Biochimica et Biophysica Acta, 1147 (1993) 262–266

BBAMEM 75936

Membrane potential determination in large unilamellar vesicles with hexakis(2-methoxyisobutylisonitrile)technetium(I)

Daniel M. Chernoff [a], Gary R. Strichartz [b] and David Piwnica-Worms [a]

[a] *Department of Radiology and* [b] *Anesthesia Research Laboratories, Harvard Medical School and Brigham and Women's Hospital, Boston, MA (USA)*

(Received 17 December 1992)

Key words: Lipophilic cationic probe; Isonitrile; Organotechnetium compound; Valinomycin; Membrane potential; Large unilamellar vesicle; Radionuclide

The accumulation of the lipophilic cation hexakis (2-methoxyisobutylisonitrile)technetium (^{99m}Tc-MIBI) within large unilamellar vesicles made from egg phosphatidylcholine was examined as a function of time and membrane potential (E_m). Equilibrium distribution occurred within minutes at 30°C. The transmembrane distribution of Tc-MIBI was measured at $E_m = 0$ mV and at a series of negative membrane potentials. The distribution of Tc-MIBI was in close agreement with the Nernst equation for passive distribution of a permeant ion across a bilayer, permitting the membrane potential to be predicted from Tc-MIBI distribution. In this respect, Tc-MIBI behaves similarly to other radioprobes of membrane potential, but with unique properties including high specific activity (10^9 Ci/mol), rapid kinetics of distribution, low potential-independent binding, and short half-life (6.02 h). The results indicate a mechanism for tissue accumulation of Tc-MIBI in vivo that may in part account for its utility in clinical imaging of ischemic myocardium.

Introduction

Hexakis(2-methoxyisobutylisonitrile)technetium(I) (Tc-MIBI or Tc-SestaMIBI) is one member of a class of low-valence technetium (^{99m}Tc) coordination compounds originally designed as myocardial perfusion imaging agents [1–4]. Chemical analysis has shown these complexes to be monovalent cations, with a central Tc(I) core surrounded by six identical ligands coordinated through the isonitrile carbon with an octahedral geometry, effectively trapping the metal ion inside a lipophilic sphere. Of these radiopharmaceuticals, Tc-MIBI has shown the most clinical promise to date and has gained approval from the USFDA for use in the noninvasive assessment of coronary artery disease by external scintigraphic imaging [4–6].

Cellular transport studies of a series of ^{99m}Tc-alkylisonitrile complexes in cultured chick myocyte preparations have shown that the rate of influx and net uptake of the compounds are affected by both lipophilicity and cationic charge [7]. Subsequent semi-quantitative data from cultured chick myocyte and mouse fibroblast preparations have shown that depolarization of the plasma or mitochondrial membrane potentials inhibits net uptake of Tc-MIBI and causes release of previously-accumulated Tc-MIBI. It has been hypothesized [8–9] that the membrane potential dependence of Tc-MIBI uptake is determined by passive transmembrane equilibration of the cation complex in accordance with the Nernst equation:

$$[\text{Tc-MIBI}]_i = [\text{Tc-MIBI}]_o \exp(-E_m F/RT) \quad (1)$$

where $[\text{Tc-MIBI}]_i$ and $[\text{Tc-MIBI}]_o$ refer to the internal and external concentrations, respectively, of Tc-MIBI; E_m is the transmembrane electrical potential and $RT/F = 26$ mV at 37°C. In this respect, Tc-MIBI would resemble other lipophilic, cationic permeant probes of membrane potential such as tetraphenylphosphonium (TPP) and derivatives [10–13], oxacarbocyanine dyes [14], safranin-O [15], or rhodamine-123 [16–18].

The purpose of the present study was to test the hypothesis that Tc-MIBI is a quantitative radioprobe of membrane potential. The extremely high specific activity of Tc-MIBI (up to 10^9 Ci/mol), short half-life (6.02 h), high-energy gamma-emission photon (140 keV), and ready availability as a kit formulation could

Correspondence to: D. Piwnica-Worms, Department of Radiology, Brigham and Women's Hospital, 75 Francis Street, Boston, MA 02115, USA.

