# Interspecies Difference in Drug Protein Binding–Temperature and Protein Concentration Dependency: Effect on Calculation of Effective Protein Fraction

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**Abstract** □ The effects of temperature and protein concentration on the binding of thiopental to bovine and rat serum albumin and to rat plasma were examined using flow and equilibrium dialysis techniques. The effects of temperature and protein concentration were peculiar to each species. The binding of thiopental to rat serum albumin or rat plasma was sensitive to temperatures between 4 and 37°, whereas the binding to bovine serum albumin was sensitive to a protein concentration difference of 0.1–4.44%. Therefore, the effective protein fraction is affected by various sets of binding parameters of albumins determined under various temperatures and protein concentrations. Thus, a semipredictive method for plasma or tissue binding may be unsuccessful unless proper binding parameters are used.

**Keyphrases** □ Thiopental—effects of temperature and protein concentration on plasma and tissue binding, interspecies differences □ Effective protein fraction—effects of temperature and protein concentration, interspecies differences □ Drug protein binding—effects of temperature and protein concentration, interspecies differences □ Dialysis—equilibrium and flow techniques, effects of temperature and protein concentration on plasma and tissue binding, interspecies differences

Drug protein binding has an important role in drug distribution, disposition, and excretion. Numerous reports have dealt with drug protein binding under various conditions of drug level, protein concentration, and temperature. Some authors (1–3) adopted experimental conditions different from physiological ones, such as a lower temperature or lower plasma protein concentration. In addition, they discussed *in vivo* situations based on the inadequate experimental data obtained in this way.

## BACKGROUND

Recently, important critical information on these points was reported (4–6). Shen and Gibaldi (6) provided significant criticism on a previous report (7) that dealt with the application of *in vitro* drug protein binding data to the prediction of an *in vivo* situation utilizing a physiologically based pharmacokinetic model. To construct a physiologically based pharmacokinetic model, both tissue and plasma protein binding are important. However, tissue and organs are not as accessible as plasma for the determination of drug protein binding.

Dedrick and Bischoff (7) proposed the concept of the effective protein fraction to depict tissue binding, taking into consideration that little information was available on the binding of barbiturates to tissues. They calculated effective protein fractions based on the binding data of various rabbit organ homogenates at an initial drug concentration of 0.5 m*M* using the binding parameters (8) of 1% bovine serum albumin for barbiturates. However, Shen and Gibaldi (6) concluded that the use of the effective protein fraction in developing pharmacokinetic models for drug distribution might not be feasible for the following reasons:

1. Binding parameters were assumed to be constant over the concentration range of both bovine serum albumin and rabbit organ homogenate.
2. Interspecies differences were not considered.

Nevertheless, since the concept of the effective protein fraction is useful for the prediction of drug distribution in tissues where there is a shortage of information on tissue binding, the effects of protein concentration and temperature on drug protein binding were studied here using bovine and rat serum albumin and rat plasma. By considering these effects and interspecies differences, a semipredictive method for plasma protein or tissue binding was tested by measuring binding levels in plasma or a homogenized tissue sample.

Thiopental, which was studied previously (6, 7), was chosen as the model drug for the present study. Flow dialysis was used to assess protein binding of thiopental to bovine and rat serum albumin and rat plasma, while equilibrium dialysis was used for the rat plasma and homogenized rat tissue samples.

## EXPERIMENTAL

**Chemicals**—Rat serum albumin[1] and bovine serum albumin[2] were Fraction V powder. Stock albumin solutions were prepared by dissolving serum albumin powder in 0.05 *M* tromethamine buffer (I) adjusted to pH 7.4 with hydrochloric acid. Thiopental sodium[3] was prepared fresh daily.

**Quantitation of Thiopental**—Standards were carried through the entire procedure. A previous method (9) was used with slight modification (*i.e.*, dual-wavelength measurement[4] and *n*-heptane were employed). The recoveries from extraction were >90% and linear within the concentration range used.

**Rat Plasma and Tissue Sample**—Plasma was obtained by centrifuging, 30 min after intravenous injection of heparin, the fresh blood of a male Wistar rat (250–300 g) fed a normal laboratory diet. After the body was perfused with cold saline, the muscle was excised, rinsed in ice-cold saline, blotted, weighed, cut into small pieces, and homogenized by a motor-driven polytef glass homogenizer in 0.05 *M* I to yield 25% (w/v) homogenate. Rat plasma and homogenized muscle sample were predialyzed through cellulose dialysis tubing against 100 volumes of I at pH 7.4 for 18–24 hr at 4° with stirring. Little volume change was found during predialysis.

**Dialysis**—Flow dialysis of serum albumin solutions and rat plasma were carried out according to a reported method (10) using I at pH 7.4. Flow rates of I were 25 ml/hr at 37° and 12 ml/hr at 4°, respectively. Thiopental permeability through cellulose dialysis membrane remained almost constant before and after each experiment, which was checked by carrying out dialysis using I at pH 7.4 instead of protein solution.

