6

METHODOLOGICAL PROBLEMS IN DRUG-BINDING STUDIES

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HISTORICAL REMARKS

When the clinician Bennhold, who can be called the "father of protein binding," performed his first experiments in protein binding about 60 years ago, he used a rather curious method. He put a warm liquid gelatin solution in test tubes. After the gelatin solidified, he added a small amount of a dye-stuff, dissolved either in water or in plasma, to the top of the gelatin layer. Several hours later, he observed how far the dye-stuff had penetrated the gelatin and used this as a criterion to estimate how much of the dye-stuff was bound to the plasma.

Figure 6-1 is an original illustration from a publication of Bennhold (Bennhold, Kylin, and St. Rusznyak 1938). The test tube on the left contains the yellow dye-stuff dissolved in water at the top of the gelatin layer. In the center, the same dye-stuff was dissolved in the plasma of a patient with renal failure. The upper horizontal lines indicate the border between gelatin and dye-stuff solution. The lines marked with roman numerals indicate the zones of diffusion of the dye-stuff after 24, 48, 72, and 96 hours. It can be seen that in the central tube, on account of binding to the plasma proteins, the dye-stuff did not proceed as fast as from the aqueous solution or from the uremic plasma.

Compared to the methods we use now, this was certainly a rather primitive way to determine protein binding. But if one reads the 1938 book, in which Bennhold reviewed his findings, it is surprising to see the basic facts about binding he reported. As Figure 6-1 shows, he was familiar with the fact that binding to uremic plasma is lower than to normal plasma.

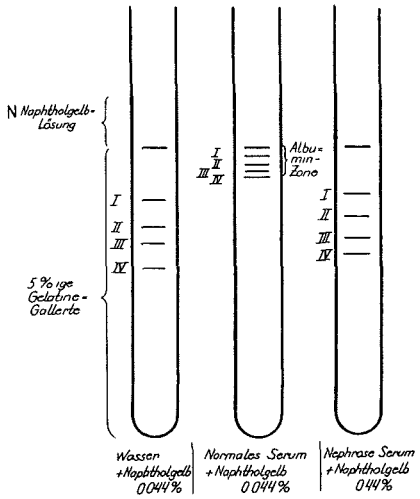

FIGURE 6-1
Original reproduction from Bennhold, Kylin, and St. Rusznyak (1938). Reprinted with permission.

DIRECT METHODS

Consider now the present methods. In general, protein binding of a drug means that there is an equilibrium between the free and the bound amount of the drug. This equilibrium is quite unstable, and even a small alteration caused by the techniques of determination may induce significant errors. With the following group of methods, which can be called "direct" methods, this risk is very small because they do not affect the equilibrium significantly.

Spectrophotometry

In some drugs, the binding to proteins induces a shift of their absorption spectra. Therefore, the difference in optical density, before and after drug and protein solution are mixed, can be used to determine binding. This can be performed in the visible as well as in the ultraviolet range. Binding can also influence the emission of a fluorescent drug or the fluorescence of certain groups at a protein molecule, e.g., the tryptophan group of albumin, can be decreased by binding.

Optical Rotatory Dispersion and Circular Dichroism

If optical rotation is measured at different wavelengths, the resulting curve is normally plane. A light absorbing drug, however, alters this curve at the wavelength of its absorption, a phenomenon called the "cotton effect." Moreover, the right and the left circulating components of plane polarized light may be absorbed differently, causing "circular dichroism." These effects also may be used to determine protein binding.

Nuclear Magnetic Resonance and Electron Spin Resonance

Nuclear magnetic resonance is based on the fact that the proton nucleus of a hydrogen atom behaves like a little magnet if it is brought into a strong magnetic field. Binding changes the resonance signal, and this can be used for the determination of binding. Electron spin resonance may be used in a similar way.

Advantages and Disadvantages of the "Direct" Methods

Considering the advantages and disadvantages of these methods, it has to be stressed once more that they do not interfere significantly with binding equilibrium. Moreover, they allow the measurement of even very fast changes in binding. Thus, for example, the kinetic of the interaction between drug molecules and the proteins can be determined in a stopped flow equipment recording the alteration of light absorption. Furthermore, these methods are useful to study binding forces or binding sites. On the other hand, they have the disadvantage that these techniques are often limited to special cases and that the values gained are—at least primarily—relative. Therefore, they are very suitable to record changes in binding or to study binding sites, but not for routine determination of binding.

INDIRECT METHODS

Another group of methods including equilibrium dialysis, ultracentrifugation, ultrafiltration, gel filtration, and—less important—electrophoresis and some biological methods can be called indirect methods. They have in common that free and bound amounts of the

drug are separated by different techniques. However, complete separation is not necessary. It is satisfying just to gain a protein-free part of the original solution which contains the free drug in a concentration corresponding to equilibrium concentration. Then, binding can be calculated indirectly by the following equation:

$$c_{bound} \rightleftarrows c_{total} - c_{free}$$

The reliability of these methods depends mainly on the question how close the separation technique comes to the requirement not to influence the binding equilibrium. The importance of this requirement is demonstrated in an extreme manner by a technique which was formerly used by some investigators. To obtain the protein-free portion, they precipitated the proteins using trichloroacetic acid, or heavy metal salts. Table 6.1 shows the difference if the binding of tolbutamide to plasma is either determined by equilibrium dialysis or by precipitation of the proteins. Obviously, precipitation alters the tertiary structure of the proteins and thus decreases the binding of tolbutamide considerably.

