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Internalization of the lipophilic fluorescent probe trimethylamino-diphenylhexatriene follows the endocytosis and recycling of the plasma membrane in cells

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The lipophilic fluorescent probe trimethylamino-diphenylhexatriene (TMA-DPH) has been shown previously to behave as a marker of plasma membrane in living cell systems, and it has therefore been widely used in membrane fluidity studies via fluorescence anisotropy measurements. However, progressive internalization of this probe in cells could lead to unsuitable interferences, when long incubations times were required. The mechanism of this internalization had not yet been elucidated. We present here fluorescence-intensity kinetic results and fluorescence micrographic data on L929 cells and on mouse bone-marrow macrophages, which allow us to identify the mechanism as fluid-phase pinocytosis: the probe remains associated with the plasma membrane throughout its internalization-recycling flow and it is finally concentrated in lysosomes. The study was facilitated by the partition equilibrium property of TMA-DPH between plasma membranes and the external aqueous medium, which allowed to immediately distinguish the internalized fraction of the probe from the peripheral labelling, by simply washing cells. This conclusion is confirmed by the features of the influence of temperature on TMA-DPH internalization.

Introduction

Trimethylamino-diphenylhexatriene (TMA-DPH) is a lipophilic fluorescent probe with a high quantum yield in phospholipidic membranes and a very low one in aqueous media [1]. Additional properties result from the presence of the trimethylamino cationic group. It was first shown in our laboratory [2,3] and further confirmed by other groups [4–7], that in the presence of living cells, TMA-DPH is rapidly and specifically incorporated into the plasma membrane, remaining anchored by ionic interactions between the cationic moiety and negatively charged phospholipid heads. This property has made it a widely used tool for evaluating membrane fluidity in intact cells, by fluorescence anisotropy measurements. However, once incorporated in the plasma membrane, TMA-DPH is further partially internalized, more or less rapidly, depending on the cell type. The mechanism of the internalization had not hitherto been elucidated, and it became important to understand it better for experiments involving long incubation times with the probe, as for example in a recent work on phagocytosis by macrophages [8].

Investigating this internalization mechanism was facilitated by another property of TMA-DPH, namely its amphiphilic character: in interaction with cells, the probe undergoes a partition equilibrium between the membranes and the external aqueous medium (> 90%) [8–11,6]. Accordingly, washing the cells entirely extracts the probe from the plasma membrane towards the external medium, with the loss of the fluorescence. Conversely, the internalized fraction of the probe is preserved from this effect. Moreover, because of this property, the fluorescence intensity of TMA-DPH in the plasma membrane is proportional to the cell density in a given cell system. Thus, a simple comparison between the fluorescence intensity before and after washing en-

Abbreviations: DMEM, Dulbecco's modified Eagle's medium: M-CSF, macrophage colony stimulating factor; PBS, phosphate-buffered saline (without Ca^{2+}, Mg^{2+}); TMA-DPH, trimethylamino-diphenylhexatriene.

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ables an immediate evaluation of the internalized fraction. Using this approach, we carried out fluorescence-intensity kinetic studies and present here fluorescence micrographic data, on L929 cultured mouse fibroblasts and mouse bone-marrow macrophages, which unambiguously correlate the internalization of TMA-DPH with fluid-phase pinocytosis. This process allows cells to exchange external solutes with internal compartments of the vacuolar system; it involves a high-rate internalization-recycling flow of plasma membrane via pinocytic vesicles (pinosomes) [12–15]; TMA-DPH simply remains associated with the plasma membrane throughout this two-way movement.

Materials and Methods

Cell cultures and processing

L929 cells. Mouse fibroblasts from the L929 line (Flow Laboratories, D. Dutscher, France) were grown as monolayers in 75 cm^2 plastic culture flasks (Costar, D. Dutscher, France), using high-glucose (4.5 g/l), high-carbonate (3.7 g/l) Dulbecco's Modified Eagle's Medium (DMEM, Seromed, Intermed, France) supplemented with 10% foetal calf serum (Cytosystem, Vietech, France), 1 mM sodium pyruvate (Gibco, France), 2 mM l-glutamine (Seromed, Intermed, France), $5 \cdot 10^{-5}$ M β-mercaptoethanol (Merck, F.R.G.) and antibiotics: penicillin 50 U/ml, streptomycin 50 μg/ml (Seromed). The seeding density was $2 \cdot 10^5$ cells/ml and the volume, 30 ml per flask. The cultures were kept in water-saturated atmosphere, of 8% CO_2 in air, at 37 °C. These particular conditions were necessary to obtain an appropriate cell supernatant for the preparation of bone-marrow-derived macrophages: L929 cell supernatant is known to contain the macrophage-colony-stimulating factor (M-CSF) [16]. The supernatant was collected after 9 days' incubation and stored at −20 °C after centrifugation and filtration. When the L929 cells were used as a model in the experiments, they were recovered after 4 days' culture (80% of confluency) by mild trypsination; they were counted with a Nageotte hemacytometer and their viability (> 95%) was assessed by trypan-blue exclusion.

