Activities of ACTH-Potentiating Substances Isolated from Rat Serum on Steroidogenesis in Isolated Rat Adrenal Cells

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Abstract—Peptides that potentiate the response of adrenal cells to ACTH1-24 were isolated from rat serum. ACTH-potentiating activity was found in fractions of 9,000-40,000 in molecular weight (APS-Fr) and of smaller molecular weight (SM-Fr) on Sephadex G-100 gel filtration of the serum extract. The peptides were isolated from APS-Fr by preparative acid polyacrylamide gel electrophoresis and named d1, d, d2, e, f and g. Their molecular weights, estimated by Sephadex G-75 gel filtration, were 41,000, 33,000, 24,000, 17,500, 17,500 and 16,000, respectively. All of these peptides were glycopeptides. The isoelectric point of peptide d was 8.45 and some of the others, such as f and g, were more basic. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis revealed that these peptides were decomposed into various fragments. ACTH-potentiating activity was highest in peptide d1 and lowest in peptide e. The maximum activity of peptide d was observed at 3 x 10^-8 M when steroidogenesis was induced by 9 x 10^-12 M ACTH1-24. While these peptides did not show any ACTH-like activity at any stage of isolation, the fractions of these peptides eluted from a Sephadex G-75 column showed more or less ACTH-like activity. These peptides therefore seemed to possess latent ACTH-like activity. The molecular weight of SM-Fr was smaller than ACTH1-24. The ACTH-potentiating activity of SM-Fr was low, and SM-Fr did not show any ACTH-like activity. SM-Fr therefore seems to be the smallest structure which has ACTH-potentiating activity. The similarity of these peptides to proopiomelanocortin-related substances was discussed.

ACTH1-24-induced steroidogenesis in isolated rat adrenal cells is potentiated by the extract obtained from ACTH-free rat serum by the OUSO G-32 adsorption method (1). On Sephadex G-100 gel filtration of the serum extract, most of the ACTH-potentiating activity is found in the fractions from 9,000 to 40,000 in molecular weight (APS-Fr). A small portion of the activity is also found in the range of smaller molecular weights (SM-Fr) (1). The potentiating substance in the serum may be produced mainly by the pituitary because the potentiating activity in the serum is decreased markedly by hypophysectomy (1). Furthermore, it has been suggested that APS-Fr affects ACTH-induced steroidogenesis in the adrenal cells at a step between the binding of ACTH to its receptor and the formation of cyclic AMP (2).

Since APS-Fr is a combination of fractions ranging widely in molecular weight as described above, it seems likely that APS-Fr comprises some substances which have ACTH-potentiating activity. An attempt was therefore made to separate these substances. In this study, several peptides which had ACTH-potentiating activity were isolated from the APS-Fr, and the activities of these peptides were examined.

Materials and Methods
Male adult rats of the Donryu strain were used. Trunk blood was collected by decapitation and centrifuged to obtain the serum, which was then stored at -12°C until use. Remaining ACTH activity in the
serum was eliminated by thawing the serum and leaving it to stand at room temperature before use. Siliconized glassware, and plastic tubes and pipettes were used throughout the experiments.

**ACTH preparations:** ACTH$_{1-24}$ (Cortrosyn, M.W. 3,294) was purchased from Daiichi Pharmaceutical Co. The Third International Working Standard (5 subcutaneous units or 1.5 intravenous units/approx. 50 μg/vial) (3) was used for porcine ACTH$_{1-39}$ (M.W. 4,567). Each μU (intravenous unit) of this standard ACTH has a potency equivalent to approx. 10 pg of synthetic β$_{1-39}$ porcine ACTH, when assayed by the isolated adrenal cell method (4).

Preparation of isolated rat adrenal cells and measurement of ACTH-potentiating activity have been described previously (1, 2).

**Measurement of protein content:** The method of Lowry et al. (5) was used on a micro-scale, the final volume being 0.5 ml. The amounts of all the samples were expressed in terms of protein content.