Abbreviations: Tc-MIBI, hexakis(2-methoxyisobutylisonitrile)technetium(I); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; TPP, tetraphenylphosphonium; TMA, tetramethylammonium; TPB, tetraphenylborate; MLV, multilamellar vesicle; LUV, large unilamellar vesicle.

make it a useful membrane potential probe in vitro as well as in vivo. The agent contains no titratable proton, thereby eliminating direct effects of pH on the agent as a confounding factor in membrane potential measurement. Furthermore, Tc-MIBI is not metabolized significantly in vitro or in vivo [2].

In this study, equilibrium transmembrane distribution of Tc-MIBI is measured in a well-defined nonbiologic membrane system composed of unilamellar phosphatidylcholine vesicles in which a stable potassium (K^+) diffusion potential can be established in the presence of the K^+ ionophore, valinomycin [19–22]. This simple 'model membrane' system permits manipulation of membrane potential by varying the ratio of intra- to extravesicular K^+, and avoids the possible confounding influence of intracellular organelles and active transport processes in the quantitative determination of the equilibrium distribution of lipophilic cations.

Materials and Methods

Lyophilized egg phosphatidylcholine was obtained from Avanti Polar Lipids (Birmingham, AL). Valinomycin, potassium glutamate, potassium hydroxide, tetramethylammonium (TMA) chloride, TMA hydroxide, and sodium tetraphenylborate (TPB) were purchased from Sigma (St Louis, MO). Hepes buffer was from Calbiochem (San Diego, CA). ^{14}C-labelled inulin was purchased from New England Nuclear (NEN, Billerica, MA). Synthesis of the radiolabeled compound [^{99m}Tc]MIBI was performed using a one-step kit formulation (kindly provided by T.R. Carroll, E.I. duPont, Medical Products Division, North Billerica, MA) as described elsewhere [8,9].

Solutions

Standard intravesicular buffer consisted of (in mM): 160 K-glutamate, 10 Hepes, titrated to pH 7.5 with KOH. Nominally K^+-free buffer consisted of (in mM): 160 TMA-Cl, 10 Hepes, titrated to pH 7.5 with TMA-OH. Glutamate was chosen as the internal counterion and TMA as the external cation after preliminary experiments using only internal KCl and external NaCl indicated a rapid ($t_{1/2} < 1$ h) dissipation of membrane potential as measured by Tc-MIBI accumulation (data not shown).

Inulin was dissolved in a solution containing 10 mM Hepes titrated to pH 7.5 with TMA-OH prior to the preparation of vesicles. Valinomycin, TPB, and Tc-MIBI were dissolved in ethanol prior to addition to buffers. Final ethanol concentration was less than 0.5% (v/v).

Preparation of vesicles

LUVs (Large Unilamellar Vesicles) were prepared from egg phosphatidylcholine using the techniques described by Hope et al. [19]. In brief, multilamellar vesicles (MLVs) were prepared by hydrating 200 mg of egg phosphatidylcholine lipid film in 2 ml standard buffer containing 5 μCi/ml of [^{14}C]inulin (0.4 mmol/l) as a marker for trapped intravesicular volume. The lipid dispersion was frozen and thawed five times to increase the trapped volume of the vesicles and to obtain equilibrium transmembrane solute distributions [22]. The resulting frozen and thawed MLVs were then repeatedly (10 ×) extruded through two stacked polycarbonate filters of nominal 100 nm pore size (Amicon, Richmond, CA) at room temperature using a high-pressure extrusion device (Lipex Biomembranes, Vancouver, BC). The resulting LUVs exhibited trapped volumes of 1.5 μl per μmol phospholipid [21] and a mean diameter of 114 ± 27 nm as determined by sizing on a Coulter counter (Coulter Instruments, Hialeah, FL). Stock preparations were stable for at least 2 weeks at 5°C as determined by stability of vesicle size and trapped volume, and by ability of the vesicles to retain trapped inulin.

