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AN ETHIDIUM ION SELECTIVE ELECTRODE AND ITS APPLICATION TO DNA-ETHIDIUM BINDING

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A liquid membrane electrode has been made which is selective for ethidium ion. The membrane is formed in a capillary by a 3-nitro-o-xylene solution of an ethidium-tetraphenyl borate complex. The electrode emf (vs saturated KCl-calomel reference) has a linear dependence upon the logarithm of ethidium concentration from 2 μM to 0.5 mM. The electrode is used here to measure free ethidium ion in mixtures with calf thymus DNA. The binding isotherms obtained are in general agreement with a control photometric titration and with literature results. Direct measurement of free ethidium concentration by convenient potentiometric methods is useful in the study of ligand binding to nucleic acids and to related compounds.

The binding of ethidium ion to DNA and to related substances has been widely studied, and extensive comparisons of binding isotherms obtained by equilibrium dialysis and by various photometric methods have been published (1,2). Equilibrium dialysis requires a membrane which is impermeable to one of the reactant species. Photometric methods require the value of a spectroscopic parameter characteristic of the complexed state of the ligand (e.g. molar absorptivity or fluorescence intensity). Such a parameter is difficult to evaluate precisely, and the subsequent analysis for model suitability is very sensitive to its value (2,3). We have been studying the energetics of ethidium binding to DNA and to dinucleosides, and there is a pressing need for more direct measurement of free ligand concentration. A substantial literature exists describing the theory and characteristics of electrodes which selectively measure the concentration of a wide variety of ions (4). In this communication we describe the construction of an ethidium ion selective electrode, and its use to obtain an ethidium-DNA binding isotherm.

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METHODS

The electrode used here is of the type usually called liquid membrane or liquid ion exchanger (4). A complexing agent is used to solubilize, in a water immiscible solvent, the ion species whose concentration is to be measured. The water insoluble phase is the "membrane", and it forms a junction between the solution in which the ion is to be measured and (in our case) a silver-silver bromide electrode in saturated KBr. The complex was precipitated upon mixing 40 ml of 0.025 M ethidium bromide (Sigma Chemical Co.) with 20 ml of 0.05 M sodium tetraphenyl borate (Aldrich Chemical Co.). The precipitate was filtered and dried, mp. 112-115°. The membrane solution was 0.5 percent by weight of the complex dissolved in 3 nitro-o-xylene (Aldrich Chemical Co.). 2 mm capillary glass was drawn with a Narishige PE-2 "electrode puller"; the resultant tips were 40 micrometers in diameter, and were smoothed with carborundum paper. A 5 mm length of the capillary tip was rendered hydrophobic by dipping in 19 percent (v/v) dimethyl dichloro silane-toluene solution for 10 minutes, followed by 5 minute washes in toluene and acetone, successively. The capillary tips were "loaded" by dipping into the liquid membrane solution. The silver-silver bromide wire electrode and saturated KBr were then added at the large end of the electrode, and the top sealed with parafilm. The ohmic resistance of the electrode membranes was typically 5 megohm. All measurements reported here were made in a buffer solution which was 0.1 M NaCl, 1 mM EDTA, and 0.01 M trihydroxymethyl aminomethane, pH 7.1. A saturated KCl-calomel fiber junction electrode was used as reference; cell measurements were made with a Corning model 110 meter. Strip chart records were obtained to permit quantitative examination of response time. When not in use, the electrodes were stored in 2 μM ethidium bromide.

Results and Discussion

A. Electrode Characteristics

Figure 1 shows the response of two electrodes to ethidium concentration. The data were obtained by successive additions of 1 mM ethidium bromide to 3 ml of buffer. These data were used to standardize the electrode by linear regression on the function shown as eq. 1,

$$E = E' + m \log[L] \qquad (1)$$

in which E is the voltage associated with the ethidium concentration L; E' and m are used here simply as adjustable parameters, but can be interpreted thermodynamically and theoretically (4). Table I summarizes the results obtained. For a singly charged ion under our conditions (23°), "Nernstian" response would require that m = 58.4 mv. We have found that whereas E' is sensitive to the dimensions of the membrane, m is much less variable, although the latter does increase slowly over the first few days after an electrode is prepared. The electrodes reached a stable reading within 30 seconds

