THE DISTRIBUTION OF 1,6 DIPHENYL HEXATRIENE FLUORESCENCE IN NORMAL HUMAN LYMPHOCYTES

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SUMMARY A study of the distribution of the fluorescent probe 1,6 diphenyl hexatriene in living intact human tonsil lymphocytes showed that it corresponded to the distribution of the phospholipids, except that the fluorescent intensity of the probe in the plasma membrane was enhanced by about 15% relative to its chemical distribution. The lifetime of the probe at 37° was 9.6 ns in the plasma membrane but only 8.6 ns in the whole cell. After correcting for light scattering depolarization, the mean value for the polarization ratio of the probe in the plasma membrane at 37° was 0.244 ± 0.009 (7) compared to 0.217 ± 0.008 (9) for the living cell, and hence the microviscosity of the plasma membrane was greater.

INTRODUCTION

1,6 diphenyl hexatriene (DPH) is being widely used as a probe to study the dynamic properties of the lipids in cell membranes (1-4). In many instances this is done with intact cells (5-7) but no study has so far been reported showing the quantitative location of the probe fluorescence within the cell membrane systems. While it becomes increasingly clear that there is very little partitioning of this label between different phases (8,9) there is no clear evidence about its cellular distribution. This may be important, particularly when different cells are compared, or when conclusions are drawn about microviscosity changes in the cell plasma membranes.

Pagano et al (10) using tritiated DPH, reported that 50% of the probe was located on an electronmicrograph within ± 0.15 μm of the mouse erythrocyte plasma membrane, but only 16% within a similar distance of a mouse thymocyte plasma membrane. In two ways these measurements were unsatis-

Abbreviation used is DPH, 1,6 diphenyl 1,3,5 hexatriene.

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factory. Firstly, there is no fundamental reason for the chemical and fluorescent distribution of DPH to coincide, unless the lifetime of the probe is the same in all its cell environments. Secondly, the finding that 50% of the probe was not located on the mouse erythrocyte membrane suggests some redistribution of the probe during the preparation of the electron-micrograph.

MATERIALS AND METHODS

(a) Cells and DPH-labelling

1,6 diphenyl hexatriene and preservative free tetrahydrofuran were obtained from Aldrich and ^{3}H-adenosine monophosphate was obtained from the Radiochemical Centre, Amersham, U.K. Tonsil lymphocytes were prepared from enlarged but non-inflamed tonsils, freshly removed from children and young adults at operation. The tonsils were chopped with a scalpel and shaken in Dulbecco's phosphate buffered saline, without calcium or magnesium. The resulting suspension was filtered through nylon gauze. The cells were centrifuged at 200 g for 15 minutes and resuspended in Dulbecco's phosphate buffered saline. After washing, the erythrocytes were removed by lysis with tris buffered ammonium chloride (11). The cells were resuspended in physiological saline, buffered with 10 mM tris at pH 7.5 (20°C). Any clumps were removed by filtration through nylon gauze. After washing once more in tris buffered saline, they were suspended in a 2 x 10^{-6}M colloidal suspension of 1,6 diphenyl hexatriene (DPH) in tris buffered saline. The diphenyl hexatriene suspension was prepared by rapidly injecting 0.1 ml of a 2 x 10^{-3}M solution of DPH in tetrahydrofuran into 100 ml of stirred tris buffered saline as described by Shinitzky and Inbar (1). The cells were labelled for 30 minutes at 37°C and then centrifuged and resuspended in tris buffered saline.

The cell viability was >90% as judged by trypan blue exclusion, and contamination by monocytes or polymorphonuclear leucocytes <1%.

(b) Preparation of cell membranes

The cells were disrupted at 0°C at a cell concentration of about 2 x 10^7 cells ml^{-1} by pumping the suspension at 400 ml hr^{-1} through a cell disruption pump (12). This ruptures the cells but not the subcellular particles by forcing the suspension through a small orifice against a spring loaded needle at a pressure of about 28 kg cm^{-2}. The membrane suspension was kept at 0° - 6°C and was then subjected to the differential centrifugation scheme used for pig lymphocytes by Allan and Crumpton (13). Nuclei and unbroken cells were removed by centrifuging at 300 g (average) for 15 minutes, and mitochondria by centrifuging the supernatant at 4,000 g (average) for 15 minutes. The post mitochondrial supernatant was centrifuged at 20,000 g for 30 minutes, giving the plasma membrane sample. The supernatant was retained. The pellets were homogenously suspended in known volumes of tris buffered saline.

The samples were assayed for 5'-Nucleotidase as described by Avruch and Wallach (14), using ^{3}H-adenosine monophosphate, except that the buffer used was 0.1M glycine/sodium hydroxide, pH 8.5 (20°C) and the enzyme was precipitated with 0.3 ml 5% zinc sulphate and 1 ml 0.15M barium hydroxide solution.

Protein was assayed as described by Lowry et al (15), using bovine serum albumin as a standard, except that the samples and standards were initially suspended in a total volume of 0.5 ml, containing 5% sodium dodecyl sulphate. Phospholipids and cholesterol were extracted from the samples by the method of Folch et al (16). Phospholipid was assayed as described by McClare (17), and cholesterol by using the enzyme cholesterol oxidase as described by Richmond (18) except that the method followed was that for "free cholesterol" in the British Drug Houses (BDH) booklet accompanying the assay kit (BDH 25043K).

