Pregnenolone Sulfate Binding in the Guinea Pig Adrenal Cortex: Comparison with Pregnenolone Binding†

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ABSTRACT: A pregnenolone sulfate binding component, present in the soluble fraction of the guinea pig adrenal cortex, has been identified and partially characterized. The binding activity was mostly lost after treatment of cytosol with trypsin, but was retained when trypsin inhibitor was present along with the trypsin. Binding was destroyed at 60 °C but was little affected by sulfhydryl reactants. Binding was also maintained over a broad pH range of 4–9. The equilibrium association constant at 0 °C was 3×10^7 M^{-1}. On sucrose density gradient analysis the binding protein had an s value ranging from 3.2 to 3.7. The pregnenolone sulfate binding protein had an apparent molecular weight of 22 000–26 000 as determined by gel filtration with Sephadex and Ultrogel. Steroid competition studies revealed a high degree of specificity for the binding protein. Pregnenolone sulfate binding activity was present in liver and kidney cytosols as well as serum. The latter binding activities were not destroyed at 60 °C and had different s values on sucrose density gradient analysis (liver and kidney 0.75–1.95; plasma 3.7–4.4). In addition, the serum binding component had a different elution volume than that of adrenal cytosol on gel filtration. Cytosols of brain and gut did not demonstrate pregnenolone sulfate binding. Studies comparing the pregnenolone sulfate binding activity with the previously reported pregnenolone-binding activity of the adrenal cortex [Strott, C. A. (1977) *J. Biol. Chem. 252,* 464–470] revealed that these two binding factors are distinct entities.

We have been examining adrenocortical steroid–protein interactions in an attempt to identify specific proteins that might be involved in the regulation of steroid synthesis. It is generally accepted that the regulation of adrenal steroidogenesis occurs between cholesterol and pregnenolone[1] (Stone & Hechter, 1954; Karaboyas & Koritz, 1965; Garren et al., 1971). A previous report from this laboratory described some of the properties of a specific pregnenolone-binding protein in the soluble fraction of the guinea pig adrenal cortex (Strott, 1977). In that paper it was recorded that pregnenolone sulfate was an effective competitor with pregnenolone for binding. This was of interest because pregnenolone and pregnenolone sulfate have an important commonality; i.e., both are products of the cholesterol side chain cleavage enzyme system.

In the studies reported here, we have examined the binding of pregnenolone sulfate in the soluble fraction of the guinea pig adrenal cortex and have attempted to compare the pregnenolone sulfate binding activity to the previously reported pregnenolone-binding activity (Strott, 1977). From the latter studies, we have concluded that pregnenolone and pregnenolone sulfate are bound to distinct proteins.

Materials and Methods

Steroids. Cystalline steroids were purchased from Research Plus Steroids, Steraloids, or Schwarz/Mann. The melting point for all steroids was examined and, in each instance, was found to be consistent with data reported in the literature. [7-^{3}H]-Pregnenolone (15.8 Ci/mmol), [7-^{3}H]pregnenolone sulfate, ammonium salt (25 Ci/mmol) and [7-^{3}H]dehydroepiandrosterone sulfate, ammonium salt (10 Ci/mmol) were purchased from New England Nuclear. Each radioactive steroid was found to cochromatograph with its authentic standard using thin-layer chromatography. All labeled and nonlabeled steroid stock solutions were made up in either absolute ethanol or methanol and stored at −15 °C.

Protein Standards. Cytochrome *c*, chymotrypsinogen A, ovalbumin, bovine serum albumin, and aldolase were purchased from Boehringer.

Enzymes and Chemicals. RNase, DNase, trypsin, and trypsin inhibitor were purchased from Sigma. All chemicals were reagent grade.

Buffers. The following buffer systems were used: 0.25 M sucrose, 50 mM phosphate, 3 mM $MgCl_2$, pH 7.4 (buffer A); 10 mM Tris, 1.5 mM Na_2EDTA, pH 7.4 (buffer B); 50 mM Tris, 1.5 mM Na_2EDTA, 0.02% NaN_3, pH 7.4 (buffer C); 0.1 M citric acid and 0.2 M dibasic sodium phosphate were mixed in appropriate proportions to produce solutions with pH values of 4.0, 5.0, 6.0, and 7.0 (buffer D); 0.2 M Tris and 0.2 M HCl were mixed in appropriate proportions to produce solutions with pH values of 8.0 and 9.0 (buffer E).

Glassware. All glassware used in steps following the preparation of cytosol and serum was acid-washed, rinsed exhaustively with water, and siliconized with Dri-film SC-87 (Pierce Chemical Co.).

Preparation of Cytoplasmic Supernatant Fraction and Serum. Male guinea pigs (NIH strain) weighing between 600 and 800 g were used within 24 h after being received in the laboratory. The animals were killed by decapitation and blood

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[1] The trivial steroid names used are: pregnenolone, 3β-hydroxy-5-pregnen-20-one; pregnenolone sulfate, 20-oxo-5-pregnen-3β-yl sulfate; pregnenolone acetate, 20-oxo-5-pregnen-3β-yl acetate; 17-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; 21-hydroxypregnenolone, 3β,21-dihydroxy-5-pregnen-20-one; pregnendione, 5-pregnene-3,20-dione; progesterone, 4-pregnene-3,20-dione; 17-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 11-deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; dehydroepiandrosterone sulfate, 17-oxo-5-androsten-3β-yl sulfate; androsterone sulfate, 17-oxo-5α-androstan-3α-yl sulfate; epiandrosterone sulfate, 17-oxo-5α-androstan-3β-yl sulfate; etiocholanolone sulfate, 17-oxo-5β-androstan-3α-yl sulfate; testosterone, 17β-hydroxy-4-androsten-3-one; cholesterol, 5-cholesten-3β-ol; cholesterol sulfate, 5-cholesten-3β-yl sulfate; estradiol, 1,3,5(10)-estratriene-3,17β-diol; estrone, 3-hydroxy-1,3,5(10)-estratriene-17-one; estrone sulfate, 17-oxo-1,3,5(10)-estratrien-3-yl sulfate.