Bone-marrow-derived macrophages. Bone-marrow-derived macrophages were chosen as models instead of peritoneal macrophages, because they are easy to obtain at high yields and at a high level of purity.

Bone-marrow stem cells were isolated from femora of 6–8-week-old male Balb/c mice. These cells were seeded at a density of $2 \cdot 10^5$ nucleated cells/ml in a medium with a composition similar to that above, but with the addition of 25% L929-conditioned supernatant and 5% horse serum (Gibco). They were cultured in teflon bags (Heraeus, Hanau, F.R.G.) using the hydrophobic surface of the plastic film to prevent adherence [17,18] and kept in an 8% CO_2 atmosphere at 37 °C. Well differentiated macrophages were harvested between day 8 and 11, at a yield of 1.0 to $1.2 \cdot 10^6$ cells/ml, and the purity and the viability level was over 90%, as indicated by the number of adherent cells. We should mention here that successful preparation depended greatly on the quality of the foetal calf serum.

TMA-DPH cell labelling and fluorescence microscopy

The cells were allowed to adhere for 2 h on quartz cover-slips, at a density of $(1–3) \cdot 10^5$ cells/ml and then incubated for various times at 37 °C in DMEM/10% foetal calf serum, containing $2 \cdot 10^{-6}$ M TMA-DPH (Molecular Probes, Oregon, U.S.A.) from a $4 \cdot 10^{-3}$ M stock solution in dimethylformamide (Prolabo, France, spectroscopic grade). The cover-slips were then immersed and gently shaken for a few seconds in Ca^{2+}-Mg^{2+}-free PBS (thereafter referred to as PBS), at room temperature, which, according to the partition equilibrium, resulted in the loss of the peripheral membranar staining. When peripheral staining was desired, a washing solution containing TMA-DPH $2 \cdot 10^{-6}$ M in PBS was used instead, which prevented the partition equilibrium from being displaced. The fluorescence microscopic observations of the samples were carried out as rapidly as possible to limit further intracellular evolution of the labelling features. The equipment consisted in a Leitz Ortolux II photomicroscope with Ploemopack system for epi-illumination, set at 340–380 nm for excitation and 430 nm for observation. The original magnification was 600 × ; 400 ASA T-Max Kodak films were used for recordings.

In order to characterize the subcellular location of TMA-DPH in lysosomes, a double-labelling technique was used with acridine orange (Euchrysin) as a specific marker for low pH compartments [19,20]. The cells were first incubated with TMA-DPH ($2 \cdot 10^{-6}$ M) as before, and then treated with acridine orange (Merck) 1 μg/ml in PBS for 5 min, which was long enough for a clear lysosomial coloration. In fact, the concentrations and durations reported in the references quoted were reduced to prevent any contribution of acridine in the TMA-DPH observation. After the samples had been washed, the TMA-DPH fluorescence was observed first, and then the filters were switched for acridine orange fluorescence excitation (515–560 nm) and observation (580 nm). In addition, a gap of 5 s or more was left between the two observations, to allow TMA-DPH fluorescence to be completely photobleached [21] before the acridine orange labelling was examined. Under these conditions, no interference could occur between the two stainings. The micrographs were recorded on 400 ASA T-MAX Kodak Films.

Kinetic TMA-DPH internalization assays and fluorescence intensity measurement

After careful homogenization and counting, the cell suspension was adjusted to $2.5 \cdot 10^5$ cells/ml and seeded

in 6-well culture clusters, 35 mm in diameter (Costar), at a rate of 2 ml/well, for cell adherence (37 °C, e.g. 2 h). The layers were then washed and incubated with TMA-DPH $2 \cdot 10^{-6}$ M in DMEM/10% foetal calf serum, at selected temperatures and for various times. At the end of this step, the supernatant was quickly removed and the layers were washed five times for about 3 s, with 2 ml PBS, at room temperature. This ensured total extraction of TMA-DPH from the plasma membrane [8], but did not noticeably modify the internalized probe fraction. The cells were quantitatively recovered, from each well, in 2 ml PBS, after gentle scraping with a rubber policeman or a 'cell lifter' (Costar), which left them undamaged. They were immediately transferred to 1 cm quartz cuvettes, and assayed for fluorescence intensity with a Perkin-Elmer MPF 66 fluorimeter (excitation, 360 nm; emission, 435 nm). The whole operation took less than 4 min after the end of incubation. The 6 one-culture-cluster wells served to determine one point on the kinetic curves. The fluorescence intensity measurements were performed in PBS because the culture medium and serum interfered with the spectral properties of TMA-DPH. The contribution of background noise in these measurements was negligible.

Kinetic TMA-DPH exocytosis studies

In order to investigate TMA-DPH conveyance from subcellular locations back to the plasma membrane, the following assays were carried out. After incubation of the cells with TMA-DPH, the supernatant was replaced by TMA-DPH-free medium with 10% foetal calf serum, and re-incubated for various times, at 37 °C. The fluorescence intensity was measured after a single washing with PBS, as described above.

