A Relationship between Thyroid Function and a Naturally Occurring Inhibitor of Hypophysial Proteinases*

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The proteinases of the pituitary gland have received increasing attention in recent years and much of the work has been directed toward finding a physiological function for the enzymes. Adams and Smith (1) described both peptidase and proteinase activities in the gland. Ellis (2) purified one of the proteinases and described a number of its physical and chemical properties. A peptidase that cleaves bovine growth hormone at a methionyl bond has also been studied by Ellis (3). Reichert (4) found a difference between mammalian and fish hypophysial proteinases and has suggested that the enzymes possessing an alkaline pH optimum may be characteristic of the mammal. Kobayashi et al. (5) noted a correlation between proteolytic activity of the pituitary gland and testicular growth, suggesting that the enzymes may be involved in the release of the gonadotropins. Related to this is the report by Reichert (6) of increased proteolytic activity in the ovary upon gonadotropic stimulation.

A substance in serum that inhibits the fragmentation of growth hormone and prolactin has been found. In this communication we wish to present evidence which shows that the concentration of this inhibitor is influenced by the physiological status of the thyroid gland.

EXPERIMENTAL PROCEDURE

Hormone Preparations—1. Bovine Growth Hormone. The bovine growth hormone used as substrate in the study of the inhibitor in serum was prepared by the procedure suggested by Hays and Steelman (7) which combines steps developed by Campbell and Davidson (8) and Wilhelmi et al. (9). A detailed description of the method is presented here since the complement to the electrode vessel, a normal pattern was obtained. Incubation Procedure—An 0.5% solution or suspension of the hormone was prepared in 0.05 M phosphate buffer, pH 7. The sample was then divided into 0.2-ml aliquots. Most of the hormone preparations were quite insoluble at pH 7, and it was necessary to make as smooth a suspension as possible in order to obtain reproducible results. In practice, the hormone was first mixed with a small portion of the buffer and made into a smooth paste with the aid of a glass rod. The sample was then diluted to the final concentration.

To each 0.2-ml aliquot of the suspension were added 2 μl of serum that had been diluted 1:10 with the phosphate buffer. A drop of toluene was added and the tubes were stoppered and incubated for 8 hours at 37°. After incubation aliquots, usually 25 μl, were analyzed by disk electrophoresis. For comparison of the relative amounts of inhibitor in different sera, our bovine growth hormone and diluted serum were used.

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Incubations at pH 4 were also carried out in the same manner except that 0.14 M acetate buffer, pH 4, was substituted for the phosphate buffer.

As reported previously (10), the degradation of growth hormone by contaminating proteinases was noted to take place in a stepwise fashion or by a decrease in the intensity of all the bands. Serum inhibited both these reactions. The over-all decrease in intensity of the bands required a shorter incubation time and was the measurement usually employed.

Maintenance of Rats and Collection of Sera and Pituitary Glands—Mature male Holtzman rats weighing 200 to 250 g were used. The animals were fed a basal diet of Purina mink chow to which various supplements were added. Propylthiouracil was used at 0.03% of the diet; thyroid powder (U.S.P. desiccated) at 0.1% and 1%; and 2,4-dinitrophenol at 0.02%. Just before death, the rats were anesthetized with ether and blood was withdrawn from the left renal vein and allowed to clot. The serum was collected by centrifugation and kept at 5° until used. The serum was found to be unstable so it was used within 18 hours after collection. For electrophoretic analysis of the serum alone, 2 μl were used. Samples of human serum, obtained from blood drawn by venous puncture, were treated in the same manner. The concentration of protein in the serum was determined by the method of Sutherland et al. (13).

The anterior lobe of the pituitary gland of the rat was freed from the posterior lobe and frozen until used. Since the growth hormone and prolactin were found to undergo fragmentation even when frozen, the glands were analyzed as soon as possible after collection. For electrophoretic analysis the anterior lobe was homogenized in 0.05 M phosphate buffer, pH 7.5, using a microhomogenizer and a buffer to tissue ratio of 0.02 ml of buffer per mg of tissue. An aliquot of 25 μl was used per column.