Accumulation of probes within vesicles

Stock LUVs were passed through 30 × 1 cm Sephadex G-50 gel filtration columns equilibrated with either standard buffer or K-free buffer both to remove the untrapped inulin and (in the case of the K-free column) to replace extravesicular K^+ with the impermeant cation TMA^+. Because of overlap of the LUV and free inulin elution peaks, only the first 800 μl following the dead volume were collected when 200 μl of stock LUVs were loaded on the column. This permitted a typical efficiency of separation of free from trapped inulin of > 99.9% (data not shown), with recovery of roughly one-half of the loaded vesicles.

The resulting 160 mM K^+ and K^+-free LUV stocks were then mixed in appropriate proportions to establish the desired K^+ gradient(s). Phospholipid concentrations were typically 10 μmol egg phosphatidylcholine/ml, yielding a trapped volume (ratio of intravesicular to total volume) of 1.5%. Valinomycin was used at a concentration of 1 μg per μmol phospholipid. ^{99m}Tc-MIBI was used at concentrations of 100–200 μCi/ml (200–400 pmol/l).

Quantitation of probe accumulation

Spin-column method. Experiments assessing the time-dependence of Tc-MIBI uptake into vesicles were performed by passing 30 μl aliquots from the incubating mixtures through Sephadex G-50 gel filtration 'spin columns' packed in disposable 1 ml syringes. Vesicles were eluted by centrifugation at 300 × g and 4°C for 3 min, untrapped probe remaining within the spin column [19]. Spin columns were prepared with K^+-free media to inhibit loss of trapped Tc-MIBI during centrifugation. Control experiments using vesicles alone or

probe alone showed elution of 50–70% of vesicles loaded on a spin column and retention of > 99.9% of free probe (data not shown). Spin column eluants were then mixed with 5 ml of Aquasol-2 scintillation fluid (NEN), vortexed, and counted in a Beckman LS8000 liquid scintillation counter. Triplicates of each sample were prepared in this manner.

Counting was performed with a wide energy window immediately after obtaining samples and again after a 4 day delay, allowing the ^{99m}Tc (half-life 6.02 h) to decay to ^{99}Tc (half-life $3 \cdot 10^5$ years) in the interim. The residual counts at 4 days, after background subtraction, were assigned to the [^{14}C]inulin marker for determination of trapped volumes; the [^{14}C]inulin counts were then subtracted from the first set of counts to determine the ^{99m}Tc activity. All ^{99m}Tc counts were decay-corrected to a common time-point. Counting efficiencies for ^{99m}Tc and ^{14}C were 19% and 20%, respectively.

Dialysis method. For quantitative estimation of Tc-MIBI distribution as a function of membrane potential, an equilibrium dialysis technique was chosen, avoiding potential problems with transmembrane fluxes of probe while vesicles are passing through the spin columns. 300 μl volumes of the incubating mixtures were loaded into Centricon-10 micropartition cells (W.R. Grace, Amicon Division, Danvers, MA) and centrifuged in an angled rotor at $5000 \times g$ for 10 min at 5°C. Control experiments (not shown) verified that the 10 kDa cut-off dialysis membrane in these cells efficiently separate free from vesicle-associated Tc-MIBI. Cells were pre-coated with bovine serum albumin by filling with a 2 mg% solution of albumin for 10 min, then decanting and air-drying; this procedure reduced nonspecific adsorption of Tc-MIBI to the plastic vessel walls by approx. 98% (data not shown).

By sampling the activity of Tc-MIBI in the ultrafiltrate and retentate (methods as described for spin-column technique, above), and calculating the trapped aqueous volume in the retentate, the equilibrium intravesicular activity of Tc-MIBI and hence an intra/extravesicular activity ratio was determined. No correction was made for background binding of Tc-MIBI to the LUV membranes per se. Small corrections were made for partial reflection of Tc-MIBI from the dialysis membrane as determined by measuring the ratio of Tc-MIBI activity per unit volume (eluant/retentate) in micropartition cells containing Tc-MIBI but without LUVs; this ratio ranged between 88% and 94%.

