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### Research Article

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# Binding of Digitoxin and Some Related Cardenolides to Human Plasma Proteins

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**ABSTRACT** Tritium-labeled digitoxin, digitoxigenin, digoxin, and digoxigenin of established purity and chemical authenticity were used to study the binding of these compounds to human plasma proteins. 97% of digitoxin in plasma was nondialyzable. Continuous flow paper electrophoresis of plasma containing digitoxin and dialysis experiments in which human serum albumin competed for the glycoside with plasma or plasma protein fractions demonstrated that digitoxin was almost exclusively bound by albumin. Equilibrium dialyses revealed that the interaction was characterized by a single binding site on the albumin molecule and an association constant of  $9.62 \times 10^4$  liter/mole at 37°C. At 1°C the association constant was  $4.64 \times 10^4$  liter/mole. The interaction therefore was endothermic; the gain in enthalpy of 3.5 kcal/mole and the free energy change of  $-7.06$  kcal/mole was derived from a large change in entropy of 33.8 cal/mole per °K. The direction of these thermodynamic changes suggested the formation of a hydrophobic bond between digitoxin and albumin. Quenching of the fluorescence of albumin by digitoxin indicated that the conformation of albumin was altered by the binding process.

Digitoxigenin, its mono- and didigitoxosides, digoxin, and digoxigenin competed with digitoxin for its binding site on albumin. The affinity of the mono- and didigitoxosides for the site was equal to that of digitoxin, but that of digitoxigenin was only one-third as great. The ability of the digitoxose residues of the glycosides to enhance binding to albumin was also observed with digoxin,

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which was more extensively bound by the protein than digoxigenin.

At concentrations of 2 µg/ml or less in plasma, only 23% of digoxin was bound. Albumin, which interacted with digoxin with an apparent association constant of  $9 \times 10^2$  liter/mole at 37°C, was entirely responsible for the binding. Lowering the temperature from 37° to 1°C decreased the fraction of digoxin bound to albumin by two-thirds.

The marked difference in avidity of digitoxin and digoxin for serum albumin is reflected by the higher plasma concentrations, lower rate of urinary excretion, and longer half-time of digitoxin as compared to those of digoxin when these compounds are administered to man.

## INTRODUCTION

Interaction of digitoxin and certain other cardiac glycosides with a constituent of serum has been known since 1913 when Oppenheimer reported that the toxic effects of these compounds on the isolated frog heart were markedly attenuated when they were dissolved in rabbit, ox, or horse serum rather than in Ringer's solution (1). Subsequent investigators (2-4) demonstrated that the protective action of serum was related to binding of the glycosides by albumin, and that the globulins did not participate in this reaction.

Using equilibrium dialysis methods, Farah (4) studied the ability of serum albumin of various animal species to bind digitoxin and observed that the extent of binding varied directly with the concentration of the protein and the ligand. From his data, it can be calculated that in a system at 4°C containing 1.0 g/100 ml of human albumin and 29 µg/100 ml of digitoxin, 85% of the glycoside is bound. More recently, Spratt and Okita (5) reported failure of digitoxin-<sup>14</sup>C and -<sup>3</sup>H to migrate

with albumin during electrophoresis of plasma on starch block. On the basis of these observations and results of dialysis experiments performed with serum proteins fractionated by ammonium sulfate precipitation, they questioned the specificity and avidity of binding of digitoxin by albumin.

Additional information on the association of digitoxin with the plasma proteins is needed since, as in the case of other compounds (6-8), such an association influences the distribution, metabolism, and excretion of the glycoside in man (9). The availability of pure crystalline human serum albumin and of digitoxin-<sup>3</sup>H with a high specific activity (9) facilitated reexamination of the problem. This report concerns the specificity and nature of the interaction between digitoxin and human serum albumin and the physical constants that govern it. In the course of the investigations, data were obtained on binding to albumin of digitoxigenin, the mono- and didigitoxosides of digitoxigenin, digoxin, and digoxigenin; these data are also presented.

## METHODS

The two lots of digitoxin-<sup>3</sup>H used in these studies were prepared by tritium exchange and had specific activities of 581 and 991 mc/mole. The methods employed in preparing and purifying the compound and establishing its chemical purity and specific activity have been presented previously (9). Digitoxigenin-<sup>3</sup>H (528 mc/mole), digoxin-<sup>3</sup>H (528 mc/mole), and digoxigenin-<sup>3</sup>H (3.0 c/mole) were prepared, purified, and analyzed by similar methods. The specific activities of these compounds were determined by converting them to acetate derivatives (digitoxigenin acetate, digoxin tetraacetate, and digoxigenin diacetate) with acetic-<sup>14</sup>C anhydride of known specific activity and subsequently assaying the tritium and <sup>14</sup>C activities of the radiochemically pure derivatives in a liquid scintillation spectrometer. The unlabeled digitoxin<sup>1</sup> was 99.6% pure. Other cardenolides were obtained from commercial sources.<sup>2</sup>

Plasma was obtained from normal individuals and patients with cardiac disease being treated with digitoxin. Blood was collected in syringes moistened with heparin solution. Heparin in concentrations of 10 mg/ml was found not to interfere with binding of digitoxin by albumin.

Continuous flow paper (curtain) electrophoresis of 100 ml of fresh normal plasma to which was added 2 μg of digitoxin and 6.0 × 10<sup>6</sup> dpm of digitoxin-<sup>3</sup>H was performed in a Spinco model CP apparatus<sup>3</sup> at 4°C. The plasma was first dialyzed against 2 liters of triethylamine-acetic acid buffer at a pH of 9.0, and the dialysate was used in the electrophoretic run (10), which was carried out at 600 v and 95 ma while the plasma was applied to the paper at the rate of 1.5 ml/hour. 30 fractions were obtained. An aliquot of each was sub-

mitted to microzone electrophoresis on cellulose acetate, and the proteins were identified after staining with Ponceau S red. Fractions with similar electrophoretic patterns were combined to form 10 fractions of 8-80 ml. Electrophoresis on cellulose acetate was performed with an aliquot of each of the 10 combined fractions, and the protein in the bands was stained and estimated densitometrically. The digitoxin in 2 ml of each combined fraction was extracted with 10 ml of ethyl acetate. 5 ml of the extract was transferred to a counting vial; the ethylacetate was evaporated, and the tritium activity was assayed using a toluene phosphor.<sup>4</sup> This method of extraction results in complete recovery of digitoxin, digoxin, and their gensins from plasma and solutions of plasma proteins.

Electrophoresis on starch gel of plasma containing digitoxin-<sup>3</sup>H was performed by previously published methods (11) using hydrolyzed starch.<sup>5</sup> Half the block was stained to identify the protein bands. The other half was sectioned into 1 cm segments. Each of the segments was incubated at 37°C with an amylase preparation,<sup>6</sup> and the contained digitoxin was extracted with 10 volumes of purified dichloromethane (9). The dichloromethane was washed in sequence with 1/10 volumes 0.1 N NaOH and 1/10 volumes 0.1 M acetic acid before its tritium content was determined.

Equilibrium dialysis was performed with bags 5 cm in length made from cellulose tubing,<sup>7</sup> 27/32 inch diameter when inflated. The bags were washed with 0.05 M Tris-0.1 M NaCl buffer at pH 7.4 and stored in the buffer at 4°C until used. 3 ml of plasma or a solution of human serum albumin in Tris-NaCl buffer containing 40,000 dpm of digitoxin-<sup>3</sup>H/ml was pipetted into a bag, which was placed in a 125 ml Erlenmeyer flask containing 25 ml of Tris-NaCl buffer and digitoxin. The concentrations of the glycoside ranged from 0.01 to 12 μg/ml; in a typical run, dialysis with each of six concentrations was performed in triplicate. Albumin concentration was varied, but in experiments designed to obtain data regarding association constant and binding sites, a concentration of 6.9 mg/ml was used in order to minimize osmotic effects and to provide an analytically favorable difference at equilibrium between tritium concentration in the bag and in the dialysate.

