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Spectrofluorometric measurements of the dispersion state of pyrenedodecanoic acid and its uptake by cultured cells and liposomes

Shimon Gatt [a] and Eitan Fibach [b]

[a] *Department of Membrane Biochemistry and Neurochemistry, The Hebrew University-Hadassah School of Medicine and* [b] *Department of Haematology, Hadassah University Hospital, Jerusalem (Israel)*

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Pyrene dodecanoic acid (P12), a medium-chain fatty acid to which the fluorescent probe pyrene is covalently linked, showed a considerable increase in fluorescence when the probe was introduced into a hydrophobic environment. Also, when closely packed in an aggregate, an energy transfer between two adjacent molecules of pyrene occurred, resulting in a shift of the peak of the emission spectrum from 378 nm ('monomeric') to 475 nm ('excimeric'). These two respective properties were utilized for the following: (a) A spectrofluorometric measurement of the critical micellar concentration (CMC) of the pyrene fatty acid, defined as the concentration at which the 475 nm emission peak appeared as a consequence of the aggregation of P12 molecules in aqueous solution to form micelles; the CMC of P12 was found to be in the range of 1 to 2 μM. (b) The penetration of P12, from an aqueous solution or dispersion, into unilamellar phospholipid vesicles was determined by monitoring the increase of the fluorescence at 378 nm. The fluorescence increase was time-dependent and proportional to the respective concentrations of P12 or phospholipid vesicles. Substituting the neutral phosphatidylcholine with the negatively-charged phosphatidylserine vesicles resulted in a slower rate as well as lesser total uptake of P12. (c) The uptake of P12 by cells was accompanied by an increase in the monomeric fluorescence emission intensity. Using cells in suspension, this could be followed continuously in a spectrofluorometer equipped with a recorder. The uptake was found to be time-dependent and proportional to P12 concentration.

Introduction

Uptake of medium and long chain fatty acids by cells has been studied extensively. In most studies radioactively-labelled fatty acids were complexed to albumin and incubated with the respective cells [1]. This mimicked physiological conditions where the fatty acids are mostly linked to proteins; e.g., in serum, over 99% of the unesterified acids ('free fatty acids') are complexed with albumin [2,3]. In some studies, fatty acids were dispersed in medium devoid of albumin, and the uptake of molecules which dissociated from the micellar dispersion was followed [4].

Using radioactively-labelled fatty acids (e.g., Ref. 5) required extensive washing of the cells, extraction of their lipids and counting. In previous

Abbreviations: P-12, 12-(1-pyrene)dodecanoic acid; MEL, murine erythroleukaemia cells; PBS, phosphate-buffered saline; CMC, critical micellar concentrations; SUV, small unilamellar vesicles; PS, phosphatidylserine; PC, phosphatidylcholine; E/M, emission of excimers/emission of monomers.

Correspondence: E. Fibach, Department of Haematology, Hadassah University Hospital, Jerusalem 91120, Israel.

studies we followed uptake by cell suspension of fatty acids to which a fluorescent probe had been linked covalently. The latter provided a sensitive label for measuring uptake of the respective fatty acid [6]. The analytical approach using fluorescent derivatives of fatty acids was, however, similar to that employed with the radioactive acids. Thus, following incubation, the cells were washed, their lipids extracted with organic solvents and chromatographed on columns or thin-layer plates of silica or alumina and the fluorescence of the total extract or respective lipid fractions measured.

In this paper we show that uptake of a fluorescent fatty acid derivative pyrenedodecanoic acid (P12), by liposomes and cells, can be measured directly and followed continuously in a spectrofluorometer, obviating the need for separating, washing and extracting the cells. For this purpose the following properties of the pyrene fatty acid were utilized: (a) Below the critical micellar concentration (CMC) only monomers exist [7] and these are strongly quenched in aqueous media. Transfer of these molecules to a hydrophobic environment (e.g., liposome or cell membrane, as shown in this paper) or even attached to the surface of albumin, as demonstrated in a previous publication [8], causes a considerable increase in their fluorescence. (b) Above the CMC, aggregation of molecules in solution occurs. An energy transfer between adjacent molecules of the pyrene fatty acid causes a shift in the fluorescence-emission from the monomeric (378 nm) to an eximeric, longer wavelength (peak at 475 nm) [9–12]. Upon addition of liposomes or cells, a considerable increase in the monomeric emission occurred as a consequence of incorporation of P12 molecules into their bilayered membranes.

