Steroid–Protein Interactions. XIV. Interaction between Human α_1-Acid Glycoprotein and Progesterone*

Manik Ganguly, Robert H. Carnighan,† and Ulrich Westphal

ABSTRACT: The α_1-acid glycoprotein (AAG, orosomucoid) of human blood serum forms a relatively strong association complex with progesterone. The affinity constant decreases with increasing temperature. The strength of interaction is pH dependent; it has a maximal value at pH 8. The binding affinity between AAG and various Δ^4-3-keto steroid hormones follows the "polarity rule." Precipitation of AAG by alcohol–acetone results in a "delipidated" preparation which cannot be distinguished from the nonprecipitated material by chemical and physicochemical criteria except for its higher binding affinity for progesterone. The association constant for the progesterone–AAG complex after solvent precipitation of AAG is 10.8×10^5 M^{-1}. The number of progesterone binding sites for the delipidated AAG is $n = 1$.

Recombination of the delipidated AAG with the original lipid material results in a preparation with the progesterone binding characteristics of the nonprecipitated AAG.

The long-term aim of this series[1] is the elucidation of noncovalent interaction between steroid compounds and proteins to form dissociable complexes. This includes the relationship of chemical structure of the two interacting components to their binding affinity and capacity; it is also concerned with some of the biological consequences of such interactions.

The objective of the present paper is an investigation into the interaction between progesterone and the α_1-acid glycoprotein.[2] It has been found previously (Westphal *et al.*, 1961) that AAG of human blood (Weimer *et al.*, 1950; Schmid, 1953) forms a dissociable complex with progesterone the association constant of which is greater than that of the HSA–progesterone complex. Our interest in this interaction was prompted by certain similarities observed (Westphal, 1964) between the binding of progesterone to AAG and the complexing of corticosteroid hormones and progesterone with the corticosteroid binding globulin.[2] Both AAG and CBG (Daughaday, 1958; Seal and Doe, 1962, 1964, 1966; Sandberg *et al.*, 1966; Muldoon, 1967) are glycoproteins with a relatively high carbohydrate content. The steroid complexes of the two glycoproteins, *i.e.*, progesterone–CBG and progesterone–AAG, resemble each other in their dependency of stability on temperature. The association constants at 4° are several times greater than those at 37°, in contrast to the progesterone complex of HSA (Seal and Doe, 1966). Similarly, the pH optimum of the affinity constant is at about pH 8 for both the cortisol–CBG (Westphal, 1967b) and the progesterone–AAG complex. The number of binding sites for the steroids tested is one in both cases. In an analogous way as for the complexes of corticosteroid hormones with CBG (Slaunwhite *et al.*, 1962), the biological activity of progesterone is markedly reduced by association with AAG (Westphal and Forbes, 1963).

Attempts to elucidate the chemical basis of the strong interaction between CBG and corticosteroid hormones are handicapped by the poor availability of CBG which occurs in normal human plasma at a concentration of approximately 23–45 mg/l. (Doe *et al.*, 1964; Seal and Doe, 1966; Westphal, 1967a; Muldoon, 1967). In contrast, about 750 mg of AAG is present in 1 l. of normal human serum (Winzler, 1960). In view of the similarities in the interactions of AAG and CBG with steroid hormones, the present studies were undertaken in order to characterize the steroid binding properties of AAG.

Materials

The chemicals used in the present work were reagent grade or of the highest purity that could be obtained. CM-cellulose and DEAE-cellulose were purchased from the Eastman Kodak Co.; Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc., New Market, N. J. Thiobarbituric acid, an Eastman preparation, was recrystallized several times from distilled water, preheated to 80°. Orcinol, reagent grade from Fisher Scientific Co., and 2-amino-2-methyl-1,3-propanediol, Eastman practical grade, were recrystallized from

* From the Biochemistry Department, University of Louisville School of Medicine, Reynolds Building, Louisville, Kentucky 40208. *Received December 12, 1966.* This work was supported by grants from the National Institute of Arthritis and Metabolic Diseases (AM-04040 and AM-06369) and a research career award (U. W.) from the Division of General Medical Sciences (GM-K6-14,138) of the U. S. Public Health Service, and by a contract from the U. S. Army Medical Research and Development Command (DA-49-193-MD-2263).

† Present address: Vanderbilt University Medical School, Nashville, Tenn.

[1] For publication XIII see Westphal (1967a).

[2] Abbreviations used: AAG, α_1-acid glycoprotein or orosomucoid; HSA, human serum albumin; CBG, corticosteroid binding globulin; ACD, acid–citrate–dextrose.

benzene. Crystallized HSA was purchased from Nutritional Biochemical Corp. Human ACD–plasma was obtained from whole blood.[3] All dialyses were carried out in seamless regenerated cellulose tubing (Visking Co., Chicago, Ill.) of various sizes which had been prewashed with distilled water, then with a solution of 0.002 M NaEDTA and 0.002 M ascorbic acid in 0.05 M phosphate buffer (pH 7.4) for 24 hr on a shaking machine, and finally rinsed with four changes of double-distilled, deionized water over a period of 24 hr. For the immunoelectrophoresis experiments, special Agar-Noble of Difco Laboratories, Detroit, Mich., was used. The antiserum against human AAG, produced in rabbit, was furnished by Behringwerke A. G., Marburg, Germany, through Lloyd Brothers, Inc., Cincinnati, Ohio.

Two progesterone-4-^{14}C preparations were applied: one from Nuclear-Chicago, sp act. 20.8 mc/mmole, radiochemical purity verified to be 98.9%; the second from New England Nuclear Corp., Boston, Mass., sp act. 46.0 mc/mmole, radiochemical purity verified to be 99.4%. Testosterone-4-^{14}C (sp act. 3.70 mc/mmole) and corticosterone-4-^{14}C (sp act. 44.4 μc/mg) were obtained from Tracerlab.[4] New England Nuclear Corp. furnished cortisol-4-^{14}C (sp act. 24.8 μc/mg), estrone-6,7-^{3}H (sp act. 2.85 c/mmole), and estradiol-6,7^{3}-H (sp act. 5.6 c/mmole). Cortexone-4-^{14}C was prepared from the acetate (New England Nuclear Corp.; sp act. 9.17 μc/mg) by hydrolysis as previously described (DeVenuto *et al.*, 1958). The radiochemical purity of these steroids was periodically determined and found to be between 94 and 100%.

Methods

Preparation of α_1-Acid Glycoprotein. The AAG used in our experiments was prepared by a method developed in our laboratory. The phenol precipitation of Michon (1962) was applied initially; two of the subsequent chromatographic steps resembled those described by Bezkorovainy and Winzler (1961).

Phenol Precipitation. Outdated human blood (12 pints) was centrifuged for 30 min at 4°, 3000*g*, and the supernatant was clarified by centrifugation at 6000*g*, 4° for 30 min. To the plasma thus obtained (2360 ml) solid sodium chloride was added to give a 20% solution. An equal volume of a 5% aqueous phenol solution was added dropwise with constant stirring over a period of 4 hr at 4°. After standing for 16 hr at 4°, the suspension was centrifuged for 30 min at 4°, 6000*g*, and the supernatant was dialyzed in Visking cellophane bags with continuous flow of distilled water for 96 hr or until free of phenol and chloride ions. The protein material was then lyophilized (yield 1.6 g).

