Proteolytic Inhibitors in Serum
I. Effect of Food Intake and of Hypophysectomy*

JACKLYN B. MELCHIOR AND ROBERTA. SLIWINSKI
(Biochemistry Department, Graduate School and Stritch School of Medicine, Loyola University, Chicago, Ill.)

It has been known for many years that serum possesses the property of inhibiting several proteolytic enzymes (6, 10, 12, 20, 23). Many theories as to the role of the serum inhibitors have been proposed (9); however, at present their source and function remain unknown.

In 1908, Brieger and Trebling (1) reported that the antiproteolytic activity of serum was increased by malignant disease. Since that time many attempts have been made to use serum inhibitor levels to aid in diagnosis of cancer (3, 4, 6, 20-23). In general, it has been concluded that significant changes occur in the antiproteolytic activity in some, but not all, cases of cancer, and that similar changes occur in other, apparently unrelated, conditions. It is thus apparent that, although a correlation exists, the presence or absence of malignancy is not the only variable in determining the level of such inhibitors in serum.

With this in mind, a systematic study of the effect of a variety of conditions on the antiproteolytic activity of serum has been undertaken. It is felt that only after a clarification of this aspect of the problem can the relationship of the proteolytic inhibitors of serum to the cause or the effect of cancer be understood.

The present paper deals specifically with the effect of restriction of food intake and of the endocrine balance on the inhibition of chymotrypsin or trypsin by serum.

METHODS

Enzymes.—Crystalline trypsin of bovine origin, containing not more than 50 per cent MgSO4 or "Tryptar" (lyophylized crystalline trypsin), were procured from the Armour Laboratories and were dissolved in 0.001 M HCl to give solutions of 75 or 50 µg/ml, respectively. Crystalline chymotrypsin, salt-free, obtained from Nutritional Biochemical Company, was dissolved in 0.001 M HCl to give a solution of 15 µg/ml. The enzyme solutions were always used immediately after preparation.

Substrates.—Hammersten casein (Nutritional Biochemical Company) was dissolved in a 0.1 M tris-hydroxymethylaminomethane (tris) buffer, pH 7.6. A 4 per cent solution was used for trypsin, while a 1.5 per cent solution proved more satisfactory for chymotrypsin.

Inhibitors.—The animals used throughout this work were albino rats, procured from the Loyola Medical School Animal Colony or from the Hormone Assay Company of Chicago. Unless otherwise noted, they were fed ad libitum throughout. The hypophysectomized animals and their controls received the diet recommended by Astwood,1 and the others were given fox chow pellets.

Blood was drawn from the tail of the rats, permitted to clot, and centrifuged in the cold. The serum was diluted with a buffer containing 0.075 M tris and 0.075 M NaCl, pH 7.6, and used on the same day. In routine measurements a 1-200 dilution was used.

The procedure consisted of mixing 1.0 ml. each of substrate, enzyme, and either serum diluted with buffer or buffer alone in a 12-ml centrifuge cone. The reaction was carried out at 37° C., and all solutions were at or near this temperature before being mixed. Proteolysis was stopped by the rapid addition of 3.0 ml. of 10 per cent trichloroacetic acid (TCA). After standing overnight, the tubes were centrifuged, and the optical density of the supernates was determined in a Beckman Model DU Spectrophotometer equipped with a photomultiplier attachment, with a wave length of 2760 Å and a slit width of 0.085 mm. Values were corrected for suitable blanks (i.e., tubes in which TCA was added before the enzyme), and the data were expressed as µg. tyrosine released by the enzyme. Values for tyrosine were read from a standard curve. All measurements were made in duplicate.

* Supported by a grant-in-aid from the Illinois Division of the American Cancer Society.

Received for publication June 10, 1954.
In the chymotrypsin assay, the serum and substrate were mixed, and the reaction was started by the addition of enzyme. In the trypsin assay, however, the enzyme was mixed with diluted serum and pre-incubated before starting the reaction by the addition of substrate. Both the pre-incubation and proteolysis periods were timed exactly. The proteolysis period was 15 minutes in both assay procedures.

Measurement of chymotrypsin inhibitor by casein proteolysis requires little comment. Typical results are shown in Chart 1 and were obtained routinely with either rat or human serum.

