THE DETERMINATION OF ASSOCIATION CONSTANTS OF DIFFUSIBLE COMPLEXES BY THE DIFFERENTIAL DIALYSIS METHOD

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1. Introduction

The measurement of the rate of dialysis has been developed by Colowick and Womack [1] for determination of the association constant of an macromolecule–ligand complex. The method is rapid, but is limited to nondiffusible binding molecules. Therefore, an adaptation of the procedure to diffusible complexes is proposed, the differential dialysis method.

2. Differential dialysis method

The binding constant of a diffusible complex may be determined, provided the dialysis rate of the complex is clearly different from that of the free ligand. This requirement is met by many metallo-organic complexes. The apparatus is that described by Colowick and Womack. At the beginning of the determination, labeled ligand in trace amounts is introduced into the solution in the upper chamber. When a first measurement has given the number N_F of cpm corresponding to the diffusion rate of the free ligand, an excess of binding molecule is added, in order to obtain the number N_B of cpm corresponding to the diffusion rate of the complex. Then small amounts of unlabeled ligand are successively added, giving a series of N_i values, which permits one to calculate the part f_i ligand according to an equation derived from Fisk's law (see mathematical appendix):

$$f_i = \frac{N_i - N_B}{N_F - N_B}$$

It is noted that the case of a non-diffusible complex is only a limit case, when N_B equals zero.

The values f_i are used to calculate the concentrations c_i of free and b_i of bound ligand. Then, with a Scatchard-type plot [2], association constants are computed. Table 1 shows that they are consistent with those reported in the literature. With the same method, the fraction of ligand in the free state may be directly estimated in presence of a competitive ion

Table 1

Complex	Association constant	Values found in the literature	References
	M^{-1}	M^{-1}	
Ca · ATP	0.4×10^4	0.4×10^4; 1.4×10^4; 10^4	3 4 5
Ca · ADP	0.7×10^4	0.65×10^3	6
Ca · EGTA	10^5	1.3×10^5	7[a]
	2.1×10^5	3.3×10^5	7[b]

[a] For pH = 6.20. (Calculation M. Makinose).
The value 10^5 corresponds to pH = 6.15.

[b] For pH = 6.40.
The value 2.1×10^5 corresponds to pH = 6.30.

or molecule. Complicated calculations are avoided. When radioactive compounds are used, the method has a resolution of two orders of magnitude lower than with selective electrodes, ignores the interferences of perturbing ions, and needs only very simple devices. The precautions to be followed are those mentioned by Colowick and Womack. Moreover, it is convenient to check, with the help of the calculated association constant, whether trace amounts of ligand are really entirely bound in the presence of an excess of binding molecule.

Mathematical appendix

When the label in the lower chamber of the dialysis cell has reached the steady state, it is proportional to the radioactivity of the upper chamber and to that of the aliquots taken from the fraction collector [1]. If the labeled substrate diffuses only in the free form, we have:

$$N_F = S D_S \quad (1)$$

where N_F is the number of cpm counted in the aliquot, S the concentration of the active substrate in the upper chamber, and D_S the diffusion coefficient of the free form. If the label diffuses only in the bound form, we have:

$$N_B = S D_B \quad (2)$$

where D_B is the diffusion coefficient of the bound form. If the label diffuses in both forms, (1) and (2) become:

$$N_i = f_i S D_S + (1-f_i) S D_B \quad (3)$$

where f_i is the fraction of the total substrate which is in the free form. Therefore:

$$N_i = f_i N_F + N_B - f_i N_B \quad (4)$$

and

$$f_i = \frac{N_i - N_B}{N_F - N_B} \quad (5)$$

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