Purification and Characterization of High-Molecular-Weight Forms of Adrenocorticotropic Hormone of Ovine Pituitary Glands†

Teh H. Lee* and Mang S. Lee

ABSTRACT: A highly purified preparation of high-molecular-weight adrenocorticotropic hormone (ACTH) was prepared from ovine pituitary glands by dilute acetic acid extraction, oxycellulose fractionation, Sephadex gel filtration, and affinity chromatography on immobilized α_p(1-39)ACTH antibodies. Two ACTH peptides of molecular weights of 24 000 and 34 000 were detected by sodium dodecyl sulfate-acrylamide gel electrophoresis in this preparation. It appeared that the immobilized antibodies adsorbed two forms equally well and could not distinguish between them under the conditions used. These two ACTH peptides were found to be present in crude extracts of ovine pituitary glands, indicating that they were not artifacts produced by the purification procedure. The high-molecular-weight forms of ACTH were found to be susceptible to degradation by tissue enzymes. They could be easily destroyed during the extraction, if precautions were not taken. Moreover, they were poorly adsorbed by oxycellulose which had been used for the adsorption of ACTH activity from crude preparations by most investigators. These properties probably accounted for the fact that high-molecular-weight forms of ACTH remained undetected until very recently.

It has been reported by many investigators that ACTH[1] exists in high-molecular-weight forms in addition to the well-characterized α(1-39)ACTH in pituitaries, plasmas, tumors, and established cell lines. These peptides were designated "big" and "intermediate" ACTH (Yalow and Berson, 1971; Coslovsky and Yalow, 1974) and assigned apparent molecular weights on the basis of gel-filtration elution volumes (Orth et al., 1973; Eipper and Mains, 1975) and sodium dodecyl sulfate-acrylamide gel electrophoretic mobilities (Lee and Lee, 1976; Mains and Eipper, 1976). Although there is evidence to suggest that they might be glycoproteins (Eipper et al., 1976), the chemical nature of high-molecular-weight forms of ACTH has not been firmly established. The "big" ACTH of human plasmas and tumors could be converted by a limited tryptic digestion to an ACTH-like fragment, immunologically indistinguishable from α_h(1-39)ACTH (Yalow and Berson, 1973; Hirata et al., 1975). The conversion was accompanied by the appearance of the biological activity (Gewirtz et al., 1974). Two forms of "big" ACTH have been detected and both appear to play a precursor role in the biosynthesis of α(1-39)ACTH (Mains and Eipper, 1976), but only one gene product translated by AtT-20 tumor cell mRNA in a cell-free system was detected (Jones et al., 1977). The "intermediate" ACTH, on the other hand, does not appear to serve as a precursor for the biosynthesis of α(1-39)ACTH (Mains and Eipper, 1976). It should be noted that these studies were made either using unfractionated crude tissue extracts or tumor cell cultures and that there were uncertainties and limitations inherent to the methodology used. To obtain definitive proof of biosynthetic pathway and the exact knowledge of structural interrelationship of these ACTH peptides for a full understanding of the biosynthesis and physiological function of ACTH, one must rely on the isolation from normal pituitaries, and chemical and biochemical characterization of each of these ACTH peptides. This communication reports the partial purification and some chemical behaviors of high-molecular-weight forms of ACTH of commercial ovine pituitary glands and the determination of their molecular weights by sodium dodecyl sulfate-acrylamide gel electrophoresis.

Experimental Section

Material. Frozen ovine pituitary glands were purchased commercially and stored at −20 °C. Oxycellulose (8-12% COOH) was a product of Eastman Chemical Products, Inc. Human serum albumin (1× crystallized), pepsin (porcine stomach mucosa, 2500 U per mg), ribonuclease (bovine pancreas, 5× crystallized, salt free), aldolase (rabbit muscle), and carbonic anhydrase (bovine erythrocytes, lyophilized, salt free) were commercial products and used without purification. The ovine prolactin was our own preparation (36 IU of pigeon cropsac activity per mg).

Radioimmunoassay. The procedure of Berson and Yalow (1968) was followed. The assay of most of our ACTH preparations was kindly provided by Dr. Yalow and her staff.

Extraction of Glands. We observed that pituitary extracts prepared by extraction of glands under mild conditions, i.e., saline and distilled water extraction, invariably showed very little or no high-molecular-weight forms of ACTH upon size distribution analysis of their RIA-ACTH activity, unless the glands after homogenization were heated immediately in a boiling water bath. In contrast, the heating was not necessary when a more drastic condition, such as glacial acetic acid extraction, was employed. Moreover, it was noted that the content of high-molecular-weight forms of ACTH of frozen pituitary glands decreased steadily during storage at −10 °C. These observations together with those made by other investigators (Yalow and Berson, 1973; Hirata et al., 1975) appear to suggest that high-molecular-weight forms of ACTH are very

† From Protein Hormone Research Laboratory, Veterans Administration Hospital, and the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, New York 10468. *Received November 1, 1976.* This work was supported by funds from the Research Service of the Veterans Administration. A preliminary report was presented at the 67th Annual Meeting of American Society of Biological Chemists, San Francisco, California, and the Vth International Congress of Endocrinology, Hamburg, Germany.