**Isolation of ACTH-potentiating substance from rat serum:** Serum extract having ACTH-potentiating activity was prepared from rat serum by the QUSO G-32 adsorption method (1). The maximum ACTH-potentiating activity was observed with an amount of the serum extract corresponding to 500 μl of rat serum (1). The serum extract was partially purified by Sephadex G-100 gel filtration. ACTH-potentiating activity was found in fractions 24-29 (APS-Fr) and fractions 36-39 (SM-Fr) (1). Each of these fractions was combined and concentrated on a Diaflo membrane (YM-2, Amicon Co.) and lyophilized. The maximum ACTH-potentiating activity was observed with 5 μg of APS-Fr corresponding to 110 μl of rat serum (2). ACTH-potentiating substance was isolated from the lyophilized APS-Fr by preparative acid polyacrylamide gel electrophoresis. The electrophoresis was carried out on a slab gel, measuring 11.3 x 13.8 x 0.2 cm, by the method of Reisfeld et al. (6). The separating gel was 15% acrylamide and its length was 10.5 cm in a total length of 11.3 cm. After a current of 40 mA per gel was run for 20 min, 2.5-2.7 mg of APS-Fr was dissolved in 300 μl of 40% sucrose in the electrophoretic buffer and applied to the stacking gel. Electrophoresis was then carried out with a current of 40 mA per gel for 6 hr. The middle part of the gel was cut vertically with a width of 6 mm and stained with Coomassie brilliant blue solution (2.5 g of Coomassie Brilliant Blue R250, 227 ml of methanol, 46 ml of acetic acid, and water up to 500 ml) for 30 min. It was then destained with 10% ethanol in 7% acetic acid (destaining solution). From the stained gel strip, positions of the peptide bands were determined. The part of the gel corresponding to the band of each peptide was cut out horizontally to form a strip of gel. Each gel strip was cut to 1-1.5 mm$^2$, placed in 10 ml of 0.15% acetic acid and left to stand overnight at 4°C. The extract was then transferred to a plastic centrifuge tube, and extraction was repeated three times. The combined extract was centrifuged to remove small fragments of the gel and concentrated on a Diaflo membrane. About 60% of the amount of APS-Fr used for the electrophoresis was recovered as the sum of the isolated peptides. To determine the purity of the isolated peptides, acid polyacrylamide gel electrophoresis (6) was carried out on micro-slab gel, measuring 5.0 x 8.5 x 0.1 cm, and run at a current of 20 mA for 110 min.

**Sephadex G-75 gel filtration:** The concentrated extract of each isolated peptide was lyophilized and subjected to Sephadex G-75 gel filtration. The peptide was applied to a column (1 x 55.5 cm) equilibrated with 0.2 M ammonium acetate buffer, pH 4.5. The elution was carried out at a flow rate of 3.25 ml/hr at 4°C, and 1-ml fractions were collected. The eluate was monitored for ultraviolet (UV) absorbance at 280 nm (Mini-UV monitor II, Atto Co.). Egg albumin (M.W. 45,000, Sigma Chemical Co.), soybean trypsin inhibitor (M.W. 21,500, Sigma Chemical Co.), cytochrome C (M.W. 12,384, Sigma Chemical Co.) and ACTH$_{1-24}$ were used as molecular weight markers. The relative elution volume, $V_e/V_o$, was used as a function of the molecular weights, where $V_e$ is the elution volume of each peptide and $V_o$ is the void volume of the gel column.