Protein-bound Iodine—The method of Zak et al. (14) was employed.

Use of Thyroxine—A 10⁻³ M solution of thyroxine was made by suspending 4 mg of the sodium salt in 0.25 ml of 0.05 M phosphate buffer, pH 7.5, adding 25 μl of 1 N NaOH to dissolve the material, and finally bringing the volume to 2.5 ml with additional buffer. Higher dilutions were made if needed. An aliquot of 10 μl of the solution of thyroid hormone was then added to 0.2 ml of a 0.5% solution of growth hormone or prolactin in the same phosphate buffer. Since pH altered the rate of fragmentation of the pituitary hormones, an equal amount of alkali was used in the sample of thyroxine was added to control samples of the pituitary hormones. This was done by adding 25 μl of 1 N NaOH to 2.5 ml of phosphate buffer, pH 7.5, and using 10 μl of this buffer-NaOH mixture or a higher dilution, with 0.2 ml of a 0.5% solution of pituitary hormone. To determine whether thyroxine itself altered the electrophoretic pattern of bovine growth hormone, thyroxine was added to a sample of the hormone just before electrophoresis.

Ultrafiltration—Ultrafiltration was carried out by the procedure described by Peterson and Sober (15).

Fractions from Human Serum—The fractions were purchased from the California Corporation for Biochemical Research. They were designated as γ-globulins (Fraction II), β-globulins (Fraction III), α-globulins (Fraction IV), and albumin (Fraction V). The material was dissolved in 0.05 M phosphate buffer, pH 7, to make a 0.5% solution. Aliquots of 10 μl were added to 0.2 ml of a suspension of bovine growth hormone.

RESULTS

Inhibition of Fragmentation by Normal Serum—Fragmentation of the pituitary hormones was inhibited at pH 7 by serum from the human and the rat. As shown in Fig. 1, the inhibition could be detected by prevention of an over-all decrease in intensity or the bands of growth hormone or by inhibition of the stepwise fragmentation. Every attempt was made to use serum that was not contaminated with lysed red cells. There was always the chance, however, that a few red cells, whole or disrupted, were present in the serum. For this reason a lysate of washed red cells was tested for its effect on the degradation of bovine growth hormone. The lysate was made so that, after removal of particulate material by centrifugation, the solution had an optical density of 3.3 (1-cm light path) at 540 mp. An aliquot of 2 μl of the whole suspension was then added to 1 mg of bovine growth hormone and incubated for 5 hours at 37°. No inhibition of acceleration of the degradation was seen. Since the amount of hemoglobin corresponding to this optical density could not go undetected, we feel certain that unnoticed contamination by red cells has not influenced the results.

The effectiveness of serum as an inhibitor varied with the hormone preparation used. This is summarized in Table I. The bovine growth hormone and ovine prolactin prepared in these laboratories were most effectively inhibited. The addition of 2 μl of a 1:10 dilution of serum to 0.2 ml of a 0.5% solution of hormone strongly inhibited the breakdown both at room temperature and at 37°. For the same degree of inhibition to be seen in the growth hormone preparations obtained from the National Institutes of Health,1 2 μl of whole serum were needed. As with other inhibitors (10), an effect with serum was most difficult to demonstrate with human growth hormone prepared by the method of Raben.

Serum did not inhibit the disappearance of the bands of growth hormone (10) when the incubation was carried out at pH 4. The serum was also ineffective at pH 10.

1 Gift from the Endocrinology Study Section, National Institutes of Health. Preparations from this source are designated "NIH."
Inhibitor of Hypophysial Proteinases

Vol. 238, No. 10

Bovine GHf ........................

Human GH (Raben). ...............

Human GH, .......................

Ovineprolactin ....................

