TABLE III

N.M.R. SHIFTS FOR PROTON ON SUBSTITUTED CARBON ATOM IN STEROIDS

Compound	Proton location	Substituent and orientation	Proton conformation	Shift, c.p.s.
Androsterone	C_3	3α-OH	Equatorial	91
Epiandrosterone	C_3	3β-OH	Axial	113
5-Isoandrosterone	C_3	3α-OH	Axial	113
3α-Hydroxypregnane-11,20-dione	C_3	3α-OH	Axial	116
11β-Hydroxyprogesterone	C_{11}	11β-OH	Equatorial	78
9α-Fluorocorticosterone acetate	C_{11}	11β-OH	Equatorial	77
Corticosterone	C_{11}	11β-OH	Equatorial	82
11β-21-Dihydroxy-4,17-(20)-pregnadiene-3-one	C_{11}	11β-OH	Equatorial	78
11α-Hydroxyprogesterone	C_{11}	11α-OH	Axial	95
11α-Hydroxypregnane-3,20-dione	C_{11}	11α-OH	Axial	95
11α-Hydroxyallopregnane-3,20-dione	C_{11}	11α-OH	Axial	100
19-Nortestosterone	C_{17}	17β-OH	(Axial)	110

steroid framework, the n.m.r. method may very well be unrivaled. Although based on empirical correlation with laboriously established known structures, the characteristic chemical shift values associated with axial and equatorial protons should be helpful in cases where a rapid determination of the orientation of a substituent is desired.

The ability to work with a few milligrams of material will allow practical application as well as purely academic studies, although it should be noted that when the sensitivity is pushed to the limit, the esthetic appearance of the spectra will deteriorate, and information associated with the weaker lines will be lost. Samples between 10 and 50 mg. should permit spectra similar to those displayed in the accompanying figures to be obtained. Under these conditions, application of nuclear magnetic resonance techniques to identification problems and structural determinations in steroid chemistry should prove of considerable value in conjunction with the established chemical and physical methods.

Acknowledgment.—One of us, M.T.R., is indebted to the Upjohn Company, Kalamazoo, Michigan, for a grant in support of research on steroids, and to Mr. G. B. Spero and Dr. E. R. Garrett of that company for their help in making samples of several steroids available. We are also indebted to Mr. R. C. Jones and Mr. L. F. Johnson for their expert operation of the spectrometer.

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Steroid–Protein Interactions. V. Comparison of Spectrophotometric and Equilibrium-dialysis Procedures for Determination of Binding Constants[1]

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RECEIVED MAY 29, 1958

The validity of a spectrophotometric procedure for the determination of interaction between proteins and Δ^4-3-ketosteroids was examined by a comparison with the established method of equilibrium dialysis. Values of $r/[S]$, *i.e.*, moles of Δ^4-3-ketosteroid bound per mole human serum albumin, per mole of free steroid, have been calculated from spectrophotometric data for testosterone, progesterone, desoxycorticosterone and cortisol. These binding values were found to be in agreement with those determined by equilibrium dialysis.

Introduction

It has long been known that the absorption spectra of certain molecules are altered in the presence of proteins.[2] This spectral change is indicative of an interaction between the two components; it usually consists of a decrease of the extinction coefficient and may also be connected with a hypsochromic or bathochromic shift of the absorption maximum. This phenomenon has been observed and utilized particularly in the visible range of the spectrum; it constitutes the basis of spectrophotometric methods to measure intermolecular interactions, *e.g.*, with dyestuffs.[2] Analogous findings also have been reported for the ultraviolet range.[3–5]

Recent observations have shown that the ultraviolet absorption spectra of Δ^4-3-ketosteroids are altered in the presence of various proteins.[6] In some cases, the absorption maximum is shifted toward shorter wave lengths; in all cases where interaction takes place, the extinction coefficient ϵ is decreased. The extent of the reduction of ϵ has been found to be approximately proportional to the strength of interaction determined by various other methods.

The spectrophotometric procedure for the determination of interaction between Δ^4-3-ketosteroids and proteins[6] is a convenient technique which permits a rapid analysis of binding behavior in a system free of interference by components other than the solvent and the two interacting solutes. However, in contrast to other methods, *e.g.*, equilibrium dialysis, electrophoresis, solvent distribution, ultracentrifugation and others, the spectrophotometric method does not give information on binding by a physical separation of the free and bound portion of the molecules under investigation. It appeared necessary, therefore, to test the validity of the use of spectral data as an indicator of strength of interaction. In the present study, values for binding

(1) For paper IV of this series see ref. 8.