Some years ago, we had to decide what technique was best for our binding studies. To compare the different methods for their reliability, we measured the binding of several drugs to pooled plasma by these methods. As expected, we obtained somewhat different values for each drug according to the method. However, this only demonstrated to us that there are differences between the methods. It did not tell us whether values were right or wrong. Of course, one cannot test different methods if the only means to determine the true values are limited available methods. However, there is one value for binding that can be predicted exactly without any method of determination: zero binding. If protein binding is determined in drugs dissolved in a nonbinding medium, all methods should give zero for binding, if correct. This was the principle for our comparative study. I will now describe it to you (Kurz et al. 1977).

TABLE 6.1
Extent of Binding of Tolbutamide (10^{-4} mol/l) to Bovine Plasma Determined by Different Methods (percent)

Precipitation of proteins	40-50
Equilibrium dialysis	96

Equilibrium Dialysis

The principle of equilibrium dialysis is based on the fact that a semipermeable membrane allows only the diffusion of the small drug molecules and acts as a barrier to the proteins and the protein/drug complexes (see Fig. 6-2). Therefore, at equilibrium, the drug concentration at the protein-free side of the membrane must correspond with the free drug concentration in the protein solution.

Error of the Method

In Table 6.2, several drugs are listed according to their molecular weight. The error of the dialysis method was estimated as already reported. The values demonstrate that beside the high molecular weight suramine, the error in dialysis was below 1 percent. However, the high error of nearly 60 percent for suramine was found to be due to an insufficient length of diffusion. Normally, we incubated the dialysis bags for 14 hours, and this was sufficient time for most of the drugs to achieve equilibrium. As can be seen from the graph in Figure 6-3, suramine needs about 150 hours to reach equilibrium under these conditions. Therefore, in summary, one can say that if there is sufficient time for diffusion, even drugs of relatively high molecular weight can be determined by equilibrium dialysis.

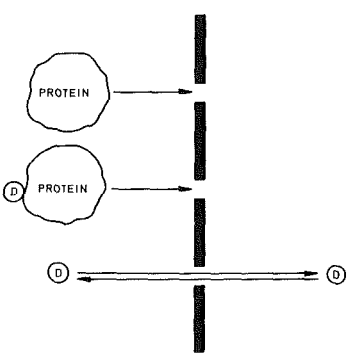

FIGURE 6-2
Attitude of protein molecules, drug molecules (D), and drug-protein complexes toward a dialyzing membrane.

TABLE 6.2
Dialysis of Drugs in Phosphate Buffer 0.05 M pH 7.4

Substances	M.W.	$c_0 - c_x$ (percent)
Isoniazid	121	0.5 ± 0.8
p-Aminosalicylic acid	153	0.7 ± 0.4
Sulfaguanidine	214	0 ± 0.3
Nitrofurantoin	238	0.5 ± 0.4
Sulfadimethoxine	310	0.7 ± 0.1
Chloroquine	320	0.5 ± 0.3
Meticillin	420	0.3 ± 0.4
Chloramphenicol monosuccinate	436	0 ± 0.3
Tetracycline	444	0.3 ± 0.2
Rolitetracycline	526	0.5 ± 0.1
Streptomycin	581	0.8 ± 0.1
Suramine	1297	58.5 ± 0.6

Notes:

Drug solution was inside the dialysis bags. The concentration of the drugs was determined after 14 hr of dialysis in the fluid outside the bag. The values ($\bar{x} \pm s_{\bar{x}}$) reflect the difference between the calculated concentration at equilibrium (c_0) and the measured concentration (c_x) in percent of c_0. N = 3. The drugs are listed according to their molecular weight (M.W.).

Source:

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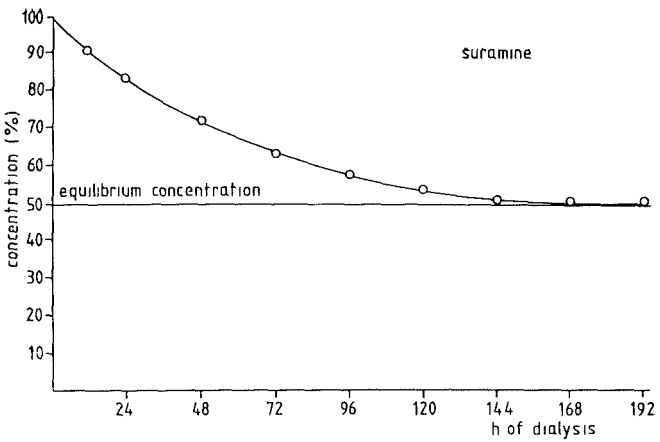

FIGURE 6-3
Concentration of suramine in dialysis bags with increasing time of dialysis.
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Adsorption of the Drugs to Dialysis Membranes

Drugs can be bound to the dialyzing membranes to a considerable extent. The values of Table 6.3 show that, among the drugs tested, there are great differences ranging from zero up to more than 50 percent for streptomycin or tolonium chloride. The cells used in modern devices for equilibrium dialysis are mostly made of plastic material such as teflon, for example. Lipophilic drugs are absorbed very well by this material. Furthermore, the drugs dissolved in the plastic material may redissolve and thus appear in the solutions of the following experiments. Therefore, cells of stainless steel should be used.