The flasks were stoppered with silicone rubber stoppers, and were shaken mechanically in a water bath at 37°C for 20-24 hr. Preliminary experiments with systems containing only buffer and digitoxin-<sup>3</sup>H either inside or outside the bag demonstrated that dialysis attained equilibrium within 14 hr. At 1°C equilibrium was established in 20 hr. Unless stated otherwise all dialyses were performed at 37°C and with a 0.15 M Tris-NaCl buffer at pH 7.4.

Human serum albumin was obtained from two commercial sources. One of these preparations<sup>8</sup> was recrystallized four times, and has been shown to contain 1.83-2.50 moles of free fatty acids per mole of albumin (12). The other preparation,<sup>9</sup> also crystallized, contained 0.06-0.74 moles of fatty acids per mole of protein (12). The albumin in each new lot was analyzed by electrophoresis on starch gel to exclude the presence of protein contaminants before it was used. Immunoelectrophoresis of each preparation revealed a single, clean-cut

<sup>1</sup> Kindly supplied by Dr. James A. Dingwall, Squibb Institute for Medical Research, New Brunswick, N. J.

<sup>2</sup> Digitoxigenin, digitoxigenin monodigitoxoside, and digitoxigenin didigitoxoside were obtained from Boehringer Mannheim Corp., New York, N. Y.; digoxin ("Lanoxin"), from Burroughs Wellcome & Co., Tuckahoe, N. Y.; digoxigenin, from K & K Laboratories Inc., Plainview, N. Y.

<sup>3</sup> Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.

<sup>4</sup> Liquifluor, Pilot Chemicals, Inc., Watertown, Mass.

<sup>5</sup> Starch-Hydrolyzed, Connaught Medical Research Labs., Toronto, Canada.

<sup>6</sup> Cotazym, Organon, Inc., West Orange, N. J.

<sup>7</sup> Dialyzer tubing, Arthur H. Thomas Co., Philadelphia, Pa.

<sup>8</sup> Lot Nos. 7749, 6941, and 9216, Nutritional Biochemicals Corp., Cleveland, Ohio.

<sup>9</sup> Lot Nos. 9 and 23, Pentex Inc., Kankakee, Ill.

precipitin arc corresponding to albumin and no evidence of other plasma proteins. By reweighing after storage in a vacuum dessicator for 5 wk, moisture content of the albumin was found to be 2% or less.

At the conclusion of dialysis and after the contents of the flasks reached room temperature, 2 ml of the bag solution and 2 ml of the dialysate were each extracted with 10 ml of ethyl acetate. 5 ml of extract was pipetted into a counting vial, and after the ethyl acetate was evaporated with the aid of a heating lamp and fan, tritium content was determined. The standard undialyzed protein solution that was used to prepare the bags was similarly assayed. Ethyl acetate extracts of solutions of albumin and of Tris-NaCl buffer produced no quenching of tritium activity when added to known quantities of digitoxin-<sup>3</sup>H.

In some experiments, digitoxin in ethyl acetate extracts of bag contents and dialysate was also measured by the *m*-dinitrobenzene color reaction (9). No significant or systematic discrepancies between the isotopic measurements and the colorimetric data were encountered, and the calculated values for fraction of digitoxin bound agreed within 1%. To check on intactness of the digitoxin, randomly selected extracts were dried and chromatographed on paper in a solvent system containing benzene, methanol, and water (4:2:1; v/v) for 6 hr or cyclohexane, dioxane, methanol, and water (4:4:2:1) for 24 hr (9). Radioscans of the chromatograms showed single peaks corresponding to digitoxin. In some instances, a quantity of digitoxin-23-<sup>14</sup>C was added to part of the extracts before they were dried and chromatographed.<sup>10</sup> The area of the chromatogram containing digitoxin was eluted and assayed for <sup>3</sup>H and <sup>14</sup>C. The tritium activity, when corrected for losses on the basis of recovered <sup>14</sup>C, agreed closely with the directly measured tritium content of the extracts. These experiments further established the absence of quenching and again indicated that the digitoxin-<sup>3</sup>H was not chemically degraded during dialysis. Randomly selected dialysates were analyzed for protein with Folin-Ciocalteu phenol reagent (14); these analyses revealed no leakage of protein from the bag except in the rare instances of obvious loss of bag contents.

Only data derived from dialysis flasks that yielded recovery of tritium within 95–105% were used. An average recovery of 97% indicated that adsorption of digitoxin by the bag was slight if it occurred at all. The fraction of digitoxin within the bag that was bound by protein was calculated as follows: fraction bound = (cpm/ml bag – cpm/ml dialysate)/cpm per ml bag. From the fraction bound and the concentrations of tritium activity within the bag and within the dialysate, the molar concentration of unbound digitoxin, *A*, and the moles of digitoxin bound per mole of albumin,  $\bar{v}$ , were calculated. The molecular weight of albumin was regarded as 69,000 (15). The association constant, *k*, for the interaction of digitoxin and a single binding site on the albumin molecule and *n*, the number of binding sites for digitoxin on the albumin molecule, were determined by solving the equation (16, 17):  $\bar{v}/A = kn - k\bar{v}$ . This was accomplished graphically as advocated by Scatchard (16) by plotting experimentally derived values for  $\bar{v}/A$  against corresponding values for  $\bar{v}$  and extending the line defined by the points to intersect the axes of the graph. At  $\bar{v}/A = 0$ ,  $\bar{v} = n$ , and at  $\bar{v} = 0$ ,  $\bar{v}/A = kn$ .

The physical constants governing the interaction were determined by standard means (17). The free energy of

binding,  $\Delta F^\circ$ , was calculated from the relationship:  $\Delta F^\circ = -RT \log_e k$ , in which *R* is the gas constant (1.987 cal/degree per mole); and *T* is the temperature in °K. The enthalpy change,  $\Delta H^\circ$ , was calculated from the observed association constants, *k*<sub>1</sub> and *k*<sub>2</sub>, at two different temperatures, *T*<sub>1</sub> and *T*<sub>2</sub>, by the van't Hoff formula:  $\log_e (k_2/k_1) = \Delta H^\circ/R (1/T_1 - 1/T_2)$ . The entropy change,  $\Delta S^\circ$ , was derived from the equation:  $\Delta F^\circ = \Delta H^\circ - T \Delta S^\circ$ .

To study the effects of pH on binding, a 0.15 M phosphate buffer at pH 6.0 and 0.15 M Tris-NaCl buffer at pH 9.0 were substituted for the usual Tris-NaCl buffer. The effects of possible competing ligands, digitoxose,<sup>11</sup>  $\beta$ -angelica lactone,<sup>12</sup> which is structurally identical to the lactone ring of the cardenolides, and other cardenolides were assessed by adding the competing substance to the buffer outside the bag. The  $\beta$ -angelica lactone was distilled at 10 mm Hg before use; the fraction boiling at 87°C was collected. Because it was found that penicillin and streptomycin slightly interfered with binding of digitoxin by albumin, these agents were not added to the dialysis flasks in any of the experiments reported. The possible influence of the fatty acids bound to serum albumin on the binding of digitoxin was investigated by using albumin freed of fat by the isoctane-acetic acid extraction method of Goodman (18) and by the charcoal extraction method of Chen (12).

The methods used to study binding of digitoxigenin, digoxin, and digoxigenin to serum albumin were similar to those used for digitoxin except for the use of diatol<sup>13</sup> as the phosphor in assaying tritium activity of digoxin-<sup>3</sup>H and digoxigenin-<sup>3</sup>H. Equilibration times for these compounds were similar to those for digitoxin.

Optical rotary dispersion was determined with a Cary model 60 spectropolarimeter using a cell with a 0.2 mm light path. Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer.

