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Research Article

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The Binding of Androsterone Sulfate, Etiocholanolone Sulfate, and Dehydroisoandrosterone Sulfate by Human Plasma Protein *

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The 17-ketosteroids appearing in largest quantity in the urine of human subjects are dehydroisoandrosterone, androsterone, and etiocholanolone.[1] These steroids are not excreted to any appreciable extent in unconjugated form, but are conjugated as sulfates and glucuronides (1, 2). Dehydroisoandrosterone is excreted mainly as its sulfate congener, whereas androsterone and etiocholanolone appear in the urine mainly as glucuronides. Nevertheless, the latter two compounds are also excreted as sulfates, and the concentration of these sulfates in urine may equal or exceed that of dehydroisoandrosterone sulfate (1, 2). It has been established that dehydroisoandrosterone sulfate (D-S) is the main 17-ketosteroid in human peripheral plasma (3–5). Androsterone sulfate (A-S) has been identified in smaller quantity in plasma (4–6), and etiocholanolone sulfate (E-S) is present in the smallest concentration of the three conjugates (7). Since the urinary concentration of these steroid sulfates does not appear to be a simple reflection of their usual plasma levels and there is present evidence that A-S is cleared by the kidney at a more rapid rate than D-S (8), it became pertinent to study the excretion of these compounds by the kidney. The glomerular filtrate presented to the kidney tubules may be ideally considered as a protein-free ultrafiltrate of plasma containing only that fraction of the conjugates not bound to plasma proteins. As the initial portion of this investigation it was decided to study the binding of these steroid sulfates to human plasma proteins. Since D-S has been previously shown to be strongly bound to bovine serum albumin at physiological concentrations (9), particular attention was paid to the binding of these three steroid sulfates to human serum albumin.

Methods

Steroid sulfates

E-S was synthesized as its sodium salt. Etiocholanolone was reacted with chlorosulfonic acid in pyridine solution. The pyridinium salt of E-S that was obtained from this reaction was dissolved in hot water and converted to its sodium salt by the addition of solid sodium chloride. The E-S that precipitated out of solution was repeatedly recrystallized from small quantities of water. The final compound melted at 150 to 151° C. It required a 15-hour run to separate it from D-S and A-S in the Schneider and Lewbart no. 2 system (10). After solvolysis the resulting compound and its acetate derivative showed paper chromatographic mobilities identical to the original etiocholanolone and its acetate.

D-S and A-S were purchased from commercial sources.[2] Elemental analysis of these crystalline compounds was provided by their supplier and closely agreed with theoretical calculations of these values. The melting point of these compounds also agreed with values in the literature. In addition, both substances behaved as would be anticipated in two paper chromatographic systems. After solvolysis the free steroids ran identically to authentic samples of the appropriate compounds.

Tritiated steroid sulfates

Twenty-mg samples of E-S and A-S were each labeled by the Wilzbach procedure (11). The labeled compounds were purified by repeated chromatography in several different paper chromatographic systems. The E-S and A-S had specific activities of 11 mc per mg and 4 mc per mg, respectively. Radiochemical purity was assured in two ways. First, each of the purified

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[1] Dehydroisoandrosterone, 3β-hydroxy-Δ5-androstene-17-one; androsterone, 3α-hydroxy-5α-androstan-17-one; etiocholanolone, 3α-hydroxy-5β-androstan-17-one.

[2] Purchased from Mann Research Laboratories, New York, N. Y.

compounds was rerun in two different paper chromatographic systems in mixture with their respective unlabeled steroid. When each of the paper strips was automatically scanned for radioactivity, only a single, symmetrical peak was in evidence, which ran identically to the unlabeled compound. Second, each of the purified tritiated compounds was added to known portions of their respective unlabeled compounds and the specific activity determined. This specific activity stayed within the range of experimental error following chromatography of the conjugates. In addition the specific activity remained appropriate following solvolysis and chromatography of the unconjugated compounds.