Results

Time-course of Tc-MIBI accumulation

Previous studies have shown that LUV preparations exhibiting a K^+ diffusion potential (inside negative) can stably accumulate lipophilic cations such as tetraphenylphosphonium [23], leading to high internal/external concentration ratios of the agent that are quantitatively related to the membrane potential by the Nernst equation. Here we investigated the ability of LUVs to accumulate Tc-MIBI in a similar, potential-dependent manner. LUV preparations (160 mM internal K^+ concentration) with either 160 mM or 3 mM external K^+ were incubated both with and without valinomycin, and the apparent transmembrane distribution of Tc-MIBI was measured as a function of time (Fig. 1). Stable accumulation of Tc-MIBI required the presence of (1) a transmembrane K^+ gradient; and (2) valinomycin to clamp the membrane potential at the potassium equilibrium (Nernst) potential by increasing the membrane permeability to K^+ [24,25]. In the presence of 160 mM K_i/3 mM K_o and valinomycin ($E_m \cong -100$ mV), there was considerable accumulation of Tc-MIBI into the LUVs (Tc-MIBI$_i$/Tc-MIBI$_o \cong 50$); in contrast, little accumulation was seen in the absence of a K^+ gradient or in the absence of valinomycin.

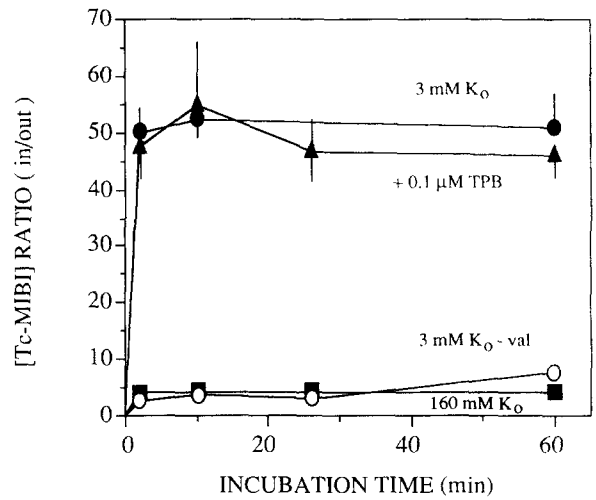

Fig. 1. Accumulation of Tc-MIBI measured as a function of time after addition of radioprobe to LUV suspension in the presence of valinomycin (1 μg/μmol phospholipid) under the following conditions: K_i 160 mM/K_o 3 mM (closed circles); same conditions, but in the absence of valinomycin (open circles); K_i 160 mM/K_o 160 mM (closed squares); K_i 160 mM/K_o 3 mM + 0.1 μM tetraphenylborate (closed triangles). Data are represented as mean ± S.E.(when larger than symbol) of three determinations.

The rate of accumulation of Tc-MIBI could not be determined in these experiments, however, equilibrium uptake appeared to be achieved by the earliest obtainable time point (2 min). Accumulation ratios remained stable over 2 h. Addition of tetraphenylborate (TPB), a lipophilic anion known to lower the activation energy for transmembrane diffusion of cations, had no measurable effect on the steady-state accumulation of Tc-MIBI in the presence of a membrane potential, as expected. This is in contrast to findings in whole-cell preparations, in which Tc-MIBI accumulation is enhanced in the presence of TPB [33] (see Discussion).