**SDS polyacrylamide gel electrophoresis:** The electrophoresis was carried out on
micro-slab gel using a method based on that of Swank and Munkres (7). Acrylamide gels contained 0.1% sodium dodecyl sulfate (SDS), 0.075% N,N,N’,N’-tetramethyl-ethylenediamine (TEMED), 0.07% ammonium persulfate, 0.1 M H₃PO₄, 8 M urea, and Tris (hydroxymethyl)methylamine (Tris) at a final pH of 6.8. The separating gel was 12.5% and the stacking gel was 5% (acrylamide : N,N’-methylenedisacrylamide (bis)=10:1). The peptide marker (M.W. range 2,512–16,949, BDH Co.) or each peptide isolated from the acid polyacrylamide gel was dissolved in a solution containing 1% SDS, 8 M urea, 1% 2-mercaptoethanol and 0.01 M H₃PO₄ adjusted to pH 6.8 with Tris, and heated at 60°C for 10 min. After a current of 15 mA per gel was run for 20 min, the samples were applied. Electrophoresis was carried out with a current of 15 mA per gel until the bromophenol blue (BPB) marker reached the separating gel and then electrophoresed with 30 mA per gel. The gel was stained for 20 min and then destained. Molecular weight was estimated from the mobility (distance moved by the peptide/distance moved by BPB) of the peptide using a calibration curve for molecular weight markers which was obtained by plotting log₁₀ of molecular weights of the marker standards against their electrophoretic mobilities.

**Concanavalin-A affinity chromatography:**
A concanavalin-A agarose (Marzen Petroleum Co.) column was prepared in a Sepacol mini-column (Seikagaku Kogyo Co.) with silicon-rubber tubing and a stopper under the column. The bed volume was 0.5 ml and the dead space 0.5 ml. Chromatography was carried out on the basis of the method of Orth and Nicholson (8). The column was equilibrated with 4 ml of 0.1 M ammonium acetate solution, containing 1 mM CaCl₂, 1 mM MgCl₂, 1 mM MnCl₂ and 0.1% bovine serum albumin (BSA). Each lyophilized peptide was dissolved in 1 ml of this buffer and applied to the column. Molecular weight was estimated from the mobility (distance moved by the peptide/distance moved by BPB) of the peptide using a calibration curve for molecular weight markers which was obtained by plotting log₁₀ of molecular weights of the marker standards against their electrophoretic mobilities.

**Isoelectric focusing:** Isoelectric focusing (IEF) was performed in a slab gel measuring 11×11×0.1 cm, in a flat-bed multielectrophoretic chamber, using a constant wattage power supply (Atto Co.). One milliliter of polybuffer 96 (pH 6–9, Pharmacia Fine Chemicals), 2.0 ml of glycerol and 4.5 ml of water were added to 7.5 ml of the stock solution which consisted of 9.7% acrylamide and 0.3% bis. The mixed solution was then thoroughly de-aerated and 0.1 ml of 0.1 M ammonium persulfate and 5 μl of TEMED were added. The gel solution was then poured into the casting assembly. After polymerization, the glass plates were removed and the gel slab, adhering to Gelbond film, was aged overnight at 4°C. The anolyte buffer was 0.25 M Hepes and the catholyte buffer was 1 M NaOH. A pH Calibration Kit (pH 3–10, Pharmacia Fine Chemicals) was used as an isoelectric point marker. After prefocusing at a constant wattage of 5 W for 30 min at 5°C, the marker and samples were applied. IEF was carried out at a maximum wattage of 8 W and a maximum voltage of 2000 V for 2 hr at 5°C. The gel with Gelbond film was fixed overnight in an aqueous solution of 10% triflicarboxylic acid and 5% sulfoacetic acid (fixing solution). The gel was then stained with Coomassie blue solution diluted with an equal volume of the fixing solution. A mixture of equal volumes of the destaining solution and the fixing solution was used for the destaining to prevent the escape of peptides from the gel.

