Immunochemical Investigations of Human Pituitary Growth Hormone

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Earlier immunochemical investigations of growth hormone (somatotropin) isolated from bovine and human pituitaries showed that these two protein hormone preparations form species-specific antibodies, as evidenced by the results of precipitin ring tests with rabbit antiserum and of anaphylactic shock experiments in guinea pigs (1, 2). It was further demonstrated that the rabbit antiserum exerts in hypophysectomized rats an antihormone activity specific for the particular growth hormone concerned. This paper presents quantitative data on the antigen-antibody complex of human growth hormone. In addition, the growth hormone content of human pituitary glands has been quantitatively determined on the basis of the immunochemical reaction of the hormone.

EXPERIMENTAL

Antigen—Growth hormone was isolated from human pituitaries by a method previously outlined (3, 4). The homogeneity of the preparation has been demonstrated by electrophoresis, by studies in the ultracentrifuge and by terminal group analysis (3-6).

Production of Rabbit Antiserum—Young albino rabbits weighing initially approximately 2.4 to 2.8 kg were immunized according to a schedule essentially the same as that followed previously (2), except that the booster shots were given in 0.9% sodium chloride solution instead of as an alum precipitate. It was observed that high antibody titers could be obtained with a total dose of 7 or 8 mg of human growth hormone. Only sera obtained from the first two bleedings were used in the present experiments.

Preparation of γ-Globulin—The antibodies to normal serum proteins that were contained in the antiserum were absorbed out by the repeated addition of small amounts of normal human serum in 1:10 dilution. The γ-globulin was prepared from this absorbed antiserum by a modification of the method originally described by Horvai and Smetana (7). The procedure was as follows: 25 ml of rabbit antiserum were adjusted to pH 8.5 and mixed with 87.3 ml of a 0.4% solution of rivanol (2-ethoxy-6,9-diaminoacridine lactate), which had been adjusted to the same pH. The resulting precipitate was removed by centrifugation at room temperature, and for every 15 ml of the clear supernatant fluid, 2 ml of saturated ammonium sulfate solution were added. The resulting solution was adjusted to pH 5.5 and poured onto an Amberlite cation resin (IRC-50) column that had been equilibrated with a 0.2 M phosphate buffer of pH 5.5 containing 0.15 M (NH₄)₂SO₄. Under these conditions the rivanol and any contaminating hemoglobin pigment were strongly adsorbed onto the resin, whereas the γ-globulin passed through unadsorbed. This eluate was thoroughly dialyzed first against running water and then against distilled water in the cold, and was then lyophilized. The yield was of the order of 300 mg for 25 ml of the serum. The preparation retained immunological activity over long periods of time when stored at 2° in a desiccator. The γ-globulin dissolved very readily in sodium chloride or phosphate buffer (pH 7.4); the very small amount of residue which remains is removed by centrifugation.

The preparation obtained by this method, when it was subjected to free electrophoresis, was found to contain γ-globulin to the extent of 73%. This main component had a mobility, in Veronal buffer of pH 8.9 and 0.1 ionic strength, of \(-1.15 \times 10^{-5}\) sq. cm per second per volt. Two additional electrophoretic components, with mobilities of \(-3.56 \times 10^{-4}\) and \(4.20 \times 10^{-4}\) sq. cm per second per volt, respectively, accounted for the remainder. The preparation of γ-globulin obtained by means of the rivanol procedure is sufficiently pure to be used satisfactorily for the quantitative precipitin tests and, moreover, the procedure has the special advantage of being relatively easy to perform. For the purposes of this paper, this γ-globulin fraction will be called γ-globulin.

Precipitin Reaction—The procedure followed for the quantitative precipitin reactions was essentially that of Heidelberger and Kendall as outlined by Kabat and Meyer (8). To increasing amounts of antigen in serological tubes was added a reconstituted γ-globulin solution (12 mg per ml) in the amount of 0.5 ml per tube. In the single instance where a comparative study between absorbed antiserum and γ-globulin was made, absorbed antiserum (0.5 ml) was used instead of this γ-globulin solution. The pH of the media was maintained at 7.5 by the addition of NaCl, in varying amounts so that the volume did not exceed 1.5 ml.