Chromatography on CM-cellulose. The lyophilized Michon fraction (1.4 g) was dissolved in 10 ml of 0.025 M acetate buffer (pH 4.1) and applied to a CM-cellulose column (4.1 × 31 cm) equilibrated with the same buffer. The column was eluted at 4° with 1500 ml of 0.025 M acetate buffer (pH 4.1) and then with 1500 ml of 0.05 M phosphate buffer (pH 7.3) at the rate of 1 ml/min. The eluate was collected in 20-ml fractions and the absorbance was measured at 278 mμ in a Zeiss spectrophotometer Model PMQ II and plotted. The fractions containing the symmetric peak eluted by the acetate buffer were pooled, dialyzed at 4° for 72 hr with three daily changes of deionized water, and lyophilized (yield 1.2 g).

Gel Filtration over Sephadex G-200. The material purified over CM-cellulose (1.0 g) was dissolved in 15 ml of 0.1 M Tris-HCl buffer in 1.0 M NaCl (pH 8.0) and applied to a Sephadex G-200 column (4.1 × 47 cm). The column was eluted at 4° with 1000 ml of the Tris-HCl–NaCl buffer at the rate of 40 ml/hr. Absorbance of the eluate (20-ml fractions) was measured at 278 mμ and plotted. The material forming the symmetric peak was pooled, dialyzed for 72 hr at 4° with three daily changes of deionized water, and lyophilized (yield 900 mg).

Chromatography on DEAE-cellulose. The protein from the Sephadex G-200 column (800 mg) was fractionated twice on a DEAE-cellulose column (4.1 × 50 cm) using gradient elution. The gradient was prepared in eight chambers of a Buchler Varigrad, applying 220 ml of 0.005 M phosphate buffer (pH 8.0) (starting buffer) in the first chamber, and 440 ml of 0.05 M phosphate (pH 5.5) (limit buffer) in the eighth chamber. Each of chambers 2–7 received 27.5 ml more of the limit buffer, and 27.5 ml less of the starting buffer, than the preceding chamber, so that the seventh chamber had 165 ml of limit buffer and 55 ml of starting buffer. To each of the first seven chambers, 220 ml of double-distilled water was added to make a total volume of 440 ml. The eluate was collected in 20-ml fractions. Absorbance at 278 mμ and pH of the eluates were measured. The major portion of the protein was eluted in a symmetrical peak between pH 6.3 and 6.1. This material was dialyzed at 4° for 96 hr with three daily changes of deionized water and lyophilized; 600 mg of AAG preparation, type C, were obtained.

Removal of Lipids by Ethanol–Acetone Precipitation. All operations were performed in a cold room at 4°, using precooled solutions. The chromatographically purified AAG, type C (200 mg), was dissolved in 50 ml of 0.1 M acetate buffer (pH 4.1) and 200 ml of an alcohol–acetone mixture (9:1, v/v) was added. The precipitate formed was kept for 2 hr with occasional shaking, centrifuged at 6000*g* for 30 min, washed with 20 ml of cold absolute ethanol, recentrifuged for 30 min at 6000*g*, finally washed with 20 ml of cold ether, and centrifuged for 30 min. The precipitate was dried in a vacuum desiccator at 4° for 18 hr; the yield was 180 mg of AAG preparation, type P.

[3] Outdated blood was obtained through Grant No. NC-29-55 of the National Red Cross Blood Program. Grateful acknowledgment is made to Miss Evelyn Fleming, Administrative Director of the Louisville Regional Blood Center, for the efficient cooperation.

[4] The authors are indebted to the Endocrinology Study Section, Department of Health, Education, and Welfare, for supplying the radioactive corticosterone through Tracerlab, Boston, Mass.

Ultracentrifugal Analysis. Sedimentation studies were performed at 20° in a Spinco Model E ultracentrifuge at 59,780 rpm. The AAG (type C) was dissolved in 0.1 M NaCl solution and the $s^0_{20,w}$ value was determined by extrapolation of the sedimentation coefficients to zero concentration. For other AAG preparations, the sedimentation coefficients were compared at 1% concentration in 0.1 M NaCl.

Electrophoretic Analysis. Paper electrophoresis was carried out in the LKB electrophoresis apparatus (Model No. 3276B) in Veronal buffer (pH 8.6, μ = 0.125) at 180 v for 10 hr. The protein was dissolved in this buffer to a concentration of 5%, and 15 μl was applied to the strip. After the electrophoresis, the strips were cut lengthwise, one-half was stained in Amido Black solution for protein and the other half in the periodic acid–fuchsine–sulfite reagent for carbohydrate (Koiw and Gronwall, 1952). Moving-boundary electrophoresis was carried out in a Beckman Model H Tiselius apparatus at 1° using a 1% solution of the protein in 0.12 M Veronal buffer (pH 8.6). Immunoelectrophoresis was performed with the LKB 6800 A Immunophor apparatus on agar gel. Antisera to human AAG, prepared in the rabbit, were used.

Chemical Analysis. Hexose, hexosamine, and fucose were determined by the methods summarized by Winzler (1955), sialic acid by the procedure of Warren (1957), and nitrogen by Lang's (1958) modification of the micro-Kjeldahl technique. The amino acid analyses were performed with a Technicon automatic amino acid analyzer, Model NC-1; type B Chromobeads were employed. Since valine did not consistently separate from glucosamine, the value for valine was verified by an analysis of an aliquot sample on a Beckman automatic amino acid analyzer, Model No. 120.

Measurement of Radioactivity. A stock solution (0.20 μg of progesterone-4-^{14}C/ml) was prepared in benzene containing 10% methanol. Aliquots were dried under a stream of cotton-filtered nitrogen and dissolved in the aqueous solvents to a concentration of 0.02 μg/ml. The mixtures were warmed at 45° for 3 hr with gentle shaking to facilitate the dissolution of the progesterone in the aqueous media. Radioactivity was measured in glass vials in a Packard Tri-Carb scintillation counter with the scintillation solution of DeMoor and Steeno (1963). In some experiments the ^{14}C concentrations were determined by solid-state counting in a Nuclear-Chicago gas-flow counter using previously described techniques (Pearce *et al.*, 1956) at a "reliable error" of ±2.5% or less.

Equilibrium Dialysis. The binding affinity of the progesterone–glycoprotein complexes was determined in duplicate using Visking bags containing 5 ml of protein solution (0.5 mg/ml). Two bags were placed in each bottle to which 20 ml of outside solution containing 0.02 μg of progesterone-4-^{14}C/ml was added. The dialysis systems were equilibrated for 48 hr at 4°; this period of time is more than sufficient for complete equilibration. The radioactivity of the inside solution of each bag was then determined in triplicate 1-ml samples to obtain the total steroid concentration inside the bag. Quadruplicate 1-ml samples were counted for the outside solution to obtain the concentration of the unbound steroid. The concentration of the bound steroid is given by the difference between total concentration inside the bag and concentration of unbound steroid. Duplicate 1-ml samples of the outside solutions before equilibrium dialysis were counted for a check of the recovery of radioactivity.

