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CARBOXYFLUORESCEIN AS A PROBE FOR LIPOSOME-CELL INTERACTIONS

EFFECT OF IMPURITIES, AND PURIFICATION OF THE DYE

EVELYN RALSTON [a,*], LEONARD M. HJELMELAND [b], RICHARD D. KLAUSNER [a], JOHN N. WEINSTEIN [a] and ROBERT BLUMENTHAL [a]

[a] *Laboratory of Theoretical Biology, National Cancer Institute and* [b] *Developmental Pharmacology Branch, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD 20205 (U.S.A.)*

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Impurities in 5(6)-carboxyfluorescein can affect phospholipid vesicle stability and apparent rates of carboxyfluorescein transfer into cells. Thorough purification and characterization of the dye are thus important to many applications with vesicles and/or cells. The dye can be purified by adsorption chromatography on a hydrophobic gel, following treatment with activated charcoal and precipitation from ethanol-water. The 5- and 6-carboxy-isomers can be separated from each other (though for most purposes it is not necessary to do so) by synthesis, crystallization, and hydrolysis of the diacetate derivatives. Purification is monitored by thin-layer and high pressure chromatography.

Some of the most useful information on liposome-cell interaction [1] is obtained by monitoring transfer of water soluble markers from liposomes to cells. Hagins and Yoshikami [2] and Weinstein et al. [3] introduced the fluorescent dye carboxyfluorescein ** as such a marker. This polar, highly water-soluble dye has since been used by a large number of laboratories for this and other purposes. Carboxyfluorescein has been used for following liposome-cell [2–12], cell-cell [13], and liposome-liposome [14] interactions. It has also been employed as a marker for monitoring enzymatic attack on intact liposomes [15] or perturbation of the lipid bilayer structure by serum lipoproteins and apolipoproteins [16,17]. The concentration dependent self-quenching of carboxyfluorescein fluorescence permits leakage from liposomes to be monitored continuously, and a distinction can be made between endocytosis of liposomes, binding of liposomes to the cell surface, and direct transfer of liposome contents to the cytoplasm [3].

The carboxyfluorescein used in most of the studies noted above was obtained from Eastman (Rochester, NY), then treated with activated charcoal and recrystallized (hereafter termed 'partial purification'). The resulting carboxyfluorescein appears homogeneous on silica gel thin-layer chromatograms eluted with

* Present address: Laboratoire de Chimie biologique, Université libre de Bruxelles, 1640 Rhode St. Genese, Belgium.

** Some confusion exists concerning the exact structure of carboxyfluorescein. Originally sold as '6-carboxyfluorescein' (Kodak catalog No. 49), the dye was renamed '4(5)-carboxyfluorescein' (Kodak catalog No. 50) after it was realized that the preparation was, in fact, a mixture of two isomers. The correct name appears to be '5(6)-carboxyfluorescein'. In Chemical Abstracts the 6-isomer of the closed, lactone form is listed as 3',6'-dihydroxy-3-oxo-spiro(*iso*-benzofuran-1(3H),9'-(9H)xanthen)-6-carboxylic acid (registry number [3301-79-9]).

Abbreviations: DOPC, di-L-oleoylphosphatidylcholine; DPPC, di-L-palmitoylphosphatidylcholine.

butanol/acetic acid/water (80 : 20 : 20, v/v). However, some remaining contaminants were brought to our attention when a series of dipalmitoylphosphatidylcholine preparations were grossly leaky and became turbid within minutes after sonication. The problem was traced to a particularly contaminated batch of starting material (Eastman, No. C48), but qualitatively similar impurities were later found in all batches tested. In this paper, we describe the effects of these contaminants, show how they can be detected, and present two routes of purification, one by adsorption chromatography, the other by synthesis and subsequent hydrolysis of carboxyfluorescein diacetate. By the former procedure, one obtains a mixture of the two isomers. By the latter, the two isomers are separated, and 5-carboxyfluorescein is obtained.

Partial purification of the crude carboxyfluorescein is achieved by treatment with activated charcoal in boiling ethanol, followed by filtration through Whatman 50 paper and precipitation at −20°C to 0°C from ethanol/water (1 : 2, v/v). After extensive washing with H_2O and drying, an orange powder is obtained. The relatively insoluble acid is then brought into aqueous solution (usually 200 mM, pH 7.4) by titration with 1 or 10 N NaOH. This carboxyfluorescein still contains impurities, as shown by TLC (with $CH_3Cl/CH_3OH/H_2O$, 65 : 25 : 4, v/v) (Fig. 1A). With one of the most contaminated Eastman batches as starting material, about 8% of the total fluorescence (λ_{ex} = 479 nm, λ_{em} = 520 nm) of the partially purified carboxyfluorescein was due to impurities. These can be removed by a single pass over a hydrophobic column of LH-20 Sephadex (Pharmacia, Uppsala, Sweden). Typically, 50 ml of 250 mM carboxyfluorescein at pH 7.4 can be purified on a 40 × 5 cm LH-20 column by elution at room temperature, with distilled water. Carboxyfluorescein elutes as a well-defined dark orange-red band, preceded by one brown, non-fluorescent band close to the solvent front. Other impurities trail behind. The concentration of dye at the peak is about 200 mM and its color is orange-red, without the dark-brown tinge of impure carboxyfluorescein solutions *. Essentially complete removal of the contaminants is demonstrated by TLC

