INHIBITORY EFFECTS OF ANTIRAT PITUITARY SERUM (APS) ON HYPOPITUITOMIZED RATS INJECTED WITH PITUITARY HORMONES

By

Ludwik Anigstein, Edward G. Rennels and Dorothy M. Anigstein

ABSTRACT

This study concerns the effects of the rat pituitary antiserum (APS) and of bovine growth-hormone (STH) antiserum upon the organs of hypophysectomized rats. Hormonal stimulation was produced by the injection of rat pituitary homogenate or purified hormones. By using the tibial width test and organ weights, hormonal stimulation and the degree of inhibition by antiserum were evaluated. It was found that APS, when injected in conjunction with rat pituitary homogenate into hypophysectomized rats, inhibited the activities of growth and gonadotrophic hormones. Similar inhibitory effect was obtained when APS was injected in conjunction with bovine STH into hypophysectomized rats. Bovine STH antiserum was found to inhibit stimulatory effect of rat pituitary. Serological studies by complement fixation and precipitin tests exhibited specific antibodies in APS and in bovine STH antiserum against their homologous antigens. The Ouchterlony technique revealed some common components in rat and bovine pituitary antigens.

Early attempts to produce specific antibodies against the anterior lobe of pituitary gland and the serological differentiation between the anterior and the posterior lobes by tissue specific antigens were initiated by Witebsky & Behrens (1932). These studies were followed by those of Kestner (1938) who immunized rabbits with extracts of the anterior lobe of bovine hypophysis and injected the antiserum into intact rats. It was found the specific «dynam action» based on oxygen consumption was considerably lower in rats injected with the antiserum. Although the basic idea of an antipituitary serum was first
conceived by Legardii-Laura (1919, 1923), this author used the posterior lobe of the hypophysis as antigen for the antiserum in his attempt to control diabetes. The possible biological influence of an antagonistic or inhibitory factor, acting as pituitary antibody led to the concept of substitution therapy and »simulated hypophysectomy« by physiological suppression of the hypophysis (Thompson & Cushing 1937).

The action of specific antisera on the pituitary gland can be interpreted either in the light of the antigen-antibody reaction or as antihormone activity. The latter concept was expressed by Collip (1935) in his well known antihormone theory which states that every hormone may be antagonized by a specific antihormone. Strong support of the antibody nature of the inhibitory substance was given by Ehrlich (1934, 1935) who showed that the injection of rabbits with gonadotrophic extracts resulted in the formation of specific antibodies identical with complement fixing antibodies.

The analysis of antgonadotrophic substances by Twombly (1936), particularly of the precipitins formed by the hormone and the antisera also speaks in favour of the antibody concept. Furthermore, the observation by Gordon & Charipper (1938) that splenectomy, especially when combined with blockade of RES inhibits antihormone formation, added considerably to the immunological concept of antihormones. In the same category is the inhibition of antgonadotrophic production in rabbits after cortisone treatment (Hamburger 1952), this being similar to the known inhibitory effect of cortisone upon formation of true antibodies. However, recent observations by Lederer (1954) indicate that cortisone and corticotrophin (ACTH) not only failed to decrease but actually accelerated the formation of thyrotrophic antihormone in the rabbit (Sonenberg 1958, p. 246). Nevertheless, the prevalent opinion of investigators considers antihormones as belonging to the complexity of antigen-antibody reactions and appearing in the serum of animals treated with hormones (Schmidt 1955). It is characteristic for the nature of the growth-promoting hormone that it appears to be chemically an unremarkable protein with multiple biological activities (Ketterer et al. 1957).

In the immunological studies of the pituitary gland two types of antigens have been used by various authors, namely, the total tissue homogenate or the purified pituitary hormones. Whereas antisera of high potency were obtained when pituitary homogenate was used as antigen, the high purification of the hormones used as antigen did not always result in satisfactory production of antibodies. For example, Heijkenskjöld & Gemzell (1958) were unable to demonstrate antigenic properties of human growth hormone, but Hayashida & Li (1958) have obtained physiologically active antisera against highly purified bovine growth hormone. Recently antibodies in good titre were obtained to bovine, porcine and human growth hormones by Fishman et al. (1959).

Previous observations of the authors on the biological properties of the antirat
pituitary serum (APS) injected into rats indicated a relative suppression of body-weight (Anigstein et al. 1958).

The purpose of the present investigations was to study the experimental conditions under which the hormones present in the tissue homogenate of the rat pituitary can be influenced by the homologous antiserum. Parallel observations were made on the activities of antibodies elicited by purified bovine growth hormones used as antigens. Consequently, it was possible to compare the immunological relationships of the rat pituitary tissue and APS with the purified growth hormone preparations and their antisera. The experimental assay used in this study gave us the opportunity to investigate not only the effect of the pituitary antiserum (APS) upon the growth stimulation of hypophysectomized rats, but also its antigenadotrophic activities. Thus an increase of ovarian and uterine weights in animals given pituitary homogenate is the reflection of gonadotrophic stimulation, while an increase in thyroid weight similarly reflects thyrotrophic activities. Inasmuch as these organs were weighed routinely, it was possible to detect stimulation by the hormones or inhibitory effects by antihormones by comparison with the weights of the organs of hypophysectomized control rats. In addition to the above in vivo studies, parallel serological tests in utilizing complement fixation, precipitin ring tests and agar diffusion techniques (Ouchterlony 1958) were utilized.