was collected in a chilled beaker for later separation of the serum. The adrenal glands were immediately removed and placed in buffer A on ice. The extraneous fat and fibrous material were removed and the tissue was weighed. The tissue was minced with a razor blade and placed in buffer B on ice (1 g/5 mL) and homogenized with a polytron Pt-10 homogenizer in a 4 °C cold room. The tissue was homogenized with a low-speed 6-s burst of the polytron followed by a 15-s rest period. This step was repeated five times. The glass tube containing the tissue was maintained in an ice bath throughout. The homogenate was spun at 6000 rpm in a Sorvall GLC-2 centrifuge in the cold room for 10 min. The clear portion of the supernatant was decanted into a clean tube with a disposable pipet and respun as before for 30 min. The clear portion of the supernatant was transferred to a polycarbonate tube with a disposable pipet and centrifuged at 235 000*g* at 2 °C for 60 min which yielded the soluble cytoplasmic fraction (cytosol). Care was taken at all times to avoid any gross lipid contamination which floated on top of the supernatants. Since the adrenal gland of a mature guinea pig (mean weight 700 g) has a cortex to medulla ratio of approximately 91:1 (Hartman & Brownell, 1949), the contribution of medullary cytosol to the total adrenal cytosol was ignored. When tissue other than the adrenals was used, it was processed in a similar fashion. The collected blood was allowed to clot at 0 °C and the serum was removed following centrifugation at 1000 rpm in a refrigerated centrifuge.

Formation of Pregnenolone Sulfate Macromolecular Complex. An aliquot of the ethanolic solution containing [^{3}H]pregnenolone sulfate was added to tubes containing buffer B (for the pH studies either buffer D or E was used). To this mixture was added an appropriate amount of either cytosol or serum to give the desired final protein and pregnenolone sulfate concentration (final ethanol content was less than 1% v:v). Samples were blended gently with a Vortex mixer and incubated at 0 °C for 60 min. A time study revealed that equilibrium was achieved by 60 min (vide infra). For steroid competition studies, a 1000-fold molar excess of the nonlabeled steroid was added with labeled pregnenolone sulfate.

Binding Assay. Separation of bound from free pregnenolone sulfate was achieved by a Sephadex G-25 column (0.6 × 14 cm). Columns were prepared by packing 5-mL siliconized glass pipets to the 1-mL mark with Sephadex swollen in buffer B and degassed. Following packing, the columns were washed with buffer B. A 0.1-mL sample volume was applied and the columns were eluted with buffer B. Six-drop fractions were collected directly into counting vials with the bound pregnenolone sulfate emerging in the void volume. The entire assay was performed in a 4 °C cold room.

Sucrose Density Gradient Analysis. Samples (200 μL) were layered on 4.8-mL linear gradients of 5–20% sucrose. The sucrose was dissolved in buffer B for routine studies and in either buffer D or E for the pH studies. Cytochrome *c* (s = 1.8), chymotrypsinogen A (s = 2.6), ovalbumin (s = 3.6), and bovine serum albumin (s = 4.4) were used as marker proteins (Smith, 1968). The gradients were centrifuged for 15–16 h at 40 000 rpm in a Beckman 50.1 rotor at 2 °C. Following centrifugation, the polyallomer tubes were placed in a piercing unit and fractions collected directly into counting vials.

Fractionation of Cytosol by Gel Filtration. 1. Sephedex G-150 was swollen in buffer B over boiling water for 5 h and the fines removed. The swollen gel was cooled to 4 °C and degassed. A column (1.6 × 70 cm) was prepared in a 4 °C cold room using the same buffer. The column material was allowed to settle for 3 days under flow prior to application of any sample. Sample volumes of 2 mL were applied and eluted with buffer C by ascending chromatography. One and seven-tenths milliliter fractions were collected at a flow rate of 15.36 mL/h.

2. Ultrogel AcA 44 (LKB) was deaerated and a column (2.5 × 70 cm) prepared in a 4 °C cold room using buffer C. The column material was allowed to settle for 3 days under flow prior to application of any sample. Sample volumes of 3 mL were applied and eluted with buffer C by ascending chromatography. Two and five-tenths milliliter fractions were collected at a flow rate of 25 mL/h.

Polyacrylamide Gel Electrophoresis. Electrophoresis was performed using a continuous buffer system consisting of 50 mM Tris-HCl (pH 7.4) and 1.5 mM Na_2EDTA. Photopolymerization and electrophoresis were performed at 0–4 °C. Tube gels were made 5% T and 2% C. Samples with added sucrose were carefully layered in a volume of 25 μL and electrophoresis carried out at 1 mA/gel. Following electrophoresis gels were sectioned transversely into 1-mm slices for the determination of radioactivity in each slice.

Enzymatic Release Studies. Aliquots of cytosol (1 mL) were equilibrated with [^{3}H]pregnenolone sulfate and incubated for 60 min at 0 °C with (a) no addition, (b) 1 mg of trypsin, (c) 1 mg of trypsin plus 1 mg of trypsin inhibitor, (d) 1 mg of RNase, and (e) 1 mg of DNase plus 5 μL of 1 M $MgSO_4$.