Presentation of Binding Data. The interaction of steroid with AAG was expressed in two ways. The association constant (K) and the number of binding sites (n) for progesterone complexes of pure AAG preparations were determined by standard procedures (Edsall and Wyman, 1958). For less complete characterization and for comparison of different AAG preparations for their steroid binding affinity, a method was used involving equilibrium dialysis at one single concentration of steroid and of protein under a given set of conditions. Both procedures are subsequently described.

STANDARD PROCEDURE. The number of binding sites for progesterone in human AAG and the association constant of the complex were determined at 4° (pH 7.4) by equilibrium dialysis, using seven or eight different concentrations of progesterone-4-^{14}C at constant protein concentration. The data were evaluated (Edsall and Wyman, 1958) by the graphic method of Scatchard (1949) or that of reciprocal plots (Klotz *et al.*, 1946). Both procedures are based on the equation

$$\bar{\nu} = \frac{nK[S]}{1 + K[S]} \qquad (1)$$

where $\bar{\nu}$ is the average number of molecules of steroid bound per molecule of protein; n, the number of binding sites for the steroid on the protein molecule; K, the association constant for each binding site, and [S], the molar concentration of unbound steroid. For eq 1 to be valid, the assumption is made that the n binding sites are equivalent and independent, each having the same association constant K. Use of eq 1 appears justified for the binding systems studied since not more than one binding site has ever been observed for the progesterone–AAG complex in the present and other unpublished work.

If eq 1 is valid, plots of $\bar{\nu}/[S]$ against $\bar{\nu}$ will be linear (Scatchard, 1949); the intercepts on the abscissa and on the ordinate give n and nK, respectively. Plots of $1/\bar{\nu}$ against $1/[S]$ (Klotz *et al.*, 1946) also give straight lines; the values for n and K can be obtained from the intercept and the slope. Determination of K and n by the two procedures gave the same values. Because of the difficulties of extrapolating reciprocal plots to the ordinate, the Scatchard procedure was preferred for the evaluation of the binding data for AAG (type C) and AAG (type P) plus lipid.

DETERMINATION OF BINDING AFFINITY AT ONE SINGLE CONCENTRATION. By assuming the special case of $n = 1$, and absence of other binding sites of lower affinity, the binding affinity between protein and steroid was ex-

pressed as

$$\frac{\bar{\nu}}{[S]} = \frac{[S]_{bd}}{[P][S]} \quad (2)$$

where $\bar{\nu}$ is the average number of molecules of steroid bound per molecule of protein present, and [S], $[S]_{bd}$, and [P] are the molar concentrations of unbound steroid, bound steroid, and total protein, respectively; the dimension is M^{-1}. Equation 2 is an approximation of (Edsall and Wyman, 1958)

$$k_{assoc} = \frac{[S]_{bd}}{[P]_{unbd}[S]} \quad (3)$$

where $[P]_{unbd}$ is the molar concentration of unbound protein. The following rationale is given for the use of eq 2 instead of eq 3 in our short procedure of determining binding affinity. In all our equilibrium dialysis experiments, the molar ratio of total progesterone applied to total glycoprotein applied is approximately 1:100. Therefore, the theoretical maximum of bound protein is 1% of total protein. Since only a portion of the total progesterone (from maximally 93 to 25% or less, depending on binding affinity) is bound to AAG, the concentration of actually bound protein is even smaller than 1% of total protein and can therefore be neglected in relation to total protein. The concentration of unbound protein, $[P]_{unbd}$, then becomes equal to that of total protein, [P], and eq 2 and 3 are identical for the purposes of the present study. This approximation is realistic since the range of error in the determination of $[S]_{bd}$ and [S] by equilibrium dialysis, using radiolabeled steroids for sensitive quantification, is greater than that arising from equating $[P]_{unbd}$ and [P].

For routine characterization of steroid binding affinity and for comparison of different AAG preparations by this method, all determinations of binding data by equilibrium dialysis were done under identical conditions as described above, and binding affinity was expressed as $\bar{\nu}/[S]$ by eq 2. This is done in order to distinguish the binding parameters, obtained at single concentrations of steroid and of protein, from the association constants (K) which were determined by the more complete procedure as outlined above. A molecular weight of 41,000 was used for AAG (Winzler, 1960).

Influence of Temperature on Progesterone Binding Affinity. The AAG preparation used for this particular experiment was prepared as follows (all operations were done at 4°). The dialyzed and lyophilized product from the Michon precipitation was purified by CM-cellulose chromatography as described above. The lyophilized material had an average $\bar{\nu}/[S]$ value of 2.1×10^5 M^{-1}. An amount of 300 mg was dissolved in 300 ml of cold 0.01 M acetate–0.04 M sodium chloride buffer adjusted to pH 4.65, and 1200 ml of cold absolute ethanol was added slowly. After a period of 4 hr, the protein was collected by centrifugation, suspended in 100 ml of absolute ethanol, recentrifuged, suspended in 100 ml of ether, recentrifuged, and dried in a vacuum desiccator.

The AAG preparation thus obtained (I-117) was dissolved in 0.05 M phosphate buffer (pH 7.4) to a concentration of 0.5 mg/ml and placed in dialysis bags in 5-ml portions. For each temperature, duplicate bottles were set up each having one bag and 10 ml of outside solution containing 0.02 μg of progesterone-4-^{14}C and 15 μg of streptomycin per ml. Streptomycin at this concentration has no significant influence on the AAG–progesterone interaction. The dialysis systems were equilibrated for 48–52 hr, except for the 50° experiment in which the time was limited to 24 hr, a period sufficiently long for equilibration. Tests for thermal stability of AAG were done by exposing a 0.5-mg/ml solution to 60° for various periods of time, and subsequently measuring the progesterone binding affinity by equilibrium dialysis at 4° for 48 hr under the usual conditions.

The temperature dependency of the interaction between progesterone and HSA was determined in a similar way, using the same albumin preparation (Cutter 262-89) that had been applied in previous studies (Westphal, 1957, 1961). The inside solutions contained 2.0 mg of albumin/ml, the outside phase 0.02 μg of progesterone-4-^{14}C/ml; the over-all molar ratio of albumin to progesterone thus was 230:1. Equilibration times were 48, 48, 40, 22 hr, at 4, 24, 37, and 45°, respectively. To test for possible heat denaturation, a duplicate albumin sample was heated at 45° for 24 hr, cooled to 4°, and its $\bar{\nu}/[S]$ value was determined at 4°.

