Purification and Properties of Interstitial Cell-stimulating Hormone from Sheep Pituitary Glands*

PHIL G. SQUIRE AND CHOH HAO LI

From the Hormone Research Laboratory and the Department of Biochemistry, University of California, Berkeley, California

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Early investigations on the purification and characterization of the hypophysial interstitial cell-stimulating hormone yielded highly active products from sheep (1-3) and swine (4, 5) pituitary glands. The preparations appeared to be homogeneous according to the physical and biological criteria of purity then available.

The importance of the early ovine and porcine preparations is attested by the fact that most of what we know of the biological action of ICH was learned from studies involving the use of these early preparations, and yet it is probably fair to state that no practical method for obtaining the hormone in a state of purity that would satisfy modern criteria was available at the time that the present investigation was initiated; therefore, it seemed profitable to re-examine the problem of the purification of ICH, with use of purification procedures which have been developed since the earlier work and application of the newer criteria of purity. There evolved from this re-examination an improved purification procedure as well as new information concerning the physical, chemical, and biological properties of the hormone. In the course of these studies, we have obtained two chromatographically distinct fractions with high ICH activity. A brief outline of the essential steps in the purification procedure and some of the properties of one of the fractions, which was designated β-ICH, has already been presented (6).

It is the purpose of this communication to present more complete experimental details as well as data relative to yields and purity of fractions obtainable by the new procedure.

EXPERIMENTAL

Purification Procedure

Acetone Desiccation—A kilogram of whole frozen sheep pituitary glands is taken through an acetone desiccation step as previously described (1-3) except that the acetone is chilled to −15° before use. The yield from this step is 225 gm.

Initial Extractions—The product of the previous step is extracted for 2 to 4 hours with 2.5 l. of 0.5 per cent NaCl. 12 ml. of glacial acetic acid dissolved in 50 ml. of water are added to bring the pH to 4.55 ± 0.05. The suspension is extracted for an additional hour under these conditions and then centrifuged at 15,000 r.p.m., to yield 2000 ml. of supernatant fluid.

Alcohol and Sulfosalicylate Fractionation—The pH of the supernatant fluid is adjusted to 7.15 ± 0.05 by the addition of approximately 150 ml. of NaOH. Ethyl alcohol is added to make a final concentration of 40 per cent; the solution is cooled to −15° during this addition and then centrifuged at −15°. The supernatant fluid is saved for the purification of FSH. The precipitate is suspended in 0.5 l. of water containing crushed ice, and the suspension is stirred at 0° until all the protein lumps are broken up. An inactive precipitate is removed by centrifugation at 18,000 r.p.m. To the resulting supernatant fluid (Fraction A, 12 gm.) is added 5.5 gm. of NaCl and 55 ml. of 0.2 M sulfosalicylate buffer. The pH is adjusted to 3.60 ± 0.05 and the precipitate is removed by centrifugation at 2400 r.p.m.

Ammonium Sulfate Fractionation—Solid (NH₄)₂SO₄ is added to the supernatant fluid obtained after precipitation with sulfosalicylate to make a concentration of 1.30 M. The pH is adjusted to 6.9 ± 0.1 with 5 N NaOH, and the precipitate that forms is removed by centrifugation at 2400 r.p.m.; additional ammonium sulfate is added to the supernatant fluid to bring the salt concentration to 2.0 M. The precipitate (Fraction B) is removed by centrifugation at 2400 r.p.m. The supernatant fluid is saved for the purification of FSH.

Chromatography on IRC-50 Resin—A sample of Fraction B is adsorbed onto an Amberlite IRC-50(XE-97) column that has been equilibrated with 0.2 M potassium phosphate at pH 5.9 (protein load, not more than 5 mg. per ml. of resin). An inactive fraction (Fig. 1, tubes 9 to 21) which passes through unadsorbed is discarded. The active ICH fraction (Fraction C) is eluted with 0.2 M potassium phosphate buffer of pH 6.9 (Fig. 1, tubes 144-165). A sample of Fraction C is then applied to a similar column that has been equilibrated with a 0.2 M potassium phosphate buffer of pH 6.13 (protein load, not more than 3 mg. per ml. of resin) and is eluted with 0.2 M potassium phosphate at pH 6.9. The unadsorbed protein appearing after the passage

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1 The abbreviations used are: ICH, interstitial cell-stimulating hormone; FSH, follicle-stimulating hormone; and TSH, thyroid-stimulating hormone.

