Interaction of Dantrolene Sodium with Human Serum Albumin

J. J. VALLNER [x], L. A. STERNSON, and D. L. PARSONS

Abstract □ The binding of dantrolene sodium to human serum albumin was studied by fluorescence quenching and difference spectrophotometry. The association constant was calculated from each method of measurement and was large. This binding affinity may be of importance in the clinical setting, since competitive displacement of anionic drug by concurrently administered agents can occur. Consequently, the displacement of dantrolene from albumin was examined with a wide range of drugs. To gain insight into the characteristics of drug–albumin binding, the interaction of drug with cationic, anionic, and nonionic surfactants was also studied. Additions of drug to solutions of either the anionic or nonionic surfactant failed to result in a perturbation with the difference spectral technique. However, dantrolene added to the cationic resin produced a difference spectrum analogous to that observed with the drug–protein interaction.

Keyphrases □ Dantrolene sodium—binding to human serum albumin, fluorescence quenching and difference spectrophotometry, interaction with cationic, anionic, and nonionic surfactants □ Albumin, human serum—binding to dantrolene sodium, fluorescence quenching and difference spectrophotometry □ Fluorescence quenching—study of binding of dantrolene sodium to human serum albumin □ Difference spectrophotometry—study of binding of dantrolene sodium to human serum albumin □ Surfactants—cationic, anionic, and nonionic, interaction with dantrolene sodium □ Drug–protein binding—dantrolene sodium to human serum albumin

Most drugs are bound to serum albumin. Protein binding is an important factor in the distribution, metabolism, excretion, and activity of any administered chemical substance; only the fraction of drug unbound exerts pharmacological activity (1). The binding interaction is generally thought to occur through noncovalent forces, *i.e.*, hydrogen bonds and electrostatic, van der Waal, or hydrophobic interactions.

The binding equilibrium is often described by an association constant or constants together with the number of sites exhibiting each association constant. Such information is useful in establishing a proper dosage regimen (1). It is also of importance when two or more drugs that strongly bind to protein and compete for the same binding sites are administered concurrently. Higher free (unbound) drug concentrations are thereby produced and may precipitate a toxic response. Such increases in pharmacological activity of the displaced drug often necessitate an adjustment in dosage.

The binding to human serum albumin of a new drug should be examined, especially if the drug is an acidic molecule, because competitive binding with serum protein among acidic drugs has been shown to be particularly significant (2, 3). Recently, dantrolene sodium[1], 1-[[5-(4-nitrophenyl)furfurylidene]amino]-hydantoin sodium hydrate, a new muscle relaxant which apparently acts directly and selectively on skeletal muscle (4–6), was released for use in the United States.

Despite possessing a pKa of about 7.5 (7), dantrolene sodium has limited solubility at pH 7.4. The acidic and hydrophobic nature of the dantrolene molecule suggests that it may have a high affinity (binding constant) for serum albumin. However, the measurement of this affinity is made more difficult by the associated low solubility of dantrolene, as was demonstrated previously with various lipophilic drugs (8). The interaction of dantrolene with albumin was determined by difference spectrophotometry and fluorescence quenching measurements. Such techniques can detect small changes with suitable accuracy, and they appeared to be the most advantageous techniques for studying the interaction with protein of a drug with limited water solubility.

The propensity for other drugs to displace dantrolene from albumin binding sites also was determined from difference spectrophotometric measurements and may be of clinical importance in consideration of multiple drug therapy.

EXPERIMENTAL

Materials—The human serum albumin used had been investigated previously for purity[2] (9). Warfarin sodium[3], clofibrate[4], decamethonium bromide[5], propranolol hydrochloride[6], tolazoline hydrochloride[7], tolbutamide[8], isopropamide iodide[9], prochlorperazine[9], and ethacrynic acid[10] were used as received. Phenobarbital, secobarbital, and aspirin were USP grade; other materials were reagent grade. All solutions were prepared in deionized water.