## RESULTS

*Fraction of digitoxin bound in plasma.* Dialyses at pH 7.4 and 37°C of multiple samples of fresh normal plasma containing added digitoxin ranging in concentration from 0.01 to 12  $\mu$ g/ml demonstrated that 97% of the glycoside in the plasma was in bound form. An identical value was found with plasma from two patients who had been maintained on the drug and whose plasma digitoxin concentrations were 1.0–1.9  $\mu$ g/100 ml. The total protein concentration in the plasma samples was 7.1–7.3 g/100 ml, and the albumin concentration was 4.7–4.9 g/100 ml. Although most of these dialyses were performed with 0.15 M Tris-NaCl buffer as dialysate, the use of a Krebs-Henseleit buffer at pH 7.4 (19) produced no change in the results.

*Albumin as the major digitoxin-binding protein in plasma.* The results of continuous flow paper electrophoresis of plasma containing digitoxin-<sup>3</sup>H are presented in Fig. 1. The content of digitoxin in the various combined fractions closely paralleled the content of albumin.

<sup>11</sup> Nutritional Biochemicals Corp., Cleveland, Ohio.

<sup>12</sup> K & K Laboratories, Inc., Plainview, N. Y.

<sup>13</sup> A mixture of 500 ml toluene, 500 ml dioxane, 300 ml methanol, 104 g naphthalene, 6.5 g 2,5-diphenyloxazole, and 130 mg *p*-bis[2-(5-phenyloxazolyl)]benzene.

<sup>10</sup> Digitoxin-23-<sup>14</sup>C was a kind gift of Dr. G. Rabitzsch, Deutsche Akademie der Wissenschaften zu Berlin, Berlin-Buch, Germany.

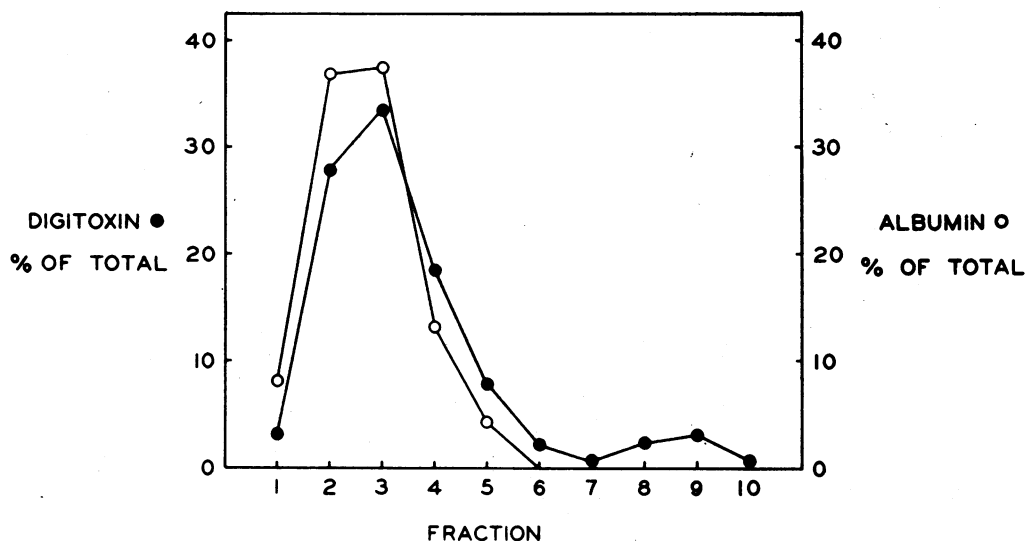


FIGURE 1 Results of continuous flow electrophoresis on paper of 100 ml of plasma containing 4  $\mu\text{g}$  of digitoxin- $^3\text{H}$  demonstrating migration of the glycoside with albumin. The amounts of digitoxin (closed circles) and albumin (open circles) in the fractions are expressed as per cent of total recovered digitoxin and albumin. Each fraction was a combination of three adjacent subfractions that exhibited a similar protein pattern on microzone electrophoresis on cellulose acetate. Anode to left.

Fractions 1-5 contained all the albumin and 91% of the digitoxin. Fractions 6-7 contained  $\alpha$ -,  $\beta$ -, and  $\beta_2$ -globulins and 3% of the digitoxin. Only  $\gamma$ -globulins were present in fractions 8-10 which contained the remaining 6% of the glycoside.

Electrophoresis on starch gel demonstrated a strikingly different pattern. The proteins migrated in the usual manner, but the digitoxin remained at the origin and fanned out slightly to either side of it (Fig. 2). The albumin band contained almost no digitoxin.

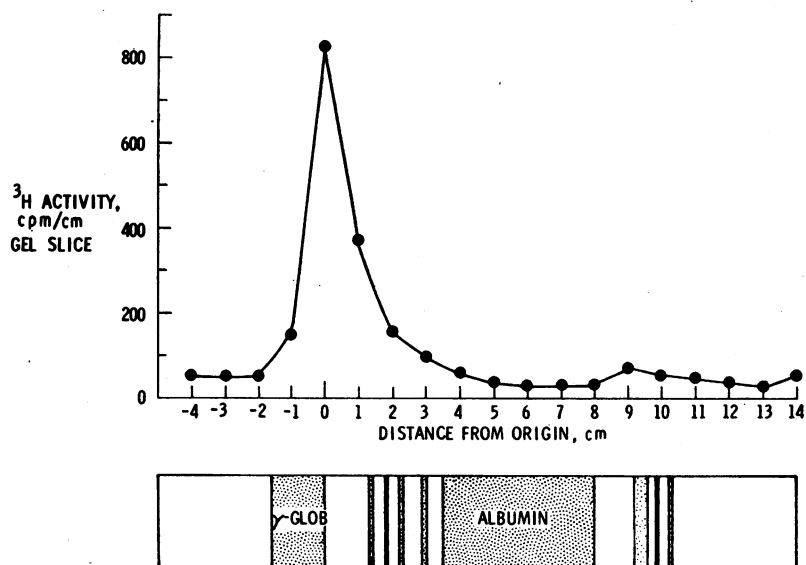


FIGURE 2 Results of electrophoresis on starch gel of 0.4 ml of plasma containing digitoxin- $^3\text{H}$  at a concentration of 2.7  $\mu\text{g}/100$  ml. The starch block was cut in two; half was stained for identification of proteins (lower panel). The other half was cut into 1 cm segments, and tritium content of each slice was measured (upper panel). Anode to right.

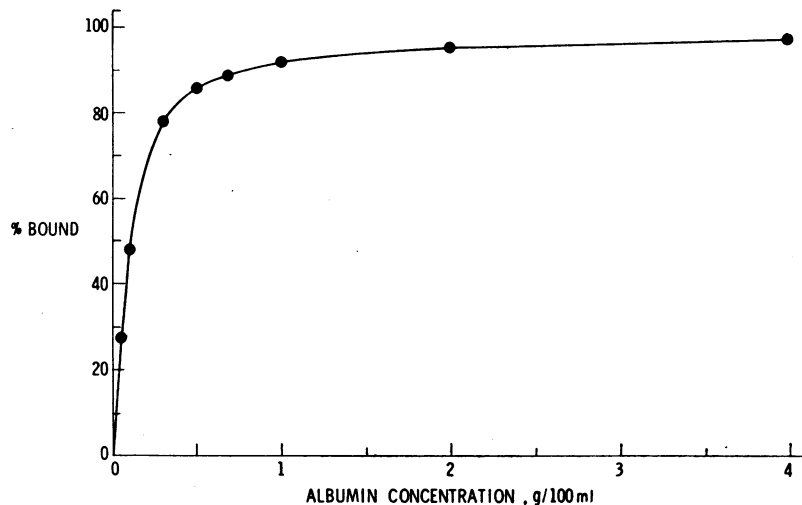


FIGURE 3 Relationship between the fraction of digitoxin bound to human serum albumin<sup>8</sup> and the concentration of albumin. The dialyses were performed at 37°C, pH 7.4, and with digitoxin, 2  $\mu\text{g/ml}$ , in the dialysate.

