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Research Papers

PROBLEMS OF DIALYSIS TECHNIQUES IN THE STUDY OF MACROMOLECULE BINDING OF DRUGS

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SUMMARY

The binding of methyl orange to bovine serum albumin has been investigated by means of equilibrium and differential dialysis.

In order to obtain reproducible and exact results, the binding of the ligand to the membrane must always be studied.

A significant binding gives with equilibrium dialysis, as well as with the differential dialysis, erroneous results. The correction as proposed by Klotz et al. was an amelioration. But the most correct binding curve is obtained by including the membrane binding in the total binding phenomenon.

INTRODUCTION

The binding of different ligands to macromolecules, and especially to biological macromolecules is the subject of numerous investigations.

A great number of physicochemical methods are described in order to study the interaction between a ligand and macromolecules (Meyer and Guttman, 1968). Most of these techniques consist in the determination of the unbound ligand. The amount of bound drug can be calculated from the difference between the unbound and the total amount of the drug in the system.

A frequently used method to determine the free species of a drug is dialysis, in its various forms: either the 'classical' equilibrium dialysis (Patel and Kostenbauder, 1958; Patel and Foss, 1964) or the 'dynamic' dialysis (Meyer and Guttman, 1968, 1970; Brown and Crooks, 1973) in which the kinetic process of dialysis is followed.

In dialysis, the semipermeable membrane gives a separation between the free species, which is a relatively small molecule, and the macromolecule together with ligand–macromolecule complex. The impermeability of the membrane to macromolecules results in a selective diffusion even when a concentration gradient exists.

Applying the method of dialysis to the binding of methyl orange to bovine serum albumin, the technique was further examined. In the literature (Klotz et al., 1946; Meyer and Guttman, 1970; Shikama, 1968) we found different data about the binding of the dye to bovine serum albumin. The differences in the parameters n and K (Table 1), which

TABLE 1

BINDING CONSTANTS FOR THE INTERACTION MO–BSA

n = the number of binding sites; K = the intrinsic association constant (1/M)

	pH	n	K
Shikama, 1968	6.9	16	$3.8 \cdot 10^3$
Klotz et al., 1946	5.68	22	$2.27 \cdot 10^3$
Meyer and Guttman, 1970	7.3	22	$2.08 \cdot 10^3$

characterize the binding are due to the circumstances of dialysis used in the various studies. This paper will show the effects of membrane binding and pH on the binding constants for the interaction between bovine serum albumin and methyl orange.

MATERIALS AND METHODS

Materials

Bovine serum albumin (BSA) fraction V (Sigma Chemical Company) was used throughout this study. Methyl orange (MO), was of reagent grade (Merck) and molecular weight 327.34. The dye was determined spectrophotometrically in 0.1 N hydrochloric acid with a Unicam SP 1800 spectrophotometer at a wavelength of 560 nm. Dialysis tubing was Visking cellulose membrane (20/32) with an average pore size of 2.4 nm.

Procedure

The tubes were cut to a fixed length. They were heated in distilled water at 90°C for 1 h and subsequently washed several times with distilled water to remove any contaminating substances in the cellophane. Knotted at one end, they were dried by pressing between filter paper and then inflated. After filling with suitable quantities of the fluid to be dialysed the bags were tightly tied and placed in wide-mouth glass-stoppered glass tubes containing phosphate buffer. The glass tubes were then fixed in a tumbling waterbath and the temperature was maintained at 25°C during the whole experiment.

The dialysis bags were weighed before and after the experiment to check the dialysis procedure by volume changes. No volume change may occur because of the presence of the phosphate buffer which acts as swamping electrolyte.

In order to determine the adsorption of methyl orange to the cellophane membranes, the dialysis tubes were filled with solutions of the dye under the same conditions as for dialysis. The bags were placed in glass tubes containing the buffer solutions and put in the waterbath at 25°C. After dialysis the two compartments were analyzed.

RESULTS AND DISCUSSION

Binding by the dialysis membrane

When the system consisted only of methyl orange, phosphate buffer and the cellulose membrane, it was found that the methyl orange concentration decreased very rapidly due

to binding of the methyl orange by the dialysis membrane.

Fig. 1 shows the adsorption of methyl orange by the membrane as a function of time. After 120 min the adsorption remained constant. The adsorption is also dependent on both the concentration of methyl orgaine and the size of the membranes. By keeping the size of the dialysis bags constant throughout the different experiments the binding by the membrane is only a function of the concentration of methyl orange. The amount of methyl orange adsorbed (Fig. 2) increased with increasing concentrations of the dye and finally reached saturation. The relative amount adsorbed decreased as the concentration of methyl orange increased. The binding by the membrane in the presence or absence of albumin is not the same, especially for the lower concentrations of methyl orange. This becomes very clear where the absorbed amount is expressed as a per cent of the total amount present.