On the other hand, degree of adsorption depends considerably on the drug concentration. Suxamethonium is a good example of this effect. The graph of Figure 6-4 shows the extent of suxamethonium adsorbed to cellulose membranes at different drug concentrations. Adsorption is relatively low at high drug concentrations and rises to nearly 100 percent at very low drug concentration. Therefore, if adsorption is not taken into account, one would find a concentration-dependent binding for suxamethonium that does not exist. If drug concentration cannot be determined in both compartments of a dialyzing cell, it is always advisable to use blanks for correction.

TABLE 6.3
Adsorption of Drugs to Dialysis Bags and to Nitrocellulose Centrifuge Tubes

Substances	Adsorption to	
	Dialysis Bags	Centrifuge Tubes
p-Aminosalicylic acid	1.9-3.7	< 0.1
Barbital	0	0.8
Chloramphenicol	3.8-7.0	0.1
Chlordiazepoxide	0.6-4.2	4.6
Chlorimipramine	5.8	0.6
Chloroquine	2.5-5.3	1.1
Desipramine	0.8-13.3	0.9
Hexobarbital	0	1.9
Isoniazid	3.9-9.7	< 0.1
Meticillin	0	< 0.1
Methohexital	4.0	9.9
Nitrofurantoin	5.6-10.1	5.5
Pentobarbital	1.0	1.2
Phenylbutazone	1.3	< 0.1
Promazine	6.9	10.3
Rolitetracycline	14.8-26.1	0.8
Streptomycin	54.2-57.2	< 0.1
Sulfadimethoxine	7.0-13.7	0.9
Sulfaguanidine	0	0.5
Sulfinpyrazone	11.4	0.1
Suramine	12.2-16.7	1.0
Tetracycline	13.6-15.8	1.3
Thiamylal	2.1	15.3
Thiobutabarbital	2.7	3.0
Thiopental	0.1-2.6	8.1
Tolonium chloride	53.7	1.9

Notes:

The values (means from three measurements) show the adsorbed amount in percent of the total amount of the drug. All drugs used were in 10^{-4} M solution at pH 7.4.

Source:

Kurz et al. 1977. Reprinted with permission.

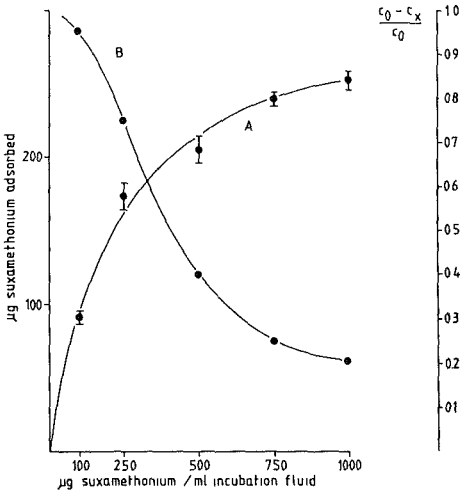

FIGURE 6-4
Adsorption of suxamethonium to dialysis membranes. One-half gram of dialysis membrane was cut into pieces and shaken for 1 hr in a solution containing suxamethonium at different concentrations. The dots represent mean values from three measurements. (A) = absolute amount of suxamethonium adsorbed; (B) = relative amount of suxamethonium adsorbed; c_0 = concentration of suxamethonium before incubation with membrane material; c_x = concentration after incubation.
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Alteration of Protein Solution

Dilution of the protein concentration during dialysis may be caused by the fact that proteins attract water from the buffer compartment by their osmotic activity. Using dialysis cells, the alteration in protein concentration is very small. Working with dialysis bags, dilution can be almost completely eluded, providing that the pressure inside the bags is slightly higher than that outside.

Donnan Effect

The Donnan effect may cause a difference between drug concentration in the protein-free compartment and the free drug concentration in the plasma. The error caused by this mechanism is mostly related to drugs that are both highly ionized and slightly bound.

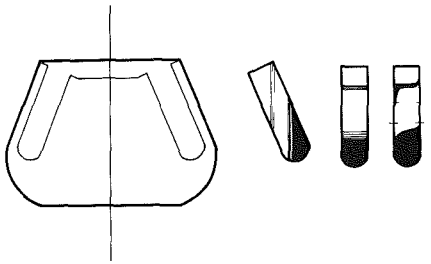

FIGURE 6-5
Ultracentrifugation.

