ACTA CHEMICA SCANDINAVICA 17 (1963) S165–S169

The Effect of Hypophysectomy on the Concentration in Serum of an Inhibitor of Hypophyseal Proteinases

U. J. LEWIS, J. N. FISHER and W. P. VANDERLAAN

Division of Endocrinology, Scripps Clinic and Research Foundation, La Jolla, California, USA

The fragmentation of growth hormone by hypophyseal proteinases is inhibited by rat serum. Hypophysectomy causes an initial two-fold rise in the amount of inhibitor in the serum of the rats. Administration of bovine growth hormone or bovine thyrotropin does not prevent the increase. The inhibitor from human serum was partially purified by chromatography on DEAE-cellulose and by preparative disc-electrophoresis.

The serum of normal rats has been found[1] to contain a substance which inhibits the fragmentation[2] of growth hormone by hypophyseal proteinases. The inhibitor was shown to be heat-labile and non-dialyzable. The concentration of the inhibitory substance was also found to be influenced by the function of the thyroid gland[1]. The inhibitor was high in early stages of induced hypothyroidism and abnormally low in rats made hyperthyroid. In this communication we report that hypophysectomy causes an initial increase in the concentration of the inhibitor in serum. A method for partially purifying the inhibitory substance in human serum is also described.

MATERIALS AND METHODS

Electrophoretic analysis. The fragmentation of growth hormone was observed by electrophoretic analysis on columns of polyacrylamide. The method has been named "disc-electrophoresis" by its inventors, Ornstein and Davis[3]. The apparatus is available commercially from Canal Industrial Corporation, Bethesda, Maryland. The use of the technique in the study of pituitary hormones has been described[1,2,4].

Preparative disc-electrophoresis. Initial attempts to isolate the inhibitor from whole serum were unsuccessful because of instability of the substance. We found that a commercially available fraction of human serum (α-globulins, Fraction IV, California Corporation for Biochemical Research, Los Angeles, California) was rich in the inhibitor and at this stage of purification the substance was not inactivated during subsequent processes of isolation.

Disc-electrophoresis can be used as a preparative method by cutting out sections of unstained columns and eluting the protein. Usually an identical column is stained for the purpose of locating the components that are resolved by the electrophoresis. This was not necessary

for the α-globulin-fraction. By adding bromphenolblue to the sample, the albumin was stained blue and since transferrin has a reddish-brown color, we had two markers for determining where to cut sections from the columns of gel.

Columns 1 cm in diameter and 7 cm in length (small pore gel[3]) were used. To each column 2 mg of the α-globulin-fraction was added. Electrophoresis was carried out exactly as described by Ornstein and Davis[3]. When the buffer-front was within 1 cm of the end of the column, the polyacrylamide gel was removed from its glass tube and 4 sections were cut from the gel (Fig. 1). The protein in the sections of gel was eluted electrophoretically into dialysis sacking as described previously[4]. The recovered protein was dialyzed overnight against water and then lyophilized. All processes were carried out at 5°C.

Chromatography on DEAE-cellulose. The inhibitor in the α-globulin-fraction was adsorbed by DEAE-cellulose that had been equilibrated with 0.005 M phosphate buffer, pH 7. Many electrophoretically slowly migrating components, including transferrin, were eluted from the column with 0.04 M K_2HPO_4. The inhibitor together with albumin were then removed with 0.2 M K_2HPO_4. A 3-fold purification was achieved by this procedure.

Bovine growth hormone. This hormone has been found[2] to be contaminated with varying amounts of proteinases depending on the procedure used in its isolation. The preparation used in these studies was isolated by a combination of the methods of Campbell and Davidson[5] and Wilhelmi *et al.*[6] as suggested by Hays and Steelman[7]. The kind and amount of contaminating proteinases make a convenient system for studying the fragmentation of the hormone.

Incubation procedure. Bovine growth hormone readily undergoes fragmentation[2] by contaminating proteinases when allowed to stand at neutrality or at weakly alkaline conditions and the degradation is inhibited by the addition of serum[1]. These observations form the basis of the test used to detect the inhibitor in serum. The test is carried out by first thoroughly suspending the hormone in 0.05 M phosphate buffer, pH 7, to give a concentration of 5 mg/ml. Then to 0.2 ml of this suspension is added 2 μl of a 1:10 dilution of whole serum prepared in the same phosphate buffer. A drop of toluene is added, and the tubes are stoppered and placed at 37°C for 8 hours. After incubation 25 μl-aliquots are analyzed by disc-electrophoresis to determine the degree of degradation of the growth hormone.