The tubes were incubated under a table lamp (approximate temperature 37°) for 1 hour, and stored in a refrigerator (2°) for 72 hours. Mercaptoethanol in a concentration of 1:10,000 was added to prevent bacterial growth. The precipitates were then removed by centrifugation in the cold for 30 minutes, and the supernatant fluids were transferred to individual tubes for the determination of excess antigen and antibody. The precipitates were washed first with 1.0 ml and then 0.5 ml of chilled 0.9% sodium chloride solution, with centrifugation for 15 minutes in the cold being carried out after each washing. The washed precipitates were then dissolved in 0.18 ml of 0.01 M NaOH and the protein was determined by means of the micro method of Lowry et al. (9).

The supernatant fluids were tested for excess of antigen and
FIG. 1. Precipitin reaction between human growth hormone and antiserum to human growth hormone, as determined by the Ouchterlony technique. Antiserum, 6 μl, in central well; 1, 2.5 μg of human growth hormone; 2, 5 μg of human growth hormone; 3, 7.5 μg of human growth hormone; 4, 10 μg of human growth hormone. Figs. 1 to 3 were photographed at the end of 24 hours.

FIG. 2. Precipitin reactions as determined by the Ouchterlony technique. 1, human growth hormone, 15 μg; 2, γ-globulin, 210 μg; 3, human pituitary extract, 18 μl; 4, antiserum to human growth hormone, 10 μl.

antibody by both the precipitin ring test and the Ouchterlony agar-gel diffusion technique (10). The point at which no excess of antigen or antibody was detectable in the supernatant fluid was taken as the equivalence point.

Agar-gel Technique—The technique used for the precipitin tests in agar was a micro modification of the well known Ouchterlony procedure (10). The agar plate was prepared by setting a microscope slide in a suitable frame and pouring onto it 5 ml of 0.9% molten agar. Holes were then made in the set agar according to a predetermined pattern (cf. Figs. 1 and 2). With small holes used to hold antigen and antibody, it was possible to perform these agar-gel diffusion studies with as little as 0.01 ml of the antigen and antiserum solutions. For the tests to determine excess of antigen and antibody in the supernatant fluids, larger cups holding as much as 0.10 to 0.15 ml of solution in a regular Petri dish were used. The precipitin bands were clearly visible within 12 to 24 hours and could easily be photographed.

RESULTS

Precipitin Tests in Agar—The single continuous antigen-antibody precipitin band obtained from the agar-gel diffusion studies, carried out with varying concentrations of human growth hormone (Fig. 1), clearly demonstrates the immunological homogeneity of the antigen. That the purified human growth hormone and the isolated γ-globulin are identical with their native counterparts, i.e., growth hormone as it exists in the pituitary gland and the antibody present in the antiserum, respectively, is evident from the precipitin band seen in Fig. 2.

Growth hormone isolated from monkey pituitaries (3, 4) cross-reacted fully with antiserum to human growth hormone (Fig. 3). It can also be seen from Fig. 3 that growth hormone from sheep (11) or beef (12) pituitaries did not cross-react with the antiserum to human growth hormone. This result is in complete agreement with the earlier findings (13) obtained by means of precipitin ring tests.

Quantitative Precipitin Tests—Quantitative determinations of the amounts of the precipitates obtained from the region of antibody excess to the region of antigen excess for a human growth hormone/γ-globulin system are presented in Table I and Fig. 4. It can be noted that the equivalence point for human growth hormone is located at 50 μg. In keeping with the postulate of Heidelberger and Kendall (14), the ratio of antibody to antigen, when plotted against the antigen concentration, gave a straight line. By extrapolation, the value for R, i.e., the ratio of Ab to An at the equivalence point, is obtained. There is close agreement between the extrapolated value for R, 2.5, and the observed value of 2.25 (Table I). Total inhibition of precipitation could be achieved at a concentration of 400 to 500 μg of antigen.