Expression of results

As pointed out previously [9–11,6] and corroborated in this work, the fluorescence intensity of TMA-DPH incorporated into the plasma membrane of intact cells, i.e., the fluorescence intensity measured after a short incubation without cell washing, is directly proportional to the cell density, and, for a given cell density, to the actual plasma membrane area, provided the measurements are performed in the same cell system.

This suggested a way of calibrating the internalized fraction in relation to the cell surface staining, by the ratio: fluorescence intensity after washing/fluorescence itnensity without washing. The fluorescence intensities of unwashed cells were measured after replacing the incubation medium by PBS containing TMA-DPH at the same concentration, to avoid extracting the probe from the plasma membrane. The incubation in this case was very short ($\leqslant 1$ min) to enable us to neglect the contribution from internalization.

Results

General properties of TMA-DPH in interaction with the cell systems studied

L929 cells had already been used as a model in earlier studies with TMA-DPH [2,9]. In interaction with bone-marrow macrophages TMA-DPH was found to display the same general properties. When the probe was added to macrophage suspensions or adherent layers. The fluorescence intensity increased very rapidly, within approx. 10 s, and continued to increase thereafter, but at a much lower rate. This biphasic behaviour was previously shown to correspond respectively to the incorporation into the plasma membrane and to the further internalization into the cells.

When the TMA-DPH concentration varied, for instance between $1.0 \cdot 10^{-7}$ M and $3 \cdot 10^{-6}$ M, the fluorescence intensity due to plasma-membrane staining increased proportionally.

This fluorescence intensity evolved proportionally also when the cell concentration varied between $1.0 \cdot 10^{5}$ and $2 \cdot 10^{6}$ cells/ml, which confirmed again that the incorporation into the plasma membrane was ruled by a partition equilibrium [9]. In point of fact, when the macrophages were adequately washed, immediately after TMA-DPH incorporation (i.e., approx. 10 s after the addition of the probe), the fluorescence intensity dropped to zero (the background level was negligible under these measurement conditions, indicating the absence of any contribution from scattered light, or aspecific fluorescence).

However, when the washing was done later, residual fluorescence intensity, which increased with the incubation time, was observed, and this corresponded to the progressive internalization of the probe. So, in Fig. 1, the fluorescence spectra of TMA-DPH in Balb/c bone-

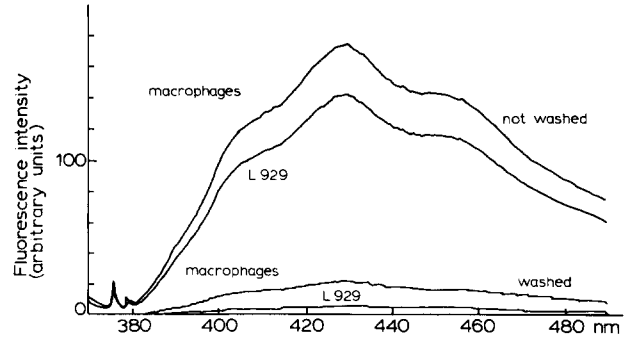


Fig. 1. TMA-DPH fluorescence spectra in washed and unwashed cells. L929 fibroblasts and Balb/c bone-marrow macrophages, $5 \cdot 10^{5}$ cells were incubated at 37 °C in culture clusters with 2 ml DMEM/10% foetal calf serum for 2 h for cell adherence, and then for 10 min with TMA-DPH, $2 \cdot 10^{-6}$ M, in the same medium. After washing of the layers with PBS containing TMA-DPH at the same concentration (unwashed cells), or TMA-DPH-free PBS (washed cells), the cells were recovered by gentle scraping in 2 ml of the same PBS solutions, and the fluorescence spectra were taken with a MPF 66 Perkin-Elmer spectrofluorimeter (excitation wavelength, 360 nm).

