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Continuous spectrofluorometric measurements of uptake by cultured cells of 12-(1-pyrene)-dodecanoic acid from its complex with albumin

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Aqueous dispersions of 12-(1-pyrene)-dodecanoic acid (P12), a medium-chain fatty acid to which the fluorescent probe pyrene has been covalently linked, shows a considerable increase in fluorescence when the probe is introduced into a hydrophobic environment. This enables the uptake of P12 by liposomes and cells to be followed directly in a spectrofluorometer, without separating the cells from the P12-containing medium. In the present study, we show that complexing P12 to albumin produced a very high fluorescence emission intensity. This made direct measurements of the uptake by cells of albumin-bound P12 impossible. Such direct measurements could, however, be made using albumin which had been interacted with trinitrobenzenesulphonic acid (TNBS). The yellow trinitrophenyl (TNP) residues, which were thereby covalently linked to the albumin, quenched the fluorescence of pyrene in the TNP–albumin/P12 complex. Upon release of the P12 molecules from this complex and their subsequent uptake by cells, fluorescence increased. This technique was utilized for the continuous monitoring of the uptake of P12 by different cell types and cells at various stages of maturation.

INTRODUCTION

At low concentrations, fatty acids exist in a monomolecular solution in water. Above a certain concentration (critical micellar concentration, CMC) aggregation occurs and micelles are formed (Shinoda, 1963). In biological fluids, fatty acids are mostly linked to proteins; thus, in serum, over 99% of the non-esterified acids ('free fatty acids') are complexed to albumin (Goodman, 1958; Spector, 1975). Uptake of fatty acids by cells most probably occurs when single molecules, which dissociate from the albumin, reach the plasma membrane and insert into the outer layer. Subsequently, translocation into the inner layer occurs, followed by conversion to the coenzyme-A derivatives and incorporation into the neutral lipids and phospholipids in the cell interior. In the absence of albumin, single molecules of the fatty acids, obtained by dissociation from micelles (Spector, 1968), incorporate into the plasma membrane.

For most studies of transport, radioactively labelled fatty acids have been employed (e.g. Morand *et al.*, 1982*a* and references therein). The use of these compounds required separation, extensive washing of the cells, extraction and counting the radioactivity of the lipid extract. In previous studies, we replaced the radioactive tracer with a fluorescent probe, which provided a sensitive label that could be observed microscopically and quantified (Morand *et al.*, 1982*b*, 1984). The analytical approach using fluorescent derivatives of fatty acids was similar to that employed with the radioactive acids. Thus, following incubation, the cells were centrifuged, washed extensively, their lipids extracted with organic solvents and chromatographed on columns or thin-layer plates of silica or alumina. Recently, we devised procedures in which both the dispersion state and uptake of 12-(1-pyrene)-dodecanoic acid (P12) by liposomes and cells could be followed and measured directly in a spectrofluorometer, obviating the need for separating, washing and extracting (E. Fibach & S. Gatt, unpublished work). For this purpose, two properties of the pyrene fatty acid were utilized: (i) its fluorescence, which is quenched in water, increases considerably when the pyrene is introduced into a hydrophobic environment, such as micelles, liposomes or cells; (ii) when irradiated at 343 nm, single molecules of P12 emit mainly at 378 nm, but, when present in an aggregated form, an energy transfer occurs between adjacent pyrene molecules and the emission shifts to a longer wavelength with a peak at 475 nm (Charlton *et al.*, 1976; Sengupta *et al.*, 1976; Doody *et al.*, 1978; Roseman & Thompson, 1980). Appearance of the latter, 'excimeric', emission peak could thus be an indicator of aggregation; and its disappearance, of disaggregation into an undispersed, monomeric state.

The present study indicated that complexing P12 to albumin resulted in a considerable increase in fluorescence. Because of this, transfer of P12 from its complex with albumin into cells could not be measured by the direct procedure. To overcome this, albumin was treated with trinitrobenzenesulphonic acid (TNBS) and then complexed with P12. The yellow colour of the covalently linked trinitrophenyl (TNP) groups absorbs the photons emitted by the pyrene nucleus and therefore quenches its fluorescence. The TNP–albumin/P12 complex, thus, has

Abbreviations used: P12, 12-(1-pyrene)-dodecanoic acid; MEL, murine erythroleukaemia cells; FCS, fetal calf serum; BSA, bovine serum albumin; PBS, phosphate-buffered saline; DMSO, dimethylsulphoxide; HMBA, hexamethylenebisacetamide; TNBS, trinitrobenzenesulphonic acid; TNP, trinitrophenyl; CMC, critical micellar concentration.

