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Critical Evaluation of Use of Effective Protein Fractions in Developing Pharmacokinetic Models for Drug Distribution

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Abstract □ A critical evaluation of the concept of effective protein fractions, which was previously utilized in physiologically based pharmacokinetic models to describe the binding of barbiturates to plasma and various tissues, is presented. The calculation of effective protein fractions requires as a minimum the extrapolation of *in vitro* binding parameters from one concentration of protein to another. Based on available literature data, it is shown that thiopental binding parameters vary markedly with the concentration of bovine serum albumin and that such concentration dependency cannot be predicted readily. Similar anomalous protein binding behavior has been reported for other drugs. Consequently, the use of effective protein fractions in developing pharmacokinetic models for drug distribution may not generally be feasible. The apparent successful use of such fractions in the case of thiopental appears to be fortuitous. Errors incurred in the extrapolation of the binding parameters may be compensated for by errors introduced by the experimental method in which the original binding data were obtained as well as interspecies differences in protein binding.

Keyphrases □ Protein fractions, effective—evaluation of use in developing drug distribution pharmacokinetic models □ Pharmacokinetic models—effective protein fraction, barbiturate binding, evaluation of use, equations □ Drug distribution—pharmacokinetic models, evaluation of effective protein fraction □ Binding parameters—evaluation of use of effective protein fraction in pharmacokinetic drug distribution models

In recent years the utility of physiologically based pharmacokinetic models has been demonstrated in describing quantitatively the distribution and elimination of barbiturates (1, 2), methotrexate (3–5), and cytarabine (cytosine arabinoside) (6) in various species. These models are uniquely different from the conventional compartmental models because all parameters involved have a specific physiological and/or physicochemical basis.

The development of such models carried the exciting implication that, in principle, based on *in vitro* tissue binding and partition studies together with well-documented physiological parameters such as organ weight and blood flow rate, it may be possible to make *a priori* predictions of drug distribution in the body. Excretion parameters such as biliary and renal clearances can be obtained from appropriate animal data. With the cytarabine model, an initial attempt was made to incorporate *in vitro* enzyme kinetic data in quantitating metabolism. It is evident that the development of physiologically and anatomically realistic models offers the possibility of a systematic, rational approach in extrapolating animal data to humans by appropriate scale-up of parameters.

DISCUSSION

In general, the four-compartment model proposed for barbiturates (1) and, in particular, for thiopental (2) represents the basic framework of all physiological models. The body is divided into several well-defined anatomical regions: blood, viscera, lean, and adipose compartments. Each body region further consists of a blood pool in equilibrium with the respective tissue. Within each compartment, except for the adipose tissue, the drug is present in both freely diffusible and "protein"[1]-bound form. Mathematical resolution of the model, therefore, requires binding parameters relating the free and bound concentrations in the various compartments.

In principle, the binding relationships should be readily obtainable by *in vitro* binding studies with plasma and various tissues. Abundant information on drug–plasma protein interaction already exists in the literature, whereas tissue binding data are meager or nonexistent. Furthermore, tissue proteins are poorly defined. Tissue samples are often too insoluble, and only their homogenates or centrifugal fractions can be studied. But plasma binding studies can easily be performed with whole plasma, plasma protein fractions, or crystalline serum albumin. The latter is often preferred because it is commercially available in pure form, is well characterized, and constitutes about 50–65% of total plasma protein, *i.e.*, 3.5–5.5% by weight of plasma.

Binding data obtained with an isolated protein such as albumin can be analyzed according to the physical law of mass action. Multiple classes of noninteracting binding sites are often assumed, and

[1] The term "protein," as used in this article, refers to those components in various tissues and blood to which the drug is bound. These components include proteins and other macromolecules.

Table I Total Protein Content of Various Fresh Soft Tissues in Humans[a]

Tissue	Total Protein, %
Plasma	6.95
Red blood cell	36.8
Brain (whole)	10.5
Heart	16.0
Liver	17.0
Kidney	18.0
Muscle	18.5

[a] From Ref. 27.

parameters such as the association constant and binding capacity associated with each class of site can be calculated by graphical techniques (7, 8) and computer analysis (9, 10).

