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# Chapter 2

## Equilibrium dialysis and rate dialysis

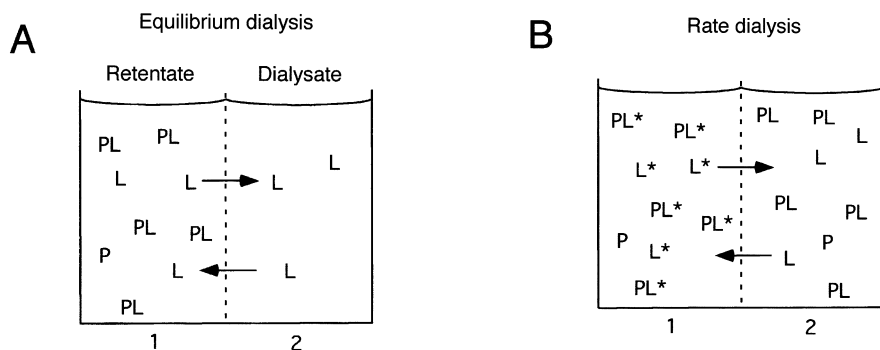
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### 1 Introduction

Studies on the interaction of low molecular mass molecules, i.e. ligands, with high molecular mass protein molecules play fundamental roles in biomedical research. Among important examples to be mentioned are drug binding to plasma proteins, especially albumin (1), vitamins to their transport proteins, as well as binding of various substances, e.g. hormones and transmitter substances to receptor proteins in their target cells. Ideally the questions to be answered from such studies are how many ligand molecules,  $N$ , may bind to a given macromolecule and with what affinities,  $K_1, K_2, \dots, K_N$  under the given set of conditions specified by, e.g. pH, temperature, ionic strength, and buffer composition. One way of approaching the answer is to measure several sets of concentrations of free ligand and protein bound ligand. The classical technique used to measure these parameters is termed *equilibrium dialysis* (2) and is based on the principle of separating the free ligand from the protein bound ligand by allowing the former to dialyse through a semi-permeable membrane until the concentration of ligand in the dialysate, i.e. the protein-free compartment, under ideal conditions, is equal to the concentration of unbound ligand in the retentate, i.e. the protein-containing compartment (*Figure 1A*). Although various other techniques based on different principles have been introduced to measure these parameters, equilibrium dialysis remains a sound and simple technique with the advantages that the interacting molecules are present under physiological conditions, i.e. in solution, and neither the ligand molecule nor the protein molecule are modified in any way. The procedures for setting up experiments and the necessary control experiments are described for ligands which absorb UV or visible light and ligands which can be obtained in radiolabelled form.

Although equilibrium dialysis is sound in its principle it possesses some inherent problems that under certain conditions may cause serious artefacts. These include, among many others, osmotic dilution of retentate and Donnan effects due to the presence of the protein on only one side of the membrane (3).



**Figure 1** Principles of dialysis techniques. In order to measure the binding strength of a ligand, L, to a protein, P, various concentration values of free ligand and protein bound ligand should be measured. (A) Equilibrium dialysis is performed with the protein present in one compartment (compartment 1 or retentate) separated from another compartment (compartment 2 or dialysate) by a semi-permeable membrane. The membrane allows dialysis of the ligand, L, but not the protein, P. At equilibrium, under ideal conditions, the concentration of ligand in compartment 2 is equal to the concentration of free ligand in compartment 1. The free ligand may be measured by spectroscopy in compartment 2 or the ligand concentration may be measured in either compartment when a radiolabelled substance is used. (B) Rate dialysis is performed with solutions containing identical concentrations of protein and ligand in either compartment. At the start of dialysis the radiolabelled ligand is present in compartment 1 only and the rate of dialysis to compartment 2 is measured. The dialysis rate constant of the free ligand is measured without protein present.

Furthermore, reliable measurement of the free ligand concentration, which is a key element in determining the affinity of a ligand, is a problem with tightly bound ligands. This is partly due to the fact that the free concentration of a high-affinity ligand is very low and thereby very inaccurately determined and partly due to the fact that impurities, whether these are light absorbing, light scattering, or radiochemical may interfere strongly with the determination of the low free ligand concentration. However, if the ligand can be obtained in radioactive form a modified dialysis procedure using the very same equipment as in the case of equilibrium dialysis may be resorted to. The principle of this dialysis technique was developed more than 20 years ago in parallel, but independently, in two different European laboratories, in Aarhus (4–6) and in Nijmegen (7–9). The technique measures the rate of dialysis of a trace amount of a ligand between two compartments containing identical concentrations of ligand and protein (*Figure 1B*). Thereby, this technique very elegantly circumvents the problems of osmotic dilution and Donnan effects and minimizes the problem of measuring very low ligand concentrations—problems that are inherent and may be troublesome in equilibrium dialysis. The modified technique is known under various names; dialysis rate method/determination (4, 5, 7, 10), rate dialysis (11), dialysis exchange method (12), dialysis exchange rate determination (13), and symmetric dialysis (8, 9). Here we will refer to the technique

as *rate dialysis* since it is a short name that simultaneously expresses the difference and the similarity to equilibrium dialysis.

Equilibrium dialysis as well as rate dialysis give binding data in the form of sets of concentrations of free ligand and the corresponding average number of ligand molecules bound to the protein. Once the binding data are in hand the next problem to be solved is the interpretation of the results. Such interpretation of the binding mechanism is not straightforward and may require computer analysis. The reader should consult the literature on the topic (14–20).

## 2 Equilibrium dialysis

### 2.1 Principle

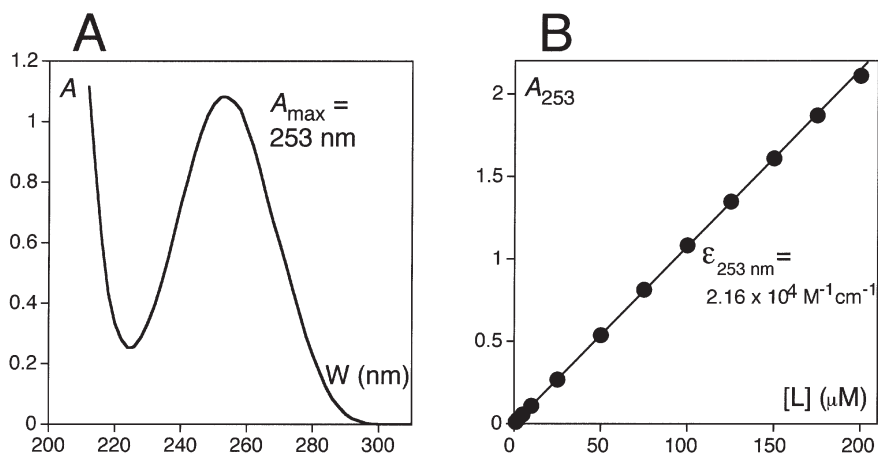
With equilibrium dialysis two buffered solutions, one containing protein called retentate or compartment 1, and the other without protein called dialysate or compartment 2 are separated by a semi-permeable membrane, i.e. a membrane that allows diffusion of a low molecular mass ligand but not the high molecular mass protein molecule to be tested (*Figure 1A*). At the start of dialysis the ligand may be introduced into either one of the compartments or in both compartments in equal concentrations. In the latter case binding of the ligand to the protein in the retentate, compartment 1, results in a net diffusion of ligand from the dialysate into the retentate. The ligand diffuses until the free ligand concentration in compartment 1,  $[L_F]_1$ , is equal to the concentration of ligand in compartment 2,  $[L]_2$ . Depending on the method used the ligand concentration may then be measured in compartment 2, with light absorbing ligands, or in both compartments, with radiochemical ligands and we may calculate the concentration of bound ligand,  $[L_B]_1$ . When the protein concentration in compartment 1,  $[P]_1$ , is known the average number of bound ligand molecules per protein molecule,  $r$ , may be obtained from the following equation:

$$r = \frac{[L_B]_1}{[P]_1} = \frac{[L]_1 - [L]_2}{[P]_1} \quad [1]$$

where  $[L]_1$  is the sum of the free and bound ligand concentrations in compartment 1. We will now consider measurement of the free ligand concentration by light absorption spectroscopy and by scintillation counting of a radioactively labelled ligand.

