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Fluorescence changes of ethidium bromide on binding to erythrocyte and mitochondrial membranes

Fluorescent probes have been used to study the physical properties of macromolecules in solution[1,2]. Recently, their use has been extended to biological membranes to ascertain the nature of the binding sites and their responses to different ionic and metabolic states. RUBALCAVA *et al.*[3] showed that 1-anilinonaphthalene-8-sulfonate (ANS) binds with enhanced fluorescence to hemoglobin-free rabbit erythrocyte membranes. This binding was found to be sensitive to the concentration and the nature of the cations in the suspending media. Similar observations have been reported for microsomes by VANDERKOOI AND MARTONOSI[4] and for mitochondria and submitochondrial particles by AZZI *et al.*[5] and CHANCE *et al.*[6] who also showed that the fluorescence responds to metabolic changes.

Ethidium bromide (2,7-diamino-9-phenylphenanthridinium-10-ethyl bromide) has been shown by LEPECQ AND PAOLETTI[7] to bind with enhanced fluorescence to native double-stranded DNA and RNA. On the other hand, binding with a low enhancement in the fluorescence occurs with single-stranded RNA, denatured DNA or polyvinylsulfate. Ethidium is a molecule in which the positive charge is delocalized throughout the conjugated double bonds of the aromatic rings. This results in the dye being soluble in solvents such as ethylene glycol, ethanol, butanol and octanol, where it shows enhanced fluorescence over that in water. From the above, LEPECQ AND PAOLETTI[7] proposed that the increase in quantum yield on binding was the result of the immersion of the dye in a hydrophobic region of the native nucleic acid where it was protected against quenching by the aqueous solvent.

It is likely that ethidium should interact with other hydrophobic surfaces of neutral or opposite charge. Fig. 1 demonstrates that the enhancement in the fluorescence of ethidium coincides with the formation of micelles of sodium lauryl sulfate. Above the critical micelle concentration, the detergent is in the form of spherical Hartley micelles with the hydrocarbon chains directed away from the water. Since ethidium is an amphipathic molecule, it is likely that it penetrates the palisade layer with the charge directed towards the aqueous phase. From the values of n (molecules of ethidium per micelle) approx. 1 molecule of ethidium binds per sodium lauryl sulfate micelle. These findings correlate with similar estimates obtained from the binding of ANS to micelles of cetyltrimethylammonium bromide and Triton X-100 (ref. 3).

This interaction of ethidium with an apolar–polar interface with enhanced fluorescence suggested its use as a membrane probe. Fig. 2 shows Scatchard plots of data on the binding of ethidium to hemoglobin-free erythrocyte membranes in hypotonic solutions and to mitochondria in two different metabolic states obtained by suspension in the presence of succinate and of succinate and antimycin A. The data were calculated using the values of relative maximum fluorescence intensity shown in Table I, obtained from a titration of a fixed concentration (1.0 μM) of ethidium with increasing concentrations of membranes[3]. The derived parameters of these plots and those of Fig. 2 are shown in Table I.

Abbreviation: ANS, 1-anilinonaphthalene-8-sulfonate.

The hemoglobin-free erythrocyte membranes gave an approx. 17-fold enhancement in fluorescence over that of aqueous ethidium. A high affinity of the probe for the membrane is shown by the low apparent dissociation constant, $\overline{K}_{app}$, emphasizing its suitability as a probe. This compares favorably with ANS ($\overline{K}_{app}$ 43 μM)[3].