Tritiated D-S, SA 1.25 mc per mg, was purchased commercially.[3] This compound met the same criteria of radiochemical purity as those cited above.

Protein solutions. Four protein solutions were studied in the following experiments. These were fresh human serum, fresh or frozen heparinized plasma, a salt-poor human serum albumin solution, and a purified human Fraction V (albumin) preparation.[4] The albumin preparations were more than 97% homogeneous by electrophoretic study. The albumin solutions were prepared in 4% concentration in 0.9% saline.

Equilibrium dialysis procedure (12). Equilibrium dialysis was carried out in 8-ml screw top vials covered with aluminum foil. The semipermeable membrane consisted of a bag made from $\frac{8}{32}$-inch NoJax Visking dialysis tubing. The serum, plasma, or 4% albumin solution was diluted with 4 vol of 0.9% saline and adjusted to pH 7.4 with 0.1 N HCl or 0.1 N NaOH. Two ml of the diluted plasma was delivered into each bag.

Each vial was prepared to contain 6 ml of 0.9% saline. This volume included small quantities of the tritiated steroid sulfate (at most 0.02 μg) and the added carrier steroid sulfate. Approximately 20,000 cpm was added to each vial; this count includes correction for the counting efficiency of the counter and quenching by 0.5 ml of saline or diluted protein solution. The dialyses were brought to equilibrium at 4° C over a 60-hour period on a wheel rotating twelve times a minute. Experiments in in which radioactive steroids were added to the inside or outside of the bag showed equilibrium to be reached in this period of time. After dialysis, 0.5 ml of solution from the inside and from the outside of the bag was pipetted into 20 ml of a liquid scintillator and counted in a scintillation spectrometer with an error of less than 5%.

The percentage of binding was determined by the following equation: per cent bound $= 100\,[1 - (D.Vr/R.Vd)]$, where R and D are the total amounts of radioactivity present inside and outside the dialysis casing, respectively, and Vr and Vd are the corresponding volumes. The concentration of bound steroid inside the dialysis casing in micromoles per liter was then calculated.

The association constant, k, and the number of binding sites, n, per molecule of protein were determined by application of the procedures cited by Edsall and Wyman (13). The data for each of the steroid sulfates were displayed on a Scatchard plot, and approximations for the k and n values were obtained graphically. These values were then further adjusted to fit the equation: $\bar{\nu} = (n_1k_1PA/1 + k_1A) + (n_2k_2PA/1 + k_2A)$, where $\bar{\nu}$ is the concentration of bound steroid, A is the concentration of unbound steroid, and P is the concentration of binding protein. P was calculated with the value of 65,000 as the molecular weight of human serum albumin. The values of n_1 and k_1 and of n_2 and k_2 are for two different sets of binding sites on the albumin molecule with no interaction between sites.

Ultrafiltration procedure. Details of this procedure have been published (12). Eleven ml of heparinized plasma of pH 7.4 was incubated with less than 0.09 μg tritiated steroid for 0.5 hour at 37° C. After equilibration a half-ml plasma sample was taken for radioactive counting, and 10 ml of plasma was transferred to a cellophane casing and spun in a Toribara ultrafiltration apparatus (14) for 40 minutes at 37° C. One-half ml of the ultrafiltrate and of the plasma remaining in the cellophane casing was transferred to scintillation solution and counted. The count in the ultrafiltrate was considered representative of the proportion of unbound steroid in the plasma. The mean count of the plasma samples taken before and after ultrafiltration was used for this calculation.

On three occasions experiments were performed to offer assurance that the steroid conjugates were not being hydrolyzed before ultrafiltration. Several times the usual amount of radioactivity of the three steroid conjugates was added to separate plasma samples, which were then incubated at 37° C for 3 hours. After ultrafiltration, the ultrafiltrate was partitioned between water and chloroform, and the organic phase was backwashed once with water. Greater than 95% of the radioactivity remained in the aqueous phase, strongly suggesting that the steroid conjugates remained intact during the analytical procedure.