### 2.2 Measurement of free ligand by UV or visible light absorption

If the ligand to be measured contains UV or visible light absorbing structures, e.g. conjugated double bonds, phenol rings, or quinoide groups, the free ligand concentration may then be spectroscopically measured in the dialysate. In order to reliably measure the concentration of the ligand in the experiment it is necessary first to record the absorption spectrum of the ligand in order to select



**Figure 2** Spectroscopic determination of the concentration of biphenylacetate. (A) Light absorption spectrum of 100  $\mu\text{M}$  biphenylacetate recorded in a quartz cuvette with a path length of 0.5 cm in the sample position and buffer in the reference position. Measurements were performed in a double beam Beckman Acta M V spectrophotometer. The spectrum shows an absorption maximum at 253 nm. (B) shows a standard curve of the absorption at 253 nm versus the concentration of biphenylacetate. The regression line for points obtained with an absorption below 1.5 has a slope of  $1.079 \times 10^4$  corresponding to an absorption coefficient of  $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ . It is characteristic for a high quality spectrophotometer that points with high absorption (around 2) falls only slightly below the regression line. This deviation is much higher in spectrophotometers of lower quality. The experimental data were used with permission from ref. 6.

the wavelength of the absorption maximum, e.g. 253 nm for biphenylacetate (Figure 2A) and measure the absorption of the ligand at 253 nm,  $A_{253}$ , over a wide concentration range to determine the molar absorption coefficient and at the same time test the quality of the spectrophotometer (Figure 2B, see Protocol 1). If the substance, as in case of biphenylacetate, obeys the Beer-Lambert law we find a strict linear correlation between  $A_{253}$ , and the concentration of the ligand (Figure 2B). We can then calculate the molar absorption coefficient,  $\epsilon_{253}$ , which is  $2.16 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$  in the present example. Some ligands do not obey the Beer-Lambert law, i.e. the absorption versus concentration relationship deviates from linearity. This may be caused by dimerization or precipitation of the ligand at high concentrations.

If the ligand itself does not significantly absorb light it may occasionally be possible to perform a chemical reaction that produces a light-absorbing substance, e.g. as described for sulfonamides which may be determined spectrophotometrically after diazotization (21).

## Protocol 1

### Measurement of drug concentration by UV or visible light absorption spectroscopy

#### Equipment and reagents

- Double beam UV/visible light absorption spectrophotometer
- Two matched quartz cuvettes with identical path lengths, e.g. 0.5 cm or 1 cm
- Buffer: 66 mM sodium phosphate buffer pH 7.4 or another appropriate for the experiments

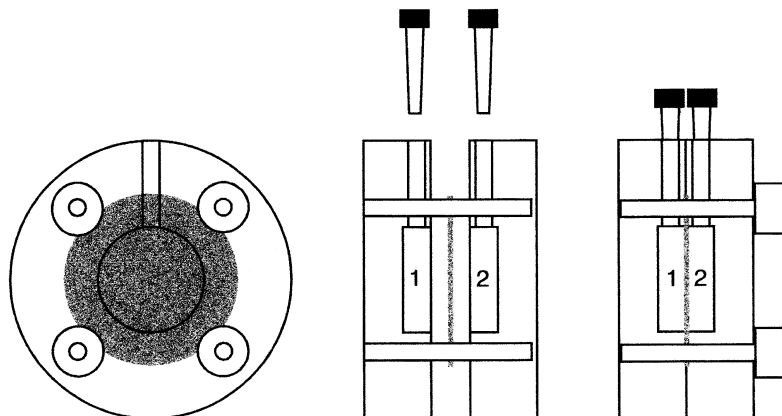
#### Method

- 1 Weigh the ligand accurately and dissolve it in the appropriate buffer in concentrations between, e.g. 1  $\mu\text{M}$  and 200  $\mu\text{M}$ .
- 2 Zero adjust the spectrophotometer with both sample and reference cuvettes.
- 3 Replace the sample cuvette with a cuvette containing the drug at an appropriate concentration<sup>a</sup> and record the absorption spectrum over a wide wavelength range, e.g. 200–500 nm, or whatever is appropriate.
- 4 Determine the maxima of the spectrum and select a maximum at a convenient wavelength, e.g. between 250–300 nm, to be used for concentration measurements.
- 5 At the selected wavelength,  $\lambda$ , measure the absorbance,  $A_\lambda$ , at various concentrations of ligand and plot  $A_\lambda$  against the concentration of the ligand,  $[\text{L}]$ . The slope of the curve is determined by linear regression. The correlation coefficient is the molar extinction coefficient,  $\epsilon_\lambda$ . A strict linear correlation should be obtained if the drug obeys the *Beer-Lambert law*,  $A_\lambda = \epsilon_\lambda \times [\text{L}] \times l$  where  $l$  is the path length of the cuvette.

<sup>a</sup>The appropriate concentration is determined on a trial and error basis.

### 2.3 Running an equilibrium dialysis experiment

A dialysis experiment can be set up according to *Protocol 2*. Various dialysis chambers may be used with various volumes from microlitres to millilitres. In each case the principle is the same; the two chambers are separated by a semi-permeable membrane. In the present example we use cylindrically formed home made polyacrylamide dialysis chambers suitable for introduction of 1 ml samples in each compartment (*Figure 3*). A chamber consists of two halves each with a compartment constructed by a cylinder formed bore 1.9 cm in diameter and 0.5 cm in depth. The two halves of the chamber are held together by four screws with a trapped dialysis membrane between the compartments. There are various types of membranes on the market. An important issue to consider is the chemical properties of the membrane. Thus, ideally the membrane should not bind the ligand or protein in question. Various types of membranes



**Figure 3** Example of a dialysis chamber. Two chamber halves each with a compartment (1 and 2) constructed by a cylinder formed bore 1.9 cm in diameter and 0.5 cm in depth. The two chamber halves are held together by four screws with a trapped dialysis membrane between the compartments (grey). The compartment volumes are 1.4 ml, convenient for introduction of 1 ml samples. An air bubble is left and serves as a stirrer when the chambers are put on a rotating device in a thermostatted cabinet.

may be tried in order to find one suitable for a given set of experimental conditions (*Protocol 3*). Furthermore, the membrane molecular cut-off should be high enough to allow the ligand molecule to pass through and low enough to retain the protein molecule. Checks should be made to ensure that the protein does not leak through the membrane (*Protocol 3*). The volume in each compartment in the present example is 1.4 ml, convenient for introduction of a 1 ml sample volume. An air bubble is left and stirs the solution when the chambers are put on a rotating plate in an air thermostat set at a constant temperature. Maintaining a constant temperature is important since the strength of many binding reactions depends upon the temperature. In general, the interaction affinity decreases with the increasing temperature.

## Protocol 2

### Setting up a dialysis experiment with spectroscopic measurement of the ligand<sup>a</sup>

#### Equipment and reagents

- Double beam UV/visible light absorption spectrophotometer
- Dialysis chambers with 1.4 ml compartments suitable for 1 ml sample volumes<sup>b</sup>
- Dialysis membrane<sup>c</sup>
- Thermostatted rotating equipment for home-made apparatus or water-bath for, e.g. Dianorm apparatus
- Two matched quartz cuvettes with identical path lengths, e.g. 0.5 cm or 1 cm

**Protocol 2** continued

- Buffer: 66 mM sodium phosphate buffer pH 7.4 or another appropriate for the experiments
- Known concentration of pure protein dissolved in the appropriate buffer
- Ligand dissolved in the appropriate buffer and diluted to various concentrations

**Method**

- 1 Cut the membranes from the dialysis tube to an appropriate size for positioning in the dialysis chambers.<sup>d</sup>
- 2 Soak the membranes in distilled water with three changes of water over about 1 h.
- 3 Soak the membranes in the appropriate buffer for the experiment with two or three buffer changes for about 30 min to 1 h.<sup>e</sup>
- 4 Dry the membranes softly with paper tissue before mounting in the chambers. Avoid too much buffer remaining on the membrane since this will dilute the samples.
- 5 Introduce 0.5 ml of the ligand solution into all compartments 1 and 2.
- 6 Introduce 0.5 ml of the protein solution into all compartments 1.
- 7 Introduce 0.5 ml buffer with no ligand into all compartments 2.<sup>f</sup>
- 8 Place the chambers on a rotating plate in a thermostatted cabinet and allow dialysis to proceed for the time necessary to achieve equilibrium. This time is determined in separate experiments as described in *Protocol 3*.
- 9 Measure the absorption in all the solutions from compartments 2 at the selected absorption maximum,  $A_\lambda$ , and calculate the concentration of the ligand from the equation  $[L]_2 = A_\lambda / (\epsilon_\lambda \times l)$  using the molar absorption coefficient, as determined in *Protocol 1*.<sup>g</sup>

<sup>a</sup> Together with the experiment several control experiments should be performed for proper measurements as detailed in Section 2.4 and in *Protocol 3*.

