Isolation of Aldosterone-Stimulating Factor (ASF) and Its Effect on Rat Adrenal Glomerulosa Cells In Vitro

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SUMMARY A protein fraction has been isolated from normal human urine which upon chronic administration produced hypertension in rats. The hypertension is associated with retention of sodium and increased circulating aldosterone. The protein fraction has been purified to homogeneity, and its molecular weight has been determined to be 26,134 daltons by equilibrium ultracentrifugation. The compound has been identified to be clearly different from ACTH, angiotensin II, and β-lipotropin. It stimulated aldosterone production from rat glomerulosa cells in vitro in a dose-dependent fashion from $10^{-5}$ to $10^{-7}$ M with a maximum stimulation at $10^{-7}$ where a fourfold increase was obtained during 2 hours of incubation. Removal of some carbohydrate moieties by insoluble neuraminidase caused a twofold increase in aldosterone production in vitro. The protein fraction has been named “aldosterone-stimulating factor” or “ASF.” Further studies are in progress to define its physiological role. (Hypertension 3: 4-10, 1981)

Key Words • aldosterone • purification • glycoprotein • insoluble neuraminidase • sialic acid • molecular weight

W E HAVE described previously the existence of a protein fraction in normal human urine that produced hypertension in rats when injected for 10 days. The hypertension was associated with sodium retention, expansion of plasma volume, and increased circulating aldosterone, all of which suggested an adrenal-mediated mechanism. This paper describes isolation of the protein, tests of its physical homogeneity by electrophoresis and ultracentrifugation, its immunochromical purity by double immunoinfusion (Ouchterlony technique), and its biological activity by effect on aldosterone production from rat adrenal glomerulosa cells in vitro. Because of its unique features, we would like to call the protein “aldosterone stimulating factor (ASF).”

Materials and Methods

Purification of ASF

The starting material for purification was prepared as described earlier. Throughout the purification procedure, a cocktail of bactericidal- and protease-inactivating agents (n-ethylmaleimide, 5 mM; phenylmethylsulfonylfluoride, 2 mM; and NaN₃, 1 mg/ml) were added to all solutions at points of storage to prevent degradation of the protein, except where used for test of biologic activity. The purification was achieved by the steps described below.

Affinity Chromatography

ConA-sepharose 4B (obtained from Pharmacia Fine Chemicals) as labeled contained 10 mg of ConA/g of sepharose 4B. It was equilibrated with buffer (15 vols) containing 10 mM NaCl, 10 mM sodium acetate, 1 mM magnesium chloride, and 1 mM calcium chloride at pH 6.0 and 5°C prior to use. The protein was dialyzed against the same buffer prior to application to the column (1 ml of protein/ml gel bed), and eluted with 0.01 M alpha-methylmanoside added to the buffer at 5°C. Elution profiles were monitored by optical absorbancy at 280 nm and by tests of ASF activity. Active fractions, which coin-
cided with the absorbancy profile, were pooled and concentrated by ultrafiltration (Amicon × 10,000). This fraction was then dialyzed against neutral 0.05 M NaCl containing the inhibitors.

**Gel Filtration**

Concentrates of the protein collected from ConA-Sepharose columns were chromatographed on columns of Sephadex G-50 as an additional test of monodispersity prior to analysis by ultracentrifugation, 1 ml being applied to a 1.2 × 20 cm column equilibrated with 0.05 M NaCl, and inhibitor cocktail being added to portions kept for physical studies.

**Polyacrylamide Gel Electrophoresis**

Discontinuous polyacrylamide gel electrophoresis was conducted using 7% gel and tris-glycine buffer at pH 8.5. The enzyme-treated ASF was layered in sucrose. At the end of electrophoresis, the gels were washed with distilled water, fixed in sulfosalicylic acid, stained with Coomasie blue R (0.2% aqueous), and excess stains were removed by mixture of acetic acid/ethanol/water (2:3:1) and, finally, by acetic acid (10%).

**Physical Characterization**

Ultracentrifuge studies were carried out using a Beckman Spinco Model E equipped with electronic temperature and speed controls and a photometric scanning optical system for analysis of homogeneity and molecular size of the protein. Sedimentation coefficients were determined at 60,000 rpm and 20°C, at protein concentration corresponding to 1.0 and 2.0 absorbancy units. The midpoints of the absorbancy and refractive index were taken as the position of the protein boundaries, and were plotted in the usual fashion.