The binding to the membrane can be treated as an adsorption phenomenon of the Langmuir-type and may be considered as analogous to binding of one group of binding sites on a macromolecule (Meyer and Guttman, 1970; Pedersen et al., 1977):

$$r^* = \frac{n^* K^* D_F}{1 + K^* D_F} \tag{1}$$

where r^* is the amount of ligand molecules bound per amount of membrane and K^* is

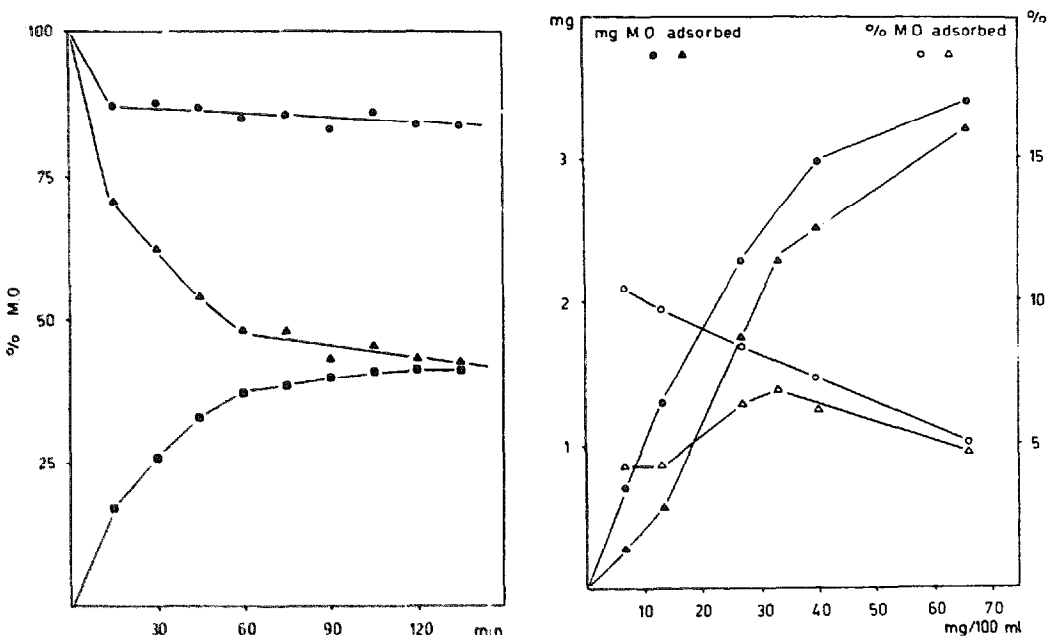

Fig. 1. Loss of MO to the dialysis membrane. ●, MO in inside and outside of dialysis bag; ▲, MO inside of the dialysis bag; ■, MO outside of the dialysis bag. Initial conc., 60 μg MO/ml.

Fig. 2 Adsorption of MO to the dialysis membrane. ●○, in the absence of albumin; ▲△, in the presence of albumin. Albumin concentration = $1 \cdot 10^{-4}$ ml^{-1}. Length of the membrane 11 cm (Visking 20/32).

the association constant for binding to the membrane, n^* is the amount of binding sites and D_F is free-drug concentration.

For the binding of methyl orange to the dialysis membrane (Fig. 3) we found the following parameters: $n^* = 0.015$ and $K^* = 0.557$. They indicate that the ligand has a lower affinity for the membrane than for the specific binder of interest, namely the protein.

Ligand–protein binding

To study the binding between methyl orange and albumin the technique of dialysis was used under various forms. They all appear very simple but they did not always result in the same binding curve. The differences were due to binding of the dye by the membrane.

When using equilibrium dialysis and having the possibility to measure the two compartments (method A), the binding to the membrane is easily excluded and the obtained binding curve represents only binding to the macromolecule.

But when measuring only the protein-free side (method B), the ligand seemed to have a greater affinity for the protein. For such cases Klotz et al. (1946) proposed to make a correction, by using a control tube, where the macromolecule was replaced by buffer (method C). After determination of the concentration of the ligand in the control tube