MATERIAL AND METHODS

1. Hormones and immunization procedures

Rat pituitary homogenate was used for a dual purpose, namely for the immunization of rabbits (Anigstein et al. 1958) and a source of hormones for injection of hypophysectomized rats. In the preparation of the rat pituitary homogenate for the immunization of rabbits, anterior lobes (7.2 mg average) of large male rats were removed after the vascular system of the rat had been perfused with Gey's solution. The glands were weighed, frozen and at the time of use they were homogenized in glass tissue grinders. Albino male rabbits (2-3 kg each) were injected twice weekly for 3 weeks subcutaneously and intra-abdominally with rat pituitary homogenate emulsified in Freund's complete adjuvants (Difco). About 10-20 mg of the rat pituitary homogenate were used per injection per rabbit. Two weeks after the last injection the rabbits were bled. In other series of experiments purified growth hormones were used as antigens for immunization of rabbits as well as for serological tests. The bovine somatotrophin (Somar A) produced by Armour Pharmacutical Company and supplied by courtesy of Dr. J. A. Hubata was injected once or twice weekly subcutaneously (about 5 mg per dose) into rabbits in Freund's adjuvant suspension following in principle the technique of Hayashi & Li (1958). In addition, highly purified bovine growth hormone (STH) supplied by courtesy of Dr. C. H. Li was used as antigen for immunization of rabbits.

The preparation of globulin (used in experiments 2 and 5) by saturated ammonium sulfate precipitation was based on techniques described by Kabat & Mayer (1948).
2. Experimental animals

Immature albino female Holtzman rats (Sprague-Dawley strain) were used for the evaluation of hormone activity, the effect of antiserum and for tibia test assays. All experiments were preceded by hypophysectomy of the rats performed by one of us (E. G. R.) by parapharyngeal approach without cannulation of the trachea. Ether anesthesia was used. The operation was carried out on the 28th day of age of rats (60-65 g body weight). Any rats showing an increase in weight indicating only partial hypophysectomy were discarded. After a 14 day postoperative period the injections were begun and continued for the next 4 days. On the day following the last injection the rats were autopsied, the organs (ovaries, uterus, thyroid and adrenals) removed, freed of connective tissue and weighed on a Roller-Smith torsion balance. A careful examination of the sellar region of the hypophysectomized rat was routinely made as a further check of the completeness of the operation. In addition, both tibiae were dissected and prepared for measurements.

3. The tibia test as a bioassay for growth hormone

A generalized decrease of epiphyseal activity in the long bones of cats and dogs following hypophysectomy was first described by Dott & Fraser (1923). This observation supplemented by Lucke & Hückel (1933) on the stimulating effect of the growth hormone on the tibial cartilage in rats and similar observations by Freud et al. (1939) on hypophysectomized rats, became the basis of the classical bioassay of the pituitary growth hormone (Evans et al. 1943) in which the width of the epiphyseal cartilage of rats reflects growth stimulation (Li & Evans 1948).

In the present study the tibia test, about three times more sensitive than the body-weight method, was used for the quantitative evaluation of the activity of growth hormone in hypophysectomized rats. In the tibia test the procedures outlined by Greenspan et al. (1949) were followed in principle. On the day following the last injection the rats were sacrificed, both tibiae were dissected and split, fixed in 10% formalin and treated with 2% silver nitrate. Eight to ten individual measurements of the thickness of epiphyseal cartilage of both tibiae were made and the data statistically analyzed. At the end of each series the level of probability *P* was obtained from the Fisher *t* test table of significance of differences between means.

The pituitary homogenate used as stimulatory factor in hypophysectomized rats was collected from male albino rats weighing in excess of 500 g. The anterior lobe was separated, weighed and kept frozen in separate vials. At the time of use the material was thawed and ground in the proper amount of sterile Gey's solution in a glass homogenizer. For example, in Experiment 1, four vials were prepared containing 17.67 to 18.63 mg pituitary tissue each, and placed in deep freeze. On each of the four days of the experiment, the tissue from one vial was thawed, ground in 4 ml sterile Gey's solution, approximately 0.45 mg in 0.1 ml. This dose was given subcutaneously per rat twice daily, totalling for the four days of experimentation, approximately 3.6 mg per rat.

The only departure from this procedure occurred in the preparation of the pituitary material used in Experiment 6. In this case the glands were removed from rats and weighed after the vascular system had been perfused with Gey's solution.

4. Serological methods

Complement fixation tests were performed as previously described (Anigstein et al. 1958). Ring tests and diffusion-in-gel techniques were used for detecting precipitating antibodies.
Antigens for the ring tests were diluted serially from 1 mg/ml to 0.0005 mg/ml in phosphate buffered saline, and placed in 0.1 ml amounts in precipitin tubes. The undiluted serum was delivered to the bottom of the tube through the antigen dilutions and the interfacial tests read after 1, 3, and 24 hours at room temperature.

The techniques used for Ouchterlony double diffusion-in-gel, were based on standard procedures (M. W. Wilson and B. H. Pringle 1954) using 2% washed filtered Noble agar (Difco) in 0.85% buffered saline pH 7.4, containing 0.1% merthiolate, 25 ml to a standard Petri plate. Antigens and antisera in 0.1 ml amounts were placed 1.5 cm apart in wells 6 mm in diameter. Plates were read or photographed after 8 to 14 days at room temperature. The antigens used were: rat anterior pituitary homogenate (prepared as for immunizing rabbits), 20 mg/ml in merthiolate saline; growth hormones (Li & Armour), 100 µg/ml; undiluted rat serum and beef globulin (Armour). All rabbit antisera were undiluted.

Rat liver was homogenized, washed in saline and the sediment was used in the amount of 20 mg/ml of merthiolate saline.

EXPERIMENTS AND RESULTS

Preliminary experiments were devoted to the adjustment of dosages of rat pituitary homogenate and of APS to appropriate levels for evaluation of the stimulating effects of the homogenate and of the inhibitory effects of APS. In evaluating the rat organ weights at autopsy and the tibial response it was found necessary to reduce the original homogenate dosage from the total of 10.8 mg/rat to approximately 3.6 mg/rat.