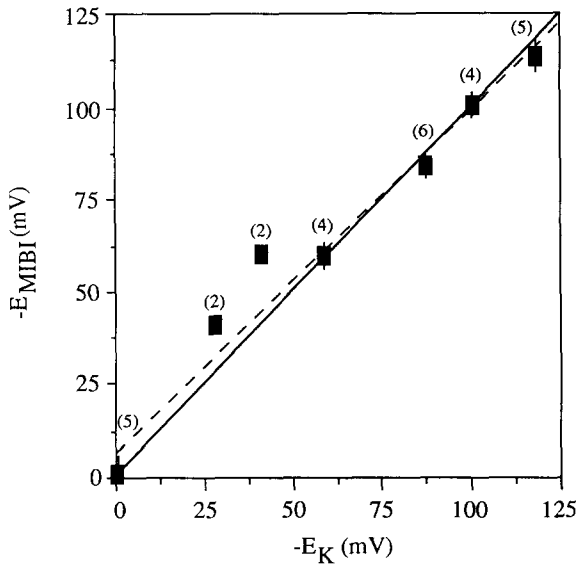

Fig. 2. Membrane potential estimated from Tc-MIBI distribution (E_{MIBI}) into large unilamellar vesicles is plotted against the potassium diffusion potential (E_K). Solid line is the line of identity (predicted Nernstian distribution). The dashed line represents a linear regression of the data ($-E_{MIBI} = -0.92$; $E_K + 6.8$ mV; $r = 0.97$). Numbers in parentheses indicate number of independent determinations at each potential.

Membrane-potential-dependence of Tc-MIBI accumulation

Studies of Tc-MIBI accumulation in cultured mouse fibroblasts [8] and chick heart cells [9] have shown that hyperpolarization of plasma membrane and mitochondrial membrane potentials (by exposure to nigericin) increases cellular accumulation of the agent. Conversely, depolarization (produced by raising the concentration of extracellular K^+ in the presence of valinomycin) both decreases steady-state accumulation and causes release of previously accumulated Tc-MIBI. We evaluated quantitatively the membrane-potential-dependence of Tc-MIBI accumulation in LUVs by varying the concentration gradient of K^+ across the LUV membrane. LUVs were incubated for 10 min in the presence of Tc-MIBI, valinomycin, and in solutions of varying K^+ concentration. Aliquots were loaded onto Centricon centrifugation dialysis cells to separate free- from trapped Tc-MIBI. The apparent membrane potential based on equilibrium Tc-MIBI distribution (E_{MIBI}) was plotted against the K^+-diffusion potential (E_K) (Fig. 2). The calculated membrane potential closely approximated the K^+-diffusion (membrane) potential over the range tested. Linear regression analysis yielded a slope for E_{MIBI} versus E_K of 0.92 (55.2 mV per decade change in K_o; $r = 0.97$). E_{MIBI} at zero potential was determined to be -1.1 ± 6.8 mV (mean $\pm$ SE, $n = 5$), consistent with a low level of nonspecific binding to the LUV membrane. As expected, inversion of the K^+ gradient across the LUV membrane (inside positive) excluded Tc-MIBI (data not shown).

Discussion

The use of permeant lipophilic cations to measure electrical potentials across biologic membranes is well established [26]. ^3H- or ^{14}C-labelled probes such as tetraphenylphosphonium have been used to estimate membrane potential in a wide variety of biologic systems not readily accessible by microelectrode techniques [27,28]. Optical probes such as cyanine dyes [26] have similarly found application in optically accessible systems. The data presented in this report indicate that the organotechnetium complex, Tc-MIBI, provides quantitative estimates of membrane potential.

Applications in vitro

As an agent for in vitro measurement of membrane potential in a variety of biologic systems, Tc-MIBI has several unique properties which may prove useful. These include: (1) the low level of potential-independent adsorption of Tc-MIBI to the lipid bilayer; (2) the high energy of its gamma photon, which permits direct counting of the gamma emissions from samples without the use of fluorescent cocktails; (3) short half-life, simplifying double label experiments as exemplified in this report; (4) extremely high specific activity (more than six orders of magnitude greater than is typical for ^3H- or ^{14}C-labelled agents), enabling use of Tc-MIBI at far lower concentrations and thereby reducing the likelihood of untoward biochemical or toxic effects of the agent; and (5) faster transmembrane kinetics compared to agents such as TPP, which may allow more rapid determination of E_m under selected circumstances. In this regard, concurrent addition of a lipophilic anion such as tetraphenylborate, commonly used to increase the membrane transport rates of cationic probes, would not be required with Tc-MIBI. Furthermore, the lack of any effect by TPB on Tc-MIBI accumulation in LUVs confirms previous conclusions from our laboratory that the enhancement by TPB of intracellular Tc-MIBI accumulation in whole cell preparations is mediated by TPB-induced alterations in mitochondrial transport, and not by effects on the plasma membrane [33].