Protein Determination. Trichloroacetic acid precipitable material was assayed according to the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Results

Pregnenolone Sulfate Binding Components in Different Tissues. Adrenal, liver, kidney, brain, and gut cytosol preparations (8–15 mg/mL protein) were incubated with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate and analyzed by sucrose density gradient centrifugation. A representative study is shown in Figure 1. The adrenal had a peak of radioactivity corresponding to an s value ranging from 3.2 to 3.7 (n = 8). The liver and kidney had superimposable radioactive peaks corresponding to an s value ranging from 0.75 to 1.95 (n = 6). No demonstrable radioactive peaks were seen with the brain and gut cytosol preparations (n = 3). When serum preparations (10–15 mg/mL protein) were incubated with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate and analyzed in a similar fashion, a peak of radioactivity corresponding to an s value ranging from 3.7 to 4.4 (n = 6) was found (data not shown).

To determine that the radioactive peak observed with adrenal cytosol was unaltered [^{3}H]pregnenolone sulfate the following experiment was performed. Fractions of the sucrose density gradient containing the radioactive peak were pooled and extracted with 1-butanol. The extract was mixed with crystalline pregnenolone sulfate and recrystallized from methanol. The specific activity in the recovered crystals remained constant through three crystallizations, indicating that the radioactive peak with adrenal cytosol was composed of pregnenolone sulfate.

Effect of Temperature on Pregnenolone Sulfate Binding. Adrenal, liver, and kidney cytosol preparations and serum samples were maintained at 30 °C for 30 min and 60 °C for 10 min as well as at 0 °C. Following centrifugation of the 60 °C samples to remove any flocculent material, all samples were incubated with [^{3}H]pregnenolone sulfate at 0 °C for 60 min and then analyzed by sucrose density gradient centrifugation (Figure 2). It can be seen that the 60 °C treatment markedly reduced [^{3}H]pregnenolone sulfate binding in the adrenal cytosol (Figure 2A), while there was no apparent effect of the heat treatment on binding in serum (Figure 2B), liver (Figure 2C), and kidney (Figure 2D).

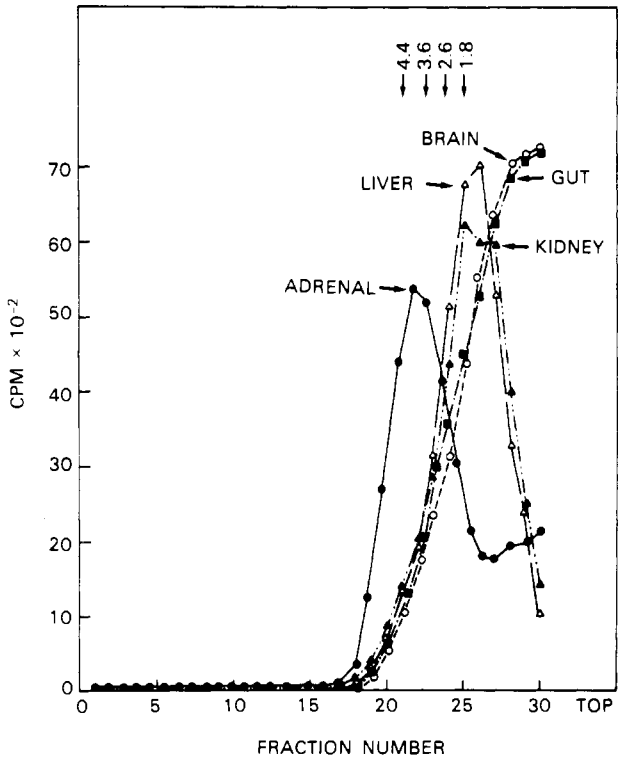

FIGURE 1: Sucrose density gradient patterns of guinea pig adrenal, liver, kidney, brain, and gut cytosols (8–15 mg/mL protein) incubated for 60 min at 0 °C with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate. Samples (200 μL) were layered on 5–20% sucrose gradients (4.8 mL) and centrifuged at 149 000g for 15–16 h at 2 °C. The arrows indicate the optical density peaks for the standard proteins, cytochrome c (s = 1.8), chymotrypsinogen A (s = 2.6), ovalbumin (s = 3.6), and bovine serum albumin (s = 4.4).

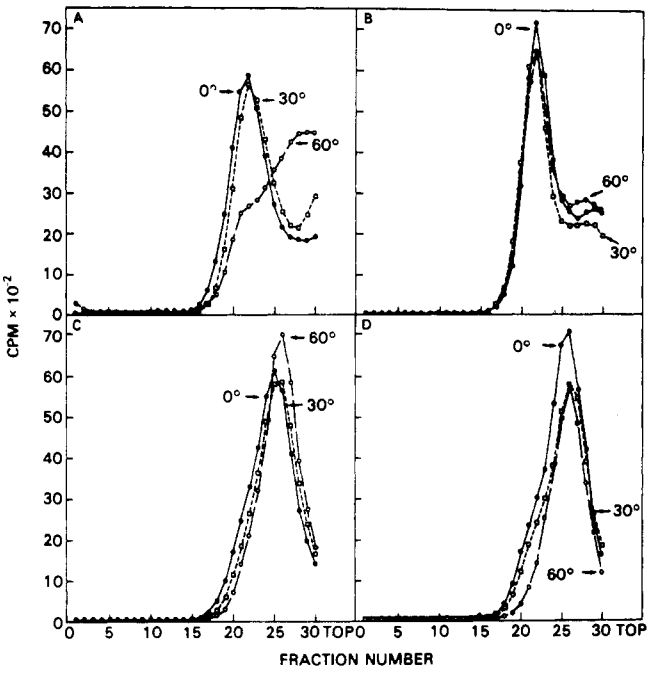

FIGURE 2: Sucrose density gradient patterns of (A) adrenal, (C) liver, and (D) kidney cytosols and (B) serum (8–15 mg/mL protein) incubated for 60 min at 0 °C with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate following the preincubation of cytosol and serum at 0 °C, 30 °C for 30 min, and 60 °C for 10 min. Samples (200 μL) were layered on 5–20% sucrose gradients (4.8 mL) and centrifuged for 15–16 h at 2 °C.