Materials and Methods

Dispersion of P12. The required quantity of 12-(1-pyrene)dodecanoic acid (P12) (Molecular Probes, Junction City, OR) in chloroform/methanol (2:1, v/v), was evaporated to dryness under nitrogen, 2–4 mole excess of KOH was added, the tube was heated 10 min at 60°C and phosphate-buffered saline (PBS) was added to the required volume.

Preparation of phospholipid vesicles. Solutions of phospholipids, in mixtures of chloroform and methanol were evaporated under nitrogen and further dried in a high vacuum for about 2 h. PBS was added and the dispersion stirred on a cyclomixer and sonicated, under nitrogen for 3 min at 4°C using a Heat System Sonicator 350 equipped with a microprobe. The dispersion was then centrifuged in an Eppendorf tube for 2 min at about $10^4 \times g$ at 4°C and the clear supernatant collected.

Cells. The HL-60 cell line, originally established from the peripheral blood of a patient with acute promyelocytic leukaemia [13] and the murine erythroleukaemia (MEL) cells established by Friend et al. [14] were maintained in alpha-minimal essential medium supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY). Cells were cultured every 3–4 days and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Measurement of fluorescence of P12. Fluorescence of aqueous solutions or dispersions of P12 in the absence or presence of small unilamellar vesicles (SUV) and cells, was measured in Perkin-Elmer LS-5 spectrofluorometer and a R 100-A recorder. The excitation was at 343 nm and emission at 378 nm ('monomeric') or 475 nm ('excimeric'). Scans between 370 nm and 550 nm were recorded.

Results

Fluorometric measurements of the dispersion state of P12

Irradiation of an aqueous solution of monomeric P12 (< 1 μM) at 343 nm resulted in an emission peak at 378 nm and a second peak of lesser intensity at 405 nm (Fig. 1A). When the concentration of P12 was increased another peak, at 475 nm, appeared. The latter peak reflects the formation of micellar aggregates and is the consequence of an 'energy transfer' between adjacent pyrene molecules in the aggregate. This excimeric peak was already evident at 1 to 2 μM. Further increase in the concentration of P12 caused only a small increase in the monomeric peak, while the excimeric peak increased considerably; the ratio between the peak heights (E/M ratio) is demon-

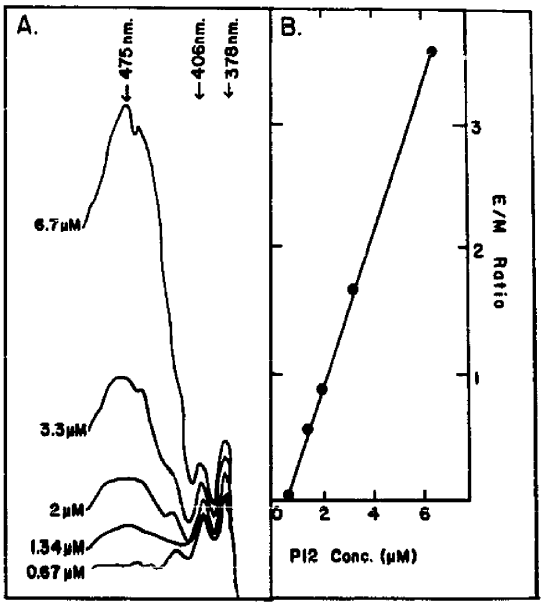

Fig. 1. Fluorescence of P12 in aqueous solution. (A) P12 was dispersed in PBS at the indicated concentrations, excited at 343 nm and the emission spectra, between 370 and 500 nm, were scanned. (B) The ratios of emission at 475 nm over emission at 378 nm (E/M) were calculated and plotted as a function of P12 concentration.

strated in Fig. 1B. The 'critical micellar concentration' (CMC) of P12, defined as the concentration at which micellar aggregates start to form, was between 1 to 2 μM.

Spectrofluorometric monitoring of uptake of P12 by unilamellar liposomes

We have previously shown that the fluorescence of P12 which is highly quenched in water increased considerably when the fatty acid was linked to albumin, probably because association of the pyrene nucleus with the hydrophobic environment of the protein [8]. It was expected that a similar increase would occur upon incorporating P12 molecules into the hydrophobic environment of the membrane of a liposome or cell. Fig. 2 shows a continuous recording, by the spectrofluorometer, of the time-dependent increase in the fluorescence emission at 378 nm upon mixing P12 with SUV of phospholipids, indicating the incorporation of molecules of P12 into the bilayer vesicle. In Fig. 2A, 0.5 and 1 μM of P12 and 5 μM PC-SUV were used. The fluorescence increased in a hyperbola-like fashion and its intensity was related to the respective P12 concentration. In Fig. 2B, 1 μM P12 was administered to 5 or 20 μM PC-SUV. Although the latter concentration exceeded the former 4-fold, the fluorescence increased only 2.5-fold. Fig. 2C compares uptake of 1 μM P12