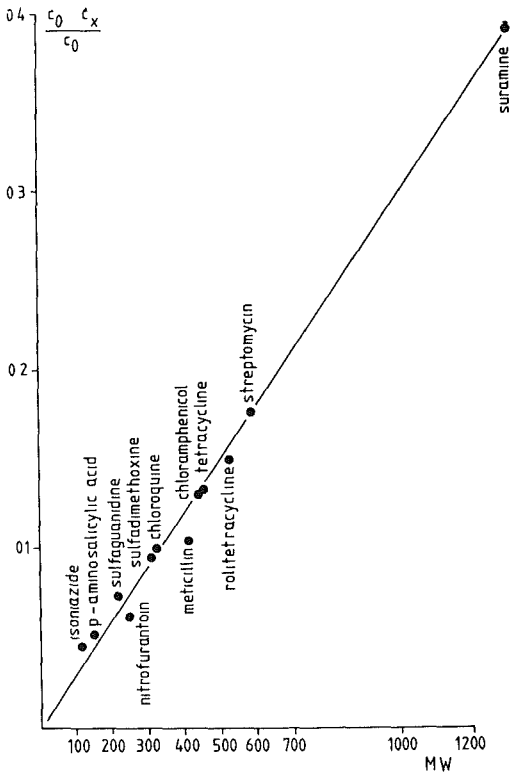

FIGURE 6-6
Drug sedimentation depending on molecular weight. c_0 = concentration of drugs before centrifugation; c_x = concentration of drugs in the uppermost ml of centrifuge tubes after 15 hours of centrifugation at 100,000 · g. The dots are mean values of four experiments.
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Ultracentrifugation

The ultracentrifugation technique makes use of the fact that sedimentation of solutes depends upon their molecular weights. Therefore, after centrifugation, the protein molecules are expected to be at the bottom of the centrifuge tube, whereas the supernatant should contain only the low molecular weight drug molecules. Actually, the situation is somewhat different as is demonstrated in Figure 6-5. While spinning in the rotor, the proteins accumulate at the side of the centrifuge tube. If the tubes are taken out of the rotor and brought into vertical position, they do not resemble the second tube. The real situation is represented by the tube on the right. Moreover, the lipoproteins accumulate at the top. Therefore, only the mid-portion of the supernatant can be used for the analysis of free drug concentration.

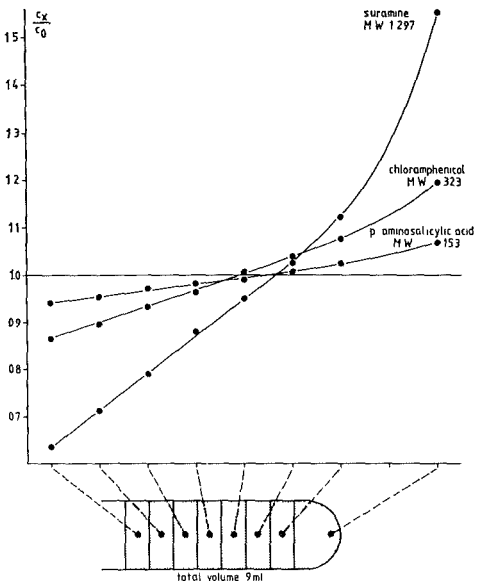

FIGURE 6-7
Concentration gradients in aqueous solutions of suramine, chloramphenicol, and p-aminosalicylic acid produced in the centrifuge tubes after 15 hours centrifugation at 100,000 · g. After centrifugation, the solution was removed in fractions of 1 ml beginning from the top, and drug concentration in each fraction was determined. c_0 = drug concentration without centrifugation; c_x = drug concentration after centrifugation. The dots are mean values of four experiments.
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TABLE 6.4

Substances	a) Dialysis in methylcellulose solution 0.25%	b) Ultracentrifugation (A) in methylcellulose solution 0.25%	b) Ultracentrifugation (B) in phosphate buffer 0.05 m	Difference A - B
Sulfaguanidine M.W. 214	0.7 ± 1.2	11.5 ± 0.7	7.6 ± 0.6	+3.9
Nitrofurantoin M.W. 238	0.73 ± 0.4	13.8 ± 0.5	10.2 ± 0.5	+3.6
Sulfadimethoxine M.W. 310	0.0 ± 3.6	12.1 ± 0.2	10.5 ± 0.2	+1.6
Suramine M.W. 1297	0.1 ± 0.9	33.0 ± 0.4	43.1 ± 0.7	-10.1

Notes:

a) Binding of four drugs to methylcellulose 0.25% determined by equilibrium dialysis.

b) Decrease in concentration of these drugs after ultracentrifugation (15 hours, 100,000 · g) in phosphate buffer or in methylcellulose solution. The decrease is given in percent of the initial concentration before ultracentrifugation. The values are means of 3–4 experiments.

All values are in percent.

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Error of the Method

The values in Figure 6-6 show that with ultracentrifugation the error starts at about 5 percent and increases up to about 40 percent for suramine. The error is mostly caused by the fact that in addition to the proteins, the drug molecules show some sedimentation.