Time Study and Binding Affinity of the Adrenal Cytosol Binding Component for Pregnenolone Sulfate. Adrenal cytosol (2 mg/mL protein) was mixed with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate and incubated at 0 °C. At various times, a 0.1-mL aliquot was removed and assayed for binding by gel filtration. A binding equilibrium was reached by 60 min and remained constant for at least 4 h (data not shown).

Incubation of adrenal cytosol (1 mg/mL protein) with increasing concentrations of [^{3}H]pregnenolone sulfate resulted in saturation of the binding sites (Figure 3). On the basis of heat sensitivity it appeared that essentially all the pregnenolone sulfate binding by the Sephadex column technique was specific. The equilibrium association constant (K_a) at 0 °C was 3.0×10^7 M^{-1} (n = 5). A Scatchard plot is also shown in Figure 3. The concentration of pregnenolone sulfate binding sites as estimated from the Scatchard analysis was approximately 10×10^{-12} mol per mg of protein.

Enzymatic Digestion Studies and Effect of SH-Reacting Reagents on the Adrenal Cytosol Pregnenolone Sulfate Binding Component. Adrenal cytosol was incubated with [^{3}H]pregnenolone sulfate alone, and with one of the factors (dithiothreitol, N-ethylmaleimide, DNase, RNase, trypsin, and trypsin plus trypsin inhibitor) and analyzed by sucrose density gradient centrifugation. Trypsin treatment markedly decreased binding, while the addition of trypsin inhibitor maintained binding equivalent to a control sample (data not shown). DNase and RNase had no appreciable affect on binding (data not shown). Binding was not enhanced by the presence of dithiothreitol (data not shown) while N-ethylmaleimide caused only a slight reduction in binding (data not shown).

Effect of Hydrogen Ion Concentration on Pregnenolone Sulfate Binding. [^{3}H]Pregnenolone sulfate binding to adrenal cytosol was analyzed by sucrose density gradient centrifugation at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. In this study adrenal glands were homogenized in either buffer D at pH 4.0, 5.0, 6.0, and 7.0, or buffer E at pH 8.0 and 9.0. The gradients were made with the same buffer systems at the appropriate pH. [^{3}H]Pregnenolone sulfate binding was qualitatively the same at all pH values (Figure 4). Although this kind of study cannot be evaluated quantitatively, there appeared to be somewhat less binding at pH 4.0 and 5.0.

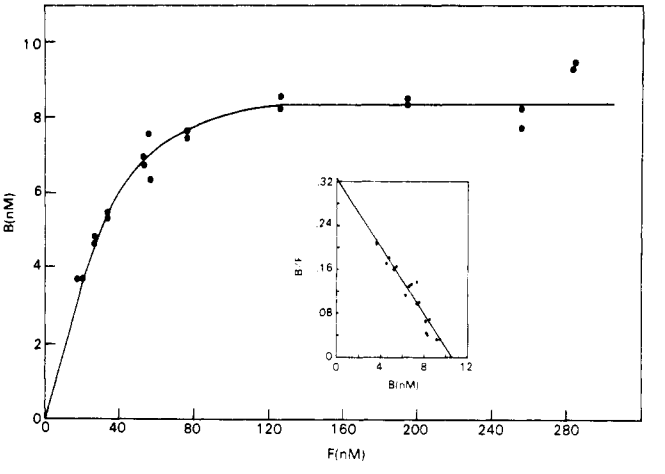

FIGURE 3: Saturation curve and Scatchard plot of [^{3}H]pregnenolone sulfate binding by adrenal cytosol. Aliquots of cytosol (1 mg/mL protein) were incubated with increasing concentrations of [^{3}H]pregnenolone sulfate for 60 min at 0 °C. The bound [^{3}H]pregnenolone sulfate was measured using a Sephadex G-25 column (assay details are given under Materials and Methods). The saturation curve plotted represents total [^{3}H]pregnenolone sulfate binding. On the basis of heat sensitivity, essentially all the pregnenolone sulfate binding was considered specific. B refers to bound and F to free [^{3}H]pregnenolone sulfate. B/F refers to the ratio of the two moieties.