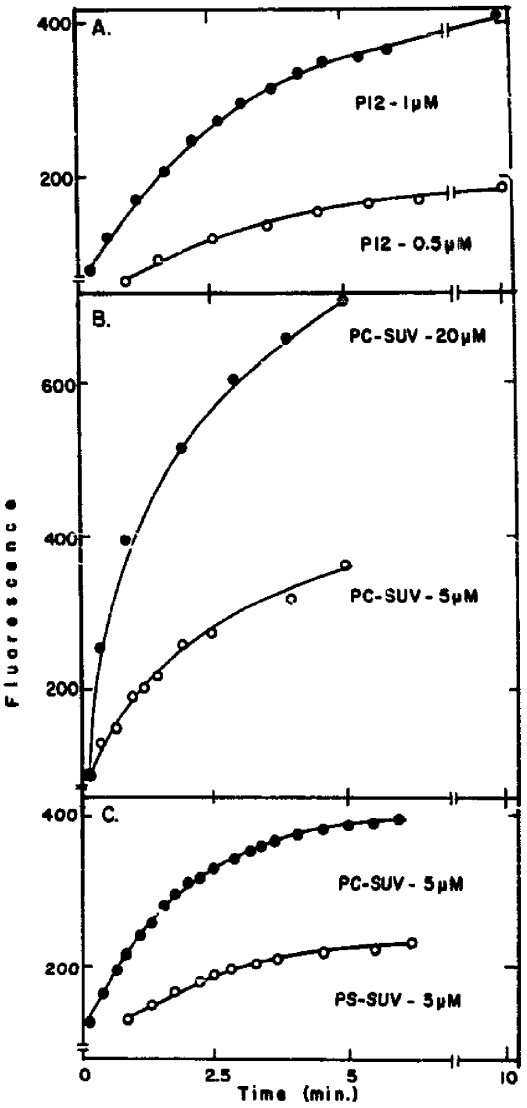

Fig. 2. Kinetics of influx of P12 into unilamellar phospholipid veiscles as measured by increase of its fluorescence. (A) P12, 0.5 or 1 μM, was added to sonicated vesicles of PC (10 μM) and the fluorescence at 378 nm recorded. (B) P12 (1 μM) was added to vesicles contained 5 or 20 μM of PC. (C) P12 (1 μM) was added to PC or PS vesicles (5 μM each).

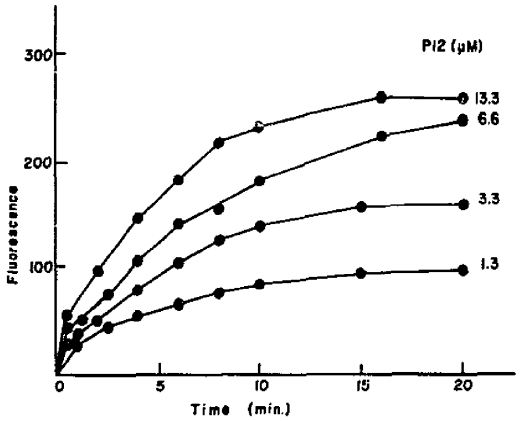

Fig. 3. Kinetics of uptake of P12 by cells as measured by the increase of its fluorescence. P12, at the concentrations shown on the respective curves, was dispersed in PBS containing 5 mM glucose, 10^6 HL-60 cells were added and the fluorescence at 378 nm was measured after various incubation times.

into PC or PS vesicles (5 μM each). While the former vesicles are neutral due to the zwitterionic form of the PC, the latter have a net negative charge. Incorporation of P12 into the PS vesicles was slower and the quantity incorporated less than that observed with the corresponding PC vesicles.

Spectrofluorometric monitoring of P12-uptake by cells

Fig. 3 shows the time-dependent increase in the fluorescence emission at 378 nm as a consequence of uptake of P12 by cells. The concentrations used (from 1.3 to 13.3 μM) were all in the micellar region and the increasing fluorescence intensities at 378 nm suggests a monomeric distribution of the P12 molecules following uptake by and incorporation into the cells. This is further analysed in Fig. 4.