(c) Fluorescence Measurements

The degree of fluorescence polarization of DPH in the samples was measured with an Elscint Microviscosimeter MV-1 at 37° ± 0.3°C. The excitation light was 365 nm.

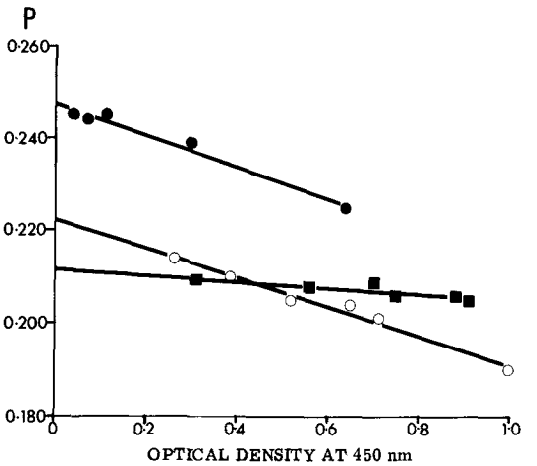
The fluorescent lifetimes of DPH in the live cells, and the 20,000 g plasma membrane vesicles were measured directly at 37°C using an Ortec Single Photon Counter and Applied Photophysics optics. The resulting spectra were deconvoluted, giving single exponential decay lifetimes of 8.6 ns for the live cells, and 9.6 ns for the plasma membrane. Fluorescence intensity measurements were performed using a Perkin-Elmer Fluorescence Spectrophotometer MPF-3L with a thermostatically controlled sample holder and a ratio stabilized light source. The % of DPH in a sample was calculated from:

$$\frac{\text{Intensity of sample fluorescence} \times \text{volume} \times 100}{\text{Intensity of fluorescence in homogenate} \times \text{homogenate volume.}}$$

RESULTS AND DISCUSSION

The Figure shows the fluorescence polarization values obtained at 37°C for the living tonsil cells, the unfractionated cell homogenate and the 20,000 g plasma cell membrane preparation. Fluorescence polarization readings were plotted as a function of the optical density of the suspension at 450 nm, to correct for light scattering depolarization. This causes little effect on the whole cells, but is very much larger for the small plasma membrane vesicles. The value of the fluorescence polarization taken was that of the linearily extrapolated value at zero optical density.

As can be seen from the diagram the fluorescence polarization of the homogenate is slightly greater than that for the whole cells. In 3 experiments the polarization increased between 0.005 to 0.011 on cell rupture. By contrast, the polarization of the probe in the isolated plasma membrane was appreciably greater than that in the whole cell. Mean polarization values and standard deviations at 37°C for the whole tonsil lymphocytes were



Figure

Values of the degree of fluorescence polarization, P, vs. optical density at 450 nm of DPH embedded in:

■ Live lymphocytes

o Lymphocyte unfractionated homogenate

● Lymphocyte plasma membrane vesicles

All measurements are at 37°C λexc = 365 nm.

0.217 ± 0.008 (9), and for the plasma membranes 0.244 ± 0.009 (7). The figures in parenthesis show the number of samples studied.

The table shows the comparative distribution of the DPH fluorescence, phospholipids and the two plasma membrane markers 5'-Nucleotidase and cholesterol. These were all obtained in one experiment, but the distributions were found to be similar for all the markers in at least two other experiments. If the cells were labelled in 0.1% sodium azide the distribution did not change. As the DPH recovery was always rather low, about 90%, and the phospholipid and cholesterol rather high (up to 113%), the results in the table have been normalised to facilitate comparison. The actual experimental results are given by multiplying the figure by the recovery/100. The fluorescence distribution corresponds quite well to the phos-

TABLE

Sample	Membrane DPH Fluorescence	Phospholipid Mole %	5' Nucleotidase Activity	Cholesterol Mole %	Protein Weight %
300g pellet	19.0	19.0	11.3	12.6	37.5
4,000g pellet	24.5	26.1	22.5	19.1	8.5
20,000g pellet	24.1	22.4	34.3	35.3	6.7
Supernatant	32.3	32.5	31.9	33.0	47.2
Recovery	92.4	112.7	102.7	106.4	110.6

Percentage distribution of the fluorescence of 1,6 diphenyl hexatriene compared to the distribution of phospholipid, 5' Nucleotidase, cholesterol and protein in human tonsil lymphocytes subject to differential centrifugation.

pholipid distribution, although its percentage was always 2.2 to 4.6% higher in the 20,000 g pellet than the phospholipid.

In comparison to the plasma membrane markers there is too much fluorescence in the 300 g pellet and too little in the 20,000 g pellet for the DPH to be situated exclusively in the plasma membrane.

The two plasma membrane markers in the table are purified five-fold (35/6.7) relative to the protein concentration in the original homogenate, whereas the mitochondrial and endoplasmic reticulum marker enzymes were not increased relative to their value in the original homogenate (Johnson and Robinson, manuscript in preparation). From electronmicrographs and the enzyme assays Johnson and Robinson estimated that over 90% of the 20,000 g pellet was plasma membrane. From the mean of four experiments it was calculated that 62% of the fluorescence was associated with the plasma membrane (i.e. 5'-nucleotidase or cholesterol) but only 54% of the phospholipid. These results are quite consistent with DPH being chemically evenly distributed among the phospholipids, since the lifetime of the probe is longer in the plasma membrane than its average value in the whole cell, and hence the fluorescence of DPH in the plasma membrane should be proportionally brighter.

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