The measurement of the trypsin inhibitor, however, is known to be inherently more difficult. A previous worker, having observed sigmoid curves when the inhibitor units were plotted against serum concentration, have resorted to running a series of dilutions in order to determine the inhibitor concentration in a single sample (3, 21, 22). McCann and Laskowski (13) have pointed out several empirical factors which can contribute to the shape of the curves obtained. It is apparent from the nature of trypsin (16) that one of the difficulties arises from the relatively slow rate of combination of the inhibitor with the enzyme (5). If the complication of a variable inhibition occurring during the proteolysis period is to be avoided, a pre-incubation of enzyme with inhibitor at pH levels which favor the self-digestion of the trypsin is necessary.

When the effect of varying the pre-incubation time was studied, curves such as shown in Chart 2 were always obtained. This has included determinations on human and rat serum, and the use of phosphate as well as tris buffers. It is probable that the decrease in inhibitor as pre-incubation time is increased beyond 5 minutes is an artifact arising from a more rapid self-digestion of trypsin in the control tubes. Previous workers have failed to correct for the change in activity of trypsin upon pre-incubation in absence of inhibitor and, hence, have not observed this effect (5). The self-digestion of trypsin is known to be affected by many factors (9), and it is reasonable to assume that one or more substances present in the serum may protect the trypsin, resulting in an apparent decrease in inhibitory power. The fact that McCann and Laskowski did not obtain a sigmoid curve when a partially purified preparation of serum inhibitor was used (13) would appear to lend support to the present explanation of the nonlinearity of the results. This effect, plus the measurable rate of combination of enzyme and inhibitor, leads to an optimum time of pre-incubation.

![Chart 1](chart1.png)

**Chart 1.**—Effect of serum on the activity of chymotrypsin.

![Chart 2](chart2.png)

**Chart 2.**—Effect of pre-incubation time on trypsin and on serum antitryptic activity. Curve 1, trypsin activity in control tubes. Curves 2 and 3, inhibitor activity with 0.01 and 0.005 ml. rat serum, respectively. One unit is defined as the activity equivalent to 1 µg. of tyrosine in the test system described in the text.

The plot of trypsin inhibition against serum concentration for a sample of rat serum for two different pre-incubation periods is shown in Chart 3. It can be seen that a 5-minute pre-incubation results in a curve approaching linearity more closely than the 30-minute period and, in fact, permits determination of trypsin inhibitor level within a few per cent by a single measurement in
the region of 0.005-ml. serum. It is noted that the system described fulfills the requirements for linearity as given by McCann and Laskowski (13). However, as is evident from the above discussion, the shape of the curve is a function of an unknown number of variables and, hence, may vary from serum to serum.

On the other hand, a consideration of our results has led to the conclusion that the antitryptic and antichymotryptic activities reside in the same substance. In the experiments reported here both values were obtained on each animal, and in no case was there evidence to invalidate this conclusion. This is in complete agreement with the work of Shulman (18). Typical results are shown in Table 1. A comparison of the per cent changes occurring after hypophysectomy with the curves of Charts 1 and 3 indicates that the relative decrease in chymotrypsin and trypsin inhibition is compatible with the assumption that the same entity is responsible for both effects. Although the substrate requirements for the two enzymes are markedly different (15), it has been demonstrated repeatedly that many trypsin inhibitors occurring in nature will also inhibit chymotrypsin (11). The only other explanation for the results obtained with serum would seem to be the less likely possibility of several substances all having the same relative inhibitory power toward the two enzymes.

It is thus apparent that determination of the extent of inhibition of either trypsin or chymotrypsin is a valid measure of the concentration of this substance in serum. Because of the greater simplicity of the antichymotrypsin assay, we have elected to report this quantity for the experiments described below. For brevity the quantity measured will be referred to as antitryptic activity.

**RESULTS**

**Effect of fasting.**—A group of 24 young adult male rats were chosen for this experiment. After establishing normal values for the antitryptic activities of the group, one half of the animals were deprived of all food, although permitted water ad libitum. During the course of the fast the animals lost about 30 per cent of their body weight, and their blood became thick and difficult to draw from the tail. However, the data (Chart 4) show clearly that the fast exerted no detectable effect on the inhibitor level of the serum.

**Effect of hypophysectomy.**—Two groups of hypophysectomized rats and suitable controls were assayed for serum antitryptic activity (Table 1). The inhibitor level was decreased significantly in every case.

Since removal of the pituitary gland invariably results in a decrease in the levels of the inhibitor, it was of importance to test the possibility of reversing the effect with pituitary material. A group of twelve hypophysectomized animals was selected, and the inhibitor level of each rat determined. A homogenate was prepared in 0.25 M sucrose from a pituitary gland taken from a freshly killed rat and injected subcutaneously into each of four rats. A similar homogenate prepared from rat liver was injected into another group of four, while the remaining four received injections of sucrose only. Injections were given daily. The results (Chart 5) indicate clearly that pituitary contents specifically reverse the effect of hypophysectomy.