The possibility that the disparity in the results of the two types of electrophoresis was related to an affinity of digitoxin for the starch gel was investigated by allowing human serum albumin and starch gel to compete for the glycoside in a dialysis system. The bag contained digitoxin-<sup>3</sup>H and albumin at a concentration of 4.0 g/100 ml, and the dialysate consisted of starch gel that had been diluted with an equal volume of Tris buffer to prevent setting. After equilibration, the concentration of digitoxin in the bags was 28% less than in starch-free control systems, and in the dialysate it was almost three times greater. With lower concentrations of albumin, displacement of digitoxin from the bags was even greater.

Additional data on the avidity of albumin for digitoxin relative to other plasma proteins was obtained by competitive dialysis in which the bag contained albumin at a concentration of 4.2 g/100 ml and the dialysate contained plasma protein fractions (Cohn) adjusted to concentrations approximating those in normal plasma (20) and 4  $\mu\text{g}$  of digitoxin/100 ml. In none of the systems containing Cohn fractions was the concentration of glycoside in the bag lowered by more than 5% of that in similar systems containing no protein in the dialysate. Relative to the amount bound by albumin, concentrations of digitoxin bound by the various Cohn fractions were as follows: fraction II, 0.3%; fraction III, 0.6%; fraction III-0, 1.5%; fraction IV-1, 1.8%; fraction IV-4, 1.1%. It is of importance in interpreting the data that all the fractions except fraction II contain significant amounts of plasma albumin.

When normal plasma diluted with Tris-NaCl buffer (dialysate) was dialyzed against a solution of albumin

(bag), the distribution of digitoxin in the systems (initial concentration: 2  $\mu\text{g/ml}$ ) between bag contents and dialysate was related entirely to the concentration of albumin on either side of the membrane.

*Quantitative aspects of the interaction between digitoxin and albumin.* The fraction of digitoxin bound by albumin varied with the concentration of the protein (Fig. 3). At a concentration of 4 g/100 ml, 97% of the glycoside in a milliliter of the protein solution was bound, and even at an albumin concentration of 1 mg/ml, 50% was bound.

Fig. 4 is a Satchard plot of representative data from multiple dialyses at 37°C and pH 7.4 with the albumin<sup>8</sup> containing 1.83–2.50 moles of free fatty acid per mole. The regression line derived from the data (21) intersected the  $\bar{v}$  axis at 1.13 (SE:  $\pm 0.06$ ) and the  $\bar{v}/A$  axis at  $9.62 (\pm 0.18) \times 10^4$ , indicating a single major binding site for digitoxin on the albumin molecule and an association constant of  $9.62 \times 10^4$  liter/mole. Because of the limited solubility of the glycoside and consequent invariable decrease in recovery when its concentration in the dialysate exceeded 12  $\mu\text{g}/100$  ml, it was not possible to obtain values for  $\bar{v}$  greater than 0.67. The possibility that additional points with higher values for  $\bar{v}$  might have revealed the existence of additional binding sites with weaker affinity for digitoxin could, therefore, not be excluded.

Lowering the temperature resulted in progressive decrease in binding (Table I, Fig. 5). The free energy change,  $\Delta F^\circ$ , for the reaction at 37°C was  $-7.06$  kcal/mole. From the changes in  $k$  with temperature, changes in enthalpy,  $\Delta H^\circ$ , and entropy,  $\Delta S^\circ$ , associated with the reaction were calculated. The value of 3.43 kcal/mole for

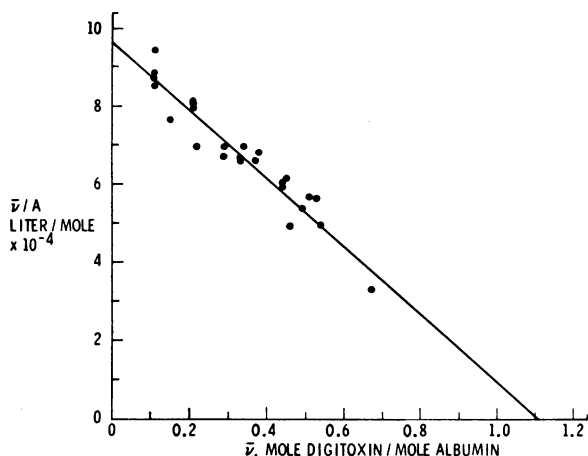


FIGURE 4 Scatchard plot of data obtained from equilibrium dialyses of digitoxin and human serum albumin<sup>8</sup> at pH 7.4 and 37°C. Each point represents the average value of three or more dialyses.  $\bar{v}$  denotes the number of moles of digitoxin bound per mole of albumin; A is the concentration of unbound digitoxin in mole per liter. The intercept of the regression line on the  $\bar{v}$  axis represents the number of binding sites for digitoxin on the albumin molecule; the intercept on the  $\bar{v}/A$  axis is  $kn$ , the product of the association constant and the number of binding sites.

$\Delta H^\circ$  calculated with data from the temperature decrement 37°–1°C agreed closely with value of 3.54 kcal/mole calculated with data from the decrement 37°–20°C. Since  $\Delta H^\circ$  was positive, the change in free energy was derived from a large change in entropy; the value for  $\Delta S^\circ$  at 37° was 10.49 kcal/mole or 33.8 cal/mole per °K. The reaction, therefore, was spontaneous (negative  $\Delta F^\circ$ ), endothermic (positive  $\Delta H^\circ$ ), and was associated with a large gain in entropy.

The albumin<sup>9</sup> preparation with a lower fatty acid content manifested significantly greater affinity for digitoxin; at 37°C,  $k$  was  $11.37 (\pm 0.18) \times 10^4$  and the value for  $n$ , the number of binding sites, was  $0.94 \pm 0.03$ . An approximately similar degree of decreased affinity for the glycoside with decreasing temperature was observed, and values for  $\Delta H^\circ$  and  $\Delta S^\circ$  were in close agreement with those determined for the albumin that contained more fat (Table I).

Because of the positive enthalpy and large change in entropy associated with binding, evidence of a conformational change in the albumin was suspected. The optical rotary dispersion spectrum of a solution of albumin,<sup>9</sup> 1 mg/ml in Tris-NaCl buffer at pH 7.4, revealed the anticipated Cotton effect with a peak of dextrorotation at 198  $m\mu$  and maximum levorotation at 233  $m\mu$  (12, 22). Addition of digitoxin (final concentration: 12  $\mu\text{g}/\text{ml}$ ) to the albumin solution produced no changes in the optical rotation of the protein.

The fluorescence spectrum of albumin has been shown

TABLE I  
Physical Constants for Interaction of Digitoxin with Human Serum Albumin

	Temperature °C	Albumin preparation	
		A*	B†
$k$ , liter/mole $\pm \text{SE} \times 10^{-4}$	37	$9.62 \pm 0.18$	$11.37 \pm 0.18$
	20	$6.89 \pm 0.09$	$8.74 \pm 0.21$
	1	$4.64 \pm 0.11$	$6.20 \pm 0.18$
$n \pm \text{SE}$	37	$1.13 \pm 0.06$	$0.94 \pm 0.03$
	20	$1.20 \pm 0.09$	$1.02 \pm 0.07$
	1	$1.02 \pm 0.10$	$1.08 \pm 0.11$
$\Delta F^\circ$ , kcal/mole	37	– 7.06	– 7.16
	20	– 6.49	– 6.59
	1	– 5.84	– 6.00
$\Delta H^\circ$ , kcal/mole		3.43	2.84
$\Delta S^\circ$ , cal/mole per °K		33.8	32.3

\* See footnote 8.