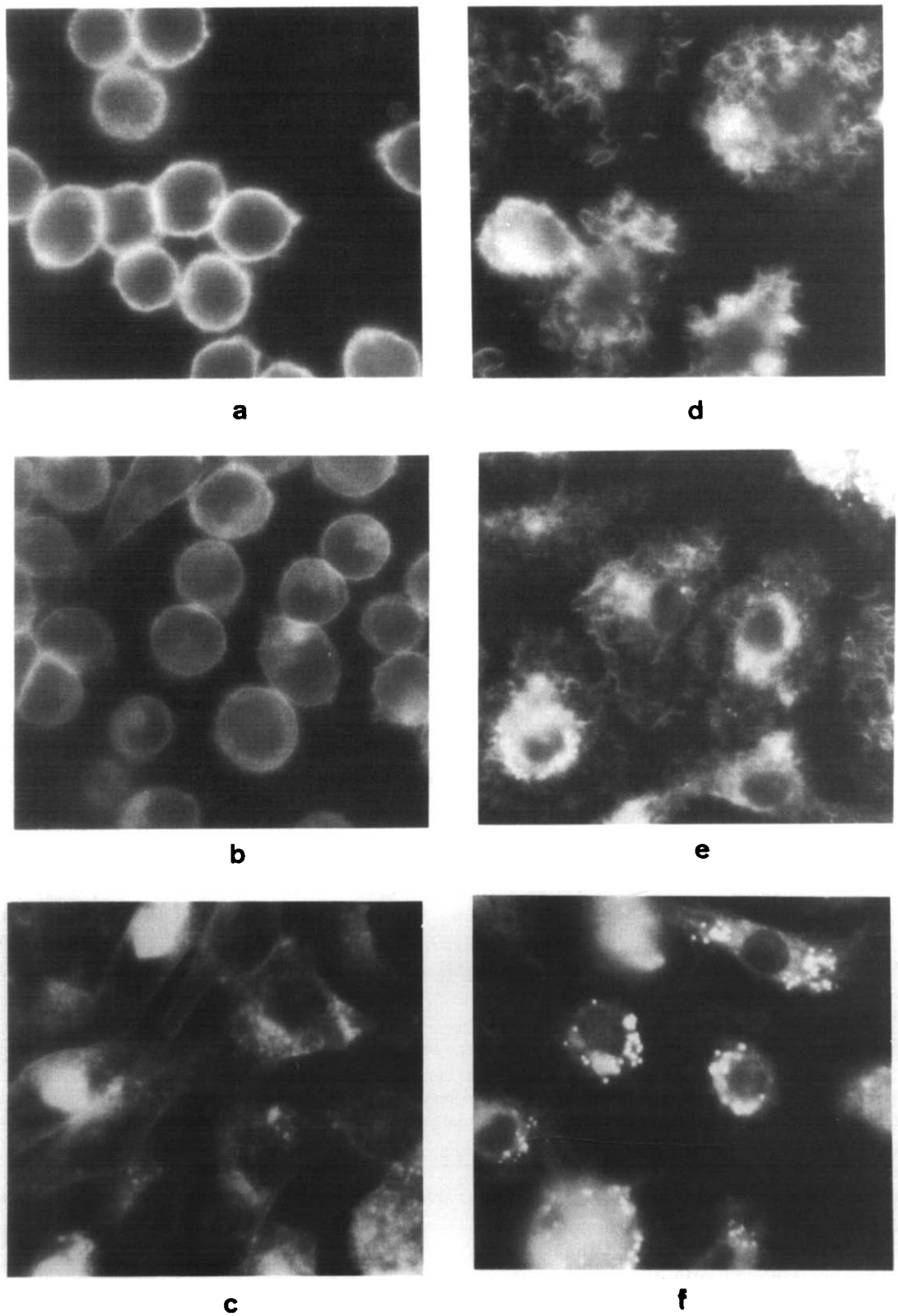


Fig. 2. Labelling features of L929 cells and Balb/c bone-marrow macrophages by TMA-DPH. The cells were allowed to adhere on cover-slips at a density of $(1-3)\cdot 10^5$ cells/ml. After incubation with TMA-DPH ($2\cdot 10^{-6}$ M) at 37°C for various times, they were washed with PBS containing TMA-DPH at the same concentration (unwashed cells) or TMA-DPH-free PBS (washed cells). The micrographs in this figure, represent the labelling features for some selected incubation times: (a) L929, 5 min, unwashed; (b) L929, 5 min, washed; (c) L929, 90 min, washed; (d) Balb/c macrophage, 5 min, unwashed; (e and f) Balb/c macrophages, 30 min, unwashed, respectively, 90 min, washed. Excitation 340–380 nm; observation 430 nm; magnification ×600.

marrow-macrophages and in L929 cells were compared before and after cell washing, for a 10 min incubation at 37° C. The figure shows that by that time the internalized part of the fluorescence intensity was about 3-times higher in macrophages than in L929 cells, which recalls earlier data on pinocytosis [22].

The internalized TMA-DPH fraction, in L929 cells and in macrophages, assessed from the fluorescence intensity after cell washing, was also shown to be proportional to TMA-DPH and cell concentrations, indicating an evolution parallel with that of the plasma-membrane fluorescence.

A very important point regarding the further interpretation of the internalization mechanism, is that the fluorescence intensity measured after washing was not influenced by the care taken in recovering cells by scraping. This operation was usually done cautiously so as to prevent cell disruption. However, it was noticed that significant cell disruption, for instance increasing turbidity 3–4-times, when it was done intentionally, albeit with moderation, had no effect at all on the internalized fluorescence intensity of TMA-DPH. This indicates that TMA-DPH is present inside the cells only within closed structures (vesicles), in which it is protected from partitioning with the external buffer upon cell disruption; otherwise, rupture should result in a considerable decrease in fluorescence intensity (which is the case with strong cell homogenisation). Furthermore, the stability of the fluorescence intensity in the presumed vesicles supposes that TMA-DPH is present in the internal leaflet of their membrane, in equilibrium with engulfed solute. This observation suggests that TMA-DPH had been taken up from the external medium by the formation of plasma membrane vesicles, in other words, by pinocytosis.

