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DIFFERENTIAL EFFECTS OF ERYTHROCYTE LYSATES ON SPECTRA OF POTENTIAL-SENSING CARBOCYANINE DYES

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Summary

A recent report by Hladky, S.B. and Rink, T.J. ((1976) J. Physiol. 263, 287—319) demonstrates the binding of a thiocarbocyanine dye (di-S-C_3(5)) to an intracellular constituent from human erythrocytes. Evidence presented below shows that the binding of carbocyanine dyes depends on the particular type of dye used and on the species of erythrocyte from which cellular constituents are prepared. It is recommended that dyes which show minimal binding to intracellular components be used for investigations where carbocyanine dyes are employed as probes of membrane potential.

Cyanine dyes (Fig. 1) have been used with increasing frequency in experimental systems where rapid, non-invasive procedures for monitoring membrane potentials are desired. Recent applications include work with suspensions of erythrocytes [1—4], ascites tumor cells [5, 6], microorganisms [7—10], protozoa [11], mitochondria [12, 13] and membrane vesicles [3, 14]. In every case, manipulations of the membrane potential have been shown to in-

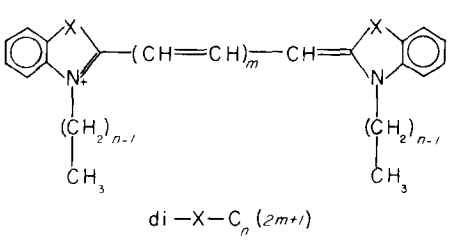

Fig. 1. Structure of a generalized carbocyanine dye (3,3'-dialkyl-X-*m*carbocyanine) with shorthand notation of Sims et al. [3]. X = S: thiocarbocyanine, X = O: oxacarbocyanine, *m* = 1: carbocyanine, *m* = 2: dicarbocyanine.

duce characteristic changes in the fluorescence of the dye-cell suspensions. Conditions which induce an interior negative membrane potential or hyperpolarization of an existing potential lead to a pronounced decrease in suspension fluorescence, while depolarizing events cause an increase in fluorescence. Work by Hoffman and Laris [2] and Sims et al. [3] has shown that observed fluorescence changes are due to a change in distribution of dye between the cellular and extracellular compartments and that cell-associated dye apparently exhibits a marked fluorescence quenching.

While these general observations apply to a wide variety of systems studied, the precise mechanism by which cyanine dyes respond to changes in membrane potential is still not understood. One possibility is that hyperpolarization causes positively-charged dye molecules to become associated with the cell membrane and/or with cellular constituents in response to an increase in monomeric dye concentration within the cell. Hladky and Rink [1] have concluded that a significant proportion of a thiocarbocyanine dye (designated as di-S-C_3(5) in the shorthand notation of Sims et al. [3]) is bound to an intracellular protein, presumably hemoglobin, following potential-induced uptake by human erythrocytes. For intact cell preparations negligible amounts are considered to be free within the cell or bound to the outside surface of the membrane. Since most reported studies have been conducted with di-S-C_3(5), the observation is of possible significance beyond the erythrocyte system.

On the other hand, Kimmich et al. [4] report that 88—100% of the amount of an oxacarbocyanine (di-O-C_3(5)) dye which is bound to intact pigeon erythrocytes for given conditions, can be bound to erythrocyte ghosts which have been largely depleted of their hemoglobin and exposed to the same conditions. These observations suggest that compared to thiocarbocyanines, binding to hemoglobin may not be as significant a factor in the fluorescence response of oxacarbocyanine dyes, which otherwise show a similar fluorescence behavior. For this reason, we undertook an investigation of the absorption spectra for these two dyes in the presence and absence of various mammalian and avian erythrocyte lysates in order to ascertain if differences exist in their spectral properties following exposure to hemoglobin.

Heparinized blood from either human, chicken or rat was washed 3 times in a 140 mM NaCl/20 mM Tris·Cl/1 mM $CaCl_2$/1 mM $MgSO_4$, pH 7.4, solution. The third wash was centrifuged and the supernatant and top layer of cells were aspirated away. The packed erythrocytes were resuspended in the same solution to a 20% hematocrit. A small sample of cells was then transferred to 15 ml of lysing solution containing 40 mM KCl and 4 mM Tris·Cl, pH 7.4, and vortexed. (For the rat erythrocyte the lysing solution consisted of 2 mM NaH_2PO_4/3 mM Na_2HPO_4/1 mM $MgSO_4$/1 mM $CaCl_2$, pH 7.4.) The cell lysate was left at room temperature for 15 min and then osmolality was restored by adding 2.0 ml of 1 M KCl and 3.0 ml of distilled water. The mixture was left at room temperature for another 15 min and then centrifuged at 900 × g for 5 min. Supernatants were saved for absorption analysis. The final lysate concentration was 2.5 μl cell volume/ml for human and chicken erythrocyte preparations and 1 and 10 μl/ml for rat erythrocyte preparations. These lysate concentrations were chosen to approximate conditions described in Hladky and Rink [1]. 25 μl of 100 μM stock solution of either dye (di-S-C_3(5) or di-O-

