions (Smith et al., 1983; Levy et al., 1987); cytosolic magnesium ions (Levy et al., 1988), and oxygen (Taylor & Deutsch, 1988) has been described recently.

One basis for the measurement of membrane potential involves the determination of the distribution of ions that are in electrochemical equilibrium across the membrane. As discussed by Deutsch and Kula (1978), this distribution can be related to the membrane potential by the Nernst equation if (1) the ion is passively distributed in its charged form only and (2) a true equilibrium is established. Any direct pumping of the ion or coupling of its transport to any other ions that are not in equilibrium invalidates its application, and binding to any components of the system introduces an error in the calculated membrane potential. Most ion distribution measurements utilizing this approach have been based on radiolabeled probes such as [^{14}C]thiocyanate (Deutsch & Kula, 1978; Pollard et al., 1976; Holz, 1979; Hock, 1980) and [^{3}H]MTPP$^+$ and [^{3}H]TPP$^+$ (Maloney et al., 1975; Kashket, 1981). More recently, ion-selective electrodes have been used in such studies without the need for radiolabeled ions (Kamo et al., 1979; Kumar & Nicholas, 1981; McCarthy et al., 1981). Kirk et al. (1988) have recently described the use of NMR measurements of the transmembrane distribution of hypophosphite ion in erythrocytes to obtain similar information. This approach takes advantage of the significant chemical shift difference which is found to characterize intra- and extracellular ion resonances, eliminating the need for a physical separation of cells from bathing solution.

The analogous use of a fluorinated anion probe is potentially advantageous as a consequence of the inherently greater sensitivity of ^{19}F for detection, with the consequent improvement in time resolution, or the possibility of utilizing lower cytocrits. The simplest such anion, F^-, is unsuitable for such an application as a consequence of its metabolic activity (Curnutte et al., 1979; Stein et al., 1985; Suketa et al., 1977) and the low solubility product of calcium and magnesium fluorides. Thus, intracellular F^- resonances are frequently not observed (Selinsky et al., 1988). Instead, we report here on the successful evaluation of the trifluoroacetate anion for determining the membrane potential of erythrocytes. The pK of −0.26 for this ion (Perrin et al., 1981) renders it nontitratable in physiological systems, while the three equivalent fluorine nuclei offer a potential sensitivity increase of 37.7 over the detection of a phosphorus nucleus. Additionally, the parallel measurement of the distribution of a neutral analogue, trifluoroacetamide, provides an independent means of determining the ratio of intra/extracellular volume that can be directly used to convert the trifluoroacetate intensity measurements into concentration ratios, allowing the direct and rapid estimation of the membrane potential from the NMR measurement alone.

Materials and Methods

Blood was obtained as outdated red blood cells from the American Red Cross or as a donation from a human volunteer. The plasma and buffy coat were discarded and the cells washed

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[1] Abbreviations: H_t, hematocrit; NMR, nuclear magnetic resonance; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; TFA, trifluoroacetate; TFM, trifluoroacetamide.

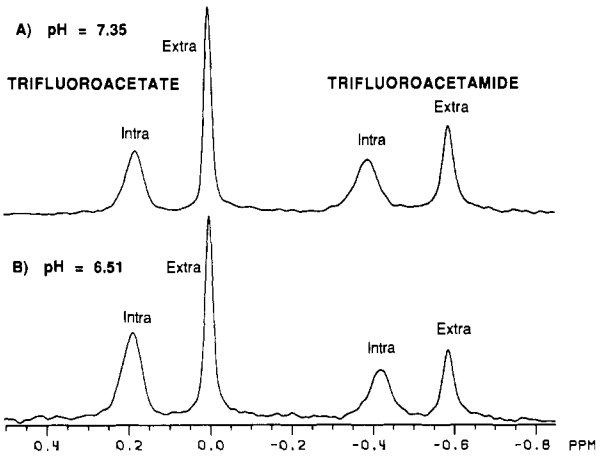

FIGURE 1: ^{19}F NMR spectra of erythrocytes suspended in 145 mM NaCl, 10 mM phosphate, 7 mM glucose, 2 mM trifluoroacetate, and 1.5 mM trifluoroacetamide, adjusted to pH = 7.35 (A) and 6.51 (B). The hematocrits for the two solutions were determined to be 58.6% (A) and 54.5% (B).