Other general features to be mentioned in this section are:

(i) the innocuity of TMA-DPH in macrophages, as in other cell systems earlier studied, which may be inferred from the perfect stability of adherence properties, in the presence of TMA-DPH concentrations as high as $5 \cdot 10^{-6}$ M, for more than 3 h;

(ii) the absence of perturbing metabolizing effects inside the cells, as already pointed out in [5,6] and evidenced here by the results of the kinetic studies (see Discussion);

(iii) the insensitivity of the TMA-DPH fluorescence intensity to pH changes, between pH 2 and 9, recently demonstrated (Duportail, G. and Kuhry, J.G., unpublished data) using small unilamellar phosphatidylcholine vesicles. This point is important, since it is shown here that TMA-DPH accumulates in lysosomes.

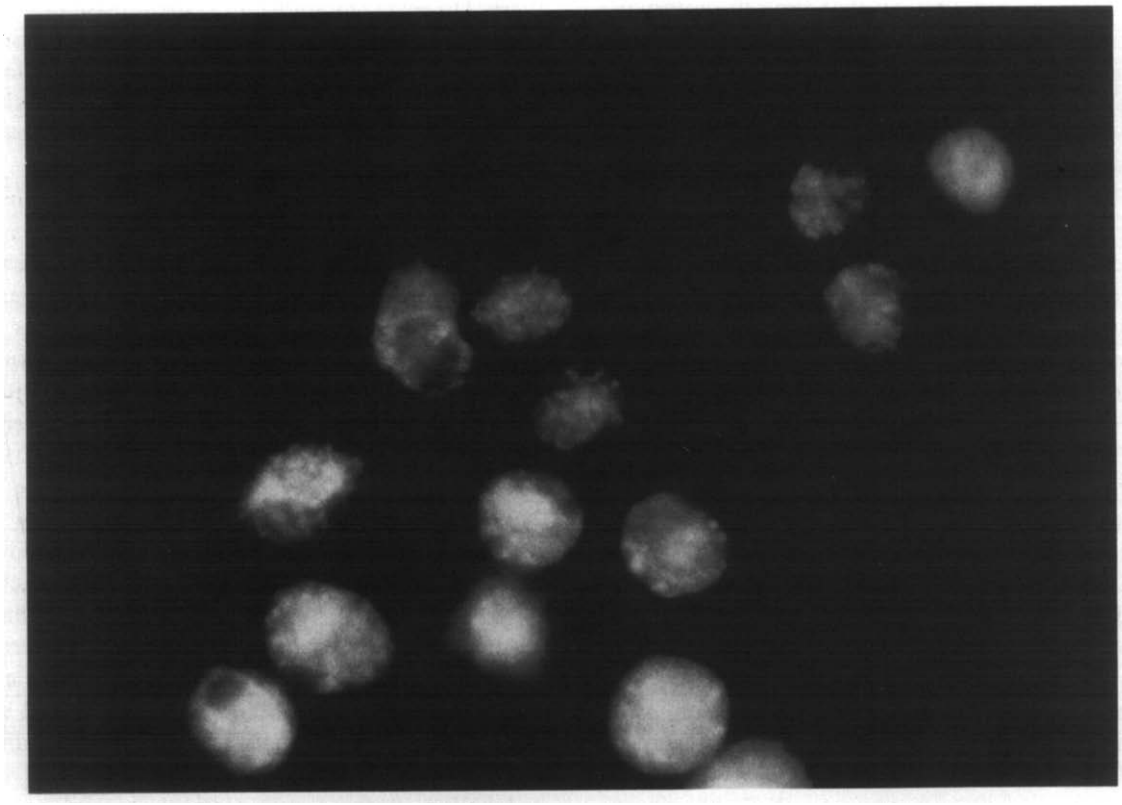
Labelling features

The fluorescence microscopy technique used in this study takes advantage of the low depth of field of high-magnification objectives (×60) to focus on restricted zones of the cells, singling out particular structures. However, in addition to the unavoidable scattered light, this technique was further impeded by the considerable photobleaching [21] of TMA-DPH under the UV illumination of the microscope excitation light, which ruled out the observation of the labelling features for longer than 2 s.