2 A detailed account of the many experiments, successful and unsuccessful, on which this new purification procedure is based may be found elsewhere (7).
FIG. 1. Chromatography of Fraction B on IRC-50 resin. The 602-mg. sample was applied to the column containing 330 ml. of resin equilibrated with 0.2 M potassium phosphate at pH 5.88, and elution with buffer of pH 6.9 was begun at tube 15. Fraction C was recovered from tubes 144 to 165. (Tube volume, 17 ml.).

FIG. 2. Chromatography of Fraction C on IRC-50 resin. The 180-mg. sample was applied to the column containing 250 ml. of resin equilibrated with 0.2 M potassium phosphate at pH 6.13. Elution with buffer of pH 6.9 was begun at tube 81. Fraction D-2 was recovered from tubes 164-177 and Fraction D-3, from tubes 178-193.

FIG. 3. Zone electrophoresis of 250 mg. of Fraction D-2 in acetate buffer of pH 4.2 and ionic strength 0.10 for 72 hours at 3 volts cm. -1. As indicated, Fraction E-3 was recovered from segments 16 to 29, and E-2, from segments 30 to 34.

FIG. 4. Zone electrophoresis of 150 mg. of Fraction D-3 in acetate buffer of pH 4.2, ionic strength 0.10 for 72 hours at 3.0 volts cm. -1. As indicated, Fraction E-3 was obtained from segments 19 to 29 and E-2, from segments 30 to 34.

of 1 retention volume is inactive.5 The major portion of the ICH activity is eluted in an asymmetric peak with 0.2 M potassium phosphate at pH 6.9. As indicated in Fig. 2, the sharp portion of the peak is designated Fraction D-2, and the trailing shoulder, D-3.

Zone Electrophoresis on Starch—An inactive component with a high mobility is removed from Fractions D-2 and D-3 by zone electrophoresis6 on starch (9, 10) which was carried out for 72 hours at 3 volts per cm. in an acetate buffer of pH 4.2, I/2 = 0.2, with a protein load of 250 mg. of Fraction D-2 or 150 mg. of Fraction D-3. Two active fractions (E-2 and E-3) are recovered from this step7 (Figs. 3 and 4).

Removal of Starch by Chromatography—Any starch that is present as a contaminant in the E fractions which were obtained from zone electrophoresis can be removed by means of adsorption from an IRC-50 column equilibrated with 0.2 M potassium phosphate buffer and subsequent elution at pH 6.9. A protein load of 1 mg. per ml. of resin is used. In addition to removing the starch, this step provides a more complete separation of two chromatographically distinct ICH fractions designated

5 The amount of protein load is crucial. An inactive protein fraction passes through the column with the passage of 1 retention volume. This is followed by a “tail” which has ICH activity. The fraction of total activity in this “tail” increases with the amount of protein applied to the column. With a load of 3 mg. per ml. of resin, it would amount to about 10 to 15 per cent of the total activity. (See Fig. 1 and reference (6) for an elution diagram from an experiment in which the load was 4 mg. per ml. on a 330 ml. column.)

6 Starch segments obtained from preparative zone electrophoretic experiments were extracted once with water, and then twice with 0.2 M potassium phosphate buffer of pH 6.9, since extraction with this latter buffer leads to higher recovery of protein.

7 Chromatography on CM-cellulose (11) was investigated as an alternative method for the purification of Fraction D-2. It was found that the proteins in this fraction were completely adsorbed from pH 5.7 acetate buffer of ionic strength 0.03. The protein could then be eluted as a single, almost symmetrical peak by applying a mild NaCl gradient. Bioassay data showed that the center of the peak had a higher specific activity than either edge, an indication that some resolution had been achieved. Since, however, better resolution was attained by zone electrophoresis, this latter method was used for further purification of Fraction D-2.
Tube Number

FIG. 5. Upper diagram, chromatography of 200 mg of Fraction E-3 (from D-3) on IRC-50 resin. Adsorption from 0.2 M potassium phosphate at pH 6.13. Elution with 0.2 M potassium phosphate at pH 6.9. Fraction F-2 is obtained from tubes 100-109, F-3 from tubes 110-119. Optical density at 278 mμ; ΔΔΔΔΔ, pH. Lower diagram, rechromatography of Fraction F-3 (from D-3), yielding β-ICH.