* Correspondence should be addressed to T. H. Lee, Veterans Administration Hospital, Bronx, New York 10468.

[1] Abbreviations used: ACTH, adrenocorticotropic hormone; RIA-ACTH, ACTH activity measured by radioimmunoassay; HSA, human serum albumin; BSA, bovine serum albumin; HGH, human growth hormone; PTH, parathyroid hormone; Bis, methylenebisacrylamide; SD, standard deviation; DEAE, diethylaminoethyl.

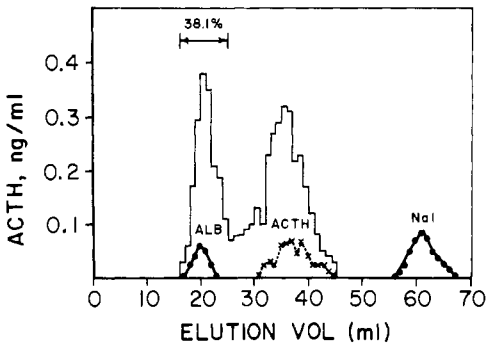

FIGURE 1: Size distribution pattern of RIA-ACTH activity of 0.1 N acetic acid extract of ovine pituitary glands. A column with a bed volume of 50 mL of Sephadex G-50 (fine) was used. The column was equilibrated and developed with 0.05 M NaH_2PO_4-Na_2HPO_4-0.25% BSA-0.5% 2-mercaptoethanol, pH 7.6, buffer at 5 °C. Fractions of 1 mL were collected. [^{131}I]HSA (● — ●), [^{125}I]α_p(1-39)ACTH (X - - - X), and Na^{125}I (● — ●) were used as markers. Radioactivity and RIA-ACTH activity (bars) of all fractions were measured.

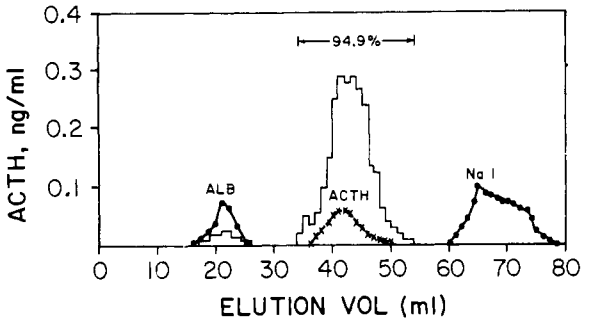

FIGURE 2: Size distribution pattern of RIA-ACTH activity which was eluted from oxycellulose. Same conditions and notations specified in Figure 1 were used.

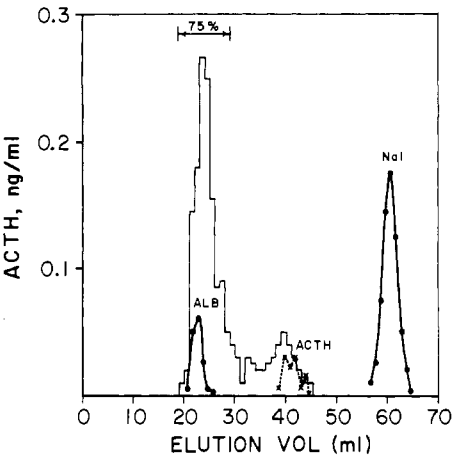

FIGURE 3: Size distribution pattern of RIA-ACTH activity which could not be absorbed by oxycellulose. Same conditions and notations specified in Figure 1 were used.

susceptible to degradation by enzymes in pituitaries. For this reason, heating at 85-95 °C was employed in the preliminary extraction of glands and size distribution analysis of RIA-ACTH activity was performed on the product obtained at each step of the extraction and the purification procedure.

The glands were homogenized in 0.1 N acetic acid-0.5% 2-mercaptoethanol (7 mL of solvent per g of glands). The homogenate was heated immediately in a vigorously boiling water bath for 10 min with constant shaking. After rapid cooling, it was stirred for 1 h and centrifuged at 25 000g for 30 min. The residue was extracted with the same solvent (3 mL per g of glands), omitting the homogenization and heating. The combined supernatant fluids were lyophilized. The yield and the specific activity are 5.87 ± 0.48 g of extract per 100 g of frozen ovine pituitary glands and 1.35 ± 0.28 μg of RIA-ACTH per mg of the extract, respectively (mean ± SD of three experiments). Figure 1 shows the size distribution of RIA-ACTH activity of the extract. It should be noted that, although "big" ACTH was later shown to consist of two high-molecular-weight forms, they could not be resolved by the Sephadex G-50 column used in the size distribution analysis.

