vestigated, seven (*L. casei, L. arabinosus, L. pentosus, L. brassicae, L. fermentatus, L. brevis* and *L. pentoaceticus*) showed no stimulation at concentrations of analogs below the inhibitory range. In almost all of the other cases stimulation was noted with all of the analogs.[31]

(31) The data were insufficient to classify *S. faecalis* R. while *L. dextranicum* 8086 gave only a slight stimulation. *L. lycopersici* was the only organism which was stimulated by some but not all analogs.

LOS ANGELES, CALIFORNIA

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, UNIVERSITY OF UTAH]

The Binding of Uncharged Molecules to Proteins. II. Testosterone and Bovine Serum Albumin[1,2]

BY JOHN A. SCHELLMAN,[3] RUFUS LUMRY[4] AND LEO T. SAMUELS

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The binding reaction between bovine serum albumin and testosterone has been investigated as a function of steroid concentration, protein preparation, temperature and hydrogen-ion concentration by means of dialysis and partition techniques. The data are interpreted in terms of a linear relation between the reciprocal of the average number of molecules of substrate bound per protein molecule and the reciprocal of substrate concentration. This relation, which introduced the average association constant and a measure of the heterogeneity of binding sites, is shown to apply generally in cases of low substrate concentration. Total binding increases with purity of preparation and with temperature. The amount of binding increases with *p*H and is reduced in the presence of thiocyanate ion, methyl orange anions and zinc cations. The free energy of binding is the same as observed with charged and uncharged organic molecules. The enthalpy change is positive so that binding depends on a large positive entropy change. An explanation for this fact, and in general for "configurational adaptability," is discussed as the result of local relaxation in the "tertiary" folding of the protein. Rough data for the binding of cortisone and estradiol are given and a tentative correlation of the various observations is presented.

In another publication we have shown that a large variety of steroids form stable complexes with serum albumin.[5] These studies plus those of Klotz, *et al.*, on substituted azo benzenes,[6] adenosine[7] and sulfonamides[8]; Bischoff and co-workers on steroids[9]; and the work of Carsten and Eisen[10] on neutral dinitrophenyl compounds have established the binding of uncharged organic molecules to serum albumin as a quite general phenomenon. In the present paper we attempt to correlate the binding of steroids with that of anions to determine the mode of interaction, and to indicate the significance of the empirical isotherm. Since it is known that commercial serum albumin contains traces of strongly bound small molecules varying with the method of preparation,[11] the results reported here may not necessarily be compared with those obtained using other preparations. To some extent the studies reported here are of but relative quantitative significance just as are most other studies with serum albumins.

(1) Reported before the Biochemical Division at the Atlantic City Meeting of the American Chemical Society, September, 1952.

(2) This investigation was supported in part by a research grant C-307 from the National Cancer Institute of the National Institutes of Health, Public Health Service, in part under a research grant from the American Cancer Society and by the United States Atomic Energy Commission under Contract No. AT (11-1)-82 Project 4.

(3) United States Public Health Service Fellow.

(4) Merck Fellow in the Natural Sciences during part of this investigation. School of Chemistry, University of Minnesota, Minneapolis, Minn.

(5) K. Eik-Nes, J. A. Schellman, R. Lumry and L. T. Samuels, *J. Biol. Chem.*, **206**, 411 (1953).

(6) I. M. Klotz and J. Ayers, THIS JOURNAL, **74**, 6178 (1952).

(7) I. M. Klotz and F. Walker, *ibid.*, **70**, 943 (1948).

(8) I. M. Klotz and J. Urquhart, *J. Biol. Chem.*, **173**, 21 (1948).

(9) F. Bischoff and R. E. Katherman, *Federation Proc.*, **11**, 188 (1952).

(10) M. E. Carsten and H. N. Eisen, *ibid.*, **12**, 187 (1953).

(11) J. L. Oncley and H. M. Dintzis, Paper presented at the 122nd Meeting of the American Chemical Society, Atlantic City, N. J., September, 1952.

Experimental

The determination of an accurate isotherm in these binding studies, as in other adsorption studies, requires the determination of free and combined substrate over a considerable range of concentration. For this reason two experimental techniques have been developed for use with steroids and similar molecules: dialysis equilibrium such as employed by other investigators in protein binding[12]; and partition analysis, a technique previously employed by Karush[13] in dye-binding studies.