<sup>b</sup> Home-made apparatus of polyacrylamide or various commercial equipment can be used from, e.g. Amika Corp, Dianorm GmbH or Spectrum Medical Industries.

<sup>c</sup> The chemical properties and molecular cut-off depends upon the protein and ligand in question, e.g. cellophane or acetylcellulose with molecular cut-off at 10–20 kDa. Membranes are available from Dianorm GmbH and Spectrum Medical Industries.

<sup>d</sup> 3 cm in diameter in the present case.

<sup>e</sup> If necessary, the membranes may be boiled for 15 min, in a buffer, e.g. sodium bicarbonate in order to extract light absorbing or light scattering impurities before they are put in buffer.

<sup>f</sup> All compartments thus contain 1 ml solutions. Compartments 1 contain the protein in half of the original concentration and all the compartments contain the drug in half of the original concentrations.

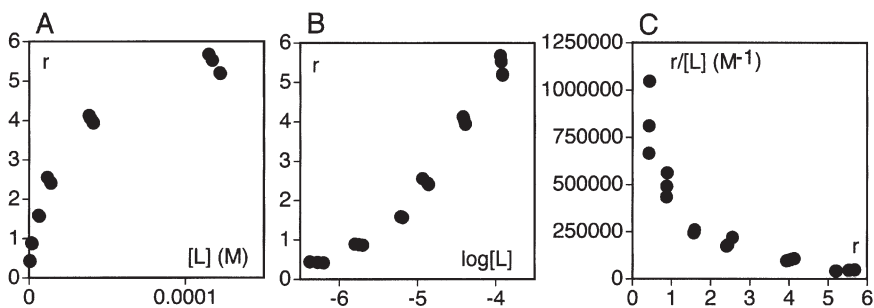
<sup>g</sup> It is important to include chambers with no protein and no ligand in order to check for leaching of light absorbing or light scattering impurities from the membrane or protein preparation during dialysis since the presence of these will give an overestimation of the free ligand concentration and thereby an underestimate of the affinity. If the problem cannot be circumvented by boiling the membranes, the ligand may be purified by HPLC before measurements.

At each measured free ligand concentration in compartment 2,  $[L]_2$ , the bound ligand concentration in compartment 1,  $[L_B]_1$ , is calculated from:

$$[L_B]_1 = 2 \times ([L_i]_2 - [L]_2) \quad [2]$$

where  $[L_i]_2$  is the initial ligand concentration present in compartment 2 at the start of the dialysis. The measured average number of bound ligand molecules per protein molecule,  $r$ , at that concentration of free ligand is then obtained from Equation 1. A binding isotherm for the specific set of ligand and protein is thus characterized by a set of values of free ligand concentrations,  $[L]$ , and their corresponding  $r$  values. From the law of mass action it follows that there is only one value of  $r$  for any given free ligand concentration no matter which protein concentration is present. The set of values obtained from the dialysis experiments together represent the experimentally determined binding isotherm for the ligand–protein interaction under the chosen conditions specified by pH, temperature, ionic strength, and buffer composition used.

The results may be plotted in several ways. The average number of bound ligand molecules per protein molecule,  $r$ , can be plotted against the free ligand concentration  $[L]$  on a linear scale (linear plot, *Figure 4A*) or on a logarithmic scale (logarithmic plot, *Figure 4B*). The latter plot gives a more even distribution of the experimental points especially at low ligand concentration. Alternatively  $r/[L]$  can be plotted versus  $r$  (Scatchard plot, *Figure 4C*). The various plots each have their advantages and disadvantages. However, all of them may be used to obtain an overview of the quality of the experimental data. The experimental reproducibility generally tends to decrease as the free ligand concentration approaches zero. This is not obvious in the linear plot (*Figure 4A*). The logarithmic plot shows this slightly better but it is seen especially in the Scatchard plot



**Figure 4** Various plots of experimental data from binding of flurbiprofen to human serum albumin measured by equilibrium dialysis. (A) A linear plot of the average number of bound ligand molecules per protein molecule,  $r$ , versus the free concentration of ligand,  $[L]$ . This plot may erroneously indicate saturation of the protein molecule with ligand at high ligand concentrations. (B) A logarithmic plot. This plot does not erroneously indicate saturation at high ligand concentrations. (C) A Scatchard plot. This plot clearly shows that at low ligand concentrations (close to the y-axis) the experimental accuracy decreases. The intercept with the y-axis gives the first stoichiometric binding constant. However, the Scatchard plot may also tend to erroneously suggest saturation of the protein molecule at high ligand concentrations. The experimental data were used with permission from ref. 6.

(Figure 4C), where the measurements tend to scatter close to the y-axis when the ligand concentration approaches zero.

## 2.4 Running control experiments for equilibrium dialysis

In order to obtain reliable measurements of the binding affinity of a ligand to a purified protein it is necessary to run several control experiments (detailed in *Protocol 3*) and to consider other sources of errors.

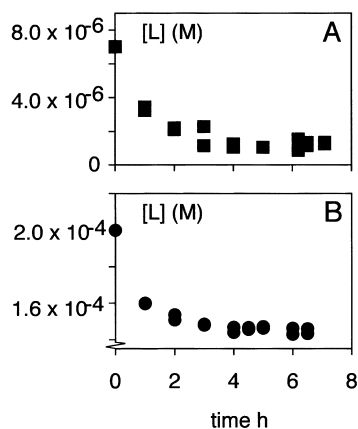
- (a) The purified protein may contain bound ligands which may interfere with binding of the ligand in question and should be removed before analysis.
- (b) Determination of the time taken for establishment of binding equilibrium in the system used.
- (c) Light absorbing or light scattering impurities may accumulate during dialysis and interfere with measurement of the ligand.
- (d) The ligand may bind to the membrane and/or chamber walls during dialysis.
- (e) Protein may leak through the dialysis membrane.
- (f) Retentate may be diluted due to the osmotic pressure from the presence of a high protein concentration.
- (g) Charged ligands may be unequally distributed across the membrane due to the Donnan effect.
- (h) Ligand may be unstable during dialysis.
- (i) Bacterial growth.

### 2.4.1 The protein may initially contain ligands that interfere with measurement of the analysed ligand

The first thing to ascertain is whether the purified protein, initially, contains the ligand that is to be analysed because this will disturb the measurements, as the total concentration of ligand will be unknown. Also, the presence of other types of ligands may interfere with the measurements since these other ligands could compete for binding with the analysed ligand. Albumin purified from serum, contains e.g. fatty acids (1) that may be removed by a defatting procedure. EF-hand  $\text{Ca}^{2+}$  binding proteins may carry  $\text{Ca}^{2+}$  as well as other divalent cations which should be removed by passage through a chelating agent like Chelex 100 (22), etc. It is up to the investigator to suggest which types of ligands the protein preparation may contain, from accumulated knowledge about the protein.

