IMMUNOLOGICAL STUDIES OF A BOVINE GROWTH HORMONE PREPARATION

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SUMMARY

The NIH-B2-GH preparation of ox growth hormone (GH) was separated by chromatography on DEAE-cellulose into six fractions. Five of these fractions when assayed in hypophysectomized rats showed GH activity ranging in potency from 0.25 to 2.5 times the starting material. Growth activity could not be correlated with the concentration of any single component revealed by starch gel electrophoresis.

Antisera produced to NIH-B2-GH had antihormone activity and produced two precipitin lines in Ouchterlony diffusion tests. One of these lines was associated with serum γ-globulin and was shared by all five fractions. The other line was present in only two of the fractions, and these contained the more anionic components.

It is suggested that the more cationic growth-active components present in bovine and ovine GH preparations do not readily produce precipitating antibodies and that this may complicate the results of precipitin and gel diffusion tests when heterogeneous GH preparations have been used to prepare the antisera.

INTRODUCTION

Antibodies to purified bovine growth hormone (GH) preparations have been produced in rabbits by a number of investigators (Hayashida & Li, 1958, 1959; Fishman, McGarry & Beck, 1959; Moudgal & Li, 1961). Hayashida & Li (1958) demonstrated that the antiserum to bovine GH had antihormone activity and that it formed precipitates with as little as 1 μg. of bovine GH. These authors (Hayashida & Li, 1959) also showed that ovine GH and bovine GH appeared to be immunologically identical when studied by precipitin ring and agar gel diffusion tests against antisera to bovine GH. These results were confirmed by Moudgal & Li (1961).

It has been shown (Ferguson & Wallace, 1961, 1963) that most bovine GH preparations, as currently prepared, are electrophoretically heterogeneous and contain more than one component with growth activity. The relationship between these components and the antisera produced to them has not so far been investigated.

The bovine NIH-B2-GH preparation can be separated by chromatography on diethylaminoethylcellulose (DEAE-cellulose) into several fractions (Ferguson &
Wallace, 1963). In this paper the interactions of these fractions with antisera prepared in rabbits to NIH-B2-GH have been examined by gel diffusion and immunoelectrophoretic techniques.

MATERIALS AND METHODS

Preparation of antisera. Antisera against NIH-B2-GH were prepared in five rabbits. Two milligrams of GH in complete Freund’s adjuvant were injected at four sites, two subcutaneous and two intramuscular. Four booster injections each of 2 mg. GH in normal saline, brought to pH 9 just before injection, were administered intravenously 4, 5, 6 and 10 weeks after the initial injection. Animals were bled 1 week after the last injection. This method of producing antisera was used in order to produce a high titre with the limited amount of antigen available. Antisera to the major cathodal component of sheep GH were prepared in two rabbits by the method of Wallace (1962).

Growth hormone. The bovine GH preparation used was the preparation NIH-B2-GH issued by the National Institutes of Health, Bethesda.

The ovine GH was prepared by the method of Wallace & Ferguson (1963).

Gel diffusion. Standard Ouchterlony gel diffusion tests (Ouchterlony, 1948) and Björklund inhibition tests (Björklund, 1952) were run in agar plates, prepared according to Reisner & Sobey (1962).

Immunoelectrophoresis. Immunoelectrophoresis was carried out as described by Sobey, Reisner & Adams (1962).

Chromatography. Chromatography was carried out on a 2·3 cm. diameter column prepared from 25 g. of DEAE-cellulose (Whatman DE-50, W. & R. Balston Ltd.) washed before use as previously described (Wallace & Ferguson, 1961).

The GH (330 mg.) was dissolved in 50 ml. of sodium borate buffer, pH 8·8 (0·025 M-NaOH, 0·095 M-H₃BO₃), and the insoluble residue removed by centrifugation. The clear supernatant was added to the column and developed, first with 400 ml. borate buffer and then with a gradient formed by a pH 7·2 sodium potassium phosphate buffer, (0·05 M-NaOH, 0·085 M-KH₂PO₄). Five millilitre volumes were collected and the absorbance at 280 mμ was read for each tube. On the basis of the absorbance values the tube contents were grouped into five fractions, numbered A to E, dialysed and freeze-dried. The material insoluble at pH 8·8 (fraction R) was also dialysed and freeze-dried.

Biological assay. Growth activity was estimated by the increase in the width of the tibial epiphyseal cartilage in hypophysectomized rats (Evans, Simpson, Marx & Kibbrick, 1943). Doses (0·5 ml.) were given in 0·1 % albumin containing 1 μg. sodium L-thyroxine/ml.

Starch gel electrophoresis. This was carried out by the method of Ferguson & Wallace (1963) and Ferguson (1964).

RESULTS

Text-fig. 1 shows a plot of the absorbance at 280 mμ against elution volume after chromatography of NIH-B2-GH on DEAE-cellulose. Two distinct peaks of protein concentration occur, one eluted unadsorbed by the borate buffer and the other with
the phosphate buffer. The shapes of the peaks indicate that each contains several components. The tube contents were collected into five fractions, A to E.