Equilibrium dialyses of rat plasma and homogenized rat tissue samples were carried out according to the method of Sugiyama *et al.* (11) using I at pH 7.4. Equilibrium was established by 12 hr of dialysis at 37° and by 24 hr of dialysis at 4°, respectively. Equilibrium dialyses of homogenized tissue were carried out only at 4°.

**Data Analysis**—Results from binding experiments presented in Figs. 1–4 were subjected to curve fitting to Eq. 1 by a digital computer program (12):

$$r = \frac{n_1 k_1 C_f}{1 + k_1 C_f} + \frac{n_2 k_2 C_f}{1 + k_2 C_f} \qquad \text{(Eq. 1)}$$

where $k_1$ and $k_2$ are the association constants corresponding to $n_1$ and $n_2$, the number of primary and secondary binding sites; $C_f$ is the free drug concentration, and $r$ is the molar ratio of the bound drug to the binding protein assuming a molecular weight of 69,000. Results from binding experiments presented in Figs. 5 and 6 were subjected to curve fitting to Eq. 2 by means of a digital computer program (12). Data were weighted with the reciprocals of their variance:

[1] Pentex.
[2] Armour Pharmaceutical Co., Phoenix, Az.
[3] Tanabe, Tokyo, Japan.
[4] Hitachi 356 UV spectrometer.

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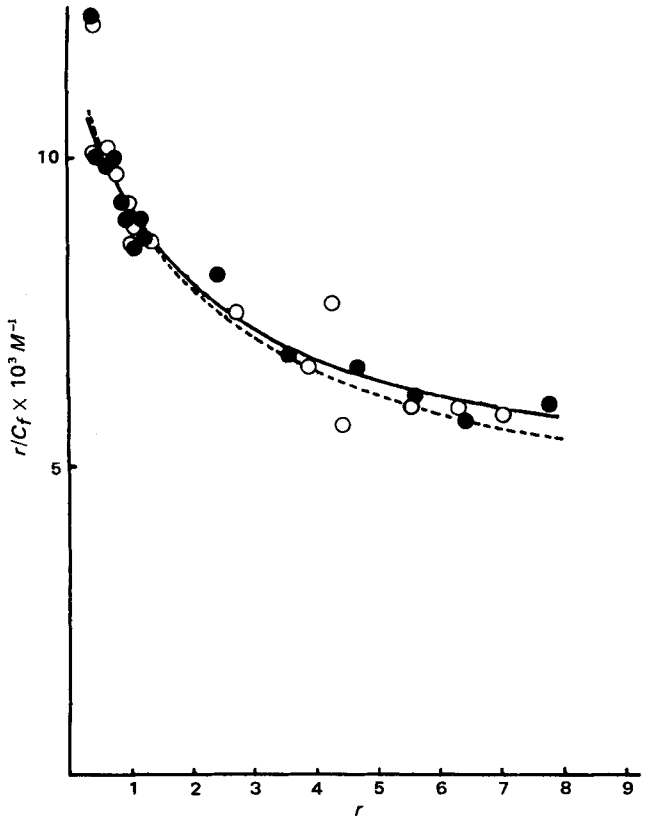


**Figure 1**—*Effect of temperature on thiopental binding to 1% (w/v) bovine serum albumin in 0.05* M *tromethamine buffer at pH 7.4 by flow dialysis [4° (●) and 37° (○)]. Each point represents the mean of three experiments. The smooth curves at 37° (- - -) and 4° (—) are the best fit to these data using the predetermined values for the number of sites* $n_1 = 1.0$ *and* $n_2 = 83.4$.

$$C_b = \frac{n_1(P)k_1C_f}{1 + k_1C_f} + \frac{n_2(P)k_2C_f}{1 + k_2C_f} \qquad \text{(Eq. 2)}$$

where $(P)$ is the protein concentration and $C_b$ is the bound drug concentration in plasma or tissue homogenates. Value $n_1(P)$ or $n_2(P)$ is presumed to be the composite parameter.

The $n_1$ and $n_2$ values obtained from experiments using each protein source at various protein concentrations and temperatures were averaged and constrained, and revised sets of $k_1$ and $k_2$ values were derived corresponding to the constrained average $\bar{n}_1$ and $\bar{n}_2$ values according to a previous report (5).

## RESULTS

**Comparison of Two Dialysis Methods**—Figure 5 displays, in the form of a Scatchard plot, the binding of thiopental to rat plasma at 4° assessed by flow and equilibrium dialyses. At low thiopental concentrations, there was a slight difference in binding, probably due to the sensitivity limit of the analytical technique, especially in the flow dialysis experiment. (Flow dialysis was employed mainly for the advantages of reduced incubation time and smaller volume of protein sample compared to equilibrium dialysis.)