Influence of pH on the Progesterone Binding Affinity. The AAG preparation I-117 was used in duplicate equilibrium dialysis experiments at 4° under the usual conditions, except that different buffer solutions were applied. Between pH 2.30 and 8.00, 0.05 M citrate–phosphate was used; comparison was also made with 0.05 M phosphate buffer (pH 7.4). At the higher pH values, 0.05 M ammediol (2-amino-2-methyl-1,3-propanediol) was adjusted with 10 N hydrochloric acid to the desired pH.

Determination of Interaction of AAG with Steroid Hormones Other Than Progesterone. The equilibrium dialysis experiments were done at 4° under the usual conditions applying solutions of 1.0 mg of AAG (0.5 mg in case of estrone and estradiol)/ml of inside solution. Progesterone-4-^{14}C, testosterone-4-^{14}C, cortexone-4-^{14}C, corticosterone-4-^{14}C, cortisol-4-^{14}C, estrone-6,7-^{3}H, and estradiol-6,7-^{3}H were added to the outside solutions at concentrations of 0.05, 0.05, 0.05, 0.01, 0.02, and 0.02 μg/ml, respetcively. The ratio of steroid to AAG was 75:1 or higher.

Measurement of Ultraviolet Absorption and Optical Rotatory Dispersion. The ultraviolet absorption spectra of the AAG preparations (types C and P) were measured at 23 ± 1° in a Zeiss PMQ II spectrophotometer. The Cary Model 60 recording spectropolarimeter was used for preliminary measurements to compare the location of trough and peak of the negative Cotton effect in the two AAG preparations (types C and P).

TABLE I: Physicochemical Characterization of AAG Preparations.

Physicochemical Characteristic	AAG (type C[a]) Homogeneous by Chromatography	AAG (type P[a]) by Alcohol–Acetone (9:1) Pptn of AAG-C
Sialic acid (%)	11.6	11.6
Hexose (%)	14.0	15.0
Hexosamine (%)	11.6	12.0
Fucose (%)	1.4	1.2
Nitrogen (%)	9.8	9.7
Amino acids[b]		
Asp	23.7	24.6
Thr	17.5	17.8
Ser	9.0	7.0
Glu	32.4	33.8
Pro	8.6	8.3
Gly	7.8	8.1
Ala	10.1	10.4
Val	9.2	9.9
CyS	4.1	4.5
Met	1.3	1.4
Ile	10.1	10.3
Leu	16.5	16.9
Tyr	11.3	12.0
Phe	10.5	10.9
Try	4[c]	
Lys	15.6	15.9
His	3.4	3.4
Arg	9.3	10.2
Sedimentation coefficient, $s^{1\%}_{20,w}$ (S)	2.76	2.74
Electrophoretic mobility in Veronal, pH 8.6; cm^2/vsec	-6.22×10^{-5}	
Paper electrophoresis	α-Globulin, homogeneous	α-Globulin, homogeneous
Immunoelectrophoresis	Homogeneous, single band	Homogeneous, single band not distinguishable from that obtained with type C
Cotton Effect		
Min (mμ)	230	230
Max (mμ)	204	204
$\bar{\nu}$/[S] (progesterone) in 0.05 M phosphate, pH 7.4, 4° ($M^{-1} \times 10^{-5}$)	2.9	10.3

[a] Corrected for moisture content. [b] Moles per mole (41,000g; Winzler, 1960) of AAG. [c] Value from Marshall and Porath (1965).

Results and Their Evaluation

Physicochemical Characterization of the AAG Preparations. The carbohydrate content and other characteristics of the pure AAG preparations are given in Table I. Comparison of the data for the glycoprotein before and after precipitation with alcohol–acetone shows that the composition is the same for the two preparations. The ultracentrifugal analysis of AAG during chromatographic purification revealed an almost symmetrical peak for the final product resulting from the DEAE-cellulose column; the sedimentation peak was somewhat less symmetrical after the Sephadex G-200 filtration (Figure 1). The schlieren patterns of the chromatographically pure AAG (type C) and of the precipitated AAG (type P) were essentially identical and indicated homogeneity with respect to size and shape. The sedimentation coefficients ($s_{20,w}$) of the two preparations in 1% solution were found to be 2.76 and 2.74, respectively. The value, $s^0_{20,w}$, at infinite dilution of AAG (type C) was found to be 3.08. No difference between the AAG preparations before and after precipitation was revealed by paper strip electrophoresis. The optical rotatory dispersion (ORD) data were identical for the AAG preparations (types C and P). Similarly, no difference between the two preparations was found in the ultraviolet absorption spectrum down to 190 mμ.

Influence of Delipidation on the Progesterone Binding Affinity of AAG. One of the difficulties encountered over the years in the studies on progesterone interaction with AAG has been the inconsistency of the $\bar{\nu}$/[S] values obtained as a measure of binding affinity. In spite of rigorous adherence to identical conditions in the equilibrium dialysis procedure, various pure AAG preparations gave $\bar{\nu}$/[S] values of wide divergence, *e.g.*, as low as 1.2×10^5 M^{-1} and as high as 7.4×10^5 M^{-1}. Discrepancies were experienced even with the same AAG preparations in different experiments without any apparent changes in experimental conditions.

It was finally found that the difficulties were compounded by the simultaneous involvement of two problems (Westphal *et al.*, 1966). First, it was observed that