The base-line level of tibial width was established in control groups of animals given Gey’s solution or APS only. In such groups the average values for the epiphysial width were found between 168 and 156 µ, these data agreeing with those obtained by Hayashida & Li (1958) for hypophysectomized rats injected with saline or growth hormone antiserum.

Experiment I (Table 1)

The purpose of this experiment was to test the activity of rat pituitary homogenate injected into hypophysectomized rats in the presence of measured amounts of homologous antiserum. Groups I and II were injected with a total of 3.6 mg of the pituitary tissue and group II received in addition 2.5 ml APS. This treatment resulted in an increase in weight of the ovaries, uterus, adrenals and thyroid as compared with the control group III. The growth stimulating effect of pituitary homogenate became particularly evident in group I, the tibia test showing 265 µ for the epiphysial width as compared with 159 µ of group III treated with normal rabbit serum. Data for the tibia were averaged for each animal of the three groups and the standard error of the group means calculated. Further statistical analysis showed probability values *P* between means of organ weights and tibial widths of different groups of rats (Table 1a).
Table 1.
Stimulatory effect of rat pituitary homogenate and its inhibition by rat pituitary antiserum (APS) as evaluated by organ weights and tibial tests in hypophysectomized rats.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Material injected</th>
<th>Number of rats</th>
<th>Body wt. g</th>
<th>Ovaries mg</th>
<th>Uterus mg</th>
<th>Adrenals mg</th>
<th>Thyroid mg</th>
<th>Tibial width μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rat pituitary homogenate&lt;sup&gt;1&lt;/sup&gt;</td>
<td>6</td>
<td>92</td>
<td>16.3 ± 2.85&lt;sup&gt;*&lt;/sup&gt;</td>
<td>160.1 ± 5.02</td>
<td>11.6 ± 0.54</td>
<td>6.6 ± 0.39</td>
<td>265 ± 4.04</td>
</tr>
<tr>
<td>II</td>
<td>Pituitary homogenate plus APS&lt;sup&gt;2&lt;/sup&gt;</td>
<td>4</td>
<td>97</td>
<td>7.9 ± 0.60</td>
<td>54.7 ± 2.15</td>
<td>13.0 ± 0.60</td>
<td>5.5 ± 0.48</td>
<td>204 ± 23.00</td>
</tr>
<tr>
<td>III</td>
<td>Normal rabbit serum</td>
<td>5</td>
<td>84</td>
<td>6.5 ± 0.85</td>
<td>30.6 ± 1.78</td>
<td>10.6 ± 0.75</td>
<td>4.9 ± 0.44</td>
<td>159 ± 8.50</td>
</tr>
</tbody>
</table>

All rats hypophysectomized at age 28 days; first injection 2 weeks postoperatively.

<sup>1</sup> Dosage: 0.45 mg pituitary homogenate per rat, s.c., 2 × daily for 4 days. Total: 3.6 mg/rat.

<sup>2</sup> Dosage: 0.5 ml APS per rat, i.p., daily for 5 days, starting on the day before homogenate injections.

<sup>*</sup> Standard error.
Table 1a.
Statistical analysis showing level of significance $P$ for differences between means of controls compared with treated groups of rats.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Thyroid</th>
<th>Adrenals</th>
<th>Ovaries</th>
<th>Uterus</th>
<th>Tibial width</th>
</tr>
</thead>
<tbody>
<tr>
<td>I vs. II</td>
<td>$&gt;0.10$</td>
<td>N.S.</td>
<td>$&lt;0.05^*$</td>
<td>$&lt;0.001^{**}$</td>
<td>$&lt;0.02^*$</td>
</tr>
<tr>
<td>I vs. III</td>
<td>$&lt;0.02^*$</td>
<td>N.S.</td>
<td>$&lt;0.05^*$</td>
<td>$&lt;0.001^{**}$</td>
<td>$&lt;0.001^{**}$</td>
</tr>
<tr>
<td>II vs. III</td>
<td>$&gt;0.3$</td>
<td>$&lt;0.05^*$</td>
<td>$&gt;0.20$</td>
<td>$&gt;0.10$</td>
<td>$&lt;0.10$</td>
</tr>
</tbody>
</table>

* Significant at 0.05 level of probability $P$.
** Highly significant at 0.01 level of $P$.
N.S. = not significant.

The stimulatory effect of the rat pituitary homogenate was also evident from its gonadotrophic action as revealed by the weights of the ovaries and the uterus (Tables 1 and 1a) with a highly significant increase of the uterus ($P < 0.001$) and a lesser increase of the ovaries ($P < 0.05$). Of other organs, the thyroid showed significant stimulation ($P < 0.02$), whereas the adrenals were not significantly affected.

As seen from Table 1a, the rat pituitary homogenate significantly stimulates the thyroid, an action which in group II is only slightly inhibited by APS. In this experiment, the largest adrenals were found in rats of group II. This would indicate that APS not only failed to inhibit the stimulatory effect of the homogenate, but on the contrary, it seems to have enhanced the slight stimulatory effect of the homogenate (group I, Table 1). The gonadotrophic effect of the rat pituitary homogenate was evidenced by the increased weights of the uterus and ovaries; however, when rats of group II were treated with APS in addition to the pituitary homogenate, a drastic suppression of the gonadotrophic effect was noted. This was most probably due to the antihormonal activity of APS. A significant suppression of the growth hormone activity was noted by the tibial test when APS was given to the hypophysectomized rats in combination with the homogenate (265–204 μ, Table 1).