Dialysis Equilibrium.—The procedure is similar to that described by Klotz, Walker and Pivan.[12] A solution of the steroid in Locke buffer[5] (ionic strength 0.155 *M*) was placed outside the dialysis bag (Visking) and a solution of albumin in the same buffer was placed within. Equilibrium was secured by rotating the sample tubes, which were completely filled to prevent protein denaturation at the air interface, in a constant temperature water-bath. Experiments demonstrated that equilibration was complete in 24 hours. Aliquots of the solutions from inside and outside the bag were extracted five times with ethyl ether. The ether was evaporated under an air jet, the steroid taken up in absolute ethanol and the ultraviolet absorption measured on a Cary spectrophotometer over the range of 2200–3000 Å. The non-linear background spectrum, probably the result of extracted phosphate, could be eliminated by the empirical functions

$\Delta_{2400} = 2.14D_{2400\text{ Å.}} - (D_{2300\text{ Å.}} + D_{2500\text{ Å.}})$ for testosterone

$\Delta_{2360} = 2.0D_{2360\text{ Å.}} - (D_{2260\text{ Å.}} + D_{2460\text{ Å.}})$ for cortisone

The D's are the optical densities at the indicated wave lengths and Δ is a factor proportionate to the concentration of the steroid at the wave length of maximum absorption in ethanol. Assuming that the protein does not affect the activity of the free steroid, the free steroid in the bag may be equated to the steroid concentration outside and the bound steroid to the difference between this and the total concentration inside the bag. All dialysis experiments were performed on 1% protein solutions. No appreciable changes in the volume contained in the dialysis bag occurred during the experiments as judged by experiments with partially filled dialysis tubes.

Partition Analysis.—The substrate was equilibrated in a two-phase system consisting of the protein solution and an

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

(13) F. Karush, *ibid.*, **73**, 1246 (1951).

immiscible organic solvent, in this case *n*-heptane (purified Skellysolve C). A typical experiment consisted of the following steps: A series of known testosterone solutions in heptane was prepared. The individual solutions were then each equilibrated with $M/15$ phosphate buffer (ionic strength 0.174 M) by gentle rocking for a period of 24 hours at the desired temperature in a device prepared for this purpose. Both solutions were analyzed; the aqueous solutions by direct reading in a Beckman DU spectrophotometer against a buffer blank at 2485 Å., the wave length of maximum absorption in buffer; the heptane solutions by evaporating off the heptane, redissolving the steroid in ethanol and reading at 2400 Å. Absorption constants calculated from standard solutions were used in the determination of concentrations and thus ultimately of the partition coefficient α which was found to be independent of concentration for all steroids investigated. Accurately measured amounts of solutions of steroid in heptane were then equilibrated with protein solutions in $M/15$ phosphate buffer and the heptane phase was analyzed. The total gain of steroid by the aqueous phase equals the loss from the heptane phase. The free testosterone in the aqueous phase was calculated from the final concentration in heptane divided by α; the bound amount was obtained by subtraction of this from the total. The results with this method were more precise than those obtained with the dialysis procedure, probably as a result of the absence of background absorption in the spectra. The method also has the advantage of considerably greater simplicity.[14] All partitioning experiments unless otherwise noted were performed with 2% protein solutions.

Several of the experiments required only the determination of total solubilities in the presence of excess solid steroid. The procedure employed for these determinations has been described elsewhere.[5]

The binding of estradiol was studied by means of the partition technique, toluene serving as organic solvent. Estradiol is so insoluble in water that it cannot be detected in aqueous solution though it is strongly bound by serum albumin. The partition coefficient was determined by equilibrating 5 ml. of estradiol solution in toluene with 1 liter of buffer solution saturated with toluene, and analyzing the toluene phase before and after equilibration. It was found to be 250 ± 25. All estradiol binding studies were carried out at *p*H 7.4 (phosphate buffer) and 25.0° in 2% protein at 0.17 M ionic strength.