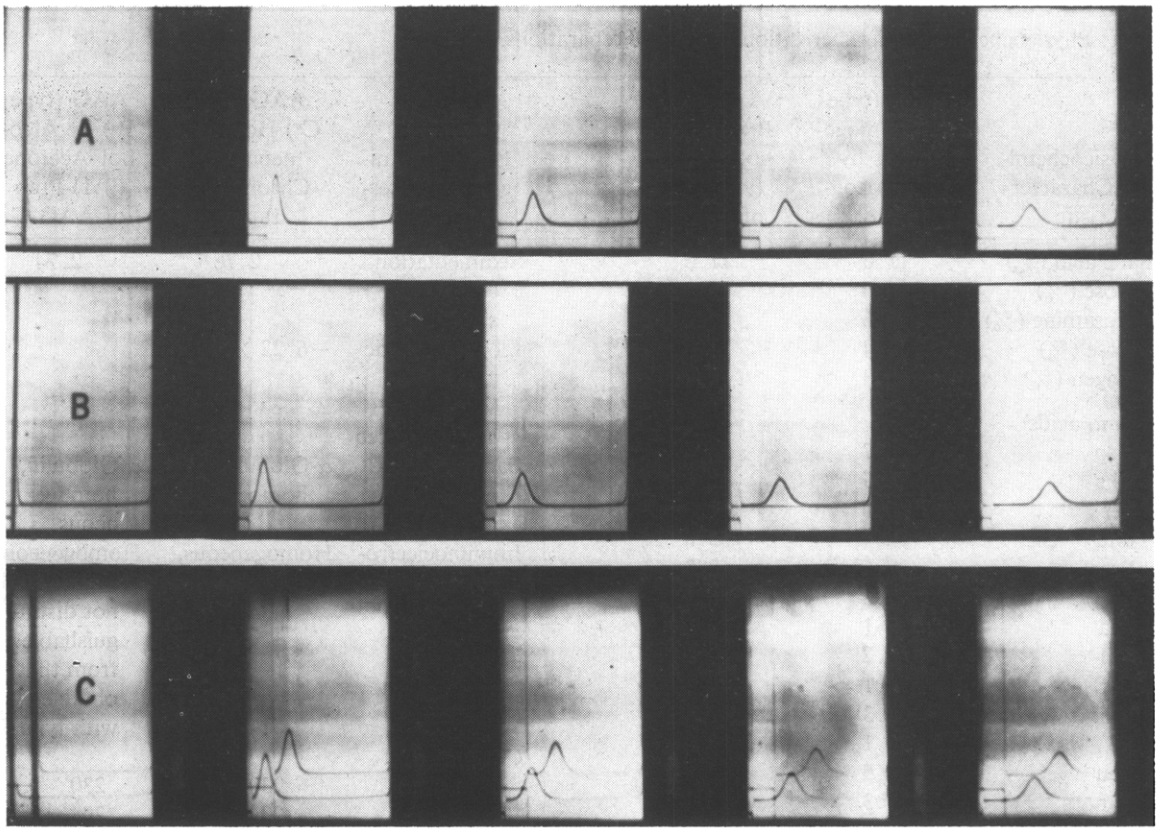

FIGURE 1: Ultracentrifugal patterns of AAG in 0.1 M NaCl. Sedimentation proceeded from left to right; exposures were taken at 3, 30, 60, 90, and 120 min after speed of 59,780 rpm was reached. Protein concentration 1%, temperature 20°, pH 7.4, and bar angle 75°. (A) AAG after Sephadex G-200 chromatography; (B) AAG after DEAE-cellulose chromatography; (C) upper curve: AAG (type C); lower curve: AAG (type P).

traces of heavy metal ions reduced the binding affinity of AAG for progesterone. Redistillation from Pyrex glass of the distilled water, subsequent deionization over exchanger columns, and rigorous elimination of contaminating ions from all components of the systems used, resulted in fairly uniform $\bar{\nu}$/[S] values, usually of about 1–2 $\times$ 10^5 M^{-1}, for the AAG preparation (type C) (J. Kerkay and U. Westphal, to be published.)

A second factor contributing to the inconsistency of the binding affinity was eliminated by delipidation of the pure AAG preparations under mild conditions. Table II shows that washing of a dry, chromatographically pure AAG preparation (type C) with 10% acetone in alcohol at 2° more than doubles the binding affinity; precipitation by the same solvent mixture from a solution in acetate buffer increases the $\bar{\nu}$/[S] value several-fold. A $\bar{\nu}$/[S] value of approximately 10.3 $\times$ 10^5 M^{-1} has been obtained in numerous similar experiments for this delipidated AAG preparation (type P); a second precipitation under the same conditions does not lead to further increase in binding affinity. The last line in Table II indicates that the increase of the binding affinity by removal of lipid is reversible. Recombination of a precipitated AAG preparation (type P, $\bar{\nu}$/[S] = 9.9 $\times$ 10^5 M^{-1}) with the dry residue of the supernatant resulted in the low $\bar{\nu}$/[S] value of 0.3 $\times$ 10^5 M^{-1}. This binding affinity is lower than that of the nonprecipitated AAG (type C) because the amount of dry residue used for recombination was disproportionately high; all of the dry residue obtained by evaporation of the solvent from the supernatant of the alcohol–acetone precipitation of 20 mg of AAG (type C) was added to only 6 mg of the precipitated AAG (type P).

The number of binding sites in the precipitated AAG preparation (type P) (Figure 2 and Table III) was found to be approximately one. This confirms earlier results (Westphal, 1964) obtained with an AAG preparation (I-117) which had been precipitated with 95% alcohol and subsequently washed with ether. The association constant (K) for this binding site in AAG (type P) was slightly higher than the previous value (Table III), presumably as a result of the improved method of delipidation. Evaluation of the binding data for AAG (type P) by the method of Scatchard (1949) gave virtually identical results as might be expected from the experimental points plotted in Figure 2 (lower curve).

Determination of number of binding sites and of association constant for the chromatographically pure AAG before precipitation (type C) by the method of reciprocal plots gave considerably lower values (Figure

TABLE II: Influence of Delipidation of AAG on Progesterone Binding Affinity.

AAG Type	Prepn	Treatment	$\bar{\nu}$/[S] in 0.05 M Phosphate pH 7.4, 4°, $M^{-1} \times 10^{-5}$
C	M-16	None	2.8
	M-16	Precipitated with alcohol–acetone (9:1) at pH 4.6	9.0
C	M-18	None	1.1
	M-18	Washed with alcohol–acetone (9:1)	2.6
	M-18	Precipitated with alcohol–acetone (9:1) at pH 4.6	8.8
C	M-18	None	1.0, 1.6
	M-18	Precipitated with alcohol–acetone (9:1) at pH 4.1	9.9, 10.3
	M-18	Precipitated twice with alcohol–acetone (9:1) at pH 4.1	10.5
P	M-36	None	9.9
	M-36	Recombined with residue from alcohol–acetone (9:1) supernatant	0.3

TABLE III: Interaction between Progesterone and AAG Preparations at pH 7.4; 4°.

Preparation	No. of Binding Sites (n)	Assocn Constant (for n = 1) ($M^{-1} \times 10^{-5}$)
AAG, I-117, alcohol precipitated	1.0	8.7
AAG, type P	0.90	10.8
AAG, type C	0.20	9.4
AAG, type P recombined with lipid from type C	0.17	8.5

2). When the AAG (type P) was recombined with the "lipid material" from the supernatant of a precipitation of an equal amount of AAG (type C) the resulting AAG had essentially the same number of binding sites and association constant as the original material before delipidation (Figure 2). Evaluation of the experimental data obtained with AAG (type C) and AAG (type P) plus lipid by Scatchard's procedure gave curved lines; their extrapolation to the ordinate gave fractional values of n which were similar for the two lipid-containing AAG preparations (Table III). The association constants, calculated for $n = 1$, were similar to those of AAG (type P) (Table III).

Influence of Method of Preparation on Progesterone Binding Affinity of AAG. Table IV shows the progesterone binding affinity for four AAG samples obtained in other laboratories by different procedures. The $\bar{\nu}$/[S] values are similar to those found for our own preparations at various stages of purity. The highest affinity was measured in preparation 1; it corresponded to the $\bar{\nu}$/[S] value observed occasionally with our best preparations before solvent precipitation. The binding affinity of preparation 1 could be further improved to reach that of our AAG (type P) when it was precipitated with alcohol–acetone in the same way as described above for AAG (type C). Filtration of AAG (type C) over Sephadex G-25 at 45° under conditions which removed progesterone from CBG (Westphal, 1967a) did not significantly change the $\bar{\nu}$/[S] value (Table IV). This result indicates that no progesterone or other steroid of endogenous origin is present in the pure AAG preparations.