Binding Studies—In all binding studies, the protein (1.45×10^{-6}–1.45×10^{-4} *M*) was dissolved in Sorensen's phosphate buffer (0.066 *M* NaH_2PO_4, 0.066 *M* K_2HPO_4) adjusted to pH 7.4. However, due to the solubility problems, stock solutions of dantrolene sodium were prepared (3.57×10^{-4} *M*) in deionized water adjusted to pH 9.0–10.0 with sodium hydroxide. Microliter[11] aliquots of drug were added to the protein solution, and such additions produced no more than 0.15 pH unit changes in the protein solutions as determined with a glass hydrogen-ion sensitive electrode.

For the competition studies, the displacing drug was dissolved in the same buffer solution as the protein, and again no significant changes in pH were observed during the binding studies.

Fluorescence—Solutions of albumin were prepared over the concentration range from 1.45×10^{-6} to 4.35×10^{-5} *M* at 22°, and each solution was carried through the following procedure. The fluorescent intensity of the native protein solution was recorded[12] at excitation and emission wavelengths of 289 and 357 nm, respectively. Microliter aliquots of dantrolene stock solution were subsequently added (to give a final concentration of drug from 3.6×10^{-6} to 9.22×10^{-5} *M* in the cell), and their effect on fluorescent intensity was

[1] Dantrium, Eaton Laboratories, Norwich, N.Y.

[2] Lot MX2519, 5-2526, Armour Pharmaceutical Co.
[3] Endo Laboratories, Garden City, N.Y.
[4] Ayerst, New York, N.Y.
[5] Burroughs Wellcome, Greenville, N.C.
[6] Inderal, Ayerst, New York, N.Y.
[7] Ciba Pharmaceuticals, Summit, N.J.
[8] Upjohn, Kalamazoo, Mich.
[9] SKF Laboratories, Philadelphia, Pa.
[10] Merck Sharp and Dohme Research Laboratories, Rahway, N.J.
[11] Hamilton Co., Reno, Nev.
[12] Aminco-Bowman spectrofluorometer.

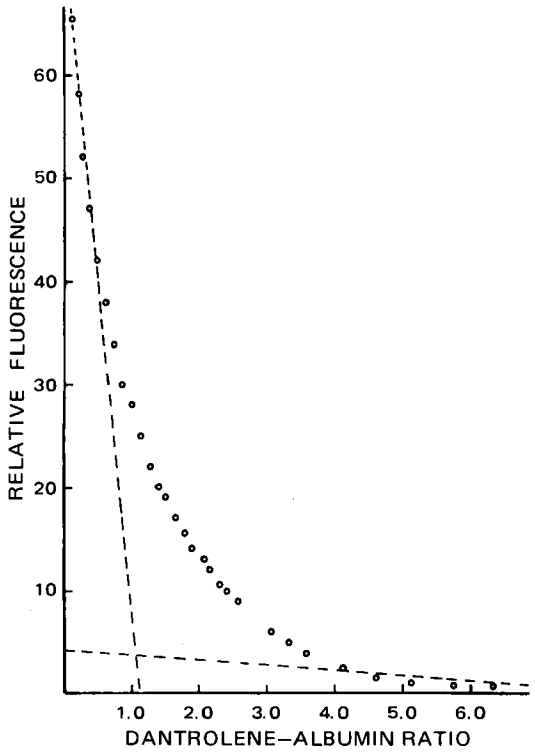

Figure 1—*Example of fluorescence quenching of human serum albumin by dantrolene. Intensity of albumin tryptophan fluorescence was monitored at 357 nm while exciting at 289 nm. Albumin concentration was 1.45 × 10⁻⁶ M.*

determined. At the selected wavelengths, the drug did not contribute to the fluorescence.

The protein concentration was kept constant throughout each quenching (binding) study so as not to contribute to the observed decreased fluorescence by having made the drug stock solution in the appropriate protein solution. Drug to protein ratios in the experiments ranged from 0.073 to 82. All dantrolene solutions were subjected to TLC on 0.25-mm silica gel 60 F-254 plates [developing solvent of chloroform–acetone (3:7)] to determine if chemical breakdown of the drug was occurring.