three times in 5 volumes of a saline buffer containing 145 mM NaCl, 10 mM phosphate (pH 7.4) or 10 mM HEPES buffer, and 7 mM glucose. Hematocrits were measured by using capillary tubes spun in a micro hematocrit centrifuge. In studies on the inhibition of the band 3 anion exchanger, the samples were incubated for 20 min with 0.5 mM 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), obtained from Sigma Chemical Co. (Labotka & Omachi, 1987). Other chemicals were obtained from Aldrich. In solution, trifluoroacetamide was found to hydrolyze to trifluoroacetate and ammonia over a period of days so the compound was freshly dissolved prior to each study.

Fluorine-19 NMR measurements were carried out at 22 °C on a Nicolet NT-360 NMR spectrometer using a 5-mm proton probe that was retuned to the fluorine resonance frequency of 339.7 MHz. Samples contained no D_2O and were run in the unlocked mode. Magnetic field drift has been measured at under 3×10^{-3} ppm/h on this magnet. Field homogeneity was adjusted by shimming on the H_2O resonance, with typical line widths of 0.05 ppm. Samples were not spun in order to prevent centrifugation effects on the cells. Interpulse delays of 30 s were used in order to ensure essentially full relaxation, and 4–32 accumulations were obtained per spectrum, depending on the concentrations of the fluorinated compounds. Spin–lattice relaxation measurements were carried out by using an inversion recovery sequence (Vold et al., 1968).

Results

The ^{19}F NMR spectra of a sample of human erythrocytes suspended in a 5-mm NMR tube containing 145 mM NaCl, 10 mM phosphate buffer (at the pH values indicated), 7 mM glucose, 2 mM trifluoroacetate, and 1.5 mM trifluoroacetamide are shown in Figure 1. Studies in which each of the fluorinated compounds was added separately allow an unequivocal assignment of the trifluoroacetate and trifluoroacetamide resonances. For both compounds, the identity of the broader, downfield resonance was determined to correspond to the intracellular species on the basis of its variation in intensity with hematocrit. Additional verification of the assignment of the trifluoroacetate resonances was made on the basis of the addition of $PrCl_3$, which specifically shifted the upfield peak. The chemical shift differences between intra- and extracellular resonances of ~0.19 ppm were found to be similar for the two compounds, suggesting either fortuitous contributions from various intracellular environments with

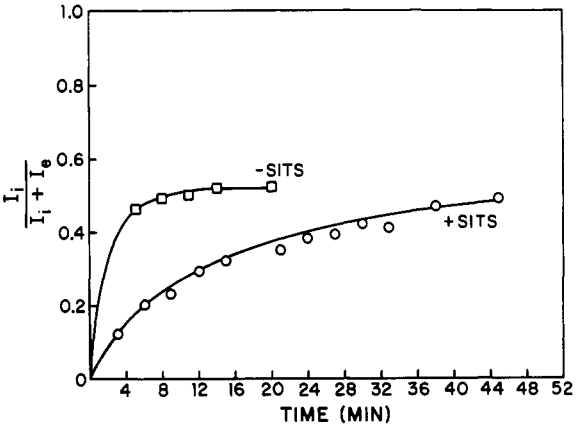

FIGURE 2: Fractional intensity of the trifluoroacetate resonance I_i as a function of time after the addition of trifluoroacetate in red cell suspensions containing either 0 or 0.5 mM SITS, as indicated.

which the molecules are in rapid exchange or dominance by the same shift mechanism, presumably the susceptibility of the red cell (Kirk & Kuchel, 1987). The shift difference in parts per million was somewhat smaller than the upfield shift of ~0.28 ppm for the intracellular hypophosphite ion observed by Kirk et al. (1988) but is larger in hertz due to the larger value of γ for ^{19}F. An inversion–recovery experiment gave $T_{1,i} = 1.67$ s and $T_{1,e} = 2.71$ s for the intra- and extracellular TFA resonances, respectively. Thus, the resonance of the intracellular TFA pool is characterized by shorter T_1 and T_2 values.