Equilibrium centrifugation was performed in accordance with guidelines established by Yphantis for the meniscus depletion method. An operating speed of 44,000 rpm was selected on the basis of a predetermined value \( S_{20, w} = 2.65 S \) as being suitable for depletion of the protein near the meniscus in solutions of densities varying from 1.0 to 1.34 g/ml. Solutions of differing densities were prepared by mixing the protein solution containing 0.05 M NaCl with equal volumes of KBr solution ranging from 0.5 to 4.0 M. Analyses employing ultraviolet absorption were carried out with protein at initial absorbancy of 0.1 at 280 nmoles, and scans were performed with the photometer set at absorbance ranges of 0.2 and 1.0 full scale. Measurements in the smaller absorbance range were approximated on the basis of a 3-week interval, with complete Freund’s adjuvant for the first three injections and with incomplete Freund’s adjuvant for the rest of the immunization period (4 months).

**Preparation of Antibody**

Antibody against ASF was raised by immunizing a goat with 150 μg of ASF (in 0.25 ml saline), at 3-week intervals, with complete Freund’s adjuvant for the first three injections and with incomplete Freund’s adjuvant for the rest of the immunization period (4 months).

**Immunochromical Double Immunodiffusion**

Microscope slides were covered with melted agar-buffer (1.5% in phosphate-buffered saline, 0.05 M). The holes were sucked out with a 1 mm flat ground injection needle. The antigen (ASF) was applied to the center hole, and the antibody was applied to the outer holes. Immunodiffusion was performed in a moist chamber for 72 hours. After immunodiffusion, the agar was washed free of nonreacted antigen and antibody in 0.9% (W/V) aqueous NaCl, then desalted by washing with deionized water. The slide was dried and stained with Coomassie blue (0.2% aqueous solution) and destained with a mixture of ethanol/acetic acid/water (50:130:875 ml).

**Biological Methods**

**Preparation of Adrenal Cells**

Adrenal cells were prepared by the method described by Douglas et al. Both adrenals were removed; the capsules (60–80 per preparation) were immediately minced finely in 5 ml of Medium 199 without K⁺; 10 ml of Medium 199/collagenase solution was added (K⁺, 3.5 mEq; collagenase, 2 mg/ml; BSA, 2 mg/ml; and DNAse 0.5 mg/ml). The mixture was gassed with 95% O₂/5% CO₂ for 30 seconds and placed in a water bath at 37°C with shaking for 30 minutes in the same atmosphere. The tissue in collagenase suspension was removed and the tissue bits allowed to settle for 5 minutes. The supernatant was then drawn off using a syringe with polyethylene tubing. The cell suspension was passed through a double layer of gauze into a plastic tube (50 ml). Adrenal

\*N = molecular weight of the protein; \( \varphi \) = partial specific volume; \( \rho \) = density of the solution; \( \omega \) = angular velocity; \( r \) = distance from the center of rotation; \( \nu \) = partial molal volume.
cells were harvested (900 rpm at 5°C for 10 minutes) and suspended in Medium 199 containing K⁺, 3.5 mEq; BSA, 2 mg/ml; and DNAse, 0.5 mg/ml. They were then sedimented again, and resuspended in incubation medium containing K⁺ (5 mEq/ml) and BSA (2 mg/ml). The cells were then counted after dye exclusion using Trypan blue (4%).

**Incubation of Adrenal Cells with AII or ASF**

The cell suspension was dispensed into plastic tubes at a level of 10⁷ cells/0.9 ml of incubation medium (Medium 199), K⁺ (-5 mEq) and BSA (-2 mg/ml). Either AII or ASF was added in doses ranging from 10⁻⁷ to 10⁻⁴ M in triplicate for each dosage. They were incubated under 95% O₂ and 5% CO₂ for 120 minutes at 37°C with slow shaking (100 strokes/min). Concentrations (Molar) of ASF or AII were prepared from lyophilized materials. Aldosterone produced at the end of the incubation was then determined by radioimmunoassay.