(2) I. M. Klotz, Chapter 8, "Protein Interactions." In "The Proteins," H. Neurath and K. Bailey, Eds., Vol. I, part B. Academic Press, Inc., New York, N. Y., 1953, p. 727.

(3) H. Theorell and R. Bonnichsen, *Acta Chem. Scand.*, **5**, 1105 (1951).

(4) N. B. Madsen and C. F. Cori, *J. Biol. Chem.*, **224**, 899 (1957).

(5) R. C. Warner, *ibid.*, **229**, 711 (1957).

(6) U. Westphal, *Arch. Biochem. & Biophys.*, **66**, 71 (1957).

between Δ^4-3-ketosteroids and human serum albumin have been determined independently by spectrophotometry and by equilibrium dialysis.

Phosphate buffer of *p*H 7.6, ionic strength 0.1, prepared from reagent grade sodium salts and deionized distilled water, was used as solvent. The steroids[7] were recrystallized several times from undiluted or aqueous ethanol, methanol or acetone. The crystalline human serum albumin preparation[8] was the same as that employed in previous studies.[6]

Steroid solutions ranging from 1 to 4 $\times$ 10^{-5} M were prepared in buffer as previously described.[6] The concentrations of the albumin solutions varied between 0.6 and 6 $\times$ 10^{-5} M, based on a molecular weight of 69,000 which was used in our previous studies. The solutions were prepared and the optical densities determined with the necessary precautions[6] at the wave lengths of maximal absorption (see Table II) in aqueous solution.[6,9] Steroid-free albumin solutions of a concentration equal to that of the experimental samples were used as blanks. Water of 25.0 $\pm$ 0.01° was circulated through the lamp housing of the Beckman DU spectrophotometer. The spectrophotometer was used without a photomultiplier attachment. One-centimeter cells were employed and the extinction coefficients were calculated from the familiar equation

$$\epsilon = \frac{1}{cd} \log (I_0/I)$$

where c is the molar concentration of steroid, d, the thickness of the absorption cell in cm., I_0, the intensity of the light passing through the solvent, and i, the intensity of the light passing through the solution.

The procedure was similar to that of Klotz, Walker and Pivan[10] and of Schellman, Lumry and Samuels.[11] Ground glass stoppered tubes contained 20 ml. of protein-free outside fluid and the dialysis bags[12] 10 ml. of a 4 $\times$ 10^{-5} M (0.276%) albumin solution. The concentration of the steroids was adjusted so that after equilibration the inside solution was approximately 2 $\times$ 10^{-5} M in total steroid. In order to facilitate equilibration, the experiments were started at an inside total steroid concentration twice that of the outside solution. Equilibrium was attained by shaking the sample tubes gently in a constant temperature water-bath at 25 $\pm$ 0.02°. Tubes containing the complete system were set up in triplicate; duplicate control tubes contained all components except the steroids. Equilibration was found to be complete in 24 hr. No changes in the volume inside the dialysis bags occurred during equilibration.

After equilibration, the steroid concentration in the outside fluid was determined by measuring the optical density, using the outside fluids of the control samples as reference solutions. Assuming that the albumin does not affect the activity of the free steroid, the steroid concentration outside may be equated to the concentration of the free steroid inside the dialysis bag. The total amount of steroid inside then equals the difference between the total quantity of steroid applied in the experiment and the amount determined in the outside fluid. It was ascertained in a number of experiments that this analysis gave the same complete recovery as exhaustive extraction of inside and outside phases with redistilled ethyl ether, evaporation of the ether under a nitrogen jet, and determination of the ultraviolet absorption of the steroid in methanol solution in the Beckman DU spectrophotometer at 241 mμ; in this case, the control samples were extracted similarly and the resulting methanol solutions were used as blank solutions in the spectrophotometric measurements.

(7) For the steroids used in this study the authors are greatly indebted to Ciba Pharmaceutical Products, Summit, New Jersey; Merck and Co., Rahway, New Jersey, and Schering Corporation, Bloomfield, New Jersey.

(8) The authors are greatly indebted to the Cutter Laboratories, Berkeley 10, California, for supplying the albumin.

(9) U. Westphal and B. D. Ashley, *J. Biol. Chem.*, **233**, No. 1 (1958).

(10) I. M. Klotz, F. M. Walker and R. B. Pivan, THIS JOURNAL, **68**, 1486 (1946).

(11) J. A. Schellman, R. Lumry and L. T. Samuels, *ibid.*, **76**, 2808 (1954).

(12) Seamless cellulose tubing, 20/32 in. inflated diameter, obtained from The Visking Corporation, Chicago 38, Illinois.