Results

The data in Table I were obtained from equilibrium dialysis studies using plasma, serum, and the two human albumin preparations. The results obtained from these four protein preparations were equivalent, and the variation fell within the range of experimental error for this procedure. It appears, from these data, that the binding characteristics of human serum can be accounted for by its albumin content. The data plotted in Figure 1 were obtained by equilibrium dialysis of the salt-poor albumin solution. In this Figure, the per-

[3] Dehydroisoandrosterone-7α-H^3 sulfate was purchased from New England Nuclear Corp., Boston, Mass.

[4] Salt-poor human serum albumin was obtained from the American National Red Cross. The purified Fraction V preparation was provided by the Protein Foundation Laboratories, Jamaica Plain, Mass.

TABLE I

The binding of androsterone sulfate (A-S), etiocholanolone sulfate (E-S), and dehydroisoandrosterone sulfate (D-S) to human plasma, serum, and two albumin preparations

	Tritiated steroid plus amount of carrier steroid*	Plasma	Serum	Salt-poor albumin	Fraction V
	μg		% bound		
A-S	0	98	98	98	97
	50		97	97	
	100	96	96		
	500		87	83	84
	1,000	78	81	80	
	2,500	63	64	65	59
E-S	0	98	98	98	97
	50	97	97	97	
	100	96	96		95
	500	84	84	84	81
	1,000	76	77	79	
	2,500	65	63	67	60
D-S	0	96	95	95	95
	50		94	94	
	100	93	93		92
	300	89			88
	500	86	88	84	87
	1,000	79	83	81	
	2,500		68	68	62

* The amount of steroid added to the 8-ml total volume of the dialysis system.

centage of the steroid sulfate that was bound was plotted against the total amount of steroid sulfate added to the dialysis vials. A-S and E-S showed almost identical data; that is, at low steroid concentration about 98% of the conjugate was bound. With increasing steroid concentration the proportions bound decreased in similar fashion. For D-S, the initial portion of the binding curve was clearly different from that for E-S and A-S. About 95% of the D-S was bound at low steroid concentration, and this percentage remained lower than that for the other two steroids until greater than 1 to 2 μmoles of D-S was added to the dialysis system, at which point, all three curves become superimposed.

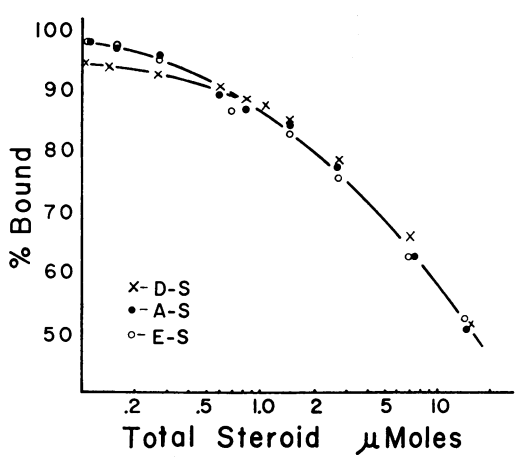

FIG. 1. EQUILIBRIUM DIALYSIS OF THREE STEROID SULFATES, DEHYDROISOANDROSTERONE SULFATE (D-S), ANDROSTERONE SULFATE (A-S), AND ETIOCHOLANOLONE SULFATE (E-S). Two ml of an 0.8% human serum albumin solution was dialyzed against 6 ml of saline. The abscissa values represent the total amount of steroid sulfate added to the dialysis system.

Since the binding curves for A-S and E-S were not found to be different, further experiments were carried out to determine whether either of these steroids differed in its ability to displace the other from the albumin binding sites. In Table II are presented data in which tracer quantities of A-S or of E-S were dialyzed with different carrier quantities of these steroids. It will be noted that tritiated A-S or E-S was displaced equally well by any of the tested quantities of either substance.