† See footnote 9.

to be sensitive to conformational changes in the protein (12, 22, 23). When excited with light at a wave length of 285  $m\mu$  both albumin preparations exhibited a peak of fluorescence extending from 290 to 475  $m\mu$  with a maximum at 340–345  $m\mu$  (Fig. 6). As noted previously by Chen (12), one of the albumin preparations<sup>9</sup> was definitely more fluorescent. Addition of digitoxin (20 mg/ml) to 2-mg/ml solutions of either albumin preparation in either distilled water (pH 5.2) or Tris-

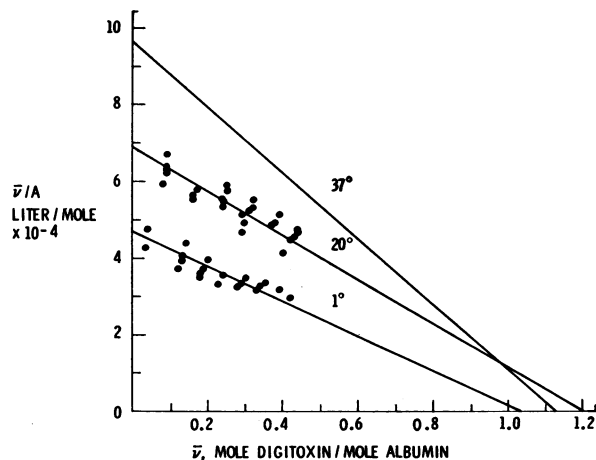


FIGURE 5 Scatchard plots of data obtained from equilibrium dialyses of digitoxin and human serum albumin<sup>8</sup> at 20°C and at 1°C showing decrease in the association constant with decrease in temperature. The upper regression line is derived from data obtained at 37°C. See Fig. 3 for symbols and interpretation.

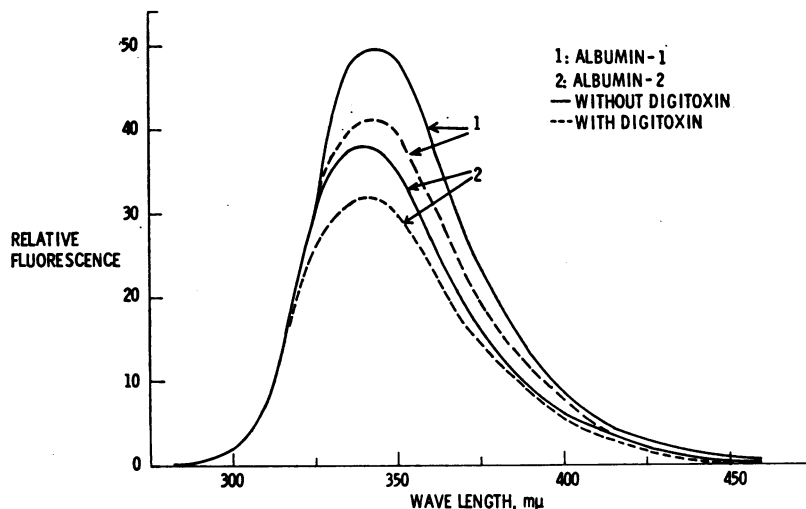


FIGURE 6 Fluorescence spectra of human serum albumin with and without digitoxin. Wavelength of exciting light: 285  $m\mu$ . Protein concentration: 2 mg/ml water. Digitoxin concentration: 20  $\mu\text{g/ml}$ . Albumin-1 was a preparation low in free fatty acid<sup>8</sup>; albumin-2 contained 1.8 to 2.3 mole of fatty acid per mole albumin.<sup>8</sup>

NaCl buffer at pH 7.4 resulted in consistent reduction in relative fluorescence in all solutions that was most apparent at wave lengths between 325 and 425  $m\mu$  (Fig. 6). At the peak fluorescent wave length of 340–345  $m\mu$ , the degree of quenching approximated 15%.

*Effects of pH, electrolytes, and various compounds on binding of digitoxin.* Substitution of a 0.15 M phosphate buffer or Krebs-Henseleit buffer at pH 7.4 for the routine Tris-NaCl buffer used in the dialyses produced identical data for  $k$  and  $n$ . Changes in pH from 6.0 to 9.0 also were without effect. Reliable data could not be obtained at higher pH because of poor recovery that was probably related to alkaline instability of digitoxin (13).

The addition of potassium chloride (10 mmoles/liter) or calcium chloride (2.9 mmoles/liter) to the standard 0.15 M Tris-NaCl buffer at pH 7.4 had no effect on binding.

In attempts to identify the structural features of digitoxin critical to its binding with albumin, dialyses were performed with various substances added to the dialysate. Digitoxose at a concentration of  $4.7 \times 10^{-6}$  mole/liter (three times the highest digitoxin concentration) did not affect binding, nor did  $\beta$ -angelica lactone at a concentration of  $5.5 \times 10^{-5}$  mole/liter. Digitoxose, itself, at a concentration of 25  $\mu\text{g/ml}$  was only 4% bound to albumin.

The didigitoxoside and monodigitoxoside of digitoxigenin, digitoxigenin, digoxin, and digoxigenin in decreasing order of activity interfered with binding of digitoxin, (Table II, Fig. 7). Because radioisotopically labeled digitoxosides of digitoxin were not available, the competitive binding effects of these compounds were

used to determine their association constants (17). The concentration of either the di- or monodigitoxoside in the bag and in the dialysate was determined from the difference between the concentrations of extracted *m*-dinitrobenzene-reacting substances (total glycoside concentration) and digitoxin. The apparent association constants were  $9.7 \times 10^4$  liter/mole for the monodigitoxoside and  $9.7 \times 10^4$  liter/mole for the didigitoxoside.

Removal of bound fatty acids by isoctane-acetic acid extraction or charcoal extraction from the albumin preparation<sup>8</sup> with the higher fat content did not alter its avidity for digitoxin. The association constant remained

TABLE II  
*Effects of Related Cardiac Glycosides and Genins on Association Constant ( $k$ ) of Digitoxin and Albumin at 37°C and pH 7.4*

Compound	Initial concentration in dialysate		$k$
	$\mu\text{g/ml}$	mmoles/liter	
Digitoxin didigitoxoside	20.3	32	$4.19 \pm 0.09$
Digitoxin monodigitoxoside	16.2	32	$4.50 \pm 0.16$
Digitoxigenin	12.0	32	$7.34 \pm 0.14$
	18.0	48	$5.87 \pm 0.24$
Digoxin	8.0	10	$8.23 \pm 0.18$
Digoxigenin	4.4	10	$9.37 \pm 0.33$
	40.0	94	$6.47 \pm 0.18$

The number of binding sites,  $n$ , for digitoxin in these experiments ranged from 0.93 to 1.10.



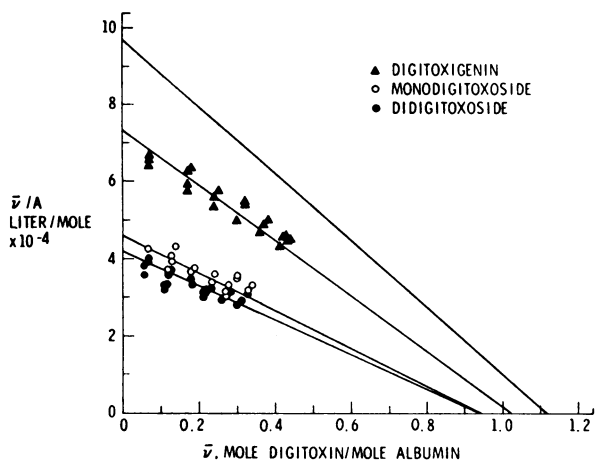


FIGURE 7 Scatchard plots showing the effects of digitoxigenin, digitoxigenin monodigitoxoside, and digitoxigenin didigitoxoside on the interaction of digitoxin and human serum albumin. Each competing ligand was added to the dialysate at a concentration of  $3.2 \times 10^{-5}$  mole/liter. Dialyses were performed at  $37^\circ\text{C}$  and pH 7.4. The upper line is derived from dialyses with no competing ligand in the system and is the same as the regression line in Fig. 3.

remarkably similar to that of the parent protein,  $9.75 (\pm 0.20) \times 10^4$  liter/mole and  $n$  continued at one ( $0.90 \pm 0.05$ ).