F-2 and F-3 (see Figs. 5 and 6). Fraction F-3 (from D-3) has previously been designated α-ICH (6).

Table 1 presents the yield and activities of various fractions obtained from 1 kg of fresh sheep pituitary glands. From this table, it can be estimated that Fractions D-2 and D-3 contain about 23 per cent of the original activity. The final highly purified F-fractions contain 12 per cent of the original activity; since the fraction we have designated β-ICH is only one of the active F fractions (e.g. F-3 from E-3 from D-3, and so on), the yield of this particular fraction represents only about 3 per cent of the total ICH activity.

Preparations of β-ICH were tested extensively for contamination by other pituitary hormones. The ascorbic acid-depletion method (14) was used as a test for adrenocorticotropic hormone contamination, the tibia test (15) for growth hormone, the local crop sac method (16) in pigeons for lactogenic hormone, the ovarian weight and histological tests (17, 18) for FSH, and the iodine uptake test of Bates and Cornfield (19) for TSH contamination. All tests were designed to detect contamination at the 0.1 per cent level, and the bioassay data indicated no detectable contamination at that level.

The assay method used to estimate the biological potency of the ICH preparations was essentially that described by Greep et al. (12), except that rats of the Long-Evans strain were used. Several aspects of the bioassay procedure were studied during the development of the purification procedure; details, including the basis for the choice of the subcutaneous route of injection, have been described elsewhere (13). We have adopted, as an aid in representing bioassay data, a laboratory unit defined as that amount of ICH which will result in an increase of ventral prostate weight amounting to 100 per cent above the controls, as measured by this assay method.

The minimal effective dose of β-ICH in hypophysectomized female rats, as defined by Simpson et al. (18), was found to be 0.002 mg.

The biological potency of β-ICH was studied in hypophysectomized male rats (12). Histological examination gave significant evidence of "repair" of the interstitial cells at a total dose of 0.5 μg of ICH.10 There is a statistically significant increase (at the 10 per cent level) in the ventral prostate weight at the same dose level. The mean ventral prostate weight of rats receiving a total dose of 2 μg was more than double that of the controls, but no further enhancement of weight was observed even when the dose was increased to 100 μg. The negligible increase in testicular weight and the complete lack of change in the seminal vesicle weights after injections of ICH
within this dose range bear out the findings made with the older preparations (20).

**Homogeneity Studies**

**Chromatography**—A sample of \( \beta \)-ICH was submitted to chromatography on an analytical (9-ml) column of Amberlite IRC-50 resin, under the same conditions as those used for the purification of the E fractions. Approximately 90 per cent of the protein emerged as a single sharp symmetrical peak, with the remaining 10 per cent, presumably denatured, emerging with the subsequent alkali wash. The chromatographic diagram is presented in the lower portion of Fig. 5. The diagram from similar analytical chromatography of Fraction F-2 (from D-2) is presented in the lower portion of Fig. 6. In both instances a considerable increase in homogeneity is evident. Furthermore, in each instance the pH of the peak tube is the same as that of the parent fractions.

**Zone Electrophoresis**—\( \beta \)-ICH was submitted to zone electrophoresis11 on starch in the small (0.7 X 40-cm.) trough (10) in the pH 4.2 acetate buffer of 0.1 ionic strength. The protein distribution pattern, given in Fig. 7, shows a sharp symmetrical \( \beta \)-ICH-active peak (cf. Table II for bioassay) preceded by a smaller peak devoid of \( \beta \)-ICH activity. The protein content represented by the faster-moving inactive peak represents 10 per cent of the total. The presence of this small inert fraction suggested that denaturation of the \( \beta \)-ICH had occurred. In order to determine whether this was true, the experiment was repeated in triplicate, and the protein was recovered from the segments containing the active \( \beta \)-ICH and rerun under the same conditions. The resulting pattern also showed the presence of a small inert component (7 per cent of the total) with a higher mobility. Apparently the process of zone electrophoresis and subsequent recovery of protein results in the conversion of some of the \( \beta \)-ICH to an inert product with a higher mobility. Such an interpretation is not unique; it has been made in a similar situation in studies with growth hormone (21), \( \beta \)-lactoglobulin (22), and glucagon (23).