In view of the lack of information on tissue binding of the barbiturates and thiopental, Dedrick and Bischoff (1) introduced the concept of "effective protein fractions" to deal with the mathematical implications of tissue binding. Through rigorous examination of the concept of an effective protein fraction, it became clear that care must be exercised when *in vivo–in vitro* correlation of protein binding is attempted. The general practice in binding experiments is to vary the total drug concentration over the range of pharmacological interest while keeping the albumin concentration constant. The extent of binding, *i.e.*, the bound and/or unbound concentration, is then measured. The binding parameters obtained from such a study are often assumed to be applicable at all other albumin levels.

It will be shown that this assumption may at times be incorrect. The significance of this problem will be illustrated with readily available thiopental binding data. It will also be demonstrated that such unpredictable deviations affect the calculation of the various effective protein fractions.

Effective Protein Fraction—A detailed study of the binding of thiopental to 1% bovine serum albumin, using the method of ultrafiltration, was reported (11). The binding data can be described by a two-term, Langmuir-type equation:

$$x = \frac{B_1K_1C}{1 + K_1C} + \frac{B_2K_2C}{1 + K_2C} \qquad \text{(Eq. 1)}$$

The same definitions as used by Dedrick and Bischoff (1) are adopted here, where x = bound concentration, C = free concentration, B = maximum binding capacity, and K = equilibrium association constant. The subscripts denote the two different classes of noninteracting binding sites. The following values were assigned (2): B_1 = 18,400 μmoles/liter, B_2 = 305,000 μmoles/liter, K_1 = 0.060 liter/μmole, and K_2 = 0.000625 liter/μmole.

In the absence of additional information, Bischoff and Dedrick explored the approximation that the binding characteristics for all proteins in the body are the same as those of bovine serum albumin. They then had to consider the question of how much protein is present in the different tissues. The normal total protein content of various soft tissues ranges from 10 to 20% by weight (Table I). Obviously, various kinds of proteins have different binding affinities toward thiopental and not all proteins would be accessible to the drug. Furthermore, tissue components other than proteins can be responsible for binding.

Based on the total protein content of various tissues listed in Table I, thiopental concentrations in blood and tissue compartments were simulated using Bischoff and Dedrick's (2) model. The same dose and input function were used. The predicted blood and muscle concentrations are compared to the experimental data of Brodie *et al.* (12) in Fig. 1 and a substantial overestimate is apparent. Less obvious, but equally significant, is that neither the time required to attain distribution equilibrium nor the predicted tissue-to-blood ratios agree with the experimental data. High metabolic clearance values have to be assigned to bring the blood concentration profile back into the proper perspective. However, even under these conditions the rank order of the tissue-to-blood distribution ratio cannot be corrected. It is apparent that only part of the tissue proteins are *effective* in binding the drug.

Goldbaum and Smith (11) reported the fraction of thiopental bound to various rabbit organ homogenates at an initial concentration of 0.5 m*M*. Dedrick and Bischoff (1) showed that for each data point a mass balance equation can be written:

$$C_t = fC + (1 - f)x \qquad \text{(Eq. 2)}$$

where C_t, the total drug concentration, was assumed to be that present initially, *i.e*, 0.5 m*M*; and C and x, free and bound drug concentrations, respectively, are related by Eq. 1. The term f is a dimensionless parameter defined as the fraction of water in tissue (2). To be more precise, the term $(1 - f)$ is an estimate of the effective fraction of protein and f is simply the remaining fraction of a volume of tissue homogenate. Free drug is assumed to be distributed homogeneously in this nonprotein fraction. Therefore, Eq. 2 was rewritten as:

$$C_t = (1 - f_p)C + f_px \qquad \text{(Eq. 3)}$$

where f_p is the effective protein fraction. The bound concentration, x from Eq. 1 has units of millimoles of drug bound per kilogram of protein. To convert this into the proper units of concentration (*i.e.*, millimoles of drug bound per liter of tissue homogenate), a density of unity has been assumed. By rearranging Eq. 3, an algebraic expression for f_p can be obtained:

$$f_p = \frac{C_t - C}{x - C} \qquad \text{(Eq. 4)}$$

Free drug concentration can be calculated from the fraction bound data of Goldbaum and Smith (11), since:

$$C = (1 - \text{fraction bound})C_t \qquad \text{(Eq. 5)}$$

By combining Eqs. 1, 4, and 5, f_p values for each rabbit tissue studied were calculated (Table II). When these values were compared to those provided in Ref. 1 (Table C2), it immediately became apparent that all of the present values except for the undiluted plasma were one-fifth those calculated by Dedrick and Bischoff (1). Subsequently, referring back to Goldbaum and Smith's original paper (11), it was noted that the rabbit blood and tissue samples were homogenized and diluted to five volumes with phosphate buffer before ultrafiltration. This explains the fivefold difference between the sets of values.