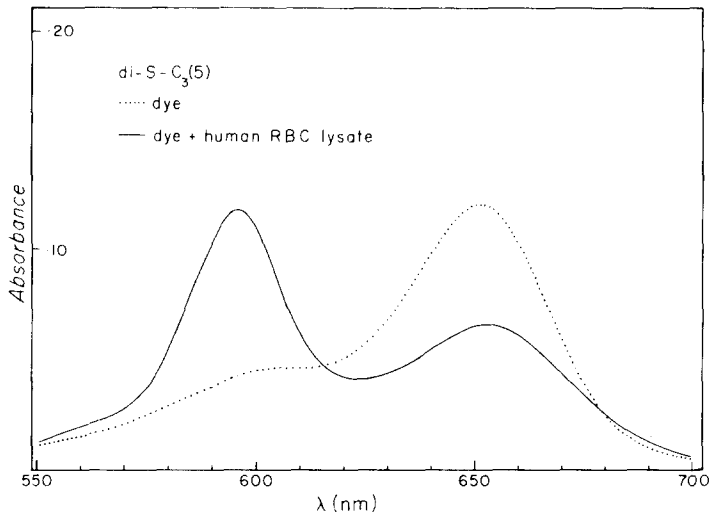

Fig. 2. Absorption spectra of di-S-C_3(5) in the absence (dotted curve) and presence (solid curve) of human erythrocyte (RBC) lysate. Final dye concentration, 1 μM; lysate concentration, 2.5 μl cell volume/ml buffer. Buffer solution was 140 mM KCl/4 mM Tris·Cl/1 mM $MgSO_4$/1 mM $CaCl_2$, pH 7.4. All spectra were run at room temperature (20°C).

C_3(5) in 100% ethanol) were added to 2.5 ml of the diluted lysate in the sample cuvet (bringing the final dye concentration to 1 μM), and run against a matched standard containing the same concentration of lysate solution on a Cary-118 C split beam recording spectrophotometer. These spectra were compared to spectra obtained on dye mixed with buffer but without cell lysate (140 mM KCl/4 mM Tris·Cl/1 mM $MgSO_4$/1 mM $CaCl_2$, pH 7.4). Di-S-C_3(5) was scanned from 550—700 nm while di-O-C_3(5) was scanned from 500—650 nm. There were no significant absorption changes in either dye when the buffer was varied (substituting 140 mM NaCl for KCl; substituting 4 mM Tris·Cl for 5 mM PO_4; or omitting both NaCl and KCl).

Fig. 2 shows the absorption spectrum of di-S-C_3(5) in the absence and presence of human erythrocyte lysate. In the absence of lysate there is a single absorption peak at 652 nm (corresponding to the monomer absorption maximum) and a small shoulder at approximately 605 nm. When dye is added to the lysate solution there is a 45% decrease in absorbance at 652 nm while a new peak appears at 597 nm (presumably the dimer absorption maximum [15]). Fig. 3 shows the absorption spectrum of di-O-C_3(5) under the same conditions. In the absence of lysate there is a single peak at 580.5 nm. In the presence of lysate there is no significant change in the position or amplitude of the absorption maximum. In addition, there are no new peaks apparent at any other wavelength. Fig. 4 shows that there are no significant changes in the absorption spectrum of di-S-C_3(5) in the presence and absence of lysate from chicken erythrocytes. No spectral shifts were found with di-S-C_3(5) and rat erythrocyte lysate or with di-O-C_3(5) and chicken or rat erythrocyte lysate (results not shown).

Assuming that any significant dye binding to protein would be reflected by spectral shifts, our results indicate that cyanine dye binding to cellular