This is demonstrated by the graph in Figure 6-7. Three drugs with different molecular weights were centrifuged in a protein-free buffer solution. After ultracentrifugation, the drug solution was removed in fractions and the drug concentration was measured. For all of the three substances a concentration gradient was found. The gradient is strong for the highly molecular suramine, but even for the p-aminosalicylic acid, with a molecular weight of 153, a slight gradient developed.

Sedimentation is influenced by the viscosity of a solution. Therefore, the results given in Figure 6-7 do not exactly represent the conditions as if a drug is dissolved in plasma. To check this influence of viscosity, we centrifuged some drugs in a methylcellulose solution of the same viscosity as plasma. As the results given in Table 6.4 show, sedimentation also occurs. However, the influence of viscosity on sedimentation differed with the molecular weight of the drugs. Sedimentation of the low molecular sulfaguanidine was increased. This effect turned smaller with increasing molecular weight of the drugs and changed to a decrease of sedimentation for the high molecular suramine. This means that blanks made with drug/buffer solutions may give wrong values for correction of the error caused by sedimentation of the drugs.

Adsorption of Drugs to the Centrifuge Tubes

The extent of adsorption of drugs to the wall of the centrifuge tubes is demonstrated in Table 6.3. Compared with adsorption to the dialysis bags, adsorption to nitrocellulose tubes is somewhat less. This may be different using tubes of another material. However, the adsorption varies also considerably from drug to drug. In ultracentrifugation technique, it is also advisable to use blanks to correct this error.

Ultrafiltration

The principle of ultrafiltration consists in using a filter membrane with a pore size allowing the passage of water and the small drug molecules, but not of the proteins or the drug/protein complexes, as is demonstrated in Figure 6-8.

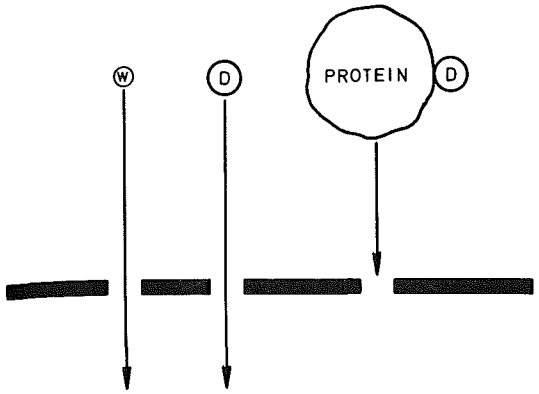

FIGURE 6-8
Attitude of water molecules (W), drug molecules (D), and drug/protein complex at an ultrafilter membrane.

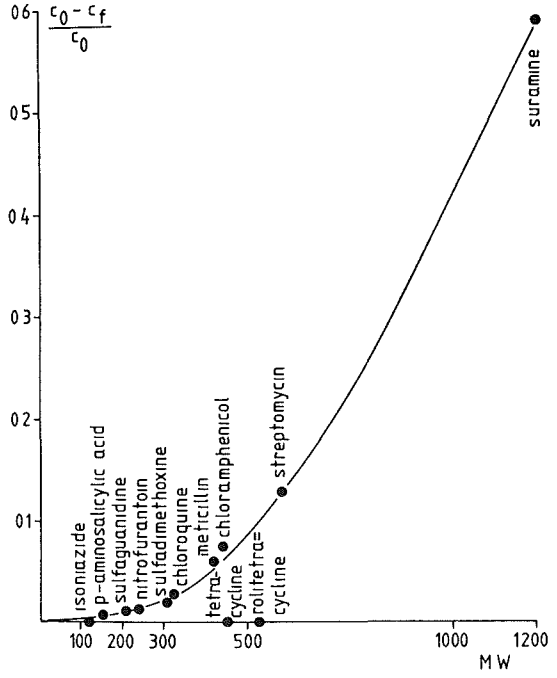

FIGURE 6-9
Inhibition of filtration in aqueous solution of drugs depending on the molecular weight. c_0 = concentration of drug before filtration; c_f = concentration of the drug in the ultrafiltrate after filtration. The volume of ultrafiltrate was < 20 percent of the total volume. The dots correspond to the mean values of three experiments.
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Error of the Method

As can be seen from the values in Figure 6-9, up to a molecular weight of about 300, the error in ultrafiltration is relatively small and comparable to the error in equilibrium dialysis. But with increasing molecular weight of the drug, the error increases up to 60 percent for suramine.

"Sieve Effect"

The error in ultrafiltration is mainly caused by what is called the "sieve effect." The model in Figure 6-10 explains the mechanism of this effect. In an ultrafilter membrane the pores do not have a uniform diameter but vary in size. There are pores wide enough to allow free passage of a drug molecule. But, in part, there are also smaller pores allowing just the passage of the tiny water molecules. This means that within a given time, more water molecules may be filtered than a corresponding number of drug molecules. In consequence, the drug concentration in the ultrafiltrate must become lower than in the unfiltered solution.

This effect may be reinforced if protein molecules partially obstruct the pores, thus hindering the passage of drug molecules but not the passage of the water molecules. Figure 6-11 demonstrates the mechanism of this effect.