**Thiopental Binding to Bovine Serum Albumin**—The effects of temperature and protein concentration on thiopental binding to bovine serum albumin were examined. As shown in Fig. 1, there was little, if any, temperature dependency of thiopental binding to 1% (w/v) bovine serum albumin between 4 and 37°, especially at low thiopental concentrations. Thiopental binding to 1 and 4% (w/v) bovine serum albumin at 37° is shown in Fig. 2. At low thiopental concentrations, there was a protein concentration dependency of thiopental binding to bovine serum albumin. In addition, thiopental binding to a range of bovine serum albumin at an initial thiopental concentration of 1 m*M* was examined (Fig. 2, inset). This relationship resembles that presented previously (6). Thus, it seems that bovine serum albumin is sensitive to albumin concentration and practically insensitive to temperatures between 4 and 37°.

**Thiopental Binding to Rat Plasma**—Figure 6 presents the data on thiopental binding to undiluted rat plasma at 4 and 37°. Figure 7 represents thiopental binding to undiluted rat plasma and 50 and 25% (v/v) diluted rat plasma at 37°. These data imply that the characteristics of thiopental binding to rat plasma are contrary to those of bovine serum albumin, *i.e.*, very sensitive to temperatures between 4 and 37° and practically insensitive to protein concentration within at least a fourfold dilution of rat plasma.

**Table I—Thiopental Binding to Rat Serum Albumin and Bovine Serum Albumin**

| Serum Albumin Concentration | Temperature | $k_1$[a] | $k_2$[a] | $n_1$[b] | $n_2$[b] |
|---|---|---|---|---|---|
| Rat | | | | | |
| 1% (w/v) | 4° | 40.9 | 1.31 | 1.0[c] | 8.0[c] |
| 1% (w/v) | 37° | 19.4 | 0.806 | 1.0[c] | 8.0[c] |
| 4% (w/v) | 37° | 20.1 | 0.990 | 1.0[c] | 8.0[c] |
| Bovine | | | | | |
| 1% (w/v) | 4° | 5.84 | 0.068 | 1.0[c] | 83.4[d] |
| 1% (w/v) | 37° | 6.31 | 0.0654 | 1.0[c] | 83.4[d] |
| 4% (w/v) | 37° | 1.62 | 0.636 | 1.0 | 11.6 |

[a] Association constant ($\times 10^3\ M^{-1}$). [b] The number of binding sites. [c] Predetermined and constrained (see text).

**Thiopental Binding to Rat Serum Albumin**—Thiopental binding to 1 and 4% (w/v) rat serum albumin and to 1% (w/v) rat serum albumin at 4 and 37° is shown in Figs. 3 and 4, respectively. These data suggest similar binding characteristics of rat serum albumin to those of rat plasma, *i.e.*, sensitive to temperatures between 4 and 37° and practically insensitive to protein concentrations between 1 and 4% (w/v).

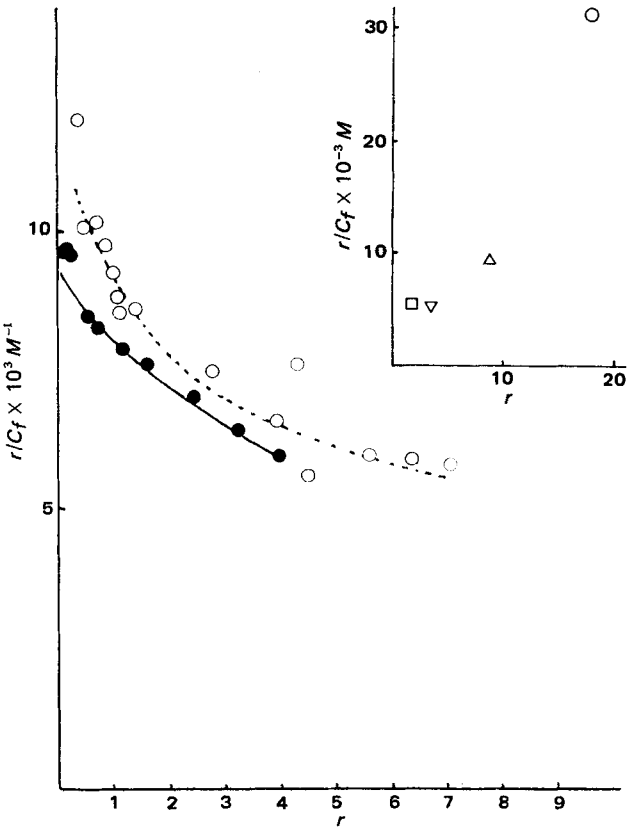


**Figure 2**—*Effect of protein concentration on thiopental binding to bovine serum albumin in 0.05* M *tromethamine buffer at pH 7.4 and 37° by flow dialysis [1% w/v (○) and 4% w/v (●)]. Each point represents the mean of three experiments. The data points and the smooth curve for 1% (w/v) bovine serum albumin are the same as those in Fig. 1. The smooth curve for 4% (w/v) bovine serum albumin (—) is the best fit to these data. [Inset: Scatchard plot for the binding of a single thiopental concentration (1 m*M*) to a bovine serum albumin concentration range in 0.05* M *tromethamine buffer at pH 7.4 and 37° by flow dialysis. Each data point represents the mean of two experiments. Key: ○, 0.1% (w/v); △, 0.5% (w/v); ▽, 1.74% (w/v); and □, 4.44% (w/v)].*

