Fluid Shifts and Other Factors Affecting Plasma Protein Binding of Prednisolone by Equilibrium Dialysis

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Abstract □ The effects of drug stability, radioactive tracer purity, buffer composition, protein concentration, and fluid shifts on the nonlinear plasma protein binding of prednisolone were examined by equilibrium dialysis. Prednisolone exhibits a concentration-dependent degradation; however, the limited extent of this does not affect protein binding. Impure tritiated prednisolone used as a tracer produces incorrect, low fractional binding values with the binding parameters generated for transcortin affected more than those for albumin. Isotonic sodium phosphate and Krebs original Ringer phosphate buffers yield similar fractional binding of prednisolone and identical protein binding parameters. Fractional binding of the steroid decreases with total plasma protein concentration, but the association constants remain constant over a twofold dilution of plasma proteins. Further dilution increases these parameters. A time-dependent colloidal osmotic fluid shift during dialysis causes dilution of plasma protein concentrations and diminished drug binding. Theoretical simulations show that the osmotic fluid shifts produce the largest changes in fractional binding for compounds that are bound by low-capacity proteins with low association constants ($K < 10^6$ M^{-1}). A mathematical equation was developed to correct bound drug concentrations and fraction bound for protein dilution caused by this effect. The fluid shifts can be prevented by the addition of dextran (mol. wt. 70,000) to the dialysis buffer in a concentration of 55% of the total protein concentration. Multiple factors can diminish the nonlinear prednisolone binding as artifacts during equilibrium dialysis, but the changes are relatively modest.

Keyphrases □ Plasma protein binding—prednisolone, equilibrium dialysis, effects of drug stability, radiotracer purity, buffer composition, and fluid shifts □ Prednisolone—plasma protein binding, equilibrium dialysis, effects of drug stability, radiotracer purity, buffer composition, and fluid shifts □ Equilibrium dialysis—plasma protein binding of prednisolone, effects of drug stability, radiotracer purity, buffer composition, and fluid shifts

Determinants of the plasma protein binding of ligands can be separated into two categories: authentic and experimental. Authentic factors include drug concentrations, plasma protein concentrations, competitors or inhibitors of binding that may be present in plasma, and other natural physiological factors such as pH. Experimental determinants are due to the manipulations that are necessary to obtain *in vitro* measurements. These factors are often characteristic of the experimental method employed. The most widely used method of determining the extent of drug plasma protein binding is equilibrium dialysis. Protein binding by this method can be affected by drug stability, radioactive tracer purity, time of equilibration, dilution, temperature, pH, buffer composition, and colloidal osmotic fluid shifts caused by plasma proteins.

The interaction of glucocorticoids with proteins and the examination of factors affecting binding have been the subject of numerous publications over the past 25 years. These steroids are bound in plasma principally by transcortin, albumin, and α_1-acid glycoprotein. Hydrocortisone, corticosterone (1), and prednisolone (2) are bound strongly to transcortin, but this protein is easily saturated due to its low binding capacity (2, 3). Albumin has a large capacity, but low affinity for binding these steroids. α_1-Acid glycoprotein exhibits both weak affinity for binding hydrocortisone (4) and prednisolone (5), as well as low binding capacity. Its contribution to overall steroid binding in plasma can be neglected (5).

Saturation of transcortin binding sites results in a nonlinear relationship between the fraction bound and the plasma prednisolone concentration. There is a decrease in the fraction of prednisolone bound at higher drug concentrations. Thus, one concern in measuring prednisolone binding by equilibrium dialysis has been the effects of dilution of the original plasma drug concentration, which shifts binding to a greater fraction bound than exists at the original *in vivo* concentration (6, 7). In addition, lower plasma albumin concentrations produce less drug binding, but increase the number of binding sites or affinity constants (*i.e.*, $N{\cdot}K$), of several drugs (8) including hydrocortisone (9, 10). The net movement of fluid from buffer to plasma that occurs during equilibrium dialysis results in a dilution of the plasma proteins. Thus, fluid shifts that occur during equilibrium dialysis may yield unpredictable changes in prednisolone binding. Equations for correcting for such dilution effects on thyroxine binding were originally proposed by Oppenheimer and Surks (11).

The composition of the buffer used in equilibrium dialysis can have a marked influence on drug binding. For example, the fractional binding of quinidine to albumin is higher when isotonic phosphate rather than Krebs–Ringer phosphate buffer is used (12, 13).

Impure radioactive tracer or degradation of ligand during dialysis may lead to inaccurate results (14). Impurities in a radioactive tracer, more likely than not, bind differently than the ligand being examined. Degradation products of an unstable ligand may also either interfere with the protein binding of the ligand itself or decrease the amount of ligand in the dialysis system. The latter also occurs if the drug binds nonspecifically to the membrane or cell.

The purpose of the present study was to assess the effects of drug stability, radioactive tracer purity, time of equilibration, buffer composition, protein concentration, and volume shifts on prednisolone binding by equilibrium dialysis. A mathematical approach to correct for protein dilution caused by fluid shifts was developed. Physically preventing fluid shifts from occurring by adding dextran to the buffer was examined. Simulations were performed to assess the expected degree of perturbation in binding caused by fluid shifts for drugs and proteins with diverse association constants.

EXPERIMENTAL

Equilibrium Dialysis—Prednisolone[1] was added to pooled human plasma, treated with charcoal to remove endogenous steroids, to yield concentrations ranging from 0 to 1000 ng/mL (2.8 μM). Total plasma protein and albumin concentrations were measured chemically[2]. Acrylic[3] dialysis cells, checked to ensure that no leakage occurred, and dialysis membrane[4] with a 12,000–14,000 molecular weight cutoff were used. Tritiated prednisolone[5] (41 Ci/mmol) was purified by HPLC (15). Plasma (0.8 mL) with trace amounts

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Technicon Auto Analyzer, Tarrytown, N.Y.
[3] Plexiglas.
[4] Spectrapor II; Spectrum Medical Industries, Inc., Los Angeles, Calif.
[5] Amersham Corp., Arlington Heights, Ill.