Experiments were repeated with all solutions and with the sample compartment maintained at 40°.

Difference Spectrophotometry—UV–visible spectra of dantrolene sodium (2.97×10^{-5} *M*) solutions were recorded[13] and showed maxima[14] at 392 nm ($a_m = 2.15 \times 10^4$) and 311 nm ($a_m = 1.40 \times 10^4$). For difference spectroscopy studies, the tandem cell technique was employed (10) in the split beam mode[13]. This technique is capable of measuring small differences from the baseline, due often to binding of the small molecule to the protein, provided that attention is paid to the particular requirement of keeping both protein and drug concentrations the same in the reference and sample compartments.

The baseline was obtained by placing the protein solution in one cell of the reference beam and buffer alone in the other cell of the reference beam; the sample compartment contained exactly the same solutions. Dantrolene was added to the buffer cell in the reference beam and the protein cell in the sample beam (at equal concentrations) to give final concentrations of drug ranging from 2.9×10^{-6} to 3.8×10^{-5} *M*.

To maintain a constant protein concentration throughout the titration, each addition of drug to the protein solution in the sample compartment was accompanied by the concomitant addition of an equal volume of albumin solution at a concentration twice that in the cell (*i.e.*, for titration of 1% albumin solutions, a 2% albumin solution was used to adjust protein concentrations). Similarly, drug concentration was maintained equally in both beams during titration by the addition of an equal volume of buffer to the cell containing drug and buffer in the reference compartment. Resulting difference spectra were subsequently recorded.

Drug–Surfactant Interactions—Difference spectroscopy studies were repeated in an identical manner to that already described, except that a 0.025% cetrimonium bromide, 0.025% sodium lauryl sulfate, or 0.1% octoxynol[15] solution was used in place of the protein.

Displacement Experiments—The competition between a number of acidic drugs for protein-bound dantrolene also was examined by difference spectroscopy. At a selected protein concentration of 3.62×10^{-5} *M* and a dantrolene concentration of 1.11×10^{-5} *M* (where it was determined that the drug was present mainly on a single class of sites), the effects on the difference spectrum after addition of various displacing drugs were examined. For this segment of the experiment, only the dantrolene–albumin difference spectrum resulting from perturbation in the visible wavelength region was investigated. The specific requirement for the variety of displacing drugs added was that they had no absorbance of their own above 340 nm.

Table I—Results of the Quenching of Native Human Serum Albumin Fluorescence by Dantrolene[a]

Albumin Concentration, *M*	Mole Ratio (*n*)	Association Constant × 10^{-5} M^{-1}
4.35×10^{-5}	0.95	3.9
2.90×10^{-5}	1.32	3.3
1.45×10^{-5}	1.68	2.3
7.25×10^{-6}	1.60	4.3
1.45×10^{-6}	1.15	5.4

[a] Excitation was at 289 nm and emission was at 357 nm in 1-cm cells at 22 ± 1°.

RESULTS

Dantrolene sodium was shown to bind to protein by the techniques of fluorescence quenching and difference spectroscopy. Fluorescence quenching measurements were based on the fact that albumin contains a single tryptophan residue that emits radiation at 357 nm when irradiated at 289 nm. Interaction of small molecules with human serum albumin, which results in a perturbation of the environment surrounding the tryptophan, is often sensed by reduction in fluorescent intensity attributed to the amino acid. Addition of dantrolene sodium (in concentrations ranging from 2.92×10^{-6} to 9.22×10^{-5} *M*) to protein solutions resulted in a quenching of the native fluorescence of the protein.