Fig. 4A summarizes the variations in the E/M ratios, as a function of the concentration of the P12. At all P12 concentrations, uptake by the cells increased considerably the fluorescence intensity of the monomeric emission (378 nm) while the fluorescence of the excimers (475 nm) was in-

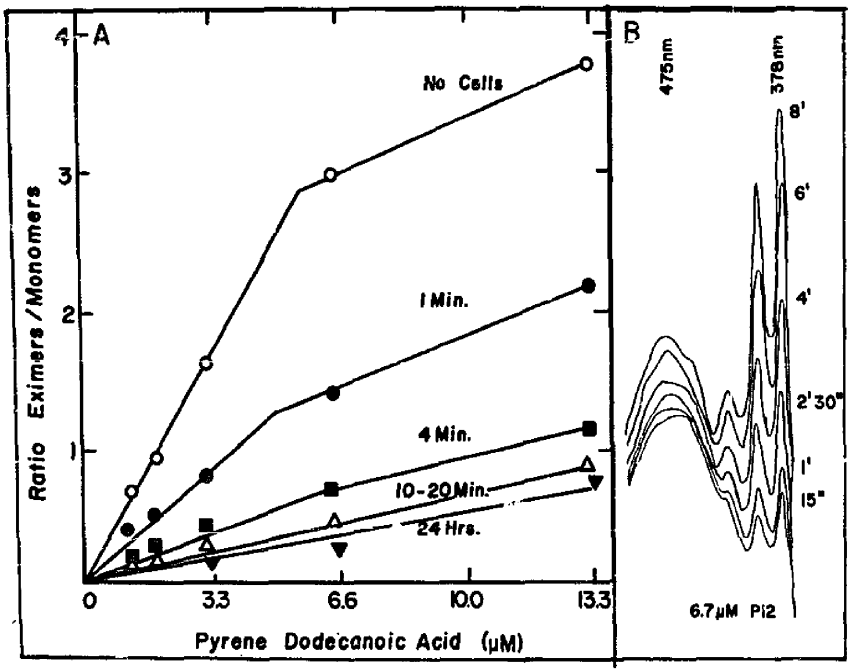

Fig. 4. Changes in emission spectra as a result of uptake of P12 by cells. HL-60 cells (10^6/ml) were exposed to the indicated concentrations of P12 in PBS. (A) The emission at 475 nm (excimers) and 378 nm (monomers) were measured at various intervals and the E/M ratio calculated. (B) Scan of emission spectra of 6.7 μM P12 in PBS following the indicated periods of incubation.

creased to a much smaller extent. This is illustrated in Fig. 4B which shows the time-related scans of emission spectra due to uptake of one concentration of P12 (6.7 μM) by the cells. With increasing incubation time, the fluorescence at 378 nm increased over 10-fold, while that at 475 nm increased only by 35% above its base value, at zero time. Thus, the E/M ratio, which was close to 3 at zero time, (see Fig. 4A) decreased to about 0.4 after 8 min. This again emphasized the monomolecular distribution of P12 and its metabolic products (e.g., neutral- and phosphoacylglycerols) within the cell.

The uptake of P12 at concentrations below its CMC could similarly be continuously measured and recorded spectrofluorometrically. Fig. 5 depicts the recordings of the time-related changes in the fluorescence at 378 nm of solutions of 0.2 and 0.4 μM P12 following addition of cells. After 25 min the increase in the fluorescence was 2-fold greater in cells incubated with 0.4 μM relative to those containing 0.2 μM P12. The rate of P12-uptake could be calculated by determining the change in the fluorescence per minute. The results (inset) indicate that the initial rate of uptake of P12 at 0.4 μM was twice the rate of 0.2 μM; at either concentration, the rate decreased with time and saturation was reached after about 30 min. The saturation was a result of depletion of P12 from the incubation medium; following addition of fresh P12 the uptake was resumed (data not show).

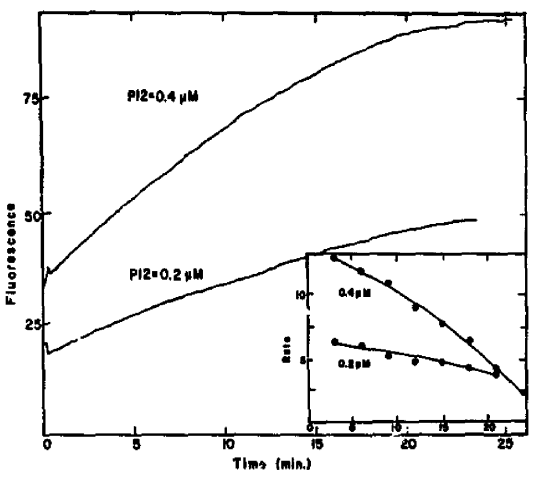

Fig. 5. Spectrofluorometric recording of the uptake of P12 by HL-60 cells. P12, 0.2 or 0.4 μM, was dispersed in PBS containing 5 mM glucose, HL-60 cells were added and the emission at 378 nm was recorded continuously. In the inset the rate of uptake was calculated as the change in fluorescence per min.