The rate of uptake of both trifluoroacetate and trifluoroacetamide by the cells was sufficiently rapid so that NMR observations made several minutes after addition of the ions indicated that the intra/extracellular ion ratio had reached equilibrium. Additional broadening of the trifluoroacetamide resonance as the temperature was raised indicates that the transmembrane exchange rate is just below the threshold required to obtain well-resolved peaks. Alternatively, there is no indication of additional exchange broadening of the trifluoroacetate resonances at higher temperature, indicating that the transmembrane exchange rate for the ion falls well into the slow-exchange limit. As pointed out by Kirk et al. (1988) in studies of hypophosphite ion distribution, several mechanisms exist for the movement of anions across the red cell membrane. Addition of 0.5 mM 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS), an inhibitor of the band 3 anion exchanger, significantly retarded the rate of entry of TFA into the cells (Figure 2). However, under analogous conditions no significant uptake of hypophosphite ion was observed. Hence, it appears that the band 3 anion exchanger is a significant, but not the sole vehicle for uptake of TFA by the red cells.

For ions meeting the criteria outlined in the introduction and assuming equivalent intra- and extracellular activity coefficients, the membrane potential can be determined from the ratio of intra- to extracellular ion concentrations according to the Nernst equation:

$$E_m = RT/F \ln ([C]_i/[C]_e) \quad (1)$$

However, the conversion of intensity data, such as that shown in Figure 1, to concentration data requires that the ratio of intra/extracellular volume also be determined. As discussed by Kirk et al. (1988), the volume ratio can be expressed in terms of two parameters: the hematocrit H_t and a parameter α corresponding to the fraction of the intracellular volume that is occupied by the cytosol. The fractional intracellular volume available to the ion is then given by the product αH_t and the

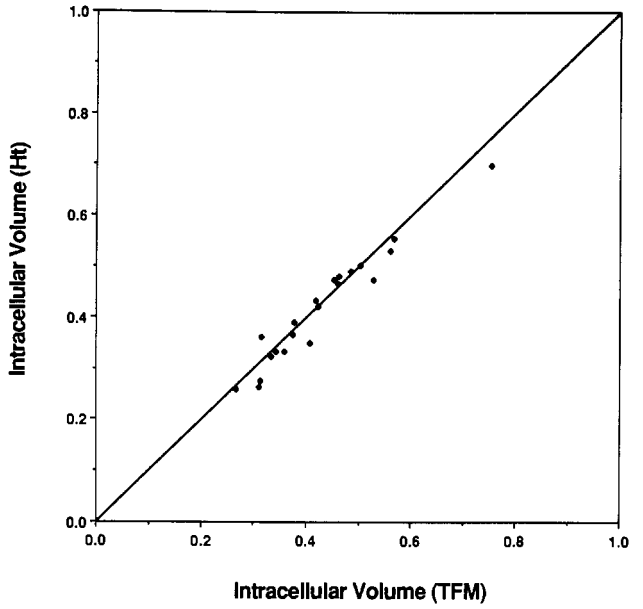

FIGURE 3: Plot of red cell volume determined on the basis of hematocrit measurements [$V_i = \alpha H_t$ with $\alpha = 0.717$ (Kirk et al., 1988)] (ordinate) vs fractional intracellular volume based on measured intensity of ^{19}F resonances of trifluoroacetamide (abscissa). The straight line in the figure corresponds to $V_i(H_t) = V_i(TFM)$.