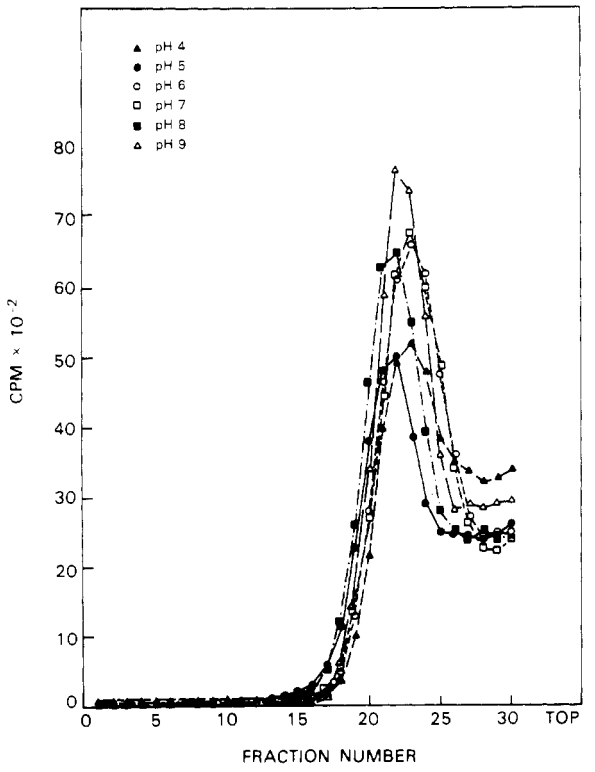

FIGURE 4: Sucrose density gradient patterns of adrenal cytosol (6–12 mg/mL protein) incubated for 60 min at 0 °C with 8.7×10^{-9} M [^{3}H]-pregnenolone sulfate at pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0. Adrenal glands were homogenized and gradients were prepared with the same buffer systems at the appropriate pH. Details are given under Materials and Methods. Samples (200 μL) were layered on 5–20% sucrose gradients (4.8 mL) and centrifuged at 149 000*g* for 15–16 h at 2 °C.

Steroid Specificity for the Adrenal Cytosol Pregnenolone Sulfate Binding Component. A number of steroids were tested for their ability to compete with [^{3}H]pregnenolone sulfate for the binding component in adrenal cytosol. These studies were not intended to be a careful examination of relative potencies, but simply to determine at a single dose the ability of a nonlabeled ligand to compete with the labeled ligand. A large concentration of the competing steroid was chosen because the binding protein had a relatively low affinity and large capacity (when compared with the classical steroid receptors). In these studies a 1000-fold molar excess of nonlabeled steroid was added along with the [^{3}H]pregnenolone sulfate and incubations carried out for 60 min at 0 °C. Binding was analyzed by gel filtration (Table I). Pregnenolone acetate proved to be as effective a competitor as pregnenolone sulfate with dehydroepiandrosterone sulfate being somewhat less effective. Progesterone, pregnendione, and epiandrosterone sulfate were moderately effective competitors. Pregnenolone caused about 50% inhibition of [^{3}H]pregnenolone sulfate binding.

A Study Comparing Pregnenolone Sulfate and Dehydroepiandrosterone Sulfate Binding in Adrenal Cytosol. Because dehydroepiandrosterone sulfate could compete with pregnenolone sulfate, binding of [^{3}H]dehydroepiandrosterone sulfate was examined and compared with [^{3}H]pregnenolone sulfate binding at two temperatures. Aliquots of cytosol were maintained at 0 °C or heated to 60 °C for 10 min (the latter samples were centrifuged to remove any flocculent material). Following this, the radioactive steroids were added and the samples incubated for 60 min at 0 °C. The samples were analyzed by sucrose density gradient centrifugation (Figure 5). There was a small shoulder of radioactivity with [^{3}H]dehydroepiandrosterone sulfate which was, if anything, enhanced following 60 °C heat treatment of the adrenal cytosol. Whereas, the large radioactive peak with [^{3}H]pregnenolone sulfate disappeared following similar heat treatment. This finding indicated that dehydroepiandrosterone sulfate was not normally bound by the same factor that bound pregnenolone sulfate.

TABLE I: Competition between [^{3}H]Pregnenolone Sulfate and Various Nonlabeled Steroids for Binding to the Adrenocortical Cytosol Binding Component[a]

nonlabeled steroid	% [^{3}H]pregnenolone sulfate bound[b] expt 1	expt 2
pregnenolone sulfate	10	8
pregnenolone acetate	12	11
dehydroepiandrosterone sulfate	18	16
progesterone	25	30
pregnendione	38	38
epiandrosterone sulfate	42	35
pregnenolone	55	43
androsterone sulfate	48	52
etiocholanolone sulfate	45	48
17-hydroxyprogesterone	62	55
21-hydroxypregnenolone	86	64
cholesterol sulfate	73	78
dehydroepiandrosterone	72	70
testosterone	64	73
11-deoxycorticosterone	83	77
11-deoxycortisol	86	82
17-hydroxypregnenolone	127	120
cholesterol	108	118
estradiol	91	118
estrone	98	117
estrone sulfate	99	107
cortisol	100	111

[a] One-milliliter cytosol (~1.0 mg/mL protein) samples were incubated with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate and 8.7×10^{-6} M of a nonlabeled steroid at 0 °C for 60 min. A 0.1-mL aliquot was then applied to a Sephadex G-25 column (0.6 × 14 cm) and eluted with buffer B. The void volume containing the steroid-bound fraction was collected directly into counting vials and the radioactivity determined. [b] % [^{3}H]pregnenolone sulfate bound = [^{3}H]pregnenolone sulfate + nonlabeled steroid (cpm)/[^{3}H]pregnenolone sulfate (cpm) × 100.

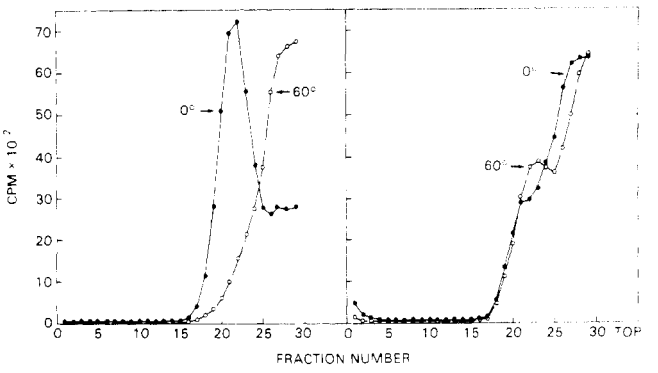

FIGURE 5: Sucrose density gradient patterns of adrenal cytosol (9 mg/mL protein) incubated for 60 min at 0 °C with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate (left panel) and 2×10^{-8} M [^{3}H]dehydroepiandrosterone sulfate (right panel) following the preincubation of cytosol at 0 and 60 °C for 10 min. Samples (200 μL) were layered on 5–20% sucrose gradients (4.8 mL) and centrifuged for 15–16 h at 2 °C.