**Results**
ACTH-potentiating activity in the isolated peptides: About ten peptide bands were revealed by the acid polyacrylamide gel electrophoresis. Figure 1 shows a typical stained gel strip. These stained peptide bands were named a, b, c, … i from the top of the
These peptides were extracted from the resulting gel as described previously. ACTH potentiating activity was found in the peptides of d1, d2, e, f and g as shown in Fig. 2. Peptide d1 had the highest activity and e had the lowest. As the bands d1 and d2 were often very adjacent and looked like one band, they were treated as one band in some experiments; this band was represented by d. The purity of the isolated active peptides was determined by micro acid polyacrylamide gel electrophoresis. As shown in Fig. 3, each peptide showed a single band corresponding to each band of APS-Fr. Figure 4 shows a dose-response relationship for peptide d. The range between minimum and maximum effective doses was narrow. The ACTH-potentiating activity of peptide d reached maximum at about 500 ng in 0.5 ml incubation medium. Figure 5 shows the effects of peptides d and f on the steroidogenesis of adrenal cells in response to various concentrations of ACTH1-24. The ED50 was

estimated from the figure, taking into account empirically the fact that the maximum response to ACTH1-24 alone should be the same as that with addition of peptide d or f (2), although the response to ACTH1-24 alone had not reached maximum at that point. The ED50 for ACTH1-24 was approx. $1.5 \times 10^{-10}$ M, but in the presence of peptide d, it decreased to $8.4 \times 10^{-12}$ M, and in the presence of f, it decreased to $1.1 \times 10^{-11}$ M. The correct ED50 for ACTH1-24 seems to be somewhat lower than that described above, because the response of the cells should be increased in further incubation at relatively higher doses of ACTH1-24. Even when this correction was taken into account, the affinity of the adrenal cells to ACTH1-24 was increased over 10 times by the addition of peptide d or f. When ACTH1-39 was used in place of ACTH1-24, the affinity of the adrenal cells to ACTH1-39 was increased only twice by the addition of d (Fig. 6). Figure 7 shows a comparison of the log-dose response curves for ACTH1-24 and ACTH1-39. The relation of the curve for ACTH1-39 to the curve for ACTH1-24 (Fig. 7) was similar to the relation of the curve for ACTH1-24 in the presence of peptide d or f to the curve for ACTH1-24 (Fig. 5). Comparison of the results in Figs. 5, 6 and 7 suggests the presence of a contaminating ACTH-potentiating substance in ACTH1-39.

**Estimation of molecular weights of the isolated peptides:** Molecular weights of the peptides isolated from the acid polyacrylamide gel were estimated by Sephadex G-75 gel filtration. Each peptide showed a single peak of UV absorption on gel filtration. The molecular weights of these peptides, estimated from the calibration curve for molecular weight markers, were as follows: d1, 41,000; d, 33,000; d2, 24,000; e, 17,500; f, 17,500; g, 16,000 (Fig. 8). The alternative method of estimating the molecular weights, SDS polyacrylamide gel electrophoresis, was applied to the isolated peptides, and it was revealed that these peptides were dissociated into various fragments by the SDS treatment. Table 1 shows the apparent molecular weights of these fragments estimated from their electrophoretic mobilities on the gel. As can be seen in the Table, the pattern of
Table 1. Apparent molecular weights of the fragments of isolated peptides on SDS polyacrylamide gel electrophoresis

| Sample | Molecular weight (10^3) | ACTH-potentiating activity |
|--------|-------------------------|-----------------------------|
|        |                         |                             |
| APS-Fr (8)* | 22.6(4)**     | 14.7(8)                     | 12.6(8)          | 10.5(8)          | 8.5(8)          | 6.6(8)          | 4.6(8)          | 3.2(8)          | +               |
| Peptide a (6) |                         | 11.3(8)                     | –                |
| b (8)   |                         | 14.5(5)                     | 10.6(8)         | –                |
| c (9)   |                         | 10.4(9)                     | –                |
| d₁ (5)  |                         | 15.1(1)                     | 13.2(1)         | 8.3(5)           | 4.3(5)           | +               |
| d (9)   | 22.8(1)                 | 15.5(2)                     | 13.3(9)         | 7.9(9)           | 4.4(9)           | +               |
| d₂ (5)  |                         | 13.6(5)                     | 8.3(5)          | 4.4(5)           | +               |
| e (9)   |                         | 13.4(9)                     | 9.5(3)          | 7.5(9)           | 6.0(5)           | 4.5(9)           | +               |
| f (12)  | 15.7(3)                 | 12.2(8)                     | 7.5(12)         | 4.7(12)          | 2.8(3)           | +               |
| g (12)  |                         | 12.8(9)                     | 7.5(12)         | 6.3(12)          | +               |
| h (9)   |                         | 12.5(9)                     | 7.3(9)          | –                |
| i (4)   |                         | 12.6(4)                     | 7.2(4)          | 6.2(3)           | –                |