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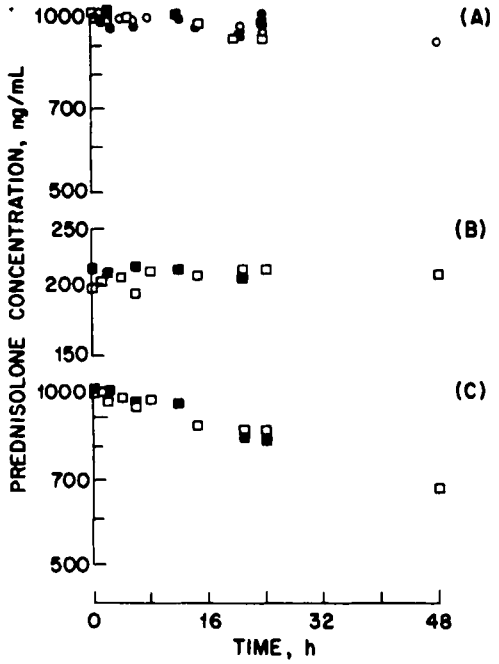

Figure 1—*Stability of prednisolone as a function of time at 37°C. Key: (A) Stability profiles for prednisolone in normal (○) and nephrotic (□) serum contained in glass tubes and normal (●) and nephrotic serum (■) in the dialysis cells. The initial concentration of each set of samples was 1000 ng/mL. (B) Stability profiles for prednisolone in normal serum contained in glass tubes (□) and normal serum in the dialysis cells (■). The initial concentration of each set of samples was 200 ng/mL. (C) Stability profiles for prednisolone in isotonic phosphate buffer, pH 7.4, contained in glass tubes (□) and in the dialysis apparatus (■) at an initial concentration of 1000 ng/mL.*

(10,000 cpm) of tritiated prednisolone was dialyzed against isotonic sodium phosphate buffer, pH 7.4, (0.8 mL) at 37°C for 16 h. Postdialysis plasma and buffer volumes were measured using a syringe[6]. Aliquots (0.5 mL) of plasma and buffer were added to water (2.5 mL) and scintillation cocktail[7] (10 mL) and counted with a scintillation counter[8]. Counts per minute were converted to disintegrations per minute using the external standard ratio method (16). Experiments were done in duplicate or triplicate.

Stability and Membrane Binding—The stability of prednisolone was assessed in normal and pooled nephrotic patient serum and in isotonic phosphate buffer, pH 7.4. Prednisolone was dissolved in each of these fluids to produce concentrations of 200 or 1000 ng/mL. Serum and buffer samples were gently shaken in glass tubes maintained at 37°C with samples of each solution taken at 0, 1, 2, 4, 6, 8, 14.5, 21, 24, and 48 h after placement into a water bath.

To determine the stability and extent of adsorption of prednisolone in the presence of dialysis membranes and dialysis cells, aliquots of the above solutions were added to the dialysis apparatus. The cells were gently shaken in a water bath at 37°C, with removal of samples at 0, 2, 6, 12, 21, and 24 h after initiation of the study.

All samples were frozen (−20°C) immediately on removal from the water bath. Prednisolone concentrations were determined in all samples using the HPLC technique of Rose and Jusko (15).

Radioactive Tracer Purity—Equilibrium dialysis experiments were carried out using tritiated prednisolone, which had been maintained in a freezer (−20°C) in benzene-ethanol (9:1) for 6 months. The material was purified by HPLC, and the dialysis experiments were repeated. The same plasma was used for both experiments.

Equilibrium Rate—The time required to obtain equilibration of free prednisolone concentrations between the plasma and buffer compartments of dialysis cells was evaluated at both low (50-ng/mL) and high (1000-ng/mL) plasma prednisolone concentrations. Plasma, with prednisolone added, was dialyzed against either isotonic sodium phosphate buffer or isotonic buffer with dextran added. At various times after the initiation of dialysis, plasma and buffer samples were removed from the cells. The volumes of the samples were measured and aliquots placed in scintillation vials for counting of the radioactive tracer. Samples were obtained over a 24-h period. Fractional binding of prednisolone was calculated at each sampling time.

[6] Unimetrics Corp., Anaheim, Calif.
[7] Aquasol; New England Nuclear, Boston, Mass.
[8] Packard.

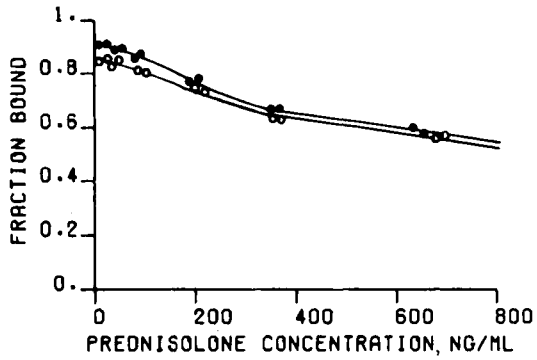

Figure 2—*Fraction bound* versus *postdialysis plasma prednisolone concentration before (○) and after (●) purification of the tritiated prednisolone tracer by HPLC.*

Influence of Buffer Composition—Krebs original Ringer phosphate, pH 7.4 (0.14 M), was prepared according to Dawson *et al.* (17). Isotonic sodium phosphate buffer, pH 7.4 (0.14 M), was composed of 0.054 M Na_2HPO_4, 0.013 M KH_2PO_4 and 0.075 M NaCl. Both buffers were prepared in distilled deionized water. Plasma, with prednisolone added, was dialyzed against the Krebs and phosphate buffers.

Effect of Protein Concentration—The total protein concentration of pooled human plasma (total protein = 5.5 g/dL, albumin = 3.7 g/dL) was defined as 100%. This plasma was diluted with various volumes of isotonic sodium phosphate buffer, pH 7.4, to yield total protein concentrations of 87.5% (4.8 g/dL), 75% (4.1 g/dL), 50% (2.8 g/dL), and 25% (1.4 g/dL). Prednisolone was added to each protein dilution (0–1000 ng/mL) and dialyzed as described previously.

Fluid Shifts—*Mathematical Correction*—The concentration of drug bound was corrected for protein dilution due to fluid shifts by:

$$D_B = (D_{Te} - D_F) \cdot \frac{V_{pe}}{V_{pi}} \qquad \text{(Eq. 1)}$$

where D_F and D_B are the free and protein-bound plasma prednisolone concentrations, D_{Te} is the total plasma prednisolone concentration at equilibrium, and V_{pi} and V_{pe} are the initial and equilibrium plasma volumes. The fraction of prednisolone bound to plasma proteins (F_B) can be calculated by:

$$F_B = \frac{(D_{Te} - D_F) \cdot \frac{V_{pe}}{V_{pi}}}{\left[(D_{Te} - D_F) \cdot \frac{V_{pe}}{V_{pi}}\right] + D_F} \qquad \text{(Eq. 2)}$$

The derivation of these equations is provided in the *Appendix*.