Plate 1, fig. 1, shows the stained patterns obtained after four of these fractions and fraction R had been submitted to starch gel electrophoresis. There was not enough of fraction A to carry out many experiments and only limited data are available for it. Material moving towards the cathode was present largely in fractions B and C, although some also occurred in fraction D, and consisted of two electrophoretic components, both of which have been shown to possess growth activity (Ferguson & Wallace, 1961). Fraction E contained mostly material which moved towards the anode, together with traces of cathodal components. The distribution of components in fraction R resembled those of fraction E.

Table 1 shows the results obtained when the growth activity of each of the fractions was assayed by the increase in the tibial epiphysial cartilage width in hypophysectomized rats. Fraction R was more than twice as active as the starting material, and fraction D was only about one quarter as potent. The other three fractions, B, C and E, had approximately the same potency as NIH-GH. Since fractions R and E contain only small amounts of the cathodal components there must be at least one other growth-active component present in the NIH-B2-GH preparation. In terms of weight about 80% of the applied material, containing all the biological activity, was recovered. Of this recovered activity approximately a third was present in fraction E and a third in fraction R.

The result of immunoelectrophoresis in agar gel is shown in Pl. 1, fig. 2, and demonstrates the presence of at least three antigenic components.
In Ouchterlony diffusion plates, wide diffuse precipitation bands common to fractions B, C, D, E, R, Armour bovine γ-globulin Fraction II (BGG) and bovine GH were found. In addition, sharper bands, common to bovine GH, fraction E and fraction R were present (Pl. 2, figs. 1 and 2). Björklund inhibition of the plates with BGG or whole cattle serum removed the diffuse bands common to all fractions but did not remove the lines common only to fractions E, R and bovine GH (Pl. 2, figs. 3 and 4).

Table 1. *Yield and growth activity of chromatographic fractions from NIH-B2-GH*

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Total dose (μg.)</th>
<th>Width of tibial epiphysial cartilage (μ) Mean ± s.e.</th>
<th>Relative potency (i.u./mg.)</th>
<th>Weight recovered mg.</th>
<th>Activity recovered %</th>
<th>Activity recovered (i.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>B</td>
<td>80</td>
<td>260 ± 10</td>
<td>1-5</td>
<td>8</td>
<td>2-4</td>
<td>—</td>
</tr>
<tr>
<td>C</td>
<td>80</td>
<td>246 ± 11</td>
<td>1-0</td>
<td>40</td>
<td>12-0</td>
<td>40</td>
</tr>
<tr>
<td>D</td>
<td>80</td>
<td>218 ± 22</td>
<td>0-24†</td>
<td>23</td>
<td>6-9</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>80</td>
<td>250 ± 8</td>
<td>1-1†</td>
<td>92</td>
<td>27-6</td>
<td>101</td>
</tr>
<tr>
<td>R</td>
<td>20</td>
<td>249 ± 9</td>
<td>2-5</td>
<td>65</td>
<td>19-5</td>
<td>163</td>
</tr>
<tr>
<td>NIH-B2-GH</td>
<td>80</td>
<td>259 ± 6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>20</td>
<td>199 ± 4</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>80</td>
<td>246 ± 6</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Fractions A, B, C, D, E and R, six animals per dosage level. NIH-B2-GH, fourteen animals per dosage level.
† Separate potency figures calculated from another assay. Data for standard not shown.

Table 2. *Inhibition of the biological action of bovine GH by antiserum*

<table>
<thead>
<tr>
<th>Group*</th>
<th>Total dose NIH-B2-GH (μg.)</th>
<th>Antiserum (ml.)</th>
<th>Width of tibial epiphysial cartilage (μ) Mean ± s.e.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>—</td>
<td>—</td>
<td>132 ± 4</td>
</tr>
<tr>
<td>2</td>
<td>40</td>
<td>—</td>
<td>226 ± 6</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>0-88</td>
<td>194 ± 7</td>
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<tr>
<td>4</td>
<td>—</td>
<td>—</td>
<td>156 ± 6</td>
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<tr>
<td>5</td>
<td>20</td>
<td>—</td>
<td>191 ± 9</td>
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<tr>
<td>6</td>
<td>80</td>
<td>—</td>
<td>230 ± 12</td>
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<tr>
<td>7</td>
<td>40</td>
<td>2-64</td>
<td>131 ± 4</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>2-64†</td>
<td>138 ± 7</td>
</tr>
</tbody>
</table>

Group 3 significantly different from group 1 (P < 0-01) and from group 2 (P < 0-001); groups 7 and 8 significantly different from groups 5 and 6 (P < 0-001).

* Six rats per group.
† Antisera adsorbed with bovine γ-globulin.

When ovine GH was tested by Ouchterlony diffusion against antiserum to bovine GH, no precipitation bands were obtained. Also, when ovine GH and bovine GH were tested against antiserum to the major cathodal component of ovine GH, no precipitin lines were formed. These results are shown in Pl. 2, figs. 5 and 6. Diffusion experiments were carried out between pH 7 and 9 and over a range of both antiserum and antigen dilutions.