Experiment 2 (Tables 2 and 2a)

In this experiment, the globulin fraction of APS was prepared and used in conjunction with the homogenate (Table 2, group 2). The serum was fractionated in order to concentrate the antibody content, to increase the purity of the preparation and decrease the toxicity of the antiserum. A total of 27 hypophysectomized rats, arranged in four groups was used in this experiment. Six rats were treated with rat pituitary homogenate alone (group 1), five rats were injected with rat pituitary homogenate plus the globulin fraction (APS C)
**Table 2.**
Stimulatory effect of rat pituitary homogenate and its inhibition by APS G and STH antiserum as evaluated by organ weights and tibial tests of hypophysectomized rats.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Material injected</th>
<th>Number of rats</th>
<th>Body wt. g</th>
<th>Ovaries mg</th>
<th>Uterus mg</th>
<th>Adrenals mg</th>
<th>Thyroid mg</th>
<th>Tibial width μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rat pituitary homogenate†</td>
<td>6</td>
<td>97</td>
<td>20.5 ± 2.57*</td>
<td>186.2 ± 5.35</td>
<td>11.5 ± 0.50</td>
<td>0.2 ± 0.34</td>
<td>288 ± 16.58</td>
</tr>
<tr>
<td>II</td>
<td>Rat pituitary homogenate plus APS G‡</td>
<td>5</td>
<td>95</td>
<td>10.0 ± 0.64</td>
<td>63.0 ± 4.32</td>
<td>12.5 ± 1.13</td>
<td>5.3 ± 0.19</td>
<td>213 ± 12.45</td>
</tr>
<tr>
<td>III</td>
<td>Rat pituitary homogenate plus STH antiserum§</td>
<td>10</td>
<td>84</td>
<td>11.8 ± 0.31</td>
<td>72.7 ± 12.81</td>
<td>11.2 ± 0.31</td>
<td>5.4 ± 0.22</td>
<td>212 ± 5.56</td>
</tr>
<tr>
<td>IV</td>
<td>Normal rabbit serum</td>
<td>6</td>
<td>84</td>
<td>7.7 ± 0.63</td>
<td>83.7 ± 1.81</td>
<td>10.9 ± 0.21</td>
<td>5.7 ± 0.19</td>
<td>165 ± 3.17</td>
</tr>
</tbody>
</table>

All rats hypophysectomized at age 28 days; first injection 2 weeks postoperatively.
1 Dosage: 0.41 mg pituitary homogenate per rat, subcutaneously, 2 × daily, for 4 days. Total: 3.3 mg/rat.
2 Gamma globulin of rat pituitary antiserum (APS) 0.5 ml i. p. daily for 4 days.
3 Bovine somatotrophin STH antiserum 0.5 ml . p. 2 × daily for 4 days.
* Standard error.
Table 2a.
Statistical analysis of Table 2 showing significance $P$ for differences between means of controls with treated groups of rats.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Thyroid</th>
<th>Adrenals</th>
<th>Ovaries</th>
<th>Uterus</th>
<th>Tibial width</th>
</tr>
</thead>
<tbody>
<tr>
<td>I vs. II</td>
<td>&gt; 0.05</td>
<td>&lt; 0.01**</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.01**</td>
<td></td>
</tr>
<tr>
<td>I vs. III</td>
<td>&gt; 0.05</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.001**</td>
<td></td>
</tr>
<tr>
<td>I vs. IV</td>
<td>&gt; 0.20</td>
<td>&gt; 0.10</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.005**</td>
<td>&lt; 0.001**</td>
</tr>
<tr>
<td>III vs. IV</td>
<td>&gt; 0.50</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.025*</td>
<td>&lt; 0.001**</td>
<td></td>
</tr>
<tr>
<td>II vs. IV</td>
<td>&gt; 0.10</td>
<td>&lt; 0.05*</td>
<td>&lt; 0.001**</td>
<td>&lt; 0.02*</td>
<td></td>
</tr>
</tbody>
</table>

* Significant at 0.05 level of probability $P$.
** Highly significant at 0.01 level of $P$.

...of APS (group II) and ten rats (group III) received in addition to the homogenate the somatotrophin antiserum prepared by immunization of rabbits with purified bovine growth hormone (Armour). Finally, the last group of six rats (group IV) injected with normal rabbit serum served as controls (Table 2).

The main significance of the data presented in Table 2 relates to growth hormone and to gonadotropic activity of the hormone contained in the rat pituitary homogenate. Whereas no significant differences between any of the group means in the weights of the thyroid and the adrenals were found, the homogenate stimulated considerably the uterus and the ovaries as manifested by the increased weights of these organs. Inasmuch as the lowest tibial response (165 $\mu$) was shown by group IV, the highest growth stimulation was obtained in group I injected with rat pituitary homogenate alone and showing a tibial width of 288 $\mu$. Between these two extremes of suppressed or stimulated growth activity are the intermediate values of groups II and III. A highly significant inhibition of tibial plate growth (213 $\mu$) was achieved by the globulin APS G which also gave an almost complete inhibition of gonadotropic stimulation as indicated by weights of ovaries and uteri (Table 2).

Similarly to the action of APS G, the bovine somatotrophin antiserum inhibited the stimulating effect of the pituitary homogenate as seen from the tibial test as well as from the ovarian and uterine weights. The suppression of ovarian and uterine growth of group III as compared with group I indicates a probable contamination of this somatotrophin with gonadotropic hormones.

It is of interest to note that whereas APS G and somatotrophin antiserum both exerted the same degree of inhibition on the tibial plate growth, they differed markedly in their effect on the body-weight of the rats (95 g as compared to 84 g). Since it is recognized that the tibial response is the more sensitive measure of growth hormone activity, this may indicate a greater
inhibitory capacity of STH antiserum. The failure of both APSG and STH antiserum to inhibit more completely the tibial response may be due to the presence of other hormones in the pituitary homogenate capable of influencing the tibial reaction.