### 2.4.2 Check for establishment of binding equilibrium

A certain time is necessary for the system to establish binding equilibrium. The time needed to reach this may be determined as follows (*Protocol 3A*). A set of chambers are set up with identical concentrations of ligand and protein. The ligand concentration is measured in compartment 2 at various times after the



**Figure 5** Check for establishment of equilibrium with dialysis of phenylbutazone bound to human serum albumin. Initial concentrations of phenylbutazone in compartment 2 are  $7 \mu\text{M}$  (A, ■) and  $200 \mu\text{M}$  (B, ●), respectively. Equilibrium is established within 5–6 h. The experimental data were used with permission from ref. 6.

start of dialysis. The time chosen for dialysis should be sufficient to attain equilibrium and not significantly longer due, possibly, to difficulties with bacterial growth (see later). Figure 5 shows the results of an experiment for the binding of phenylbutazone to human serum albumin. The initial concentrations in compartments 1 and 2 were  $7 \mu\text{M}$  (Figure 5A) and  $200 \mu\text{M}$  (Figure 5B), respectively, with  $30 \mu\text{M}$  albumin at  $37^\circ\text{C}$  pH 7.4,  $66 \text{ mM}$  phosphate buffer. In this system equilibrium is established within 5–6 h. Equilibrium may be established more quickly by adding the ligand to the protein side, the retentate (23).

#### 2.4.3 Check for extraction of light absorbing or light scattering impurities from the dialysis membrane or protein preparation

During dialysis a certain degree of turbidity may accumulate in the solutions due to extraction of impurities from the dialysis membrane or from the protein preparation. Turbidity of the solution will give a certain light absorption or light scattering that disturbs the measurement of the ligand. Therefore experiments should include a few chambers with only buffer in both compartments and a few chambers with buffer and protein but no ligand in order to check that the light absorption, at the given wavelength in these compartments, is negligible compared to the absorption in the chamber with the lowest ligand concentration analysed (Protocol 3B). If the absorption, however, is not negligible try to extract the impurities from the dialysis membrane by boiling it in a buffer. However, the impurities may also come from the protein sample and it may thus be necessary to purify the solution in compartment 2 after dialysis and before measurement of the ligand, e.g. by HPLC. Otherwise the ligand can be measured by scintillation counting if available in radiolabelled form (see Section 2.5).

#### 2.4.4 Check for binding of the ligand to chamber walls and membrane

Test whether the ligand binds to the chamber walls or to the membrane (24) by introducing known concentrations of ligand into the compartments with mounted membrane and dialyze for the same time as in the actual experiment. At the end of dialysis the concentration of ligand measured in the compartments should be the same, within experimental limits, as introduced into the compartments (*Protocol 3C*).

#### 2.4.5 Check for protein leakage through the membrane

It is important to check the dialysis membrane for its ability to retain the protein in compartment 1. Leakage of only a few per cent of the protein may lead to significant disturbances. To test for leakage, known concentrations of protein are introduced into compartment 1 and the concentration in compartments 1 and 2 are measured after dialysis (*Protocol 3D*). Measurements can be done by UV light absorption around 280 nm or in the case of turbid samples by using a dye binding assay (25, 26).

#### 2.4.6 Osmotic dilution of retentate

Osmosis may be a problem if high concentrations of protein are used due to volume changes during dialysis (27–29). This may be monitored by measuring the concentration of protein in the retentate at various times. If osmosis is a problem the protein concentration will decrease during dialysis. Lowering the protein concentration may help to solve the problem. Theoretical expressions that correct for osmotic dilution effects have been published (3).

#### 2.4.7 Donnan effects

In general proteins possess a certain net charge at the given pH at which the experiment is performed. As an example albumin has about 20 negative charges at pH 7.4 (30). This will give an unequal distribution of a charged ligand across the membrane due to the Donnan equilibrium. At an albumin concentration of 30  $\mu\text{M}$  in a 66 mM sodium phosphate buffer the ratio between the concentrations in each of the compartments for a ligand with one negative charge is 1.002. This unequal distribution will increase with increasing protein concentration and decreasing buffer concentration. Thus, the Donnan effect may be minimized by lowering the protein concentration, increasing the buffer concentration or, if possible, by changing the pH to values near the isoelectric point of the protein. Expressions that correct for the Donnan effect have been presented (3). The magnitude of the Donnan effect may be measured by analysing the distribution of a charged ligand that is known not to bind to the protein.

#### 2.4.8 Instability of ligand during dialysis

It is important that the ligand is stable under the conditions of the experiment. Instability may be a problem with ligands that contain several conjugated double

bonds as these compounds can be unstable in daylight. Protection from light may solve the problem. Usually structures with phenol rings like those in, e.g. many anti-inflammatory drugs are stable (6). Some ligands may participate in chemical reactions e.g. acetylsalicylic acid (aspirin), which acetylates albumin (31) and thereby is converted to salicylic acid during dialysis.

#### 2.4.9 Bacterial growth

Bacterial growth may be a problem if dialysis is performed for a very long time at 37 °C. However, the system can be checked for the time taken to adjust to equilibrium and the shortest possible time compatible with achievement of equilibrium should be chosen. This time may be 5–6 h where bacterial growth usually presents no major problem. Otherwise the solution can be sterilized by filtration or antibiotics may be added, e.g. gentamicin or kanamycin (32). In the latter case, check that the antibiotic does not interfere with binding of the ligand.

### Protocol 3

#### Control experiments for equilibrium dialysis using spectroscopic measurement of the ligand

##### Equipment and reagents

- See *Protocol 2*

##### A Check for time to obtain binding equilibrium

- 1 Set up several dialysis chambers with the highest and lowest ligand concentrations used in the experiment and stop the dialysis at various time intervals, e.g. at each hour for 6–8 h.
- 2 Measure the ligand concentration in compartments 2 as described in *Protocol 2*.
- 3 Plot the ligand concentrations in compartments 2 *versus* time. Equilibrium is established when the concentration does not change further with time. It is important to note whether turbidity interferes with the measured absorbances.

##### B Check for extraction of light absorbing or light scattering substances from the membrane or protein preparation during dialysis (turbidity)

- 1 Set up a few chambers with pure buffer in compartments 1 and 2 and a few chambers with protein present in compartments 1 at the same concentration as used in the experiments and buffer in compartments 2.
- 2 Dialyse for the same time as used for the main experiment (as determined above, *Protocol 3A*).
- 3 Measure the absorption,  $A_{\lambda}$ , in compartments 2 and compare them with the measured absorbances obtained in the main experiment.<sup>a</sup>

**Protocol 3** continued**C Check for binding of ligand to chamber walls and membrane**

- 1 Dilute the ligand to appropriate concentrations, e.g. the same concentrations as used in the experiments (*Protocol 2*).
- 2 Set up dialysis experiments as described in *Protocol 2* with 0.5 ml ligand present in all compartments 1 and 2 and 0.5 ml pure buffer introduced into all compartments 1 and 2. No protein is present. Dialysis is performed for the same time as in the experiment (as determined above, *Protocol 3A*).
- 3 Measure the ligand concentrations in compartments 2 after dialysis. Ideally they should be the same as introduced into the compartments.

**D Check for protein leakage through the membrane**

- 1 Set up chambers with a known protein concentration in compartments 1 and pure buffer in compartments 2.
- 2 Measure the protein concentration in both compartments after dialysis for the time used in the experiments. Measurement of the protein concentration may be performed by UV light absorption spectroscopy near 280 nm; or for turbid samples, by using a protein assay kit based on dye binding as described by, e.g. Lowry (25) or Bradford (26). The protein concentration in compartment 2 after dialysis should be negligible.

<sup>a</sup>The contribution to the absorbance from the turbid solution should be negligible otherwise it is necessary to purify the ligand by HPLC or if possible extract impurities from the membrane before dialysis by boiling the membranes in a buffer.

## 2.5 Measurement of ligand concentration by using a radiolabelled ligand

Spectroscopic measurement of the ligand concentration can only be used when the ligand does contain light absorbing structures or reacts with a substance that gives a compound that may be spectroscopically determined. If this is not the case another way of measuring the ligand concentration should be used. If the ligand can be obtained radiolabelled the concentration can be measured by scintillation counting. The use of radiolabelled ligands possesses advantages as well as disadvantages. First of all it is very important that the radiochemical purity of the ligand is as high as possible. The concentrations of free and bound ligand may then be measured by using the following relationships:

$$[L_F] = \frac{A_2}{A_1 + A_2} [L_T] \text{ and } [L_T] = [L_i]_1 + [L_i]_2 \quad [3]$$

$$[L_B] = \frac{A_1 - A_2}{A_1 + A_2} [L_T] \text{ and } [L_T] = [L_i]_1 + [L_i]_2 \quad [4]$$

where  $A_1$  and  $A_2$  are the measured radioactivities in compartments 1 and 2 and  $[L_i]_1$  and  $[L_i]_2$  are the initial concentrations introduced into compartments 1 and

2, respectively. In the case of very high affinity ligands it is advisable to check the results at low concentrations of ligand with rate dialysis since this technique is especially suited for analysing high affinity ligands (see Section 3).