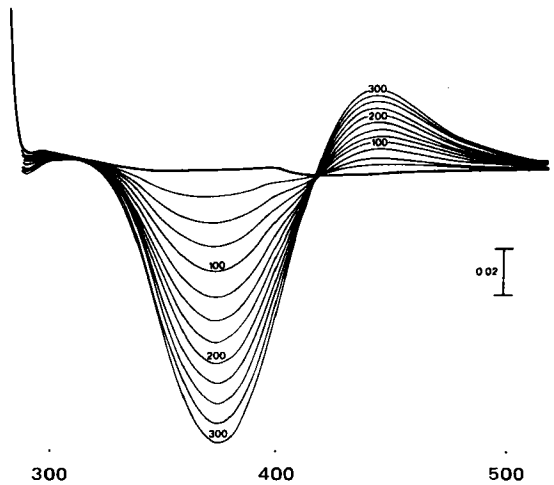

Figure 2—*Difference spectrophotometric titration of 1.45×10^{-4} M human serum albumin with dantrolene sodium. Each curve represents the addition of 25 μl of dantrolene solution (3.57×10^{-4} M) to 3 ml (initially) of buffer in the reference compartment and to 3 ml of protein in the sample compartment. The numbers 100, 200, and 300 denote microliters of dantrolene added for the difference spectrum to be obtained.*

[13] Cary 118 spectrophotometer, Cary Instruments, Monrovia, Calif.
[14] J. J. Vallner, unpublished observations.
[15] Triton X-100, Rohm and Haas.

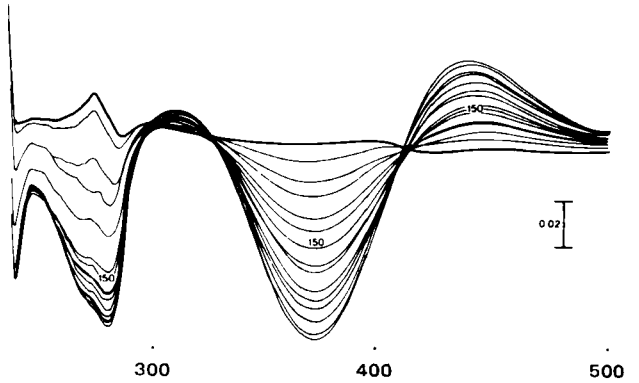

Figure 3—*Difference spectrophotometric titration of 7.25 × 10^{-5} M human serum albumin with dantrolene sodium. Interaction in the UV region is clearly indicated. (See caption of Fig. 2.)*

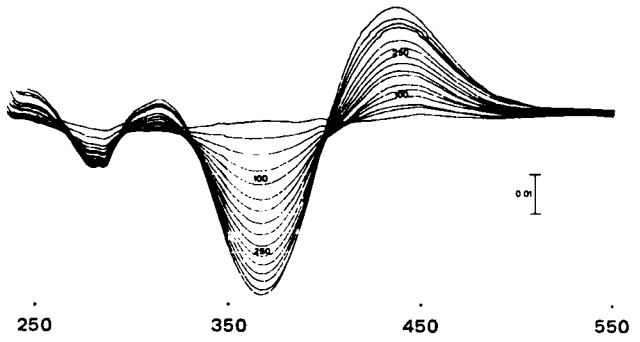

Figure 5—*Difference spectrophotometric titration of 1.45 × 10^{-5} M human serum albumin with dantrolene sodium. (See caption of Fig. 2.)*

A typical titration curve for the drug–albumin interaction is shown in Fig. 1. Extrapolation of the linear portions of these experimental titration curves gave the stoichiometric point from which the mole ratio (n) of dantrolene bound to protein was obtained (11). Association constants for the interaction of drug with protein were also calculated from the titration curves (Table I).

Quenching titrations also were performed at 40° at albumin concentrations of 1.45 × 10^{-5} and 4.35 × 10^{-5} M. At this higher temperature, the magnitude of the binding constants was reduced 30 ± 1%.

It was previously observed that chemical breakdown of dantrolene occurs in solution over 48 hr. To determine if degradation of dantrolene takes place during binding studies, all dantrolene solutions were subjected to TLC analysis upon conclusion of the experiment. Development of chromatograms with acetone–chloroform (7:3) plus 1% acetic acid revealed a single spot (R_f 0.51) attributable to the parent drug molecule. In two instances, a second, less intense spot (R_f 0.08) was also observed, representing the solution breakdown product. In these two instances, the data were discarded.