Experiment 3 (Table 3)

It was of interest to compare the activity of the purified bovine growth hormone (somatotrophin, Armour) with the rat pituitary homogenate used in previous experiments. Two dose levels of STH were used, namely 100 μg and 400 μg per rat in the standard assay procedure. A series of 16 hypophysectomized rats was divided into three groups of which group I was the non-injected control. There was only a slight increase in the tibial plate width (186 μ) in rats injected with 100 μg per rat (group II), but when the dose was increased to 400 μg, the value of the tibial width rose to 223 μ. Although 400 μg per rat showed a significant stimulation, it was considerably less than that produced by the pituitary homogenate used in previous experiments. Further analysis of somatotrophin (Armour) showed no significant effects on the adrenal, ovarian, uterine or thyroid weights thus indicating that the degree of contamination with ACTH, FSH and TSH was too low to be detected in this type of assay at these dosages. It seems therefore that hormonal contamination which is not revealed by such direct means, may be more readily detected by a bioassay in which physiological inhibition of these hormones by antiserum is utilized as the index of contamination (group I and III, Table 2).

Experiment 4 (Table 4)

In this experiment 18 hypophysectomized assay animals were divided in three groups of which group I served as the uninjected control. Two dosage levels (48 μg and 96 μg) of the highly purified bovine STH preparation were given to the test animals (groups II and III). This preparation was without effect on the weights of ovaries, uterus, adrenals and thyroid, but a highly significant stimulation of tibial growth was observed in both injected groups with a higher degree of stimulation in the group treated with the higher dosage. Also a graded effect of the STH preparation on the body weight of rats was found.

Experiment 5 (Table 5)

The purpose of this experiment was to test the inhibitory capacity of APS globulin given in conjunction with rat pituitary homogenate or with purified somatotrophin. As seen from Table 5 it was found again that the dose of pituitary homogenate used (3.3 mg/rat) gave significant stimulation of tibial plate (293 μ as compared with 159–171 μ for basal values in our previous experiments). When the highly purified bovine somatotrophin was given (group
Table 3.
Effect of bovine somatotrophin (growth hormone, STH) on organ weights and on tibial growth of hypophysectomized rats.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Material injected</th>
<th>Number of rats</th>
<th>Body wt. g</th>
<th>Ovaries mg</th>
<th>Uterus mg</th>
<th>Adrenals mg</th>
<th>Thyroid mg</th>
<th>Tibial width μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>4</td>
<td>82</td>
<td>7.3 ± 0.32*</td>
<td>24.0 ± 0.70</td>
<td>10.7 ± 0.89</td>
<td>5.4 ± 0.18</td>
<td>171 ± 1.22</td>
</tr>
<tr>
<td>II</td>
<td>STH 100 μg/rat**</td>
<td>6</td>
<td>86</td>
<td>7.2 ± 0.49</td>
<td>25.6 ± 1.45</td>
<td>9.9 ± 0.31</td>
<td>5.3 ± 1.84</td>
<td>186 ± 6.67</td>
</tr>
<tr>
<td>III</td>
<td>STH 400 μg/rat***</td>
<td>6</td>
<td>88</td>
<td>8.3 ± 0.46</td>
<td>33.5 ± 1.50</td>
<td>11.2 ± 0.59</td>
<td>4.9 ± 2.00</td>
<td>223 ± 10.35</td>
</tr>
</tbody>
</table>

Hypophysectomy at age 28 days. First injection of hormone 2 weeks postoperatively.
* Standard error. **12.5 μg 2 × daily for 4 days, s.c. ***50 μg 2 × daily for 4 days, s.c.
† Armour Co.

Significance test.

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Ovaries</th>
<th>Uterus</th>
<th>Tibial plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>I vs. III</td>
<td>&gt; 0.05</td>
<td>&lt; 0.01</td>
<td>&lt; 0.005</td>
</tr>
</tbody>
</table>

Significant at 0.05 level. Highly significant at 0.01 level.
Table 4.
Effect of highly purified beef growth hormone† on hypophysectomized rats.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Material injected</th>
<th>Number of rats</th>
<th>Body wt. g</th>
<th>Ovaries mg</th>
<th>Uterus mg</th>
<th>Adrenals mg</th>
<th>Thyroid mg</th>
<th>Tibial width μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>6</td>
<td>71</td>
<td>8.0 ± 0.58*</td>
<td>32.3 ± 2.79</td>
<td>10.6 ± 0.49</td>
<td>5.7 ± 0.54</td>
<td>168 ± 7.99</td>
</tr>
<tr>
<td>II</td>
<td>STH</td>
<td>7</td>
<td>79</td>
<td>8.4 ± 0.21</td>
<td>36.6 ± 1.73</td>
<td>11.0 ± 0.58</td>
<td>5.6 ± 0.22</td>
<td>211 ± 6.37</td>
</tr>
<tr>
<td>I</td>
<td>STH</td>
<td>48 μg³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>STH</td>
<td>5</td>
<td>89</td>
<td>7.1 ± 0.31</td>
<td>25.2 ± 0.63</td>
<td>11.7 ± 0.29</td>
<td>5.9 ± 0.26</td>
<td>283 ± 6.74</td>
</tr>
<tr>
<td></td>
<td>STH</td>
<td>96 μg²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All rats hypophysectomized at age 28 days; first injection of hormone 2 weeks postoperatively.

