traction and chromatography is essential for good results. The capillary column with an immobilized stationary phase and the possibility of solvent washing was also a big advantage in this assay.

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Binding of Sulfadimethoxine to Isolated Human Blood Protein Fractions

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Abstract □ The binding of sulfadimethoxine to selected human blood protein fractions and to fresh serum has been examined by means of a new equilibrium dialysis technique which minimizes experimental error and permits the evaluation of low-level binding. Certain α-globulin fractions, containing mixtures of proteins, were found to bind the drug. Scatchard analysis of the binding of sulfadimethoxine to fresh serum, calculated as though all of the binding is due to albumin, gives a different result from that obtained with isolated albumin. This may be a reflection of the contribution of the α-globulins to the overall binding of sulfadimethoxine in fresh serum. Although sulfadimethoxine is amphoteric, it did not bind to the α_1-acid glycoprotein. The drug behaves as an acidic compound when binding to the blood proteins.

Keyphrases □ Sulfadimethoxine—blood fraction binding, equilibrium dialysis, humans □ Blood fraction binding—humans, sulfadimethoxine, equilibrium dialysis

Sulfadimethoxine is a long-acting sulfanilamide antibacterial drug which is extensively bound to the proteins of the blood (1, 2). It has been shown that the antibacterial activity and rate of metabolic *N*-acetylation of sulfanilamide drugs depend on the concentration of free, unbound drug in the plasma (3, 4). Nonlinearity in the pharmacokinetics of sulfadimethoxine has been associated with dose-dependent changes in the percentage of the drug bound to plasma proteins (5). Furthermore, the volume of distribution of sulfadimethoxine is altered significantly by small changes in the concentration of plasma albumin (6).

Albumin is the most important of the blood proteins in the binding of sulfanilamide drugs (2, 6–11), although the involvement of other plasma proteins has been implied (12). Sulfadimethoxine is an amphoteric compound (13) with an acidic center at the sulfonamide (pK_a 6.7) and a basic center at the aniline-like primary amino group (pK_b 11.98). The isoelectric point is at pH 4.36. Albumin is effective in binding acidic drugs such as warfarin (14–16), fenoprofen (17, 18) and phenylbutazone (19). Unlike these compounds, basic drugs such as imipramine (20), alprenolol (21), propranolol, (22) and lidocaine (23) bind significantly to α_1-acid glycoprotein and lipoproteins in addition to albumin. The individual contributions of different proteins to the binding of drugs becomes significant in disease states which lead to changes in the concentration of one or more of the blood proteins (24–27). The present study was undertaken to determine whether proteins other than albumin are involved in the overall binding of the amphoteric drug sulfadimethoxine.

The binding of sulfadimethoxine to isolated human plasma protein fractions was measured by a newly developed equilibrium dialysis technique (28). This method employs calibration and control procedures that minimize artifactual errors and permit statistical evaluation of control and test data. This stringent control technique also permits correction to be made for concentration-dependent binding to the dialysis membrane or other equipment; thus, it is possible to demonstrate statistically significant binding at low levels (<10%).

EXPERIMENTAL SECTION

Equilibrium dialysis was performed using 20-cm strips of dialysis tubing[1], 1-cm diameter, 4.8-nm pore diameter, with a molecular weight cut-off of 12,000. These membranes were immersed in boiling water and then stirred for 2 h as they cooled. The tubing was then stirred with 70% methanol for 30 min, stored in 50% methanol overnight, rinsed with distilled water, and soaked in phosphate buffer (pH 7.4) for 2–3 h prior to use. The membranes were used immediately after preparation. The tubing was tied with a double knot at one end, and then filled with 2 mL of protein solution in phosphate buffer (pH 7.4) containing sulfadimethoxine. The protein solutions were as follows: Cohn Fraction I (fibrinogen)[2] 0.34% (w/v); Cohn Fraction II (α-globulin)[3], 0.74% (w/v); Cohn Fraction IV-1 (α-globulin)[3], 0.81% (w/v); Cohn Fraction IV-4 (α-globulin)[3], 0.81% (w/v); Cohn Fraction V (albumin)[3], 4% (w/v); Cohn Fraction VI (α_1-acid glycoprotein)[3], 0.1% (w/v); lyophilized serum[3], 7.3%; and fresh serum[4], reduced to half volume by ultrafiltration. Except for the fresh serum, the protein solutions were prepared initially at double strength in buffer and then diluted with an equal volume of buffer containing sulfadimethoxine. The fresh serum, concentrated to half volume, was diluted with