Scatchard[15] has emphasized the complications resulting from protein studies in salt solutions since many inorganic anions are bound by serum albumin. The ions of phosphate buffer at the concentrations used here are known to compete with organic anions.[13,16] Experiments discussed below, however, did not reveal such competition in the systems used. Penicillin was employed as an antibiotic in all dialysis experiments and in high-temperature partition studies. Though this substance has been reported to be a competitor in binding studies[17] no unequivocal confirmation of this fact was observed with the concentrations used here (100 units/ml.).

All experiments were run in triplicate. The dialysis experiments on testosterone demonstrated a standard error of about 5%. The partition experiments on this steroid were reproducible to ±2%. The estradiol experiments, however, involved a larger error because of the high partition coefficient.

Materials.—Since different amounts of binding were observed with different preparations of bovine serum albumin (BSA) it is necessary to distinguish the three preparations[18] employed: A. Lot G. 10106 Bovine fraction V amorphous powder: ash, 0.97%; moisture, 4.63%; electrophoresis analysis in *p*H 7.7 phosphate buffer: albumin, 93.1%; globulin, 6.9%.

B. Lot G. 212112 crystallized bovine albumin: ash, 0.32%; moisture, 1.34%; electrophoresis analysis in *p*H 8.6 barbiturate buffer: albumin, 97%; globulin, 3%.

C. Lot G. 370295 crystallized bovine albumin: ash, 0.21%; moisture, 0.76%; electrophoresis analysis in *p*H 8.6 barbiturate buffer: albumin, 98%, globulin, 2%.

$CuCl_2$, $ZnCl_2$, KSCN and all buffer materials and other salts were of C.P. grade. Heptane (Skellysolve C) was redistilled after washing with sulfuric acid and water. Methyl orange and toluene were of reagent grade. Testosterone, estradiol and cortisone were obtained as pure compounds.[19] Melting points were checked and found to correspond with the reported values.

Results

Interpretation of Binding Data.—The well-known equation[20] governing multiple association between a polyvalent substance such as a protein and an univalent substrate in the absence of interaction among valences of the larger molecule is

$$r = \frac{S_{\text{Bound}}}{P_{\text{Total}}} = \frac{S\sum_{i=1}^{n} K_i + 2S^2 \sum_{i=1}^{n-1}\sum_{j>i}^{n} K_iK_i + \dots}{1 + S\sum_{i=1}^{n} K_i + S^2 \sum_{i=1}^{n-1}\sum_{j>i}^{n} K_iK_i + \dots} \quad (1)$$

wherein S is the concentration of substrate, K_i is the association constant for the ith site, and n is the number of sites. The quantity r is the average number of molecules bound to a protein molecule. If the K_i are all equal, (1) reduces to

$$\frac{1}{r} = \frac{1}{nK} + \frac{1}{S} + \frac{1}{n} \quad (2)$$

and a plot of $1/r$ *vs.* $1/S$ yields a straight line from which values of n and K may be calculated. However, examination of (1) demonstrates that (2) is not a unique consequence of identical sites but will always be observed at the very low substrate concentrations frequently employed in binding studies with proteins. This fact may be shown in the following way. Inverting (1) and expanding the right-hand side in powers of S by long division, (3) is obtained

$$\frac{1}{r} = \frac{1}{S\sum_{i=1}^{n} K_i} + \left(1 - \frac{2\sum_{j>i}^{n} K_i K_j}{\left(\sum_{i=1}^{n} K_i\right)^2}\right) + R(S) + R'(S^2) + \dots \quad (3)$$

in which R, R', etc., are functions of first and higher powers of S and thus negligible in very dilute solution. On recalling the identity $\left(\sum_{i=1}^{n} K_i\right)^2 = \sum_{i=1}^{n} K_i^2 + 2\sum_{j>i}^{n} K_iK_j$, and the definitions

$$\langle K\rangle = \frac{\sum_{i=1}^{n} K_i}{n}, \quad \langle K^2\rangle = \frac{\sum_{i=1}^{n} K_i^2}{n}$$

(14) Though the extremely low solubility of heptane in water minimizes competition between partitioning solvent and substrate in these experiments, some competition may exist. Its extent should be small relative to that provided by toluene or hexanol, both of which are known to be weak competitors with dye anions.[13]

(15) G. Scatchard, *Am. Scientist*, **40**, 61 (1952).

(16) I. M. Klotz, H. Triwush and F. M. Walker, THIS JOURNAL, **70**, 2935 (1948).