† Dosage: 6 μg s.c. 2 × daily for 4 days. Total: 48 μg per rat.
‡ Dosage: 12 μg s.c. 2 × daily for 4 days. Total: 96 μg per rat.
†† This preparation was kindly supplied by Professor C. H. Li of the Hormone Research Laboratory, Berkeley, Calif., U.S. A.
* Mean standard error.
** Highly significant.
Table 5.
Effect of APS G† on organ weights and tibial width of hypophysectomized rats treated with pituitary homogenate or STH.∗

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Material injected</th>
<th>Number of rats</th>
<th>Body wt. g</th>
<th>Ovaries mg</th>
<th>Uterus mg</th>
<th>Adrenals mg</th>
<th>Thyroid mg</th>
<th>Tibial width μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rat pituitary homogenate†</td>
<td>5</td>
<td>92</td>
<td>17.7 ± 1.40</td>
<td>136.4 ± 17.70</td>
<td>11.6 ± 0.54</td>
<td>5.9 ± 0.35</td>
<td>293 ± 15.60</td>
</tr>
<tr>
<td>II</td>
<td>STH‡</td>
<td>5</td>
<td>83</td>
<td>7.6 ± 0.95</td>
<td>32.2 ± 1.43</td>
<td>10.6 ± 0.45</td>
<td>5.1 ± 0.30</td>
<td>229 ± 7.38</td>
</tr>
<tr>
<td>III</td>
<td>STH plus APS G§</td>
<td>6</td>
<td>84</td>
<td>8.2 ± 0.24</td>
<td>42.3 ± 0.14</td>
<td>10.9 ± 0.50</td>
<td>4.4 ± 0.29</td>
<td>185 ± 6.72</td>
</tr>
<tr>
<td>IV</td>
<td>Rat pituitary homogenate plus APS G§</td>
<td>4</td>
<td>94</td>
<td>11.3 ± 0.60</td>
<td>51.0 ± 0.75</td>
<td>11.8 ± 0.31</td>
<td>5.3 ± 0.45</td>
<td>269 ± 17.39</td>
</tr>
</tbody>
</table>

† Antirat pituitary serum globulin.
∗ Somatotrophin highly purified bovine growth hormone (supplied by C. H. Li).
N. S. = not significant.
Hypophysectomy at age 28 days.
† Dosage: 0.41 mg/rat 2 × daily for 4 days. Total: 3.3 mg/rat.
‡ Dosage: 5 μg 2 × daily for 4 days s.c. Total 48 μg/rat.
§ Dosage: 0.5 ml i.p. initially, 0.2 ml/day for 3 days.

Significance test – P values.

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Ovaries</th>
<th>Uterus</th>
<th>Tibial width</th>
</tr>
</thead>
<tbody>
<tr>
<td>I vs. IV</td>
<td>&lt;.005</td>
<td>&lt;.005</td>
<td>N. S.</td>
</tr>
<tr>
<td>II vs. III</td>
<td>&gt;.001</td>
<td>&gt;.001</td>
<td>&gt; 0.05</td>
</tr>
</tbody>
</table>
II), no stimulatory effect was noted on organs weight although a marked stimulation of tibial width was produced (229 µ as compared with our base line). The combination of rat pituitary homogenate and APS globulin resulted in a marked inhibition of ovarian and uterine weights and a reduction of tibial width (269 against 293 µ) indicating the growth inhibitory capacity of the globulin preparation. The same dosage of APS globulin produced a significant inhibition of the growth stimulating effect of purified bovine somatotrophin (185 µ compared with 229 µ).

Experiment 6 (Table 6)

This experiment was designed to test the inhibitory capacity of STH antiserum as measured by its ability to counteract the stimulatory effect of rat pituitary homogenate on the one hand or its homologous antigen (STH Li) on the other hand.

It can be seen from the results of this experiment that the injected pituitary homogenate again caused a significant growth of the ovaries and the uterus indicating gonadotrophic hormonal activity which has been slightly inhibited by the STH antiserum. This inhibitory effect may reflect a possible contamination of the growth hormone with gonadotrophic hormones, even though no stimulation of the ovaries and the uterus resulted from administration of STH in groups III & IV.

Contrary to our previous experience, there seems to be in this series a significant stimulation of the adrenals in rats of groups I and II treated with pituitary homogenate. The same applies to the thyroid which showed some increase in groups I and II treated with pituitary homogenate. It should be noted that the rats used for the pituitary material were perfused (Gey’s solution) whereas in earlier experiments perfusion was not performed. The only other difference between this and earlier experiments was the fact that the assay animals used in this series were smaller at the start of the experiment although their terminal weights were comparable to those of previous series. Perhaps these differences may have contributed to the greater effect of the homogenate on the adrenals and on the thyroid than previously established.

In regard to the tibial width values, the stimulatory effect of the homogenate was similar to that previously observed. No inhibitory effect of the STH antiserum was noted when combined with pituitary homogenate (group II). A lower but significant level of tibial stimulation was found in group IV injected with purified STH. When STH antiserum was given in conjunction with STH, a complete inhibition of the growth stimulation was observed.

Serological studies. – Studies were made on antisera produced in rabbits using rat anterior pituitary tissue and bovine growth hormone (STH Armour and STH Li) as antigens to investigate their serological relationships. Complement fixation, precipitin ring tests and Ouchterlony double diffusion-in-gel techni-
**Table 6.**

Effect of STH\(^*\) rabbit antiserum on organ weights and tibial width of hypophysectomized rats treated with rat pituitary homogenate and/or with STH.