FIG. 3. Cross-reaction between antiserum to human growth hormone and monkey, bovine, and ovine growth hormones. 6 μl of antiserum to human growth hormone contained in central well; 1, human growth hormone, 2 μg; 2, monkey growth hormone, 2 μg; 3, bovine growth hormone, 5 μg; 4, ovine growth hormone, 5 μg.
TABLE I
Precipitin reaction of human growth hormone with γ-globulin isolated from rabbit antiserum to human growth hormone

<table>
<thead>
<tr>
<th>Antigen added A (μg)</th>
<th>Total protein precipitated B (μg)</th>
<th>Difference (B - A) (μg)</th>
<th>Antibody to antigen ratio (B - A)/A</th>
<th>Antibody in supernatant fluid</th>
<th>Antigen in supernatant fluid</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>15</td>
<td>12.5</td>
<td>5.0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5.0</td>
<td>30</td>
<td>25.0</td>
<td>5.0</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>10.0</td>
<td>53</td>
<td>43.0</td>
<td>4.3</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>25.0</td>
<td>100</td>
<td>73.0</td>
<td>3.0</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>40.0</td>
<td>140</td>
<td>100.0</td>
<td>2.5</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>50.0</td>
<td>161</td>
<td>111.0</td>
<td>2.2</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>60.0</td>
<td>171</td>
<td></td>
<td></td>
<td>+</td>
<td>±</td>
</tr>
<tr>
<td>70.0</td>
<td>190</td>
<td></td>
<td></td>
<td>+</td>
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<td>80.0</td>
<td>157</td>
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<tr>
<td>120.0</td>
<td>127</td>
<td></td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 4. Precipitin curve for human growth hormone and γ-globulin from antiserum to human growth hormone. 6 mg of γ-globulin per tube; total volume of reactants, 1.5 ml. O, precipitin curve; □, antibody to antigen versus antigen.

The precipitin curves obtained from the reaction of human growth hormone with both the prepared γ-globulin and the absorbed antiserum are presented in Fig. 5. With the absorbed antiserum, neither a clear cut equivalence point nor a zone of excess antigen could be observed. It is evident from the precipitin curve for the human growth hormone/γ-globulin system that some contaminating antibodies have been removed in the course of the preparation of the γ-globulin.

The precipitin curves obtained with purified human growth hormone and with the growth hormone present in the crude pituitary extract as antigens can be seen in Fig. 6. It is interesting that there is very little difference in immunological behavior between the two. Moreover, the precipitin curve obtained for human growth hormone dissolved in normal human serum (Fig. 7) is very similar to that obtained for human growth hormone alone.

![Table I](http://www.jbc.org/)

![Fig. 4](http://www.jbc.org/)

![Fig. 5](http://www.jbc.org/)

![Fig. 6](http://www.jbc.org/)
FIG. 7. Precipitin curve showing recovery of human growth hormone (HGH) added to normal human serum. \( \triangle - \triangle \), human growth hormone added to normal human serum; \( \circ - \circ \), HGH alone; 6 mg of \( \gamma \)-globulin per tube.

FIG. 8. Precipitin curve showing cross-reaction between monkey growth hormone (MGH) and \( \gamma \)-globulin isolated from antisera to human growth hormone (HGH) (6 mg of \( \gamma \)-globulin per tube). \( \triangle - \triangle \), monkey growth hormone; \( \circ - \circ \), human growth hormone.