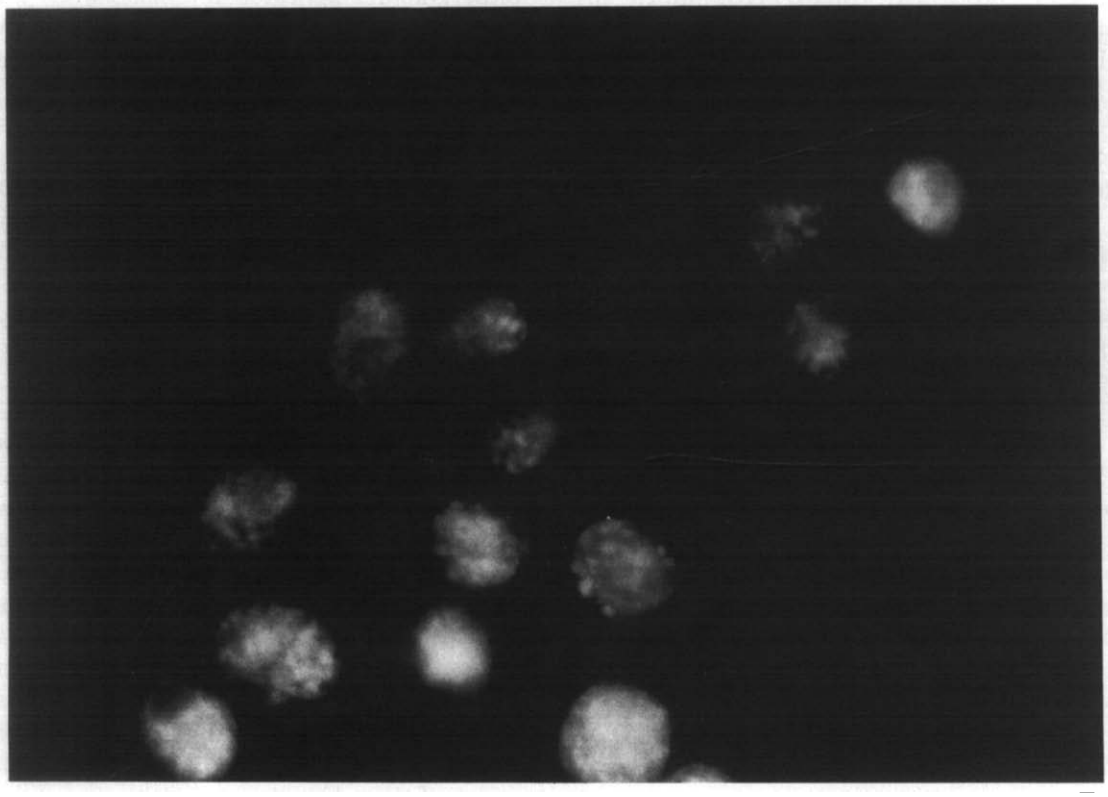
Fig. 2 compares aspects of the labelling steps of L929 cells, and Balb/c bone-marrow macrophages. The specificity of the peripheral labelling in the early stage of incubation with TMA-DPH (5 min) apepars clearly on freshly adhered L929 cells in Fig. 2a, as does the effect of cell washing on the same preparation on Fig. 2b. Similar observations were not possible with macrophages because they strongly and repidly adhered to the support, reducing the cell thickness, and because their membranes displayed such an intricate structure (Fig. 2d). Nevertheless, plasma-membrane staining could be inferred by comparing the outlines of washed (Fig. 2f) and unwashed cells (Fig. 2d) and from the fact that the dark non labelled nuclei were not distinguishable after short incubations (5 min) (Fig. 2d). The internal labelling features for L929 (90 min of incubation) are shown in Fig. 2c and those for macrophages (30 and 90 min of incubation) in Figs. 2e, 2f. The aspect was discontinuous and granular, and the nuclei remained dark and unlabelled. Some large, particularly brilliant granules were observed in the perinuclear region. In general, these granules appeared for incubations lasting more than 10 min. After 90 min (Fig. 2f), they were so bright that they appeared in visible-phase contrast microscopy, due to absorption of the excitation light (not represented). They were identified as lysosomes with the double-labelling technique using acridine orange, a specific marker [19,20] of the acidic vacuolar compartment (see Materials and Methods). Careful examination of Fig. 3 shows that, in many cases, the two labellings were superimposable. But, because of the aforementioned difficulties, photographs were not easy to obtain and direct observations were much more convincing.

TMA-DPH internalization and exocytosis kinetics

Fig. 4 represents the evolution of the intracellular fluorescence intensity of TMA-DPH, i.e., after cell washing, in L929 cells and Balb/c bone-marrow macrophages for increasing incubation times and various temperatures. For standardization, the results were expressed as % of the fluorescence intensity corresponding to the plasma membrane labelling, which was measured without cell washing, immediately after the addition of TMA-DPH. The curves exhibit a biphasic aspect; they could be fitted by two exponential functions using a 'Statistic Analysis System' with a non-linear least-squares fitting. For instance in the case of macrophages at 37° C, the expression was:



a



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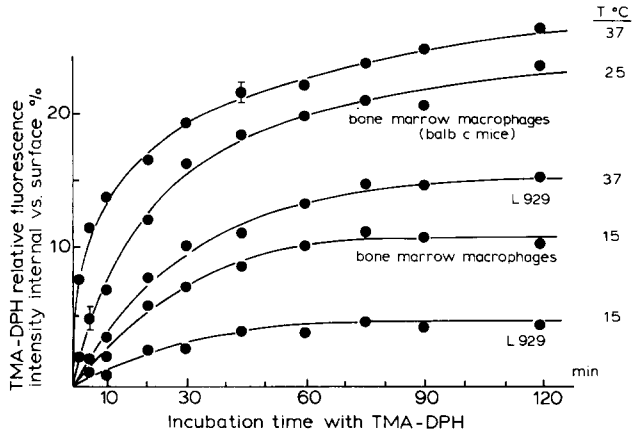