Purification of Crude Pituitary Extract. All purification steps were carried out at 5 °C and employed solvents containing 0.2% 2-mercaptoethanol, except it was specified otherwise.

Amberlite CG50 [H^+] Adsorption. Three to four grams of the pituitary extract was dissolved in 300 mL of 0.1 N acetic acid. After the removal of insolubles by centrifugation, the solution was acidified to pH 2.8-3.0 with HCl and passed through a column of Amberlite CG50 [H^+] (65 mm i.d. × 50 mm). The column was washed with 500 mL of 0.1 N acetic acid and eluted with 500 mL of 50% acetic acid. Under these conditions, ACTH was adsorbed tightly while large amounts of inert proteins were either not adsorbed or could be removed by subsequent washings. The elution of the adsorbed activity by 50% acetic acid was essentially quantitative. The results of a series of experiments indicated that the recovery of RIA-ACTH in the adsorbed fraction (50% acetic acid eluate) and that in the unadsorbed fraction (including washings) were 108.1 ± 20.2 and 5.4 ± 4.0, respectively, and that a two- to threefold increase of the specific activity was achieved. Size distribution analyses of the RIA-ACTH of the adsorbed and the unadsorbed fraction showed no changes in the distribution pattern as compared with the original pituitary extracts.

Oxycellulose Fractionation. Ever since Astwood et al. (1951) demonstrated that ACTH peptide could be adsorbed onto oxycellulose to achieve a 10- to 20-fold purification, fractionation by oxycellulose became the method of choice for the purification of this peptide. When the Amberlite CG50 [H^+] adsorbed fraction of the pituitary extract was fractionated by this procedure, the adsorption of RIA-ACTH onto oxycellulose was not complete and a considerable amount of the activity was left in the unadsorbed fraction. The average recovery of the RIA-ACTH activity in the unadsorbed fraction and that in the adsorbed fraction were 28.6 ± 6.2 and 57.7 ± 9.5%, respectively. When the adsorbed and the unadsorbed fractions were analyzed for the size distribution of the RIA-ACTH activity, we found that the oxycellulose adsorbed fraction contained almost exclusively α_0(1-39)ACTH (Figure 2) and that the unadsorbed fraction contained "big" ACTH as a major component and α_0(1-39)ACTH as a minor component (Figure 3). Apparently oxycellulose selectively adsorbed α_0(1-39)ACTH and left the high-molecular-weight forms of ACTH virtually unadsorbed.

Sephadex G-100 Gel Filtration. The unadsorbed fraction obtained from oxycellulose fractionation was purified by gel filtration on a column of Sephadex G-100 in 0.1 N acetic acid. [^{131}I]HSA and [^{125}I]α_p(1-39)ACTH were employed as molecular size markers. Under the conditions employed, the "big" ACTH emerged together with [^{131}I]HSA in an elution volume about halfway between the void volume of the column and the elution volume of [^{125}I]α_p(1-39)ACTH. There were considerable inert materials eluted both at the void volume and after the "small" ACTH marker; therefore, an appreciable degree of purification was achieved. The gel-filtration pattern and the

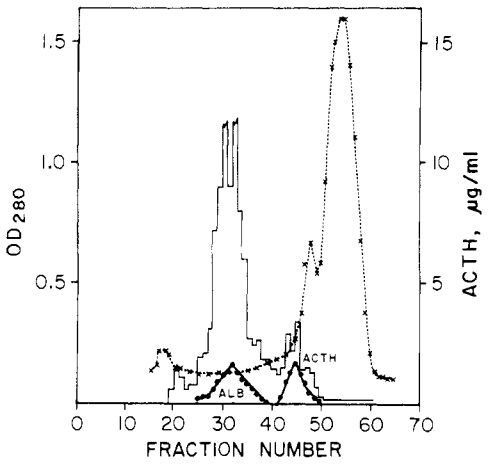

FIGURE 4: Sephadex G-100 gel-filtration pattern of ovine pituitary extracts from which "small" ACTH had been removed by oxycellulose treatment. A column, 50 × 1000 mm, containing 1900 mL of Sephadex G-100 was used. The column was equilibrated and developed with 0.1 N acetic acid–0.2% 2-mercaptoethanol at 10 °C and the effluent was collected in a refrigerated box at 5 °C. One thousand milligrams of the ACTH preparation were fractionated and fractions of 25 mL were collected. [^{131}I]HSA and [^{125}I]α_p(1–39)ACTH were used as markers. OD at 280 nm (X—X), radioactivity (●—●), and RIA-ACTH activity (bars) of all fractions were measured. Pooled fractions, S-100-I, S-100-II, S-100-III, and S-100-IV, were obtained by combining tubes 15–25, 26–40, 41–49, and 50–60, respectively.