**Incubation of ASF with Neuraminidase**

Insolubilized neuraminidase (Sigma Chemical Company) was used to remove sialic acid from the protein. The insoluble enzyme preparation was filtered and washed twice with water, and then washed with acetate buffer (0.01 M) at pH 5.0, and suspended at a concentration of 5 mg/ml in the buffer. A known aliquot (0.5 or 1 ml) was then added to 1.5 ml of ASF in 0.05 M acetate buffer, pH 5.0. The mixture was allowed to incubate for 10 minutes at 37°C with mild shaking. The enzyme was then removed by centrifugation. The supernatant was collected, and the effect of the treatment was assessed by: 1) in vitro assay of ASF activity; 2) polyacrylamide gel electrophoresis; and 3) analytical gel filtration through Sephadex G-75.

**Statistical Analysis**

The data are presented as mean ± SE. Statistical analysis was done by Dunnet's test.

**Results**

Details of prior purification up to affinity chromatography involved benzoic acid adsorption, DEAE cellulose chromatography, and gel filtration as published previously. The partially purified material showed a single band on polyacrylamide gel electrophoresis; and the molecular weight was on the order of 800 daltons. The nonsedimentable component was found to increase at the expense of high molecular weight component on standing at room temperature, and therefore appeared to arise from degradation, conceivably from a bacterial enzyme. Further, the low molecular component was found to have no aldosterone-releasing activity as assessed by assays on the adrenal cell preparations, while the major nonpermeating component retained much of the original activity. This observation necessitated renewed preparation and further purification of the compound under conditions preventing degradation by addition of proteolytic enzyme inhibitors.

ConA-sepharose affinity chromatography was selected for further purification because of earlier indications that the protein contained carbohydrate. All but about 15% of the protein was absorbed by the ConA, and approximately 65-70% of the protein applied to the column was recovered on elution with α-methylmanoside. The nonabsorbed fraction possessed no ASF activity, and the eluted fraction possessed full activity of the starting material, in that it induced a fourfold increase in aldosterone production/2 hrs in vitro when added in a dose of 10⁻⁷ M (dry wt/m.w.) to collagenase-dispersed adrenal glomerulosa cells. When the ConA-purified protein was subjected to gel filtration it eluted as a single peak in anticipated position corresponding to 25,000 daltons, but only when stored with protection of the inhibitor cocktail.

With either freshly purified or "protected" protein, ultracentrifugation showed a single symmetrical peak on sedimentation at 60,000 rpm, and with exception of slight uncertainty in the baseline all of the ultraviolet-absorbing material sedimented in position coincident with the Schlieren boundary at a rate of s₂₀,₅₀ = 2.65S. The sedimentation rate was on the order of magnitude expected for a protein of about 2.5 × 10⁶ daltons, as had been suggested by the gel filtration and ultrafiltration used in purification. To obtain a more precise value of the molecular weight, the solution was analyzed by equilibrium ultracentrifugation under conditions of meniscus depletion. In this method of analysis, the centrifuge is operated at a speed just sufficing to deplete the protein from solution near the meniscus and cause it to accumulate at the bottom of the cell until equilibrium is obtained between diffusion and sedimentation. At equilibrium, an exponential gradient characteristic of the molecular size of each sedimentable component is set up at the bottom of the cell.

When components of varying size exist, logarithmic plots of the concentration of protein vs distance squared become curvilinear. As indicated by examination with both Rayleigh interference and ultraviolet absorbance optics after establishment of equilibrium in solutions of density ranging from 1.0 to 1.4 g/ml, the protein was found to be homogeneous (fig. 1) in that no curvilinearity of the plots were indicated as tested statistically by the method of orthogonal polynomials. A high degree of homogeneity was also indicated by observation that the slopes of the logarithmic plots decreased in a
FIGURE 1. Schlieren pattern formed by sedimentation of the purified protein after 7.5 and 35 minutes at 60,000 rpm. Solvent: 0.05 M NaCl and 5 mM TRIS-HCl at pH 7.4, temp. 25°C. The sedimentation rate corresponded to a coefficient (s_m) of 2.65.

highly linear fashion with increasing density, as though the protein was not only uniform in size but also uniform in density. Extrapolation of the slope vs density plot to the intercept corresponding to zero slope (where density of the protein can be assumed to equal that of the medium) gave a value of 1.32 g/ml. The inverse of this density corresponds to a value of 0.756 for the partial specific volume (v), a value needed to obtain the buoyancy term (1 - v) in the sedimentation equation

$$M = \frac{2RT}{(1 - v)\omega^2} \cdot \frac{2.303d (\log C)}{dr^2}$$

From the estimated v, the conditions of centrifugation and the slopes

$$\frac{d \log A}{dr^2} = \frac{d \log C}{dr^2}$$

of the plots in figure 2, a weight average molecular weight of 26,189 ± 184 (SEM) was calculated. A similar value of 26,569 was obtained from measurements of concentration gradient with the interference optical system in an analysis employing 1% KBr as solvent and 40,000 rpm as operating speed.