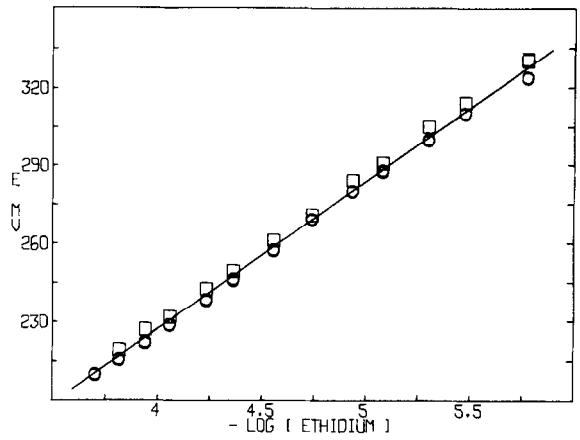

Figure 1. Electrode standardization. Results for two electrodes are shown. The circles are data obtained at 5 hours and 5 days with one electrode; the squares are data for another electrode taken two days after its preparation. The data have been normalized by subtracting E' values for each separate data set (Table I) from E observed. The line drawn was calculated by linear regression on the 39 observations shown.

after an addition of ethidium ion, and in this respect compare favorably with glass electrode pH measurements.

Selectivity of the ethidium electrode was examined briefly by carrying out a series of potential measurements upon ethidium-proflavin mixtures. Selectivity coefficients (4) of 0.01 and 0.019 were measured at 1 and .5 mM proflavin, respectively.

B. Titration of DNA with ethidium ion.

As a test of the electrode, we have observed the binding of ethidium to calf thymus DNA (Sigma Chemicals, type I), using both potentiometric measurement and absorption photometry.

1. Potentiometric method. The electrodes were dipped in 3 ml of 10 to 50 μM ethidium in buffer. DNA, 1.29 mM in DNA phosphate (ε_{260} = 6600 M^{-1} cm^{-1}) was added serially by syringe and the cell voltage recorded. In another series of measurements, serial additions of ethidium were made to 3 ml of DNA solution. The data were analyzed for applicability to a single class of sites model by two methods, both employing the Marquardt non linear regression

Table I. Electrode Standardization Parameters

electrode	E', mv	m, mv	std. dev.
a, 5 hrs	256.9(.79)	56.7(.77)	1.0182
a, 5 days	266.8(.32)	58.5(.30)	0.3041
b, 2 days	270.9(.63)	57.3(.62)	0.8411

23°, 0.1 M NaCl, 0.001 M EDTA, 0.01 M tris, pH 7.1. Quantities in parentheses are single parameter percent relative standard deviations, by linear regression on eq. 1. 13 observations in each data set; data are shown in fig. 1.

procedure (5). In the first method, free ethidium in the mixtures (L) was calculated using equation 1 and values of E' and m for the electrode in use. The ratio of bound ethidium to DNA phosphate was calculated from the relation $r = (L_o - L)/P_o$, where L_o and P_o are total ethidium and DNA phosphate, respectively. r and L values were then fitted to eq. 2, where n is the limiting

$$r = \frac{nL}{K+L} \qquad (2)$$

value of r at saturation and K is the dissociation constant for the postulated single class of sites (2). Typical results are given in line 1 of Table II. In the second method of data analysis, the complete set of standardization and titration data was fitted simultaneously to eq. 3. All parameters

$$E = E' + m \log\{\tfrac{1}{2}(L_o - K - nP_o + [(K - L_o + nP_o)^2 + 4KL_o]^{1/2})\} \qquad (3)$$

are defined in the text and in equations 1 and 2; the independent variables are L_o and P_o. Typical values of n and K are given in line 2 of Table II. The associated values of E' and m were 253.1 and 56.15 mv, respectively.