**Binding of digitoxigenin.** Dialyses with plasma demonstrated that at equilibrium concentrations of digitoxigenin in the plasma of less than  $5 \mu\text{g/ml}$ , 94% of the compound was bound. In solutions of albumin at 4.0 g/100 ml, the extent of binding was identical. The fraction bound decreased at lower concentrations of albumin, but even at a concentration of 0.69 g/100 ml, which was used for determination of the binding constants, 78% of the genin was bound. At concentrations of the genin greater than  $6.0 \mu\text{g/ml}$  the degree of binding decreased, and at  $170 \mu\text{g/ml}$ , only 41% was bound.

On equilibrium dialysis it was possible to obtain data with values for  $\bar{v}$  as high as 2 moles of digitoxigenin per mole of albumin.<sup>8</sup> The Scatchard plot (Fig. 8) was curvilinear, and was extrapolated to  $\Sigma kn$  of  $4.06 \times 10^4$  and  $n$  of 3. Successive approximations (17) led to the conclusion that albumin contains a single primary binding site with  $k$  of  $3.36 \times 10^4$  liter/mole and two secondary binding sites, each with  $k$  of  $3.5 \times 10^8$  liter/mole. The curve defined by these constants fit the experimental data closely (Fig. 8).

Binding was enhanced at  $1^\circ\text{C}$ . The apparent association constant increased to  $4.6 \times 10^4$  liter/mole with no change in the number of binding sites. The altered affinity was related entirely to increase in  $k$  of the primary binding site to  $3.9 \times 10^4$  with no evident change in the higher order sites. The enthalpy change associated with binding at the primary site was  $-0.7$  kcal/mole, and the

change in entropy was 18.5 cal/mole per  $^\circ\text{K}$ . Thus the reaction was spontaneous and exothermic and was associated with a moderate increase in entropy.

The albumin with a low fat content manifested significantly less affinity for the genin. The association constant for the single primary binding site was  $1.85 \times 10^4$  liter/mole. Two higher order binding sites, each with  $k$  of  $3 \times 10^8$  liter/mole, were also present. Extraction of fat from the lipid-rich protein lowered its affinity for digitoxigenin; a Scatchard plot of dialysis data using this protein was superimposable on the data obtained with the albumin preparation originally low in bound fatty acid.

The addition of digitoxin at an initial concentration of  $12 \mu\text{g/ml}$  to the dialysate interfered with binding of the genin. Decrease in  $k$  of the primary binding site for the genin to  $2.8 \times 10^4$  liter/mole with no change in the avidity of the two weaker binding sites indicated that digitoxin competed with the genin only for the stronger binding site. Digoxin ( $24 \mu\text{g/ml}$ ) and digoxigenin ( $40 \mu\text{g/ml}$ ) depressed binding of digitoxigenin only slightly.

**Binding of digoxin.** At a concentration of  $0.005 \mu\text{g/ml}$  in plasma,  $23 \pm 2\%$  (mean  $\pm$  SD) of the digoxin was bound. With increasing concentrations, the fraction bound decreased (Fig. 9); at  $30 \mu\text{g/ml}$ , only  $10 \pm 1\%$  was bound.

Human serum albumin<sup>8</sup> at a concentration of 4.0 g/100 ml manifested an affinity for digoxin identical with that of plasma. Lowering the protein concentration

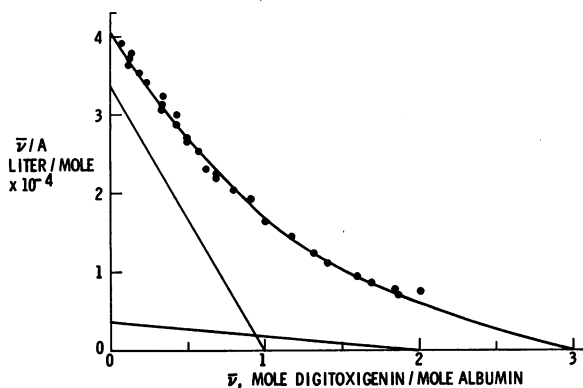


FIGURE 8 Scatchard plot of data obtained from equilibrium dialyses of digitoxigenin and human serum albumin<sup>8</sup> at pH 7.4 and  $37^\circ\text{C}$ . Each point represents average values of three or more dialyses. The curve was derived from the equation  $\bar{v} = [n_1 k_1 A / (1 + k_1 A)] + [n_2 k_2 A / (1 + k_2 A)]$ , where  $n_1$  and  $n_2$  represent the number of sites within each of two classes, primary and secondary, and  $k_1$  and  $k_2$  are the respective association constants characteristic of a site in each class (17). Other symbols are defined in Fig. 3. The curve that fitted the experimental data best was derived with the following assumed values:  $k_1 = 3.36 \times 10^4$ ,  $n_1 = 1$ ,  $k_2 = 3.5 \times 10^8$ , and  $n_2 = 2$ . The straight lines represent hypothetical plots for each of the two classes of binding sites.

resulted in decreased binding (Fig. 9); thus, in an albumin solution of 4.0 g/100 ml,  $23 \pm 2\%$  of digoxin at a concentration of  $2 \mu\text{g/ml}$  was bound, and in an albumin solution of 1.0 g/100 ml,  $13 \pm 3\%$  was bound. Competitive dialysis experiments with albumin at 4 g/100 ml on one side of the membrane and individual plasma protein fractions (Cohn) at normal plasma concentrations on the other side revealed insignificant binding by the globulin fractions.

Because of the low avidity of digoxin for albumin and limited solubility of the glycoside, data for a Scatchard plot could not be obtained. The highest value for  $\bar{v}$  was 0.03. The points clustered closely to the  $\bar{v}/A$  axis and indicated an apparent association constant of approximately  $9 \times 10^5$  liter/mole. Reduction in temperature markedly decreased the binding (Fig. 9). At  $1^\circ\text{C}$ , only  $7 \pm 2\%$  of digoxin at a concentration of  $2 \mu\text{g/ml}$  was bound in a 4 g/100 ml solution of albumin. Change in pH between 6.0 and 9.0 had no effect. Digitoxin, digitoxigenin, and, to a lesser degree, digoxigenin, when added to the dialysate, interfered with the binding of digoxin.

**Binding of digoxigenin.** Digoxigenin was bound to albumin<sup>8</sup> but to a lesser degree than digoxin. In a  $1 \mu\text{g/ml}$  solution of digoxigenin at an albumin concentration of 4 g/100 ml, only  $14 \pm 2\%$  of the genin was bound; at lower albumin concentrations the following values for fraction bound were obtained: 2.0 g/100 ml,  $10 \pm 1\%$ ; 1.0 g/100 ml,  $7 \pm 2\%$ ; 0.5 g/100 ml,  $5 \pm 1\%$ ; 0.1 g/100 ml,  $1.1 \pm 1\%$ .