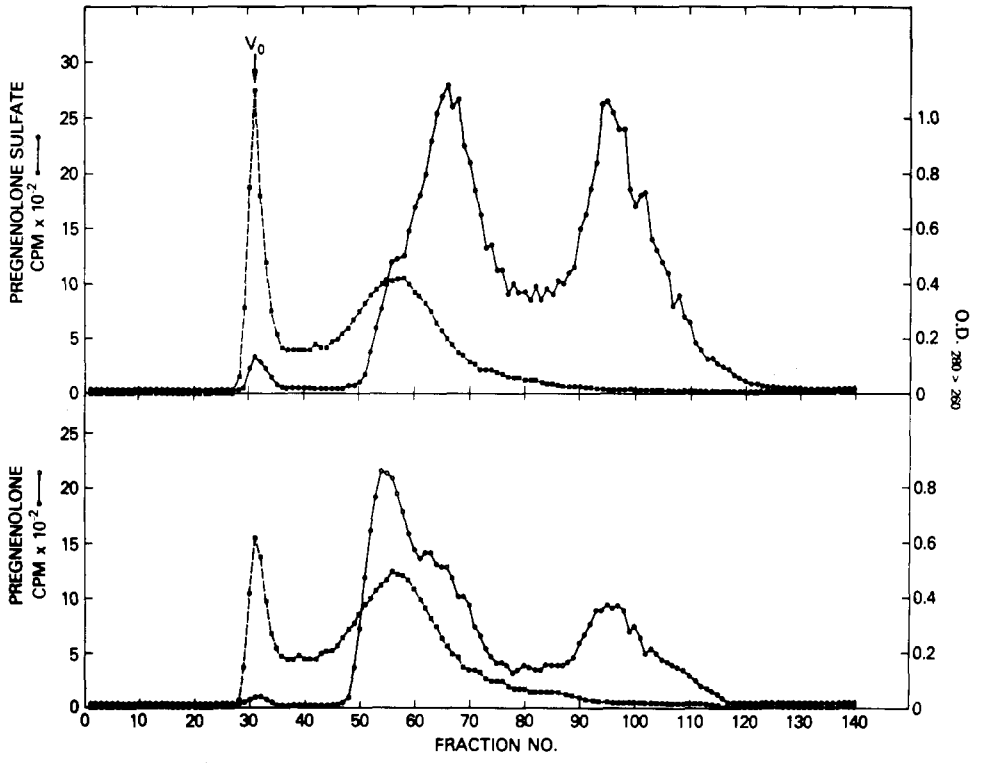

FIGURE 6: Sephadex G-150 elution profiles of adrenal cytosol (8–11 mg/mL protein) incubated for 60 min at 0 °C with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate (top panel) and 1×10^{-8} M [^{3}H]pregnenolone (bottom panel). Details are given under Materials and Methods. Protein content per fraction (■- - -■) was plotted as the optical density (OD) at 280 nm being greater than the OD at 260 nm. V_0 refers to the void volume.

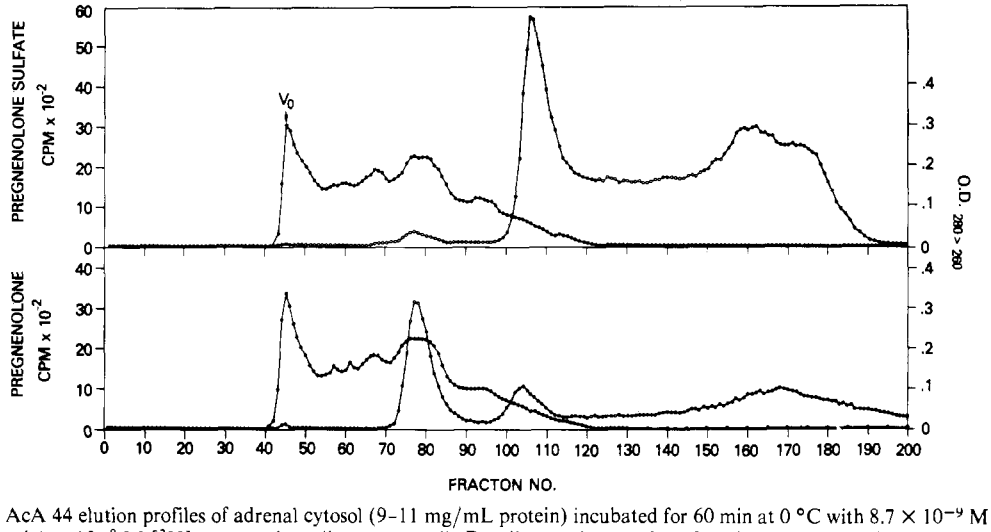

FIGURE 7: Ultrogel AcA 44 elution profiles of adrenal cytosol (9–11 mg/mL protein) incubated for 60 min at 0 °C with 8.7×10^{-9} M [^{3}H]pregnenolone sulfate (top panel) and 1×10^{-8} M [^{3}H]pregnenolone (bottom panel). Details are given under Materials and Methods. Protein content per fraction (●—●) was plotted as the optical density (OD) at 280 nm being greater than the OD at 260 nm. V_0 refers to the void volume.