Interactions between serum albumin and dantrolene were also examined by difference spectrophotometry. Difference absorption spectra for the association of drug with protein in phosphate buffer (Figs. 2–6) are characterized by positive peaks at 443 ± 3 and 315 ± 1 nm and negative peaks at 371 ± 4 and 285 ± 3 nm. The absorption spectrum of dantrolene in buffer solution (pH 7.4) is characterized by peaks at 392, 311, and 223 nm. Families of curves were generated by the incremental addition of dantrolene to protein solutions of fixed concentration: 0.05, 0.1, 0.25, 0.5, and 1% in albumin. At the 1% protein level, difference peaks in the UV region were obscured by excessive absorption by protein.

Adequate precautions (as described under *Experimental*) were taken to maintain equivalent concentrations of drug and protein in both the sample and reference beam so that the curves must be the result of an interaction between dantrolene and albumin.

Absorbance titration curves (Fig. 7) were generated by plotting the change in absorbance (measured as difference in intensity of the 445-nm peak and 370-nm trough) *versus* the drug concentration at a constant protein level. Increasing concentrations of drug produced linearly increasing absorbance changes (r = 0.998 ± 0.001). The curve remained linear and did not level off (saturate) at higher drug concentration. The absence of a typical saturation curve (concave downward) may be due to solubility limitations rather than to the drug–albumin interaction itself. The solubility of dantrolene in aqueous solution at pH 7.4 is very low, so the drug concentration range over which binding was studied could not exceed the solubility limit (about 1 × 10^{-4} M). This concentration may not be high enough to saturate available binding sites, so a leveling off of the curve may not be observed.

Double reciprocal plots of the slope of titration curves described in Fig. 7 *versus* the protein concentration were constructed as shown in Fig. 8 and gave a straight line (r = 0.996 ± 0.001) with an intercept corresponding to the change in absorbance of dantrolene in the presence of an infinite amount of albumin. The association constant for the interaction was estimated from this plot to be 4.34 × 10^4 M^{-1}. (The amount of bound and free drug could not be determined due to linear increases in the change in absorbance *versus* added dantrolene concentration plots. Thus, the method of analysis was exactly analogous to that used in Michaelis–Menten kinetics.)

To elucidate the characteristics of the binding occurring between dantrolene and albumin, the interaction of drug with surface-active agents was studied. Difference spectra were generated as already described, except that surfactant was substituted for protein. Studies were conducted using anionic (sodium lauryl sulfate), cationic (cetrimonium bromide), and nonionic (octoxynol) surfactants at levels above the critical micelle concentration (CMC). The addition of drug to solutions of either the anionic or nonionic surfactant failed to result in a perturbation of the baseline, even at drug concentrations of 3 × 10^{-5} M. Addition of drug to the solution of cationic resin produced a difference spectrum (Fig. 9) characterized by a peak at 430 nm and a trough at 365 nm. The intensity of the spectrum was proportional to the concentration of drug added. This bathochromic shift of the dantrolene difference spectrum was analogous to the shift observed with the drug–protein interaction.

The propensity for other drugs to affect the interaction of dantrolene with albumin was also investigated by difference spectrophotometry. Studies were carried out at a constant concentration of dantrolene and protein, known to generate a significantly intense

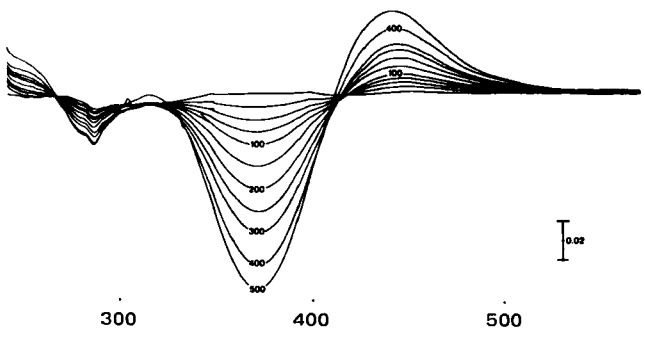

Figure 4—*Difference spectrophotometric titration of 3.63 × 10^{-5} M human serum albumin with dantrolene sodium. (See caption of Fig. 2.)*