Attention was then drawn to the fact that fraction bound values

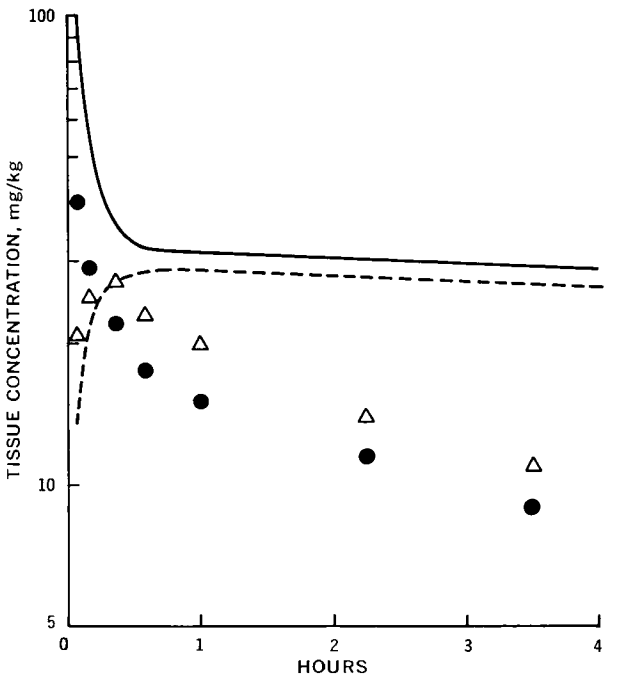

Figure 1—*Predicted concentrations of thiopental in blood* (——) *and muscle* (– – –) *using total protein concentration in various tissues with the four-compartment physiological model of Bischoff and Dedrick (2). Blood* (●) *and muscle* (△) *concentrations of thiopental in a dog obtained from Brodie* et al. *(12) are included for comparison.*

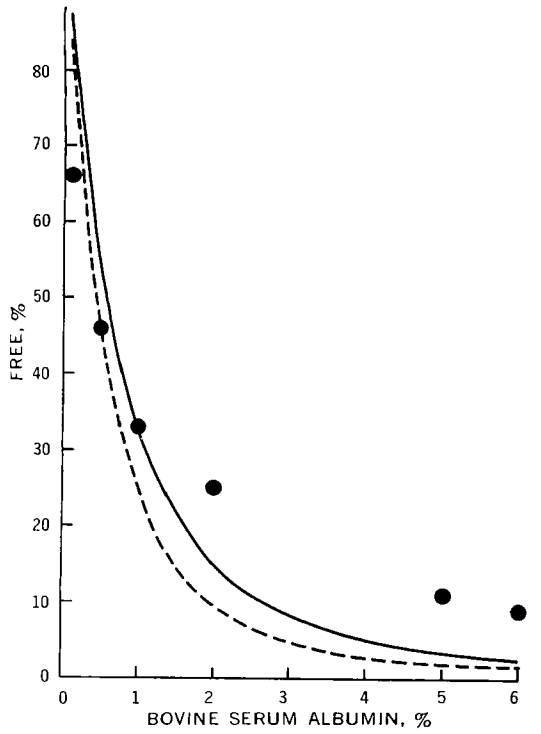

Figure 2—*Effect of bovine serum albumin concentration on the percent of unbound thiopental. Key:* ●, *data obtained from Goldbaum and Smith (11);* ——, *predicted values at a total drug concentration of 1 m*M*;* ***and*** – – –, *predicted values at a total drug concentration of 0.5 m*M.

for both diluted (five times) and undiluted plasma were available in the paper by Goldbaum and Smith (11). One would expect that values of effective protein fractions calculated for both diluted and undiluted plasma should be identical. However, after calculating an f_p value and applying a correction factor of 5, the diluted plasma data yielded a fraction value of 0.04255. This new effective protein fraction for plasma is about three times larger than that calculated from the undiluted plasma data—*viz.*, 0.0148. The discrepancy prompted a close examination of the thiopental binding data.