The error caused by the sieve effect increases with increasing pressure of filtration. Therefore, the pressure ought to be as low as possible to keep the error small.

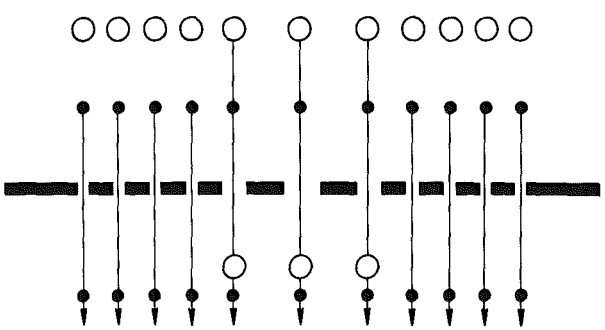

FIGURE 6-10
"Sieve effect" at an ultrafilter membrane. Black circles = water molecules, white circles = drug molecules.

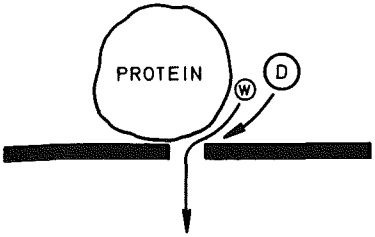

FIGURE 6-11
"Sieve effect" by protein molecules partially obstructing the pore of an ultrafilter membrane. D = drug molecule, W = water molecule.

Dependence on the Relative Volume of Ultrafiltrate

The concentration of the drug in the ultrafiltrate is influenced by the relation between the starting volume of the solution and the volume of the ultrafiltrate. The graph in Figure 6-12 demonstrates that with continued filtration of a drug/plasma solution, the concen-

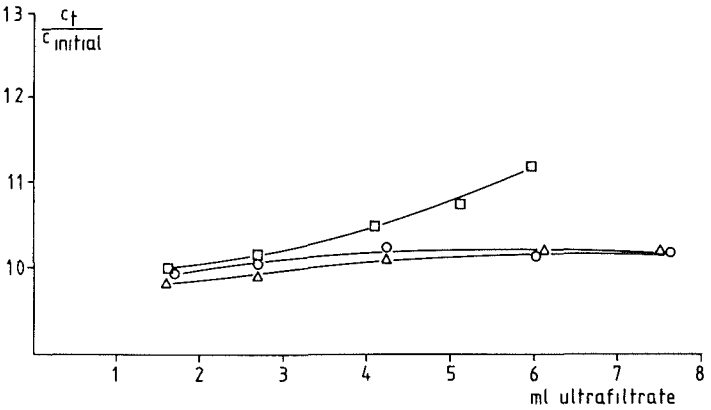

FIGURE 6-12
Change of concentration of sulfaguanidine in the ultrafiltrate at different lengths of filtration. $c_{initial}$ = concentration of the drug which was measured in the exterior solution at the preceding equilibrium dialysis. c_t = concentration of the drug in the ultrafiltrate which was removed in fractions during filtration. o phosphate buffer; Δ methylcellulose solution; □ plasma.
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tration of the drug in the ultrafiltrate increases. In this experiment, the initial volume of the drug/plasma solution was 20 ml. The graph shows that up to a relative volume of 2 ml of the ultrafiltrate, the error is small but then increases considerably. This means that in practice, the amount of the ultrafiltrate should not exceed 10 percent of the initial volume in order to keep the error small. The graph also suggests that this effect is caused by the proteins, because dissolving the drugs in buffer or a methylcellulose solution, the concentration did not increase with continued filtration.

Adsorption of Drugs to the Filter Membrane

In ultrafiltration, another error can be caused by the adsorption of the drugs to the filter membrane. It takes place in a way similar to that discussed for dialysis.

Gel Filtration

For the gel filtration technique, the drug/protein solution is given on top of a sephadex column. The principle of this method is that the dissolved substances pass the column with different speeds according to their molecular size. Thus, the proteins and the drug/

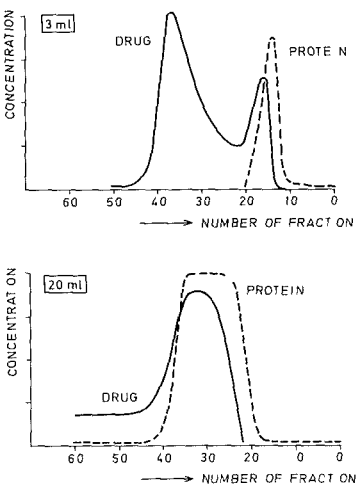

FIGURE 6-13
Concentration in the eluate of a plasma/drug solution at a sephadex gel column. Formation of "plateau regions" after increasing the volume of the plasma/drug solution from 3 ml to 20 ml.

protein complexes appear first in the eluate, whereas the free drug molecules follow later, as is shown in the upper part of Figure 6-13. However, free and bound drug are thus separated within the column and consequently equilibrium must be affected. Therefore, this method in its simple application did not give correct values for binding. Nichol and Winzor (1964) found that using a relatively large volume of the drug/plasma solution, plateau regions are formed for the drug as well as for the proteins. From the values measured in these plateau regions, binding can be calculated with much better reliability.