Table 2 shows the results of two biological assays carried out to test the ability of the antiserum to inhibit the biological activity of the NIH-B2-GH. The GH was
dissolved (see Methods) and the solution divided into two equal volumes. To one volume was added antiserum and to the other the same volume of normal rabbit serum. A precipitate formed in the GH and antiserum solution, but this was allowed to remain. At a total dose of 0.88 ml. the antiserum significantly depressed the tibial cartilage width response to 40 \( \mu g \) GH, but the response was still greater than the control value. When a threefold amount of antiserum (2.64 ml.) was given with 40 \( \mu g \) GH, total suppression was obtained and tibial cartilage width was not significantly different from control levels. There was no difference between unabsorbed antiserum and antiserum absorbed with BGG.

**DISCUSSION**

It has been shown that the preparation NIH-B2-GH is heterogeneous and contains at least three components with biological activity (Ferguson & Wallace, 1961, 1963). Some of this heterogeneity arises from the presence of serum protein since absorption of the antiserum to NIH-B2-GH with bovine serum \( \gamma \)-globulins removes a number of precipitating antibodies.

The source of the antigen responsible for the single precipitin band remaining after absorption with bovine serum \( \gamma \)-globulins is not certain. It does not appear to be associated with the active cathodal fractions of the GH preparation, since it is shown by fractions E and R, which contain only a trace of these components. Fractions B and C, which contain high concentrations of the cathodal components, give no precipitin band. Neither does it appear to be associated with the major anodal components in fraction R, since these are present in greater concentration than in fraction E, and yet the precipitin line formed in diffusion tests is stronger to fraction E. It could arise from the other components active in growth tests present in the preparation or from a tissue-specific antigen of pituitary origin which followed the biologically active material during purification. Hayashida & Li (1958) found that the prolactin present in their GH preparation was sufficient to cause detectable precipitating antibodies. However, electrophoretic examination of preparation NIH-B2-GH and chromatographic fractions prepared from it, failed to show prolactin. If present, it must occur in amounts of less than 1 \( \mu g /mg \). since amounts of prolactin greater than 0.5 \( \mu g \) can be detected after starch gel electrophoresis.

It is also possible that the antigen is a bovine serum protein normally present in very low concentration, but concentrated during purification to such an extent that absorption with serum failed to remove it, or that it arises from the alteration of serum or pituitary proteins during the preparation of GH. Whatever its source there is no evidence that it was concentrated by chromatography since the precipitin band formed against fraction E appears no stronger than that formed against NIH-B2-GH.

The growth activity of preparation NIH-B2-GH can be completely inhibited by the antisera prepared against it but our results do not show whether the antibodies produced against the different active components are immunologically identical.

Although the antiserum prepared to the cathodal component of ovine GH was shown by the haemagglutination technique (Boyden, 1951) to contain antibodies which combined with both ovine and bovine GH, no precipitin bands could be formed
against these preparations. Neither did the antiserum to bovine GH produce precipitin bands with ovine GH. These results also suggest that the more cationic growth-active components of bovine and of ovine GH do not readily form precipitating antibodies.

The results presented in this paper illustrate the difficulties of establishing immunological identities between hormones from different species when heterogeneous preparations are used to prepare the antisera. They also suggest that the use of such antisera to demonstrate, by precipitin tests, the presence of a particular hormone in any extract from tissue, or to measure its concentration, could lead to erroneous results.

We wish to thank the Endocrine Study Group of the National Institutes of Health, Bethesda, for the gift of GH used in these studies and Dr K. A. Ferguson for carrying out the starch gel electrophoresis.

We wish also to thank Academic Press Inc., N.Y., for permission to reproduce data contained in Table 1, Text-fig. 1 and Pl. 1, fig. 1.

REFERENCES


DESCRIPTION OF PLATES

PLATE 1

Fig. 1. Starch gel electrophoresis of chromatographic fractions from bovine NIH-B2-GH. 0.5 mg. samples; 0.025 M-tris buffer system; 22–23 v/cm. for 5 hr.

Fig. 2. Immunelectrophoresis of bovine growth hormone (BGH); 1% and 2% BGH run for 90 min. 90 v and 15 mA in tris-EDTA-borate buffer, pH 8.9. Anti-BGH in the trough.

PLATE 2

Figs. 1 and 2. Agar double diffusion (Ouchterlony) plates showing the reactions between anti-bovine growth hormone serum (ab BGH) and 2% concentrations of bovine growth hormone (BGH), bovine γ-globulin (BGG), and chromatographic fractions B, C, D, E and R.

Figs. 3 and 4. Agar double diffusion (Björklund inhibition) plates showing a single residual reaction between anti-BGH serum and BGH and chromatographic fractions E and R, after prior absorption of plates. Otherwise identical with 1 and 2, with 1% BGG.

Fig. 5. Ouchterlony plate showing the absence of a reaction between antibodies to the major cathodal component of growth hormone (ab SGH) and either sheep growth hormone (SGH) or BGH.

Fig. 6. Ouchterlony plate showing reactions between anti-BGH serum and BGH and no reaction between anti-BGH serum and SGH.