Influence of Temperature on Progesterone Binding Affinity. The effect of temperature on the progesterone–AAG interaction is shown in Figure 3 (curve A). The $\bar{\nu}$/[S] value at 4° is more than five times that at 50°. The low binding affinity at 50° is not the result of irreversible denaturation of the glycoprotein molecule as the thermostability tests at 60° indicate (Table V). No change in association constant is seen after 12-hr exposure at 60°, and the slight decrease after 24 hr is still within experimental error.

For comparison, the temperature dependency of the progesterone–albumin binding is represented in curve B of Figure 3. The slope of the declining curve is considerably smaller than in the case of AAG. The test for thermostability showed that heating of the albumin for 24 hr at 45° did not affect the binding affinity measured at 4°.

Influence of pH on Progesterone Binding Affinity. As Figure 4 shows there is a steady rise in binding affinity from acidic pH up to pH 8 where a maximal value is reached; further increase in pH results in reduced association. The $\bar{\nu}$/[S] values in the ammediol buffer are at a somewhat lower level than those measured in phosphate buffer. It is not known whether this indicates a specific effect of the propanediol derivative, or whether this compound contained small amounts of contaminants which decreased the association constant.

Compared to the results obtained in 0.05 M phosphate (pH 7.4) a smaller binding affinity between AAG and progesterone was observed in 0.05 M Tris-HCl and in 0.05 M Veronal buffer at pH 7.4 (Table VI). No difference in the $\bar{\nu}$/[S] values was found when the phosphate buffer was replaced by NaCl–cacodylate (Table VI).

Influence of Steroid Structure on Affinity of Binding to AAG. Table VII gives the $\bar{\nu}$/[S] values for the interaction of AAG with various steroid hormones which differ in their "polarity," *i.e.*, in the number and nature of polar groups in their molecule. It is evident that the affinity

TABLE IV: Progesterone Binding Affinity of Different AAG Preparations (0.05 M phosphate, pH 7.4, 4°).[a]

Prep	Method of Preparation	Reference	$\bar{\nu}$/[S] ($M^{-1} \times 10^{-5}$)
1	From fraction VI of Cohn's alcohol fractionation	Schmid (1953)	6.6
1 ppt	Preparation 1 precipitated with alcohol–acetone (9:1) at pH 4.6	This paper	10.6
2	Chromatography, pH 4.1	Bezkorovainy and Winzler (1961)	2.6
3	Chromatography, pH 3.7, starting directly from serum	Kalous and Poncová (1965)	1.2
4	Chromatography, pH 3.7, starting from fraction VI of Cohn's alcohol fractionation	Kalous and Poncová (1965)	4.2
AAG-C	Chromatography	This paper	3.0
AAG-C_{str}	AAG (type C) filtered over Sephadex G-25 at 45°, pH 7.4	This paper	2.5

[a] The authors are indebted to Drs. K. Schmid (preparation 1), A. Bezkorovainy (preparation 2), and V. Kalous (preparations 3 and 4) for kindly supplying the pure AAG preparations.

of binding follows the polarity rule (Westphal, 1961). The interaction is weakest for the steroid with the greatest number of hydroxy and keto groups.

Discussion

Chemical nature of a glycoprotein appears to be a common characteristic of a number of serum components which have the specific ability of interacting with hormones and other plasma constituents to form dissociable complexes. The iron binding transferrin and the copper complexing ceruloplasmin are glycoproteins, as is the haptoglobin which forms distinct complexes with hemoglobin (for review, see Laurell, 1960). Urinary glycoproteins which bind cyanocobalamine have been purified (Kallee *et al.*, 1964). The thyroxin binding globulin has been shown to be a glycoprotein (Ingbar and Freinkel, 1960) with a relatively high carbohydrate content (Seal and Doe, 1964). Very similar in its chemical properties is another serum glycoprotein, the CBG (Seal and Doe, 1962, 1964, 1966; Slaunwhite *et al.*, 1966; Muldoon, 1967) which has a particularly high binding affinity for corticosteroid hormones and progesterone. The corresponding CBG of rabbit serum is also a glycoprotein (Chader, 1966). High association constants have been observed for CBG in species other than human (Seal and Doe, 1966; Westphal, 1967a), for interaction with corticosteroid hormones as well as with progesterone.

It is assumed that the biological function of CBG lies in the regulation of hormonal activity. An analogous role of AAG for the control of progesterone action is not likely to be of similar significance since the concentration of binding sites of the progesterone binding HSA is more than 100 times greater than that of AAG, and since at 37° the association constant of the progesterone–CBG complex (Seal and Doe, 1966; Westphal, 1967a)

TABLE V: Test for Thermostability of AAG.

Hr at 60°	$\bar{\nu}$/[S] at 4° ($M^{-1} \times 10^{-5}$)
0	4.2
0.25	4.2
1	4.3
6	4.3
12	4.3
24	4.0
75	2.8

TABLE VI: Influence of Nature of Buffer on the Binding Affinity between AAG and Progesterone.

AAG Type	Prepn	Solvent (M), pH	$\bar{\nu}$/[S] at 4° ($M^{-1} \times 10^{-5}$)
C	M-16	Water, 7.4	1.8
	M-16	Phosphate (0.05), 7.4	2.8
	M-16	Tris-HCl (0.05), 7.4	1.6
	M-16	Veronal (0.05), 7.4	0.5
P	M-36	Phosphate (0.05), 7.4	9.9
	M-36	NaCl (0.15) + sodium cacodylate (0.013), 7.4	9.9

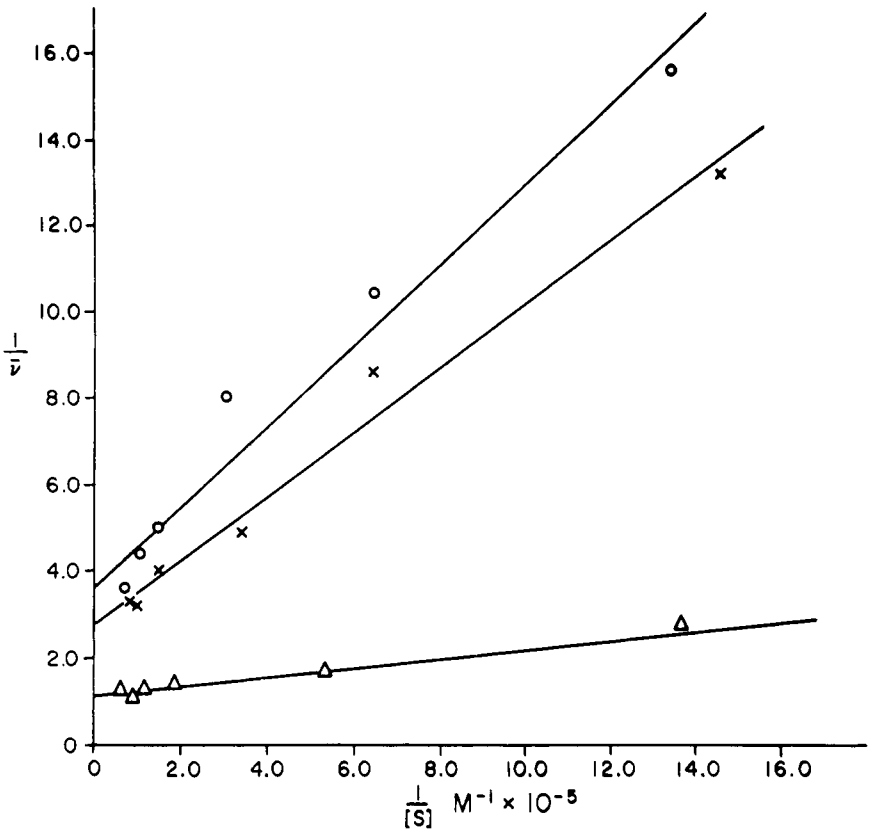

FIGURE 2: Binding of progesterone to AAG at pH 7.4, 4°. ν is the number of steroid molecules bound per total number of protein molecules; [S] represents the molar concentration of unbound steroid. Lower curve: AAG (type P); middle curve: AAG (type C); upper curve: AAG (type P) plus "lipid."