(17) I. M. Klotz, J. Urquhart and W. Webes, *Arch. Biochem.*, **26**, 420 (1950).

(18) All protein preparations were obtained from the Armour Laboratories; we are indebted to Mr. R. J. Seidel for the analyses.

(19) The steroids used were supplied through the courtesy of Ciba Pharmaceutical Products, Inc., Summit, N. J., and Merck and Co., Inc., Rahway, N. J.

(20) (a) A. von Muralt, THIS JOURNAL, **52**, 3518 (1930); (b) I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946).

with n the true number of sites, (3) takes on the form for small S

$$\frac{1}{r} = \frac{1}{n<K>} \times \frac{1}{S} + \frac{1}{n}\frac{<K^2>}{<K>^2} \qquad (4)$$

Substituting the second moment of the distribution of the K's about the mean K, $\mu^2 = <K^2> - <K>^2$, (5) results

$$\frac{1}{r} = \frac{1}{n<K>} \times \frac{1}{S} + \frac{1}{n}\left(1 + \frac{\mu^2}{<K>^2}\right) \qquad (5)$$

A similar result has been derived for polyelectrolytes.[21] Equation 5 may be converted to a form analogous to that employed by Scatchard and coworkers[22] by multiplying through by $n<K>r$. The number of binding sites n can be determined directly from plots according to (2) only in the unlikely event that the second moment is zero or at least small with respect to K.

The error in interpretation according to (2) may be considerable. Consider a protein with 10 identical sites each having association constant of unity. (2) applies here in an unambiguous way. If, however, we consider a similar protein but possessing an eleventh site with constant 5, (2) yields an apparent number of sites 6.4 and an apparent association constant of 2.3 to two significant figures.

The low water solubility of testosterone and most other steroids has restricted all measurements to regions in which (5) applies and the values calculated with the use of (2) must be considered only as relative binding parameters, as indeed they must in most other studies of the binding of other substances by serum albumin.[23] Even in studies in which heterogeneity has been detected, the sites have been divided into but two classes and the results are probably still subject to considerable error. Karush in particular has consistently emphasized the ambiguity of empirically determined values of n.[12] In the absence of knowledge of the heterogeneity of the binding sites, only $n<K>$ has theoretical significance.

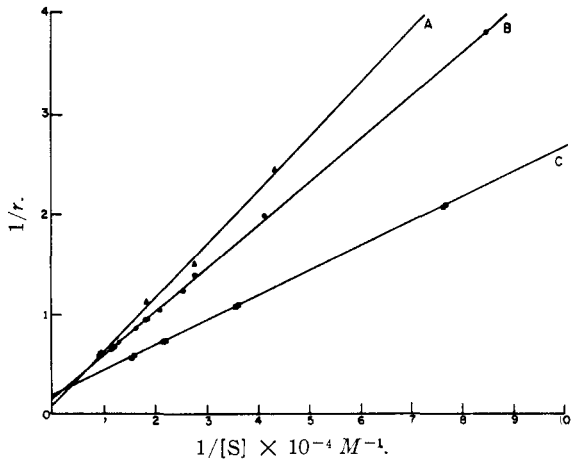

Fig. 1.—Binding of testosterone at 25° to three preparations of BSA of increasing purity dissolved in phosphate buffer, pH 7.4 ± 0.1. Preparation A was studied by dialysis; B and C were studied in partition experiments, plotted according to equation 5.

The Variability of Protein Samples.—The results of studies on the binding of testosterone at pH 7.4 ± 0.1 and 25° to three samples of BSA of increasing purity (A, B and C) are shown in Fig. 1. $n<K>$ values calculated by the application of "least squares" to unweighted data according to (5) appear in Table I, column 1.[24] Simple interpretation according to (2) suggests a decrease in number of sites (column 2) coupled with an increase in K (column 3) as purity improves. A more reasonable interpretation follows from (5) and suggests an increase in heterogeneity with purity as might be expected if the protein in its preparation has been more carefully freed of substances occupying strong binding sites. The example in the previous subsection is directly analogous to the difference between BSA samples A and B except in the values of the constants. There is as yet no reason to believe that even preparation C is completely free of impurities held at strong and important sites. Comparisons of variables in binding studies then should be confined to experiments on a single preparation.