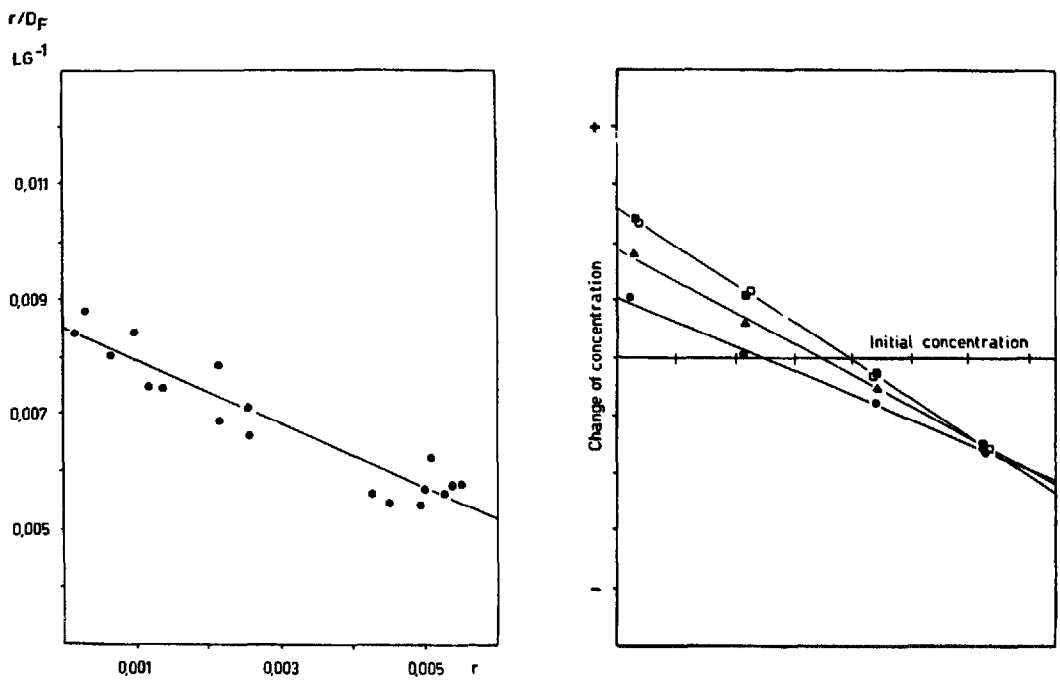

Fig. 3. Scatchard plot for the binding of MO to the dialysis membrane.

Fig. 4. Quantitative differential dialysis method. Binding MO–albumin. MO concentration = 40 mg/100 ml. Albumin concentration = $1 \cdot 10^{-4}$ ml^{-1}. Dialysis time: •, 30 min; ▲, 60 min; ■, 120 min; □, 180 min.

outside of the cellophane bag, it was possible to calculate the total amount of drug remaining in the dialysis system. This value was used by him to calculate the protein-bound amount of the dye in the protein solution.

Recently Pedersen et al. (1977) described a more correct calculation for the amount of ligand bound by the cellophane in presence of the protein (method D).

The quantitative differential dialysis method (Polderman et al., 1974) (method E) which was proposed as an alternative for the study of slowly attained equilibria and applied by us (Bontinck and Kinget, 1976) for the study of micellar binding, was applied here as well for further evaluation. In this method a drug–macromolecule solution was dialysed against a series of different concentrations of the same ligand. After a few hours the solutions were analyzed and the decrease or increase in concentration of the methyl orange was plotted against the starting concentrations. A straight line was obtained. The intersection with the X-axis gives us the value of the free ligand concentration. The slope of the line is determined by the period during which it was dialysed. When using this method the drug was only measured in one compartment.

A test solution was dialysed during 30, 60, 120, or 180 min and there was a shift in the intersection with the X-axis for some periods (Fig. 4). Which means that there is a different quantity of drug free for one constant quantity of total ligand and macromolecule. As a control, both compartments were analyzed for the different dialysis periods. After dialysing for 30 min, there was no diffusion from one compartment to the other. We noticed a decrease in the methyl orange concentration on both sides of the dialysis membrane. After establishing the mass balance we calculated the quantity of drug adsorbed to the membrane. After dialysing for 60 min an exchange had taken place. In the period between 120 and 180 min there was no difference anymore in the intersection with the X-axis. By correcting the starting concentrations for the binding to the membrane (method F) we obtained with this method a binding curve comparable with the curve of equilibrium dialysis where both compartments were analyzed.

Treatment of data

Non-cooperative ligand–protein binding may be described as analogous to the law of mass action by means of equation (Esdall and Wyman, 1958):

$$r = \sum_{i=1}^{i} \frac{n_i K_i D_F}{1 + K_i D_F} \tag{2}$$

where r denotes moles of drug bound per mol of binding protein, n_i represents the number of binding sites in the i'th class with the intrinsic association constant K_i, and D_F is the concentration of unbound ligand, i means the number of classes of binding sites.

If a model with one class of binding sites is assumed, the equation is often written in the following form:

$$\frac{r}{D_F} = nK - Kr \tag{3}$$

which on plotting r/D_F as a function of r gives the Scatchard plot.