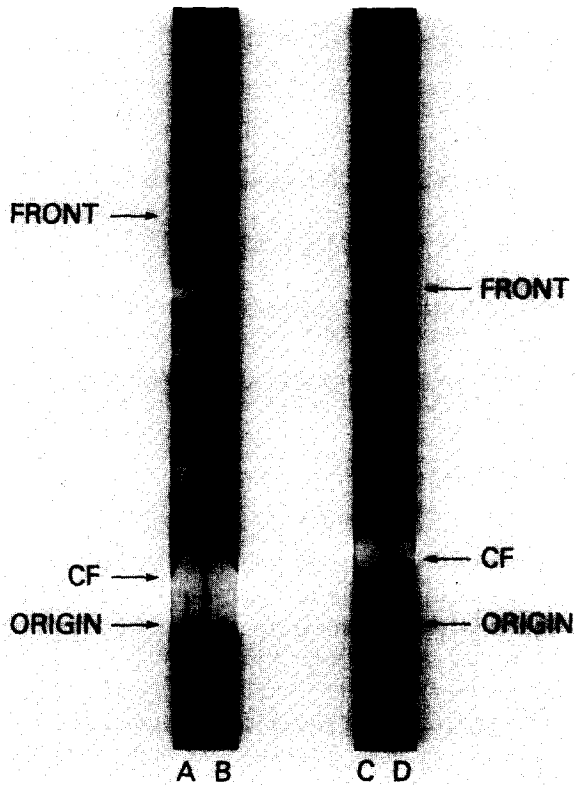

Fig. 1. Thin-layer chromatography of carboxyfluorescein: an aqueous solution of carboxyfluorescein at pH 7.4 was applied on Whatman LK6D (5 × 20 cm) plates (Kontes, Vineland, NJ) and developed with chloroform/methanol/water (65 : 25 : 4, v/v). The plates were photographed under a long wavelength fluorescent lamp. Note that lanes A and B were 1000 times more heavily loaded than C and D. (A) Partially purified carboxyfluorescein (1 μl, 10 mM). (B) Carboxyfluorescein (1 μl, 10 mM) after adsorption chromatography on LH-20. (C) Partially purified carboxyfluorescein (1 μl, 10 μM). (D) Cellular extract: red blood cell ghosts (prepared according to Cabantchik and Barzilay (personal communication), slightly modified from Ref. 20. 10^9/ml were incubated for 3 h at 37°C with 5 mM partially purified carboxyfluorescein in a final volume of 500 μl; after 5 or 6 washes with ice-cold buffer the pellet was extracted twice with 500 μl methanol. The extracts were pooled and concentrated by evaporation before TLC. The fluorescence of the spotted sample was equivalent to that of 1 μl of 1 μM carboxyfluorescein.

* Carboxyfluorescein partially purified (Cat No. C-194) and purified on LH-20 (Cat. No. C-194X) are currently available planning to market more completely purified carboxyfluorescein (Haugland, R., private communication).

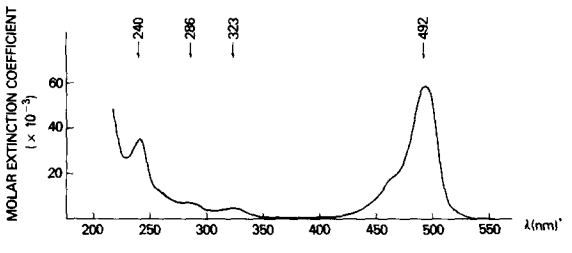

Fig. 2. Ultraviolet and visible absorption spectrum of purified carboxyfluorescein (10 μM solution). This spectrum was recorded on a Jasco 500-A Spectropolarimeter equipped with a DP-500 data processor in the laboratory of Dr. S.K. Yang, USUHS, Bethesda, MD. The approximate extinction coefficients given in the text were measured with a Gilford spectrophotometer (Model 240) equipped with a digital absorbance meter (Gilford 410).

(Fig. 1B). When directly applied to the crude, non-crystallized dye, LH-20 adsorption chromatography does not permit total purification. The recrystallization is more efficient at removing polar contaminants and the LH-20 at removing non-polar contaminants.