TABLE I: Sephadex G-100 Gel Filtration of Ovine Pituitary Extract from Which "Small" ACTH Had Been Removed by Oxycellulose Treatment.[a]

Fraction	Dry weight (mg)	Sp act. ("big & "small" ACTH) (μg/mg)	Size distribution of RIA-ACTH (% "big")
S-100-I	125.3 ± 23.2	0.68 ± 0.42	
S-100-II	165.3 ± 27.7	6.65 ± 0.63	90.8 ± 11.0
S-100-III	227.0 ± 34.1	1.75 ± 0.24	32.5 ± 4.6
S-100-IV	270.0 ± 41.2	0.10 ± 0.05	

[a] The column was developed and fractions were pooled as described in Figure 4. Values given are mean ± SD of results of three experiments.

TABLE II: Affinity Chromatographic Purification of Ovine "Big" ACTH.[a]

Fraction	RIA-ACTH[b] (μg)
AFC-I	25.8 ± 4.2
AFC-II	1.7 ± 0.4
AFC-III	2.0 ± 0.7
AFC-IV	0.1 ± 0.06
AFC-V	13.2 ± 0.9
AFC-VI	2.3 ± 0.7

[a] An aliquot of fraction S-100-II containing 150 μg of "big" ACTH was dissolved in 2 mL of buffer A (0.1 M sodium barbital, pH 8.4) and fractionated on an affinity chromatographic column containing 6 mL of wet affinity adsorbent in the same buffer. The column was washed successively with two 20-mL aliquots of buffer A and two 20-mL aliquots of buffer W-2 (0.1 N acetic acid–1 N NaCl) and eluted with two 20-mL aliquots of buffer E (0.1 N acetic acid–1 N NaCl–3 M guanidine hydrochloride). The column effluents from each aliquot of buffers were pooled into fractions listed in the table. [b] Values given are mean ± SD of results of three experiments.

RIA-ACTH activity of four fractions obtained by pooling the column effluent are shown in Figure 4 and Table I, respectively. The most active fraction, S-100-II, had a specific activity of 6.65 ± 0.92 μg per mg and contained 90.8 ± 11.0% of "big" ACTH.

Affinity Chromatography on Immobilized α_p(1–39)ACTH Antibodies. *N*-Hydroxysuccinimide ester of succinylated 3,3′-diaminopropylamino-Sepharose 4B was prepared from CNBr-activated Sepharose 4B (Cuatrecasas, 1970; Cuatrecasas and Parikh, 1972) and used as the solid phase of the affinity adsorbent. The γ-globulin fraction of a rabbit anti-α_p(1–39)ACTH serum was isolated by DEAE-cellulose chromatography (Levy and Sober, 1960). Coupling of the γ-globulin fraction to the solid phase was carried out in 0.1 M sodium phosphate buffer, pH 7.0, with a protein to solid phase ratio of 10 mg of protein per mL of wet solid phase in a total volume of 4 mL. The mixture was rotated gently for 18–24 h. After completion of the coupling reaction, the suspension was washed alternately with two washing buffers, W-1 (0.2 M sodium borate–1 N NaCl, pH 8.2) and W-2 (0.1 N acetic acid–1 N NaCl), until the OD at 280 nm of the washing was identical with that of W-1. RIA-ACTH could be adsorbed onto the affinity adsorbent in 0.1 M sodium barbital, pH 8.4, and eluted by 0.1 N acetic acid–1 N NaCl–3 M guanidine hydrochloride. It was observed that approximately 20% of the applied RIA-ACTH activity could not be adsorbed by the immobilized antibodies and that essentially the same fraction of activity remained unadsorbed when the total amount of RIA-ACTH activity applied to the adsorbent was decreased 10- to 20-fold. These observations appeared to suggest that the unadsorbed activity might represent a fraction of RIA-ACTH which had been altered during purification. The binding capacity of the affinity adsorbent was estimated to be about 20 μg of RIA-ACTH activity per mL of wet adsorbent, calculated from the difference between the total RIA-ACTH activity applied to the column and that remaining in the column effluent. However, elution by 0.1 N acetic acid–1 N NaCl–3 M guanidine hydrochloride recovered only approximately 2 μg of RIA-ACTH activity. The poor recovery could not be attributed to the presence of guanidine hydrochloride in the assay mixture because the eluate was diluted and an equivalent concentration of guanidine hydrochloride was added to the assay mixture of the standard ACTH solution. It was also unlikely that the adsorbed ACTH was incompletely eluted because the affinity adsorbent could be used repeatedly without serious losses of binding capacity. At present no satisfactory explanation could be offered.

The specificity of the immobilized antibodies was established by the observation that other peptide hormones (HGH, bovine PTH, and ovine prelactin) were not absorbed. Moreover, unlabeled ACTH ("big" or "small") was able to displace [^{125}I]α_p(1–39)ACTH, previously adsorbed on the affinity adsorbent.