[1] Fisher Scientific Co., Toronto, Ontario, Canada.
[2] ICN Pharmaceuticals Inc., Cleveland, Ohio.
[3] United States Biochemical Corp., Cleveland, Ohio.
[4] Collected from a healthy young-adult male volunteer.

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Table I—Sulfadimethoxine Binding

	Total Concentration of Sulfadimethoxine, nmol/mL				
	322.6	451.6	580.6	806.5	1096.8
Fresh serum					
% bound	95.9 ± 1.4[b]	96.9 ± 0.3	95.6 ± 0.5	92.1 ± 0.3	86.5 ± 0.4
nmole/mL bound	309.3 ± 4.4	437.4 ± 1.4	555.4 ± 2.7	742.9 ± 2.5	948.7 ± 4.7
Cohn Fraction V Albumin					
% bound	93.2 ± 0.5	90.5 ± 0.1[c]	87.2 ± 0.5	77.5 ± 0.3[c]	72.8 ± 0.04[b]
nmol/mL bound	300.7 ± 1.6	408.4 ± 0.6	506.4 ± 2.9	625.1 ± 2.2	798.8 ± 1.8
Cohn Fraction IV-1					
% bound	10.3 ± 1.1	11.8 ± 1.5	11.0 ± 1.3[d]	11.6 ± 0.8	13.7 ± 0.7
nmol/mL bound	33.2 ± 3.5	53.1 ± 6.9	64.2 ± 7.8	93.3 ± 6.5	150.3 ± 7.1
Cohn Fraction IV-4					
% bound	17.6 ± 0.8	16.0 ± 0.5	15.3 ± 1.1	12.2 ± 0.6	12.3 ± 1.0
nmol/mL bound	56.7 ± 2.4	72.4 ± 2.3	88.6 ± 6.4	98.6 ± 4.6	135.4 ± 11.1
Lyophilized serum					
% bound	17.8 ± 1.1[c]	20.8 ± 0.4	18.6 ± 0.8	16.6 ± 0.7	18.4 ± 0.4
nmol/mL bound	57.4 ± 3.5	93.7 ± 1.9	107.9 ± 4.9	133.7 ± 5.5	201.8 ± 4.2

[a] Unless otherwise noted each value is the mean of five replicates, ±*SE*; all values are significantly different from control values; $p < 0.001$. [b] $n = 3$. [c] $n = 4$. [d] $p < 0.01$.

an equal volume of buffer containing sulfadimethoxine. Albumin concentrations in fresh serum and lyophilized serum were measured by the method of Mancini *et al.* (29). The drug concentrations were 100, 140, 180, 250, and 340 μg/mL.

The following procedure was adopted for each of the solutions described above. The tubing was sealed and placed in a glass culture tube (16 × 125 mm) with a polytetrafluorethylene cap. Four milliliters of phosphate buffer (pH 7.4) were placed in each culture tube, and the solutions were dialyzed for 24 h at 37°C[5]. The tubes were rotated vertically through 360°, 12 times per minute using a rotary mixer[6]. The protein solution and dialysate were assayed for sulfadimethoxine using the Bratton-Marshall method (30), and absorbance was measured at 420 nm. Five replicates of each test concentration were dialyzed. A set of five controls was prepared for each sulfadimethoxine concentration studied and placed in the rotary mixer next to the test samples. Controls were identical to the test samples, except that the solution inside the dialysis membrane contained no protein. Glassware and buffer were autoclaved[7] prior to use. Preparation and transfer of solutions was carried out under aseptic conditions in a laminar airflow hood[8]. The dialysate was tested for the absence of protein visually (absence of frothing) and by means of a semiquantitative colorimetric indicator[9].