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a rather low fluorescence intensity. Upon release of P12 molecules and their insertion into the cell membrane, the fluorescence is dequenched and increases considerably, thereby permitting the continuous measurement of P12 uptake by the cells. This paper describes the synthesis of TNP–albumin and the procedure for continuously measuring, in a spectrofluorometer, the uptake of P12 which has been complexed to this 'yellow albumin' by various cultured cells.

MATERIALS AND METHODS

Materials

12-(1-Pyrene)dodecanoic acid (P12) was purchased from Molecular Probes (Junction City, OR, U.S.A.); bovine serum albumin (BSA; fatty acid poor) from Sigma; and trinitrobenzenesulphonic acid (TNBS) from Aldrich.

Methods

Synthesis of TNP–albumin. For trinitrophenylation, BSA (100 mg) was dissolved in 8 ml of 0.2 M-$NaHCO_3$ and 400–4000 mg of TNBS in 80–800 μl of water, respectively was added and stored overnight, in the dark. The solution was then extensively dialysed against water and freeze-dried. The optical density of the TNP–albumin was determined at 330 nm (in an acidic medium) or 420 nm (in an alkaline medium).

Formation of the TNP–albumin/P12 complex. The required quantity of P12 in chloroform/methanol (2:1, v/v), was evaporated to dryness under nitrogen, 2–4 mol excess of KOH was added, the tube was heated for 10 min at 60 °C and phosphate-buffered saline (PBS) was added to the required volume. For formation of the albumin complex, a solution of TNP–BSA was added to the tube containing P12 and KOH, the mixture was incubated for 15 min at 37 °C and PBS was added to the desired volume.

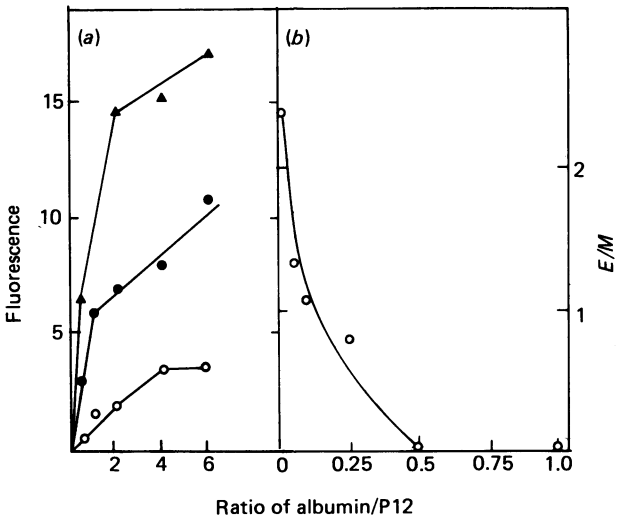

Fig. 1. Effect of albumin on the fluorescence of P12

(*a*) Complexes of P12 with BSA were prepared as described in the Methods section and the fluorescence was recorded at 378 nm. ▲, 1 μM-P12; ●, 0.5 μM-P12; ○, 0.25 μM-P12. (*b*) P12 (20 μM) was complexed to albumin, the emission was measured at 378 nm (monomers) and 475 nm (excimers) and the ratio (*E*/*M*) of the two respective values calculated.

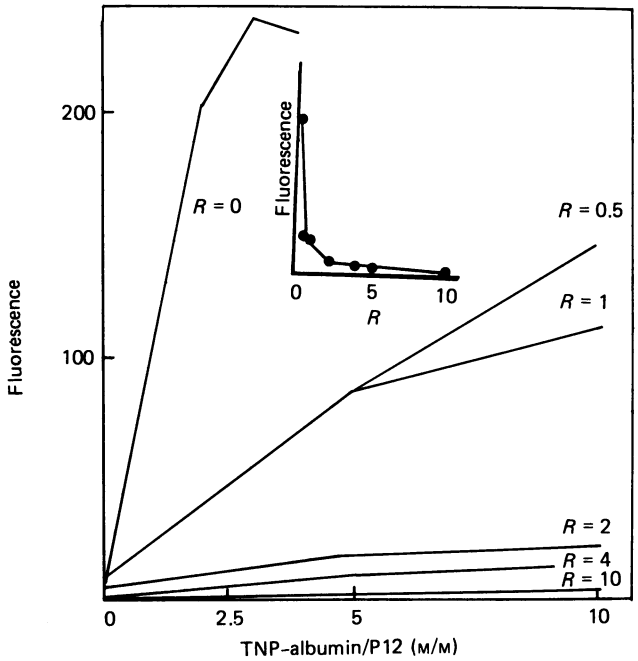

Fig. 2. Effect of TNP–albumin on P12 fluorescence

BSA was interacted with TNBS as described in the Methods section, at molar ratios of TNBS to albumin designated on the respective curves as *R*. P12 (0.2 μM) was complexed with this albumin at the ratios shown on the abscissa and the fluorescence at 378 nm was recorded. The inset demonstrates the enhanced quenching effect that an increase in the ratio of TNBS to albumin has on P12 fluorescence.