Table II summarizes the results of the affinity chromatographic fractionation of three aliquots of the Sephadex G-100 purified ACTH preparation, S-100-II. The active fractions were desalted by gel filtration on Sephadex G-25, concentrated by lyophilization, and stored as frozen solutions in 0.1 N acetic acid.

Calculation of Recovery of RIA-ACTH Activity of the Purification Procedure. The recovery of high-molecular-weight forms of ovine pituitary ACTH was calculated through

TABLE III: Summary of the Purification of "Big" ACTH from Frozen Ovine Pituitary Glands.[a]

Purification step	Dry weight (mg)	Sp act. Total (μg/mg)	Sp act. "Big" (μg/mg)	RIA-ACTH Total (μg)	RIA-ACTH "Big" (μg)	Recovery "big" (%)
0.1 N acetic acid	5870 ± 480	1.35 ± 0.28	0.41 ± 0.07	7836 ± 960	2375 ± 316	
Amberlite CG 50[b]	1445 ± 194	5.81 ± 0.66	1.71 ± 0.29	8397 ± 2001	2469 ± 737	103.9 ± 31.1
Oxycellulose[c]	1300 ± 181	1.86 ± 0.23	1.40 ± 0.14	2445 ± 647	1808 ± 84	76.0 ± 3.5
Sephadex G-100[d]	131 ± 26	6.65 ± 0.92	6.00 ± 0.81	887 ± 278	793 ± 220	33.4 ± 9.2
Affinity chromatography[e]				72 ± 11	69 ± 12	2.9 ± 0.5

[a] Based on 100 g of frozen ovine pituitary glands. Values given are mean ± SD of results of three experiments. [b] Amberlite CG 50 adsorbed fraction. [c] Oxycellulose unadsorbed fraction. [d] Sephadex G-100 gel-filtered fraction, S-100-II. [e] Affinity chromatographic fraction, AFC-V.

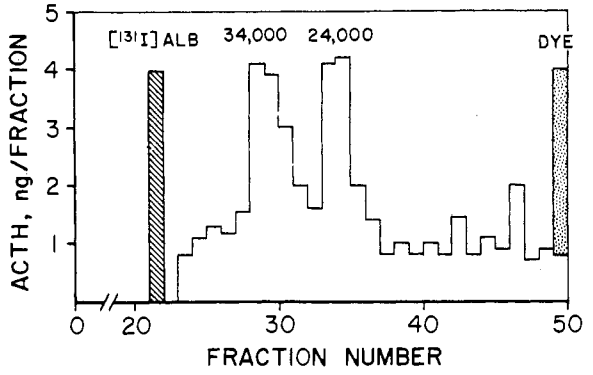

FIGURE 5: Sodium dodecyl sulfate-acrylamide gel electrophoretic pattern of purified high-molecular-weight ovine ACTH. A 10% acrylamide gel was used for the electrophoresis. Approximately 50 ng of immunoreactive ACTH activity of the purified ovine high-molecular-weight ACTH preparation was applied to the gel column. [^{131}I]HSA and bromophenol blue were used to provide a reference point for the measurement of migration and to indicate the location of the buffer front, respectively, as indicated on the electrophoretogram. The gel column was divided into 60 fractions after the electrophoresis. The RIA-ACTH activity of each fraction was measured. Two ACTH peptides of molecular weights of 24 000 and 34 000 were detected.

each step of purification. The calculation included only fractions used in the sequence of purification steps; the recoverable activity of side fractions was not included. The results were summarized in Table III.

Estimation of Apparent Molecular Weight. The affinity chromatographically purified "big" ACTH preparation was submitted to sodium dodecyl sulfate-acrylamide gel electrophoresis for the estimation of apparent molecular weight. A 10% acrylamide gel containing 0.27% cross-linker (BIS), 0.1% sodium dodecyl sulfate, and 0.1% 2-mercaptoethanol was used. The procedure of Weber and Osborn (1969) was followed with slight modifications.

In each electrophoretic run, six reference proteins of known molecular weight, i.e., aldolase, pepsin, carbonic anhydrase, ovine prolactin, ribonuclease, and cytochrome *c*, were included. Reference proteins and ACTH samples were incubated separately at 37 °C for 2 h in 0.01 M sodium phosphate, pH 7.0, containing 0.1% sodium dodecyl sulfate and 0.1% 2-mercaptoethanol. For electrophoresis of reference proteins, HSA and bromophenol blue were used to provide a reference point for the measurement of migration and to indicate the location of the buffer front, respectively. For ACTH samples, [^{131}I]HSA and bromophenol blue were used. The gel columns containing reference proteins were stained and used to calculate the relative mobility of each reference protein. Those containing ACTH samples were divided into 50-60 fractions by a Sarvant autogel divider, using 0.05 M sodium phosphate (pH 7.6)-0.25% BSA-0.2% 2-mercaptoethanol as the suspending medium. The RIA-ACTH activity of the fractions was measured. The relative mobility of the ACTH peptide, R_{ACTH}, was calculated as follows:

$$R_{ACTH} = \frac{\text{peak tube No. of ACTH} - \text{peak tube No. of [}^{131}\text{I]HSA}}{\text{peak tube No. of dye} - \text{peak tube No. of [}^{131}\text{I]HSA}}$$

The apparent molecular weight was estimated from a calibration chart, constructed from the values of relative mobility of reference proteins.