RESULTS AND DISCUSSION

For each of the protein-sulfadimethoxine systems described above, mean optical density values ($n = 5$) were calculated for tests and controls after dialysis. The percentage of binding was calculated for subtracting the mean test optical density from that of the control and expressing the result as a percentage of control, as previously described (28).

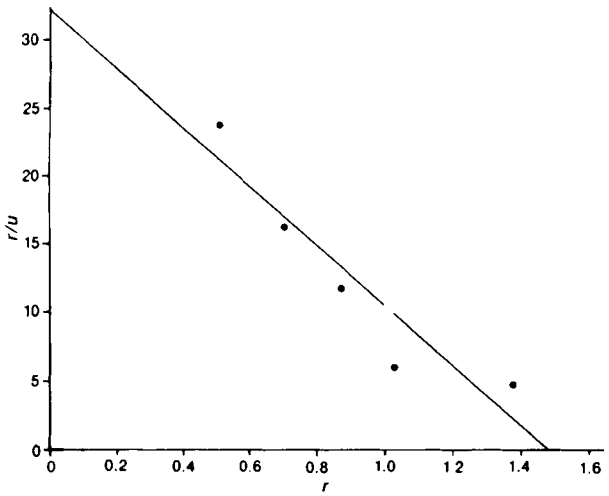

Figure 1—*A Scatchard plot of the binding of sulfadimethoxine to Cohn Fraction V albumin.*

There was a negative linear correlation between the concentration of sulfadimethoxine and the percentage binding to fresh serum (Table I; $y = -0.01316x + 102.0$; $r = -0.9475$, $p < 0.02$) and to isolated albumin (Table I; $y = -0.02807x + 102.5$; $r = 0.9863$, $p < 0.01$). This give the impression that the "degree of protein binding decreases with increasing concentration of sulfonamide" (3). However, the number of moles of sulfadimethoxine bound to fresh serum or to isolated albumin actually increased in a linear manner with increasing concentrations of drug (Table I; fresh serum: $y = 0.8223x + 62.97$; $r = 0.9978$, $p < 0.001$: albumin: $y = 0.6281x + 118.6$; $r = 0.9964$, $p < 0.001$). It is also important to realize that the concentration of free, unbound drug increased with total concentration of drug (fresh serum: $y = 0.1778x - 62.96$; $r = 0.9556$, $p < 0.02$; albumin: $y = 0.3719x - 118.6$; $r = 0.9899$, $p < 0.01$).

The results of the study of the binding of sulfadimethoxine to fresh serum (Table 1) are comparable to those published by Walker (1). Scatchard analysis (31) of the data was carried out in the same manner used by Walker who assumed that all of the binding was due to albumin (mol. wt. 69,000). In the present study, the concentration of albumin in the serum (44.25 mg/mL) was measured by radial immune diffusion (29). The five point linear regression equation ($y = -34361x + 61411$; $r = -0.8900$, $p < 0.05$) gave an x-axis intercept value of 1.8. A double reciprocal plot (32) derived from the same data ($y = 0.00002688x + 0.7377$; $r = 0.9893$, $p < 0.001$) gave a value of $n = 2.1$. This corresponds to the much quoted value of 2.0 binding sites for sulfadimethoxide per mole of albumin (1). In the study by Walker, the concentration of albumin in the serum was estimated to be 0.63 mM (43.47 mg/mL). Working from the published data (1), however, we obtained a value of 2.0 binding sites per mole only if we assumed the albumin concentration to be 40.00 mg/mL ($n = 2.1$ by Scatchard analysis; $n = 2.0$ by double reciprocal plot analysis).