Fig. 4. Comparison of TMA-DPH internalization kinetics in L929 cells and in Balb/c bone-marrow macrophages, and the influence of temperature. The cells were allowed to adhere, for 2 h, at 37 °C, in culture clusters, at a density of $2.5 \cdot 10^5$ cells/ml, in 2 ml DMEM/10% foetal calf serum. This medium was replaced by one with $2 \cdot 10^{-6}$ M TMA-DPH and the incubation continued for various times and at selected temperatures. After the layers had been washed with PBS at room temperature to extract TMA-DPH from the plasma membranes, the cells were recovered in 2 ml PBS by gentle scraping. The fluorescence intensity of the TMA-DPH internalized fraction was measured at 435 nm (excitation 360 nm) with a MPF 66 Perkin-Elmer spectrofluorimeter. The results were referred to the TMA-DPH fluorescence intensity incorporated in the plasma membranes, measured in the same assay on unwashed cells after a short incubation (<1 min). The points represent the mean from six determinations, and the error bars, typical S.D.

$$\text{F.I. } \% = 27.2 - 11.7 \exp(-0.46t) - 15.8 \exp(-0.021t)$$

with a coefficient of determination of 0.996; they display a short sharp increase in fluorescence intensity for incubation times below approx. 15 min, followed by a further continuous gradual increase. The data of Fig. 4 concern incubation times up to 120 min, but no further change in the curve trend was observed even after 4 h.

The kinetics were markedly slower at lower temperatures, indicating that the mechanisms involved were energy-dependent. At 15 °C, the gradual increase in the second part of the curve was totally suppressed, whereas in the first part, the rate was three or more times lower.

Complementary information as to how TMA-DPH might be released from the cells is shown in Fig. 5. Macrophages were pre-incubated with TMA-DPH for various times, and then kept at 37 °C in TMA-DPH-free medium. The decrease in fluorescence intensity was monitored, to trace the flow of the probe towards the external medium. The kinetics of this process were found to be almost symmetrical with those of internalization, except that the second part of the curve was quasi-horizontal instead of moderately decreasing. The kinetics were observed for more than 4 h, but, because of the totally uniform evolution, the results beyond 60 min are not shown.

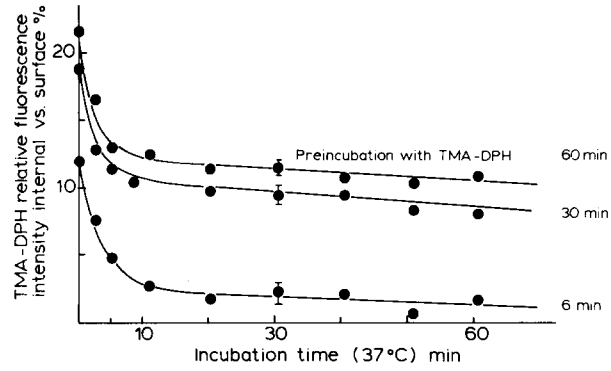

Fig. 5. TMA-DPH exocytosis kinetics in Balb/c bone-marrow macrophages. The cells were allowed to adhere in 6-well culture clusters ($2 \cdot 10^5$/ml), for 2 h, at 37 °C, and further incubated with TMA-DPH, $2 \cdot 10^{-6}$ M in DMEM with 10% foetal calf serum, for various times. After a first series of washes, the cells were reincubated at 37 °C, in DMEM, with 10% calf serum, for various times, to allow exocytosis to take place. The fluorescence intensity measurements (435 nm) were performed after a second washing. Again, the results were expressed as % of the TMA-DPH fluorescence intensity in the plasma membranes, measured in unwashed cells after brief (<1 min) incubation with the probe. The points represent the mean from six determinations and the error bars, typical S.D.

Discussion

TMA-DPH is internalized via fluid-phase pinocytosis and follows the plasma membrane throughout its internalization-recycling flow

The experimental arguments leading to this assertion are as follows:

(a) The internalization of TMA-DPH and its incorporation into the plasma membrane vary proportionally parallel with the cell concentration and with the probe concentration, which suggests a possible correlation.

(b) Cell disruption after washing, provided it is obtained by moderate treatment, does not induce any decrease in the internalized fluorescence intensity. On the grounds of TMA-DPH partition equilibrium this rules out the intracellular presence of the probe in the form of a cytosolic solution or its incorporation in any open-membrane structure, which denotes a negligible contribution of mechanisms such as flip-flop or passive diffusion. It strongly suggests that TMA-DPH is con-

Fig. 3. Comparison of labelling features of Balb/c macrophages, with TMA-DPH (a), and acridine orange (b) as lysosomial marker. The cells, adhering on cover-slips ($(1-3) \cdot 10^5$/ml) were incubated with TMA-DPH, $2 \cdot 10^{-6}$ M, at 37 °C for 20 min, washed, and treated with acridine orange (1 μg/ml) for 5 min and washed again. The observation conditions were 340–380 nm for excitation and 430 nm for observation for TMA-DPH, and 515–560 nm (excitation) and 580 nm (observation) for acridine orange. A gap of 5–10 s was left between the two observations to allow TMA-DPH to be completely photobleached; magnification ×600.

tained in closed vesicles, as mentioned above. This assumption is confirmed by the micrographic data.