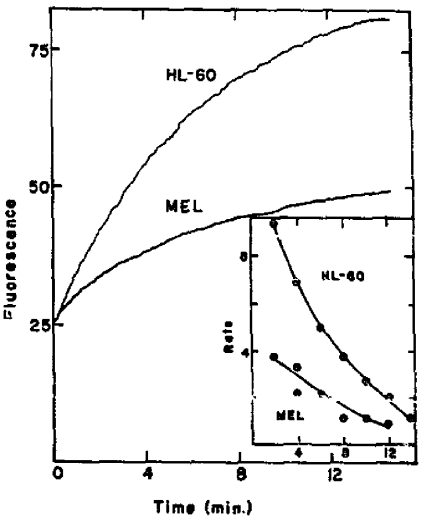

Fig. 6. Spectrofluorometric recording of uptake by P12 by HL-60 and MEL cells. P12, 0.4 μM, was dispersed in PBS containing 5 mM glucose, $0.4 \cdot 10^6$ cells per ml, HL-60 or MEL cells, were added and the emission at 378 nm recorded. The inset shows the rate of uptake of P12 (fluorescence change per min) by the two respective cell lines.

We have previously shown, using other techniques, that various cell types differ in their rate of P12-uptake; thus, HL-60 cells took up more P12 and at a faster rate than MEL cells [14]. In the experiment depicted in Fig. 6, the uptake of P12 by these two respective cell lines was measured by continuously recording the fluorescence increase. Strikingly, even at very short time intervals (less than one minute) the rate of the uptake by HL-60 cells was 2-fold higher as compared to MEL cells.

Discussion

In this paper the dispersion state as well as its uptake by liposomes and cells of a pyrene fatty acid (P12) were determined spectrofluorometrically. Evaluation of the dispersion state is based on the fact that a molecular solution of P12 emits photons at 378 ('monomers') but not at 475 nm,

while in an aggregated dispersion photons are emitted at 475 nm ('excimers') due to the energy-transfer between two adjacent pyrene molecules. That concentration which resulted in appearance of excimers was defined as the critical micellar concentration (CMC) for P12. It should be noted that while the aliphatic residue of P12 is only 12 carbon atoms long, the fused aromatic 4-ring structure of pyrene adds another eight carbon atoms to the total length, making its length between stearic (C18 : 0) and cosanoic (c20 : 0) acids. This, and the added molecular mass of 200 daltons (i.e., 50% of the total mass) are probably responsible for the rather low CMC (about 1–2 μM) defined for this acid, on the basis of appearance of the excimers.

Water has a strong quenching effect on the fluorescence emission of P12 at 378 nm and introduction of P12 into a hydrophobic environment increased considerably its fluorescence at this wavelength. Thus, complexing of P12 to serum albumin resulted in very considerable increase of the fluorescence at 378 nm [14]. The increased fluorescence intensity upon incorporating P12-molecules into a hydrophobic environment was utilized for monitoring the penetration of P12 into unilamellar phospholipid vesicles or its uptake by suspensions of cultured cells. The high fluorescence at 378 nm and its absence at 475 nm following incubating cells with P12-micelles suggested that upon penetration into the cellular membranes, molecules of P12 are diluted to an extent which decreases the chance of two molecules to become close enough for energy transfer to occur. It is most plausible that the first event which follows the association of P12 with the surface of the SUV or cell, is its insertion into the outer layer of the bilayered membrane. But the rapid rate of flipping over, suggested by Doody et al. [15] for unilamellar vesicles raises the possibility that this might also occur in the systems described in this paper.

Uptake of fatty acids by cells has been investigated extensively using radioactively-labelled molecules. This required separating and extensive washing of the cells prior to counting the cell-associated radioactivity. In contrast, uptake of a fluorescent fatty acid (i.e. P12) by cell suspensions or even liposomes could be monitored continuously, starting about 15 s after mixing the two respective components. The time-dependent increase in fluorescence, presented in relative fluorescence units, permitted following kinetics of uptake by a cell suspension as well as comparing uptake by different cell types. For determining the precise quantity of P12 which has been incorporated into the cells, their lipids could be extracted and the fluorescence emitted by their solution in an organic solvent translated to mole-quantities using a suitable standard solution of P12 in the same solvent.