Ovine GH ..........................

0.596 solution of the hormone.

inhibition.

moderately inhibitory; +, weakly inhibitory; f, questionable

inhibition from a normal rat; all sera but most markedly in the case of the hypothyroid animal.

frankly hyperthyroid rat. Note that inhibition was produced by various sera. a, Our hormone preparation incubated at 37° for 3 hours.

b, hormone incubated with serum from a hypothyroid rat (propylthiouracil for 2 weeks); c, hormone incubated with serum from a normal rat; d, incubated with serum from a frankly hypothyroid rat. Note that inhibition was produced by all sera but most markedly in the case of the hypothyroid animal.

Properties of Inhibitor in Serum—The inhibitor did not pass through a cellophane membrane during ultrafiltration or dialysis. The inhibitory substance could be detected inside the sacking in both cases; the ultrafiltrate was not inhibitory. The inhibitory action of serum decreased after standing at 5° for 2 days. When the serum was stored frozen at -10°, a decrease in inhibitory properties was also noted after 2 weeks. Lyophilized serum also lost potency when stored for 2 weeks at 5°. Heating serum at 100° for 1 minute completely destroyed the inhibitor.

Fractions of human serum were tested and only the a-globulin fraction (Fraction IV) was effective. Fractions II, III, and V were noninhibitory when tested at a concentration equal to that of Fraction IV (50 µg per mg of growth hormone).

Table I

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Degree of inhibition</th>
<th>Amount of whole serum added (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine GHf</td>
<td>+++</td>
<td>0.2</td>
</tr>
<tr>
<td>Bovine GH (NIH-B5)</td>
<td>++</td>
<td>2.0</td>
</tr>
<tr>
<td>Ovine GH</td>
<td>++</td>
<td>2.0</td>
</tr>
<tr>
<td>Ovine GH (NIH-83)</td>
<td>++</td>
<td>2.0</td>
</tr>
<tr>
<td>Ovine prolactin</td>
<td>++</td>
<td>0.2</td>
</tr>
<tr>
<td>Human GH</td>
<td>+</td>
<td>2.0</td>
</tr>
<tr>
<td>Human GH (Raben)</td>
<td>±</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* The symbols used are: +++, strongly inhibitory; ++, moderately inhibitory; +, weakly inhibitory; ±, questionable inhibition.

† The indicated amounts of serum were added to 0.2 ml of a 0.5% solution of the hormone.

‡ GH, growth hormone.

The increase in the inhibitor was noted after 1 week of feeding propylthiouracil and the concentration of the inhibitor remained abnormally high for 2 to 3 weeks. The serum was tested after feeding the drug for 3 days but no increase was observed. After 3 weeks, a gradual decline was seen until the serum contained a less than normal amount of the inhibitory substance. No such fluctuations were observed during the feeding of 1% thyroid powder.

The inclusion of 0.02% dinitrophenol in the diet of the rats produced an effect on the inhibitor which was similar to that noted with thyroid powder; that is, the amount of inhibitor in the serum decreased. A period of 3 weeks instead of 2 was required before an unequivocal lowering was observed. The value for the protein-bound iodine was 2.8 µg per 100 ml after feeding dinitrophenol for 3 weeks.

No correlation could be made between the amount of inhibitor and the total concentration of protein in the serum.

Effects of Thyroxine, Dinitrophenol, Propylthiouracil, Iodide, and Thyrotropin in Vitro—The absence of the inhibitor in serum of frankly hyperthyroid rats prompted us to test the action of thyroxine on the proteinases that contaminate growth hormone preparations. When a suspension of bovine growth hormone was made 10⁻⁴ M in L-thyroxine at pH 7.5 and incubated at 37° for 3 hours, fragmentation of the hormone was accelerated. Lower concentrations (10⁻⁵ M and lower) of thyroxine were without effect. Fragmentation of ovine growth hormone and prolactin was also accelerated by 10⁻⁴ M L-thyroxine. D-Thyroxine² speeded the proteolysis but to a lesser extent than did the natural isomer.