The [Tc-MIBI] in/out ratio at zero potential was 1.043 ($E_{MIBi} = -1.1$ mV) by the equilibrium dialysis technique, which is very close to ideal (in/out = 1), but the ratio was 4.7 by the spin column technique (Fig. 1). The difference may be attributable to large negative potentials generated transiently across LUV membranes by the zero K_o spin columns during separation, thereby promoting Tc-MIBI influx and resulting in an overestimation of Tc-MIBI background binding using the gel filtration technique. While both techniques

conformed well at large potentials, the equilibrium dialysis technique yielded superior results at zero or low potentials.

Implications for clinical use

In common with other myocardial perfusion imaging agents, Tc-MIBI distribution in myocardium reflects regional blood flow [29]. However, the membrane-potential-dependent uptake and retention of Tc-MIBI offers an additional mechanism by which this diagnostic pharmaceutical may be able to discriminate between normal and nonviable myocardium. As has been shown previously [8,9], net uptake of Tc-MIBI in cultured cells can be inhibited (and previously-accumulated Tc-MIBI released) by depolarization of mitochondrial and plasma membrane potentials; similar results have been described for TPP accumulation in fibroblasts [30]. These findings suggest that Tc-MIBI can serve as a myocardial viability agent, to discriminate between normal and injured/nonviable (depolarized) myocardium after re-establishment of flow to a previously occluded arterial territory. In vivo experiments support this hypothesis [31,32]. Further cellular and molecular studies need to be performed to define active transport mechanisms (if any) that may affect transmembrane distribution of Tc-MIBI in tissues of interest.

Acknowledgements

The authors are grateful to Steven Seltzer and Monte Blau for use of the Lipex extrusion device, and to Mary Chiu for experimental assistance. Supported by grants from the National Institutes of Health (HL 42966 and GM 15904). David Piwnica-Worms is an Established Investigator of the American Heart Association.