## Protocol 4

### Setting up dialysis experiments with a radiolabelled ligand

#### Equipment and reagents<sup>a</sup>

- Scintillation counter
- Scintillation liquid
- Radiolabelled ligand<sup>b</sup>
- Chromatographic equipment for purification of radiolabelled ligand<sup>c</sup>

#### Method

- 1 Set up dialysis experiments as described in *Protocol 2*, steps 1–8. Add a trace amount of the radiolabelled substance to the ligand solution. Enter the protein and ligand into the chambers as described in *Protocol 2*, steps 5–7. Dialysis is allowed to proceed for the time necessary to achieve equilibrium.
- 2 Withdraw equal volumes, e.g. 0.8 ml from each of the compartments at the end of dialysis and place in scintillation vials. Make a background sample by using 0.8 ml of buffer.
- 3 Add 2 ml of scintillation fluid to each sample and to the background.
- 4 Count each sample until the standard deviation is at the desired level, e.g. below 1%, i.e. with total count figures above 10 000.<sup>d</sup>
- 5 Subtract the background from each measurement and calculate the free and bound ligand concentrations from Equations 3 and 4.

<sup>a</sup>Dialysis chambers, dialysis membranes, buffer solution, protein solution, ligand solution, and thermostatted equipment are described in *Protocol 2*.

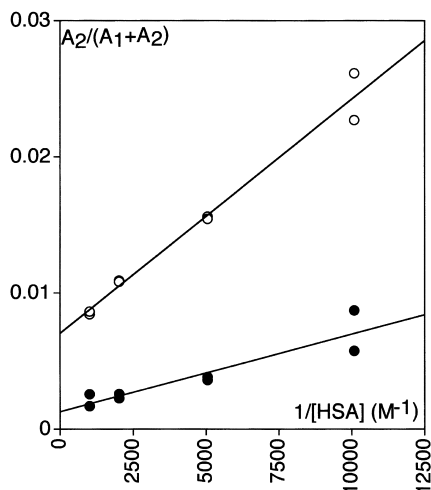
<sup>b</sup>If dissolved in an organic solvent this should be removed before use, e.g. under a stream of nitrogen, and the ligand can then be redissolved in the appropriate buffer.

<sup>c</sup>In order to obtain good results with equilibrium dialysis it is extremely important that the substance is very pure. The necessary purity depends upon the affinity. For high affinity ligands a purity around 98–99% may not be sufficient. The presence of unbound radiochemical impurities may be checked by dialysing the radiolabelled substance in the presence of increasing protein concentrations as described in Section 2.5.1. For purification thin layer chromatographic procedures as well as column chromatographic procedures may apply but this depends on the specific substance used. If purification is difficult or impossible the rate dialysis technique is highly recommended (see Section 3).

<sup>d</sup>Check that the quenching of radioactivity is equal in compartment 1 and 2 by counting equal amounts of radioactivity in the presence and absence of protein. If unequal quenching occurs the count figures should be corrected accordingly.

### 2.5.1 Control experiments for equilibrium dialysis with a radiolabelled ligand

The control experiments performed with a radiolabelled ligand should be similar to those described for a light absorbing ligand in *Protocol 3*. With equilibrium dialysis using a radiolabelled ligand it is very important to determine the radiochemical purity of the ligand and purify it if necessary, although this may be difficult (33). Even a few per cent of a radiochemical impurity may be detrimental for the analysis of high-affinity ligands. If a given ligand is 99% bound to a protein under a given set of concentrations even 1-2% of radiochemical impurity may surpass the low free ligand concentration unless the impurity binds very strongly to the protein (10). In such cases purification of the ligand is necessary in order to obtain reliable results by equilibrium dialysis. This may be done by chromatographic procedures, the nature of which depends on the specific type of substance that is measured. The amount of unbound radiochemical impurity can be checked by dialysing the radiolabelled ligand from compartment 1 to compartment 2 in the presence of varying but high concentrations of the protein in each of the compartments in order to avoid errors occurring from osmotic dilution and Donnan equilibria. At infinitely high protein concentration the amount of dialysed radiolabelled ligand should be zero. Any dialysis at this high protein concentration occurs from dialysis of an unbound radiolabelled impurity. *Figure 6* sets out an example using dialysis of decanoate with human serum albumin present in each of the chambers. A



**Figure 6** Measurement of unbound radiochemical impurity of radiolabelled decanoate. Dialysis is performed for a given time at various high concentrations of protein (human serum albumin, HSA) present in each compartment. At infinitely high protein concentration a fraction (0.7%) has dialysed from compartment 1 to compartment 2 (○) and since a similar fraction is present in compartment 1 the total amount of impurity is 1.4%. After chromatographic purification the impurity amounts to 0.3% (●). The experimental data were used with permission from refs 10 and 13.

fraction of 0.7% has dialysed into compartment 2 at infinitely high albumin concentration. An equal amount is present in compartment 1 so that the total amount of unbound impurity accounts for 1.4% of the total radioactivity. After purification of the substance by thin layer chromatography the impurity accounts for 0.3% of the total. In such a case it is better to use the rate dialysis technique, Section 3.

### 3 Rate dialysis

#### 3.1 Principle and theory

Although equilibrium dialysis is a sound technique for measuring binding of ligands the technique may give false results. It fails, especially when high affinity ligands are examined since the free ligand concentration approaches zero with increasing affinity of the ligand. With the use of radiolabelled ligands the presence of radiochemical impurities is a serious problem. Furthermore, the sources of errors occurring from osmosis and Donnan effects may become troublesome at high protein concentrations. In these cases another excellent dialysis procedure may be resorted to: rate dialysis (4–13) also called symmetric dialysis (8, 9). With this technique errors from osmosis and Donnan effects are avoided and errors due to the presence of radiochemical impurities are also minimized (10). Finally, the reproducibility at low ligand concentrations is better since with this technique we do not measure the free ligand concentration directly but a parameter that is proportional to the free ligand concentration, i.e. the dialysis rate of the free ligand. The technique uses the same equipment as used for equilibrium dialysis. The only difference is that in rate dialysis, solutions with identical concentrations of protein and ligand are introduced into both compartments with the radioactive ligand initially present only in compartment 1. Binding equilibrium is thus established in both of the compartments before dialysis has begun. The dialysis of the ligand is allowed to proceed for a given time such that it is insufficient for establishment of equal radioactivity in the compartments. The dialysis is then stopped and the radioactivity present in each of the compartments is measured.

Consider firstly, dialysis of a radioactive ligand in the system without protein present. *Figure 7* shows the dialysis of decanoate in this system. The ligand dialyses strictly according to a first order process. We may thus express the concentration difference between compartment 1 and 2 in the following way:

$$\frac{d([L^*_{T1}] - [L^*_{T2}])}{dt} = -k \times ([L^*_{T1}] - [L^*_{T2}]) \quad [5]$$

where  $[L^*_{T1}]$  and  $[L^*_{T2}]$  are the total radioactive ligand concentrations in compartments 1 and 2, respectively. If we presume that, in the presence of a protein, the equilibrium between the free ligand and the protein bound ligand is adjusted much faster than the exchange of ligand between compartments 1 and 2

and that the dialysis rate constant of the ligand,  $k$ , is independent of the concentration of the protein, we have the following expressions:

$$\frac{[L]}{[L_T]} = \frac{[L]_1}{[L_T]_1} = \frac{[L^*]_1}{[L^*_T]_1} = R \wedge \text{for } t > 0 \quad \frac{[L^*]_2}{[L^*_T]_2} = R \quad [6]$$

where  $[L^*]_1$  and  $[L^*]_2$  are the free radioactive ligand concentrations in compartments 1 and 2, respectively. In the presence of a protein the dialysis of the radioactive ligand is then presumed to follow a first order process dependent upon the concentration difference between the free radioactive ligand concentrations in compartments 1 and 2:

$$\frac{d([L^*_T]_1 - [L^*_T]_2)}{dt} = -k \times ([L^*]_1 - [L^*]_2) \quad [7]$$