**Effect of growth hormone.**—Crystalline growth hormone was dissolved in dilute base and neutralized just prior to injection into hypophysectomized female rats. The amount used (500 µg/day) was sufficient to cause growth in the animals, as

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Trypsin inhibition (per cent)</th>
<th>Chymotrypsin inhibition (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 Males, 30-60 days posthypophysectomy</td>
<td>2.0 ± 0.4*</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>12 Controls for above</td>
<td>37 ± 1*</td>
<td>40 ± 0.5</td>
</tr>
<tr>
<td>12 Females, 24 days posthypophysectomy</td>
<td>20 ± 1†</td>
<td>30 ± 0.6</td>
</tr>
<tr>
<td>12 Controls for above</td>
<td>30 ± 1†</td>
<td>55 ± 0.5</td>
</tr>
</tbody>
</table>

Data expressed as per cent inhibition produced by 0.005 ml serum and presented as the mean ± the standard error.

* 5-Minute pre-incubation period.
† 5-Minute pre-incubation period.
indicated in Chart 6. It is also apparent that the antitryptic activity of the serum was markedly increased by the injections.

The question then arose as to whether or not the growth hormone itself might be the trypsin inhibitor. However, when tested directly in the casein proteolysis system, even in amounts equivalent to the total daily dose used to elicit the effect in rats, no effect was noted.

It has also been established that the effect of the growth hormone is not instantaneous. Measurements of chymotrypsin inhibitor level up to 8 hours after a single injection showed no increase in inhibitor activity.

**DISCUSSION**

The question of the relationship of food intake to the proteolytic inhibitors of serum has appeared several times in the literature. In 1910, Rosenthal reported (17) that fasting led to a decrease in antitryptic activity of rabbit serum. On the other hand, Grob (8) and Shulman (19), among others, have attributed the rise in antitrypsin found in certain cachectic conditions including many cases of cancer as due to tissue necrosis and autolysis.

The present experiments, in which no change was noted during a fast resulting in a loss of 30 per cent of body weight, indicate that food intake and tissue wastage per se have no direct relationship to the inhibitor levels. However, it is possible that a prolonged caloric deficit would have an effect not observed in a brief, acute fast. It is known that prolonged protein and vitamin deficiencies will result in changes in pituitary ("pseudo-hypophysectomy" [14]). It seems possible that such changes might be reflected in alterations in the serum inhibitor levels.

In view of the marked decrease observed in hypophysectomy and the reversal by whole pituitary injection, it seems a warranted assumption that the level of this inhibitor in serum is related to the activity of one or more of the pituitary hormones, either directly or through the target organs. The increasing evidence for a key role of the endocrine system in many types of cancer (7) suggests the possibility that the increases associated with malignant disease may reflect associated alterations in the endocrine balance.

The results of injection of growth hormone point to this substance as one factor which has a
positive effect on the inhibitory power of serum. However, it is apparent that it is not the growth hormone per se but some metabolic product of its action that is related to the antitryptic activity of serum. It is interesting to compare these results with the work of Shulman (19), who noted a positive correlation between antitrypsin of serum and fibrinogen concentration or sedimentation rate. Campbell (2) has reported that sedimentation rate and fibrinogen concentration are increased by growth hormone injections. Thus, Shulman’s correlation appears to hold throughout another variable.

SUMMARY

Measurements of antitryptic and antichymotryptic activity of serum have been carried out employing casein proteolysis. The results led to the conclusion that only one entity is involved in these phenomena.

Fasting for 6 days had no effect on the antitryptic activity of serum. Hypophysectomy resulted in a marked decrease in the antitryptic activity, reversible by injections of whole pituitary.

Injections of crystalline growth hormone led to an increase in the inhibitory activity of serum of hypophysectomized rats. No direct effect of growth hormone on chymotrypsin was observed.

ACKNOWLEDGMENTS

The authors would like to express their sincere appreciation to the Wilson Meat Packing Company of Chicago for a gift of many of the hypophysectomized rats used in this work and to Dr. M. B. Williamson for a gift of the growth hormone.

REFERENCES

Proteolytic Inhibitors in Serum: I. Effect of Food Intake and of Hypophysectomy

Jacklyn B. Melchior and Robert A. Sliwinski


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/14/9/677

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.