Cells. The HL-60 cell line, originally established from the peripheral blood of a patient with acute promyelocytic leukaemia (Collins *et al.*, 1977), and the murine erythroleukaemia (MEL) cells established by Friend *et al.* (1971), were maintained in α-minimal essential medium supplemented with 10% (v/v) fetal bovine serum (Gibco). Cells were cultured every 3–4 days and incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 in air. Differentiation was induced in MEL cells by the addition of 280 mM-dimethylsulphoxide (DMSO) (Friend *et al.*, 1971) or 4 mM-hexamethylenebisacetamide (HMBA) (Reuben *et al.*, 1976) upon subculturing. The number of viable cells was determined by the Trypan Blue exclusion procedure. Differentiated cells were scored by the benzidine procedure (Fibach *et al.*, 1977) which detects haemoglobin-containing cells.

Measurement of P12 fluorescence. Fluorescence of aqueous solutions or dispersions of P12 was measured in a Perkin-Elmer LS-5 spectrofluorometer equipped with an R 100-A recorder. The excitation was at 343 nm and emission at 378 nm ('monomeric') or 475 nm ('excimeric').

RESULTS

Fig. 1(*a*) shows the effect of albumin on the fluorescence of P12. At concentrations less than its CMC (i.e.

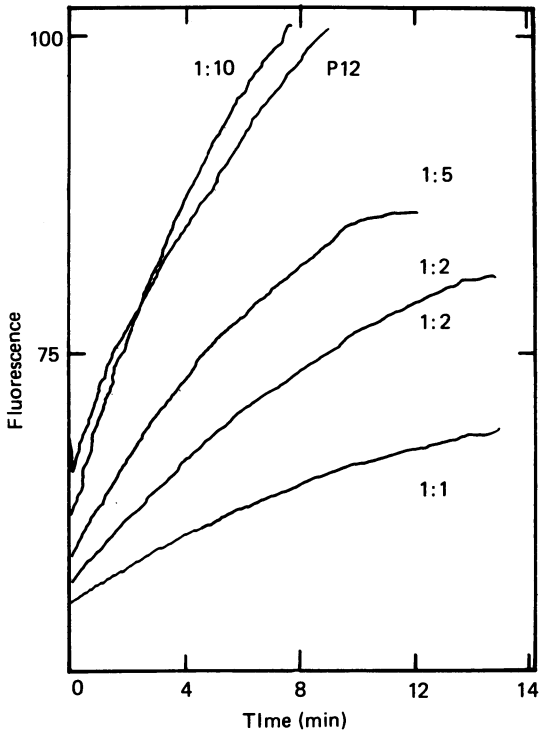

Fig. 3. Kinetics of P12 uptake by cells from complexes of varying ratios of TNP–albumin to P12

P12 (2 μM) was complexed to TNP–albumin at the indicated ratios of TNP–albumin to P12. The ratio of TNP to albumin was 5:1 in all cases. HL-60 cells (0.5×10^6/ml) were added and the changes in the fluorescence at 378 nm with time were recorded.

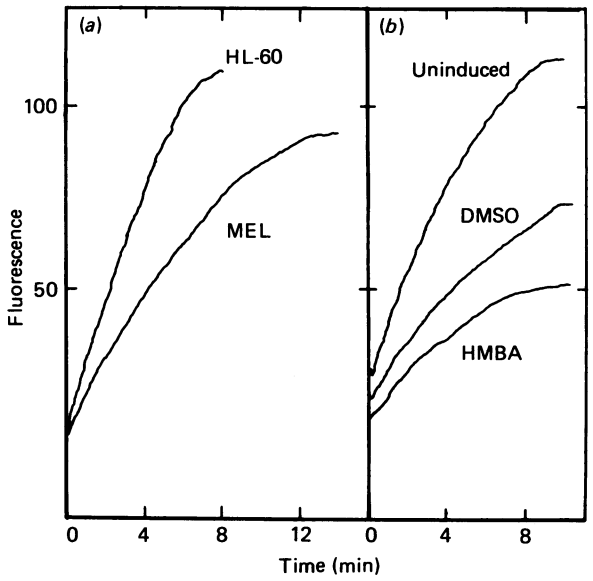

Fig. 4. Comparison of the uptake of P12 complexed to TNP–albumin by HL-60 and MEL cells, and by differentiated and undifferentiated MEL cells