Dependence of Binding Parameters on Protein Concentration—The key assumption in the calculation of effective protein fractions for thiopental lies in the dilution factor. If the fraction represents a real mass of binding material, the fivefold correction is appropriate. However, the adjustment for dilution also involves the implicit assumption that the binding parameters remain unchanged throughout the extrapolated range of albumin concentration. In the study by Goldbaum and Smith (11), the effect of varying bovine serum albumin concentration on the fraction bound of a 1 m*M* thiopental solution is also reported. Those data are reproduced here in Fig. 2 as percent of free drug *versus* percent bovine serum albumin. The validity of applying binding parameters from one protein level to another in the case of thiopental can be ascertained by simulating the fraction of free drug as a function of protein concentration utilizing the binding parameters obtained at the 1% albumin level and comparing the calculated fraction to the actual values obtained.

Given the total drug concentration and a set of binding parameters, the concentration of free drug can be calculated as follows. The effective protein fraction, f_p in Eq. 3 is replaced with P_t, which now represents the concentration of albumin. To be consistent, P_t has to be expressed in kilograms of albumin per liter of solution. By substituting Eq. 1 into Eq. 3 and expanding the resulting expression, a third degree polynomial equation in free concentration, C, is obtained:

$$(1 - P_t)\delta C^3 + [(1 - P_t)\gamma + \beta P_t - \delta C_t]C^2 + [(1 - P_t) + \alpha P_t - \gamma C_t]C - C_t = 0 \quad \text{(Eq. 6)}$$

where $\alpha = B_1K_1 + B_2K_2$, $\beta = B_1K_1K_2 + B_2K_2K_1$, $\gamma = K_1 + K_2$, and $\delta = K_1K_2$.

Table II—Calculated and Reported Values of Effective Protein Fractions

Tissue	Fraction Bound[a]	Calculated f_p[b]	Reported f_p[c]
Muscle	0.45	0.00364	0.0175
Liver	0.66	0.00719	0.0362
Red blood cell	0.40	0.00306	0.0151
Heart	0.62	0.00631	0.0301
Kidney	0.53	0.00473	0.0237
Brain	0.50	0.00429	0.0212
Plasma, undiluted	0.85	0.01480	0.0145
Plasma, diluted	0.71	0.00851	—[d]

[a] Rabbit tissue homogenate data obtained by Goldbaum and Smith (11). [b] Calculated from Eqs. 1, 4, and 5. [c] Values tabulated in Table C2 of Ref. 1. [d] An effective protein fraction based on diluted (five times) plasma was not calculated.

A similar equation was derived by Hart (13), who established that equations of this type have only one positive real root which corresponds to the actual concentration of the unbound drug. Numerical solutions of Eq. 6 at various protein concentrations, P_t, while the total concentration, C_t, is held constant can readily be found by conventional methods of successive approximation on a digital computer. Solutions were generated for total drug concentrations of 1 and 0.5 m*M*. The predicted free concentration, expressed in terms of percent free over the appropriate range of albumin concentration, is shown with the experimental data in Fig. 2. Except for coincidence with the 1% data point, the predicted curve at an initial concentration of 1 m*M* provides a poor fit of the data.

At albumin concentrations of 5 and 6%, where the percentage of free drug is very small, the differences between predicted and observed values are more than threefold. Similar experimental data at 0.5 m*M*, the initial concentration at which the rabbit tissue binding study was carried out, are not available. However, based on the comparison at a total concentration of 1 m*M*, one can certainly conclude that the binding parameters obtained with the 1% bovine serum albumin solution cannot be applied, with any degree of confidence, at any other protein concentration.

The dependency of the binding parameters on protein concentration can best be depicted by transposing Goldbaum and Smith's (11) data onto a Scatchard plot (Fig. 3). A calculated curve based on the 1% binding parameters is included in Fig. 3. The experimental points lie nearly on a straight line with a positive slope, indicating a continuous decrease in either binding capacity B or association constant K, or both, as the concentration of albumin is increased at a fixed total drug concentration. It is apparent that no one average set of binding constants, *i.e.*, no one Scatchard curve, could fit all points.