*Number of electrophoreses. **Number of visually detected bands. Average molecular weight is shown. One—two micrograms of protein of each sample was used for the electrophoresis.
Fig. 2. ACTH-potentiating activity of the peptides isolated from APS-Fr by preparative acid polyacrylamide gel electrophoresis. Each extract from the gel was concentrated, and the protein content in the concentrated extract was determined. The volumes of the concentrated extracts of isolated peptides and APS-Fr corresponding to the amounts shown in the figure and the same volumes of 0.15% acetic acid as a control were evaporated to dryness. Dried samples were incubated with $1.2 \times 10^5$ cells in the presence or absence of ACTH$_{1-24}$ for 90 min at 38°C. Total volume was 0.5 ml. Concentration of ACTH$_{1-24}$ was $9 \times 10^{-12}$ M. Each column represents the mean of values obtained from duplicate incubations. Each point represents each value in duplicate incubations. The value obtained from the incubation with the cells and each peptide was almost the same as the basal value obtained from the incubation with the cells alone. These values are not shown in the figure.

Fig. 3. Examination of the purity of the isolated peptides having ACTH-potentiating activity by micro acid polyacrylamide gel electrophoresis. Two micrograms (as protein) of each peptide or 10 μg (as protein) of starting material (APS-Fr) in 5 μl of 40% sucrose in the electrophoretic buffer was subjected to electrophoresis.
the dissociation was characteristic for each peptide. Some of these fragments were common to many peptides. However, these fragments were found not only in ACTH-potentiating peptides but also in inactive peptides.

ACTH-potentiating activity in fractions of concanavalin-A affinity chromatography of the isolated peptides: Specific binding of the isolated peptides on a concanavalin-A (Con-A) agarose gel column was examined with the elution pattern of ACTH-potentiating activity from the column. As shown in Fig. 9, ACTH-potentiating activity of peptide d, and d₂ was found in all of the excluded-washed, eluted and purged fractions on Con-A affinity chromatography. The potentiating activity of peptide e was found in the purged fraction and the activities of peptides f and g were found in the eluted and purged fractions. ACTH-potentiating activity was found in the eluted and purged fractions or purged fraction in all these peptides and there were no peptides in which the potentiating activity was found only in excluded-washed fraction on Con-A affinity chromatography. Therefore, all these peptides may be glycopeptides.

ACTH-potentiating activity of peptide e was found only in the purged fraction, and the activity in the other peptides was also found in the purged fraction as well as in the other fractions. These results suggest that these peptides have a carbohydrate chain which binds tightly to Con-A agarose gel.

Isoelectric points of the isolated peptides: The isoelectric point of peptide d was 8.45, while those of peptides f and g were more basic, judging from the results of isoelectric focusing (Fig. 10).

Other ACTH-potentiating peptide: ACTH-potentiating activity was also found in the SM-Fr on Sephadex G-100 gel filtration, besides the APS-Fr (1). ACTH-potentiating activity of SM-Fr was much lower than that of the peptides isolated from APS-Fr, the potentiating activity of 16 μg of SM-Fr being lower than that shown by 2 μg of the isolated peptides (Fig. 11). The molecular weight of SM-Fr estimated by Sephadex G-75 gel filtration was smaller than that of ACTH₁₋₂₄. The fractions of SM-Fr from Sephadex G-75
gel filtration showed relatively low ACTH-potentiating activity and did not show any ACTH-like activity (data not shown).