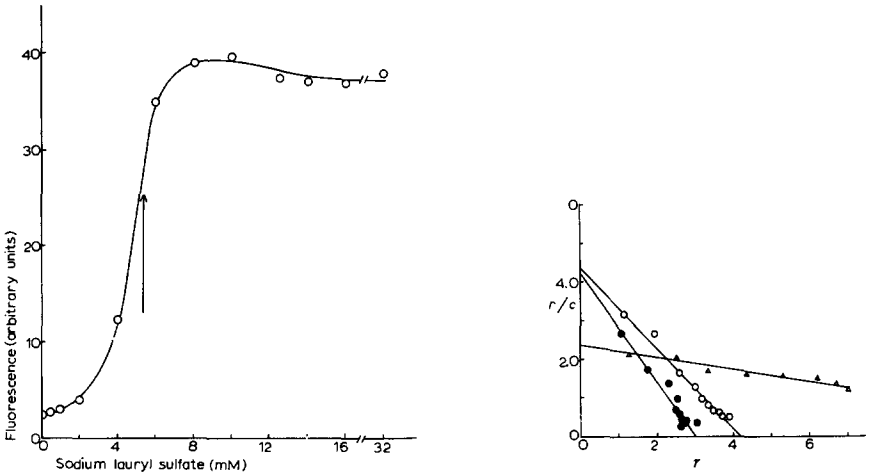

Fig. 1. Fluorescence of ethidium bromide (2 μM) as a function of sodium lauryl sulfate concentration. Fluorescence was determined in a Turner fluorimeter. The excitation wavelength, 530 nm, was selected by a narrow-pass Wratten filter 74. Emission was determined at 590 nm. The arrow indicates the critical micelle concentration of the detergent determined by surface tension.

Fig. 2. Binding of ethidium bromide to hemoglobin-free erythrocyte membranes and to mitochondria. △, hemoglobin-free erythrocyte membranes (0.31 mg protein) suspended in 20 mosM Tris–HCl buffer, pH 7.4. Incubation medium for mitochondria (0.56 mg protein): 10 mM Tris–HCl, 5 mM KCl, 5 mM Tris–phosphate, 1 mM $MgCl_2$, 0.05 mM EDTA, 250 mM sucrose, pH 7.4. ○, incubation medium *plus* 5 mM Tris–succinate. ●, incubation medium, 5 mM Tris–succinate *plus* 0.5 μg/ml of antimycin A. Temp., 25°. Fluorescence measurements as in Fig. 1. r is expressed in nmoles/mg membrane protein; c, the free ethidium concentration, in μM. The data fit the equation $r/c = \overline{K}_{app}n - \overline{K}_{app}r$ where $\overline{K}_{app}$ is the average statistical dissociation constant for the n sites.

TABLE I

DERIVED PARAMETERS FOR THE BINDING OF ETHIDIUM BROMIDE

Maximum fluorescence in arbitrary units for 1 μM ethidium under conditions where all the dye is bound (for details of determination see ref. 3). n, number of sites where the dye can bind, expressed in nmoles of dye per mg protein for data on the membranes and in molecules per micelle in the case of the detergent. Micelle molecular weight was taken as 25600 (ref. 8). Values are derived from the plots of Fig. 2 and similar data for the detergent.

System	*Maximum fluorescence*	n	$\overline{K}_{app}$ (μM)
Hemoglobin-free erythrocyte membranes	38.5	15.1	6.29
Mitochondria			
+ 5 mM Tris–succinate	27.5	4.2	0.96
+ 0.5 μg/ml antimycin	12.5	3.1	0.76
Sodium lauryl sulfate	19.5	1.3	5.0

In the case of the mitochondria, a substantial enhancement of fluorescence is obtained in the presence of substrates as compared with mitochondria whose electron

transport has been inhibited. Table I shows this as an increase primarily in the relative fluorescence intensity while the number of sites and the binding constants are only slightly altered. However, it should be emphasized that there are technical difficulties involved in the determination of the relative quantum yield in the presence of antimycin A due to the low enhancement over the aqueous ethidium. Again, the small number and high affinity of the binding sites for the ethidium make this an ideal probe for the study of mitochondrial membranes. Thus, the number of binding sites is 5-fold lower than those reported for ANS[9], and although no figures are available for the $\bar{K}_{app}$ of ANS with intact mitochondria, it would appear to be greater than that for ethidium.

The ethidium fluorescence responds to a variety of metabolic states in mitochondria. Succinate and glutamate both cause comparable fluorescence enhancements, while malate gives a smaller increase. Antimycin A, anaerobiosis and uncoupling agents all decrease the fluorescence compared with State 4 conditions. The transition from State 4 to State 3 induced by ADP causes no discernible fluorescence change. ATP, added to antimycin inhibited mitochondria, enhances the fluorescence but substantially less than succinate. This increase is, however, sensitive to oligomycin. At the concentrations used, ethidium has no detectable effect on mitochondrial respiration

The availability of membrane probes having different physical properties such as charge, lipophilicity and amphipathic nature, binding to similar or to different membrane loci, should serve materially to increase our knowledge of membrane functions and the responses of membranes to metabolic perturbations. Ethidium exhibits properties and responses to membranes different in quality and quantity to those observed with ANS, making it a valuable tool for studying the underlying membrane mechanisms.

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