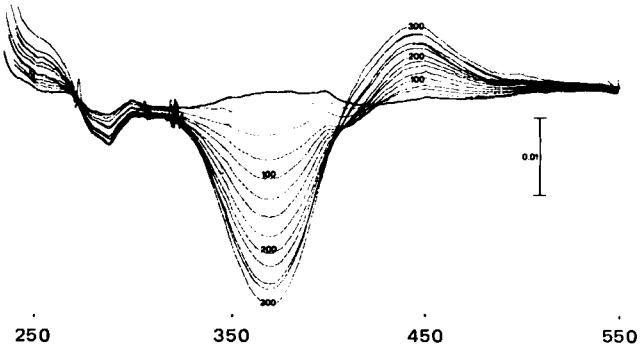

Figure 6—*Difference spectrophotometric titration of 7.25 × 10^{-6} M human serum albumin with dantrolene sodium. (See caption of Fig. 2.)*

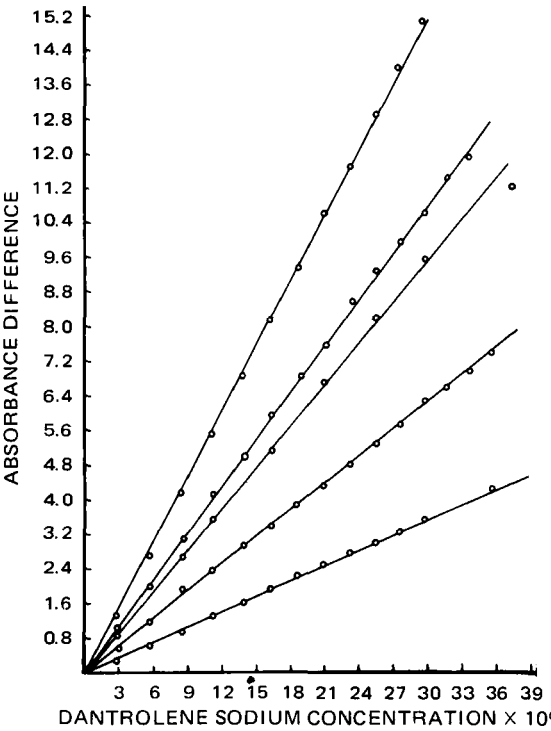

Figure 7—*Plots of absorbance difference × 10^2 (from 445-nm peak to 370-nm trough)* versus *dantrolene sodium concentration at constant protein concentrations. From the largest to smallest slope, the curves are in the order 1.45 × 10^{-4}, 7.25 × 10^{-5}, 3.63 × 10^{-5}, 1.45 × 10^{-5}, and 7.25 × 10^{-6} M human serum albumin.*

absorbance difference spectrum yet with a small enough drug to protein ratio to ensure considerable binding of dantrolene at the primary site. A drug to protein ratio of 0.32 was selected for initial studies. Displacing drug was added to both the reference beam (in buffer) and sample beam (in the dantrolene–protein solution), and the concentration of all species present was kept constant in both compartments.

The data in Table II indicate that some small, but measurable, differences (±6% changes) resulted for a number of added antagonists. These random effects are thought to be indicative of no significant displacement of the bound dantrolene. Three of the antagonists, however, did give large effects on the bound drug.

DISCUSSION

Both quenching fluorescence and difference spectrophotometric measurements support the fact that dantrolene interacts with albumin. The intensity of this interaction was calculated as an association constant from both the emission and absorption spectra. The magnitude of this constant was approximately one order of magnitude greater when calculated from quenching fluorescence data than from values obtained from difference spectrophotometric measurements. Other investigators (12) previously reported that the calculation of association constants from fluorescence measurements yielded values indicative of higher binding affinities than did values obtained by other methods.