P12 (2 μM) was complexed to TNP–albumin (5:1) at a ratio of TNBS–albumin to P12 of 1:5. (*a*) HL-60 or MEL cells (0.5×10^6/ml) were added and the fluorescence at 378 nm recorded. (*b*) MEL cells were incubated in medium containing 10% (v/v) fetal bovine serum, in the absence or presence of the differentiation inducers, DMSO or HMBA. After 4 days, cultures without inducer exhibited less than 1% haemoglobin-containing cells, while 87 and 98% of the cells incubated with DMSO or HMBA, respectively, contained haemoglobin, as measured by the benzidine cytochemical staining technique. Cells from the respective cultures were harvested, washed, added to solutions of P12 complexed to TNP–albumin and their fluorescence recorded.

0.25–1 μM), P12 formed a complex with albumin in which the hydrophobic portion of the acid is most likely attached to a hydrophobic region of the protein, as evidenced by the very great increase in the fluorescence intensity at the monomeric emission peak (378 nm). When P12, at a concentration which leads to the formation of micelles (20 μM), was complexed with albumin (Fig. 1*b*), disaggregation of the micelles and binding of the fatty acyl molecules to albumin occurred, causing a considerable decrease of the excimeric-to-monomeric (E/M) ratio.

Since complexes of P12 with albumin exhibited a high fluorescence, changes in fluorescence owing to the transfer of P12 from such complexes into cells could not be measured directly. Fig. 2 shows an experiment in which 0.2 μM-P12 was complexed to albumin which had been trinitrophenylated using various molar ratios of TNBS to albumin. The yellow colour of the covalently linked TNP groups quenched the fluorescence emission of P12 at 378 nm. As shown in the main Figure and inset, the degree of quenching depended on the ratio of TNBS to albumin, namely on the number of TNP residues linked to an albumin molecule (designated in Fig. 2 as R).

When P12, complexed to TNP–albumin, was incubated with cells, the fluorescence increased with time. This increase reflected, most likely, the release of the P12 from the complex, where its fluorescence is quenched, and its introduction into the hydrophobic cellular environment, where its fluorescence is amplified. In a separate experiment, the kinetics of uptake of P12 complexed to albumin as compared with P12 complexed to TNP–albumin were followed by washing the respective cells, following the incubation period and extracting their lipids. In either case, the fluorescence of the extracted lipids was identical, indicating that the trinitrophenylation did not change the properties of the albumin with respect to P12 binding and release for incorporation into the cell membrane (data not shown). Fig. 3 shows the kinetics of P12 uptake (2 μM) by cells from complexes of varying ratios of TNP–albumin to P12. The results indicate that the rate of uptake decreased as the ratio of TNP–albumin to P12 increased, indicating that albumin has an inhibitory effect on P12 uptake; this has been shown before by Morand *et al.* (1982*b*), using other techniques.

Fig. 4 demonstrates the differences in the uptake of P12 complexed to TNP–albumin by two cell types, HL-60 and MEL; and following erythrodifferentiation of MEL cells by two differentiation-inducing agents, DMSO and HMBA. Similar to previous findings using other techniques and much longer incubation times (Nahas *et al.*, 1987; Fibach *et al.*, 1986), uptake by MEL cells was less than by HL-60 cells and differentiation of the former further decreased the rate and quantity of P12 uptake.

DISCUSSION

Uptake of fatty acids by cells has been investigated mostly by using radioactively labelled molecules. This required separating and extensive washing of the cells before counting the cell-associated radioactivity, making continuous measurement of the uptake impossible. We have found that uptake of a fluorescent fatty acid (i.e. P12) from an albumin-free medium by a cell suspension could be monitored and recorded continuously, starting from about 15 s after mixing the two respective components (Nahas *et al.*, 1986). Under physiological conditions, fatty acids are bound to albumin and most experiments done to date on their uptake by cells were done in the presence of this protein. However, direct measurement of the cellular uptake of P12 from its complex with albumin was impossible, since the latter exhibited a high fluorescence intensity (Fig. 2), and the increase in cellular fluorescence could be detected only after separating and extensively washing the cells to remove the background fluorescence of the P12 in the medium. The fluorescence of the albumin/P12 complex could, however, be quenched using trinitrophenylated albumin; the yellow colour of this compound quenched the fluorescence of the pyrene. When P12 was released from the TNP–albumin/P12 complex and taken up by cells, fluorescence increased and could be monitored.