In many of the experiments described in this paper, uptake of P12 by cells was measured in an albumin-free medium, while in serum fatty acids are bound to albumin. Direct measurements of uptake, by liposomes or cells, of P12 from its complex with albumin is impossible since the latter exhibits a high fluorescence [8]. We have recently shown that such direct measurements could be done using albumin which had been interacted with trinitrobenzene sulfonic acid. When P12 was complexed to this derivatized albumin, the yellow color of the trinitrophenyl residues quenched the fluorescence emitted by the pyrene ring. When the complex was incubated with cells release of P12 molecules from the trinitrophenyl-albumin P12 complex and their subsequent uptake by the cells was accompanied by an increase in fluorescence which could be monitored continuously [8].

The procedures described in this paper permitted studying effects, on P12 uptake by liposomes or cells, of numerous factors such as concentration, charge, pH, temperature, absence or presence of glucose and metabolic inhibitors, by direct monitoring of the fluorescence, obviating the need for separating between the fatty acid and the SUV or cells (data not shown). Potentially, it could also be adapted for studying numerous problems related to liposomes or cells, such as uptake, release and transfer of lipids [12,16–17]. Use of quench-dequench and energy transfer could also be utilized for studying fusion processes [18–20]. This technique could also be useful for studying differences in uptake of P12 by different cell types and changes that occur following induction of cell maturation [21].

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References

1 Morand, O., Fibach, E. and Gatt, S. (1982) Biochim. Biophys. Acta 693, 143–150.
2 Goodman, D.S. (1958) J. Am. Chem. Soc. 80, 3892–3898.
3 Spector, A.A. (1975) J. Lipid. Res. 16, 165–179.
4 Spector, A.A. (1968) Ann. NY Acad. Sci. 149, 768–783.
5 Morand, O., Fibach, E., Dagan, A. and Gatt, S. (1982) Biochim. Biophys. Acta 711, 539–550.
6 Morand, O., Fibach, E., Livni, N. and Gatt, S. (1984) Biochim. Biophys. Acta 793, 93–104.
7 Shinoda, K. (1963) in Colloidal Surfactants (Shinoda, K., Makagawa, T., Tamamushi, B.T., Isemura, T., eds.), pp. 1–8, Academic Press, New York and London.
8 Gatt, S., Nahas, N. and Fibach, E. (1988) Biochem. J. 253, 377–380.
9 Charlton, S.C., Olson, J.S., Wong, K.Y., Pownall, H.J., Louie, D.D. and Smith, L.C. (1976) J. Biol. Chem. 251, 7952–7955.
10 Sengupta, P., Sackman, E., Kühnle, W. and Scholz, H.P. (1976) Biochim. Biophys. Acta 436, 869–878.
11 Doody, M.C., Pownall, H.J. and Smith, L.C. (1978) Fed. Proc., Fed. Am. Soc. Exp. Biol. 37, 1832.
12 Roseman, M. and Thompson, T.E. (1980) Biochemistry 19, 439–444.
13 Collins, S., Gallo, R.C. and Gallagher, R.E. (1977) Nature (Lond.) 20, 347–349.
14 Friend, C., Scher, W., Holland, J.G. and Sato, T. (1971) Proc. Natl. Acad. Sci. USA 68, 378–382.
15 Doody, M.C., Pownall, H.J., Kao, Y.J. and Smith, L.C. (1980) Biochemistry 19, 108–116.
16 Pownall, H.J., Hickson, D., Gotto, A.M. and Massey, J.B. (1982) Biochim. Biophys. Acta 712, 169–176.
17 Frank, A., Barenholz, Y., Lichtenberg, D. and Thompson, T.E. (1983) Biochemistry 22, 5647–5651.
18 Owen, C.S. (1980) J. Membr. Biol. 54, 13–20.
19 Struck, D.K., Hoekstra, D. and Pagano, R.E. (1981) Biochemistry 20, 4093–4099.
20 Hoekstra, D., Klappe, K., De Boer, T. and Wilschut, J. (1985) Biochemistry 24, 4739–4745.
21 Fibach, E., Nahas, N., Giloh, H. and Gatt, S. (1986) Exp. Cell. Res. 66, 220–228.