Since it was demonstrated by the experiments in agar-gel described above that monkey growth hormone cross-reacts with antiserum to human growth hormone, it is not surprising that the precipitin curve obtained with monkey growth hormone is very similar to that obtained with the human hormone (Fig. 8). The equivalence point for the monkey hormone is at 70 \( \mu \)g and the ratio of Ab to An at the equivalence point is 1.2. In a further experiment in which 6 mg of \( \gamma \)-globulin from human growth hormone was treated with an equivalent concentration of monkey growth hormone (70 \( \mu \)g) and the supernatant fluid tested for any reactivity with human growth hormone, no reactivity was observed, indicating that all the precipitable antibodies to human growth hormone had been precipitated by the monkey growth hormone. Thus, it can be concluded from these various data that the cross-reaction between these two primate hormones is complete.

Estimation of Growth Hormone Content in an Extract from Whole Human Pituitary—The quantitative precipitin test has been applied as an immunochemical assay method, to give an estimation of the growth hormone content in single human pituitary glands. In a typical experiment, a single frozen human pituitary weighing approximately 600 mg was homogenized in a Waring Blender with 50 ml of cold distilled water for 2 minutes. The mixture was transferred to a beaker, the pH was adjusted to 10.5, and the beaker was stored at 0° for 1 hour. After centrifugation, the clear supernatant fluid was diluted to a final volume of 100 ml and the pH adjusted to 7.0. For the immunochemical assay, a standard curve was established on the basis of the linear portion (0 to 20 \( \mu \)g of human growth hormone) of the precipitin curve (Fig. 4). Aliquots of the extract containing a concentration of human growth hormone in the range between 0 to 20 \( \mu \)g were used for the precipitin test;

<table>
<thead>
<tr>
<th>Pituitary sample</th>
<th>Weight of pituitary</th>
<th>GH content by immunochemical assay</th>
<th>GH content by bioassay*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>mg/100 ml</td>
<td>mg/g wet tissue</td>
</tr>
<tr>
<td>A</td>
<td>520</td>
<td>4.4</td>
<td>8.3</td>
</tr>
<tr>
<td>B</td>
<td>627</td>
<td>6.3</td>
<td>10.0</td>
</tr>
<tr>
<td>C</td>
<td>463</td>
<td>3.8</td>
<td>8.3</td>
</tr>
<tr>
<td>D</td>
<td>635</td>
<td>5.2</td>
<td>8.2</td>
</tr>
<tr>
<td>E</td>
<td>701</td>
<td>8.8</td>
<td>12.5</td>
</tr>
<tr>
<td>Average</td>
<td>5.7</td>
<td>9.5</td>
<td>8.6</td>
</tr>
</tbody>
</table>

* On the basis of increment of the width of tibial epiphyseal cartilage plate in hypophysectomized rats.

the results of the assay on five pituitary samples are given in Table II. Biological assay was also performed by the tibia test (15) in hypophysectomized rats. It can be observed that the values obtained from the immunochemical assay were usually slightly higher than those from the biological assay.

**DISCUSSION**

The agar-gel diffusion patterns and the precipitin curves obtained in this study demonstrate unequivocally the specificity of the antigen-antibody system of human growth hormone. The precipitin curves obtained for purified human growth hor-
Reactions between rabbit antiserum to human pituitary growth hormone and the hormone from ovine, bovine, simian, and human pituitaries have been investigated by the agar-gel procedure of Ouchterlony. It was found that human growth hormone behaved as an homogeneous antigen and that the antiserum cross-reacted only with the simian hormone.

A modified rivanol procedure for the preparation of γ-globulin from rabbit antiserum to human growth hormone has been described; a quantitative precipitin curve with a purified γ-globulin fraction used as the antibody was obtained for human and simian growth hormone. It was noted that the antigen in human pituitary extracts was immunochemically indistinguishable from the hormone isolated from human pituitaries, on the basis of both the quantitative precipitin tests and the agar-gel diffusion method.

The quantitative precipitin reaction has been used to estimate the growth hormone content in single human pituitaries, and an estimation of the growth hormone content in sera of acromegalic patients has been presented.

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REFERENCES

10. OUCHTERLONY, O., Arkiv Kemi, 12, 1 (1949).
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Choh Hao Li, N. R. Moudgal and H. Papkoff