TABLE II

Displacement of androsterone sulfate (A-S) and etiocholanolone sulfate (E-S) by each other from human serum albumin

	Unlabeled steroid*	Tracer tritiated steroid	
		A-S	E-S
	μg	% bound	
	0	98	98
A-S	200	91	92
	400	86	87
	500	86	85
	1,000	78	76
	2,500	65	63
E-S	200	91	89
	400	86	84
	500	83	84
	1,000	77	76
	2,500	64	64

* The amount of unlabeled steroid added to the 8-ml total volume of the dialysis system.

It should be pointed out that these albumin binding systems have a rather huge binding capacity in terms of the usual physiological concentrations of these steroids. The initial addition of 0.136 μmole (50 μg) of steroid to the dialysis systems reduced the percentage of E-S or A-S which was bound from 98% to only 97% and that of D-S from 95 to 94%. Since the dialysis system contained an amount of albumin equivalent to that

present in 0.4 ml of plasma, it is apparent that the addition of over 11,000 μg of steroid to 100 ml of plasma would increase the percentage of unbound steroid by approximately only 1%.

Steroid sulfate binding constants. Represented in Figures 2 and 3 are Scatchard plots of the A-S and D-S data. The data for E-S are not presented in a separate figure, as they would plot out in fashion almost identical to those for A-S. Two sets of binding sites per albumin molecule are evident for each of the steroid conjugates. Data for each of these two binding sites were calculated independent of the other and are represented by the two straight lines in both Figures 2 and 3, which run adjacent to the two main slopes of each curve.

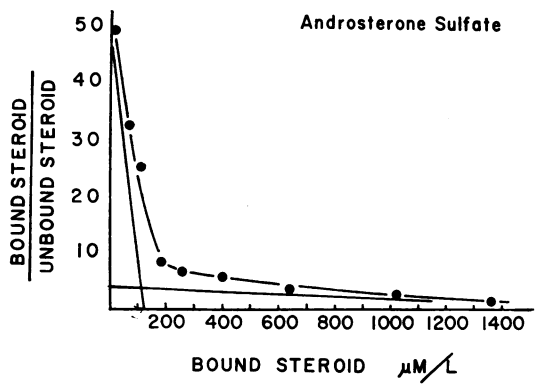

FIG. 2. SCATCHARD TYPE PLOT OF THE DATA OBTAINED FROM EQUILIBRIUM DIALYSIS OF ANDROSTERONE SULFATE. The two straight lines adjacent to the main slopes of the curve are calculated plots of each of the two sets of binding sites independent of the other.

From inspection of the Figure 2 curves, the two components for A-S differ widely in their association affinity. At physiological steroid concentrations, about twelve times as much steroid is associated with the initial binding component as with the second. The concentration of albumin in the dialysis system used for this study was 123 μmoles per L. It will be noted that this approximates the concentration of binding sites for the first binding component as represented by the abscissa intercept, i.e., $n_1 = 1$. By calculation, the association constant $k_1 = 0.36$ L per μmole or 3.6×10^5 L per mole at 4° C. The value of n_2 is approximately 16, and $k_2 = 1.9 \times 10^3$ L per mole for the second set of albumin binding sites.

For D-S, at physiological concentrations, about

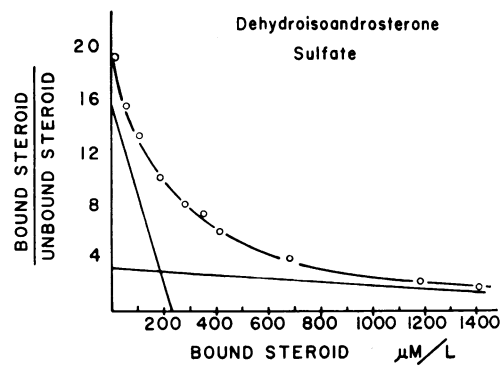

FIG. 3. SCATCHARD TYPE PLOT OF THE DATA OBTAINED FROM EQUILIBRIUM DIALYSIS OF DEHYDROISOANDROSTERONE SULFATE. The two straight lines adjacent to the main slopes of the curve are calculated plots of each of the two sets of binding sites independent of the other.

four to five times as much steroid is bound to the initial binding sites as to the second set of sites. In contrast to A-S or E-S, the abscissa intercept of the initial binding component indicates that there are two sites per albumin molecule ($n_1 = 2$). The value of k_1 is 6.3×10^4 L per mole. For the second set of binding sites n_2 is approximately 18, and $k_2 = 1.5 \times 10^3$ L per mole. The first set of albumin binding sites is clearly different for D-S than for the other two steroid sulfates. The second set of binding sites is similar for all three steroids and may well be identical.