Brunkhorst and Hess (14) observed very similar binding behavior with cortisol toward both human and bovine serum albumin. These investigators did not offer any definite explanations for the anomalous binding characteristics. They did mention the possibility that various quantities of competitive "inhibitors" may be present. Recently, Crooks and Brown (15) provided an example where the dependence of binding parameters on protein concentration is merely an artifact due to the particular buffer used. Different Scatchard plots were obtained for tolbutamide with 1 and 2% human serum albumin in tromethamine buffer. No such difference was observed when phosphate buffer was used. The investigators surmised that components of the tromethamine buffer may be competing with the drug for the same binding sites, leading to the observed dependency. In an early work of Klotz and Urquhart (16), a drastic decrease in the amount of methyl orange bound per mole of bovine serum albumin at the same free ligand concentration was observed when the protein concentration was increased fivefold, from 0.2 to 1.0%. Thus, the type of protein concentration dependency exhibited by thiopental may be more general than is now realized.

One less desirable feature of the ultrafiltration technique is that the volume of the drug–protein solution is continually reduced as part of the aqueous portion is filtered. The protein concentration increases as the filtration process progresses. Therefore, a binding equilibrium may not be attained throughout the filtration. In usual

practice, only a small portion of the solution is filtered, *i.e.*, less than 20%, so that the overall effect on protein concentration may be assumed to be negligible. Perhaps due to limitations of the assay, Goldbaum and Smith (11) chose to filter approximately one-half of the initial volume. This procedure effectively doubles the albumin concentration, and the total drug concentration remaining is different from that present initially. These changes, however, were not taken into consideration by Goldbaum and Smith. The bound concentration was simply taken to be the difference between the initial and the ultrafiltrate drug concentrations.

If one assumes, as a first approximation, that the concentration of thiopental in the ultrafiltrate represents the equilibrium free concentration in the drug–protein solution at the end of the filtration process, one can recalculate a more likely total drug concentration. The correction does not change the Scatchard plot in Fig. 3, since both bound drug and bovine serum albumin were concentrated to the same extent; *i.e.*, the variable r is not affected. The percent free data shown in Fig. 2, however, will be different. The total drug concentration after ultrafiltration will be higher than the initial concentration and will vary according to the amount of albumin present. Nevertheless, simulations based on these assumptions reveal a lack of agreement, similar to Fig. 2, between the percent free drug values as calculated from the binding parameters and those values calculated from the observed results.

Curvature in a Scatchard plot of drug–protein binding data has often been interpreted as evidence for heterogeneity of binding sites. However, Koshland (17) pointed out that the nonlinearity may also be a result of the conformational change of the macromolecule induced by an initial association with small molecules. In other words, interaction between binding sites occurs. The phenomenon is often referred to as cooperativity. There is ample evidence (18, 19) suggesting that albumin is a rather flexible molecule and can undergo extensive reversible conformational alterations. If the binding of each molecule of drug makes it more difficult for the next molecule to bind, *i.e.*, a negative cooperative effect, a downward concave Scatchard curve similar to the 1% predicted curve shown in Fig. 3 would be expected.

In fact, an opposite upward concave curve as a result of positive cooperativity has been observed with the binding of nicotinamide adenine dinucleotide to yeast glyceraldehyde 3-phosphate dehydrogenase (20). More recently, similar behavior was observed with the binding of acetylcholine to an acetylcholine specific receptor protein (21). One may perhaps infer from this discussion that a positive type of cooperative effect may explain the unusual Scatchard plot with thiopental. However, the reported positive cooperative effects have all been observed at one protein or receptor concentration and there is no reason why, in the case of thiopental, positive cooperative effects are only observed when the protein level is varied. An alternative rationalization could be that the serum bovine albumin undergoes "molecular aggregation." As the concentration of albumin increases, less of the binding surface per molecule becomes available, resulting in a decrease of affinity for the drug.