<table>
<thead>
<tr>
<th>Rat groups</th>
<th>Material injected</th>
<th>Number of rats</th>
<th>Body wt. g</th>
<th>Ovaries mg</th>
<th>Uterus mg</th>
<th>Adrenals mg</th>
<th>Thyroid mg</th>
<th>Tibial width μ</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Rat pituitary homogenate(^†)</td>
<td>6</td>
<td>87</td>
<td>18.2 ± 0.21</td>
<td>128.8 ± 24.25</td>
<td>13.6 ± 0.75</td>
<td>6.9 ± 0.23</td>
<td>294 ± 13.25</td>
</tr>
<tr>
<td>II</td>
<td>Rat pituitary plus anti-TH(^†)</td>
<td>6</td>
<td>91</td>
<td>14.5 ± 0.54</td>
<td>105.6 ± 13.20</td>
<td>14.3 ± 0.50</td>
<td>5.9 ± 0.26</td>
<td>318 ± 4.50</td>
</tr>
<tr>
<td>III</td>
<td>STH(^‡) plus anti-TH(^†)</td>
<td>6</td>
<td>81</td>
<td>7.1 ± 0.54</td>
<td>29.5 ± 1.41</td>
<td>11.4 ± 0.41</td>
<td>5.4 ± 0.21</td>
<td>156 ± 4.12</td>
</tr>
<tr>
<td>IV</td>
<td>STH(^‡)</td>
<td>5</td>
<td>86</td>
<td>6.9 ± 0.20</td>
<td>31.2 ± 0.36</td>
<td>11.1 ± 0.54</td>
<td>5.1 ± 0.13</td>
<td>208 ± 8.72</td>
</tr>
</tbody>
</table>

\(^*\) Somatotrophin highly purified bovine growth hormone (supplied by C. H. Li).
\(^†\) Bovine somatotrophin antiserum 0.5 ml i. p. daily for 4 days.
\(^‡\) Dosage: 0.41 mg/rat 2 × daily for 4 days. Total: 3.3 mg/rat.

**Significance test – P values.**

<table>
<thead>
<tr>
<th>Rat group</th>
<th>Tibial width</th>
</tr>
</thead>
<tbody>
<tr>
<td>III vs. IV</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
Complement-fixation techniques were found most suitable for evaluating APS, whereas precipitin ring tests were employed for the evaluation of bovine growth hormone antisera. The complement-fixing titers in the antisera of pituitary injected rabbits were 1:300–1:640. With the STH antisera as little as 5 to 10 µg of STH antigens, could be detected as shown by positive ring tests. These titrations are similar to those of Hayashida & Li (1958) concerning the antigenicity of bovine growth hormone.

In analysing APS (8 samples from different rabbits) by the Ouchterlony technique, distinct lines of precipitation were observed in the contact zone between the rat pituitary homogenate antigen and APS, with no line formation between APS and other rat proteins (Fig. 1). Occasionally, in some APS samples, faint lines in the diffusion zones between antisera and other rat proteins, such as liver and serum, could be seen. The usual absence of precipitation lines on contact of APS and rat proteins other than pituitary tissue seems to indicate the presence of organ specific precipitins. No precipitation lines were formed between APS and the purified growth hormone antigens.

With the bovine growth hormone (STH Armour and STH Li) antisera, the

**Fig. 1.**
Plate 7. Gel diffusion reaction between APS (4); rat pituitary homogenate (1), rat liver homogenate (2) and normal rat serum (3).
Plate 10. Gel diffusion reaction between bovine STH Armour antisera (4); bovine STH Li (1), bovine globulin (2) and bovine STH Armour (3).

Ouchterlony double diffusion-in-gel technique revealed a variety of patterns. Antisera from 5 rabbits immunized with STH Armour and 2 rabbits immunized with STH Li were studied. With antisera – STH Armour in the center well, two or three precipitation lines consistently appeared between antisera and specific antigen, with a well defined line appearing between antisera and STH Li antigen (Fig. 2). This line converged with at least one precipitation line appearing between STH Armour antigen and its specific antisera. This demonstrates the interference phenomenon of Ouchterlony (1958), indicating according to this author a reaction of identity with one or more common components in the antigens. Precipitation lines between the antisera-TH Armour and bovine protein (beef globulin, Armour) may be absent (Fig. 2) or present in a definite line not converging with lines formed between hormone antigen and antisera (Fig. 3), indicating non identity of components.

When antiserum-STH Li was placed in the center well, with STH Li and STH Armour in adjacent positions, multiple lines appeared in the diffusion zone between STH antigens and antiserum-STH Li (Fig. 4). These patterns were similar to those between diffusing antiserum-STH Armour and the growth hormone antigens, in that the lines converged in a pattern indicating
common identical components. No lines were seen in the area between diffusing antiserum-STH Li and bovine protein (Fig. 4).

In may be concluded that STH Li may contain two or more antigenic components, one or more of which show patterns of identity to those of STH Armour. Furthermore, the antiserum—STH Li was shown to be free of non specific bovine precipitins.

Precipitation lines appeared at times in the diffusion zone between rat antigens (pituitary homogenate and normal rat serum) and both bovine growth hormone antisera. The significance of these lines which appeared with some but not all antisera to bovine growth hormones, and one or both rat proteins deserve further investigation.

**DISCUSSION**

The experimental assay used in the present study gave us first of all, the opportunity to demonstrate the presence of the hormone complex contained in the rat pituitary tissue. At least two groups of hormones could be evidenced by their effect on certain organs of hypophysectomized rats, namely, the

![Fig. 3.](image)

Plate 24. Gel diffusion reaction between bovine STH Armour antiserum (4); bovine STH Li (1), bovine globulin (2) and bovine STH Armour (3).
somatotrophic and gonadotrophic complex. The activity of somatotrophin was particularly clearly seen by evaluation of the tibial measurements, whereas the effect of the gonadotrophic complex (FSH and LH) was shown by the stimulation of ovarian and uterine weights. Consequently, a gain in weight of ovaries and the uterus in animals given pituitary homogenate reflects gonadotrophic stimulation, while an increase in the weight of thyroid and adrenals similarly indicates thyrotrophic and corticotrophic activities.