**Boundary Electrophoresis**—Concurrently with the zone electrophoretic study of Fraction D-2, studies were also made by the moving boundary method12 with buffers of 0.10 ionic strength and pH values ranging from 4.20 to 8.65. In acidic buffers, two distinct peaks were observed, but at neutrality and up to pH 8.65 the protein migrated as a single peak. From our zone electrophoretic studies, it was evident that the slow component was \( \beta \)-ICH and the faster one, the inert contaminant. The pH of zero mobility of both components is between 7.0 and 7.5,13 far from the isoelectric point of 4.6 reported earlier (3). Schlieren patterns of a sample of \( \beta \)-ICH in acetate buffer of pH 4.2 are presented in Fig. 8. The presence of a small contaminant with a higher mobility is in agreement with the observations made after zone electrophoresis (cf. Fig. 7).

**Sedimentation Analysis**—Sedimentation studies14 of \( \beta \)-ICH provided additional evidence of homogeneity, since they revealed the presence of a single symmetrical peak (Fig. 9). Experiments were conducted with protein concentrations of 0.5, 0.5, 0.8, and 1.3 per cent in a buffer consisting of 0.01 M \( \text{K}_2\text{HPO}_4 \), 0.01 M \( \text{KH}_2\text{PO}_4 \), and 0.20 M \( \text{KCl} \). These experiments furnished no evidence that the sedimentation constant was concentration-dependent; therefore, the mean of the four determinations was taken as an estimate of the sedimentation constant at infinite dilution. This value (\( s_{20W} \)) with the standard deviation for the four experiments is 2.47 ± 0.02 S.

**Diffusion Studies**—Diffusion experiments were limited to a pair of duplicate runs that were carried out simultaneously in the two limbs of the cell and with the same buffer solution that was used in the sedimentation studies. The diffusion coefficient are presented in Fig. 8. The presence of a small contaminant with a higher mobility is in agreement with the observations made after zone electrophoresis (cf. Fig. 7).

**Bioassay data?**—Bioassay data obtained on samples selected from zone electrophoretic analysis of fraction F-3 (from D-3)

<table>
<thead>
<tr>
<th>Segment*</th>
<th>No. of rats</th>
<th>Ventral prostate</th>
<th>Bioassay data†</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>8</td>
<td>12.1 ± 1.65</td>
<td>75</td>
</tr>
<tr>
<td>25</td>
<td>9</td>
<td>14.9 ± 1.11</td>
<td>152</td>
</tr>
<tr>
<td>27</td>
<td>8</td>
<td>13.4 ± 0.90</td>
<td>114</td>
</tr>
<tr>
<td>31-33</td>
<td>7</td>
<td>7.9 ± 0.75</td>
<td>&lt;20</td>
</tr>
</tbody>
</table>

*See Fig. 7.
† Assayed at a total dose of 0.002 mg.
‡ Mean ± standard error.

11 The zone electrophoretic patterns of Fraction F-2 (from D-2), F-3 (from D-2) and F-2 (from D-3) were all similar to the pattern recorded in Fig. 9 except that the main peaks were not symmetrical.
12 Electrophoretic analyses by the moving boundary method and diffusion experiments were all performed in the Spinco model E analytical ultracentrifuge, equipped with the new rotor temperature indicator and control which had been calibrated at rest. All experiments were carried out at 53,700 r.p.m. in a buffer consisting of 0.010 M \( \text{KH}_2\text{PO}_4 \), 0.010 M \( \text{K}_2\text{HPO}_4 \), and 0.200 M \( \text{KCl} \). The sedimentation constants were calculated and corrected to standard conditions by the method recently summarized by Schachman (pp. 53-56 in (25)).
4.6. It is interesting that whereas the isoelectric point is very different from that of the sheep ICH prepared by the old procedure, purified \(\beta\)-ICH described in this paper revealed marked differences from the preparation obtained by the old procedure (1-3), it is quite similar to that of the porcine ICH described by Chow et al. (4) and Shedlovsky et al. (5). With the new purification procedure, it is now possible to obtain ICH in much higher yield than was possible previously. The biological activity of Fraction C is as high as what was reported before, and the yield at this stage of purification is 600 mg per kg. of glands. Up to this point, considerable effort was made to minimize any losses which might result from possible proteolytic activity and which are to be expected in any attempt to isolate a protein component present to the extent of only a small percentage of the total.