Regardless of the reasons for the deviant binding characteristic, the dependency of thiopental binding parameters on protein concentration offers a plausible explanation for the threefold difference in the value of an effective protein fraction when calculated from either diluted or undiluted rabbit plasma. Similar differences probably occur with the rest of the tissue data so that all of the effective protein fractions provided by Bischoff and Dedrick, except for undiluted plasma, may have been overestimated. The dilution factor is probably around 1.7 rather than 5.

In spite of the inappropriateness of the fivefold correction, the particular set of effective protein fractions listed by Bischoff and Dedrick provided excellent fit of the mathematical model to experimental data. When the effective protein fractions are decreased to one-third of the reported values, substantial changes in the concentration–time profiles occur. The values provided by these investigators appear to be unique. Bischoff and Dedrick followed Goldbaum and Smith in adopting the initial thiopental concentration added to the various rabbit tissue homogenates as the total drug concentration, C_t. When corrections were attempted to account for the excessive ultrafiltration, the effective protein fractions increased to about twice those of the previous values (Table II). When these new fractions were further multiplied by a dilution factor of 1.7, the values come very close to the numbers used by Bischoff and Dedrick. It appears that, by a fortuitous circumstance, the error incurred by the fivefold correction may partly be offset by an underestimation of the total drug concentration in the rabbit tissue homogenates.

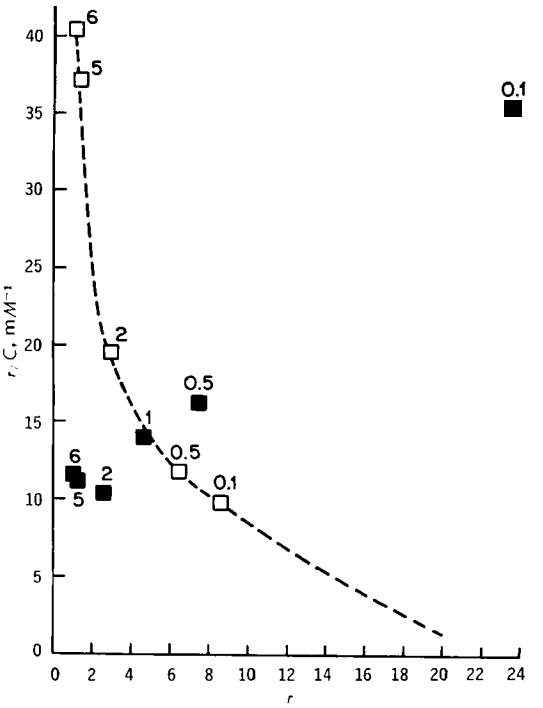

Figure 3—*Scatchard plot of fraction bound values of thiopental obtained by ultrafiltration at various bovine serum albumin concentrations. The initial concentration of thiopental was 1 m*M*. Key: – – –, calculated curve based on binding parameters obtained with 1% bovine serum albumin solution;* ■*, experimental points; and* □*, theoretical points. Numerals accompanying each point indicate the protein concentrations. The term* r *is defined as the ratio of millimoles of drug bound to millimoles of albumin, and* C *is the free concentration of drug. The molecular weight of albumin is assumed to be 68,000.*

Interspecies Differences in Protein Binding—It is well recognized that significant interspecies differences often exist with plasma protein binding. Examples include various sulfonamides (22), salicylates (23), and desipramine (24). Differences in both the quantity and binding characteristics of different plasma proteins contribute to the interspecies variability. An interesting example of the significance of interspecies differences in protein binding can be found with warfarin (25). The association constant of warfarin to human plasma albumin is six times that of canine plasma albumin. This difference has been used to explain why phenylbutazone potentiates the anticoagulant effect of warfarin in humans through protein displacement, while in dogs the enzyme-inducing effect is predominant.

In view of reported interspecies differences, it is instructive to consider the sources of the thiopental binding data used by Bischoff and Dedrick. The isotherm was based on *bovine* serum albumin. *Rabbit* organs were used for determination of tissue binding. The predicted results of the model were eventually compared to *human* and *dog* data. A total of four animal species was involved. Kane and Smith (26) studied the binding of thiopental to human plasma by equilibrium dialysis. Their study suggested that at the same free concentrations bovine serum albumin may bind five to eight times more thiopental than human plasma. Hence, interspecies variation in protein binding may be offered as another possible explanation as to why the particular set of effective protein fractions used for thiopental (1) happened to provide good prediction of thiopental distribution in dogs and humans.