**Effects of digitoxigenin and its glycosides on fluorescence of albumin.** To compare the effects of digitoxigenin, digitoxin, and the mono- and didigitoxosides of

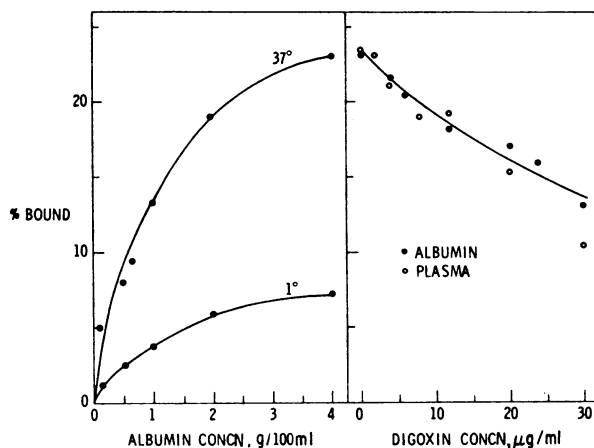


FIGURE 9 Relationship at  $37^\circ\text{C}$  and  $1^\circ\text{C}$  between the fraction of digoxin bound to human serum albumin<sup>8</sup> and the albumin concentration (left panel); the concentration of digoxin was  $2 \mu\text{g/ml}$ . Relationship between digoxin concentration and the fraction of digoxin bound to albumin (concentration: 4 g/100 ml) or existing in bound form in human plasma at  $37^\circ\text{C}$  (right).

digitoxigenin on the fluorescence spectrum of albumin, each of these compounds was added to a 6.9 mg/ml solution of albumin in water. In each instance, the quantity of compound added was calculated from the binding data to produce a value for  $\bar{v}$  of 0.67. Fluorescence was measured in the manner described earlier in the results. Except for digitoxigenin which had no effect, each of the compounds reduced the fluorescence of albumin at wavelengths between 325 and 425  $m\mu$ . The effects on the fluorescence spectrum of both albumin preparations were similar to those noted above for digitoxin and albumin in a 2 mg/ml solution (Fig. 6). The degree of quenching at the wave length, of peak fluorescence varied from 14 to 19% with no consistent differences among the compounds.

## DISCUSSION

The evidence developed in this investigation indicates that in human plasma digitoxin is extensively bound to protein and that albumin is the main carrier of the glycoside. Thus solutions of human serum albumin in normal concentration bound digitoxin as avidly as plasma. In competition for the glycoside, albumin far outstripped the globulins and on continuous flow paper electrophoresis of plasma and digitoxin, the glycoside migrated almost exclusively with albumin. The failure of digitoxin to accompany albumin and, indeed, to move from the origin in electrophoresis on starch gel was attributed to the ability of the gel to bind the glycoside and to strip it off the albumin molecule. Similar behavior of the compound in plasma during electrophoresis on a starch bed has been noted previously (5). Distortion of the pattern of distribution of ligands among the plasma proteins by electrophoretic supports has been observed previously in the case of cortisol and transcortin (24), and competition for the ligand between the support material and the binding proteins has proved to be a problem in the study of thyroxine binding (25).

It is recognized that the possible existence of a plasma protein with an avidity for digitoxin greater than albumin has not been excluded. The concentration of such a protein, however, would have to be regarded as small enough for its effects to escape detection, and therefore, its physiologic significance as compared to albumin would appear to be minor.

Binding of digitoxin to human serum albumin is fairly strong relative to the binding of most steroids to this protein. The association constants for cortisol, testosterone, progesterone, and androsterone sulfate range from  $3 \times 10^5$  to  $3.6 \times 10^5$  liter/mole (26-31). The binding is not as strong as that of thyroxine to albumin ( $k = 1.3 \times 10^6$  liter/mole) (32) or of the long-chain fatty acid anions, stearate, oleate, and palmitate, which have  $k$  values of  $6 \times 10^7$ - $1.1 \times 10^8$  liter/mole (33). The

avidity exceeds that of many pharmacologic agents (6), such as quinidine which has an association constant of  $7.7 \times 10^8$  liter/mole (34), but it is in the range of warfarin sodium, which has a  $k$  of  $8.7 \times 10^4$  liter/mole (35), and phenylbutazone with  $k$  of  $1.25 \times 10^6$  liter/mole (36).

Determination of the number of binding sites for digitoxin on the human albumin molecule is surrounded by some uncertainty because of inability to extend the Scatchard plot beyond values for  $\bar{v}$  of 0.67. At various temperatures, with changes in pH, and in competitive binding experiments, only a single binding site was manifest with equilibrium concentrations of digitoxin as high as 67  $\mu\text{g}/\text{ml}$ . These concentrations were many hundred to several thousand times greater than the concentrations of 1.0–10.0  $\mu\text{g}/100$  ml found in the plasma of patients treated with the drug (9, 37). It is apparent, therefore, that a single binding site with a  $k$  of  $9.62 \times 10^4$  liter/mole adequately characterizes the interaction of the glycoside and plasma albumin under physiologic circumstances.

The difference in the values for  $k$  obtained with the two different preparations of albumin could not be ascribed to the disparity in the quantity of fatty acid bound by the proteins since extraction of fat from the albumin with the highest fatty acid content did not alter its affinity for digitoxin. In the case of digitoxigenin, however, variation in fat content of albumin appeared to be the cause of the dissimilarity in  $k$  values for the primary binding site. Although the true nature of the interaction between albumin and a ligand is best appreciated by study of albumin that is free of bound fat and small ions (12, 18), the interaction in vivo undoubtedly occurs with the protein containing a full complement of bound fatty acid anions. The association constants obtained with the albumin preparation containing two to three molecules of free fatty acid per molecule of protein are therefore of greater physiologic relevance.

The energy changes accompanying formation of the bond provide some insight into the general mechanism of the interaction and the final state of the complex formed. The energy changes, however, may have represented a summation of effects, some of which occurred in divergent directions; it is therefore not possible to be certain of all the mechanisms involved in the binding process, but only of the final net result of possible changes in state of the solvent, the protein, and the interrelationships among them and the ligand. From the design of the dialysis experiments, in which digitoxin- $^3\text{H}$  left the protein solution in the bag to enter the dialysate, it is apparent that the interaction is reversible. The free energy change is too small for formation of a covalent bond, but it is within the range of values observed for binding of other organic molecules to pro-

tein (26–28, 34, 35, 38–40). The slight effects of pH on the association constant indicate that ionic effects are not a significant factor in the interaction. The endothermic nature of the reaction and the positive entropy change are characteristic of hydrophobic bonding (40), in which disruption of the water structure about the protein and the rupture of hydrogen bonds between water and both the protein and ligand consume heat and the freeing of water molecules produces more disorder and consequent increase in the entropy of the system.

The binding of most substances, including steroids, to protein is facilitated by low temperatures (27, 35, 38, 39, 41–43). These interactions are exothermic; part of the free energy change that favors formation of the protein-ligand complex is derived from the negative enthalpy. In this respect, the reaction between digitoxin and albumin is unusual; the free energy change of the reaction is entirely derived from the associated change in entropy, which is large enough to provide the energy for the enthalpy change and to drive the reaction.

The entropy change is much larger than the 10–20 cal/mole per  $^\circ\text{K}$  observed for many binding reactions (27, 35, 38–40). Although the processes involved in hydrophobic bonding account for the gain in entropy of many organic substances (39, 40), concomitant alterations in the structure of the protein might contribute to the increase in entropy. Thus the  $\alpha$ -helical content of human serum albumin diminishes in the process of binding sodium decyl sulfate (44).