In our studies on the binding of sulfadimethoxine to isolated albumin, the concentration of the protein was held constant at 40.00 mg/mL, an albumin concentration which is commonly employed in binding studies. The binding data cannot be compared directly because the isolated albumin was at a slightly different concentration from that in the fresh serum. The intercept values of Scatchard or reciprocal plot analysis can be compared, however, because these techniques are concerned with the number of moles of ligand bound per mole of protein. Therefore, if all the binding in serum was attributable to albumin, Scatchard plots of the respective data should give the same x-axis intercept value for serum as for isolated albumin. However, Scatchard analysis of the data (Table I), for the binding of sulfadimethoxine to isolated human albumin ($y = -22357x + 32829$; $r = 0.9511$, $p < 0.01$), gave an x-axis intercept value of 1.5. A double reciprocal plot derived from the same data ($y = 00002688x + 0.7377$; $r = 0.9893$, $p < 0.001$) gave a value of $n = 1.4$. There is a discrepancy between the corresponding values obtained with fresh serum, in the present study, and in the study by Walker (1). This suggests that (*a*) isolation of albumin from other serum constituents may alter the binding characteristics from those which prevail *in situ*, and/or (*b*) proteins other than albumin may be involved in the binding of the drug in serum.

The Scatchard plot (Fig. 1) of the binding of sulfadimethoxine to isolated albumin shows that the first four data points appear to fall on a straight line ($y = -31258x + 39244$; $r = -0.9958$, $p < 0.001$; x-axis intercept value 1.26), while the fifth point deviates considerably from this line. The study was not designed to investigate the binding of sulfisoxazole to albumin at high saturation, but the Scatchard plot does suggest that more than one binding site

[5] Thelco Model 4 Incubator; Fisher Scientific Co., Toronto, Ontario, Canada.
[6] Hematology/Chemistry Mixer Model 346; Fisher Scientific Co., Toronto, Ontario, Canada.
[7] Castle Autoclave; Fisher Scientific Co., Toronto, Ontario, Canada.
[8] Enviralab Sterility Module; Bio-Dynamics, Burlington, Ontario, Canada.
[9] Albustix; Ames Co. Division, Miles Laboratories, Rexdale, Ontario, Canada.

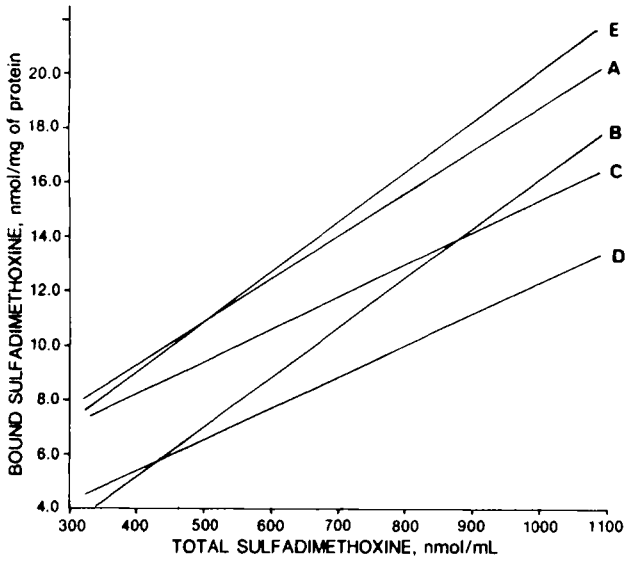

Figure 2—*The binding of sulfadimethoxine expressed in nmol/mg. Key: (A) Cohn Fraction V albumin,* $y = 0.01570x + 2.972$; $r = 0.9964$, $p < 0.001$. *(B) Cohn Fraction IV-1 α-globulin,* $y = 0.01817x - 2.1102$; $r = 0.9911$, $p < 0.01$, *(C) Cohn Fraction IV-4 α-globulin,* $y = 0.09612x + 27.69$; $r = 0.9883$, $p < 0.01$. *(D) Fresh serum, with calculations based on a total protein concentration of 72.5 mg/mL,* $y = 0.01133x + 0.8679$; $r = 0.9978$, $p < 0.001$. *(E) Fresh serum, calculated as if all the binding were due to albumin at a concentration of 44.25 mg/mL,* $y = 0.0186x + 1.422$; $r = 0.9978$, $p < 0.001$.

is involved. It is possible that at low saturation ($r < 1$), the drug binds primarily to a high affinity site, while at a higher saturation ($r > 1$), a lower affinity site will also bind sulfadimethoxine. At high saturation, however, the binding of the drug *in vivo* would be further complicated by the presence of other binding proteins.