Maintenance of rats and collection of serum. Mature, male rats weighing about 200 g were used. They were fed a diet of Purina mink chow. Hypophysectomy was performed either by

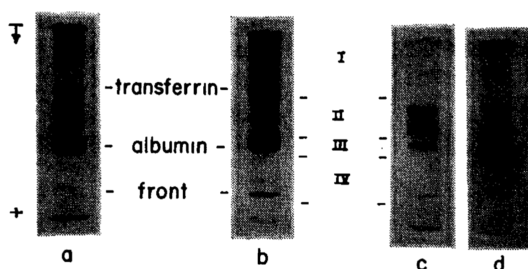

Fig. 1. Disc-electrophoretic patterns of human serum and fractions obtained from serum. *a*, a sample of whole serum; *b*, the α-globulin-fraction used as starting material for isolation of the inhibitor; *c*, the proteins that were isolated by preparative electrophoresis from section II of the column shown in *b*; *d*, albumin isolated from section III.

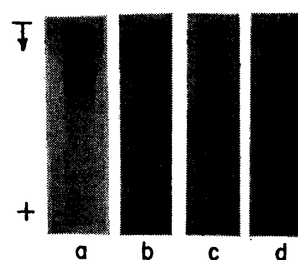

Fig. 2. Effect of rat serum on the fragmentation of bovine growth hormone. *a*, electrophoretic pattern of unincubated hormone; *b*, the same material after incubation at 37°C for 8 hours; *c*, the hormone incubated with serum from a normal rat; *d*, the hormone incubated with serum from a hypophysectomized rat.

the parapharyngeal or by the transaural[8] route. At sacrifice the rats were anesthetized with ether, and blood was removed from the left renal vein, and the sella turcica was examined for completeness of hypophysectomy. After the blood had clotted, the serum was collected by centrifugation. The inhibitor is inactivated upon prolonged standing in whole serum; therefore, the serum was used as soon after collection as was practical. Rats that were treated with growth hormone or thyrotropin received intraperitoneal injections daily. Dosage of growth hormone was 0.5 mg/day and of thyrotropin, 25 milliunits/day.

RESULTS

Inhibition by serum from hypophysectomized rats. We have found[1] that bovine growth hormone degrades in a stepwise manner when allowed to stand at room temperature, whereas an overall decrease in intensity of the bands is observed when the hormone is incubated at 37°C. Serum inhibits both reactions. In these studies we have used the higher temperature because of the shorter time required to observe fragmentation. Prevention of the decrease in intensity of the bands of growth hormone was used as the criterion for inhibition.

Fig. 2 illustrates how serum inhibits the overall decrease in the intensity of the bands of growth hormone and also how much more strongly the serum from hypophysectomized rats inhibits as compared to serum from normal rats. The rats had been hypophysectomized two weeks before sacrifice. The normals were the same age. The increase in the amount of inhibitor in the serum of hypophysectomized animals was estimated to be at least two-fold. The estimate was made by diluting the serum of hypophysectomized rats until the degree of inhibition matched that of the normal animals. Administration of bovine growth hormone or bovine thyrotropin during the two weeks following hypophysectomy did not prevent the rise in the amount of inhibitor. Normal human serum inhibited the degradation process in the same manner as did rat serum.

The protein-bound iodine[9] in the serum of normal rats was 3.2 μg/100 ml of serum; hypophysectomy reduced the value to 0.8 μg/100 ml. Administration of bovine growth hormone or thyrotropin did not restore the amount to normal limits.

Essentially the same rise in inhibitor was noted in the serum of rats that had been hypophysectomized either by the parapharyngeal or aural route. There is, perhaps, a greater possibility of damage to the hypothalamus when the rats are operated upon through the otic canal, but apparently if any such damage did occur, it did not prevent the rise in the amount of inhibitor in the serum. No increase in the inhibitor was noted after a sham operation by the transaural approach which left the pituitary gland undisturbed.

The increase in inhibitory properties of the serum was not due to a rise in total serum-protein since no more than a 5 % increase in total protein was noted in certain animals and the higher values of protein did not always correlate with increased inhibitory action. Heat-inactivated serum was not inhibitory indicating that the inhibition was not caused by an increased amount of substrate for the proteinases.

