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Studies on the Inactivation of Thyrotropin-Releasing Hormone (TRH)* (33891)

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Thyrotropin-releasing hormone (TRH)[1] has been found in the hypothalamus of several species including man (1–4). Porcine, bovine, and ovine TRH have been obtained in a high state of purity (2, 5–7). Although TRH stimulates release of thyrotropin (TSH) from the anterior pituitary *in vivo* and *in vitro*, it was never clearly shown to exist in peripheral or hypophyseal portal blood. The main difficulty in demonstrating its presence in blood is due to the fact that the bioassay methods available for its detection are not sensitive enough to detect minute quantities of TRH activity. Another difficulty is that TRH is rapidly destroyed by an enzyme present in plasma and some tissues (8, 9). This report describes some of the characteristics of the blood enzyme responsible for inactivation of TRH. The term "enzyme," as used in this report, does not imply that we are dealing with a purified enzyme system.

Materials and Methods. TRH bioassay. The TRH bioassay is an indirect procedure based on the ability of TRH, injected *in vivo*, to elicit a release of TSH from the anterior pituitary gland. The elevation of endogenous TSH increases the rate of release of labeled hormone from the thyroid gland. This is measured by an increase in blood radioactivity over that of control values. Female white Swiss mice weighing 15–20 g on arrival in the laboratory, were fed a low iodine diet for 3–4 weeks. The day before the assay the mice were injected intraperitoneally with 5 μCi of sodium iodide ^{125}I along with 0.085 μg of triiodothyronine (T3) dissolved in 0.01 *N* NaOH:1% albumin solution. Twenty-four hr later, blood samples were withdrawn from the retro-orbital sinus of the eye (55 μl of heparinized blood). Plasma or other test materials were then injected into the tail vein. Two hr later a second blood sample was taken and corresponding samples counted for radioactivity to a 2% probable error in a scintillation spectrometer. Five to six mice were used per group. To ascertain whether TSH was present, some samples were assayed by the method of McKenzie (10). The difference in counts of the initial and second hour samples from experimental groups were analyzed by the non-parametric method of Wilcoxon or by the Student's *t* test as we have previously reported (11).

Plasma fractions were obtained from the Nutritional Biochemicals Co., Cleveland,

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[1] The terminology proposed by Schally *et al.* (2) will be used in this paper.

Ohio. All plasma fractions were made up to physiologic concentrations in Krebs–Ringer bicarbonate media, pH 7.4 (12), and incubated with a known quantity of TRH for 30 min. A homogeneous preparation of porcine TRH prepared as described by Schally *et al.* (2, 5) was used in all the experiments. TRH in Krebs–Ringer medium, but without added plasma fractions, served as control. To study the effect of pH on the rate of enzyme action 0.1 *M* ammonium acetate (pH 4.5 and 5.7), 0.05 *M* sodium bicarbonate, pH 8.0, and 0.05 *M* sodium bicarbonate–carbonate pH 8.6, 9.5 and 10.8 buffers were used. Samples were neutralized before injection into the assay animals. Slices of rat liver, kidney, cerebral cortex, muscle tissues, and washed red blood cells were also incubated with TRH in Krebs–Ringer medium for 30 min at 37°. Incubation with TRH was also carried out with slices of tissue that were boiled for 1 min. After incubation the media were quickly chilled on ice, filtered if necessary, and assayed immediately for TRH. To rule out possible contamination with TSH, all samples were assayed for TSH by the McKenzie method (10).

Since rat blood is particularly rich in the enzyme(s) responsible for inactivation of TRH, it was necessary to dilute the plasma to a level at which only 60–70% inactivation occurred during a 30-min incubation period. A 1:8 dilution was found satisfactory and was used consistently as shown in the results.