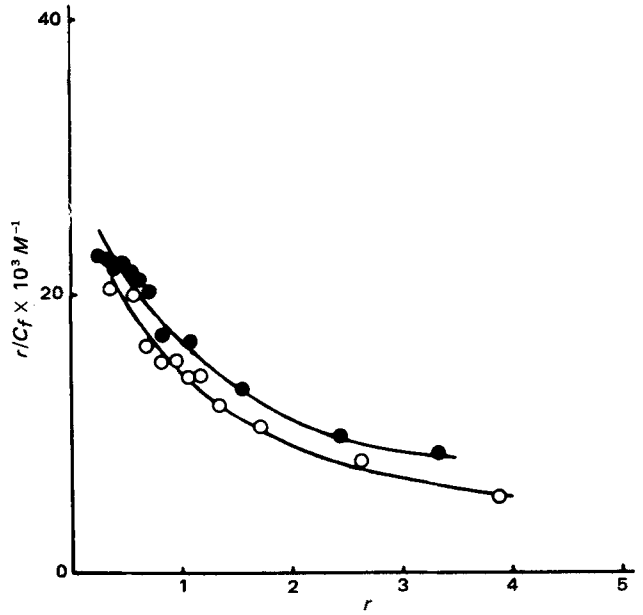


**Figure 3**—*Effect of protein concentration on thiopental binding to rat serum albumin at 37° in 0.05* M *tromethamine buffer at pH 7.4 [1% w/v (○) and 4% w/v (●)]. Each point represents the mean of three experiments. The smooth curves are the best fit to these data using the predetermined values for the number of sites* $n_1 = 1.0$ *and* $n_2 = 8.0$. *Flow dialysis was used.*

**Binding Parameters**—For the binding parameters of thiopental binding to rat serum albumin of 1% (w/v) at 4 and 37° and of 4% (w/v) at 37°, $n_1$ and $n_2$ values first were calculated without constraint and then averaged ($\bar{n}_1 = 1.0 \pm 0.221$ and $\bar{n}_2 = 8.0 \pm 3.52$). Then a revised set of $k_1$ and $k_2$ values was derived for each experiment corresponding to the constrained $\bar{n}_1$ and $\bar{n}_2$ values. In this way, the values for the number of binding sites were predetermined and constrained. The binding data for thiopental to rat plasma were analyzed in the same manner as for rat serum albumin [$\bar{n}_1(P) = 0.136 \pm 0.0251$ and $\bar{n}_2(P) = 2.42 \pm 1.12$ (m$M$)]. However, for bovine serum albumin, because of protein concentration-dependent binding, the constrained average $\bar{n}_1$ and $\bar{n}_2$ values were not proper for deriving revised sets of $k_1$ and $k_2$ values since the residual sum of squares became significantly larger than that derived without constraint. Therefore, the binding data of thiopental to 1% (w/v) bovine serum albumin at 4 and 37° were analyzed in the same manner as for rat serum albumin [$\bar{n}_1 = 1.0$(1.57 and 0.431) and $\bar{n}_2 = 83.4$(101.0 and 65.8)]

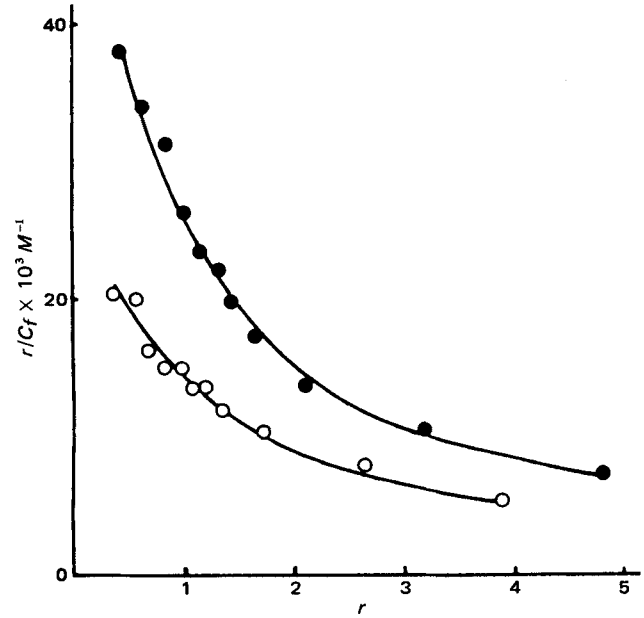


**Figure 4**—*Effect of temperature on thiopental binding to 1% (w/v) rat serum albumin in 0.05* M *tromethamine buffer at pH 7.4 by flow dialysis [4° (●) and 37° (○)]. Each point represents the mean of three experiments. The smooth curves are the best fit using the predetermined values for the number of sites* $n_1 = 1.0$ *and* $n_2 = 8.0$. *Flow dialysis was used.*