Dextran—Dextran[9] with an average mol. wt. of 70,000 was added to the isotonic sodium phosphate buffer to offset osmotic fluid shifts caused by plasma proteins. Various concentrations of dextran (0–5 g/dL) in buffer with prednisolone (0–2000 ng/mL) added were dialyzed against buffer to assess whether binding of prednisolone to dextran occurs. To develop an equation to predict the concentration of dextran needed to prevent fluid shifts, dextran (0–5 g/dL) in buffer was dialyzed against plasma (total protein = 5.5 g/dL),

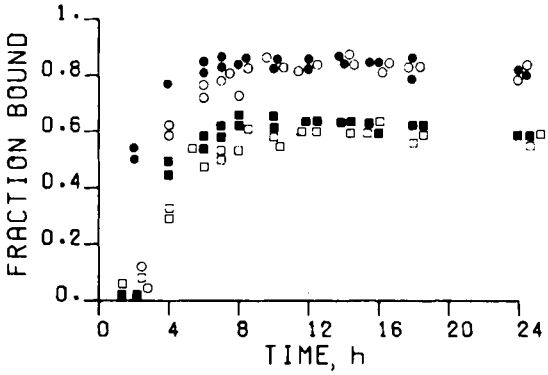

Figure 3—*Apparent fraction bound* versus *equilibration time for prednisolone plasma concentrations of 50 ng/mL (○,●) and 1000 ng/mL (□,■), dialyzed against isotonic sodium phosphate buffer (closed symbols) and phosphate buffer with dextran added (open symbols).*

[9] Pharmacia Fine Chemicals AB, Uppsala, Sweden.

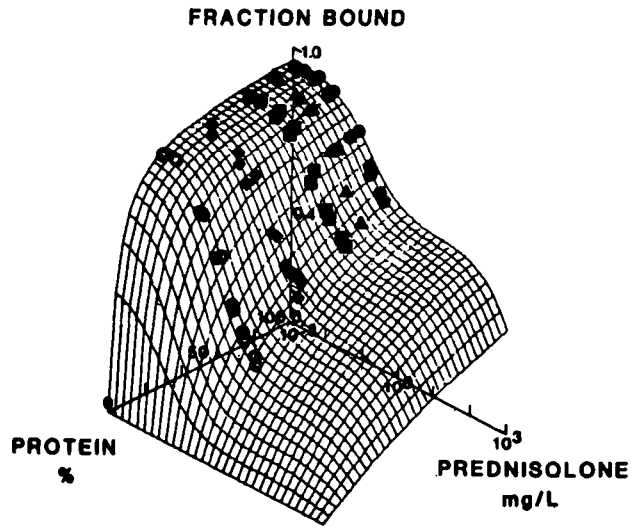

Figure 4—*Relationship between the plasma protein binding of prednisolone, unbound drug concentrations, and protein concentrations of 100% (●), 87.5% (▲), 75% (■), 50% (◆), and 25% (○).*

and postdialysis plasma and buffer volumes were measured. Mean postdialysis plasma and buffer volumes were plotted as a function of dextran concentration. The intersect of ordinary least-squares regression lines drawn through the postdialysis plasma and buffer volumes *versus* dextran concentration points occurs at the dextran concentration (3.0 g/dL) needed to offset perfectly fluid shifts caused by a total protein concentration of 5.5 g/dL. Rearrangement of the direct proportionality:

$$\frac{[\text{Dextran}]}{[\text{Total Plasma Protein}]} = \frac{3.0\ \text{g/dL}}{5.5\ \text{g/dL}} \qquad \text{(Eq. 3)}$$

yields

$$[\text{Dextran}] = [\text{Total Plasma Protein}] \times 0.5455 \qquad \text{(Eq. 4)}$$

which can be used to predict the dextran concentration required to prevent fluid shifts based on total plasma protein concentration. The predictive value of Eq. 4 was examined over a wide range of total plasma protein concentrations by employing plasma from 10 normal volunteers and 9 patients with nephrotic syndrome, a condition that results in hypoproteinemia. Total plasma protein concentrations were measured, and the appropriate dextran concentrations were determined using Eq. 4. Samples were dialyzed against buffer containing the calculated dextran concentrations, and postdialysis plasma and buffer volumes were measured. To examine the effect of dextran on prednisolone binding, plasma containing prednisolone was dialyzed against buffer in the absence and presence of dextran.

Data Analysis—When radioactive tracer methods are employed to determine the fractional binding, Eq. 2 can be written as:

$$F_B = \frac{(\text{DPM}_p - \text{DPM}_B) \cdot \frac{V_{pe}}{V_{pi}}}{(\text{DPM}_P - \text{DPM}_B) \cdot \frac{V_{pe}}{V_{pi}} + \text{DPM}_B} \qquad \text{(Eq. 5)}$$

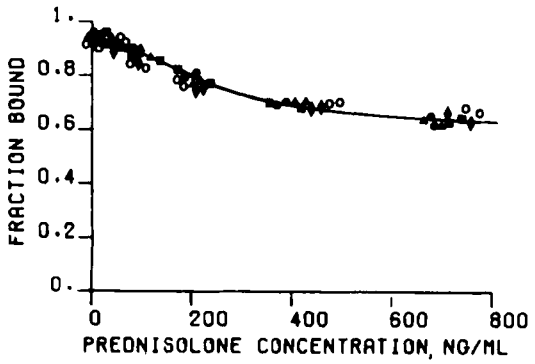

Figure 5—*Fraction of prednisolone bound* versus *postdialysis plasma prednisolone concentrations showing results of correction for protein dilutions. Symbols represent experimental data at protein concentrations of 100% (●), 87.5% (▲), 75% (■), 50% (◆), and 25% (○).*