TABLE I

THE BINDING OF TESTOSTERONE TO THREE PROTEIN SAMPLES AT 25°, pH 7.4 ± 0.1[a]

Protein	$n<K>$ × 10^{-4} M^{-1}	n (apparent)[b]	K (apparent),[b] × 10^{-3} M^{-1}
A	1.9	10	1.8
B	2.3	6	3.8
C	4.0	5	7.9

[a] The data on protein A were obtained by the dialysis method and on proteins B and C by partition analysis. [b] Calculated on the assumption of no heterogeneity among binding sites.

No saturated solubilities of testosterone in solutions of protein C could be obtained since testosterone even in the small concentrations available precipitated the protein. Dialysis of the resulting solid against buffer returned the material to solution. No difficulties were encountered in the partitioning experiments.

Effect of Ionic Strength on Steroid Solubility.—In order to make the comparison shown in Table I and other comparisons between results of dialysis and partition experiments it was necessary to establish that no large ionic strength effects occur. In Table II the effect of ionic strength on saturation solubility of testosterone is shown to be small and negligible. The more important experiments to determine the effect of ionic strength on binding

TABLE II

THE EFFECT OF IONIC STRENGTH ON THE AQUEOUS SOLUBILITY OF TESTOSTERONE, T = 25°

Ionic strength, M	0	0.05	0.1	0.25	0.5
Solubility, M × 10^6	116	112	111	98	93

(21) See for instance P. Doty and G. Ehrlich, *Ann. Rev. Phys. Chem.*, **3**, 82 (1952).

(22) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(23) In principle, the method of Sips (R. Sips, *J. Chem. Phys.*, **16**, 490 (1948); G. Halsey and H. S. Taylor, *ibid.*, **15**, 624 (1947)) offers a means for calculating a distribution function for the K's and thence the second moment of this distribution. In practice the method applies only to n larger than observed in these studies.

(24) The choice of eq. 5 or 2, especially without corrective weighing as a basis for least squares treatment, is justified only by the semi-quantitative nature of the results. The procedure will never yield "best" values of the binding parameters.

were not performed and so the quantitative comparisons in Table I may be somewhat unreliable.

Temperature Studies.—In this and subsequent experiments protein sample B was used. The effect of temperature on partition coefficients and binding parameters is shown in Table III. The free energies of association tabulated were calculated from $n\langle K\rangle = \sum_{i=1}^{n} K_i$ and thus apply to the first association between protein and substrate: P + S = PS. The results are reproducible but not readily interpreted since a maximum in $n\langle K\rangle$ is seen. While this maximum may be an intrinsic property of the binding reaction, perhaps as a result of changes in ionization with temperature, it also may be the result of competition between steroid and penicillin at the highest temperature, since the antibiotic was used only during equilibration at 37.5°. Omitting the upper point a positive enthalpy of association of about 2 kcal. may be calculated. In any event the entropy of association is positive, a characteristic of the binding of anions to serum albumin.[25]

TABLE III

THE EFFECT OF TEMPERATURE ON THE BINDING OF TESTOSTERONE *p*H 7.4, PROTEIN B

T, °C.	Method	$n\langle K\rangle \times 10^{-4}$ M^{-1}	n(apparent)[a]	$\Delta F°$, kcal./mole S	P
8.0	Partition	1.7	7	−5.5	3.13
25.0	Partition	2.3	6	−6.0	7.50
37.5	Partition	2.0	4	−6.1	9.64

[a] Calculated on the assumption of no heterogeneity among binding sites.

The Effect of *p*H.—The saturated solubility of testosterone in 1% BSA at three *p*H values (Table IV) was determined in Locke buffer solution varied to maintain a constant ionic strength of 0.155 *M*. Solubility increased with *p*H. Control experiments demonstrated no significant change in solubility of testosterone in buffer; thus the change must be attributed to increased binding. The possibility of an effect due to competition between the steroid and $H_2PO_4^-$ and $HPO_4^=$ was eliminated by control experiments in 0.5 *M* NaCl with *p*H values established by addition of NaOH. The same change in solubility was observed. An increase in amount bound with increasing *p*H has been reported by Klotz and Ayers[6] working with substituted diazobenzenes and by Levedahl working with steroids.[26] Of perhaps more interest is the observation that dye anions show a similar dependence on hydrogen-ion concentration.[27]