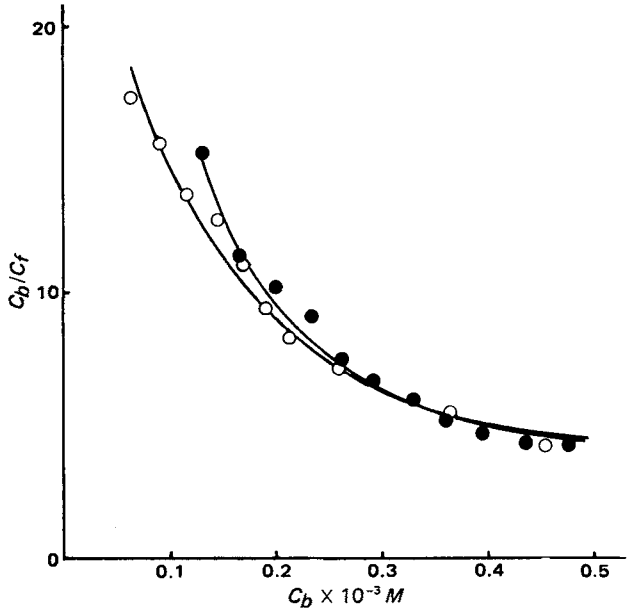


**Figure 5**—*Scatchard plots for the binding of thiopental to rat plasma at 4° by flow dialysis (●) and equilibrium dialysis (○). Each point represents the mean of three experiments. The smooth curves are the best fit to these data using the predetermined values for the binding capacities [*$n_1(P) = 0.136$ *and* $n_2(P) = 2.42$ *(m*M*)].*

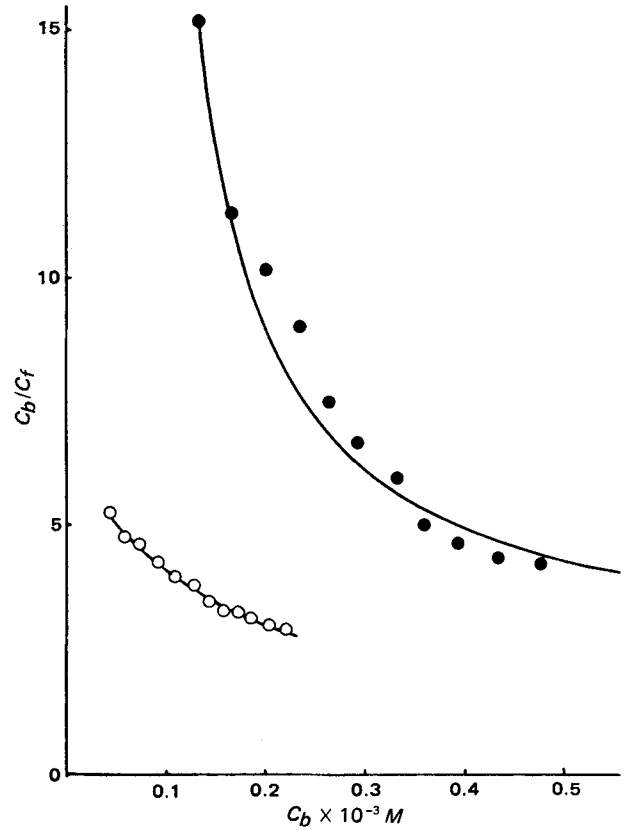


**Figure 6**—*Effect of temperature on thiopental binding to rat plasma by flow dialysis [4° (●) and 37° (○)]. Each point represents the mean of three experiments. The smooth curves are the best fit to these data using the predetermined values for the number of sites [*$n_1(P) = 0.136$ *and* $n_2(P) = 2.42$ *(m*M*)]. The smooth curve and the data points for rat plasma at 4° are the same as those in Fig. 5.*