is more than 200 times as high as that of the progesterone–AAG complex. The AAG, therefore, in spite of its relatively high affinity for progesterone, binds only a small portion of the circulating progesterone, while the major part is associated with serum albumin and CBG (Westphal, 1966).

In view of the previously experienced inconsistency in the progesterone binding affinity of AAG preparations of varying origin, rigorous purification of the glycoprotein was our first objective. The combination of fractionation procedures described above resulted in a preparation (AAG, type C) which was homogeneous by all criteria applied (Table I). The carbohydrate composition agreed with reported data (Winzler, 1960; Jeanloz, 1966). It was also in agreement with the carbohydrate content of the preparation (AAG, type P) obtained from type C by precipitation with alcohol–acetone. This delipidation procedure evidently does not affect the AAG molecule, at least as far as all measured parameters are concerned; the chemical and physicochemical properties listed in Table I were found to be virtually identical, except for the steroid binding affinity.

The amino acid composition (Table I) is in excellent agreement with the values of Marshall and Porath (1965) when the data are calculated for the same molecular weight of AAG. Agreement is also satisfactory with the amino acid analyses of Walborg and Ward (1963) and Heimburger *et al.* (1964), whereas deviations occur for several amino acids when earlier determinations are compared (reviewed by Jeanloz, 1966).

TABLE VII: Interaction of AAG with Steroid Hormones of Different "Polarity" (pH 7.4, 4°).

Steroid	O_n	=O	OH	$\bar{\nu}$/[S] ($M^{-1} \times 10^{-5}$)
Progesterone	O_2	2	0	7.4
Testosterone	O_2	1	1	4.5
Cortexone	O_3	2	1	2.0
Corticosterone	O_4	2	2	0.7
Cortisol	O_5	2	3	0.04[a]
Estrone	O_2	1	1	1.2
Estradiol	O_2	0	2	0.7

[a] The $\bar{\nu}$/[S] values determined for the cortisol complexes with AAG (preparations 1 and 3 of Table IV) were found to be $0.04 \times 10^5 M^{-1}$ for both preparations.

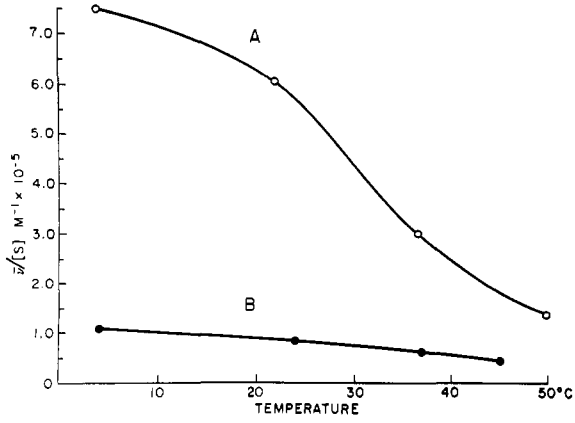

FIGURE 3: Effect of temperature on the progesterone binding affinity of AAG (curve A) and HSA (curve B) in 0.05 M phosphate buffer (pH 7.4).

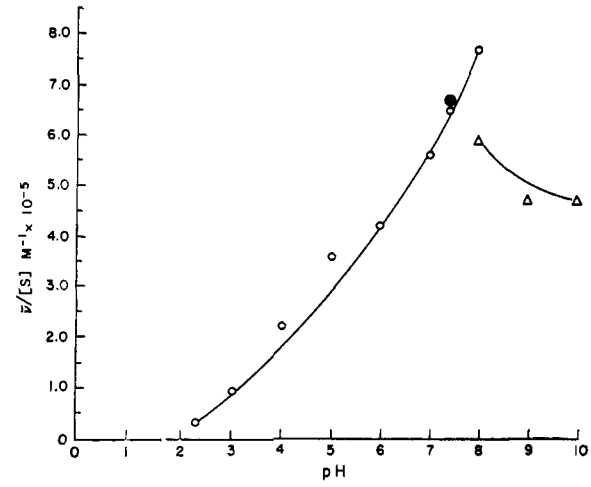

FIGURE 4: Influence of pH on the binding affinity between progesterone and AAG at 4°. (○) Citrate–phosphate (0.05 M); (●) phosphate (0.05 M); (△) 2-amino-2-methyl-1,3-propanediol (0.05 M).

Sedimentation coefficients and electrophoretic data also agree with reported data (Winzler, 1960; Jeanloz, 1966). Peak and trough of the Cotton effect were observed at the same wavelengths as described by Sarkar and Doty (1966). The exposure of AAG to 2.5% phenol at high NaCl concentration in our method of preparation also did not measurably affect binding properties. This is evident from a comparison of the association constants for progesterone and cortisol of our AAG preparations and those obtained by procedures not involving phenol precipitation (Tables IV and VII, footnote *a*). We conclude that both of the AAG preparations (types C and P) are chemically pure, and that the only detectable difference between them concerns the ability to bind progesterone.

The progesterone binding affinity of an average molecule of chromatographically pure AAG (type C) rises by precipitation with a mixture of alcohol and acetone by a factor of 3–8, depending on the original $\bar{\nu}/[S]$ value (Table II). This treatment also results in an increase of the apparent number of binding sites from a fractional value of 0.20 for AAG (type C) to $n = 1$ in type P (Table III). The association constant (K) which is calculated per binding site (n) remains essentially unaffected. These results are interpreted by the assumption that a lipophilic contamination, present in AAG (type C) firmly occupies the binding site for progesterone in the major percentage of the molecules. This blockage of the binding site apparently is so strong that the increasing quantities of progesterone do not completely displace the interfering substance; the result is a fractional value of n (Table III and Figure 2). On the average $(1 - n)$, *i.e.*, 80% of the molecules of AAG (type C) appear inactive or much less active in their binding affinity for progesterone.