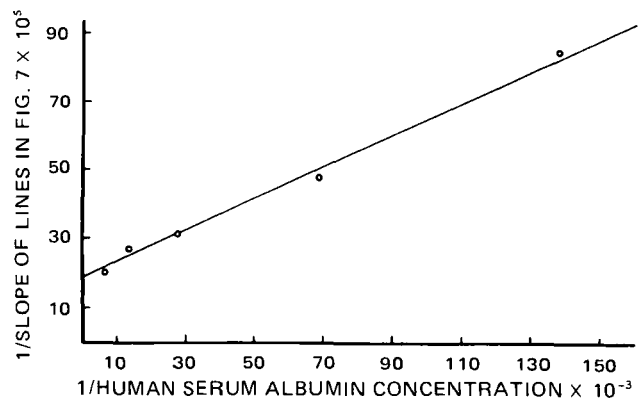

Figure 8—*Plot of reciprocal of slopes of Fig. 7* versus *reciprocal of human serum albumin concentration. (See text for explanation.)*

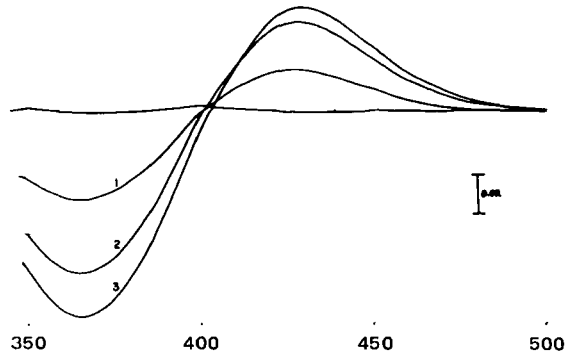

Figure 9—*Difference spectra of the interaction between cetrimonium bromide and dantrolene sodium. Cetrimonium bromide concentration was constant at 0.025%. Dantrolene concentrations were 1.11 × 10^{-5} (curve 1), 2.09 × 10^{-5} (curve 2), and 2.54 × 10^{-5} (curve 3)* M.

The native fluorescence of human serum albumin resides in a single tryptophan residue incorporated into an apparently hydrophobic region of the protein (13, 14). This region is postulated to be a high affinity site, involved in the binding of anionic drugs with the protein. Interaction of drug with this site perturbs the tryptophan environment, decreasing the fluorescent intensity of the protein. Fluorescence quenching measurements of drug–protein interactions are only sensitive to perturbations in the tryptophan residue. Secondary binding at sites remote from the fluorescent moiety are not detected.

Association constants calculated from absorption difference spectral data are more responsive to general alterations in the drug molecule environment due to interaction with protein and are, therefore, also sensitive to interactions not involving the tryptophan-containing region. Association constants calculated from difference spectral data may represent a weighted average of several binding sites. Since the tryptophan-containing region is reported to be the "high affinity site" for anionic drugs, if one assumes that additional sites have lower affinity for interaction with the drug, association constants calculated from difference spectra will be of lower value than constants obtained by monitoring fluorescence quenching.

Close examination of Figs. 2–6 suggests that dantrolene interacts with at least two sites on the protein. At a high protein concentration

Table II—Competition for Dantrolene Binding on Human Serum Albumin by a Variety of Drugs[a]

Drug Concentration, *M*	Change in Bound Dantrolene in Presence of Competition[b], %
Decamethonium bromide, 2.75 × 10^{-4}	0
Isopropamide iodide, 3.67 × 10^{-4}	0
Tolazoline hydrochloride, 3.44 × 10^{-4}	—6[c]
Prochlorperazine, 7.51 × 10^{-5}	+4
Propranolol hydrochloride, 1.10 × 10^{-4}	0
Ethacrynic acid, 1.24 × 10^{-4}	—1
Aspirin, 6.94 × 10^{-4}	—5
Secobarbital, 1.86 × 10^{-4}	+6
Phenobarbital, 2.81 × 10^{-4}	+6
Warfarin, 1.45 × 10^{-4}	—19
Clofibrate, 1.63 × 10^{-4}, 3.28 × 10^{-4}	—16, —33
Tolbutamide, 2.88 × 10^{-5}, 5.77 × 10^{-5}	+33, +44