TABLE IV

THE VARIATION OF THE SOLUBILITY OF TESTOSTERONE IN 1% SERUM ALBUMIN WITH *p*H, PROTEIN B

*p*H	6.5	7.4	8.5
Solubility, $M \times 10^6$	570	610	770

Competition with Anions.—The similarity between the binding of anion and of neutral molecule to protein suggested by the *p*H variation was further substantiated by competition experiments in which thiocyanate and methyl orange anions were added to the testosterone–BSA solution. Competition with –SCN was established by varying testosterone at fixed KSCN concentrations in *p*H 7.4 phosphate buffer at 25° using the partition method. Ionic strength was maintained constant by appropriate addition of NaCl. Results with 0.1 *M* KSCN are shown in Fig. 2.

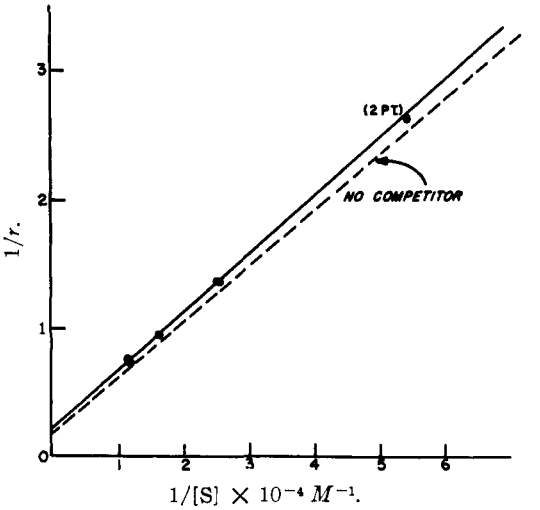

Fig. 2.—Competition between testosterone and thiocyanate ions for BSA, 25°; phosphate buffer *p*H 7.4 ± 0.1; 0.1 *M* KSCN; plotted according to equation 5; partition technique.

Although competition by –SCN was detectable at 0.015 *M* in that ion, the effect was not large at any concentration. Simultaneous control experiments without competing ion provided results identical with those previously tabulated. The slight competition observed under the circumstances shows a much greater affinity of the protein for the steroid than for the anion at the sites where competition is possible. Similar competition between aminoazobenzene and –SCN has been observed by Klotz and Ayers.[6]

Methyl orange anions in contrast to thiocyanate ions are efficient competitors for steroids. Competition of the dye molecules was determined by a more qualitative procedure in which solutions of BSA were prepared containing concentrations of dye sufficient to give values of r of 6, 10 and 15 in the absence of steroid. These concentrations were calculated from Klotz' data[12] and are thus only approximate in view of the variability of protein preparations. These solutions of dye anion and BSA were equilibrated against a standard solution of testosterone in heptane and the quantity of bound steroid was obtained in the usual manner. Within experimental precision no competition occurred at $r = 6$. At r equals 10 and 15 the experimental r values for testosterone were 1.0 and 1.0, respectively, against the control value (no dye) of 1.3. These experiments establish that competition occurs between anion and neutral molecule but probably not at all binding sites for the latter.

(25) I. M. Klotz, *Cold Spring Harbor Sym. Quant. Biol.*, **14**, 97 (1949).

(26) B. Levedahl, private communication.

(27) I. M. Klotz and J. M. Urquhart, *J. Phys. Colloid Chem.*, **53**, 100 (1949).

Competition with Cations.—The variation in binding with *p*H occurs in the region of dissociation of the imidazole groups of the protein, which suggests that these groups may participate directly in the binding. Recently, Tanford[28] has shown that several divalent cations attach themselves to the neutral imidazole groups of serum albumin. As a check on imidazole involvement two of these cations, Cu^{++} and Zn^{++}, were tested as competitors with testosterone. The experiments, carried out at *p*H 7.4, 25°, were buffer free to escape precipitation of the metal ions by phosphate. Protein and metal chlorides were dissolved in separate solutions of 0.174 *M* NaCl and titrated to *p*H 7.4 with 0.074 *M* NaOH in 0.1 *M* NaCl. The solutions were mixed and, after final dilution and *p*H adjustments, were equilibrated against standard solutions of testosterone. The results are shown in Fig. 3. Most of the experiments with these metal ions had to be discarded because of the formation of a precipitate, which occurred slowly and at a varying rate. Fig. 3 is based on experiments which remained clear during the equilibration. The copper curve consists of only four determinations. All other experiments formed precipitates, though all controllable conditions were the same.