**ACTH-like activity of the isolated peptides:**

The peptides, isolated by Sephadex G-100 gel filtration followed by acid polyacrylamide gel electrophoresis, showed only ACTH-potentiating activity, and they did not show any ACTH-like activity at any stage of their isolation. However, after the Sephadex G-75 gel filtration, ACTH-like activity was found in the resulting fractions. A typical example is shown in Fig. 12. ACTH-potentiating activity is observed as the difference between the steroidogenesis with ACTH$_{1-24}$ and peptide g, and the sum of the steroidogenesis with ACTH$_{1-24}$ (control activity) and the steroidogenesis with peptide g (ACTH-like activity). However, the ACTH-like activity of peptide g seems to consist of true ACTH-like activity and its potentiation. As can be seen in the figure, ACTH-potentiating activity was found in some fractions having low ACTH-like activity, while no clear ACTH-potentiating activity was evident in the fractions with high ACTH-like activity. However, ACTH-potentiating activity was clearly found in the diluted fraction which showed high ACTH-like activity (data not shown). Therefore, the fraction also seemed to have ACTH-potentiating activity besides ACTH-like activity, the potentiating activity probably being masked by the high ACTH-like activity. All the other peptides also showed greater or lesser degrees of ACTH-like activity after Sephadex G-75 gel filtration.
Fig. 6. Comparison of log-dose response curves for ACTH₁₋₃₉ in the presence or absence of peptide d. Twenty microliters (1.8 μg as protein) of the concentrated extract of peptide d and 20 μl of 0.15% acetic acid were evaporated to dryness. Each dried sample was incubated with 9.5x10⁴ cells and various concentrations of ACTH₁₋₃₉ for 180 min at 38°C. Total volume was 0.5 ml. Each point represents the mean of values obtained from duplicate incubations. The basal value (0.038 μg/0.5 ml) was subtracted for all the values in the figure. The value obtained from the incubation of the cells with peptide d was almost the same as the basal value. ED50 was estimated from the figure.

Discussion

Several peptides having ACTH-potentiating activity were isolated from rat serum by a series of procedures. At first, a serum extract possessing ACTH-potentiating activity was prepared from rat serum by the QUSO G-32 adsorption method (1). It was found that ACTH-potentiating substance in rat serum was produced mainly by the pituitary and that the substance was relatively stable in the serum (1). The serum extract was then partially purified by Sephadex G-100 gel filtration. The fractions in which ACTH-potentiating activity was found were named APS-Fr and SM-Fr (1). Finally, several peptides with ACTH-potentiating activity were isolated from APS-Fr by preparative acid polyacrylamide gel electrophoresis (Figs. 1, 2 and 3).

Five micrograms of APS-Fr in 0.5 ml incubation medium showed maximum ACTH-potentiating activity with 9x10⁻¹² M ACTH₁₋₂₄. However, the peptides isolated from APS-Fr showed the maximum activity with a much smaller amount. About 500 ng of peptide d in 0.5 ml (3x10⁻⁸ M) showed the maximum potentiating activity with the same concentration of ACTH₁₋₂₄ (Fig. 4).

Molecular weights of APS-Fr estimated by Sephadex G-100 gel filtration were in the range of 9,000–40,000 (1). However, the molecular weights of the active peptides isolated from APS-Fr were 41,000, 33,000, 24,000, 17,500, 17,500 and 16,000 (d₁, d, d₂, e, f and g, respectively) (Fig. 8). The smaller molecules possessing ACTH-potentiating activity in the APS-Fr seemed to be lost during acid polyacrylamide gel electrophoresis. On the other hand, these...
Fig. 7. Comparison of log dose response curves for ACTH$_{1-24}$ and ACTH$_{1-39}$. Various concentrations of ACTH$_{1-24}$ or ACTH$_{1-39}$ were incubated with $8.1 \times 10^4$ cells for 180 min at 38°C. Total volume was 0.5 ml. Each point represents the mean of values obtained from duplicate incubations. Basal value (0.053 μg/0.5 ml) was subtracted for all the values in the figure.