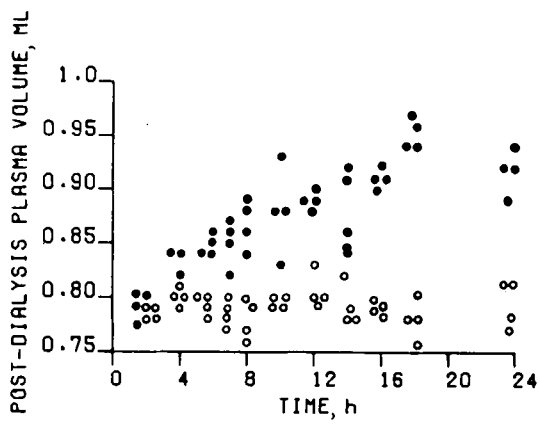

Figure 6—*Postdialysis plasma volume* versus *time where plasma was dialyzed against isotonic sodium phosphate buffer (●) and buffer with dextran (○).*

where DPM_P and DPM_B are disintegrations per minute in the plasma and buffer after background counts have been subtracted. When fluid shifts were observed, Eq. 5 was used to calculate the fraction of prednisolone bound. In the absence of fluid shifts (*i.e.*, when dextran was added to the dialysis buffer), the fraction bound was calculated by:

$$F_B = \frac{\text{DPM}_P - \text{DPM}_B}{\text{DPM}_P} \qquad \text{(Eq. 6)}$$

The plasma protein binding of prednisolone can be described by the relationship:

$$D_B = \frac{N_T K_T P_T D_F}{1 + K_T D_F} + \frac{N_A K_A P_A D_F}{1 + K_A D_F} \qquad \text{(Eq. 7)}$$

where N_T and N_A are the number of binding sites on transcortin and albumin, K_T and K_A are the association constants for transcortin and albumin, P_T and P_A are the plasma concentrations of transcortin and albumin, and D_B and D_F are the plasma concentrations of bound and free prednisolone. Least-squares estimates of $N_T K_T P_T$, K_T, and $N_A K_A P_A$ were obtained by the method of Priore and Rosenthal (18). Since P_A was measured chemically, $N_A K_A$ could be calculated. P_T was calculated assuming an N_T value of one. The total prednisolone concentration at equilibrium was generated using a variation of the methods of Behm and Wagner (6) and Rose *et al.* (7).

Statistics—The paired *t* test was used to determine whether there were any differences in the binding parameters obtained with phosphate and Krebs buffers. Regression lines for protein concentration *versus* postdialysis volumes were fitted by the weighted perpendicular least-squares method assuming error in both variables (19).

RESULTS AND DISCUSSION

Stability—The results from the stability study are presented in Fig. 1. At initial prednisolone concentrations of 1000 ng/mL, degradation in normal serum, nephrotic serum, and buffer at the end of 24 h was 6.7, 14.2, and 16.5% as determined by log-linear regression of concentration–time data. This degradation appears to be concentration dependent since no significant loss occurred in normal serum at initial drug concentrations of 200 ng/mL (Fig. 1B). The extent of this degradation does not appear to present any problem in analyzing prednisolone binding data. While the protein binding of the steroid was examined at concentrations as high as 1000 ng/mL, most samples have concentrations that were much lower and, therefore, may be subject to less degradation. There was no significant difference between the loss of prednisolone as a function of time from solutions contained in glass tubes *versus* solutions studied in the dialysis cells. This suggests that adsorption of prednisolone onto dialysis membranes and cell walls does not occur.

Radioactive Tracer Purity—The effects on binding of using aged *versus* freshly purified tritiated prednisolone tracer are shown in Fig. 2. The use of pure radioactive tracer resulted in fractional binding data higher than the data obtained using impure tracer. The binding parameters $N_T K_T P_T$, K_T, and $N_A K_A P_A$ were 5.33, $1.21 \times 10^7\ M^{-1}$, and 0.84 when impure radioactive tracer was used and 8.56, $2.16 \times 10^7\ M^{-1}$, and 0.85 when purified tracer was employed. Thus, the products of both radiolysis and of chemical degradation bind less readily, if at all, to transcortin and albumin, resulting in an increased apparent free drug concentration and incorrect, low fractional binding data. We have found that purification of tritiated prednisolone approximately every 3 months is required to obtain valid binding results.

Table I—Plasma Protein Binding Parameters for Prednisolone Related to Protein Concentration Without and With Correction for Dilution

Protein Conc., g/dL	$N_TK_TP_T$ Without Correction	$N_TK_TP_T$ With Correction	K_T, $M^{-1} \times 10^{-7}$ Without Correction	K_T, $M^{-1} \times 10^{-7}$ With Correction	$N_AK_AP_A$ Without Correction	$N_AK_AP_A$ With Correction	N_AK_A, $M^{-1} \times 10^{-3}$ Without Correction	N_AK_A, $M^{-1} \times 10^{-3}$ With Correction
5.5	11.4	13.2	2.87	3.26	0.917	1.12	1.94	2.06
4.8	11.6	14.4	3.16	3.35	0.793	1.03	1.88	1.89
4.1	8.91	12.6	2.62	3.28	0.704	1.11	1.88	2.04
2.8	6.98	13.8	3.10	4.86	0.566	1.36	2.18	2.50
1.4	4.93	15.8	4.37	7.40	0.359	1.61	2.64	2.96

Equilibration Rate—The results of the equilibration study are presented in Fig. 3. When isotonic sodium phosphate buffer is used as the dialysate, equilibration of free prednisolone occurs within 8 h and is independent of the prednisolone concentration. The inclusion of dextran in the buffer results in a slower equilibration rate (10–14 h), with the higher prednisolone concentrations requiring a longer time to attain equilibrium. This may be due to obstruction of pores in the dialysis membrane by dextran. Fractional binding values remain constant from the minimum time required to reach equilibrium of free prednisolone to at least 18 h, reinforcing our conclusion that prednisolone degradation during dialysis is minimal.

Buffer Composition—Isotonic sodium phosphate buffer and Krebs original Ringer phosphate yield similar binding of prednisolone. The mean (*SD*) $N_TK_TP_T$, K_T, and $N_AK_AP_A$ obtained were 8.59 (2.53), 1.71×10^7 (0.35×10^7) M^{-1}, and 0.92 (0.15) when isotonic phosphate buffer was used and 8.17 (3.02), 1.73×10^7 (0.48×10^7) M^{-1}, and 0.91 (0.15) when Krebs buffer was employed. There were no statistically significant differences between the binding parameters obtained using the two buffers.

These protein binding parameters agree well with previously reported values for prednisolone (2, 20–22). Chan and Slaunwhite (23) noted small decreases in the binding of hydrocortisone and progesterone to transcortin as dialysate NaCl concentrations decreased below 0.15 M. However, lower NaCl concentrations in the isotonic sodium phosphate buffer than in Krebs original Ringer phosphate (0.075 *versus* 0.118 M) did not produce this effect with prednisolone.