CONCLUSION

As is apparent from the foregoing discussion, *in vivo–in vitro* correlation of tissue protein binding is fraught with limitations and

difficulties. Since one cannot perform binding studies with homogenized tissues without dilution, care must be exercised when extrapolation of binding relationships from one protein level to another is required. Likewise, human tissue is difficult to obtain and uncertainty exists when animal tissue data are substituted. The problem of interspecies variability in binding characteristics of protein may, in some ways, be analogous to that encountered with biotransformation processes.

Therefore, the use of an effective protein fraction to account for tissue binding does not appear to be feasible if unpredictable binding behavior similar to that displayed by thiopental is found. At present, the most practical approach to handling protein binding with respect to modeling appears to be the use of equilibrium tissue-to-plasma distribution ratios as was done with the methotrexate models (3, 4). The distribution ratios can be measured or estimated from *in vivo* animal data. It must be considered, however, that the distribution ratio will vary as a function of concentration if nonlinearity in binding occurs.

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ADDENDUM

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This will be a brief commentary on the issues discussed by Shen and Gibaldi. To begin, we concur with their overall results, many of which were known to us when we published the first work, on barbiturate pharmacokinetics, that utilized the notion of "effective protein fraction." Their simulation shown in Fig. 1, where total tissue protein values are used, certainly emphasizes the fact that smaller values—effective protein fractions—are needed to predict the drug distribution properly. These types of results were what naturally led us to formulate the whole concept. Furthermore, we would now probably favor the use of "effective protein amounts" and define total concentration similarly to Krüger-Thiemer:

$$C_T = wC + \bar{p}x \qquad \text{(Eq. A1)}$$

where w is fraction water (usually approximately known) and p is the effective protein concentration. This obviates assuming unity densities, and there is no need for the two coefficients to be fractions that add to unity. The net results are similar, but fewer approximations of principles are required.

The results shown for thiopental in Fig. 2 of this Shen and Gibaldi article were also known to us; in fact, similar discrepancies are also true for the other barbiturates, even the weakly bound barbital. This was our reason for including plasma in the effective protein table rather than using the actual protein fractions, that one would hope would have worked for this tissue. In retrospect, these details perhaps should also have been published; Shen and Gibaldi, by describing this technique in greater detail, have performed a useful service for those actually wishing to use it.

To be more specific, the effective protein concept assumes that: *(a)* binding can be represented by an isotherm, $x(C)$, on a thermodynamically intensive basis (*i.e.*, per unit protein mass), multiplied by a protein concentration; and *(b)* intensive isotherms for nonspecific binding would be the same (at least approximately) for all body tissues (the mixing of data for different species was done due to the lack of more complete data and should naturally be avoided if at all possible). The first should be true if there are no interactions, say at higher protein concentrations. This, unfortunately, does not seem to be true in the physiological range for plasma and indicates a gap in our knowledge of protein binding. We were interested in the additional evidence and references provided by Shen and Gibaldi. The second could not be checked by us, since complete isotherm data were only available for plasma proteins (in fact, bovine serum albumin) and not for any tissue homogenates. It would seem that tissue differences for nonspecific binding would not be as severe as for metabolism where, presumably, very specif-

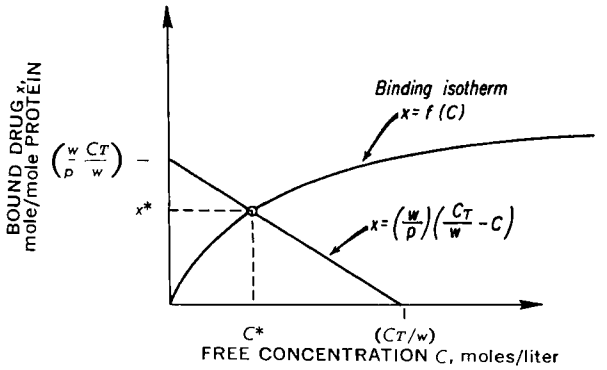

Figure A1—*Illustration of a graphical procedure for the estimation of free concentration of drug for a given total drug and protein concentration.*