Immonochemical Double Immunodiffusion

A typical immunodiffusion pattern of ASF and its antibody is shown in figure 3. As shown, one single

FIGURE 2. Logarithmic plots of the absorbance gradients formed by equilibrium ultracentrifugation of the purified protein in KBr solutions of varying density. The slopes of the plots when multiplied (2 × 2.303RT/ω²) give the reduced (buoyed) weight-average molecular weights of the protein as distributed throughout the gradient, and the degree of homogeneity of the protein is indicated by the linearity of the plots. Intercepts of the plots vary because of differences in total protein employed in the five analyses, two different preparations of the protein being used. The graph in the inset shows the values of the slopes plotted as a function of the solution density, which on extrapolation to zero slope yields the isopycnic density ($\rho_{iso} = 1.326 = 1/v$). By using the apparent v, an average value of 26,189 ± 184 (SEM) daltons was calculated from the five sets of measurements (rpm = 44,011, T = 287°C).

FIGURE 3. A typical immunodiffusion pattern of aldosterone-stimulating factor (ASF) and its antibody. Center well = antigen (ASF); outer wells = antibody, clockwise from right. Dilutions = 1:16, 1:10, 1:10, 1:4, 1:5. Stain = Coomassie blue. For detail see text.
precipitin line was obtained, suggesting the immunological homogeneity of ASF. After using a preimmunization plasma from the same goat, no precipitin line was obtained, nor, after using a similar technique, did the antibodies against ASF crossreact with ACTH, angiotensin II, renin, or β-lipotropin. This showed that ASF is immunochemically different from these known aldosterone secretogogues.

Effect of ASF and AII on Dispersed Glomerulosa Cells

The effect of AII on dispersed glomerulosa cells is shown in figure 4. When AII was added in doses of $10^{-10}$ to $10^{-6}$ M, it stimulated aldosterone production in vitro in a dose-dependent fashion with a maximum stimulation at $10^{-7}$ M, where a fourfold increase over control production was noted. Each point represents mean ± SEM of 15 tubes. The effect of ASF on adrenal cells is shown in figure 4. It stimulated aldosterone production in a dose-dependent fashion from $10^{-9}$ to $10^{-4}$ M with a maximum at $10^{-7}$ M, where a fourfold increase was noted during a 2-hour incubation period. This showed that ASF also stimulated aldosterone production from glomerulosa cells in vitro.

Effect of Neuraminidase on ASF

The neuraminidase treatment caused major changes in electrophoretic mobility of ASF. A typical polyacrylamide gel electrophoresis pattern is shown in figure 5. As shown, a significant change in mobility of ASF along with appearance of two other minor bands was noted. This suggested that neuraminidase treatment caused certain modifications of previously homogeneous ASF molecule.

When the neuraminidase-treated ASF was passed through a Sephadex G-75 column, three ultraviolet-absorbing materials (280 nmoles) were obtained. A typical fractionation pattern is shown in figure 6. A major component (A) and two minor components (B and C) were pooled separately and their biological activities were determined from the effect of aldosterone production in vitro by adrenal glomerulosa cells (table 1). A two-fold increase in aldosterone production was found in Fraction A ($47 ± 5.6$ ng/ml/2 hrs vs $23.5 ± 4$ ng/ml/2 hrs) compared to untreated ASF ($p < 0.001$) when added in equiweight concentrations (ASF - $10^{-7}$ M). The other two fractions, B and C, were inactive, i.e., the same as the cell blank.