Ultrafiltration studies. Eight different plasmas and a 4.5% human serum albumin solution were each studied on three different occasions using the Toribara apparatus. Here undiluted plasma is studied at 37° C. Both A-S and E-S showed a mean value of 98.8% of the steroid bound (range, 98.6 to 99.0%). For D-S 97.7% was bound (range, 97.6 to 98.0%). Addition of unlabeled steroid to the plasma or albumin solution in concentrations up to 500 μg per 100 ml did not significantly change the ultrafilterable proportion of any of the steroids. This is not surprising since the equilibrium dialysis experiments indicated that many times this concentration of steroid would be necessary to reduce the percentage of bound steroid sufficiently so that it would be detected by this method.

Discussion

Data have been presented on the binding of three androgen sulfates to human serum proteins.

Since the binding of these steroids to serum did not differ from that to serum albumin, it appears that these substances are primarily transported in the blood in an albumin bound form. Studies by other workers of the binding of D-S to bovine serum albumin have suggested that this association is significant in the plasma transport of this steroid conjugate (9). It has been noted that the albumin preparations used for the present study were at least 97% homogeneous by electrophoretic study. Although it is conceivable that a small quantity of a nonalbumin protein with a huge capacity for binding these steroids may account for the presented data, this is unlikely. Since the purified albumin preparations did not differ from serum in their binding curves, the contaminant binding protein would have to fractionate in an almost identical fashion to that of albumin. In addition, the binding capacity and affinity for steroid sulfate that would be required of such a contaminant would be most unusual.

It appears that both A-S and E-S are bound to the same sites of the albumin molecule with approximately the same force. Their binding curves are practically identical, and they displace each other in similar fashion from the albumin binding sites. The site with the larger association constant appears once per albumin molecule. The remaining binding sites appear approximately 16 times per albumin molecule and may well represent relatively nonspecific binding. The site representing the larger association constant for D-S appears twice per albumin molecule. The remaining binding sites for D-S appear to be similar to those for the other two steroid sulfates in mean number of sites per molecule and the value of the association constant. These calculations of course presume that human serum albumin is a homogeneous protein with identical structural and binding characteristics for each albumin molecule. This presumption has not to date been demonstrated with a reasonable degree of certainty.

A precise consideration of the excretion of these three steroid sulfates by the kidney is limited by a lack of general agreement on the true plasma levels of these steroids. There is general agreement, however, that the plasma level of D-S is appreciably greater than that of A-S (3, 4, 8, 15). From the relative urinary levels of these two steroids (1, 2) it appears that A-S is cleared by the kidney at a more rapid rate than D-S. This difference in clearance cannot be ascribed to differences in plasma protein binding. Since a larger proportion of plasma D-S is in the unbound form and therefore presumably filtered through the glomerulus, this would tend to widen the D-S/A-S ratio in the urine, rather than narrow it, as is the case.

Summary

The binding of androsterone sulfate, etiocholanolone sulfate, and dehydroisoandrosterone sulfate to human serum, plasma, and serum albumin was studied by equilibrium dialysis and ultrafiltration techniques. All three of these steroid sulfates are bound primarily to the albumin fraction of plasma. Androsterone sulfate and etiocholanolone sulfate are probably bound to the same sites on the albumin molecule with approximately the same force. Of the two sets of albumin sites for dehydroisoandrosterone sulfate, the set with greater binding affinity is different for this steroid than for the other two. Characteristics of these binding sites for all three steroid sulfates are given.

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