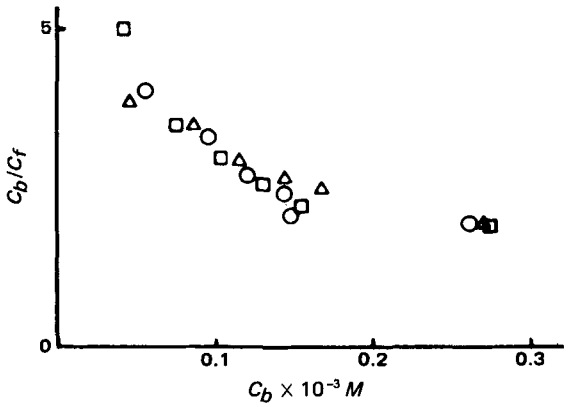


**Figure 7**—*Scatchard plots for the binding of thiopental to rat plasma at 37° by flow dialysis. Key: ○, 25% (v/v); △, 50% (v/v); and □, undiluted. Diluted rat plasma was prepared by adding 0.05* M *tromethamine buffer at pH 7.4. Dilution factors of 2 for 50% (v/v) and 4 for 25% (v/v) rat plasma were corrected respectively. Each point represents the mean of three experiments.*

and then revised sets of $k_1$ and $k_2$ values were derived corresponding to the constrained average $\bar{n}_1$ and $\bar{n}_2$ values.

For the binding data for thiopental to bovine serum albumin of 4% (w/v) at 37°, $n_1$ was assigned a value of 1.0, obtained from the data analysis of the other two experimental results of bovine serum albumin. Then a revised set of $n_2$, $k_1$, and $k_2$ values was derived corresponding to the constrained $n_1$ value since $n_1$ became negative without constraining. The residual sum of squares was not significantly larger than the residual sum of squares in the other cases. The results are summarized in Tables I and II.

**Prediction of Tissue Binding Utilizing Binding Parameters of Serum Albumin**—Dedrick and Bischoff (7) proposed the concept of effective protein fraction using the binding parameters of 1% bovine serum albumin and the bound fraction of rabbit organ homogenates at an initial drug concentration of 0.5 m$M$ previously reported (8). In the absence of additional information, Dedrick and Bischoff (7) combined these data to express thiopental content in various tissues as:

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

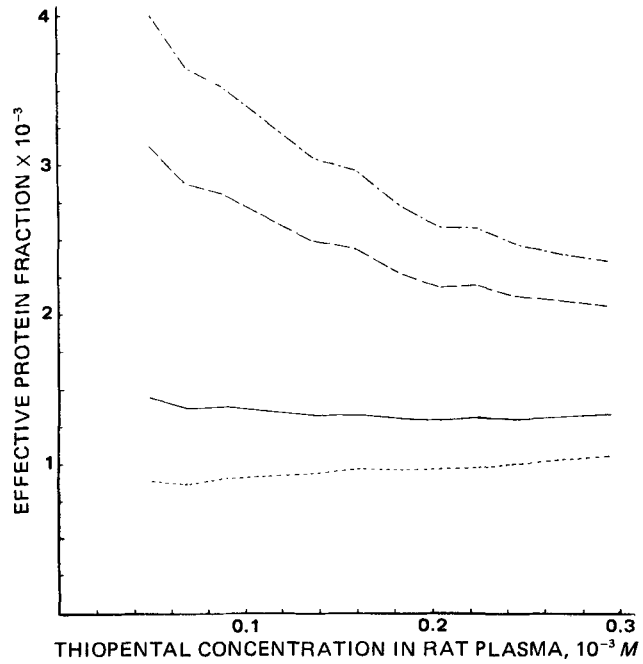


**Figure 8**—*Total thiopental concentration in rat plasma* versus *effective protein fractions* ($f_p$) *calculated according to Eq. 3 using the binding parameters of 4% (w/v) rat serum albumin at 37° (—), 1% (w/v) rat serum albumin at 4° (- - -), 4% (w/v) bovine serum albumin at 37° (- - - -), and 1% (w/v) bovine serum albumin at 37° (– –).*

**Table II—Thiopental Binding to Rat Plasma** [a]

| Temperature | Dialysis | $k_1$ [b] | $k_2$ [b] |
|---|---|---|---|
| 37° | Flow | 32.8 | 0.686 |
| 4° | Flow | 320.6 | 1.57 |
| 4° | Equilibrium | 173.3 | 1.65 |

[a] The binding capacity, predetermined and constrained (see text), was $n_1(P)$ = 0.136 and $n_2(P)$ = 2.42 (m$M$) in all cases. [b] Association constant ($\times 10^3\ M^{-1}$).

where $C_t$ is the total drug concentration [assumed to be present initially in rabbit organ homogenate, *i.e.*, 0.5 m$M$ (7)], $C_f$ is the free drug concentration, $X$ is the bound drug concentration described using the binding parameters of 1% bovine serum albumin in the form of Eq. 1, and $f_p$ is a dimensionless parameter of the effective protein fraction.