When the affinity chromatographically purified "big" ACTH preparation, AFC-V, was submitted to sodium dodecyl sulfate-acrylamide gel electrophoresis, two bands of RIA-ACTH activity, corresponding to molecular weights of 24 000 and 34 000, were detected as shown in Figure 5. The estimation of molecular weight was repeated six times. The mean molecular weights ± SD were calculated to be 34 300 ± 1100 and 24 000 ± 2200.

In order to rule out the possibility that the observed two forms of "big" ACTH might be artifacts produced by the purification procedure, a crude extract of ovine pituitary glands was submitted to sodium dodecyl sulfate-acrylamide gel electrophoresis under identical conditions. Two bands of RIA-ACTH activity, corresponding to those two forms of "big" ACTH, in addition to a band of α_0(1-39)ACTH were observed. The gel electrophoretic pattern is shown in Figure 6.

An Attempt to Detect Polysaccharides in Purified "Big" ACTH Preparation. An aliquot of the purified "big" ACTH preparation containing 10 μg of RIA-ACTH activity was lyophilized to dryness in a small glass tube. The residue was dissolved in 0.5 mL of 4 N HCl and sealed under vacuo. The tube was heated at 100 °C for 4 h. The hydrolysate was lyophilized to dryness several times with water and taken up in 100 μL of pyridine. The solution was applied to a Kieselguhr G (impregnated with sodium acetate) thin-layer plate by repeated applications. The thin-layer plate was developed with ethyl acetate-2-propanol-water (65:23.5:11.5) and sprayed with anisaldehyde-sulfuric acid reagent (Stahl and Kaltenbach, 1965). No discernible spots of sugar, except a faint smear of coloration extending from the point of application to the solvent front, were detected.

Discussion

It is apparent from Table III that the greatest loss of "big"

ACTH occurred in the purification step involving affinity chromatography on immobilized α_p(1-39)ACTH antibodies. Although the cause of the poor recovery has not been ascertained, it seems likely that a more effective eluting agent might overcome this difficulty. At present our meager supply of antiserum and the low capacity of the affinity adsorbent also severely limited the amount of material that could be purified.

The finding that high-molecular-weight forms of ACTH constitute more than one-fourth of the immunoreactive ACTH activity of pituitary glands may raise the question as to the reasons why these peptides remained undetected until very recently. The answer to this question may be sought from the properties of these peptides. Intact "big" ACTH's are very susceptible to specific and nonspecific degradation by tissue enzymes; therefore, they could be readily destroyed at the early stage of extraction from the glands, if special precautions were not taken. Furthermore, our study showed that these peptides were poorly adsorbed by oxycellulose which had been the universal adsorbent used to adsorb ACTH activity from crude pituitary extracts. For this reason, ACTH preparations used by early investigators for the isolation of α(1-39)ACTH from several species probably were devoid of high-molecular-weight forms of ACTH.

A second question which comes to our mind is whether the ACTH of 24 000 molecular weight reported here is identical with the "ACTH protein" (molecular weight 20 000) isolated many years ago (Li et al., 1942; Sayers et al., 1943). In our study, we found that the acid-acetone extraction procedure of Lyons (1937) which was used by both of these groups of investigators produced a product practically devoid of "big" forms of ACTH. Moreover, we found that the "big" forms of ovine pituitary ACTH were poorly adsorbed onto oxycellulose while Astwood et al. (1951) demonstrated that the ACTH activity of "ACTH protein" could be separated from the inert protein moiety by adsorption onto oxycellulose without prior enzymic degradation. These two lines of evidence appear to suggest that they are not identical substances.

It has been reported (Eipper et al., 1976) that [^{3}H]glucosamine and [^{3}H]mannose could be incorporated into both "big" and "intermediate" ACTH in AtT-20 tumor cell culture and that the 20 000-30 000 ACTH pool ("big" ACTH) of tumor cell extracts and similar preparations from normal pituitaries of several species could bind specifically to concanavalin A-agarose. Based on these findings, it was suggested that high-molecular-weight forms of ACTH might be glycopeptides. Although our attempt to detect sugars in the purified "big" ACTH preparation did not indicate the presence of a carbohydrate side chain, we could not rule out the possibilities that unexpected cleavage of the carbohydrate side chain might have occurred during purification and that the carbohydrate content of "big" ACTH might be too low to be detected in our test sample by the color reagent used. For this reason, the values of the apparent molecular weight estimated by sodium dodecyl sulfate-acrylamide gel electrophoresis should be viewed with reservation, on account of the well-known inaccuracy of this method for the estimation of molecular weight of glycopeptides.