By entering the relations described in Equations 6 we have,

$$\frac{d([L^*_T]_1 - [L^*_T]_2)}{dt} = -k \times R \times ([L^*_T]_1 - [L^*_T]_2) \quad [8]$$

By integrating Equation 8 in the time interval from start of dialysis  $t = 0$  to end of dialysis  $t = t$  we obtain:

$$\int_{([L^*_T]_1 - [L^*_T]_2)_{t=0}}^{([L^*_T]_1 - [L^*_T]_2)_{t=t}} \frac{1}{([L^*_T]_1 - [L^*_T]_2)} d([L^*_T]_1 - [L^*_T]_2) = \int_{t=0}^{t=t} -k \times R dt \quad [9]$$

Since the radioactive difference between the two compartments at  $t = 0$  may be calculated from the sum of radioactivities at the end of dialysis we then have the following relation for the dialysis:

$$\frac{[L]}{[L_T]} = \frac{1}{k \times t} \times \ln \frac{[L^*_T]_1 - [L^*_T]_2}{[L^*_T]_1 + [L^*_T]_2} \quad [10]$$

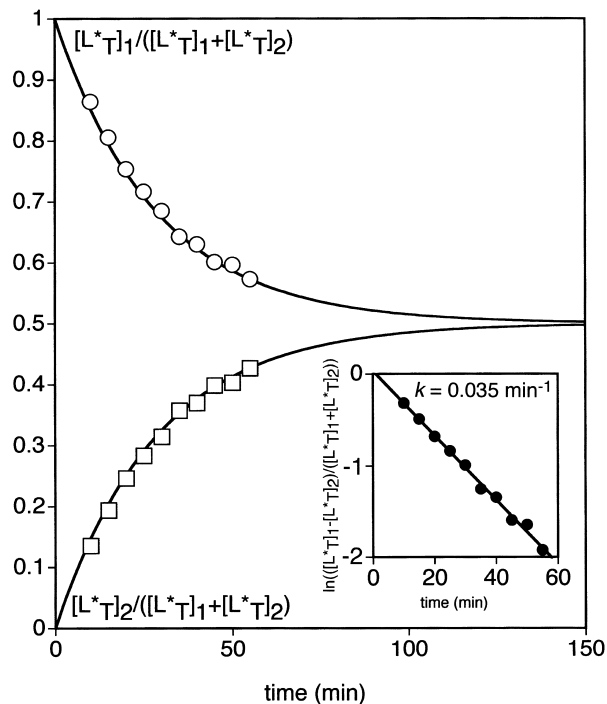
When  $A_1$  and  $A_2$  represent the radioactive counts in equal sized samples from compartments 1 and 2, respectively Equation 10 may be written as:

$$\frac{[L]}{[L_T]} = -\frac{1}{k \times t} \times \ln \frac{A_1 - A_2}{A_1 + A_2} \quad [11]$$

In order to determine the rate constant,  $k$ , we have that in the absence of a binding protein  $[L]$  is equal to  $[L_T]$ . Equation 11 then reduces to:

$$\ln \frac{A_1 - A_2}{A_1 + A_2} = -k \times t \quad [12]$$

By plotting the left side of Equation 12 on the y-axis *versus* time on the x-axis we should obtain a straight line with the slope  $-k$ . The inset in *Figure 7* shows the measurements obtained with dialysis of decanoate in the dialysis chambers as described in *Figure 3*. In this system decanoate dialyses with a rate constant of  $0.035 \text{ min}^{-1}$ . *Protocol 5* describes how  $k$  is measured. Alternatively, some authors standardise their experiment by results from equilibrium dialysis (9).



**Figure 7** Rate dialysis. With no protein present radiolabelled decanoate is initially introduced into compartment 1 and is allowed to dialyse into compartment 2 with no protein present. The relative concentrations of radioactive ligand present in compartment 1,  $[L^*T]_1 / ([L^*T]_1 + [L^*T]_2)$  ( $\circ$ ), and in compartment 2,  $[L^*T]_2 / ([L^*T]_1 + [L^*T]_2)$  ( $\square$ ), are measured at various times. The concentration of radioactive ligand present in each compartment versus time strictly follows exponential functions (solid lines). The rate constant for the process may be obtained by plotting the natural logarithm to the relative concentration difference between the compartments *versus* time (shown in the inset). The rate constant,  $k$ , as obtained from the slope of the regression line is  $0.035 \text{ min}^{-1}$ . The experimental data were used with permission from ref. 10.

## Protocol 5

### Determination of the dialysis rate constant, $k$ , for the ligand

#### Equipment and reagents

- Dialysis chambers and membranes as in Protocol 2
- Scintillation counter
- Radioactive ligand dissolved in appropriate buffer
- Buffer: 66 mM sodium phosphate buffer pH 7.4 or another appropriate for the experiments
- Scintillation liquid

**Protocol 5** continued**Method**

- 1 Incubate solutions with radioactive ligand and buffer at the appropriate temperature.
- 2 Mount the buffer-soaked and lightly dried membranes in the dialysis chambers.
- 3 Introduce all solutions with radioactive labelled ligand into compartments 1 and incubate the chambers in the air thermostat at the rotating plate for temperature equilibration.
- 4 Introduce temperature equilibrated buffer into all compartments 2. The specific time for introduction into each chamber is noted. In practice the chambers may be started with intervals of 1 min. Several chambers are set up.
- 5 Allow dialysis to proceed for various time intervals. Stop the dialysis by pipetting the solutions from compartments 1 and 2 into scintillation vials.<sup>a</sup>
- 6 Stop chambers with 5 or 10 min intervals depending on the nature of the ligand and the system used.
- 7 Add 2 ml scintillation fluid to each vial and count the radioactivity in a scintillation counter until the standard deviation of the count figures is at the desired level, e.g. below 1%, i.e. total count figures above 10 000.
- 8 Subtract the background radioactivity from each count figure.  $A_1$  represents the measurements from compartments 1 while  $A_2$  represents the measurements from compartments 2.
- 9 Plot  $\ln((A_1 - A_2)/(A_1 + A_2))$  versus time. A straight line should be obtained. Determine the slope of the line,  $-k$ , by linear regression. The dialysis rate constant is then obtained by multiplying by  $-1$ .

<sup>a</sup> If 1 ml volumes are present in either compartment it may be convenient to pipette a smaller volume for counting, e.g. 0.8 ml. The same volume should be used from both sides. Use also 0.8 ml of buffer to count as background.

### 3.2 Running experiments using rate dialysis

For rate dialysis, experiments are set up in virtually the same manner as used for equilibrium dialysis. In one set of experiments similar concentrations of protein may be present in each compartment and the concentration of ligand can vary. Before dialysis a trace amount of radioactivity is added to the solutions intended for compartments 1. Optionally a similar concentration of unlabelled substance may be added to the solutions intended for compartments 2. The time of dialysis is an important parameter. It may be necessary to run pilot experiments in order to determine which time should be chosen. Several factors should be taken into consideration. The dialysis time should be sufficiently long so that the time of dialysis can be accurately measured. Also, if a radiochemical impurity is present it is advisable to dialyse a certain fraction of radioactivity before stopping the experiment otherwise the impurity may account for too

large a fraction of the dialysed substance (10). It is reasonable to dialyse a fraction of more than about 0.2 of the initial radioactivity present in compartment 1. On the other hand the dialysis should not proceed for such a long time that the radioactive concentrations in each compartments are close to being equal. As a rule of thumb the dialysis should be stopped before a fraction of about 0.3 of the radioactivity initially present in compartment 1 has dialysed to compartment 2. *Protocol 6* outlines the details of the procedure.