(c) The kinetics of TMA-DPH internalization match those previously reported for pinocytosis in L929 cells and peritoneal macrophages [12,14,22] and in bone-marrow macrophages [15]. In those studies, the authors used horseradish-peroxidase as a fluid phase marker and [15] [^{3}H]galactose as a covalent plasma membrane marker. They also observed biphasic behaviour: the first phase (10–15 min) corresponded to the formation of pinocytic vesicles (0.1 μm in diameter) from the plasma membrane and to their internalization and flow-back, including possible interpinosomial fusion events. The kinetics of this first phase might correspond to the internalization of the entire plasma membrane in macrophages at 37°C within 30–40 min. This duration could be estimated at 40–50 min from our results. On the other hand, we show here that, after 10 min of incubation at 37°C, the internalization of TMA-DPH was 3–4-times greater in macrophages than in L929 cells, in agreement with a previous study using horseradish-peroxidase [22]. As regards eventual interpinosomial fusions, it should be mentioned that no such events could be detected with TMA-DPH: for instance, in the case of fusion between labelled and non-labelled pinosomes of equal size, the decrease in fluorescence intensity resulting from TMA-DPH dilution in the engulfed solution might be counterbalanced by the availability of fresh membrane for TMA-DPH incorporation (see comments about other fusion events at the end of the discussion). This emphasizes again the importance of TMA-DPH partition equilibrium.

One difference between the kinetics described here and those from the above-quoted works, is that the second phase of the process (long incubations), attributed to the fusion of pinocytic vesicles with lysosomes or other large vesicles of the vacuolar system (e.g., endosomes), on our curves does not reach a plateau, but continues to increase slowly. This means two things:

(i) TMA-DPH is not metabolized in lysosomes like other markers.

(ii) The incoming fluorescence intensity of TMA-DPH in lysosomes is not counterbalanced by the outgoing one. This point will be discussed further.

(d) Temperature strongly influenced the kinetics. At 15°C, the TMA-DPH uptake rate was 3-times lower than for a 15 min incubation at 37°C, i.e., the pinosome formation was markedly reduced, whereas the increase in fluorescence intensity in the second part of the curve was suppressed; this latter fact is consistent with the total inhibition [23] of pinosome-lysosome fusion below 16°C. Quantitative experiments were not easy to perform at 4°C because of partial cell detachment; however, it appeared that no significant internalization of TMA-DPH occurred ever at that temperature.

(e) Unfortunately, because of strong TMA-DPH photobleaching, the fluorescence micrographic data did not allow the detailed intracellular pathways of the probe to be followed. However, they clearly denote a vesicular structure and final incorporation into lysosomes, which are characteristics of pinocytosis. On the other hand neither nucleus, nor nuclear membranes were labelled, which confirms the absence of TMA-DPH as cytosolic solute.

(f) For simple reasons of homeostasis, the pinocytosis mechanism has to be compensated by a symmetrical exocytosis process [12,13], i.e., the plasma membrane internalized has to be recycled; the density of incoming pinocytic vesicles is balanced by the flow of returning vesicles. In fact, the curves of Fig. 5, which describe the release of TMA-DPH from cells towards the external medium, display kinetics with trends symmetrical to those of internalization, except that the fluorescence intensity in the second part of the curves remains almost constant instead of decreasing. This is consistent with the previous observation (under (c)) on internalization; both suggest that the fluorescence intensity leaving the lysosomes is considerably lower than that accumulating in them. This effect is again to be connected with the particular features of TMA-DPH, after fusion events, on the grounds of the partition equilibrium. As mentioned above, the fluorescence intensity is proportional to the probe concentration (thus inversely proportional to the vesicle volume, and to the vesicle membrane surface) which finally results in a reciprocal proportionality to the vesicle radius. Accordingly the fusion of a TMA-DPH labelled pinocytic vesicle with a non-labelled lysosome results in a decrease in fluorescence intensity by a factor $r/(R+r)$, i.e., approx. r/R, since the radius (R) of the lysosome is much larger (approx. 10 times) than that of the pincocytic vesicle (r). On the other hand, for the same reasons, the fluorescence intensity of small vesicles budding off the lysosomes will be proportional to $(r/R)^2$. This shows that the fluorescence intensity of the vesicles coming out of the lysosomes is negligible against that of the incoming vesicles, and thus explains the shape of the curves.

The conclusion then is that arguments put forward provide evidence that the mechanism by which TMA-DPH is internalized in cells is fluid-phase pinocytosis. The probe is first incorporated rapidly into the plasma membrane thanks to its positively charged group and it remains associated with the plasma membrane during its internalization-recycling process. The fluorescence intensity of the internalized fraction increases rapidly as long as the endocytosed TMA-DPH-labelled vesicles are more numerous than the exocytosed ones. The further gradual increase in fluorescence intensity merely denotes the concentration of the probe in lysosomes or in other large vesicles of the vacuolar system.

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