Eipper et al. (1976) reported that the RIA-ACTH activity of AtT-20 mouse tumor cell extracts could be resolved into four discrete forms by sodium dodecyl sulfate-acrylamide gel electrophoresis, with apparent molecular weights of 31 000, 23 000, 13 000, and 4500. Our results on the estimation of molecular weight indicated two forms of "big" ACTH with apparent molecular weights of 34 300 and 24 000. Whether

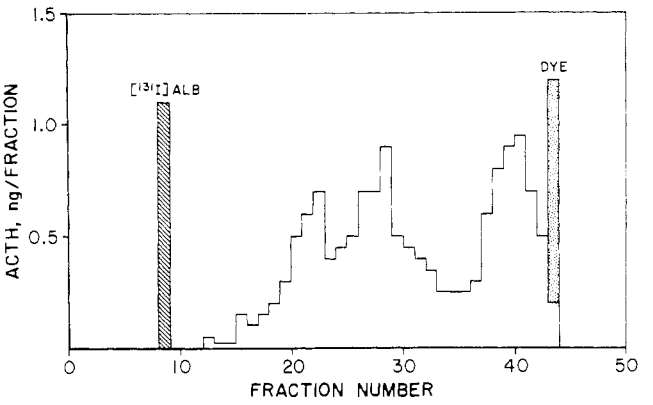

FIGURE 6: Sodium dodecyl sulfate-acrylamide gel electrophoretic pattern of a crude extract of ovine pituitary glands. Same conditions specified in Figure 5 were used.

these two forms of "big" ACTH in our purified ovine pituitary ACTH preparation are identical with those detected in AtT-20 mouse tumor cells has not been resolved at present. We were unable to detect any ACTH peptide in the molecular weight range of "intermediate" ACTH (8000-15 000) in our purified preparation, although it may be present as a minor component in ovine pituitary glands (Figures 1 and 6). This is consistent with the observation that "intermediate" ACTH was found to be a major component of high-molecular-weight ACTH only in mammals whose primary plasma glucocorticoid is corticosterone and not in those with 17-α-hydroxycorticosterone (hydrocortisone) as the predominant plasma glucocorticoid (Coslovsky and Yalow, 1974).

It is of interest to mention that the major components of RIA-ACTH activity of Furth pituitary tumor are of the molecular weight range 12 000-16 000 and that "big" and "small" ACTH constitute only a minor fraction of the total activity (our unpublished results). Other investigators (Orth and Nicholson, 1977) also reported a significant fraction of RIA-ACTH activity of ACTH-producing nonpituitary tumors to be in the molecular weight range of "intermediate" ACTH. These observations may suggest that "intermediate" ACTH may be a form of ACTH characteristic to tumor cells. If this is true, the question whether the "intermediate" ACTH detected in normal pituitaries of some species is an identical substance to the tumor "intermediate" ACTH will arise. This question, of course, cannot be answered until the hormones from both sources are isolated and characterized.

Acknowledgments

The authors thank Dr. R. S. Yalow and her staff for providing radioimmunoassay of most of our ACTH preparations; without her help this study would not have been possible. The technical assistance of Mrs. A. Tottossy is gratefully acknowledged.

References

Astwood, E. B., Raben, M. S., Payne, R. W., and Grady, A. B. (1951), *J. Am. Chem. Soc. 73*, 2969-2970.

Berson, S. A., and Yalow, R. S. (1968), *J. Clin. Invest. 47*, 2725-2751.

Coslovsky, R., and Yalow, R. S. (1974), *Biochem. Biophys. Res. Commun. 60*, 1351-1356.

Cuatrecasas, P. (1970), *J. Biol. Chem. 245*, 3059-3065.

Cuatrecasas, P., and Parikh, I. (1972), *Biochemistry 11*, 2291-2298.