Protein Concentration—Figure 4 illustrates the fraction of prednisolone bound as a function of unbound prednisolone concentration and plasma protein concentration. The surface was calculated using binding parameters obtained by fitting fractional binding data from the 75–100% protein concentration range, where no differences were found in the binding parameters (see below). The data from the 25% protein concentration deviates slightly from the predicted values. Nonlinear binding is seen at all protein concentrations examined, with less fractional binding of prednisolone at lower plasma protein concentrations. The fractional binding of prednisolone diminished as the total protein concentration decreased as a result of lower plasma transcortin and albumin concentrations. At higher prednisolone concentrations, the drug is predominantly bound to albumin. Because the affinity of albumin for prednisolone is low (10^3 M^{-1}), the diminished fractional binding is proportional to the reduced protein concentration (24). At low prednisolone concentrations, where transcortin is the major protein responsible for binding prednisolone, the change in the fractional binding is less marked owing to the high affinity of transcortin for prednisolone (24). At the middle range (100–300 ng/mL) of prednisolone concentrations, the fractional binding changes are also appreciable because of the saturation of transcortin binding. Figure 5 presents the fractional binding data shown in Fig. 4 after corrections for the protein dilutions have been made. All the corrected data, except that from the 25% protein concentration, cluster together.

Decreases in albumin concentrations in albumin solutions (9, 10) and in serum (10) have resulted in increases in the binding parameter $N{\cdot}K$. Increases in α_1-acid glycoprotein concentrations, on the other hand, result in increases in the binding affinity for hydrocortisone and corticosterone (4), but not for prednisolone (5). The computer-fitted binding parameters obtained at each protein concentration, before and after correcting for plasma dilution, are listed in Table I. Without any correction, K_T remains constant over the protein concentration range of 100–50%, but increases at the 25% protein concentration. The N_AK_A values remain constant between 100 and 75% protein concentrations, but increase at lower protein concentrations. After correcting the binding data for the plasma protein dilutions, the K_T and N_AK_A values remain constant above the 75% protein concentration, but increase at lower total protein concentrations. Inspection of Table I shows that the correction for protein dilution in the protein concentration range of 75–100% does not appreciably affect K_T and N_AK_A values.

The magnitude of the increases in K_T and N_AK_A at low protein concentration is not particularly great, yet helps explain the deviations noted at the 25% protein concentration (Figs. 4 and 5). The N_AK_A for prednisolone, which does not increase until the albumin concentration has decreased to 50% ($P_A = 2.7 \times 10^{-4}$ M), appears to be more resistant to changes in albumin concentration than hydrocortisone, which shows increases in N_AK_A at albumin concentrations $<9 \times 10^{-4}$ M (10). No change occurs in K_T until protein concentrations are reduced to 25% ($P_T = 1.1 \times 10^{-7}$ M). When diluting plasma, the possibility arises that the increases seen in the binding parameters

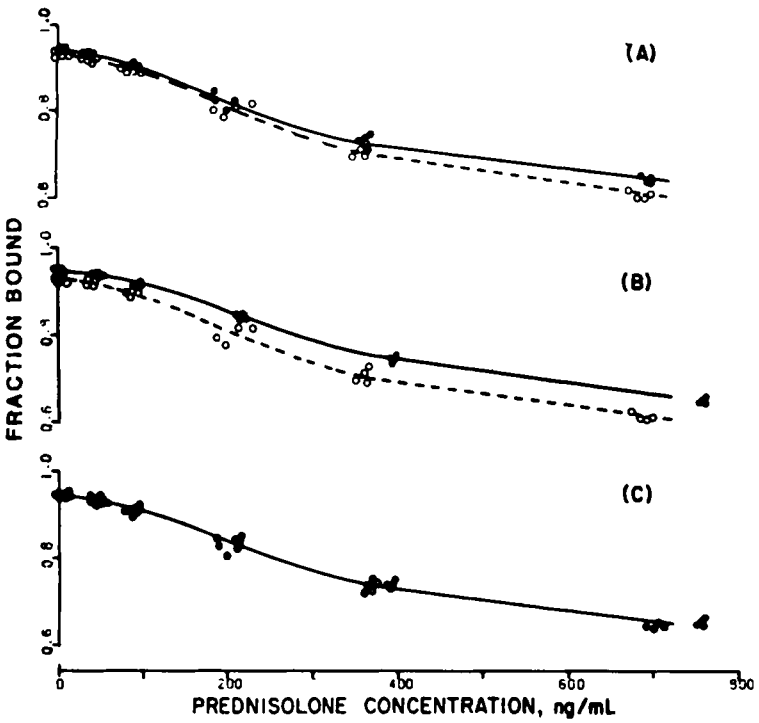

Figure 7—*Plasma protein binding of prednisolone in relation to free drug concentrations under the following conditions: (A) without (○) and with (●) mathematical correction for fluid shifts using Eq. 5, maximum difference 6%; (B) without (○) and with (●) dextran added to prevent fluid shifts; (C) with dextran added to buffer (●) and after mathematical correction (Eq. 5) for the fluid shifts that occur with buffer alone (○).*

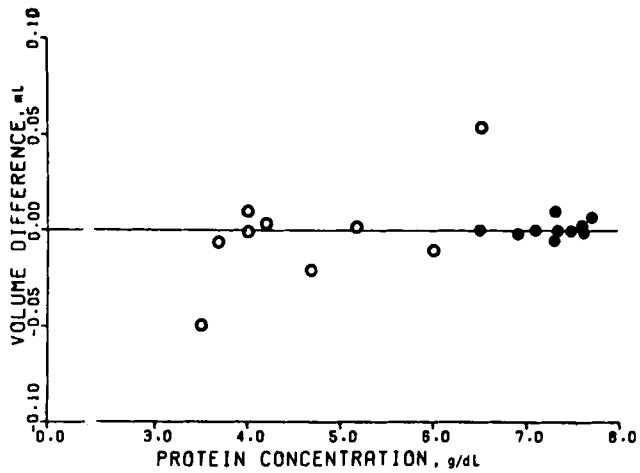

Figure 8—*Postdialysis volume differences (plasma side minus buffer side) observed when plasma containing various total protein concentrations were dialyzed against buffer, with dextran content calculated by Eq. 4. Key: (●) normal subjects; (○) patients with nephrotic syndrome; (—) no fluid shift.*

are due to lower concentrations of binding inhibitors, although these were probably removed by the charcoal. Another explanation might be that conformational changes in the albumin molecule may make available binding sites that were previously inaccessible (25). This may explain the increases in N_AK_A, but no increase occurs in the number of binding sites on the transcortin molecule. The trimer-polymer form of albumin has been shown to have a lower binding affinity for progesterone than the monomer or dimer forms (26). The binding affinity of transcortin in the rat (27) and rabbit (28) has also been shown to decrease owing to polymer formation. Whether or not human transcortin forms polymers is still being debated (29–31). A decrease in polymer formation at low protein concentrations may explain the increases seen in K_T and N_AK_A. It is important to note that K_T and N_AK_A remain constant up to a twofold dilution of the plasma proteins.