Fig. 8. Estimation of the molecular weights of the isolated peptides by Sephadex G-75 gel filtration. The relative elution volume, $V_e/V_o$, was used as a function of the molecular weights, where $V_e$ is the elution volume of the peptide and $V_o$ is the void volume of the gel column. Estimated molecular weights of the isolated peptides were as follows: $d_1$, 41,000; $d$, 33,000; $d_2$, 24,000; e, 17,500; f, 17,500; g, 16,000.
isolated peptides were decomposed into various fragments on SDS polyacrylamide gel electrophoresis. The molecular weights of these fragments were in the range of 2,800–23,000 (Table 1) and were very similar among all the peptides. This result suggests that the smaller peptides may be derived from the peptide having the largest molecular weight. It was not known which of the fragments on the SDS polyacrylamide gel had ACTH-potentiating activity, because some were common to both active and non-active peptides, but there was no fragment common to only active peptides (Table 1).

These peptides were glycopeptides as shown by Con-A affinity chromatography (Fig. 9). This result is in good agreement with the previous observation that peptide bands were stained with periodic acid-Schiff reagent on the SDS polyacrylamide gel after the electrophoresis of APS-Fr (1). The slight differences in molecular weight found among the common fragments might be due to the existence or absence of carbohydrate chains. These carbohydrate chains might play a role in the potentiating activity.
because on Con-A affinity chromatography, ACTH-potentiating activity was not found in the eluted fraction of peptide e which had the lowest activity, although the activity was found in the purged fraction. Other peptides having higher activity showed higher affinity to Con-A agarose gel (Fig. 9).

The marked migration of these peptides on acid polyacrylamide gel electrophoresis indicates that they are basic peptides, and the isoelectric points of these peptides estimated by isoelectric focusing were 8.45 or over.

Samples obtained at every stage in the isolation of these peptides from rat serum showed marked ACTH-potentiating activity and no ACTH-like activity (1, 2) (Figs. 2, 4, 5 and 11). However, after Sephadex G-75 gel filtration, these peptides did show ACTH-like activity (Fig. 12), each isolated peptide yielding a single peak of UV absorption during the filtration. Judging from
their molecular weights, estimated by the elution volumes at these peaks, the peptides did not seem to be broken down during the elution of the gel column. Also, these peptides did not seem to be contaminated with ACTH-like substance during the gel filtration. These peptides should therefore be considered to have intrinsic ACTH-like activity besides ACTH-potentiating activity, i.e., a result which suggests that a bioactive chemical structure similar to, or the same as that of the ACTH molecule, may be contained in the molecules of these peptides. A known substance which carries the ACTH molecule within its chemical structure is the precursor of ACTH, proopiomelanocortin (POMC, also referred to as pro-ACTH-β-LPH or pro-ACTH-β-endorphin, according to the cited references).

Nakanishi et al. (10) determined the primary structure of bovine ACTH-β-LPH precursor based on the nucleotide sequence of complementary DNA produced against mRNA coding for the precursor molecule. They showed that the molecular weight of the cell-free translation product of bovine ACTH-β-LPH precursor mRNA was 35K* (11) or 41K (12) depending on the system of electrophoresis used. The partial amino acid sequence of pro-ACTH-β-LPH of other species has been determined (13–21), and it is suggested that the pro-ACTH-β-LPHs of various species have very similar sequences to bovine pro-ACTH-β-LPH, although differing in minor details.