Fractionation of Adrenal Cytosol Pregnenolone Sulfate Binding Component. Adrenal cytosol (~10 mg/mL protein) was equilibrated with [^{3}H]pregnenolone sulfate and a 2-mL sample was analyzed by gel filtration using a Sephadex G-150 column (Figure 6, top panel). Pregnenolone sulfate was eluted in two major peaks (in all experiments a small insignificant radioactive peak was eluted with the void volume): the first major peak had a partition coefficient corresponding to a molecular weight of approximately 22 000 ($n = 4$); the second peak represented unbound or free pregnenolone sulfate. It can be seen that the major, bound peak has a shoulder on the leading edge suggesting a second binding factor (Figure 6, top panel). To resolve this minor binding factor, adrenal cytosol (~10 mg/mL protein) was equilibrated with [^{3}H]pregnenolone sulfate and a 3-mL sample was analyzed by gel filtration using an Ultrogel AcA 44 column (Figure 7, top panel). Again, there were two major peaks of radioactivity eluted: the first peak had a partition coefficient corresponding to a molecular weight of approximately 26 000 ($n = 6$); the second major peak represented the unbound fraction of [^{3}H]pregnenolone sulfate. In addition, there was a minor, third peak of radioactivity eluted before the first major peak. This latter peak had a partition coefficient corresponding to a molecular weight of 56 000. The area of the minor peak ranged from less than 10 up to 20% of the area of the major peak.

Studies Comparing the Pregnenolone Sulfate and Pregnenolone Binding Activities of the Adrenal Cortex. Since the K_a for the adrenal pregnenolone sulfate binding protein was

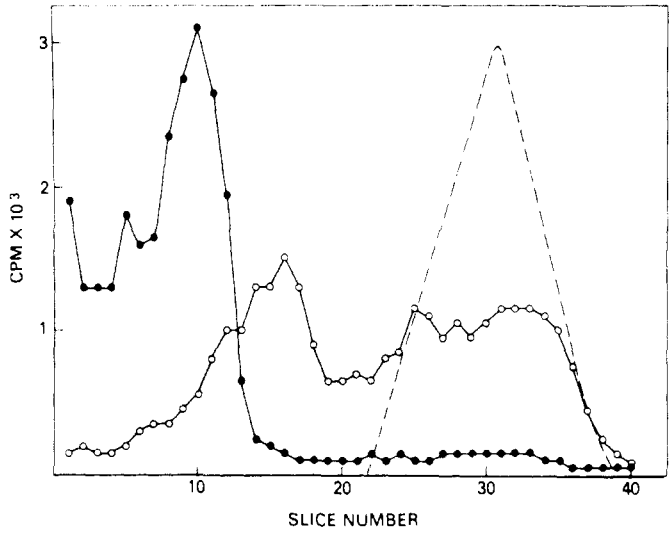

FIGURE 8: Polyacrylamide gel electrophoresis of adrenal cytosol after incubation with either [^{3}H]pregnenolone (●—●) or [^{3}H]pregnenolone sulfate (○—○). Details are given under Materials and Methods. The broken curve (- - - -) indicates where unbound [^{3}H]pregnenolone sulfate migrated in a parallel gel (sample consisted of buffer only and [^{3}H]-pregnenolone sulfate).

similar to the K_a reported for the pregnenolone-binding protein also found in guinea pig adrenal cytosol, we asked the question as to whether the pregnenolone sulfate binding protein and the pregnenolone-binding protein were one and the same. There are several pieces of evidence, however, to suggest that that is not the case.

(1) Effect of Hydrogen Ion Concentration. As shown in Figure 4, pregnenolone sulfate binding remains intact through a pH range of 4 to 9. Pregnenolone binding, on the other hand, is lost below a pH of 6 and above 8 as previously reported (Strott, 1977).

(2) Fractionation of Adrenal Cytosol Pregnenolone and Pregnenolone Sulfate Binding Proteins by Gel Filtration. As shown in Figures 6 and 7, pregnenolone sulfate (top panels) and pregnenolone (bottom panels) are predominantly bound to components with different partition coefficients, although there is some cross-reactivity. Parenthetically, the cross-reactivity observed in these studies is substantially less than the cross-reactivity we have observed with a highly specific antiserum generated against pregnenolone (DiPietro et al., 1972). The apparent molecular weight of the pregnenolone-binding protein from these studies was 55 000–56 000 as compared with an apparent molecular weight of 22 000–26 000 for the pregnenolone sulfate binding protein as indicated above.

(3) Electrophoresis of the Binding Components. When adrenal cytosol was analyzed by polyacrylamide gel electrophoresis, the radioactive peaks of [^{3}H]pregnenolone sulfate and [^{3}H]pregnenolone migrated at distinctly different rates (Figure 8). It should be noted that electrophoresis of pregnenolone sulfate is complicated by the fact that the unbound steroid is sufficiently charged to migrate in all the systems that we tried (both continuous and discontinuous), and that dissociation of the protein–steroid complex during electrophoresis is marked (Figure 8).

(4) Protamine Sulfate Precipitation. As previously reported, the pregnenolone-binding protein of adrenal cytosol was precipitable with protamine sulfate (Strott, 1977). The pregnenolone sulfate binding protein, however, was not precipitable with protamine sulfate.

(5) Steroid Competition Studies. Since pregnenolone sulfate and pregnenolone were bound to their binding proteins with essentially the same affinity, a comparison of steroid competition should be able to demonstrate distinct differences in specificity. As can be seen when [^{3}H]pregnenolone sulfate was tested (Table I), the most effective competitors were pregnenolone sulfate, pregnenolone acetate, and dehydroepiandrosterone sulfate. Whereas, when [^{3}H]pregnenolone was used (Table II), the most effective competitors were pregnenolone, pregnenolone acetate, 21-hydroxypregnenolone, and estrone. The latter steroid did not compete at all for [^{3}H]pregnenolone sulfate. A check of both tables points up several differences in terms of steroid specificity for the two binding proteins. There was one striking similarity, though, and that was the effectiveness of pregnenolone acetate to compete equally well with both [^{3}H]pregnenolone sulfate and [^{3}H]pregnenolone.