The colloidal osmotic pressure of plasma proteins tends to pull fluid into the plasma compartment of a dialysis cell, resulting in lower postdialysis protein concentrations. A decrease in the fractional binding of ligand to protein may also occur. Linear relationships were observed between postdialysis plasma ($r = 0.994, p < 0.005$) and buffer ($r = 0.966, p < 0.01$) volumes and plasma protein concentration. Fluid shifts were greatest with undiluted plasma and decreased as plasma protein concentration decreased. The decreases in protein concentration caused by these fluid shifts resulted in decreases in the fractional binding of prednisolone. As the protein binding parameters for prednisolone are constant over the range of dilutions that result from fluid shifts, this low fractional binding of prednisolone can be corrected mathematically.

Volume Shifts—*Mathematical Correction*—Equal volumes (0.8 mL) of plasma and isotonic sodium phosphate buffer were placed in each equilibrium dialysis cell. Figure 6 presents measured postdialysis plasma volumes as a function of time, showing progressive increases in the plasma volume between 2 and 14 h. Plasma volumes were static after 14 h. Mean (*SD*) plasma and buffer volumes typically found at 16 h are 0.91 (0.04) mL and 0.67 (0.04) mL, respectively. Volume shifts of similar magnitude were noted when plasma was dialyzed against Krebs original Ringer phosphate and with both hypotonic and hypertonic sodium phosphate buffers. This produces postdialysis protein concentrations that are ~86% of the initial concentrations. Equation 5 was employed to correct for this fluid shift, and Fig. 7A presents the binding results with (Eq. 5) and without (Eq. 6) this adjustment. The dilution of plasma proteins caused by fluid shifts results in lower values for the fractional binding of prednisolone. The K_T and N_AK_A were $2.83 \times 10^7\ M^{-1}$ and $2.06 \times 10^3\ M^{-1}$ without correction and $3.55 \times 10^7\ M^{-1}$ and $2.28 \times 10^3\ M^{-1}$ with correction.

Oppenheimer and Surks (11), in the first attempts to correct protein binding data for dilution of plasma proteins, corrected the free drug concentration for dilution in terms of the total volume of the dialysis system. Our method differs in that we correct the bound drug concentration for dilution of proteins using pre- and postdialysis volumes of the plasma compartment only. The bound drug concentration was corrected since this is the variable that is dependent on drug and protein concentrations (Eq. 7). Correction of the free drug concentration would appear to introduce an artifact into the system, particularly when binding is nonlinear.

Volume shifts that occur in our equilibrium dialysis system yield postdialysis protein concentrations that are ~86% of their initial value. As shown previously, protein dilutions of this magnitude have no effect on $N_TK_TP_T$, K_T, or N_AK_A. If D_F is held constant, the only changing variables in Eq. 7 are P_T and P_A. These can be corrected for by use of a dilution factor and D_B can then be calculated. This dilution factor can be expressed as the ratio of the equilibrium plasma volume (V_{pe}) to the initial plasma volume (V_{pi}). The higher D_B and F_B obtained after correction are then functions of the initial protein concentration. A dilution factor could also be calculated from directly assayed pre- and postdialysis protein concentrations. Mathematical correction for fluid shifts cannot be used if the binding parameters are highly dependent on protein concentration.

Dextran—Dextran, when added to dialysis buffer, alters the magnitude and direction of fluid shifts. Equation 4 was found to be useful for calculating the dextran concentration required to prevent fluid shifts, based on total plasma protein concentration. As shown in Fig. 8, 19 samples dialyzed against buffer containing dextran resulted in fluid shifts of <0.05 mL, while 16 resulted in shifts of <0.01 mL. No binding of prednisolone to dextran occurred.

The results of dialyzing plasma containing prednisolone against buffer alone and buffer with dextran added are shown in Fig. 7B. The fractional binding of prednisolone determined by dialyzing plasma against plain buffer is lower as a result of the dilution of plasma proteins. When dextran was added to the buffer, no fluid shifts occurred. The K_T and N_AK_A values were $2.38 \times 10^7\ M^{-1}$ and $2.06 \times 10^3\ M^{-1}$ for plain buffer and $3.54 \times 10^7\ M^{-1}$ and 2.25×10^3

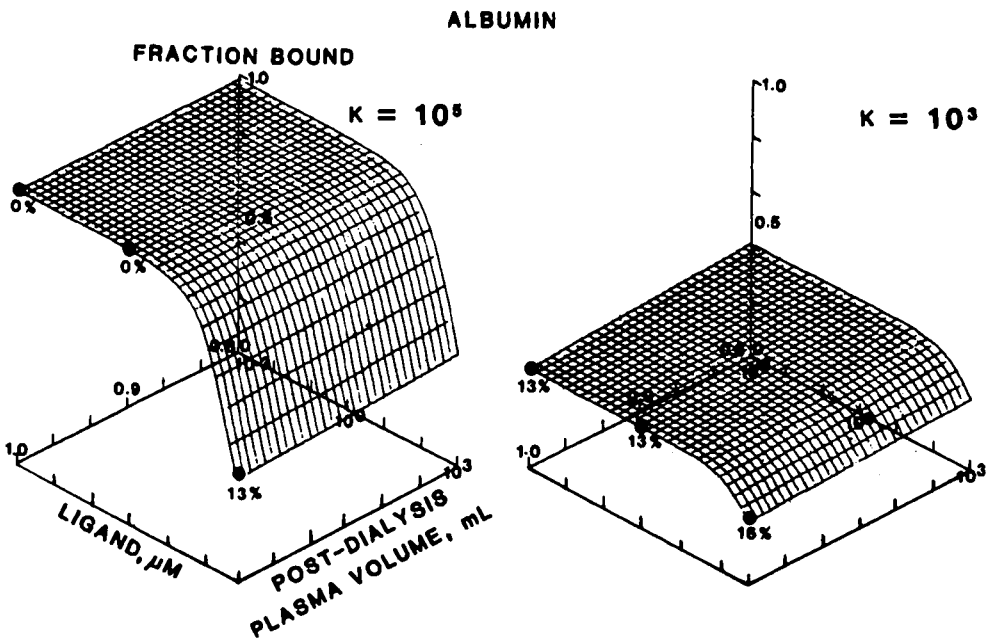

Figure 9—*Relationship between the fraction of ligand bound to albumin, free ligand concentration, and postdialysis plasma volume. Dots indicate the percent decrease in fractional binding due to volume shifts at ligand concentrations of 10^{-3}, 10^0, and 10^3 μM.*