Discussion

This study has demonstrated that a protein fraction isolated from normal human urine has been purified to homogeneity and has then stimulated aldosterone biosynthesis in vitro. This compound has been identified to be different from ACTH, AII, and β-lipotropin, the other known aldosterone secretogogues by immunochemical diffusion and by using specific blockers for AII and ACTH. Furthermore, unlike ACTH, its steroidogenic activity is not associated with increases in cyclic AMP. Its activity is unaffected by guest on April 12, 2017 http://hyper.ahajournals.org/ Downloaded from
by a specific antagonist of AI1, suggesting that it acts as a receptor site separate from AI1. It has accordingly been named "ASF."

The ultracentrifuge studies not only confirm purity and homogeneity indicated by the electrophoretic analysis, but also help to identify and eliminate a degradation process that we suspect arose from bacterial enzymes.

The high degree of homogeneity indicated by ultracentrifuge studies on the material purified in the presence of enzyme inhibitors offers hope that a precise chemical characterization can now be carried out without complication. It appeared homogeneous with respect to both molecular size and density. Although densities of simple proteins do not usually deviate much from values near that indicated by ultracentrifugation of this material, glycoproteins with high sugar content tend to have much higher densities. Binding of the protein to ConA-sepharose, its alteration by neuraminidase, and qualitative tests of sugar content indicate that the material is glycoprotein. The present assessment of its density, thus, can be taken as tentative evidence that the sugar content is uniform and moderately low.

We have reported earlier1 that ASF produced hypertension in normal rats when injected chronically for 7–10 days. The hypertension was accompanied by Na⁺ retention, expansion of plasma volume, and increased circulating aldosterone. The present study provides direct evidence for an effect of the compound on adrenal cortex to stimulate aldosterone production.

One important property of this compound is the stimulation of aldosterone production in vitro when added to isolated rat adrenal glomerulosa cells in a dose-dependent fashion (fig. 4). Further application of this in vitro assay system will greatly simplify further study.

Removal of some carbohydrate moieties by neuraminidase resulted in a twofold increase of aldosterone production by ASF in vitro (table 1). Similar increase in in vitro biological activity by another hormone (e.g., erythropoietin) after removal of sialic acid has been reported.10 The reason for the appearance of two small ultraviolet-absorptive material is not clear. One possibility is that the neuraminidase used may have been contaminated with some other proteolytic enzyme, which may have caused some nonspecific degradation of the protein.

Four humoral factors have been shown to play important roles in the regulation of aldosterone secretion. These are ACTH, potassium, sodium, and AI1. In recent years, indirect evidence from animal11-13 and human studies14 suggests the presence of an "unidentified" hormone that stimulates aldosterone production. Of particular relevance is the observation of McCaa et al.15 that hypophysectomy eliminates the increase in plasma aldosterone response of bilaterally nephrectomized dogs subjected to the stimulus of hyponatremia. Further, neither the administration of HGH or ACTH, either alone or together, led to return of the plasma aldosterone response to that seen in nonhypophysectomized animals. The studies implicate the existence of another factor, perhaps originating from the pituitary gland that controls aldosterone production. This new compound isolated from human urine appears to be a good candidate for such a factor. Further studies are now in progress to define the site and mode of action and to determine the source and mode of regulation.

![Figure 6](attachment:figure6.png)

**FIGURE 6.** A typical elution pattern of aldosterone-stimulating factor (ASF) before and after neuraminidase treatment. Left: Before treatment. Right: After treatment. Sephadex G-75 was used for gel filtration and phosphate buffer (0.01 M), pH 7.0, was used for elution.

**TABLE 1.** Aldosterone Production by Aldosterone-Stimulating Factor (ASF) After Treatment With Neuraminidase

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aldosterone production (ng/ml/2 hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells alone (100,000)</td>
<td>7.5 ± 3</td>
</tr>
<tr>
<td>ASH (10⁻⁷ M)</td>
<td>23.5 ± 4</td>
</tr>
<tr>
<td>ASF after neuraminidase treatment</td>
<td></td>
</tr>
<tr>
<td>Fraction A (10⁻⁷ M)</td>
<td>47 ± 5.6*</td>
</tr>
<tr>
<td>Fraction B</td>
<td>4.5 ± 3</td>
</tr>
<tr>
<td>Fraction C</td>
<td>6.2 ± 2</td>
</tr>
</tbody>
</table>

*P < 0.001.

ASH = aldosterone-stimulating hormone; ASF = aldosterone-stimulating factor.
Acknowledgment

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References

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