## Protocol 6

### Setting up a ligand binding experiment using rate dialysis

#### Equipment and reagents

- Dialysis chambers and membranes as in *Protocol 2*
- Radioactive ligand dissolved in appropriate buffer
- Protein dissolved in buffer at known concentration
- Buffer: 66 mM sodium phosphate buffer pH 7.4 or another appropriate for the experiments
- Ligand dissolved in buffer at various known concentrations

#### Method

- 1 Make solutions with varying concentrations of ligand. It may be convenient to have a fixed protein concentration and then vary the ligand concentration. For each experimental point more than 2 ml solutions should be made, e.g. 2.5 ml.
- 2 Split the solutions from step 1 in two tubes with 1.2 ml in each tube.
- 3 Add a trace amount of radioactive ligand to all the solutions for compartments 1.
- 4 Add a similar volume with a similar amount of unlabelled ligand to the solutions for compartments 2.<sup>a</sup>
- 5 Incubate the solutions at the temperature at which the measurements are undertaken.
- 6 Mount a set of dialysis chambers with dialysis membranes exactly as described for equilibrium dialysis, *Protocol 2*.
- 7 Introduce all solutions with radiolabelled ligand into compartments 1 and mount the cells on the rotating plate in the thermostatted cabinet. Allow an equilibration time of about 10 min.
- 8 Initiate the dialysis in a given chamber by introducing the corresponding solution for compartment 2. The dialysis in various chambers may be started with 1 min intervals.
- 9 Dialyse a fraction of between about 0.2 and 0.3 of the radioactivity from compartment 1 into compartment 2.<sup>b</sup>
- 10 Stop the dialysis by pipetting similar volumes, e.g. 0.8 ml from compartment 1 and compartment 2 into scintillation vials. Make a background with 0.8 ml of buffer.

**Protocol 6** continued

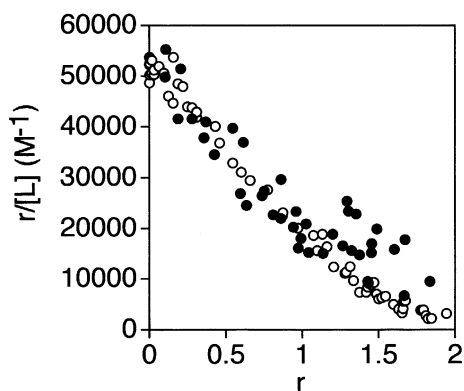
- 11** Add 2 ml scintillation solution to each vial and count the radioactivity in a scintillation counter until the standard deviation of the count figures are at the desired level, e.g. below 1%, i.e. total count figures above 10 000.
- 12** Subtract the background radioactivity from each count figure.  $A_1$  represents the measurements from compartments 1 while  $A_2$  represents the measurements from compartments 2.
- 13** Calculate the free ligand concentrations in each experiment from Equation 11 where the total ligand concentration, the rate constant, and the time of dialysis are known. Also calculate the bound ligand concentration  $[L_B]$  by subtracting the free from the total ligand concentration and calculate the average number of bound ligand molecules per protein molecule,  $r$ , by dividing with the total protein concentration.
- 14** Plot the results in one of the plots that shows the relation between  $[L]$  and  $r^c$ .

<sup>a</sup>This is especially important if the amount of added radioactive ligand is more than just a trace amount. For each experimental point we thus have two tubes containing identical concentrations of ligand and protein. However, one of the tubes contain the ligand radioactively labelled whereas the other contains the ligand unlabelled.

<sup>b</sup>The time for the dialysis thus vary with the given set of conditions used and can only be estimated from pilot experiments. In general, at the same protein concentration, the dialysis times should be longer for lower ligand/protein ratios.

<sup>c</sup>If points scatter seriously at low ligand concentrations (especially seen in a Scatchard plot) it may be necessary to run experiments at a higher protein concentration.

Rate dialysis inherently possesses significant advantages compared with equilibrium dialysis as previously mentioned. In order to obtain more precise determinations the protein concentration may be increased as seen in *Figure 8*.



**Figure 8** Binding of hexanoate to human serum albumin determined by rate dialysis. The concentration of human serum albumin is 300  $\mu\text{M}$  (○) and 30  $\mu\text{M}$  (●), respectively. The reproducibility increases with the increasing protein concentration. The experimental data were used with permission from ref. 13.

The increase in protein concentration does not affect the other sources of errors otherwise affecting equilibrium dialysis, i.e. osmosis and Donnan equilibria.

### 3.3 Rate dialysis in microchambers

Rate dialysis may also be performed in microchambers using 50  $\mu\text{l}$  of solution for one experiment, i.e. 25  $\mu\text{l}$  of solution for each compartment. Dialysis equipment suitable for this has been previously described (34). The principle is exactly the same as used for the larger chambers *Figure 3* and *Protocol 6*. Solutions identical with respect to concentrations are introduced into compartments 1 and 2 and only the solution intended for compartment 1 contains the ligand radiolabelled. The procedure used for these chambers is described in *Protocol 7*. The solutions are not stirred but remains stationary and the chamber geometry is different from the larger dialysis chambers. This means that the dialysis in practice proceeds slightly different from that in the chambers described in *Figure 3*. Empirically, the dialysis of the ligand in the microchambers may be described by a modification of Equation 11 (6):

$$\frac{[L]}{[L_T]} = - \frac{1}{k \times (t + t_0)} \times \ln \frac{A_1 - A_2}{A_1 + A_2} \quad [13]$$

Apparently the dialysis proceeds strictly according to a first order process with a time shift,  $t_0$ , that depends upon the procedure of filling, withdrawal, and rinsing of chambers and varies with the substance dialysed (6).

## Protocol 7

### Setting up rate dialysis using microchambers

#### Equipment and reagents

- Microdialysis chambers (25  $\mu\text{l}$  volumes on either side of the membrane) and microdialysis apparatus
- Radioactive ligand dissolved in appropriate buffer
- Protein dissolved in buffer at known concentration
- Buffer: 66 mM sodium phosphate buffer pH 7.4 or another appropriate for the experiments
- Ligand dissolved in buffer at various, known, concentrations

#### Method

- 1 Mount the dialysis membranes in microdialysis chambers and introduce the appropriate buffer into each of the compartments.
- 2 Make solutions, essentially as described in *Protocol 6*.<sup>a</sup>
- 3 Withdraw buffer from each of the compartments before dialysis by pipetting. The residue of buffer is removed with a strip of filter paper.

**Protocol 7** continued

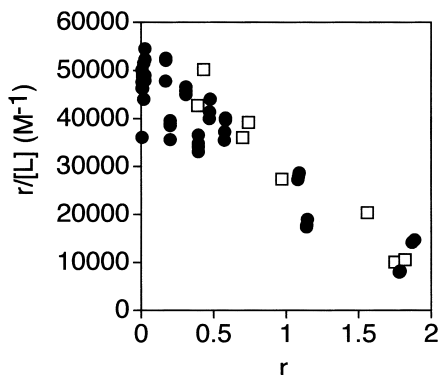
- 4 Introduce the solution intended for compartment 1 into the compartment and allow it to equilibrate with the membrane for 1-1.5 min.
- 5 Start the dialysis by introducing the solution for compartment 2 into the compartment.
- 6 Allow dialysis to proceed for a given time, e.g. 10-20 min and stop the dialysis by withdrawing the solution from compartment 1 first. Note the exact time. Rinse the chamber once by introducing and withdrawing buffer. The buffer is combined with the first solution taken out. Then withdraw the solution from compartment 2 and introduce buffer for 1 min into compartment 2 for extraction of the radioactivity in the membrane. Withdraw the buffer from compartment 2 and combine it with the first solution taken out from compartment 2.
- 7 Add 2 ml of scintillation solution to each of the vials and count the radioactivity by scintillation counting. Also establish a background by counting on equal volume of buffer and scintillation solution.
- 8 Subtract the background radioactivity from each count figure.  $A_1$  represents the measurements from compartments 1 while  $A_2$  represents the measurements from compartments 2.
- 9 Calculate the free ligand concentration from Equation 13 by using the values of  $k$  and  $t_0$  as determined below.
- 10 Measure the rate constant for dialysis of the ligand without protein present by dialysing the radioactive ligand at various times with no protein present. Plot  $\ln((A_1 - A_2)/(A_1 + A_2))$  versus time. A straight line should be obtained. Determine the slope of the line,  $-k$ , by linear regression. The dialysis rate constant is then obtained by multiplying by  $-1$ . From the regression line  $t_0$  may be determined from the intercept with the x-axis. This intercept is around  $-1$  min or  $-2$  min. The numerical value used is  $t_0$ .