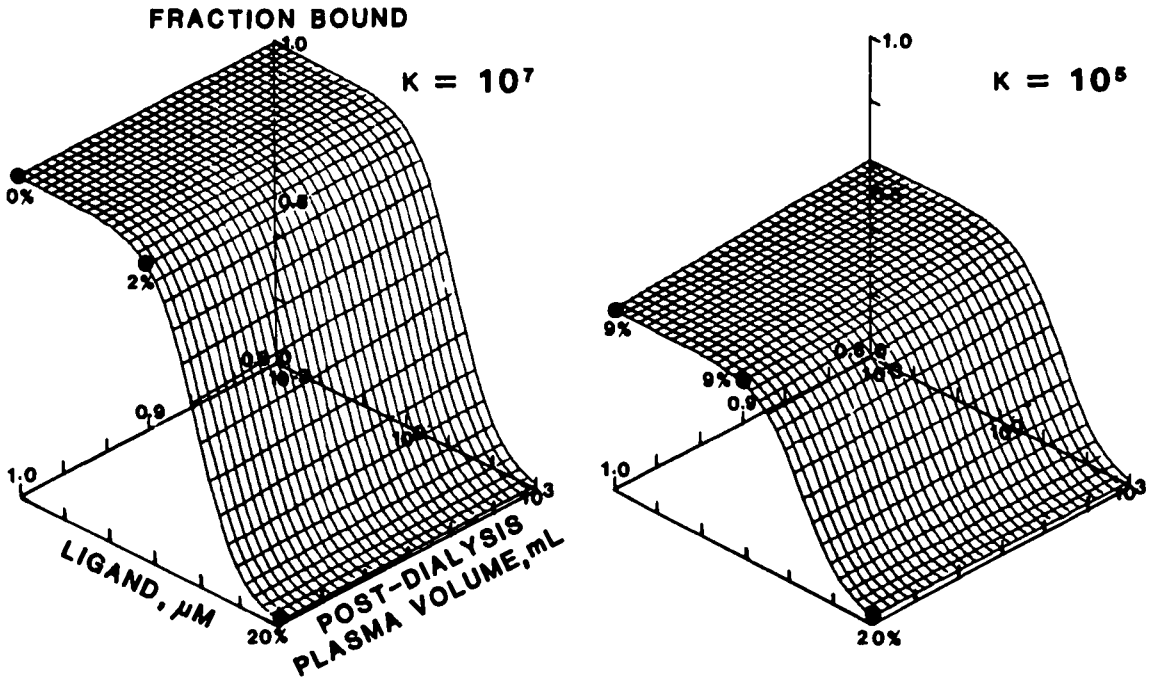

Figure 10—*Relationship between the fraction of ligand bound to α_1-acid glycoprotein, free ligand concentration, and post-dialysis plasma volume. Dots indicate the percent decrease in fractional binding due to volume shifts at ligand concentrations of 10^{-3}, 10^{0}, and 10^{3} μM.*

M^{-1} for buffer with dextran. Figure 7C shows the same data with correction for the typical volume shifts. The results with dextran and those obtained with the mathematical correction coincide on the same binding curve, although the latter are shifted slightly to the left as a result of the increased plasma volume (6, 7). The computer-fitted K_T and $N_A K_A$ were 3.54×10^7 M^{-1} and 2.25×10^3 M^{-1} for results obtained with dextran and 3.55×10^7 M^{-1} and 2.28×10^3 M^{-1} for the mathematically corrected binding data.

Dextran, when added to buffer in appropriate amounts, prevents the occurrence of any fluid shift, so that the binding results obtained are then directly related to the initial protein concentration. Inhibitors of binding also remain undiluted. The concentration of dextran (mol. wt. 70,000) required to prevent volume shifts is 55% of the total plasma protein concentration.

The use of other dialysis membranes or systems may also prevent volume shifts. Lima *et al.* (32) found that a thicker membrane with a molecular weight cutoff of 6000–8000 will diminish the fluid shift. We also examined equilibrium dialysis of prednisolone with cells that have a larger membrane-to-volume surface area[10]. Equilibration was obtained by 2 h, and no volume shifts were observed. Prednisolone binding data in these cells were identical with results obtained by mathematical correction or addition of dextran in the present system.

Calculated Effects of Volume Shifts—Simulations were employed to illustrate the effects of volume shifts on the protein binding (D_B) of ligands at various protein concentrations (P), association constants (K), and free ligand concentrations (D_F). Normal values of albumin (4.8 g/dL) and α_1-acid glycoprotein (70 mg/dL) concentrations were used to depict high and low protein concentrations. Association constants employed were 10^3 M^{-1} and 10^5 M^{-1} for albumin and 10^5 M^{-1} and 10^7 M^{-1} for α_1-acid glycoprotein. The number of binding sites per protein molecule (N) was assigned as 1 for both proteins. It was assumed that neither N nor K changed as a result of increases in the postdialysis plasma volume. The equation used for these simulations is:

$$D_B = \frac{N \cdot K \cdot P \cdot D_F \cdot \frac{V_{pi}}{V_{pe}}}{1 + K \cdot D_F} \qquad \text{(Eq. 8)}$$

where ligand concentration ranged from 10^{-9} to 10^{-3} M. Postdialysis plasma volumes (V_{pe}) ranged from 0.8 mL (no volume shift, V_{pi}) to 1.0 mL.