Results. Table I, Expt. 1 shows the results of a four-point assay of a highly purified preparation of TRH. Comparison of the slope obtained with porcine TRH indicates a parallelism with that given by the TSH standard. The dose–response relationship was linear over the range tested. The index of precision was 0.111. This TRH assay seems more satisfactory than that reported recently by Bowers *et al.* (13). Large variations in counts in the initial blood samples, often noted in that assay, has been reduced by dissolving the T3 in 1–2% bovine albumin before injection. Using this assay, we detected TRH in concentrations of less than 1 ng/0.2 ml. Experiments 2 and 3 show other dose–responses to TRH.

TABLE I. Dose–Response Relationship with a Highly Purified Porcine TRH Preparation and TSH in Mice.[a]

Sample	Dose (ng/mouse)	^{125}I release from thyroid gland of mice; changes in ^{125}I in blood (cpm ± SE at 2 hr)	*p*
Expt. 1			
Saline	—	−28 ± 7	—
Porcine[b] TRF	12	111 ± 21	.01
	48	1004 ± 199	.01
TSH	0.1 mU	100 ± 6	.01
	0.4 mU	1109 ± 64	.01
	Variance ratios *F*		
Sample	Slope	Parallel	Lambda
0.19	8.12	0.31	0.111
Expt. 2			
Saline	—	−38 ± 10	—
Porcine TRH[c]	1	365 ± 48	.01
	3	1037 ± 204	.01
	9	2827 ± 271	.01
Expt. 3			
Saline	—	−50 ± 12	—
Porcine TRH[c]	1	98 ± 18	.05
	3	451 ± 53	.01
	9	2419 ± 199	.01

[a] Treated with 0.085 μg of T3 and 5 μCi of ^{125}I.
[b] FFE step.
[c] Prepared from charcoal column.

Table II shows the percentage inactivation of TRH by the various plasma fractions obtained from porcine, bovine, and human origin. Alpha, beta, and gamma globulins inactivated 70–90% of the added TRH. Most of the fractions tested exhibited some inactivation of TRH and this may be related to their purity. In the case of albumin, purification by recrystallization was accompanied by a loss of ability to inactivate TRH. Mercaptoalbumin was almost completely devoid of inactivating enzyme(s). This may have been due to inactivation of the enzyme(s) by the heavy metal salts used in the preparation of this fraction.

Inactivation of porcine TRH by various tissues is represented in Fig. 1. Cerebral cortex, liver, and kidney tissue caused a 50–60% and muscle tissue 32% inactivation of the

TABLE II. Percentage Inactivation of TRH by Plasma Fractions of Porcine, Bovine, and Human Origin after 30-min Incubation at 37°.

Fraction	Species:	Inactivation (%) by plasma fractions Bovine	Porcine	Human
Alpha globulin		84 ± 9	76 ±11	83 ± 7
Beta globulin		95 ±10	72 ± 8	38 ±11
Gamma globulin		92 ± 8	70 ±10	52 ± 7
Albumin		40 ± 8	55 ± 7	37 ± 5
Albumin 4[a]		—	—	16 ± 3
Mercaptoalbumin		—	—	3 ± 9
Fibrinogen		52 ±12	41 ± 6	18 ± 5
Beta-lipoprotein		5 ± 3	52 ± 8	12 ± 6
Serum		100	100	100

[a] Recrystallized 4 times.

added TRH during the 30 min of incubation. Washed red blood cells caused none. Inactivation by the tissue slices could be reduced significantly if the tissues were boiled before addition of TRH and incubation.

Figure 2 shows the inactivation of porcine TRH at various dilutions of plasma with Krebs–Ringer medium. Since highly purified TRH is very expensive and in short supply, it was not feasible to increase the TRH concentration so that a 50–70% inactivation would occur. Consequently, we decided to dilute the plasma with buffer. It can be seen that approximately 60–70% inactivation would have occurred in 30 min with a 1:8 dilution of the plasma with the buffer. Enzymatic inactivation of TRH as a function of time, with a 1:8 dilution, is shown in Fig. 3.