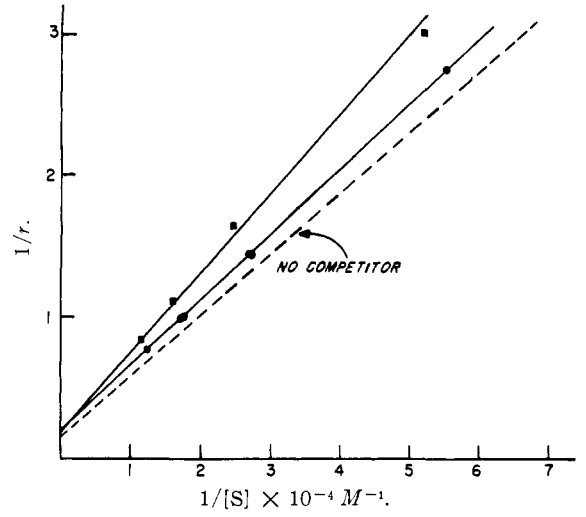

Fig. 3.—Competition between testosterone and zinc and cupric ions for BSA. Solid circles obtained with 5×10^{-4} *M* $CuCl_2$; solid squares obtained with 2×10^{-3} *M* $ZnCl_2$, 25°, *p*H 7.4 by adjustment with NaOH; ionic strength 0.174 *M*; partition technique.

Considering the low concentrations employed, the metal ions have a strong inhibitory effect on the binding of testosterone. This is most likely a direct competition for sites on the protein molecule, but not necessarily because the binding of the cations decreases the negative charge of the protein and the inhibition may be just another manifestation of the *p*H effect.

The Binding of Cortisone and Estradiol by BSA.—In a previous paper[5] a correlation between the amount of binding to BSA and the number of polar groups on the neutral substrate was demonstrated. To provide further information as to the cause of this relationship, an attempt was made to obtain full curves with cortisone (Δ^4-pregnene-11,17,21-triol-3,20-dione) and estradiol ($\Delta^{1,3,5}$-estratriene-3,17β-diol). The few experiments with both steroids gave rather large scatter but still allowed an approximation of $n<K>$ values. Partition experiments with estradiol (*p*H 7.4; 2% BSA, sample B; ionic strength 0.174 *M*; 25°) gave $n<K> = 3 \times 10^5\ M^{-1}$ with about 15% accuracy as determined by unweighted least squares. Cortisone studied by dialysis at 34° in Locke solution and 2% BSA at *p*H 7.5 gave the very rough value of $n<K> = 5 \times 10^3\ M^{-1}$ with an estimated error of 30%. Approximate $n<K>$ values were calculated in the previous paper[5] by the approximation

$$\frac{dr}{dS} = d\left(\frac{n<K>S}{1 + \left(1 + \frac{\mu^2}{<K>^2}\right)<K>S}\right)/dS \approx n<K>$$

Agreement between the approximate value for testosterone and the more exact value of this paper is satisfactory. Cortisone and estradiol gave approximate values $1.8 \times 10^3\ M^{-1}$ and $7.2 \times 10^5\ M^{-1}$, respectively. The higher approximate value for estradiol may be due to a high heterogeneity factor $\frac{\mu^2}{<K>^2}$. The smaller corresponding cortisone value is probably the result of experimental error.

Discussion

Polar Interactions.—In a previous paper data for the binding of a variety of steroids suggest the need for both polar and van der Waals interactions between steroid and BSA.[5] The competitive effect of both anions and cations found here supports the need for polar interaction but is in disagreement with the general observation that only cationic groups of BSA are required for binding dye and other anions. The present data do not disclose the method of competition by cations which may be indirect; for example, the result of a cation-produced change in protein structure. Carbonyl and hydroxyl groups of steroids are equally effective polar groups and the presence of two such groups at opposite "ends" of the molecule improve the free energy of binding corrected for solvation of the steroid by as much as 2 kcal. per mole.[5] Klotz, Burkhard and Urquhart[29] observed similar double polar interaction between methyl orange anions and human serum albumin when the groups were 12–13 Å. apart. "End" polar groups on steroids are about 20 Å. apart and it is interesting to note that only those steroids with two oppositely situated polar groups are hormonally active.