Results and Discussion

All Δ^4-3-ketosteroids are characterized by a specific light absorption of high intensity at approximately 248 mμ (in water). In the presence of certain proteins, *e.g.*, serum albumin, this absorption spectrum undergoes a change which consists essentially of a decrease in the extinction coefficient. In the range of concentrations and under the conditions employed in the present spectrophotometric studies, the steroid solutions follow Beer's law: alterations in the extinction coefficients, therefore, cannot be attributed to displacements in dimer and polymer equilibria. There is practically no change of *p*H on addition of the albumin to the buffered solutions. Thus it becomes apparent that the spectral change is the result of an intermolecular complex between the steroid and the protein. The existence of such complexes has been demonstrated by a number of different experimental procedures.[13] For the calculation of the binding constants from the spectrophotometric data, the approach of Klotz[14] was followed. It is obvious that at a given steroid concentration, increase of the albumin concentration will enhance the percentage of steroid bound to the protein. If ϵ_1 is the molecular extinction coefficient of the steroid in the absence of protein, *i.e.*, at 0% binding and ϵ_2 the molecular extinction coefficient at 100% binding, a numerical value for ϵ_2 can be obtained by graphic extrapolation to zero of the observed molecular extinction coefficient (ϵ apparent) *versus* the ratio, total steroid/total protein. This procedure is exemplified in Fig. 1 for testosterone in the presence of various concentrations of human serum albumin. By interpolation one then obtains α_1, *i.e.*, the fraction of unbound steroid, according to

$$\alpha_1 = \frac{\epsilon_{app} - \epsilon_2}{\epsilon_1 - \epsilon_2}$$

where ϵ_{app} is the molecular extinction coefficient of the steroid observed at any particular protein concentration.

The fraction α_1 permits the calculation of r, *i.e.*, the number of steroid molecules bound per mole protein, according to

$$r = [SB]/[P]$$

where [SB] is the concentration of bound steroid in moles per liter and [P] the concentration of total protein in moles per liter. If [ST] equals the molar concentration of the total steroid, then

$$r = [ST](1 - \alpha_1)/[P]$$

In Table I this calculation is given for testosterone in the presence of human serum albumin. Table I also contains values for the molar concentrations of free steroid, $[S] = \alpha_1[ST]$. From these data the convenient expression for binding strength $r/[S]$[11,15] can be calculated according to

$$\frac{r}{[S]} = \frac{1 - \alpha_1}{\alpha_1[P]}$$

where [S] is the molar concentration of the free steroid.

(13) For literature see ref. 5.

(14) I. M. Klotz, THIS JOURNAL, **68**, 2299 (1946).

(15) K. Eik-Nes, J. A. Schellman, R. Lumry and L. T. Samuels, *J. Biol. Chem.*, **206**, 411 (1954).

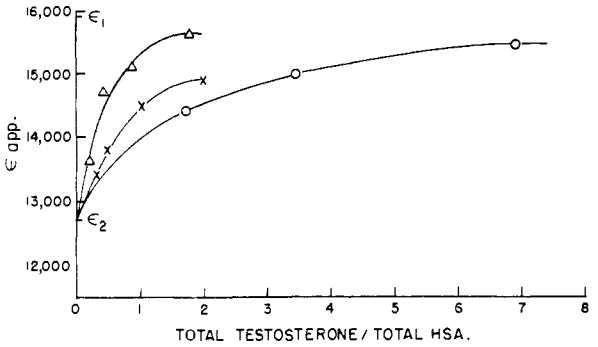

Fig. 1.—Graphic determination of ϵ_2 for testosterone in the presence of human serum albumin (HSA): ϵ_{app} = molecular extinction coefficient of steroid, observed at various concentrations of steroid and/or HSA; ϵ_1 = molecular extinction coefficient of unbound steroid; ϵ_2 = molecular extinction coefficient of completely bound steroid. Concentrations of testosterone: △, 10^{-5} *M*; x, 2×10^{-5} *M*; O, 4×10^{-5} *M*. Phosphate buffer *p*H 7.6; μ = 0.1; 25°.