fractional extracellular volume by $1 - H_t$. Alternatively, several NMR-based approaches to the determination of cell volume have been discussed in the literature. In particular, Kuchel and co-workers (Kirk & Kuchel, 1985; Raftos et al., 1988) have noted a dependence of the chemical shift difference of the neutral molecule dimethyl methylphosphonate (DMMP) on cell volume. In order to facilitate the determination of membrane potential by ^{19}F NMR, a neutral, fluorinated molecule was sought that would similarly partition according to cell volume. Trifluoroacetamide, with a pK of $\sim$11, appeared to be a reasonable candidate for such a determination. The fractional intracellular volume determined on the basis of hematocrit measurements and assuming a constant value of α is plotted vs the fractional intensity of the intracellular, fully relaxed trifluoroacetamide resonance in Figure 3. As a further measure of the partition of trifluoroacetamide based on cell volume, red cells were incubated with valinomycin and a time-dependent decrease in the intracellular resonance was observed. The limiting cell volume based on the trifluoroacetamide resonance intensity was determined to be 72% of the initial volume, in good agreement with a limiting reduction of the volume to 74% from the data of Kirk and Kuchel (1985). These results support the conclusion that the partition of this compound between intra- and extracellular compartments is simply related to cell volume. On the basis of this result, the Nernst equation can be expressed in terms of the measured intensities of the four resonances (Figure 1):

$$E_m = \frac{RT}{F} \ln \left[\frac{I_i(TFA)}{I_e(TFA)} \frac{I_e(TFM)}{I_i(TFM)} \right] \quad (2)$$

Red cell membrane potential is known to be a function of pH (Gunn et al., 1973; Freedman & Hoffman, 1979). This dependence is qualitatively evident from the data shown in Figure 1 corresponding to solutions with similar hematocrits and the pH values indicated. At pH 6.5, the ratio of intra/extracellular trifluoroacetate resonance intensities is slightly greater than 1.0, while as the pH is increased, this ratio falls to less than 1, consistent with a negative membrane potential. Data obtained as a function of pH in two studies, one utilizing

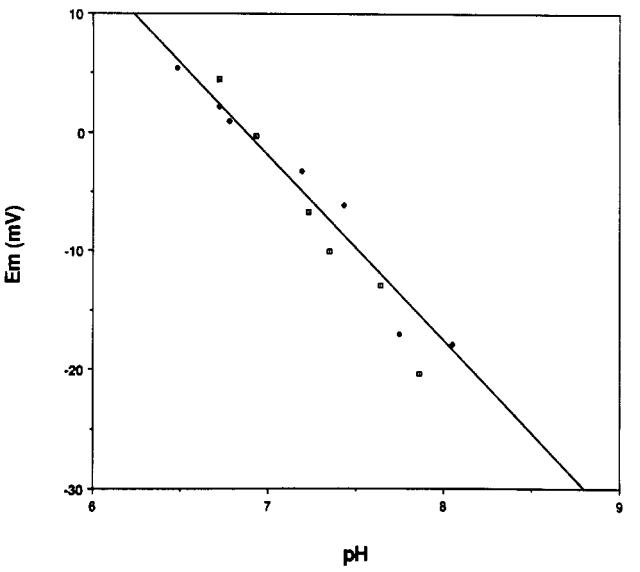

FIGURE 4: Membrane potential (in millivolts) determined as a function of intracellular pH. The two sets of data included in the figure correspond to NMR studies utilizing trifluoroacetate in which cell volume was determined from the hematocrit (⊡) or to studies in which both trifluoroacetate and trifluoroacetamide were present and eq 2 was utilized (◆). Additionally, the first study was carried out in the presence of a phosphate buffer and the second with HEPES. Susupensions also contained 145 mM NaCl and 7 mM glucose. NMR measurements were made at 22 °C.

the measured trifluoroacetate intensities and the measured hematocrit and the second based on eq 2 above, are summarized in Figure 4. These data are consistent with expectations regarding the red cell membrane potential. A linear least-squares fit of the data in the pH range indicated and corresponding to the buffer conditions in the figure legend gave

$$E_m \text{ (mV)} = 107 - 15.6(\text{pH}) \quad (3)$$

corresponding to $E_m = 0.0$ at pH 6.86 and to $E_m = 8.4$ mV at pH 7.4. The latter value is in reasonable agreement with that reported under similar conditions (Rink & Hladky, 1982; Kirk et al., 1988), and the null point is similar to the value of 6.99 obtained by Gunn et al. (1973) under somewhat different conditions.