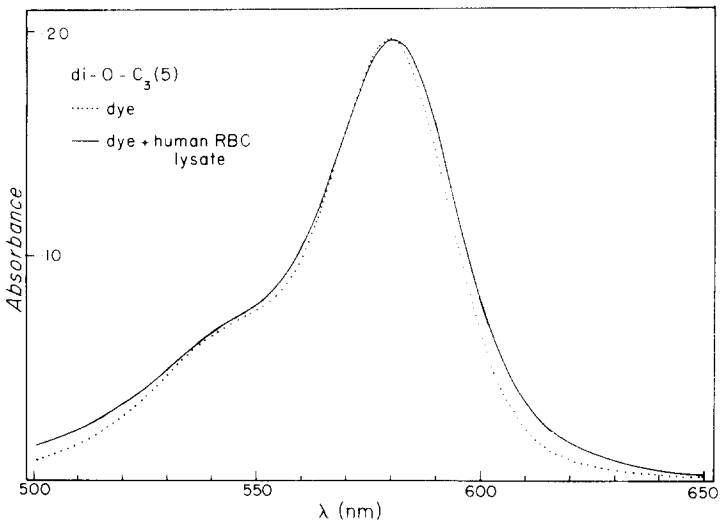

Fig. 3. Absorption spectra of di-O-C_3(5) in the absence (dotted curve) and presence (solid curve) of human erythrocyte (RBC) lysate. Conditions are identical to those described in the legend of Fig. 2.

contents depends both on the particular type of cyanine dye employed as well as on the species from which the erythrocyte lysate is prepared. Our results with di-S-C_3(5) and human erythrocyte lysate are in good agreement with the findings of Hladky and Rink [1]. In view of Hladky's work we see no reason to believe that human hemoglobin is not the major cellular constituent promoting the binding and perhaps dimerization of the thiocarbocyanine dye. Additional support for this contention is found in the work of Kay et al. [16] who showed that trace amounts of various proteins including human hemoglobin and equine myoglobin could induce new absorption peaks in the spectra of carbocyanine dyes.

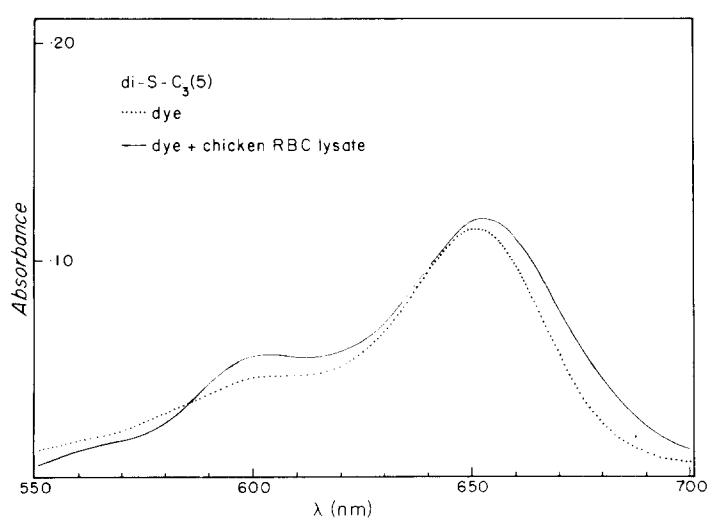

Fig. 4. Absorption spectra of di-S-C_3(5) in the absence (dotted curve) and presence (solid curve) of chicken erythrocyte (RBC) lysate. Conditions are identical to those described in the legend of Fig. 2.

On the other hand , our results also support the findings of Kimmich et al. [4] that most of the di-O-C_3(5) associated with whole cells is bound to the cell membrane rather than to the avian hemoglobin. We see no spectral changes when chicken erythrocyte lysate is mixed with di-O-C_3(5), and therefore predict that dye binding to hemoglobin is not a major factor for producing potential-induced fluorescence changes in earlier experiments with di-O-C_3(5) and intact pigeon erythrocytes. The same may be true for the use of di-S-C_3(5) with either rat or chicken erythrocytes. We are currently doing such experiments with di-O-C_3(5) and rat erythrocytes.

Even though much of the di-S-C_3(5) which is bound to the human erythrocyte is bound to hemoglobin, the significance of the molecular interaction of the dye molecule with the membrane in various potential states should not be overlooked. This interaction leads to changes in internal dye monomer concentration which, in all likelihood, controls the amount of binding to hemoglobin. To overcome the complications of dye-hemoglobin interactions Hladky and Rink [1] prepared red cell ghosts depleted of either most (resealed ghosts) or all (white membranes) of their hemoglobin. Both dye binding studies and spectra were investigated. We are currently investigating the changes in membrane and cellular distribution of di-O-C_3(5) in response to potential changes in mammalian erythrocytes. This dye exhibits no interaction with hemoglobin as detected using the spectral criteria and conditions described above. This is in marked contrast to the interaction with human hemoglobin exhibited by di-S-C_3(5).

When carbocyanine dyes do bind to hemoglobin, complications in the analysis of dye distribution among various cellular compartments arise. For this reason we recommend the use of di-O-C_3(5) or any carbocyanine dye with similar optical properties which does not bind to hemoglobin or other cellular contents, in the investigation and application of cyanine dyes as probes of membrane potential. Analysis of such situations is significantly easier, and interpretation of experiments designed to test the mechanism of potential-induced dye binding is less ambiguous.

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