An ultraviolet-visible absorption spectrum of the purified carboxyfluorescein is shown in Fig. 2. The maxima at 240 nm, 286 nm, 323 nm, and 492 nm have approximate molar extinction coefficients of $35 \cdot 10^3$, $12 \cdot 10^3$, $9 \cdot 10^3$, and $58 \cdot 10^3$, respectively.

High-pressure liquid chromatography yields an excellent analytical separation of carboxyfluorescein from its contaminants (Fig. 3), and also provides a

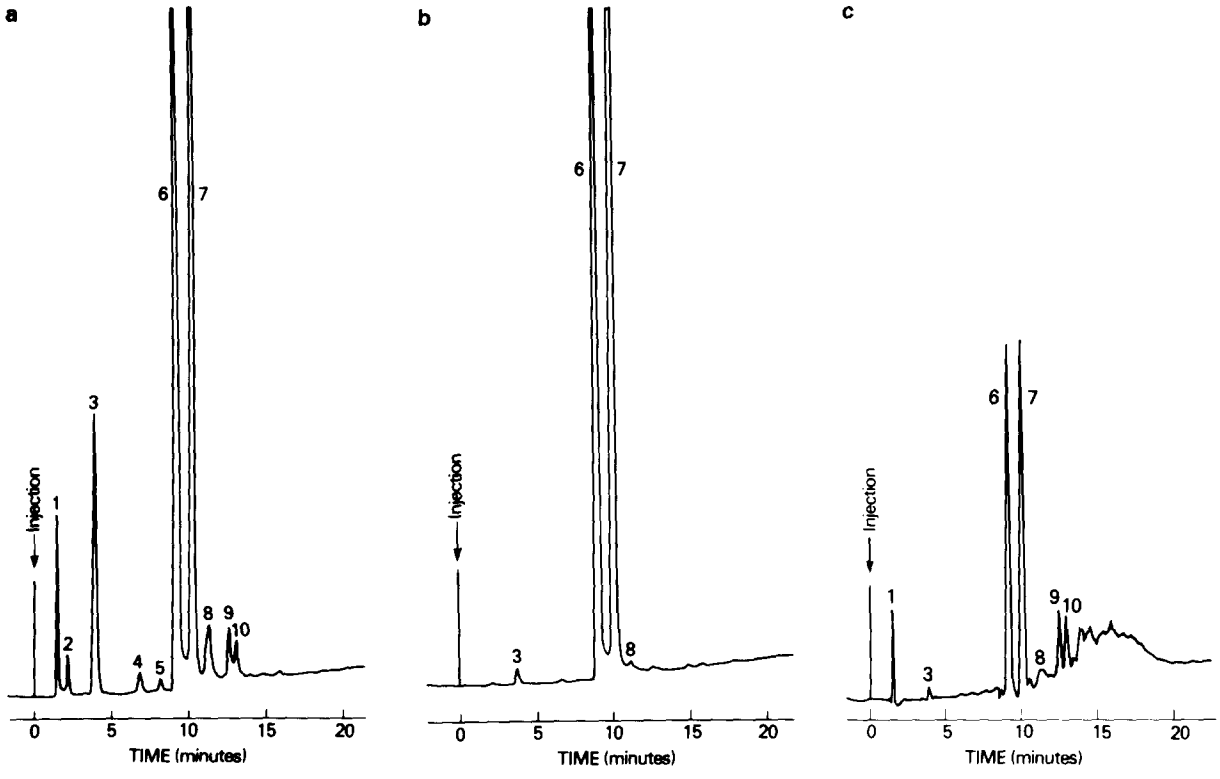

Fig. 3. High-pressure liquid chromatography (Water Associates, Milford, MA) of carboxyfluorescein: chromatography was performed at room temperature on a Micro-Bondapak C18 reverse-phase column. Column and equipment were from Waters Associates (Milford, MA). Samples (10 μl) were eluted with a linear gradient of methanol in 0.5% acetic acid. The gradient (25 to 100% methanol) was achieved in 20 min at a flow rate of 2 ml/min. Elution was followed by absorbance at 254 nm, with 0.2 A unit at full scale. (a) 10 mM aqueous solution of partially purified carboxyfluorescein. (b) 10 mM aqueous solution of carboxyfluorescein after absorption chromatography on LH-20. (c) A 25-μl sample of partially purified carboxyfluorescein, (total concentration 50 μM) encapsulated in small unilamellar dioleoylphosphatidylcholine vesicles (preparation described in Refs. 3–5).

means of monitoring purification. Peaks 6 and 7 have been recovered and identified as the 6- and 5-isomers of carboxyfluorescein, respectively, according to their NMR spectra (not shown). From batch to batch the total amounts and proportions of the impurities vary. For example, the two peaks numbered 9 and 10 in Fig. 3B appear as a single small bump in other preparations.