Figure 2 and 3 show that the rate of reaction is proportional to the enzyme concentration and time. From these figures it appears that this reaction is of the zero order and the reaction constant (K^0) appears to be approximately 1.5%/min ($Dx/dt = K^0$, where x is percentage inactivation and t is time in min).

To study the effect of temperature on the rate of enzyme action, plasma diluted 1:8 in Krebs–Ringer buffer was incubated for 30 min at the various temperatures shown in Fig. 4. The optimum temperature apparently was between 30 and 40°. Above 56° thermal inactivation of the enzyme began to occur.

Figure 5 shows that the optimum pH for inactivation of TRH by plasma appears close to neutrality. There was no inactivation of TRH when it was incubated at pH 10.8 without the addition of plasma. It was shown

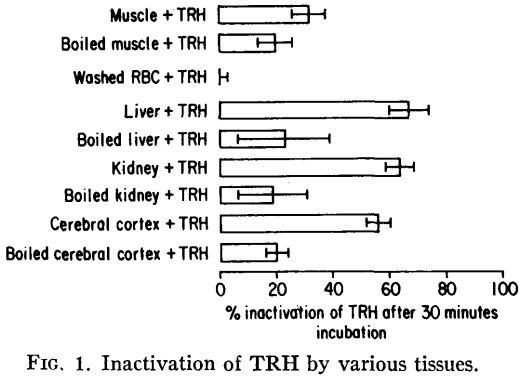

FIG. 1. Inactivation of TRH by various tissues.

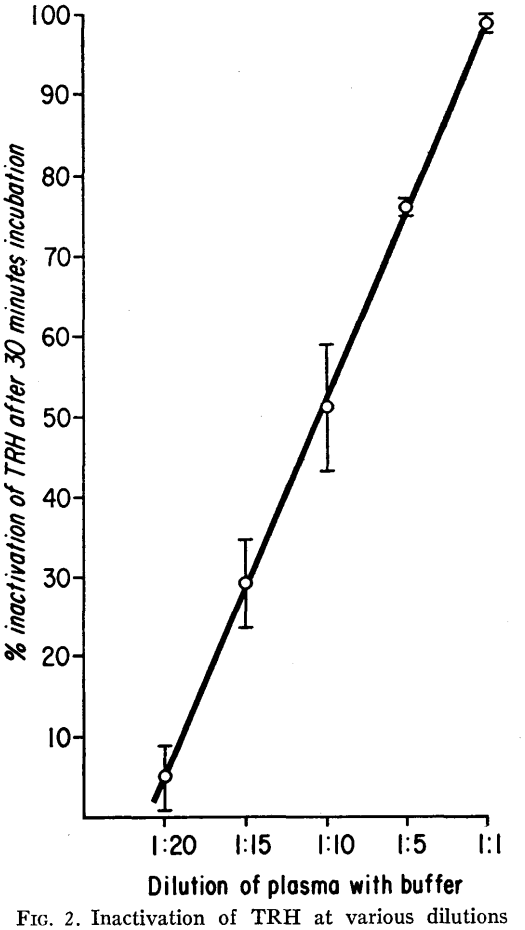

FIG. 2. Inactivation of TRH at various dilutions of plasma.