Van der Waals Interactions.—Several studies indicate the involvement of van der Waals interaction in binding to serum albumin. For instance small inorganic anions are less strongly bound than singly charged dye molecules but perhaps only as a result of the hydrophobic nature of the bulk of the dye molecule. More impressive are the observations by Karush[30] of a specificity distinction in the binding of optical enantiomorphs, a situ-

(28) C. Tanford, THIS JOURNAL, **74**, 211 (1952).

(29) I. M. Klotz, R. Burkhard and J. M. Urquhart, *ibid.*, **74**, 202 (1952).

(30) F. Karush, *J. Phys. Chem.*, **56**, 70 (1952).

ation possible only if the hydrocarbon moiety forms the third interaction point necessary for discrimination between isomers. The binding of various fatty acid ions also indicates van der Waals interactions.[31] Certainly it is to be expected that hydrocarbon portions of substrates will associate with whatever hydrocarbon groups the protein presents in the neighborhood of the binding site. The advantage to such a union, which decreases the extent of less favorable solvation, may be considerable in or at the surface of globular proteins where a low effective dielectric constant may be expected.[32]

Neutral aminoazobenzene, methyl orange ions and testosterone demonstrate approximately equal free energies for the reaction P + S $\rightleftarrows$ PS: −6.2,[6] −6.0[33] and −6.0 kcal. (Table III), respectively, (approximately 25° and *p*H 7; 1 mole/liter standard state of substrate). There is thus no advantage to the presence of a free ionized group in the substrate. It may be concluded that if van der Waals interactions are important for any one substrate in this series, they are important for all three.

Entropies of Binding and Configurational Adaptability.—The rough data for testosterone suggest a small positive heat of binding in contrast to the small negative ΔH values observed for methyl orange and aminoazobenzene (but the latter were determined at *p*H 9.2.)[6,33] The entropic advantage previously demonstrated for the union of the latter substances with BSA thus appears to be intensified for testosterone.

The statistical entropy resulting from our necessary choice of entropy changes based on $n<K>$ rather than $<K>$ assuming 10 sites is about 5 e.u./mole. A second contribution no higher than 8 e.u./mole can be estimated from the binding of chloride[34] assuming its interaction to be entirely electrostatic. The effect here is due to the diminished field in the neighborhood of the protein charge after binding.[32] The sum of these effects plus perhaps a small negative entropy change if van der Waals interactions occur does not account for the observed change somewhat larger than 20 e.u./mole. Klotz has attributed the residual entropy change for dye anion binding to a displacement of hydration water from the ionic groups during binding.[24] This hypothesis, though still tenable, is inconsistent with the larger entropy changes occurring when an uncharged molecule is bound. The proposal of Karush[35] that the residual changes are due to alteration in the structure of the protein (configurational adaptability) is more attractive. Small molecules of so-called "narcotic" substances such as alcohol or phenobarbitol are able to produce very large positive entropy changes in proteins.[36] The smaller changes produced on binding steroids may be the similar result of a very local relaxation between coiled or otherwise aggregated tubes of polypeptide in the manner discussed by Lumry and Eyring.[37] The interactions between such tubes to give the globular protein structure appear to be of a non-specific type due to van der Waals forces, ion–dipole attraction and the like. Klotz and co-workers[38] have implicated the phenolic groups of tyrosine residues and cationic groups of the protein in the formation of a binding site, and Tanford and Roberts[39] have noted that some tyrosine residues of serum albumin appear to be involved in weak bonds. It may be suggested that the steroid molecules bridge or break ammonium–phenolic cross links to reduce local strain and thus increase entropy with negligibly small enthalpy change. The globular structure of serum albumin probably is determined so thoroughly by the large number (17) of disulfide bonds that any non-specific bonds may be expected to be more exposed[40] and perhaps more strained than in other proteins studied for binding ability.

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