Dinitrophenol behaved similarly to thyroxine with the exception that the concentration of the compound had to be increased to 10⁻² M before an acceleratory effect was noted. NaI at 10⁻⁴ M enhanced the rate of proteolysis of bovine growth hormone. Propylthiouracil when added directly to growth hormone at a concentration of 10⁻⁴ M was neither inhibitory nor acceleratory. Thyrotropin had no effect when present at 50 milliunits per mg of growth hormone.

Effect of Thyroid Status on Electrophoretic Pattern of Serum—

² We wish to thank Dr. L. D. Bechtel of the Baxter Laboratories, Inc., for this sample.
The electrophoretic patterns of sera from hypothyroid, normal, and frankly hyperthyroid rats showed distinct differences as shown in Fig. 3. The serum from animals fed dinitrophenol gave a normal pattern. The pattern of serum from mildly hyperthyroid rats was between those obtained from normal and frankly hyperthyroid animals.

**DISCUSSION**

The few properties reported here for the inhibitor in serum suggest that it is proteaseous. Only when our purification studies have progressed further will we be able to compare the properties of the inhibitor with those of the proteolytic inhibitors in serum that have already been reported (16-21).

The reason for the over-all decrease in the intensity of the bands of growth hormone at 37° and a stepwise degradation at room temperature is not known. We assume that at 37° over-all proteolysis is so rapid that the more orderly fragmentation is masked. Since serum and other proteolytic inhibitors (10) slow the breakdown at both temperatures, we believe that the use of either temperature will give the same information in regard to effectiveness of an inhibitor.

The poor inhibitory action of serum in preventing fragmentation of human growth hormone may be a result of instability of the inhibitor and the slow rate of degradation of the hormone at pH 7. It was found necessary to incubate human growth hormone for at least 36 hours at 37° before noticeable fragmentation occurred. Since the inhibitor appears to be quite unstable, this long period of incubation may cause inactivation. Raising the pH of the incubation medium to accelerate fragmentation of the growth hormone was of no help for it was found that the inhibitor became less effective as the pH was raised.

The variable results obtained when determining the amount of inhibitor in the serum of thyroidectomized rats is probably a result of incomplete removal of thyroid tissue in some of the animals. Administration of propylthiouracil insures a more complete suppression of thyroid activity.

The rise in the amount of inhibitor in serum parallels the disappearance of the “thyroid-labile” protein (22, 23) in the pituitary gland of the rat. We feel that this band has been positively identified as growth hormone since, after isolation from disk electrophoretic columns, it was found (24) to be active in the tibia1 line assay for growth hormone and the material fragmented as does growth hormone from other species. Continuous et al. (25) have found a reduction in the amount of growth hormone in the hypophysis after thyroidectomy and the material fragmented as does growth hormone from other species. Contopoulos et al. (25) have found a reduction in the amount of growth hormone in the hypophysis after thyroidectomy which is consistent with the decrease in the growth hormone band noted by disk electrophoresis after thyroidectomy or feeding propylthiouracil. The inhibitor, then, can be shown to rise as growth hormone decreases and once the hormone has disappeared from the gland, the inhibitor decreases.

We do not think that the low level of inhibition observed with serum from hyperthyroid rats is a result of increased amounts of thyroxine. The concentration of thyroxine in serum is about 10⁻² μg when the protein-bound iodine is 6 μg per 100 ml. The accelerator effect of thyroxine on the proteinas in vitro was noted only at a concentration of 10⁻⁴ M. Furthermore, serum from dinitrophenol-fed rats was also low in the inhibitor and the amount of thyroxine in this serum was slightly below normal.

The potentiation of the proteolytic fragmentation of growth hormone by thyroxine, dinitrophenol, and iodide in vitro must be a nonspecific effect. In support of this is the report by Lieber-
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