TABLE I

CALCULATION OF PROTEIN-BOUND TESTOSTERONE

ϵ_1 = 15,900; ϵ_2 = 12,700

[ST] = total concn. of testosterone × 10^{-5}, moles/l.	[P] = concn. of albumin × 10^{-5} moles/l.	ε apparent at 249 mμ	α_1	[S] = concn. of free testosterone × 10^{-5} moles/l.	[SB] = concn. of bound testosterone × 10^{-5} moles/l.	r = Moles bound testosterone / Moles total albumin
2.00	6.00	13,390	0.22	0.43	1.57	0.26
2.00	4.00	13,800	.34	0.69	1.31	.33
2.00	2.00	14,520	.57	1.14	0.86	.43
2.00	1.00	14,890	.68	1.37	0.63	.63
4.16	2.40	14,410	.53	2.22	1.94	.81
4.16	1.20	14,990	.72	2.98	1.18	.98
4.16	0.60	15,440	.86	3.56	0.60	1.00

By this procedure, spectrophotometric binding constants have been determined for testosterone, progesterone, desoxycorticosterone and cortisol. Graphic illustrations of the plot $1/r$ *versus* $1/[S]$ are given in Figs. 2–4; they show that the constancy

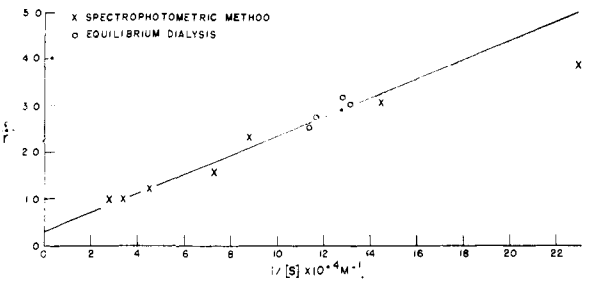

Fig. 2.—Binding of testosterone to human serum albumin: r = moles steroid bound per mole total protein; [S] = concentration of unbound steroid in moles per liter; phosphate buffer *p*H 7.6, μ = 0.1; 25°.

of this ratio is in general adequate although the precision of the values may be somewhat lower than that of corresponding figures obtained by equilibrium dialysis, as, for instance, for testosterone and bovine serum albumin.[11] Table II shows spectrophotometric data for the interaction of human

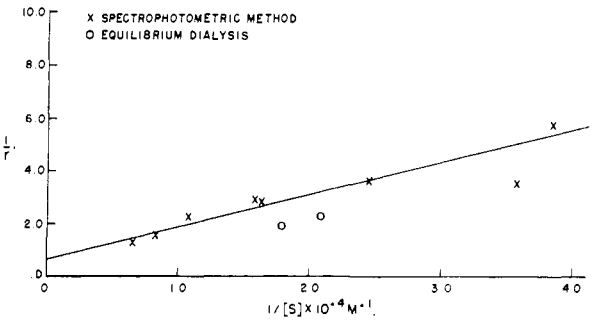

Fig. 3.—Binding of progesterone to human serum albumin. For explanations see Fig. 2.

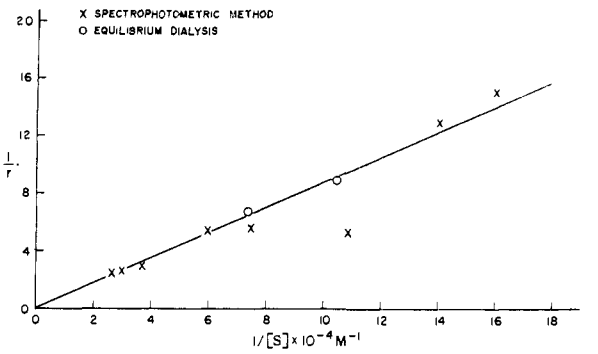

Fig. 4.—Binding of cortisol to human serum albumin. For explanations see Fig. 2.

serum albumin with the four steroid hormones studied, including average values for $r/[S]$ calculated from spectral and dialysis data (see below).

TABLE II

BINDING VALUES OF STEROIDS DETERMINED BY SPECTROPHOTOMETRIC METHOD

Phosphate buffer *p*H 7.6; μ 0.1; 25.0°

Steroid	Abs. max., mμ	ϵ_1	ϵ_2	$\epsilon_1 - \epsilon_2$	$\frac{r}{[S]} \times 10^{-4}$	n[a]	$\frac{r}{[S]} \times 10^{-4}$[b]
Testosterone	249	15,900	12,700	3200	4.1	7	4.3
Progesterone	249	16,700	13,300	3400	6.6	9	8.9
Desoxycorticosterone	249	16,300	13,000	3300	4.1	13	4.7
Cortisol	248	15,900	13,400	2500	1.3	8	1.3

[a] n = number of values of different steroid and HSA concentrations from which $r/[S]$ was averaged. [b] Average $r/[S]$ values determined by equilibrium dialysis (Table III).