Eipper, B. A., and Mains, R. E. (1975), *Biochemistry 14*,

3836-3844.
Eipper, B. A., Mains, R. E., and Guenzi, D. (1976), *J. Biol. Chem. 251,* 4121-4126.
Gewirtz, G., Schneider, B., Krieger, D. T., and Yalow, R. S. (1974), *J. Clin. Endocrinol. Metab. 38,* 227-230.
Gewirtz, G., and Yalow, R. S. (1974), *J. Clin. Endocrinol. Metab. 36,* 415-423.
Hirata, Y., Yamamoto, H., Matsukura, S., and Imura, H. (1975), *J. Clin. Endocrinol. Metab. 41,* 106-114.
Hirs, C. H. W., Moore, S., and Stein, W. H. (1953), *J. Biol. Chem. 200,* 493-506.
Jones, R. E., Pulkrabek, P., and Grunberger, D. (1977), *Biochem. Biophys. Res. Commun.* (in press).
Lee, T. H., and Lee, M. S. (1976), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 35,* 1628 (abstract).
Levy, H. B., and Sober, H. A. (1960), *Proc. Soc. Exp. Biol. Med. 103,* 250-254.
Li, C. H., Simpson, M. E., and Evans, H. M. (1942), *Science 96,* 450.
Lyons, W. R. (1937), *Proc. Soc. Exp. Biol. Med. 35,* 645-648.
Mains, R. E., and Eipper, B. A. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3565-3569.
Mains, R. E., and Eipper, B. A. (1976), *J. Biol. Chem. 251,* 4115-4120.
Orth, D. N., and Nicholson, W. E. (1977), *J. Clin. Endocrinol. Metab. 44,* 214-217.
Orth, D. N., Nicholson, W. E., Mitchell, W. M., Island, D. P., Shapiro, M., and Byyny, R. L. (1973), *Endocrinology 92,* 385-393.
Sayers, G., White, A., and Long, C. N. H. (1943), *J. Biol. Chem. 149,* 425-436.
Stahl, E., and Kaltenbach, U. (1965), in Thin-Layer Chromatography, Stahl, E., Ed., New York, N.Y., Academic Press, pp 461-469.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406-4412.
Yalow, R. S. (1974), *Rec. Prog. Horm. Res. 30,* 597-626.
Yalow, R. S., and Berson, S. A. (1971), *Biochem. Biophys. Res. Commun. 44,* 439-449.
Yalow, R. S., and Berson, S. A. (1973), *J. Clin. Endocrinol. Metab. 36,* 415-423.

In Vitro and in Vivo Enhancement of Progesterone Binding to the Uterine Progesterone Receptor by Cortisol†

Syed Saiduddin* and Hans Peter Zassenhaus

ABSTRACT: It was found that, in the presence of high concentrations of cortisol (7×10^{-7} M), the apparent K_D for progesterone binding to the rat uterine progesterone receptor decreased when compared with the calculated K_D determined without cortisol. Dilution of the cytosol showed a similar effect. The decrease in the apparent K_D was observed when the amount bound was determined by the dextran coated charcoal (DCC) adsorption method but not when binding was assayed by equilibrium dialysis. A mathematical model has been derived for the analysis of progesterone binding to the rat uterine progesterone receptor which can explain the above results. The model takes into account that the concentration of free progesterone at equilibrium (as calculated from total minus bound) is overestimated due to the binding of progesterone to low affinity material. Since this low affinity binding largely dissociates during treatment with DCC, it is not detected as bound. We show that several predictions of the model are confirmed using the rat uterine progesterone receptor. Specifically, adding cortisol to the in vitro incubations increases the amount of bound progesterone when the reactions are carried out at nonsaturating concentrations of [^{3}H]progesterone. This effect of cortisol is explained by postulating that cortisol displaces [^{3}H]progesterone from the low affinity sites, thus resulting in a higher concentration of free progesterone available for binding to the receptor. In vivo effects of cortisol on progesterone action were also examined. The inhibition by progesterone of replenishment of the rat uterine estrogen receptor was used as a bioassay for progesterone. Simultaneous injections of cortisol and progesterone resulted in greater inhibition than injections of progesterone alone. This potentiation by cortisol was dose dependent. We postulate that the potentiation is due to an increase in the level of unbound plasma progesterone because of its displacement from plasma proteins by cortisol.

In the determination of equilibrium constants for the binding between steroids and proteins, it is generally recognized that equilibrium dialysis is the most accurate method (King and Mainwaring, 1974a). For routine determinations or large numbers of samples, however, dialysis becomes unwieldy, and thus many investigators employ nonequilibrium methods (e.g., methods employing DCC,[1] gel filtration, or hydroxylapatite) to remove free from bound steroid. These methods, though quicker and simpler than equilibrium dialysis, result in a loss

† From the Department of Veterinary Physiology and Pharmacology, The Ohio State University, Columbus, Ohio 43210. *Received January 24, 1977.* Supported by a research grant HD-08576 from the National Institute of Child Health and Human Development.

[1] Abbreviations used: K_D, equilibrium dissociation constant: E_2, estradiol-17β; ER, the complex between estradiol-17β and its receptor; DCC, dextran-coated charcoal; CBG, corticosteroid binding α-globulin; R 5020, 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione; sc, subcutaneously; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; TE buffer, 0.01 M Tris-HCl-0.0015 M EDTA (pH 7.4); TTG buffer, 0.05 M Tris-HCl-25% glycerol-2.4 mM thioglycerol-0.02% sodium azide-1 mM phenylmethanesulfonyl fluoride (pH 7.4).