<sup>a</sup> One determination requires 50  $\mu\text{l}$ , i.e. 25  $\mu\text{l}$  for each compartment. Make well beyond 25  $\mu\text{l}$  per compartment for one determination, e.g. 100  $\mu\text{l}$ .

In practice this microchamber method has proven its value. *Figure 9* shows results for dialysis of salicylate by rate dialysis in microchambers (*Protocol 7*) compared with results from equilibrium dialysis performed in the macrochambers (*Protocol 2*). As can be seen there is very good agreement between the techniques under conditions where both of the techniques work.

#### **4 Rate dialysis for measurement of free ligand concentrations in complex mixtures, e.g. serum**

In many cases the biologically active form of a given drug (35) or a hormone (36) is the non-protein bound concentration rather than the total concentration. For



**Figure 9** Comparison of salicylate binding to human serum albumin. Binding experiments were performed with rate dialysis in microchambers using protein concentrations between 100–1000  $\mu\text{M}$  (●) and with equilibrium dialysis in macrochambers using 30  $\mu\text{M}$  protein (□). The experimental data were used with permission from ref. 6.

monitoring drug therapy or for analysis of hormone action it is therefore reasonable to measure the free concentration rather than the total concentration in serum. Such measurements are usually performed by equilibrium dialysis where the serum sample is dialysed against a buffer. However, as previously stated equilibrium dialysis inherently possesses some major methodological problems.

- (a) The high concentration of proteins in serum gives an osmotic pressure that may lead to dilution of the serum sample.
- (b) The high protein concentration is the basis for an unequal distribution of charged ligands due to the Donnan equilibrium.
- (c) Ligands that may compete with or even increase the protein binding of the tested ligand are dialysed away from the serum sample and could thus change the binding of the ligand in the serum sample.
- (d) Low free ligand concentrations equate with high affinity ligands. Low free ligand concentrations are usually difficult to measure due to a bad signal/noise ratio.

Of these points (a) has been dealt with thoroughly (27–29, 37–39) and expressions that correct for the errors in (a) as well as in (b) have been presented (3). However, the problems mentioned in (c) and (d) are impossible or difficult to deal with. With the availability of rate dialysis all of the problems listed are circumvented or minimized since the same concentration of protein is present in both compartments, i.e. both compartments contain the same osmotic pressure with no osmotic dilution of the serum sample. Likewise the effect of the Donnan equilibrium is omitted so that a charged ligand will be equally distributed between the two compartments. Interacting ligands will remain in the serum and not be dialysed away. Finally, measurements of high affinity ligands

can be performed more conveniently since the dialysis time within a certain frame is flexible and can be prolonged.

For measurements in serum a macro- as well as a micromethod may be applied. The principle is the same as used for measurements in pure buffer as detailed in *Protocol 6* for macrochambers and in *Protocol 7* for microchambers. The radioactive ligand may be added to the serum sample without dilution by entering the radioactive ligand, which usually is dissolved in an organic solvent like ethanol, in a test-tube and then evaporate the solvent. Serum is added to the test-tube and equilibrated for a few hours and then used for dialysis. The dialysis times depend upon the binding strength. A fraction of about 0.2–0.3 of the amount of radioactivity initially present in compartment 1 should be dialysed for the reasons given previously (see Section 3.2). The rate constant of the dialysed substance should also be measured by dialysing the substance various times in pure buffer without protein present. It is then possible to determine the fraction of free ligand from Equation 11 (macromethod) or Equation 13 (micromethod). Alternatively, the measurements may be standardized with analysis using equilibrium dialysis (9) or by measuring standard solutions with known concentrations of free and bound ligand (5, 11). If measured, the total ligand concentration may be multiplied with the fraction of free ligand and thereby give the concentration of free ligand. *Protocol 8* describes the procedure for the macrochambers and microchambers.

## Protocol 8

### Measurement of free ligand concentrations in serum using rate dialysis

#### Equipment and reagents

- Dialysis chambers as in *Protocol 2* or microchambers and apparatus as in *Protocol 7*
- Membranes as in *Protocol 2* and *Protocol 7*
- Radioactive ligand in organic solvent
- Buffer: 66 mM sodium phosphate buffer pH 7.4
- Scintillation counter
- Scintillation liquid
- Serum sample

#### A Preparation of serum sample

- 1 Place aliquots of the radioactive ligand into small test-tubes and evaporate the organic solvent over a stream of nitrogen.
- 2 Introduce the serum sample into the tube and incubate for a few hours at 37 °C under gentle shaking. For the macromethod use 1.2 ml per analysis, for the micromethod use well beyond 25  $\mu$ l, e.g. 100  $\mu$ l per analysis. A similar volume of serum sample without radioactive ligand is incubated as well at 37 °C.

**Protocol 8** continued**B Macromethod**

- 1 Mount the buffer-soaked and lightly dried membranes in the dialysis chambers.
- 2 Introduce serum samples with radioactive labelled ligand into compartments 1 and incubate the chambers in the air thermostat at the rotating plate for temperature equilibration.
- 3 Introduce the corresponding temperature equilibrated serum sample into compartment 2. The specific time for introduction is noted. In practice for analysis of several serum samples the chambers may be started with intervals of 1 min.
- 4 Allow dialysis to proceed for a specific time interval. Preferably a fraction of between 0.2-0.3 should be dialysed.
- 5 Stop the dialysis by pipetting serum from compartments 1 and 2 into scintillation vials. If 1 ml volumes are present in either compartment it may be convenient to pipette a smaller volume for counting, e.g. 0.8 ml. The same volume should be used from both sides. Make a sample of 0.8 ml serum for background counting.
- 6 Add 2 ml scintillation fluid to each vial and count the radioactivity in a scintillation counter until the standard deviation of the count figures is at the desired level, e.g. below 1%, i.e. total count figures above 10 000.
- 7 Subtract the background radioactivity from each count figure.  $A_1$  represents the measurements from compartments 1 while  $A_2$  represents the measurements from compartments 2.
- 8 Calculate the fraction of free ligand,  $[L]/[L_T]$ , from Equation 11 using the dialysis times and the rate constant.<sup>a</sup>
- 9 Measure the total ligand concentration by an appropriate method for the given ligand.
- 10 Calculate the free ligand concentration by multiplying the fraction of free ligand with the total ligand concentration.

**C Micromethod**

- 1 Mount the dialysis membranes in microdialysis chambers as described in *Protocol 7*, steps 1-3.
- 2 Introduce the radioactive serum intended for compartment 1 into the compartment and allow it to equilibrate with the membrane for 1-1.5 min.
- 3 Start the dialysis by introducing the corresponding serum sample for compartment 2 into the compartment.
- 4 Allow dialysis to proceed for a given time, e.g. 10-20 min and stop the dialysis by withdrawing the solution from compartment 1 first. Note the exact time. Rinse the chamber once by introducing and withdrawing buffer. The buffer is combined with the first solution taken out. Then withdraw the solution from compartment 2 and introduce buffer for 1 min into compartment 2 for extraction of the radioactivity in

**Protocol 8** continued

the membrane. Withdraw the buffer from compartment 2 and combine it with the first solution taken out from compartment 2. Then add 2 ml of scintillation solution to each of the vials and count the radioactivity by scintillation counting. A background is also counted.

- 5 Subtract the background radioactivity from each count figure.  $A_1$  represents the measurements from compartments 1 while  $A_2$  represents the measurements from compartments 2.
- 6 Calculate the fraction of free ligand,  $[L]/[L_T]$ , from Equation 13 using the dialysis times, the rate constant, and  $t_0$ .<sup>b</sup>
- 7 Measure the total ligand concentration by an appropriate method for the given ligand.
- 8 Calculate the free ligand concentration by multiplying the fraction of free ligand with the total ligand concentration.

<sup>a</sup>The dialysis rate constant can be measured as described in *Protocol 5* for macrochambers.

<sup>b</sup>The dialysis rate constant and  $t_0$  can be measured as described in *Protocol 7* for microchambers.

By using rate dialysis it is thus possible to measure the fraction of free ligand,  $[L]/[L_T]$ , or the free concentration of ligand,  $[L]$ , from *Protocol 8* in serum samples without having to take care of osmotic dilution and Donnan effects.

## Acknowledgements

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