Since equilibrium dialysis generally has been accepted as a reliable and accurate method for the determination of binding between small molecules and proteins,[2] this technique was adopted to test the binding values obtained by the spectrophotometric procedure. In the spectral determination of interaction between Δ^4-3-ketosteroids and proteins, standard concentrations of 2×10^{-5} *M* for steroid and 4×10^{-5} *M* for serum albumin have been used[6] for convenient comparison of the various steroids.[9] The same concentrations were employed in the dialysis equilibrium experiments; similarly, all other conditions resembled as closely as pos-

sible those of the spectrophotometric method. Table III gives the results obtained for the four steroids investigated in the present study, including the $r/[S]$ values. The average figures for $r/[S]$ from equilibrium dialysis have been included in Table II with the corresponding values obtained by the spectrophotometric procedure.

A comparison of the $r/[S]$ values given by the two methods shows generally good agreement. This becomes also apparent from Fig. 2–4 which include the reciprocals of r and [S] obtained by the spectral procedure and equilibrium dialysis. In the case of progesterone, the spectrophotometric binding value is somewhat lower than that calculated from dialysis equilibrium. Although the data of Fig. 2–4 indicate a comparatively wide range of error for the spectrophotometric procedure,[6] the small difference found by the two methods for progesterone may represent some real dissimilarity beyond the range of error. Equilibrium dialysis measures binding by physical retention, *i.e.*, by complexing the whole steroid molecule with the protein. Other things being equal, all regions of the steroid molecule should have an equal chance of interacting with the protein surface. The aliphatic nature of the methyl group, C-21, is thus likely to increase the binding of progesterone to such proteins as serum albumin; the binding value would therefore be greater than that of desoxycorticosterone with its polar hydroxy group at C-21. On the other hand, the spectrophotometric procedure measures the interaction in a different way; the chromophoric system in ring A is influenced by groupings in the steroid molecule which complex with the protein. Such influence is likely to be greater when the interacting group in the steroid is close to the chromophoric group and will be less pronounced when it is at a greater distance. This interpretation may explain why the difference between the binding values of progesterone and desoxycorticosterone is smaller when determined by the spectrophotometric method than when obtained by equilibrium dialysis. This concept is further supported by spectral observations on the interaction of human serum albumin with a number of Δ^4-3-ketosteroids containing hydroxy groups at various distances from ring A.[9] In general, the spectral influence on protein interaction of functional groups was found to decrease somewhat with increasing distance from the chromophoric system.

The data of Table II illustrate that the spectrophotometric method gives correct values for the

TABLE III

BINDING VALUES OF STEROIDS DETERMINED BY EQUILIBRIUM DIALYSIS

Phosphate buffer *p*H 7.6; μ 0.1; 25.0°, 0.4 × 10^{-4} *M* human serum albumin

Steroid	Exp. no.	Total steroid concn.[a] × 10^{-4} *M*	α_f	$\frac{r}{[S]} \times 10^{-4}$
Testosterone	1	0.2036	0.38	4.03
	2	.2081	.37	4.33
	3	.2120	.40	4.25
	4	.2252	.39	4.49
Progesterone	1	.2375	.24	8.76
	2	.2012	.24	9.12
Desoxycorticosterone	1	.1938	.39	4.53
	2	.1987	.37	4.84
Cortisol	1	.1407	.68	1.35
	2	.1960	.69	1.26

[a] Inside dialysis bag after equilibration.

interaction between Δ^4-3-ketosteroids and albumin. No exception to this conclusion was noted when the interaction of some 30 additional Δ^4-3-ketosteroids was studied with human serum albumin and β-lactoglobulin.[9] The spectral phenomenon is not limited to interactions of Δ^4-3-ketosteroids since similar reductions of ϵ were observed for mesityl oxide and isophorone in the presence of human serum albumin or β-lactoglobulin. On the other hand, the extinction coefficients of the Δ^4-3-ketosteroids were depressed by a number of small molecules containing nitrogen, including adenine, adenosine, the adenosine phosphates, cytidine, DPN^+, TPN^+ and other nucleosides, nucleotides and related compounds. These observations will be discussed in a later report. Interactions between steroids and purine derivatives have been studied recently by Scott, Munck and Engel.[16,17]

The validity of the spectrophotometric method of measuring interaction with proteins and other nitrogenous molecules has been established for compounds which contain a single conjugated system, in most cases an α,β-unsaturated keto grouping. No abnormal behavior was observed when compounds with cross-conjugations ($\Delta^{1,4}$-3-ketosteroids) were studied.[9] The method should not be extended, however, to compounds containing more than one conjugated system.

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