The possible hazards to be expected from proteolytic activity were emphasized by Adams and Smith (26). These investigators reported the presence of two proteolytic enzymes in pituitary extracts, one with an optimal pH at 8.3, the other at 3.8. In our examination of the ICH fractions obtained by the new procedure, we have detected the presence of only the latter; this enzyme is eliminated during the chromatography step, and Fraction C is therefore devoid of proteolytic activity. Since the enzyme is inactive above pH 6.5 and is inhibited by 0.2 M phosphate (26), it is evident that there are only two short periods when enzymatic activity is likely to occur, during the first extraction, and during the steps involving precipitation with sulfosalicylate. In both steps, the pH is brought to neutrality as soon as possible after centrifugation, and it is unlikely that significant enzymatic degradation will have occurred. Furthermore, considering the very mild nature of all the operations, we believe that the probability of isolating an artifact is not high.

The chromatography of Fraction C under the conditions used in these studies yields a complex peak of an appearance which suggests that two ICH-active proteins have been partially resolved. A consideration of the accumulated bioassay data can lead only to the conclusion that these two components are very nearly equal in activity. These results were confirmed in experiments (lower portion of Figs. 5 and 6) in which the evidence seemed sufficiently conclusive to suggest the hypothesis that ICH exists in the form of two chromatographically distinguishable molecular species; consequently, the component concentrated in Fraction D-2 has been designated \(\alpha\)-ICH, and the one concentrated in Fraction D-3, \(\beta\)-ICH. Physicochemical differences between these two components remain to be investigated.\textsuperscript{16}

**SUMMARY**

A new method has been developed for the purification of an interstitial cell-stimulating hormone (ICH) from sheep pituitary glands. In the course of this procedure, a highly active concentrate is prepared by fractionation with alcohol, ammonium sulfate, and sulfosalicylate. Further purification by chromatography on a resin (IRC-50) column gives rise to two active fractions, D-2 and D-3. The yield at this point in the purification procedure is about 30 times that reported previously, and it is calculated to be 30,000.

**DISCUSSION**

The study of the physicochemical properties of the highly purified \(\beta\)-ICH described in this paper revealed marked differences from the preparation obtained by the old procedure (1-3). The molecular weight is 30,000, as compared with 40,000, and the isoelectric point is approximately 7.3, as compared with 4.6. It is interesting that whereas the isoelectric point is very different from that of the sheep ICH prepared by the old procedure, the molecular weight of ICH was calculated to be 30,000.

\textsuperscript{15} Further evidence for this multiplicity of ICH components is furnished by experiments with zone electrophoresis on cellulose columns. (M. J. Jutisz and P. G. Squire, to be published.)

\textsuperscript{16} During the preparation of this manuscript, a paper has appeared on the purification of ICH and FSH (27).
the products have higher ICH activity, although neither fraction is electrophoretically pure.

By means of zone electrophoresis on starch and an additional chromatographic step, a preparation (Fraction F-3 from D-3), designated β-ICH, can be obtained that possesses a high degree of homogeneity as indicated by sedimentation analysis and chromatography on IRC-50 resin. The isoelectric point of β-ICH is estimated to be near pH 7.3. The molecular weight calculated from sedimentation and diffusion data and from an assumed partial specific volume of 0.73 is 30,000.

No contamination of β-ICH with other pituitary hormones was indicated by biological assays designed to detect such contamination at the level of 0.1 per cent; a total dose of 0.5 μg. of β-ICH produced a significant increase in the weight of the ventral prostate of young hypophysectomized rats.

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