If the antiserum contains specific antibodies acting as antihormones, the inhibition or neutralization of the pituitary hormones should be feasible and demonstrable by adequate assay. Consequently, a given antiserum was tested for its capacity to neutralize the hormones which previously served as antigens for the production of antibodies. The specificity of the antibodies for the respective hormone depends on the purity of its antigenic structure (Heijkenskjöld & Genzel 1958), but even then, the possibility is not excluded that unspecific substances play an additive role in the neutralization mechanism.

It was possible in the present study to neutralize by specific antiserum the somatotrophic and gonadotrophic hormones of the pituitary homogenate injected into test animals. However, the lower sensitivity of the adrenal and

Plate 12. Gel diffusion reaction between bovine STH Li antiserum (4); bovine STH Li (1), bovine globulin (2) and bovine STH Armour (3).
thyroid glands did not permit the demonstration of ACTH and thyrotrophin consistently in this type of assay. Only in one experiment (Table 1) was a statistically significant stimulation of the thyroid obtained although a slight increase in the thyroid weights was always found in the groups given pituitary homogenate. Similarly, adrenal gland stimulation was only occasionally seen in the groups given rat pituitary homogenate. In the last experiment (Table 6), a significant adrenal stimulation was obtained and it was noted that in this case the pituitary gland was removed after perfusion of the rats with Gey’s solution. It is possible that a greater corticotrophin (ACTH) effect noted in this experiment may be explained by the removal with the blood of enzymes capable of partially inactivating this hormone. It is probable that the assay does not give an adequate index of either thyrotrophin or ACTH and may not be used in measuring the inhibitory capacity of antisera with respect to these hormones.

It was shown that the antirat pituitary serum (APS) is capable of inhibiting in vivo the stimulatory effect of both somatotrophin and the gonadotrophin contained in the pituitary homogenate injected into the rat. This inhibition was almost complete in the case of gonadotrophic effects where neutralization of the injected hormone was obtained with either the raw APS or its globulin fraction (Tables 1, 2, 5). In the case of somatotrophic hormones, it was found that only about 50% inhibition of tibial cartilage growth could be obtained with the dosage of APS employed. This may have been the result of an inadequate dose of the antiserum, or of the action of thyrotrophin and adrenal hormones also affecting tibial growth (Geschwind & Li 1955). Higher dose levels of APS were contraindicated by the inherent toxicity of this antiserum (Anigstein et al. 1958). Preliminary attempts to reduce toxicity by absorption techniques, and by the use of the globulin fraction of APS have failed to reduce toxicity appreciably. It should be noted that while the APS was able to inhibit tibial growth considerably, it was without effect on the body-weight of the animals treated with the antiserum for 4 days. This is in contrast to the effect of STH (Armour) antiserum which inhibited body growth as well as tibial growth (Table 2) when given in combination with rat pituitary homogenate. The inhibitory activities of APS in these in vivo experiments are consistent with the serological evidence which demonstrates the presence in APS of organ specific precipitating antibodies. It suggests that some of these antibodies may be directed against the several protein hormones.

The injections of the two preparations of STH (Armour and Li) gave a good measure of their relative potencies in stimulating the tibial plate (Tables 3 and 4). In these assays no indication of any contaminating hormones was seen.

* Effects of rat pituitary antibody in tissue culture were investigated by C. M. Pomerat et al. (1958).
from examination of the organ weights. Compared to the effect obtained with STH (Li) on the tibial plate, the rat pituitary tissue appeared to contain about 96 μg of growth hormone per 3.3 mg (293 μ compared to 265–294 μ).

Antisera to both somatotrophins (STH Armour and Li) were tested for their capacity to inhibit the stimulatory effects of rat pituitary homogenate (Tables 2 and 4). It was found that STH (Armour) antiserum inhibited both the somatotrophic and gonadotrophic effects of the rat pituitary, while STH (Li) antiserum was without any significant effect. This antiserum did cause some inhibition of ovarian and uterine growth, but these effects were not statistically significant. It is important to note that this same antiserum at the same dosage completely inhibited the effect of the injected STH (Li), as seen from Table 6. It is apparent therefore that these two antisera (STH Armour and Li) differ markedly in their capacity to inhibit the hormones contained in the rat pituitary homogenate. This would indicate that the degree of species specificity may depend in part at least on the purity of the hormone and on the chemical process to which it is subjected in the purification procedure.

While it has been recently demonstrated that bovine STH is hormonally effective in the rat (Hayashi & Li 1959), the present study has shown that an antiserum to rat pituitary tissue (APS) is capable of inhibiting highly purified bovine STH Li (Table 5). This inhibition occurred even though no serological evidence of precipitating antibodies to the bovine STH (Li) was found in APS. In this connection it has been established by Van den Ende (1941) that the precipitating antibodies are not necessarily parallel to the biological neutralizing activities of the antiserum (Cruickshank & Currie 1958). It should also be noted that antisera to both of the purified preparations of somatotrophin contained precipitating antibodies against both hormone preparations. It is not surprising therefore that they neutralized in vivo their homologous antigens.

The concept of growth was used continuously in the present study as a phenomenon correlated with the biological activities of the hypophyseal growth hormone. Under the influence of the hormones contained in the pituitary, the increased dimensions of rat organs resulting from the gain in mass and in weight have been utilized as indices of the hormonal activities. In addition to metabolic processes such as protein synthesis, tissue growth is based on cell multiplication and mitosis (Weiss 1955). Whereas under normal conditions these mechanisms are controlled by the regulatory functions of the hormones, the excessive production of the latter leads to a physiological disorganization of the complex structure. The question arises whether a substance biologically antagonistic to the growth hormone in suppressing its functions can be utilized as an inhibitor of an uncontrolled cell multiplication in malignancy. In this regard the pituitary antiserum offers possibilities for the exploration of its anticellular and antihormonal functions. The practical application of these possibilities is now under investigation.
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