Error of the Method

The error in gel filtration technique is demonstrated in Table 6.5. The binding of thiopental to pooled plasma was determined comparatively by means of gel filtration, equilibrium dialysis, ultrafiltration, and ultracentrifugation. If one refers to equilibrium dialysis as the standard method, the values show that the differences between gel filtration and dialysis are within about the same order as between dialysis and other methods.

Errors in gel filtration may have the following origin: If the volume of the drug/protein solution is too small, the drug/protein complex dissociates. A volume of at least 20 ml is proposed. Some authors suggest 100 ml. The buffer used for the preparation of the

TABLE 6.5
Binding of Thiopental (10^{-4} M) to Pooled Bovine Plasma Determined by Equilibrium Dialysis, Ultrafiltration, Ultracentrifugation, and Gel Filtration

	Percent Bound ($\bar{x} \pm s_{\bar{x}}$)	Variation Coefficient (percent)
Equilibrium dialysis	82.33 ± 0.17	1.58
Ultrafiltration	91.99 ± 0.15	1.19
Ultracentrifugation	86.99 ± 0.16	1.36
Gel filtration	92.60 ± 0.26	1.54

Notes:

$N > 30$.

Variation coefficient $= \frac{s \cdot 100}{\bar{x}}$.

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TABLE 6.6
Comparison of Methods

Advantages	Disadvantages
Equilibrium dialysis:	
• Binding equilibrium almost unaffected because protein concentration not changed significantly • Suitable even for drugs with high molecular weight if sufficient time for diffusion • Less time consuming if performed in thin layers • Very good reliability	• Change in drug concentration (depending on rate of binding) • Adsorption to membrane material • Donnan effect?
Ultracentrifugation:	
• Only slight adsorption of drugs to the centrifuge tubes	• Sedimentation of the drugs starting with medium molecular weight of the drug • Relatively time consuming (12 to 16 hours) • Expensive equipment • Limited number of samples (about 10 per rotor) • Complicated technique
Ultrafiltration:	
• Suitable even for highly concentrated protein solutions, especially homogenates • Simple performance • Only small change in drug concentration if certain conditions are respected • Less time consuming	• Error by sieve effect starting with a drug molecular weight of about 300 • Increase of protein concentration during filtration • Adsorption to membrane material • Donnan effect?
Gel filtration:	
• Also suitable for drugs of high molecular weight • Less time consuming if conditions are already known	• Drugs with low affinity to protein cannot be determined accurately • Drugs which are strongly adsorbed to the gel cannot be determined • Relatively large volume of drug protein solution necessary • Preliminary study has to be made to find the optimal conditions for each drug

column and the buffer used for elution will dilute the drug/protein solution. Therefore, binding is not measured at the original drug concentration. In drugs with minor affinity to proteins, the concentration of the drug/protein complex is too low to form plateau regions. Some drugs may be adsorbed to the gel so strongly that the time for elution becomes too long. In the case of suramine, even after a time of 20 hours, no drug appeared in the eluate. A summary comparison of these methods is in Table 6.6.

Electrophoresis

Electrophoresis belongs to the rarely used indirect methods. The technique is based on the principle that the protein molecules migrate in an electrical field according to their electrical charge. Drugs bound to these proteins migrate with the same speed, whereas the free drug molecules behave differently and thus free and bound drug become separated. It is the advantage of this method that one may see immediately to what fraction of the plasma proteins binding occurs. On the other hand, binding equilibrium is affected by the separation of free and bound drug. This must be eluted by a special arrangement to achieve plateau regions.

Biological Methods

Biological methods to determine binding are limited to special cases. As an illustration, two examples will be described. If one adds an antibiotic drug to a bacterial culture, antimicrobial activity depends on the free amount of the antibiotic. This method may be used for an approximate estimation of binding and has the advantage that only small samples are necessary. Using the gasometric method of Warburg, antibiotics which bind similarly may be estimated by the decrease of metabolic activity of the bacteria.

METHODOLOGICAL PROBLEMS IN TISSUE BINDING

Within the last years, interest in the binding of drugs to tissues has increased, and this raises some special problems. Of course, tissue binding can be determined using isolated perfused organs. But this is a rather laborious and time-consuming way. Moreover, it cannot be applied to human organs. Therefore, tissues are usually cut into thin slices or homogenized in order to measure binding. This limits the methods which can be applied. Usually, ultrafiltration is the preferred method. Furthermore, there are two other problems which have to be taken into consideration.