Because of these considerations, it was suspected that binding of digitoxin is accompanied by a change in the conformation of albumin. Optical rotary dispersion provided no evidence of decrease in the helicity of the protein, but the changes in fluorescence were similar to those associated with unfolding of human serum albumin at pH 4 in the region of its N-F transition (23). The fluorescence spectrum of albumin is derived from emission by the tyrosine and the single tryptophan residues in the molecule. The tyrosine residues are directly excited by the incident light and emit light at a wavelength of 303  $m\mu$  whereas the tryptophan residue is excited by energy transfer from the tyrosine residues and emits light at 345–350  $m\mu$ . With unfolding of the albumin molecule, energy transfer from tyrosine to tryptophan decreases resulting in diminution of tryptophan emission and in a relatively greater contribution of tyrosine emission to the fluorescence spectrum (23). The quenching of tryptophan emission from albumin by digitoxin, which in either free or albumin-bound form does not absorb light between 270 and 500  $m\mu$ , therefore suggests that unfolding of the protein occurs in the process of binding and that this conformational alteration contributes to the gain in entropy. Of interest in this regard is the evidence that the binding of

at least one steroid, testosterone, to albumin occurs in a configuration parallel to the ring structure of the tyrosine side chains of the protein (42).

Although the binding site for digitoxin on the albumin molecule has not been defined by this study, it is evident from the competitive binding data that the di- and monodigitoxosides of digitoxigenin, digitoxigenin, digoxin, and digoxigenin also attach to it. Both digitoxin and digoxin have a greater affinity for the binding site than their respective genins. In the case of digitoxigenin, for which association constants could be obtained, the addition of one to three digitoxose residues at the 3 $\beta$ -position of the genin produces a threefold increase in its affinity for albumin and a change in fluorescence of the protein. With respect to its exothermic character and the magnitude of the entropy change, the interaction of digitoxigenin with albumin is similar to that of other steroids (27) and strikingly different from that of digitoxin. At equal concentrations of the protein-ligand complex, digitoxigenin did not affect the fluorescence spectrum of albumin and therefore did not induce a conformational change in the protein equivalent to that produced by the glycosides. The added binding of the digitoxose residues appears to be responsible for the unfolding of albumin and the consequent augmented entropy change and the reversal of enthalpy that accompany binding of digitoxin.

The thermodynamic differences between the interactions of digitoxigenin with albumin and digitoxin with albumin are equivalent in direction and magnitude to those associated with hydrophobic binding of an aliphatic compound (40). Digitoxose is a polar compound that is freely soluble in water, but it is a 2,6-dideoxyhexose and, therefore, possesses some aliphatic features. Although it binds weakly, if at all, to albumin, it is possible that when digitoxin binds to albumin the digitoxose residues form an additional hydrophobic bond with the protein and that formation of this bond rather than an alteration in albumin is the source of the thermodynamic differences between binding of the glycoside and binding of the genin.

Digitoxigenin is the cardioactive moiety of digitoxin; digitoxose is pharmacologically inert. In tests of acute cardiotoxicity, however, digitoxin is 2.6–2.8 times more potent on a molar basis than the genin (45, 46). The mechanism whereby the digitoxose residues potentiate the activity of the genin is unknown, although it has been attributed to increased water solubility and cell penetrability (47) and to protection of the genin from metabolic attack and inactivation at the 3 $\beta$ -hydroxyl position (48). The increase in potency conferred on the genin by the sugars does not appear to vary with the number of digitoxose residues in the saccharide side chain; the monodigitoxoside is as potent as the tridigi-

toxoside, digitoxin (45, 46). Although no conclusions can be drawn regarding the relative affinity of these compounds for their site of action, it is of interest that the association constants for the interaction of the monodigitoxoside, the didigitoxoside, and digitoxin with albumin are of similar magnitude and almost three times greater than that of the genin. Digoxin is also more potent than its genin (45, 46), and it too binds more avidly than the genin to albumin.

Digoxin differs from digitoxin only in that it contains a 12 $\beta$ -hydroxyl group, but this additional polar substituent is responsible for a hundredfold difference in the affinity of the two compounds for human serum albumin. In this respect, the compounds conform to the general rule that the avidity of steroids for albumin varies inversely with the number of polar functional groups they contain (26–28, 49). The marked disparity in the affinity of the two glycosides for human serum albumin accounts for several of the differences in their pharmacodynamic behavior.

Published data derived from nine patients maintained on 0.1 mg of digitoxin daily (9) and from eight normal subjects maintained on a daily dose of 0.5 mg of digoxin-<sup>3</sup>H (50) are presented in Table III. Because of its greater avidity for albumin, plasma concentrations of digitoxin were larger than those of digoxin and exhibited less fluctuation during the course of the day. The concentration of unbound digoxin, however, was four times greater, and consequently more of this gly-

TABLE III  
Mean Data Obtained from Nine Patients Maintained on Digitoxin and Eight Normal Subjects Maintained on Digoxin-<sup>3</sup>H\*

	Digitoxin	Digoxin- <sup>3</sup> H
Dose, mg/day	0.1	0.5
Blood concentration, $\mu\text{g}/100\text{ ml}$	0.90	0.21
Plasma concentration, $\mu\text{g}/100\text{ ml}$		
Total	1.70	0.26 $\ddagger$
Unbound	0.05	0.20
Urinary excretion, $\mu\text{g}/\text{day}$	16.4	132.0 $\S$
Half-time, days	4–6	1.3–1.8

\* The data on digitoxin were obtained from Peterson and Lukas (9); the data on digoxin-<sup>3</sup>H were derived from the paper of Marcus and associates (50). Half-times are published values for other subjects (9, 37, 51, 52).

$\ddagger$  Calculated from the blood concentration on the basis of evidence obtained in this laboratory indicating that the concentration of digoxin in the water of red blood cells is equal to the concentration of unbound digoxin in plasma. An hematocrit of 48% was assumed, and the value of 23% was used for the fraction of plasma digoxin that is bound.

$\S$  Of the 61% of the daily dose of tritium that was excreted in the urine, only this quantity appeared to be authentic digoxin.

coside was contained in the water of red blood cells. Also the rate of urinary excretion of digoxin was considerably larger, and although the mechanisms of renal excretion of the two glycosides have yet to be defined, the difference is partly attributable to the higher plasma concentration of digoxin in a form susceptible to filtration by the renal glomeruli. The difference in rates of excretion of the two compounds are in turn reflected by a difference in their persistence in the body. The half-time of digitoxin in man is 4–6 days (9, 37), whereas that of digoxin is 1.3–1.8 days (51, 52).

Hypoalbuminemia would not be expected to affect the distribution of digitoxin in the plasma to a significant extent, since even at a plasma albumin concentration of 1 g/100 ml, 92% of the glycoside is bound. The concentration of free digitoxin in the plasma, however, will be almost tripled. The fraction of unbound digoxin in plasma would be relatively less affected since only 23% is bound at an albumin concentration of 4 g/100 ml and 13% is bound at a concentration of 1 g/100 ml.

Because the quantity of unbound digitoxin in the plasma is small, hemodialysis is not expected to be an efficient means for depleting the body pool of the glycoside. For example, after an hour of *in vivo* dialysis using a dialysis unit with a 1 liter dialysate volume (53), the concentration of digitoxin in the subject's plasma measured by the double isotope dilution derivative method (9) was 1.05  $\mu\text{g}/100$  ml, whereas in the dialysate it was only 0.05  $\mu\text{g}/100$  ml. The addition of albumin or plasma to the dialysis fluid should facilitate the recovery of digitoxin. Even if the concentration of albumin in a 100 liter dialysate is as low as 0.1 g/100 ml, a concentration capable of binding 50% of digitoxin in solution, as much as 1 mg of digitoxin theoretically could be extracted from a patient with a plasma digitoxin concentration of 2  $\mu\text{g}/100$  ml. Although digoxin should be more readily removed by dialysis, the very low total plasma concentration of this drug is expected to limit the efficacy of the procedure.

Since digitoxin and digoxin are bound to the same site on the albumin molecule, the question may be posed whether these substances might displace each other from the binding site during the common therapeutic maneuver of replacing one of the glycosides with the other. The concentrations attained by these compounds in the plasma during treatment are exceedingly small fractions of the concentrations needed to demonstrate competition in the studies reported in this paper. Under therapeutic circumstances, therefore, it is most unlikely that binding of either of these glycosides to albumin will be significantly affected.

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