An alternate route to purified carboxyfluorescein takes advantage of the ease with which contaminants can be removed from the diacetate [18]. Synthesis of the diacetate had been improved [19] but difficulties in purification of the final product by crystallization from ethanol remained. We find that recrystallization from boiling isopropanol gives essentially homogeneous material. Pure carboxyfluorescein can then be regenerated from the diacetate by hydrolysis in ethanolic KOH followed by precipitation from water at low pH as previously described [19]. The dye obtained in this way contains only the 5-isomer. A more detailed protocol will be presented elsewhere (Hjelmeland, L.M. et al., manuscript in preparation).

Vesicles of dioleoylphosphatidylcholine are not grossly changed in size, state of aggregation, or leakiness by the impurities. However, the impurities can be found concentrated in those vesicles. Fig. 3c shows a liquid chromatographic profile of partially purified carboxyfluorescein encapsulated in dioleoylphosphatidylcholine. Comparison with Fig. 3a shows peaks 8, 9, and 10 to be increased relative to peaks 6 and 7. Peaks 9 and 10 fluoresce with the excitation and emission maxima characteristic of the fluoresceins.

Impurities also concentrate in cells. After incubation of red blood cell ghosts with partially purified carboxyfluorescein for 3 h at 37°C, a methanol cell extract containing the fluorescent dye recovered from the cells was compared by TLC with a similar amount (10 pmol) of the stock dye. With such small amounts of material on the plates, the impurities in the partially purified carboxyfluorescein are not detected (Fig. 1C). However, they can be seen clearly in the cell extract (Fig. 1D), showing concentration of fluorescent impurities in the cellular material. No impurities could be detected when the same experiment was done with carboxyfluorescein purified on LH-20.

One might thus expect impurities in carboxyfluorescein also to affect measurements of transfer of liposome-encapsulated dye to cells. Fig. 4 indicates that such can, in fact, be the case. Shown are dose-response curves for transfer of partially purified and LH-20 purified carboxyfluorescein from DOPC vesicles to red blood cell ghosts. The rate of transfer of fluorescence from the vesicles containing the partially purified carboxyfluorescein is significantly larger than that from vesicles containing LH-20 purified carboxyfluorescein. The difference could be explained by a faster rate of transfer into the cells of a more hydrophobic impurity. Thin-layer chromatography indicates that the impurity is enriched in the cell (not shown).

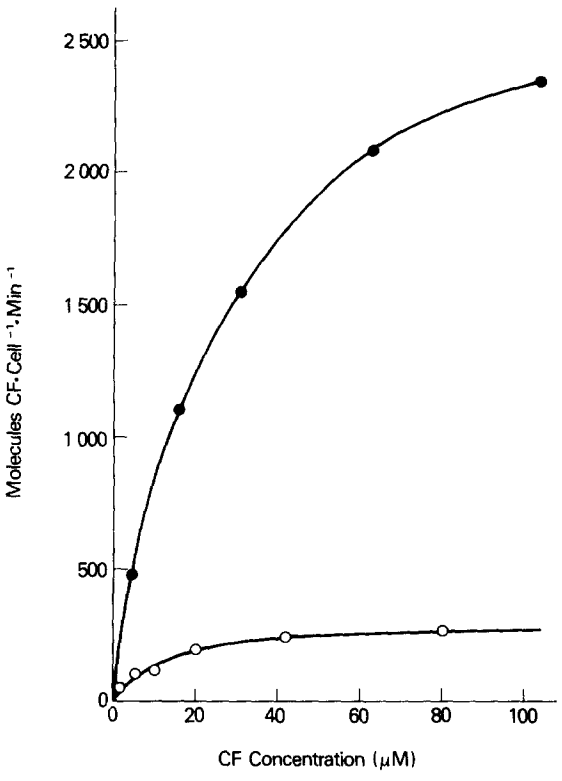

Fig. 4. Initial rate of transfer of partially purified (closed circles) and LH-20 purified carboxyfluorescein (open circles) from dioleoylphosphatidylcholine vesicles to red blood cell ghosts. The assay, based on the self-quenching properties of carboxyfluorescein, has been described in detail in Refs. 3 and 4. The data points on the ordinate represent means from fluorescence activated cell sorter histograms FACS II, Becton-Dickinson, Mountain View, CA). FACS calibration yielded 3250 molecules/channel. The abscissa represents the total concentration of carboxyfluorescein in the incubation medium after disruption of the vesicles with Triton X-100.

Finally, it is important to note that the effects on transfer are significant only because the spontaneous transfer of carefully purified carboxyfluorescein to red cell ghosts is extremely low. Transfer from similar vesicles to lymphocytes is almost an order of magnitude higher [3,4] but still in a range in which the impurities can cause significant errors. The impurities have little significance when carboxyfluorescein association with cells is large, as with ligand-specific binding [5,6] or endocytosis [12] of carboxyfluorescein-containing liposomes.

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