Discussion

Measurement of the transmembrane distribution of ions is a frequently used technique for the determination of membrane potential. It has been shown that the electrogenic permeability of the red cell membrane to chloride ion is sufficiently large to dominate the conductance and, hence, the membrane potential (Hladky & Rink, 1977). However, direct determination of the chloride ion concentrations by NMR is limited by the large line width of intracellular chloride resonances (Brauer et al., 1985). Further, the resonances of quadrupolar nuclei in cells have been difficult to interpret as a consequence of the differential broadening of line-width components corresponding to different transitions (Shporer & Civan, 1972).

Trifluoroacetate is the oxidative end product of the metabolism of several chemicals including trifluoroethanol (Blake et al., 1969) and the anesthetic halothane (Stier et al., 1972). Although such oxidative metabolism may be associated with significant toxicity due to the presence of reactive intermediates such as trifluoroacetyl chloride (Muller & Stier, 1982), most data suggest that TFA itself is relatively nontoxic (Airaksinen & Tammisto, 1968; Blake et al., 1969). Thus, doses of up to 5 g/kg wt sodium trifluoroacetate, administered by ip injection, did not elicit toxic effects in mice (Blake et al., 1969). The

low pK = −0.26 (Perrin et al., 1981) ensures that TFA is fully ionized under physiological conditions. Alternatively, trifluoroacetamide with a pK of ~11 is predominantly neutral in the pH region useful for studies of cells. In addition to the intensity ratio used here, the chemical shift difference between intra- and extracellular trifluoroacetamide resonances also appears to be related to cell volume, but is probably sensitive to other factors as well, analogous to effects observed for the ^{31}P NMR cell volume probe dimethyl methylphosphonate (Raftos et al., 1988).

As is evident from Figure 1, the ^{19}F resonances of both compounds undergo similar downfield shifts upon entering the cells. This similarity suggests that such shifts do not arise from specific interactions with cellular components but are rather from some general effect such as the susceptibility difference previously described in the literature (Kirk & Kuchel, 1987). The ^{19}F resonance of trifluoroethanol also undergoes a similar intracellular shift, but the resonances are significantly broader than those of trifluoroacetamide, presumably reflecting the greater hydrophobicity. Nevertheless, the intracellular shift of hypophosphite ion, which has also been suggested to reflect susceptibility of the red cell, is upfield rather than downfield. As noted in the introduction, the binding of ions to cellular components can alter the distribution between intra- and extracellular compartments and thus produces an error in the determination of the membrane potential. To some extent, the NMR measurement includes automatic corrections for such effects. Ions that interact strongly with macromolecules will lead to chemically shifted and/or severely broadened resonances that will not contribute to the observed peak. Alternatively, the observed signal is presumably a weighted average of solvated and weakly interacting ions. The reductions in the T_1 and T_2 values of intracellular trifluoroacetate ^{19}F resonance relative to the values for the extracellular resonance are consistent with intracellular exchange interactions which are sufficiently weak to correspond to the fast-exchange limit.

In general, anions such as TFA can traverse the cell membrane as a consequence of ion pumping, anion exchange, cotransport, or passive diffusion. Data obtained in the presence of the band 3 anion-exchange inhibitor SITS indicate that anion exchange constitutes a significant, but not the sole, means of transmembrane flux for TFA. It is probable that much of the remaining flux arises from diffusion, analogous to the case of thiocyanate (Dissing et al., 1981). However, as noted by Kirk et al. (1988), the exchange of tracer ions with chloride ions via the band 3 anion exchanger does not invalidate the use of such ions for the determination of red cell membrane potential since in red cells the chloride ions have been determined to be in electrogenic equilibrium across the membrane (Hladky & Rink, 1977). In practice, the substitution of 10 mM phosphate for 10 mM HEPES buffer did not have a significant effect on the value for the membrane potential determined by the TFA measurement, so that additional exchange interactions involving phosphate do not significantly perturb the TFA transmembrane distribution. The value of 8.4 mV obtained from a fit of the data corresponding to pH 7.4 is in good agreement with literature values (Rink & Hladky, 1982). The null point (E_m = 0) is close to that determined by Gunn et al. (1973).