When there is binding to the dialysis membrane, the membrane binding must be taken into account in the treatment of the dialysis data. For simultaneous binding to the membrane and to the macromolecule we have used the following equation:

$$r = \frac{n_1 K_1 D_F}{1 + K_1 D_F} + \frac{n_2^* K_2^* D_F}{1 + K_2^* D_F} \qquad (4)$$

where the first term represents the binding to the protein, and the last term the binding to the membrane. The membrane binding is mathematically treated as though a second macromolecule species is present.

The Scatchard plot for the binding of methyl orange to BSA is linear and is shown in Fig. 5. This is indicative for equivalent and non-interacting binding sites, i.e. the binding sites have an equivalent binding activity and the binding of one of the anions does not affect the binding of a second.

The slope of the line and the intercept on the abscissa give us the values of the binding parameters. The straight line which we obtained with equilibrium dialysis where both compartments were measured (Fig. 5) gave us a binding constant $K = 3.773 \cdot 10^3$ and a binding capacity $n = 15.04$.

The parameters (see Table 2) obtained from an equilibrium dialysis, where only the protein-free compartment is analyzed and disregards the binding to the membrane, give a higher degree of binding (method B). The same can be said of the quantitative differential

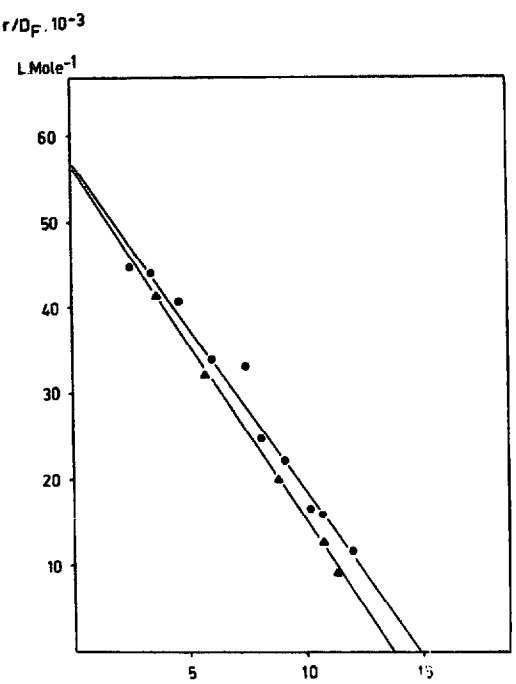

Fig. 5. Scatchard plot for the binding of MO to BSA at pH 7.3 and $t^\circ = 25^\circ C$. ●, equilibrium dialysis two compartments analyzed; ▲, method of Klotz et al., 1946.

TABLE 2

SUMMARY OF THE BINDING PARAMETERS FOR THE DIFFERENT METHODS USED AT pH 7.3 AND t° = 25°C.

Methods described in the text. n = the number of binding sites; K = the intrinsic association constant (1/M).

Method	n	K
A	15.04	$3.773 \cdot 10^3$
B	18.53	$3.419 \cdot 10^3$
C	13.81	$4.08 \cdot 10^3$
D	15.92	$3.595 \cdot 10^3$
E	18.62	$3.110 \cdot 10^3$
F	15.22	$3.531 \cdot 10^3$

dialysis method where no correction was made for loss to the membrane (method E). Correction by this method for binding to the membrane gives us parameters (method F) which are comparable with those of equilibrium dialysis where both compartments are determined.

If it is chemically impossible to analyze the protein-free side, then the correction (method C) as proposed by Klotz (Fig. 5) gives a remaining difference, due to the fact that the binding to the membrane in the presence or absence of albumin is not the same (Fig. 2).

This problem can, however, be solved by considering the membrane as another binding species. Then the binding data are calculated according to Eqn. 4 (method D). The parameters of the second term which represent the membrane binding are given by a separate study. The binding parameters for the interaction dye–BSA obtained by Eqn. 4 coincide with those of equilibrium dialysis where both sides of the dialysis system were determined.

Especially when one can only measure the protein-free compartment and when the binding to the membrane is important, this treatment of the dialysis data is the most correct one. The binding study was also performed at pH = 5.7. The binding parameters found at this pH are given in Table 3.

TABLE 3

BINDING MO–BSA AT pH 5.7 AND t° = 25°C.

Methods described in the text. n = the number of binding sites; K = the intrinsic association constant (1/M).

Method	n	K
A	21.5	$2.456 \cdot 10^3$
B	22.5	$1.84 \cdot 10^3$
F	21.4	$3.009 \cdot 10^3$

The association constants at this pH are smaller but there is a higher capacity as indicated by the higher values of n.

The pH at which the experiments are performed will also affect the degree of binding and should be mentioned in survey tables of protein binding studies.

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