Sulfadimethoxine showed low-level, but statistically significant, binding to two Cohn Fractions (33) of human plasma-serum, as shown in Table I. There were positive linear correlations between the concentration of sulfadimethoxine and the number of moles of the drug bound to Cohn Fraction IV-1 (α-globulin) ($y = 0.1472x - 17.0846$; $r = 0.9912$, $p < 0.001$) and to Cohn Fraction IV-4 (α-globulin) ($y = 0.09612x + 27.69$; $r = 0.9883$, $p < 0.01$). Unfortunately, it was not possible to carry out Scatchard analysis on these data because both α-globulin fractions are complicated mixtures of proteins (33), and there is no way of selecting a molecular weight with which to work.

Figure 2 suggests, however, that when the binding of sulfadimethoxine is expressed in terms of nanomoles bound per milligram of protein, the α-globulins have a significant capacity to bind the drug. Consequently, their contribution to the overall binding of the drug may be augmented in disease states, where plasma concentrations are elevated relative to albumin. Similar conclusions have been drawn about the role of lipoproteins in the overall binding of quinidine (34).

In Fig. 2, line E represents the binding of sulfadimethoxine to fresh serum, calculated as if all the binding were due to albumin alone. Line A represents the binding of the drug to isolated albumin. Note that the slopes of lines A and E are different and that there are larger discrepancies between binding values as the concentration of the drug increases. This may be an indication of increasing contributions by the α-globulin fractions (lines B and C) to the overall binding of the drug in serum as the concentration of sulfadimethoxine increases. Line D represents the binding of sulfadimethoxine to fresh serum based on the total concentration of protein in the serum (72.5 mg/mL). In this case, the values obtained are low because the binding proteins are diluted with protein materials which do not bind the drug.

There was no significant difference between tests and controls for the binding of sulfadimethoxine to Cohn Fractions I (fibrinogen), II (α-globulin), or VI (α_1-acid glycoprotein). These, and all of the other isolated protein fractions were comparable to their natural concentration ranges in the blood (35). Unfortunately, Cohn Fractions III^3 (β-globulin), and III-0^3 (β-lipoprotein) could not be reconstituted at or near physiological concentration and were, therefore, not examined.

Anomalous results were obtained when the binding of sulfadimethoxine to reconstituted lyophilized serum was examined. It was expected that the binding profile would be similar to that of fresh serum (Table 1), but the values obtained experimentally were ~20% of those of fresh serum. This discrepancy was undoubtedly due to low albumin concentration (15.50 mg/mL) in the reconstituted lyophilized serum. Total protein concentration was only 42 mg/mL (Biuret method), whereas normal fresh serum contains ~70 mg/mL of total protein (35).

In the experiment, care was taken to standardize the manipulative procedures in order to minimize experimental error. The use of tubes with polytetrafluoroethylene caps avoided the problem of plasticizers which can leach molecules from rubber or polyvinyl chloride-lined caps and cause displacement of drugs from protein binding sites (21, 36, 37). All solutions and glassware were sterilized before use, and solution transfers were made under aseptic conditions in a laminar airflow hood. This eliminated the possibility of microbial growth during dialysis and permitted the experiment to be conducted for 24 h without the use of preservatives, which could have influenced binding characteristics. All dialysates were checked for the absence of protein, both visually (absence of frothing) and by the use of a semiquantitative colorimetric indicator.

The concentration range of sulfadimethoxide was selected to include the reported plasma concentration obtained 4 h after an oral dose (38). Protein concentrations were held constant within normal physiological concentrations because binding parameters obtained with dilute albumin solutions have been shown to predict a higher degree of binding than those obtained by direct measurement (6–8). This phenomenon may be significant in disease states which cause hypoalbuminaemia (6), as may the role of minor binding proteins such as the α-globulins.

The plasma protein binding profile of sulfadimethoxine resembles that of an acidic drug although it is amphoteric. It did not bind to α_1-acid glycoprotein, which binds many basic drugs. This is presumably brought about by the predominance of the acidic center of sulfadimethoxine (pK_a 6.7) over the much weaker basic center (pK_b 11.98). Basic drugs, such as imipramine and propranolol, which binds strongly to α_1-acid glycoprotein, appear to have pK_b values which fall roughly within the range of 4–6. Presumably, the basic center of sulfadimethoxine is too weak to permit interaction with α_1-acid glycoprotein.