TABLE II: Competition between [^{3}H]Pregnenolone and Various Nonlabeled Steroids for Binding to the Adrenocortical Cytosol Binding Component.[a]

nonlabeled steroid	% [^{3}H]pregnenolone bound[b] expt 1	expt 2
pregnenolone	6	5
pregnenolone acetate	3	5
21-hydroxypregnenolone	7	8
estrone	20	15
pregnendione	34	27
dehydroepiandrosterone	33	42
progesterone	44	40
pregnenolone sulfate	46	47
estradiol	55	56
dehydroepiandrosterone sulfate	67	67
17-hydroxyprogesterone	77	68
17-hydroxypregnenolone	62	76
11-deoxycortisol	68	76
testosterone	70	89
estrone sulfate	70	89
11-deoxycorticosterone	83	67
cholesterol	86	68
cortisol	86	110
cholesterol sulfate	92	128

[a] One-milliliter cytosol (~1.0 mg/mL protein) samples were incubated with 5×10^{-9} M [^{3}H]pregnenolone and 5×10^{-6} M of a nonlabeled steroid at 0 °C for 60 min. A 0.1-mL aliquot was then applied to a Sephadex G-25 column (0.6 × 14 cm) and eluted with buffer B. The void volume containing the steroid-bound fraction was collected directly into counting vials and the radioactivity determined. [b] % [^{3}H]pregnenolone bound = [^{3}H]pregnenolone + nonlabeled steroid (cpm)/[^{3}H]pregnenolone (cpm) × 100.

Discussion

The data presented demonstrate for the first time the presence of a pregnenolone sulfate binding component in the guinea pig adrenal cytosol. Enzyme degradation studies revealed that the binding component was a protein. Liver and kidney cytosols and serum also contained pregnenolone sulfate binding factors which appeared to be distinct from the adrenal component. The adrenal pregnenolone sulfate binding component was heat sensitive and migrated in a sucrose density gradient with an s value of approximately 3.5 S. The serum as well as the liver and kidney cytosol factors were heat insensitive and had different s values on sucrose density gradient analysis (serum ~4.0, liver and kidney ~1.7). In addition, the serum pregnenolone sulfate binding factor was analyzed by gel filtration

and found to be clearly distinguishable from the adrenal cytosol pregnenolone sulfate binding protein (data not presented). The partition coefficient of the serum binding factor corresponded to a molecular weight of 65 000 in contrast to the adrenal cytosol binding protein which had an apparent molecular weight of 22 000–26 000 (vide supra).

The data presented also demonstrate that pregnenolone sulfate is bound to a protein that is distinct from the previously reported pregnenolone-binding protein (Strott, 1977). Each binding component had a high degree of specificity for its respective ligand. A physiological understanding of the pregnenolone and pregnenolone sulfate binding proteins is not as yet appreciated. Both binding activities appear to be confined to the adrenal gland (presumably the adrenal cortex). (It would not be unreasonable to consider that similar factors might be present in gonadal tissue, although this has not been examined.) It will be important and efforts are now in progress to purify these binding proteins in order to examine them for any enzymatic activity. If they are not enzymes, then this opens up some interesting physiological possibilities, e.g., transport proteins, etc. It should be noted, however, that once these binding proteins have been highly purified, a more careful quantitative steroid specificity examination utilizing the Scatchard analysis will need to be performed.

An intriguing aspect of the pregnenolone and pregnenolone sulfate studies is that these steroids have a certain physiological commonality: the parallel pathways, cholesterol → pregnenolone → dehydroepiandrosterone and cholesterol sulfate → pregnenolone sulfate → dehydroepiandrosterone sulfate are known to exist and the latter pathway proceeds without removal of the sulfate moiety (Roberts & Lieberman, 1970). It is still uncertain whether the enzyme systems employed in these two pathways are one and the same or whether there are distinct systems for each pathway (Hochberg et al., 1974). What appears to be certain, however, is that pregnenolone and pregnenolone sulfate are products of the cholesterol and cholesterol sulfate lyase reaction, the rate-limiting step in steroidogenesis.

References

DiPietro, D. L., Brown, R. D., & Strott, C. A. (1972) *J. Clin. Endocrinol. Metab. 35,* 729–735.

Garren, L. D., Gill, G. N., Masui, H., & Wilson, G. M. (1971) *Recent Prog. Hormone Res. 27,* 433–474.

Hartman, F. A., & Brownell, K. A. (1949) *The Adrenal Gland,* Lea and Febiger, Philadelphia, Pa.

Hochberg, R. B., Ladany, S., Welch, M., & Lieberman, S. (1974) *Biochemistry 13,* 1938–1945.

Ksaraboyas, G. C., & Koritz, S. B. (1965) *Biochemistry 4,* 462–468.

Lowry, G. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265–275.

Roberts, K. D., & Lieberman, S. (1970) in *Chemical and Biological Aspects of Steroid Conjugation* (Bernstein, S., & Solomon, S., Eds.) pp 219–290, Springer-Verlag, New York, N.Y.

Smith, M. H. (1968) in *Handbook of Biochemistry* (Saber, H. A., Ed.) p C-3, The Chemical Rubber Publishing Co., Cleveland, Ohio.

Stone, D., & Hechter, O. (1954) *Arch. Biochem. Biophys. 51,* 457–459.

Strott, C. A. (1977) *J. Biol. Chem. 252,* 464–470.