Fig. 1*a* shows the disc-electrophoretic pattern of a sample of human serum. Photograph *b* is the pattern obtained with the α-globulin-fraction of human serum used as starting material for the isolation of the inhibitor. As can be seen,

the material does not appear much different from normal serum except for the transferrin. The section designated as II had the highest concentration of the inhibitor. The disc-electrophoretic pattern of the proteins of this section after they were isolated by preparative disc-electrophoresis is shown in *c*. Either albumin was not completely separated from this section when the columns were cut, or perhaps minute quantities of albumin were adsorbed by the polyacrylamide as the albumin passed through the column. This albumin would then have been eluted and concentrated during the isolation of the protein in section II.

2 μg of protein from section II, when incubated with 1 mg of growth hormone, produced approximately the same inhibition as 22 μg of the starting material. 14 μg of material from section III gave an inhibition equivalent to 2 μg of section II.

Fig 1*d* represents the pattern obtained when the albumin-band (section III) was analyzed after isolation by preparative disc-electrophoresis. Unexpectedly, two slowly migrating bands were observed. Since these components could not have migrated to the area of albumin before the column was cut into sections, we assume that they are peptides or proteins that had been adsorbed upon the albumin and which became dissociated during the process of isolation.

DISCUSSION

The increase in the amount of inhibitor after hypophysectomy is similar to the rise seen when rats are made hypothyroid by feeding propylthiouracil[1]. These observations suggest that when the concentration of thyroxine in serum is lowered, either by loss of thyrotropin after hypophysectomy or by suppression of thyroid activity by propylthiouracil, one of the physiologic responses is the production of an increased amount of the inhibitor. If this suggestion proves to be correct, the failure of bovine thyrotropin to prevent the rise in inhibitor could be explained by the fact that the protein-bound iodine values were uneffected by administration of the thyrotropin. Related to this is the observation of VanderLaan and Greer[10] that thyrotropin does not cause a decrease in iodine content of the thyroid gland of the rat after hypophysectomy. It will be of interest to determine if thyroxine prevents the rise in the inhibitor in hypophysectomized animals. In any case we feel that the data presented here definitely indicate that the proteolytic inhibitor is under endocrine control, and this in turn suggests that control of the enzymatic fragmentation of growth hormone has physiologic significance.

The presence of some inhibitor in section III may be a result of imperfect sectioning of the columns. Another possibility is that there is more than one inhibitor and that one migrates in section II while the other travels in the area of albumin. Martin[11] has also made this suggestion to explain results he obtained during the isolation of an inhibitor of trypsin in ovine serum. It is quite logical to assume that a number of different proteolytic inhibitors are needed to control the many types of proteinases in the body.

Acknowledgments. This work was supported by Grants A-1328 and 2A-5249 from the *United States Public Health Service* and by a grant from the *Timken-Sturgis Foundation.* One of us (U. J. L.) acknowledges the receipt of a Career Development Award from the *National Institutes of Health.*

REFERENCES

1. Lewis, U. J. and VanderLaan, W. P. *J. Biol. Chem. In press.*
2. Lewis, U. J. *J. Biol. Chem.* **237** (1962) 3141.
3. Ornstein, L. and Davis, B. J. *Disc Electrophoresis,* preprinted by Distillation Products Industries, Rochester, New York, 1962.
4. Lewis, U. J. and Clark, M. O. *Anal. Biochem. In press.*
5. Campbell, J. and Davidson, I. W. F. *Abstracts of the 19th International Physiological Congress,* Thérien Frères, 1953, p. 257.
6. Wilhelmi, A. E., Fishman, J. B. and Russel, J. A. *J. Biol. Chem.* **176** (1948) 735.
7. Hays, E. E. and Steelman, S. L. In Pincus, G. and Thimann, K. V. *The Hormones,* Academic Press, Inc., New York, 1955, Vol. III, p. 203.
8. Tanaka, A. *Annual Report of Shinonogi Research Laboratory,* 1955, p. 5.
9. Zak, B., Willard, H. H., Myers, G. B. and Boyle, A. J. *Anal. Chem.* **24** (1952) 1345.
10. VanderLaan, W. P. and Greer, M. A. *Endocrinology* **47** (1950) 36.
11. Martin, C. J. *J. Biol. Chem.* **236** (1961) 2672.

Received March 18, 1963.