References

1 Jones, A.G., Abrams. M.J., Davison, A., Brodack, J.W., Toothaker, A.K., Adelstein, S.J. and Kassis A.I. (1984) Int. J. Nucl. Med. Biol. 11, 225–234.
2 Kronauge, J.F., Kawamura, M., Lepisto, E., Holman, B.L., Davison, A., Jones, A.G., Costello, C.E. and Zeng, C.-H. (1990) in Technetium and Rhenium in Chemistry and Nuclear Medicine (Nicolini, M., Bandoli, G. and Mazzi, U., eds.), pp. 677–682, Cortina International, Verona.
3 Holman, B.L., Sporn, V., Jones, A.G. et al. (1987) J. Nucl. Med. 28, 13–18.
4 Wackers, F.J., Berman, D.S., Maddahi, J., Watson, D.D., Beller, G.A., Strauss, H.W., Boucher, C.A., Picard, M., Holman, B.L., Fridrich, R., Inglese, E. and Delaoye, B. (1989) J. Nucl. Med. 30, 301–311.
5 Wackers, F.J., Gibbons, R.J., Verani, M.S., Kayden, D.S., Pellikka, P.A., Behrenbeck, T., Mahmarian, J.J. and Zaut, B.L. (1989) J. Am. Coll. Cardiol. 14, 861–873.
6 Christian, T.F., Behrenbeck, T., Pellikka, P.A., Huber, K.C., Chesebro, J.H. and Gibbons, R.J. (1990) J. Am. Coll. Cardiol. 16, 1632–1638.
7 Piwnica-Worms, D., Kronauge, J.F., Holman, B.L., Davison, A. and Jones, A.G. (1989) Invest. Radiol. 24, 25–29.
8 Chiu, M.L., Kronauge, J.F. and Piwnica-Worms, D. (1990) J. Nucl. Med. 31, 1646–1653.
9 Piwnica-Worms, D., Kronauge, J.F. and Chiu, M.L. (1990) Circulation 82, 1826–1838.
10 Grinius, L.L., Jasaitis, A.A., Kadzianskas, Y.P., Liberman, E.A., Skulachev, V.P., Topali, V.P., Tsofina, L.M. and Vlanoimirova, M.A. (1970) Biochim. Biophys. Acta 216, 1–12.
11 Deutsch, C.J., Holian, A., Holiam, S.K., Daniele, R.P., and Wilson, D.F. (1979) J. Cell Physiol. 99, 79–93.
12 Lichtstein, S., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. USA 76, 650–654.
13 Bussolati, O., Laris, P.C., Longo, N., et al. (1986) Biochim. Biophys. Acta 854, 240–250.
14 Waggoner, A.S. (1979) Annu. Rev. Biophys. Bioeng. 8, 47–68.
15 Akerman, K.E. (1979) Biochim. Biophys. Acta 546, 341–347.
16 Johnson, L.V., Walsh, M.L. and Chen, L.B. (1985) Proc. Natl. Acad. Sci. USA 77, 990–994.
17 Johnson, L.V., Walsh, M.L., Bockus. B.J. and Chen, L.B. (1981) J. Cell. Biol. 88, 526–535.
18 Davis, S., Weiss, M.J., Wong, J.R., Lampidis, T.J. and Chen, L.B. (1985) J. BIol. Chem. 260, 13844–13850.
19 Hope, M.J., Bally, M.B., Webb, G. and Cullis, P.R. (1985) Biochim. Biophys. Acta 812, 55–65.
20 Mayer, L.D., Bally, M.B., Hope, M.J. and Cullis, P.R. (1985) Biochim. Biophys. Acta 816, 294–302.
21 Mayer, L.D., Wong, K.F., Menon, K., Chong, C.P., Harrigan, R. and Cullis, P.R. (1988) Biochemistry 27, 2053–2060.
22 Mayer, L.D., Bally, M.B. and Cullis, P.R. (1986) Biochim. Biophys. Acta 857, 123–126.
23 Redelmeier, T.E., Mayer, L.D., Wong, K.F., Bally, M.B., and Cullis, P.R. (1989) Biophys. J. 56, 385–393.
24 Tosteson, D.C., Cook, P., Andreoli, T. and Hallett, M. (1967) J. Gen. Physiol. 50, 2513.
25 Lauger, P. and Neumcke, B. (1973) in Membranes, Lipid Bilayers and Antibiotics (Eisenman, G., ed.), Vol. 2, pp. 1–59, Marcel Dekker, New York.
26 Sims, P.J., Waggoner, A.S., Wang, C.H. and Hoffman, J.F. (1974) Biochemistry 13, 3315–3330.
27 Nakazato, K., Murakami, N., Konishi, T. and Hatano, Y. (1988) Biochim. Biophys. Acta 946, 143–150.
28 Aiuchi, T., Matsunaga, M., Nakaya, K. and Nakamura, Y. (1989) Chem. Pharm. Bull. (Tokyo) 37, 3333–3337.
29 Leppo, J.A. and Meerdink, D.J. (1989) Circ. Res. 65, 632–639.
30 Rugolo, M. and Lenaz, G. (1987) J. Bioenerg. Biomembr. 19, 705–718.
31 Sinusas, A.J., Trautman, K.A., Bergin, J.D., Watson, D.D., Ruiz, M., Smith W.H. and Beller, G.A. (1990) Circulation 82, 1424–1437.
32 Santoro, G.M., Bisi, G., Sciagra, R., Leoncini, M., Fazzini, P.F. and Meldolesi, U. (1990) J. Am. Coll. Cardiol. 15, 301–314.
33 Piwnica-Worms, D., Kronauge, J.F., Chiu, M.L. (1991). J. Nucl. Med. 32, 1992–1999.