Additional evidence for the considerable strength of adhesion of the lipidic contamination to AAG is given by the result of the gel filtration experiment at 45° (Table IV). Whereas this procedure completely removes corticosteroids and progesterone from the strongly binding CBG (Westphal, 1967a), filtration of AAG (type C) over Sephadex G-25 at 45° does not change the binding affinity for progesterone. This excludes the presence in AAG (type C) of endogenous progesterone and of any other steroid which may bind to the same site even if its affinity would be as high as that of progesterone to CBG; the association constant of the progesterone–CBG complex at 4° is 300–700 times greater than that of progesterone–AAG (Seal and Doe, 1966; Westphal, 1967a). The result of the gel filtration experiment at 45° is interpreted as indicating that the lipidic material is very tightly bound and remains on the AAG; removal requires precipitation with such solvents as alcohol–acetone.

If the assumption is correct that the fractional value of n for AAG (type C) is the result of inactivation (for progesterone affinity) of the major portion of the AAG molecules, then the association constant (K) of the "active" portion of the AAG molecules should be that of AAG (type P). Table III shows that this is essentially the case. Removal of the lipid by solvent precipitation of AAG (type C) transforms the 80% inactive (or much less active) molecules into type P molecules. This process is reversible; recombination of the solvent-precipitated AAG with the lipid contained in the supernatant results in an AAG preparation of the binding properties characteristic for type C.

It seems appropriate to compare the binding characteristics of pure AAG before and after solvent precipitation with analogous results obtained with human serum albumin after extraction with organic solvents under mild conditions (chloroform–methanol, 3:1, 4°). This treatment increases the association constant of HSA for progesterone 2.5 times; the number of binding sites ($n = 2$), however, remains the same (Westphal, 1966). The linear relationships evident in the reciprocal plots of the binding data (see Figure 4 in Westphal, 1966)

clearly indicate competitive binding.

If the low progesterone binding affinity of "pure" AAG preparations results from small quantities of contaminating lipids, differences in steroid binding ability observed in various samples should reflect the mode of their preparation. Table IV bears this out. The lowest $\bar{\nu}$/[S] values were found with AAG preparations 2 and 3 which were purified by chromatography, starting directly from serum. Preparations 1 and 4 have a higher steroid binding affinity; they had been subjected to alcohol fractionation which presumably removed some of the contaminating lipid. Alcohol–acetone precipitation of preparation 1 resulted in the highest binding affinity ever observed under the conditions of our test procedure, *i.e.*, that of preparation AAG (type P).

The temperature dependency of the binding affinity between progesterone and AAG (Figure 3, curve A) is very similar to that observed for the steroid complexes of the corticosteroid binding globulin, *i.e.*, the dissociation increases considerably with increasing temperature (for references, see Westphal, 1967b). In contrast, the temperature effect on the association constant of the HSA–progesterone complex is slight (Figure 3, curve B). The thermostability of AAG itself (Table V) is much greater than that of CBG which can be inactivated by exposure to 60° for a relatively short period of time (Daughaday and Mariz, 1961; Doe *et al.*, 1964). A close similarity between AAG and CBG has been found in the influence of pH on the stability of their steroid complexes; in both cases, the binding affinity had a maximal value at pH approximately 8 (Figure 4; Westphal, 1967b).

It has been consistently observed in numerous experiments that the progesterone binding affinity in 0.05 M phosphate (pH 7.4) is slightly higher than that in distilled water at the same pH. Table VI shows this result, together with $\bar{\nu}$/[S] values for some other buffers. Veronal was found consistently to decrease the binding affinity; cacodylate–NaCl was without influence. These observations led to studies on the influence of neutral salts on the steroid binding affinity of AAG which will be reported in a later publication.

A comparison of the binding affinity of different steroid hormones to AAG (Table VII) shows the validity of the "polarity rule" (Eik-Nes *et al.*, 1954) according to which introduction of electronegative groups into the steroid molecule weakens the interaction with the protein (Westphal and Ashley, 1962). The AAG–cortisol complex has a very low association constant. Estrone and estradiol are also bound by AAG; the reduction of the C-17 carbonyl group to hydroxyl decreases the affinity.

The present results indicate that the highly purified AAG, prepared by chromatographic procedures and subsequent delipidation, is well suited for a study of the chemical basis of steroid–protein interactions. This glycoprotein shares various steroid binding characteristics with CBG, a glycoprotein which has a very high affinity for progesterone. Further studies on the interaction of AAG with progesterone and other steroids will be reported.

Acknowledgment

The authors are indebted to Drs. J. A. Yankeelov, Jr., R. S. Levy, and M. Murray for their help in the amino acid analyses performed in their laboratories; to Dr. C. A. Lang for the nitrogen determination; to Mr. B. F. Van Osdol for his expert assistance in the ultracentrifugal experiments and in the electrophoretic analysis by the moving boundary method; and to Mr. J. O. Grote, Cary Instruments, for his kind assistance in the ORD measurements.

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The Study of Membrane Function by Observation of the Change in Rate of Transcellular Migration of Amino Acids*

John E. Harris and Leo Friedman

ABSTRACT: The functioning of a membrane may be observed by monitoring the transport of amino acids. We have devised an experimental system in which the factors affecting membrane function and the effects of specific compounds can be studied. A complex membrane of Ehrlich ascites cells on a Millipore filter similar to that described by D. L. Oxender and H. N. Christensen (*J. Biol. Chem. 234*, 2321 (1959)) separates two compartments of an all-glass apparatus. In each compartment is a K^+-free Krebs–Ringer bicarbonate solution containing the amino acid being studied. The larger (45 ml) compartment contains [^{14}C]amino acid, and the rate of accumulation of the radioactive label in the smaller (6.5 ml) compartment is determined by periodic sampling. Under these conditions, the addition of KCl stimulated the rate of migration of radioactive glycine, α-aminoisobutyric acid (AIB), and glutamic acid. The apparent increase in the rate of transcellular migration was the result of an increased rate in the direction of the K^+ rather than a decreased rate in the opposite direction. However, in the case of AIB, there was a decreased rate toward the K^+-containing compartment and also an increased rate in the opposite direction. The presence of 5 mM CN^- (in the glutamic acid system) inhibited the increase in rate from occurring.

Several years ago Oxender and Christensen (1959) reported transcellular concentration was a consequence of intracellular accumulation across a barrier of Ehrlich ascites cells. Their system consisted of a stainless-steel Millipore filter apparatus in which the complex cell membrane was prepared and tested. The bottom of this apparatus was coupled to a bubble-driven circulation device (Ussing and Zerahn, 1951) which formed a 35-ml compartment, and the funnel of the filter formed a 0.5-ml compartment. Oxender placed equal amounts of radioactivity in both compartments and established transcellular concentration when a difference in radioactivity was observed between the two compartments.

The capacity to follow the migration of amino acids

* Contribution No. 1073 from the Department of Nutrition and Food Science, Massachusetts Institute of Technology, Cambridge, Massachusetts. *Received May 24, 1967.* This work was supported by Grants CA-06988 and ES-00183 from the National Institutes of Health. Presented in part at the 152nd National Meeting of the American Chemical Society, New York, N. Y., Sept 11–16, 1966.