CONCLUSIONS

Sulfadimethoxine is an amphoteric drug which behaves like an acidic compound in its serum protein binding profile. Albumin is the protein largely responsible for the binding of the drug in blood, although there is also a significant amount of α-globulin binding of sulfadimethoxine in fresh serum.

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Degradation of Crystalline Ergocalciferol [Vitamin D_2, (3β,5*Z*,22*E*)-9,10-Secoergosta-5,7,10(19),22-tetraen-3-ol]

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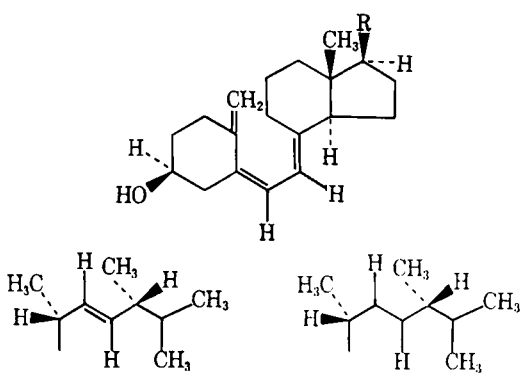
Received June 15, 1981, from the *Department of Medicinal Chemistry and Pharmacognosy, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907.* Accepted for publication October 17, 1983.

Abstract □ The products of the degradation of crystalline ergocalciferol were investigated. These studies showed that numerous acidic and neutral oxidation products were formed resulting in the complete destruction of the triene functionality. Separation of the neutral products by preparative TLC led to material identified as the Windaus ketone IIa, 2,3,3a,4,5,6,7,7aβ-octahydro-7aα-methyl-1*R*-(1α,1*R*,4*R*,5-trimethyl-2*E*-hexenyl)-4*H*-inden-4-one.

Keyphrases □ Ergocalciferol—crystal degradation products, liquid chromatography, identification of a Windaus ketone □ Liquid chromatography—determination of the crystal degradation products of ergocalciferol

Solid-gas reactions are often catalyzed by heat and/or light; thus, these reactions are of importance when studying the mechanism of drug degradation (1-6). This paper reports studies on a specific class of solid-gas reactions: solid-oxygen reactions. A review (1) reports our preliminary studies on the complex heat- and light-catalyzed solid-oxygen reactions of vitamin D_2, ergocalciferol (Ia). This paper presents these studies in greater detail.



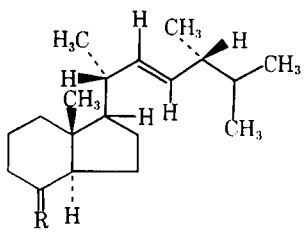
Ia, R = Ib, R =

The oxidative degradation of ergocalciferol has been known for over 40 years (7-11); however, the structures of the solid-state degradation products have not been elucidated. Thus, it is the aim of this study to isolate and unequivocally identify these products. Initially, the decomposition of ergocalciferol in room fluorescent light and air was investigated, resulting in the identification of the Windaus ketone (IIa).

EXPERIMENTAL SECTION

Reagents—Ergocalciferol[1] purchased in sealed ampules was used in all experiments. All reagents employed were of either reagent, spectral, or ACS grade. Methanol used in recrystallization of compounds was purified by reflux with magnesium and iodine followed by distillation over molecular sieves.

Apparatus—All melting points were obtained on a hot stage[2] and are uncorrected. IR spectra[3] were determined neat or as KBr pellets. NMR spectra were obtained using a 60-MHz instrument[4] with either $CDCl_3$ or acetone-d_6 as the solvent and 1% tetramethylsilane as the internal standard. Low-[5] and



Ia, R = 0
Ib, R = NNHCONH$_2$

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Kofler Hot Stage.
[3] Beckman IR-33; Beckman Instruments, Irvine, Calif.
[4] Varian Anaspect EM 360; Varian Associates, Palo Alto, Calif.
[5] Determined by Dr. I. Jardine and associates using a DuPont 21-492B mass spectrometer.

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