2. Photometric measurement. Serial additions of 1 mM ethidium bromide were made to both (.5 cm) cells in a Cary 118 spectrophotometer. The sample beam cell originally contained buffer, and the reference beam cell held 1 ml of the DNA solution used for electrode measurements. The resultant difference spectra were recorded from 400 to 600 nm. A separate series of measurements were made at high DNA/ethidium ratios to determine the difference in molar absorptivity between bound and free ethidium (2,3). Free ethidium and r were calculated

Table II. DNA-Ethidium Titration Parameters

method	n	K x 10^6, M	no. obs.	source
eq. 1,2	.19 (.02)	1.66 (.215)	10	potentiometric
eq. 3	.24 (.01)	2.97 (.206)	62	potentiometric
eq. 2	.21 (.02)	7.16 (3.34)	17	photometric, $\Delta\varepsilon$ = 3800
eq. 2	.21 (.01)	11.02 (2.96)	17	photometric, $\Delta\varepsilon$ = 3900
lit.values	.15-.25	2-7	-	refs. 2,3

Parameters obtained by non-linear regression on the function indicated in column 1. The data used in line 1 are shown in fig. 2. Quantities in parentheses are 95 percent level confidence limits.

from the relations $L = L_0 - \Delta A/\Delta\varepsilon$ and $r = \Delta A/(P_0\Delta\varepsilon)$; eq. 2 was used in the regression analysis. As has been noted by others (2,3) we found that calculated L and r are quite sensitive to the choice of $\Delta\varepsilon$. The analysis reported here was made at 465 nm and the effect of a relatively small change in $\Delta\varepsilon$ is shown by the 3rd and 4th lines of Table II.

Figure 2 shows potentiometric data in relation to the single class of sites function, eq. 2, used in the regressions. Extension of the concentration range beyond that shown in figure 2 shows systematic departure from the single class of sites model. Modifications for the excluded site model and for additional classes of sites have been employed to account for these deviations (2), but they are not considered in this report, where we are concerned chiefly with a new method of free ligand measurement.

The ethidium selective electrode described here is easily prepared, responds quickly, has a useful concentration range, and makes possible rapid and convenient measurement of free ethidium ion in small volumes. Although the electrode presumably responds to ethidium ion activity (4), we have used a standardization procedure in which non-ideal behavior is not explicitly recognized. Because of the good quality of fit to eq. 1, and because m is

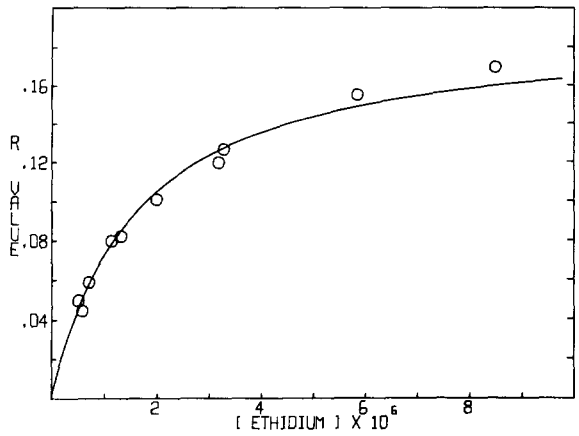

Figure 2. Potentiometric ethidium-DNA titration. Free ethidium was calculated from a standardization line (fig. 1); r was calculated as described in the text. The curve shown was calculated from eq. 2 and the parameters of line 1, Table II.

of the electrode, in the buffer used. Consideration of Table II and figure 2 shows that the electrode yields results for the binding of ethidium ion to DNA which are within the range of results reported in the literature for equilibrium dialysis and various photometric methods. The photometric methods are sensitive to the spectroscopic parameter characterizing the difference between bound and free ligand. The potentiometric method is sensitive to the quality of the function used to represent electrode response to ethidium ion, as shown by Table II. While we believe that the differences between lines 1 and 2 of Table II are in part caused by incipient deviation from the single class of sites model, it is also possible that polynomial interpolation will prove to be better than regression methods as a means of electrode standardization. Application of the ethidium selective electrode to a variety of nucleic acid binding problems appears feasible, and the development of electrodes specific for other nucleic acid-related ligands is planned. The use of ion selective electrodes to monitor the concentrations of competing ligands in a nucleic acid-ligand system is also possible, if the electrodes are sufficiently selective.

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