Simulations depicting drug binding to albumin in relation to volume changes are shown in Fig. 9. The fractional binding calculated with both association constants is linear over a wide range of ligand concentrations due to the high binding capacity of albumin. The higher association constant ($K = 10^5$ M^{-1}) yields higher fractional binding at all ligand concentrations. When $K = 10^5$ M^{-1}, an increase in the plasma volume from 0.8 mL to 1.0 mL produces <1% decrease in the fraction bound over a ligand concentration range of 10^{-3}–10^1 μM. At higher ligand concentrations, volume shifts produce progressively larger decreases in fractional binding. At a ligand concentration of 10^3 μM, a shift in plasma volume from 0.8 to 1.0 mL results in a 13% decrease (from 0.41 to 0.36) in fraction bound. When $K = 10^3$ M^{-1}, the same volume shift produces a 13% (0.05 absolute F_B) decrease in fractional binding up to a ligand concentration of 10^2 μM. A similar 16% decrease (from 0.26 to 0.22) in fractional binding occurs at a ligand concentration of 10^3 μM.

Simulations illustrating binding of ligand to α_1-acid glycoprotein are presented in Fig. 10. Saturation of this low-capacity protein at $D_F > 10^{-2}$ μM produces marked nonlinearity in the fractional binding. As expected, the higher association constant yields greater fractional binding of the drug. When $K = 10^7$ M^{-1}, volume shifts produce no change in fractional binding when ligand concentrations range from 10^{-3} to 10^{-1} μM. At a ligand concentration of 10^0 μM, the 20% increase in plasma volume results in a 2% decrease (0.94 to 0.92) in fractional binding. As the ligand concentration increases further, the percent decrease in fractional binding caused by volume shifts progressively increases. The largest absolute change in fractional binding (0.05) as a result of fluid shifts occurs at a ligand concentration of 10^1 μM. When $K = 10^5$ M^{-1}, volume shifts produce a 9% (0.05 absolute) decrease in fractional binding over a ligand concentration range of 10^3–10^1 μM. At higher ligand concentrations the absolute changes in fractional binding are extremely small.

These theoretical simulations show that changes in protein binding of a ligand are dependent on the protein concentration, the association constant, the free ligand concentration, and the magnitude of the volume shift. In general, the decrease in fractional binding is greatest with lower initial protein concentrations, lower association constants, higher free drug concentrations, and larger fluid shifts. The fluid shifts can be either corrected for mathematically or prevented with dextran.

With prednisolone, the binding results obtained by correction or dextran addition agree well. The mathematical approach requires that binding parameters remain constant and also that possible competitors or inhibitors of binding are not influenced by fluid shifts. The equations used can easily be written into a computer program. The dextran approach requires an accurate measurement of the total plasma protein concentration for each compound. For prednisolone, the fluid shifts produce a slight decrease in the fractional binding and do not present any major errors in binding results.

APPENDIX: Correction for Volume Shifts in Protein Binding

The amount of protein (AP_i) initially placed in the dialysis cell is:

$$AP_i = P_i \cdot V_{pi} \qquad \text{(Eq. A1)}$$

[10] Spectrum Medical Industries Inc., Los Angeles, Calif.

where P_i is the initial protein concentration and V_{pi} is the initial dialysate volume. After equilibration of free drug, the amount of protein (AP_e) in the plasma compartment is:

$$AP_e = P_e \cdot V_{pe} \qquad \text{(Eq. A2)}$$

where P_e is the postdialysis protein concentration and V_{pe} is the postdialysis dialysate volume. Assuming there is no loss of protein from the protein compartment:

$$AP_i = AP_e \qquad \text{(Eq. A3)}$$

and therefore:

$$P_i \cdot V_{pi} = P_e \cdot V_{pe} \qquad \text{(Eq. A4)}$$

Rearranging:

$$P_e = P_i \cdot \frac{V_{pi}}{V_{pe}} \qquad \text{(Eq. A5)}$$

In the absence of volume shifts, the concentration of bound drug (D_{Bi}) for m classes of binding sites can be predicted by:

$$D_{Bi} = \sum_{i=1}^{m} \frac{N \cdot K \cdot P_i \cdot D_F}{1 + K \cdot D_F} \qquad \text{(Eq. A6)}$$

given the number of binding sites per protein molecule (N), the association constant (K), the free drug concentration (D_F), and P_i. When volume shifts occur, the same equation applies, except for the change in protein concentration. Thus, the bound drug concentration (D_{Be}) is given by:

$$D_{Be} = \sum_{i=1}^{m} \frac{N \cdot K \cdot P_e \cdot D_F}{1 + K \cdot D_F} \qquad \text{(Eq. A7)}$$

Substituting for P_e:

$$D_{Be} = \sum_{i=1}^{m} \frac{N \cdot K\left(P_i \cdot \frac{V_{pi}}{V_{pe}}\right) D_F}{1 + K \cdot D_F} \qquad \text{(Eq. A8)}$$

If it is assumed that N and K do not vary with changes in P, then:

$$D_{Be} \cdot \frac{V_{pe}}{V_{pi}} = \sum_{i=1}^{m} \frac{N \cdot K \cdot P_i \cdot D_F}{1 + K \cdot D_F} = D_{Bi} \qquad \text{(Eq. A9)}$$

Note that the same value for the independent variable, D_F, is used with and without volume shifts. However, the measured D_F would differ from the D_F obtained had no fluid change occurred. This causes the corrected binding curve to shift up and to the right when plotting fraction bound *versus* equilibrium total drug concentration (see Fig. 7).

The fraction of drug bound at the initial protein concentration is calculated by:

$$F_{Bi} = \frac{D_{Bi}}{D_{Bi} + D_F} \qquad \text{(Eq. A10)}$$

From Eq. A9:

$$D_{Bi} = D_{Be} \cdot \frac{V_{pe}}{V_{pi}} \qquad \text{(Eq. A11)}$$

Substituting for D_{Bi}:

$$F_{Bi} = \frac{D_{Be} \cdot \frac{V_{pe}}{V_{pi}}}{D_{Be} \cdot \frac{V_{pe}}{V_{pi}} + D_F} \qquad \text{(Eq. A12)}$$

The values of D_{Te} and D_F are obtained experimentally. Since:

$$D_{Te} = D_{Be} + D_F \qquad \text{(Eq. A13)}$$

then:

$$D_{Be} = D_{Te} - D_F \qquad \text{(Eq. A14)}$$

thus:

$$F_{Bi} = \frac{(D_{Te} - D_F) \cdot \frac{V_{pe}}{V_{pi}}}{(D_{Te} - D_F) \cdot \frac{V_{pe}}{V_{pi}} + D_F} \qquad \text{(Eq. 2)}$$

as presented in the text.

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