[a] The dantrolene concentration was 1.15 × 10^{-5} *M* (where it is assumed that most of the drug is bound to the primary class of site; see *Discussion*) and the albumin concentration was 3.025 × 10^{-5} *M* (drug/protein = 0.317). [b] Each number given is an average of three separate experiments. [c] Numbers reflecting ±6% change in bound dantrolene are assumed to be indicative of no real displacement effect.

(Fig. 2), a distinct isosbestic point is maintained throughout the titration of protein with drug, suggestive of a single binding site. At lower concentrations of protein (0.25 and 0.50%), however, a more complicated family of difference spectra was recorded. Curves obtained at lower concentrations of drug pass through the isosbestic point; when the drug to protein ratio exceeded 1:3, the crossover point shifted to shorter wavelengths and the distinct isosbestic point was lost. The general shape of the difference spectra underwent no significant changes with titration by drug.

These results suggest that dantrolene interacts with a high affinity site on human serum albumin which becomes occupied at some drug to protein ratio, so additional drug must bind to secondary sites. Association constants calculated from difference spectra *(i.e.,* from plots of absorbance in the visible region *versus* concentration) then represent a weighted average of these multiple sites.

Secondary binding sites were detected also at lower protein concentrations from difference spectra generated in the UV region. Titration of albumin with drug yielded a family of spectra with troughs at 285 nm, which were saturable and contained an isosbestic point at 268 nm.

Plots of absorbance changes (445 minus 370 nm) *versus* dantrolene concentration (Fig. 7) at constant protein levels failed to show saturation of binding; *i.e.,* the curves remained linear and did not level off. This failure to observe saturation may result in part from the very limited solubility of dantrolene. Lack of saturation also may be due to multiple binding sites of equal affinity, but this possibility seems remote. Alternatively, self-association between dantrolene molecules through stacking or charge transfer complexation could account for the observed lack of saturation. Initial solvent studies carried out in these laboratories support the contention that dantrolene molecules can stack in a hydrophobic environment.

When dantrolene was dissolved in a solution containing the cationic detergent cetrimonium bromide (Fig. 9), the spectral shift was qualitatively very similar to that when drug became bound to protein. Very little effect on the difference absorption spectrum was seen at cetrimonium bromide concentrations below the CMC (0.02% w/v) (15) or in the presence of sodium lauryl sulfate or octoxynol. The shift toward longer wavelengths when dantrolene becomes bound to albumin and the effect seen when drug interacts with cetrimonium bromide suggest that the protein binding site for dantrolene consists of a cationic charge near a large hydrophobic site to accommodate the unsaturated rings.

Competition studies showed that a significant reduction of dantrolene binding occurred in the presence of the tightly bound warfarin (16, 17) and clofibrate (8), while basic drugs and the relatively weakly bound acidic drugs did not displace any dantrolene. The probability that warfarin, clofibrate, and dantrolene share some common binding site is thus enhanced. The situation with tolbutamide as the competing drug is somewhat peculiar. Tolbutamide has a binding constant of about $4–7 \times 10^4$ liters/mole (18, 19) and, as compared to clofibrate, would be expected to displace dantrolene. However, the presence of tolbutamide increased dantrolene binding. It can be speculated that tolbutamide may cause either a change in albumin conformation or a change in conformation during competition with the dantrolene molecule. Further work is underway to examine this increased binding phenomenon and the seemingly rapid breakdown of dantrolene in solution.

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ACKNOWLEDGMENTS AND ADDRESSES

Received May 30, 1975, from the *School of Pharmacy, University of Georgia, Athens, GA 30602*

Accepted for publication September 3, 1975.

The authors thank Dr. T. Higuchi and Dr. J. H. Perrin for helpful advice and discussion.

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