Thus, the values of the effective protein fraction for tissues were calculated at an initial drug concentration of 0.5 m$M$, but the assumption (7) that the values of the effective protein fraction were constant at all drug concentrations has not been proved. In the present study, comparable precise information on plasma and tissue binding were available, so the relationship between the effective protein fraction and the drug concentration was investigated. Figure 8 displays the $f_p$ values calculated according to Eq. 3 at various thiopental concentrations in rat plasma. The $f_p$ values based on the binding parameters of other than 4% (w/v) rat serum albumin at 37° proved to be dependent on thiopental concentrations in rat plasma. In particular, the binding parameters of bovine serum albumin gave decreasing $f_p$ values with increasing thiopental concentrations in rat plasma. Hence, a $f_p$ value calculated at one thiopental concentration in rat plasma could not readily be extrapolated to another. However, since the concept of the effective protein fraction is useful only when little information is available, the predicted thiopental binding to rat plasma or tissue homogenate based on a single $f_p$ value and on the binding parameters of serum albumin was compared with the observed data.

Figure 9 shows the simulation curves calculated according to Eq. 3 using the $f_p$ values at thiopental concentrations of 0.160 m$M$ in rat plasma (A) and 0.391 m$M$ in muscle homogenate (B). As can be expected from the results shown in Fig. 8, the simulated curves based on the binding parameters other than 4% rat serum albumin at 37° diverted slightly from the observed data (Fig. 9A). In Fig. 9B, the simulated curves based on the binding parameters of bovine serum albumin rather than of rat serum albumin seem to give better prediction for rat muscle homogenate thiopental binding. However, at low thiopental concentrations (those of pharmacological interest), a single $f_p$ value can give good prediction with both serum albumins. Therefore, the effective protein fraction should be calculated from the bound fraction of plasma or tissue homogenate samples measured within a drug concentration range (especially free drug concentration) of pharmacological interest. The $f_p$ value calculated at a pharmacologically meaningless drug concentration will give a wrong prediction when compared to the observed data for meaningful concentrations (Fig. 9B inset) and may lead to failure in construction of physiologically based pharmacokinetic models.

## DISCUSSION

As already pointed out (6), extrapolation of thiopental binding data for one concentration of bovine serum albumin to another will produce errors because of protein concentration-dependent binding. Thus, in the present study, the values of effective protein fractions (Fig. 8) decreased 42% for 4% bovine serum albumin and 35% for 1% bovine serum albumin with increasing thiopental concentrations in rat plasma. Similar results were obtained by calculating the values of effective protein fractions from the binding data of salicylate to human plasma (13) as well as the binding parameters of salicylate to human serum albumin (14). The calculated values of effective protein fractions of 1% human serum albumin at salicylate concentrations of 100 and 300 μg/ml were nearly equal, but the effective protein fractions of 0.1% human serum albumin changed twofold. If it is assumed that the association constants of 0.1% human serum albumin are equal to those of 1% human serum albumin, the effective protein fractions of 0.1% human serum albumin become nearly equal at 100 and 300 μg/ml of salicylate. Thus, the effects of protein concentration-dependent drug protein binding on the calculation of effective protein fraction will be ascribed to the change of association constants and/or the number of binding sites. Therefore, protein concentration-dependent drug protein binding greatly affects the effective protein fraction.