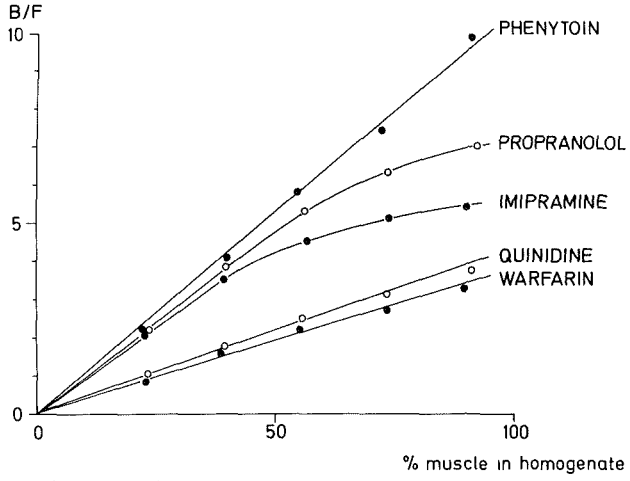

FIGURE 6-14
Dependence of binding to rabbit skeletal muscle homogenate on the dilution of the homogenate. B/F = quotient of bound to free drug. Source: Kurz and Fichtl 1983. Reprinted with permission.

Dilution of the Homogenates. Undiluted homogenates are of a paste-like consistency and, therefore, difficult to handle. Therefore, in general, a 50 percent diluted homogenate is used. But this means that the experimental data have to be converted into the values for

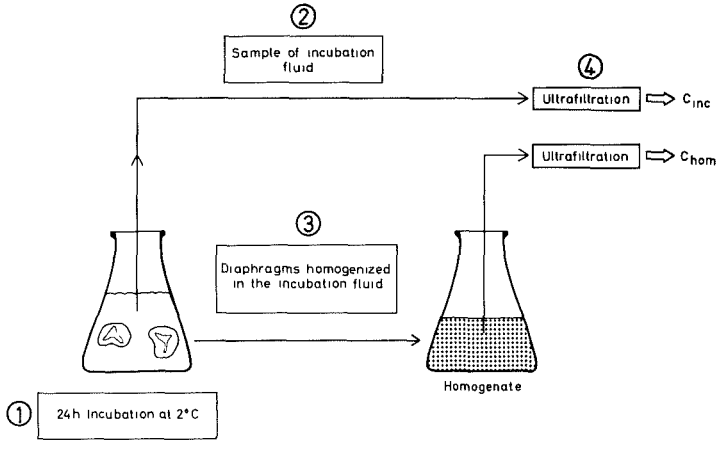

FIGURE 6-15
Comparison of binding to intact rat diaphragms and to its homogenates. Source: Kurz and Fichtl 1983. Reprinted with permission.

TABLE 6.7
Influence of Homogenization on Binding of Drugs to Rat Diaphragms

Drug	c_{hom}/c_{inc}		Percentage Increase of Amount Bound
Chlordiazepoxid	0.82	$p < 0.01$	7.8
Chlorpromazine	0.94	n.s.	
Mecamylamine	1.00	n.s.	
Methantheline	0.95	n.s.	
Phenobarbital	1.00	n.s.	
Phenprocoumon	0.94	n.s.	
Phenytoin	0.97	n.s.	
Promethazine	0.96	n.s.	
Propranolol	0.81	$p < 0.01$	7.4
Quinidine	0.79	$p < 0.05$	6.1
Salicylic acid	1.00	n.s.	
Sulfadimethoxine	1.01	n.s.	
Sulfaguanidine	1.01	n.s.	
Sulfamethoxydiazine	0.96	n.s.	
Thiopental	1.03	n.s.	

Notes:
c_{hom} = concentration of free drug in homogenate.
c_{inc} = concentration of drug in incubation fluid before homogenization.

Source:
Kurz and Fichtl 1983. Reprinted with permission.

the undiluted homogenate to get the true values for binding. The graph in Figure 6-14 shows the dependence of binding on the dilution of the homogenate. There is a linear relationship between binding and the "bound to free" concentration of the drug. In these cases, binding for the undiluted homogenate may be simply gained by extrapolation. However, for propranolol and imipramine such a procedure would give erroneous results. This means that extrapolation cannot

be recommended as a routine method; one has to clear up the influence of dilution beforehand.

Alteration of the Binding Properties of Tissues by Homogenization. In general, if a tissue is homogenized the cells will break. This raises the question whether a structural alteration of the tissue is caused which changes the binding properties. To determine this, we compared the binding of drugs to thin intact skeletal muscle represented by rat diaphragms with the binding of the drugs to the homogenate of these muscles (Kurz and Fichtl 1983). Figure 6-15 illustrates the procedure. The diaphragms are first incubated in an isotonic and buffered solution of the drug until equilibrium. Then, a sample of the incubation fluid was removed and the drug concentration measured in its ultrafiltrate. Thereafter, the diaphragms were homogenized together with their incubation fluid and again the drug concentration was measured in the ultrafiltrate of the homogenate. If no difference is found between both concentrations, the ratio between the concentration in the ultrafiltrate of the incubation fluid and the ultrafiltrate of the homogenate should equal 1, and this would indicate that binding was not affected by homogenization.

Table 6-7 shows the results of this experiment. For most of the drugs, no significant alteration of binding by homogenization could be detected. There was only a slight change for propranolol, chlordiazepoxide, and quinidine. Disregarding this fact, it can be said that binding accomplished with homogenates or slices of muscles may give results closely related to the results in the intact tissue.

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