The generally accepted processing of POMC is that AtT-20/D-16v mouse pituitary tumor cells synthesize pro-ACTH-β-endorphin which cleaves to form an ACTH biosynthetic intermediate (which further cleaves to form a 16K N-terminal fragment + a glycosylated ACTH₁₋₃₉ or ACTH₁₋₃₉)

*Molecular weight estimated by SDS polyacrylamide gel electrophoresis is expressed by the use of K (=10³) in order to distinguish it from molecular weight estimated by gel filtration.
and a β-lipotropin (LPH)-like molecule (a β-endorphin-like molecule + a γ-LPH-like molecule). The apparent molecular weight from SDS polyacrylamide gel electrophoresis (K*) and from gel filtration in 6 M guanidine HCl are as follows: pro-ACTH-β-endorphin, 31K or 27,000 (glycosylated form) and 29K or 25,800 (non-glycosylated form); ACTH biosynthetic intermediate, 23K or 17,600 (glycosylated form) and 20K or 15,400 (non-glycosylated form); (3-LPH, 11.7K or 8,200; 16K fragment, 16K or 11,200; glycosylated ACTH<sub>1-39</sub>, 13K or 6,700; non-glycosylated ACTH<sub>1-39</sub>, 4.5K or 4,500; γ-LPH, 8K or 4,600; β-endorphin, 3.5K or 3,500 (22-28).

Similar molecular weights of POMC-related peptides have been obtained in various species (29-33). The presence of two glycosylation sites have been revealed on the 16K N-terminal fragments isolated from pig (34) and human (35) pituitaries.

The isoelectric point calculated for pro-ACTH-β-endorphin is 8.37 (36). The molecular weights (K) and isoelectric points (pI) of three glycosylated forms of POMC separated from rat intermediate lobes have been shown to be 34K, pI 8.2 (form I); 36K, pI 8.2 (form II); and 35K, pI 7.3 (form III) (30). It has also been reported that the isoelectric point of human big ACTH (M.W. 20,000-40,000) is higher than 10.0, while that of small ACTH is about 6.3 (37).

The N-terminal of POMC is relatively stable in serum. It has been demonstrated that immunoreactive (IR) human N-terminal peptide has a longer metabolic clearance time and a longer half-time of disappearance than IR human ACTH in rat serum (38).

POMC is produced by many different tissues, particularly the anterior and intermediate lobes of the pituitary. Recently, a trypsin digest of the N-terminal peptide of POMC, prepared from mouse pituitary tumor cells, and a bovine α<sub>2</sub>-melanotropin (MSH) synthetic peptide, representing a portion of the N-terminal peptide, have been shown to have a marked effect on the potentiation of adrenocortical response to ACTH (39, 40).

In another study, a naturally-occurring human pro-γ-MSH glycopeptide potentiated the ACTH-induced steroidogenic response of isolated perfused rat and human adrenergic cells (41).

These findings regarding POMC-related peptides are similar to the results obtained from the isolated peptides in their molecular weights, isoelectric points, peptide glycosylation, production by the pituitary, stability in serum, ACTH-potentiating activity and ACTH-like activity.

In order to determine whether the peptides isolated from rat serum are POMC-related peptides or not, the immunoreactivities of the peptides to antiserum against ACTH and the N-terminal fragment, must first be examined.

SM-Fr also showed ACTH-potentiating activity, although its activity was much lower than those of the peptides isolated from APS-Fr (Fig. 11). The molecular weight of SM-Fr was smaller than that of ACTH<sub>1-24</sub>, and it did not show any ACTH-like activity. SM-Fr therefore seems to be the minimum structure which has ACTH-potentiating activity in serum.

In addition, the potentiation of ACTH<sub>1-39</sub>-induced steroidogenesis by the addition of the isolated peptide was not so remarkable as that of ACTH<sub>1-24</sub>-induced steroidogenesis (Figs. 5 and 6). The possibility of contamination by ACTH-potentiating substance in the Third International Working Standard used for ACTH<sub>1-39</sub> is suggested from the results shown in Figs. 5, 6 and 7.

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