The determination of membrane potential and cell volume by ^{19}F NMR measurements of cells in the presence of trifluoroacetate and trifluoroacetamide can presumably be extended to other cell types, analogous to the radiotracer and ion-selective electrode measurements. Such applications are limited by the magnitude of the chemical shift difference between intra- and extracellular resonances, by possible additional partitioning of the compounds into intracellular organelles, and in systems in which anion exchange with chloride dominates the transmembrane flux, but the chloride ion distribution is not determined by the membrane potential. The introduction of extracellular lanthanide shift reagents could help to produce the needed chemical shift difference. The effects of distribution of the ions among intracellular organelles will depend on the rates of exchange and NMR "visibility" of the compartmented ions. In general, the high sensitivity and narrow line widths of the trifluoroacetate ion make ^{19}F NMR studies of such ions an attractive strategy for probing the intracellular distribution of such ions as well as the intra/extracellular distribution described here.

Acknowledgments

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Exposure of Endogenous Phosphatidylserine at the Outer Surface of Stimulated Platelets Is Reversed by Restoration of Aminophospholipid Translocase Activity†

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ABSTRACT: Phosphatidylserine (PS) in the plasma membrane of nonactivated human platelets is almost entirely located on the cytoplasmic side. Stimulation of platelets with the Ca^{2+} ionophore A23187 or combined action of collagen plus thrombin results in a rapid loss of the asymmetric distribution of PS. Also, treatment with the sulfhydryl-reactive compounds diamide and pyridyldithioethylamine (PDA) causes exposure of PS at the platelet outer surface. PS exposure is sensitively measured as the catalytic potential of platelets to enhance the rate of thrombin formation by the enzyme complex factor Xa-factor Va, since this reaction is essentially dependent on the presence of a PS-containing lipid surface. In this paper we demonstrate that endogenous PS, previously exposed at the outer surface during cell activation or sulfhydryl oxidation, can be translocated back to the cytoplasmic leaflet of the membrane by addition of dithiothreitol (DTT) but not by nonpermeable reducing agents like reduced glutathione. Treatment of platelets with trypsin or chymotrypsin, prior to addition of DTT, inhibits the inward transport of exposed PS. Moreover, severe depletion of metabolic ATP, as obtained by platelet stimulation with A23187 in the presence of metabolic inhibitors, though not inhibiting PS exposure at the outer surface, blocks the translocation of endogenous PS to the internal leaflet of the plasma membrane. These results strongly indicate the involvement of a membrane protein in the inward transport of endogenous PS. Recently, an aminophospholipid-specific translocase in the platelet membrane was postulated on the basis of the inward transport of exogenously added PS (analogues) [Sune, A., Bette-Bobillo, P., Bienvenue, A., Fellmann, P., & Devaux, P. F. (1987) *Biochemistry 26*, 2972-2978]. The present data demonstrate for the first time the inward translocation of endogenous PS, most likely supported by the same membrane protein responsible for transport of exogenous PS.

The molecular architecture of biological membranes is highly asymmetric. This phenomenon is not only restricted to the orientation of membrane proteins but also pertains to the lipid distribution with respect to both phospholipid class and fatty acid composition (Op den Kamp, 1979). Detailed information is available on the nonrandom orientation of lipids in the erythrocyte and platelet membrane (Verkley et al., 1973; Gordesky et al., 1975; Zwaal et al., 1975; Chap et al., 1977; Schick et al., 1976): while phosphatidylcholine and phosphatidylethanolamine are present in variable amounts in both membrane leaflets, a most extreme distribution is observed for phosphatidylserine (PS)[1] and sphingomyelin, which are almost exclusively located in the inner and outer leaflets, respectively. In erythrocytes, this highly asymmetric orientation of lipids is essential for normal homeostasis. It has been demonstrated that an increased exposure of PS at the outer surface of the red cell is a signal for sequestration by the reticuloendothelial system (Tanaka & Schroit, 1983; Schwartz

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[1] Abbreviations: PS, phosphatidylserine; DTT, dithiothreitol; diamide, diazinedicarboxylic acid bis(dimethylamide); PDA, pyridyldithioethylamine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; S2238, D-phenylalanyl-L-pipecolyl-L-arginine *p*-nitroanilide dihydrochloride.

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