When comparing the kinetics of P12 uptake, complexed at various ratios to TNP–albumin, a decrease was noted with high ratios of albumin to P12, indicating that albumin has an inhibitory effect on P12 uptake. This confirms data which we have reported previously using different measuring techniques (Morand *et al.*, 1982*a*; Nahas *et al.*, 1987).

The differences in the initial rates of P12 uptake by HL-60 and MEL cells (Fig. 4*a*) or by undifferentiated and differentiated MEL cells (Fig. 4*b*), respectively, fully agree with previous findings where longer incubation periods and different techniques were used to determine its incorporation (Fibach *et al.*, 1986; Nahas *et al.*, 1987). This, most probably, reflects differences in cellular lipid metabolism of the above cells.

The procedures described in this paper permit the study of the effects of numerous factors on P12 uptake by cells (concentration, charge, pH, temperature, absence or presence of glucose and metabolic inhibitors), without the need to separate P12 and the cells (data not shown). Potentially, they could also be adapted for studying numerous problems related to liposomes or cells, such as uptake, release and transfer of lipids (Roseman & Thompson, 1980; Pownall *et al.*, 1982; Frank *et al.*, 1983). Quench–dequench and energy transfer systems could also be utilized for studying fusion processes (Owen, 1980; Struck *et al.*, 1981; Hoekstra *et al.*, 1985). This technique could also facilitate monitoring the uptake of other pyrene-containing biochemicals which form complexes with albumin or other proteins that could be trinitrophenylated, by different cell types and at various stages of cell maturation (Fibach *et al.*, 1986).

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REFERENCES

Charlton, S. C., Olson, J. S., Wong, K. Y., Pownall, H. J., Louie, D. D. & Smith, L. C. (1976) J. Biol. Chem. **251**, 7952–7955

Collins, S., Gallo, R. C. & Gallagher, R. E. (1977) Nature (London) **20**, 347–349

Doody, M. C., Pownall, H. J. & Smith, L. C. (1978) Fed. Proc. Fed. Am. Soc. Exp. Biol. **37**, 1832

Fibach, E., Reuben, R. C., Rifkind, R. A. & Marks P. A. (1977) Cancer Res. **37**, 440–444

Fibach, E., Nahas, N., Giloh, H. & Gatt, S. (1986) Exp. Cell Res. **66**, 220–228

Frank, A., Barenholz, Y., Lichtenberg, D. & Thompson, T. E. (1983) Biochemistry **22**, 5647–5651

Friend, C., Scher, W., Holland, J. G. & Sato, T. (1971) Proc. Natl. Acad. Sci. U.S.A. **68**, 378–382

Goodman, D. S. (1958) J. Am. Chem. Soc. **80**, 3892–3898

Hoekstra, D., Klappe, K., de Boer, T. & Nwischut, J. (1985) Biochemistry **24**, 4739–4745

Morand, O., Fibach, E., Dagan, A. & Gatt, S. (1982*a*) Biochem. Biophys. Acta **711**, 539–550

Morand, O., Fibach, E. & Gatt, S. (1982*b*) Biochim. Biophys. Acta **693**, 143–150

Morand, O., Fibach, E., Livni, N. & Gatt, S. (1984) Biochim. Biophys. Acta **793**, 95–104

Nahas, N., Fibach, E., Giloh, H. & Gatt, S. (1986) in Enzymes of Lipid Metabolism (Freysz, L., Dreyfuss, H., Massarelli, R. & Gatt, S., eds.), pp. 453–458, Plenum Press, New York

Nahas, N., Fibach, E., Giloh, H. & Gatt, S. (1987) Biochim. Biophys. Acta **917**, 86–91.

Owen, C. S. (1980) J. Membr. Biol. **54**, 13–20

Pownall, H. J., Hickson, D., Gotto, A. M. & Massey, J. B. (1982) Biochim. Biophys. Acta **712**, 169–176

Reuben, R. C., Wife, R. L., Breslow, R., Rifkind, R. A. & Marks, P. A. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 862–866

Roseman, M. & Thompson, T. E. (1980) Biochemistry **19**, 439–444

Sengupta, P., Sackman, E., Kuhule, W. & Scholz, H. P. (1976) Biochim. Biophys. Acta **436**, 869–878

Shinoda, K. (1963) In Colloidal Surfactants (Shinoda, K., Makagawa, T., Tamamushi, B. I. & Isemura, T., eds.), pp. 1–96. Academic Press, New York and London

Spector, A. A. (1968) Ann. N.Y. Acad. Sci. **149**, 768–783

Spector, A. A. (1975) J. Lipid Res. **16**, 165–179

Struck, D. K. Hoekstra, D. & Pagano, R. E. (1981) Biochemistry **20**, 4093–4099

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