The present study shows that there was little, if any, temperature effect

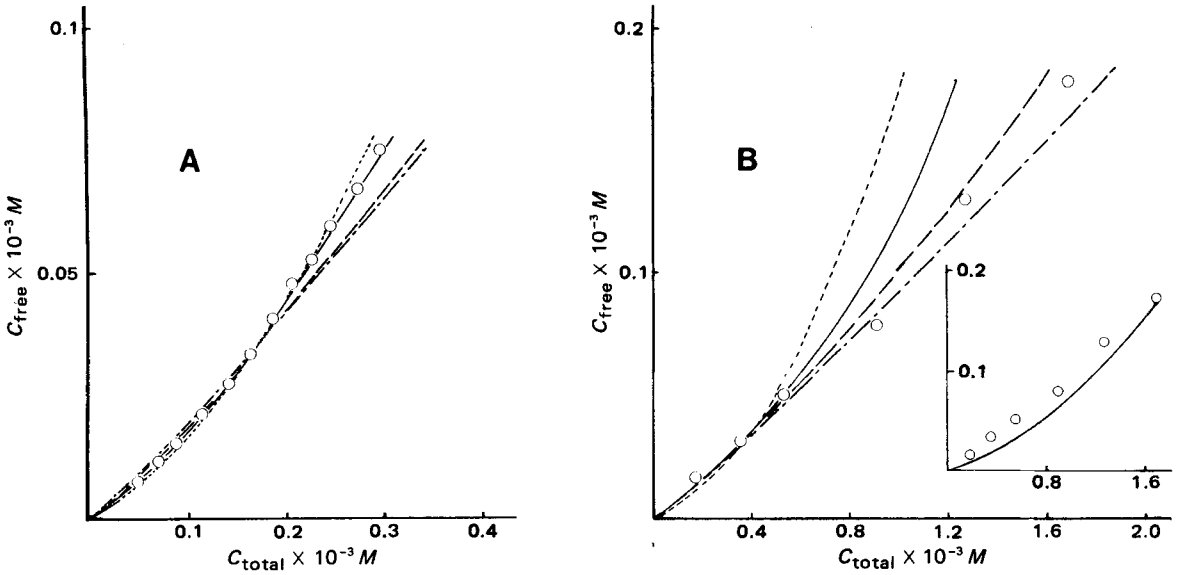


**Figure 9**—*Simulation curves calculated according to Eq. 3 using the $f_p$ values for thiopental concentration of 0.160 mM in rat plasma (A) and of 0.391 mM in muscle homogenate (B) and the binding parameters of 4% rat serum albumin at 37° (—), 1% rat serum albumin at 4° (- - -), 4% bovine serum albumin at 37° (- - - -), and 1% bovine serum albumin at 37° (– –). Each point (○) represents the mean of three experiments. [B, inset: simulation curves calculated according to Eq. 3 using the binding parameters of 4% rat serum albumin and the $f_p$ values for thiopental concentrations of 0.391 mM ($f_p$ = 0.00357) and 1.86 mM ($f_p$ = 0.00534).]*

on thiopental binding to bovine serum albumin but that temperature greatly affected thiopental binding to rat serum albumin and rat plasma. As shown in Fig. 8, because of the temperature effect on thiopental binding to rat serum albumin, the binding parameters obtained at 4° different from body temperature gave concentration-dependent effective protein fractions. Similar results are expected for the binding of salicylate to human serum albumin (5) whose association constants are highly dependent on temperature.

The binding parameters of rat serum albumin gave a better fit to the observed rat plasma binding data than those of bovine serum albumin (Figs. 8 and 9A). The binding parameters of bovine serum albumin gave a better fit to the observed muscle homogenate binding data than those of rat serum albumin (Fig. 9B). Thus, interspecies and tissue differences in drug protein binding (other than temperature and protein concentration effects) should be considered.

The concept of the effective protein fraction is not applicable to the *in vivo* situation when there is a significant protein concentration effect on drug tissue homogenate binding or when the binding profile of the tissue homogenate differs greatly from that of serum albumin. Furthermore, the homogenated tissue sample does not always reflect the *in vivo* situation. The concept of the effective protein fraction is not a perfect method for predicting tissue binding. However, since it is difficult to measure tissue binding in a direct and perfect manner at present, appropriate use of the effective protein fraction will facilitate the development of useful physiologically based pharmacokinetic models.

Consequently, *in vitro* binding experiments for predicting *in vivo* situations would be best performed at approximate body temperature, using physiological protein levels, and at free drug concentrations in the pharmacologically useful range. In addition, interspecies differences in drug protein binding should be considered.

## REFERENCES

(1) C. F. Chignell and D. K. Starkweather, *Pharmacology*, **5**, 235 (1971).

(2) D. T. Witiak and M. W. Whitehouse, *Biochem. Pharmacol.*, **18**, 971 (1969).

(3) J. N. McArthur, P. D. Dawkins, and M. J. H. Smith, *J. Pharm. Pharmacol.*, **23**, 32 (1971).

(4) C. J. Bowmer and W. E. Lindup, *Biochem. Pharmacol.*, **27**, 937 (1978).

(5) J. F. Zarolinski, S. Keresztes-Nagy, R. L. Mais, and Y. T. Oester, *ibid.*, **23**, 1767 (1974).

(6) D. Shen and M. Gibaldi, *J. Pharm. Sci.*, **63**, 1698 (1974).

(7) R. L. Dedrick and K. B. Bischoff, *Chem. Eng. Prog. Symp. Ser.*, **64**, 32 (1968).

(8) L. R. Goldbaum and P. K. Smith, *J. Pharmacol. Exp. Ther.*, **111**, 197 (1954).

(9) B. B. Brodie, L. C. Mark, E. M. Papper, P. A. Lief, E. Bernstein, and E. A. Rovenstein, *ibid.*, **98**, 85 (1950).

(10) S. P. Colowick and F. C. Womack, *J. Biol. Chem.*, **244**, 774 (1969).

(11) Y. Sugiyama, T. Iga, S. Awazu, and M. Hanano, *J. Pharmacobiol. Dyn.*, **2**, 193 (1979).

(12) T. Nakagawa and Y. Koyanagi, "SALS, a computer program for statistical analysis with least squares fitting," Library Program of the University of Tokyo Computer Center, Tokyo, Japan, 1978.

(13) A. H. Anton and W. T. Corey, *Acta Pharmacol. Toxicol., Suppl. 3*, **29**, 134 (1971).

(14) S. W. Boobis and C. F. Chignell, *Biochem. Pharmacol.*, **28**, 751 (1979).