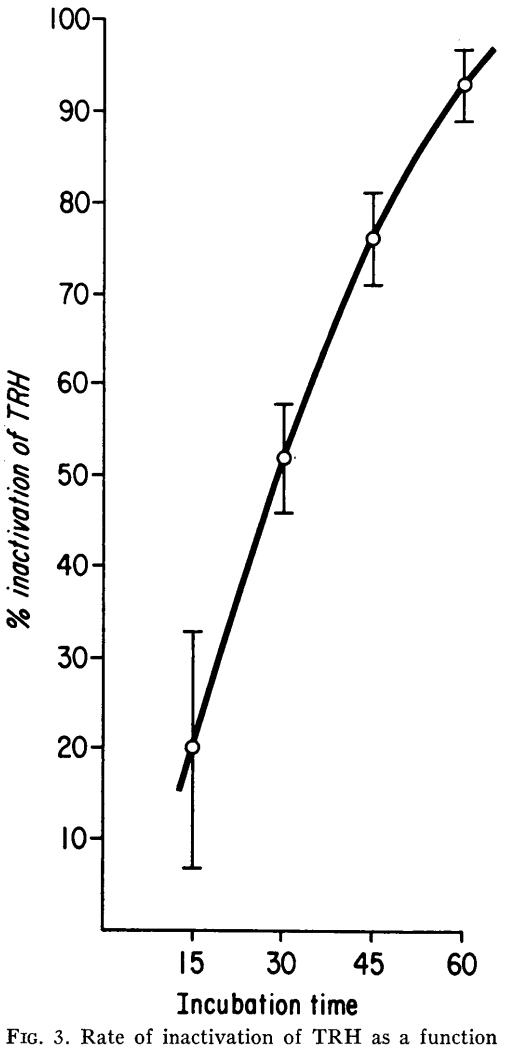

FIG. 3. Rate of inactivation of TRH as a function of time.

previously that TRH is very stable at pH 2.5–6 (2, 5, 6).

Discussion. Inactivation of TRH by blood was reported previously by Bowers *et al.* (8) and Guillemin (9). We extended these original observations and present in this report some characteristics of the enzyme system. Inactivation of TRH by plasma appears to be due to an enzyme most probably associated with globulin fractions. Since the plasma fractions used in this study were not highly purified, it is difficult to ascertain whether or not the fractions, other than globulins, were contaminated with this enzyme during their preparation. This enzyme may be in fact associated with several plasma fractions. However, purification of albumin by recrystallization lowered its ability to inactivate TRH. Incubation of TRH with slices of rat liver, kidney, cerebral cortex, and muscle resulted in loss of TRH activity in the media. Destruction of TRH may be associated with release of a specific inactiviating enzyme into the medium or may be due to blood within the tissue. The binding of TRH by the tissues or plasma fractions cannot be completely excluded. Tissue binding could presumably lead to the dissappearance of biological activity, as measured by our assay system, from the test solutions. However, since washed red blood cells did not inactivate TRH to any significant extent, it is more likely that a specific enzyme which inactivates TRH is present in liver, muscle, kidney, and cerebral cortex. The optimum pH of the enzyme(s) is about 7 and the optimum temperature is about 36°. Some residual capability of these tissues to inactivate TRH

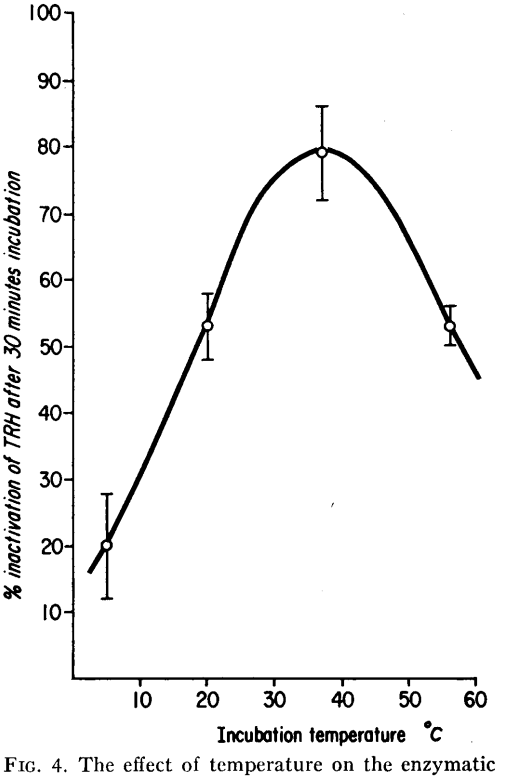

FIG. 4. The effect of temperature on the enzymatic inactivation of TRH.

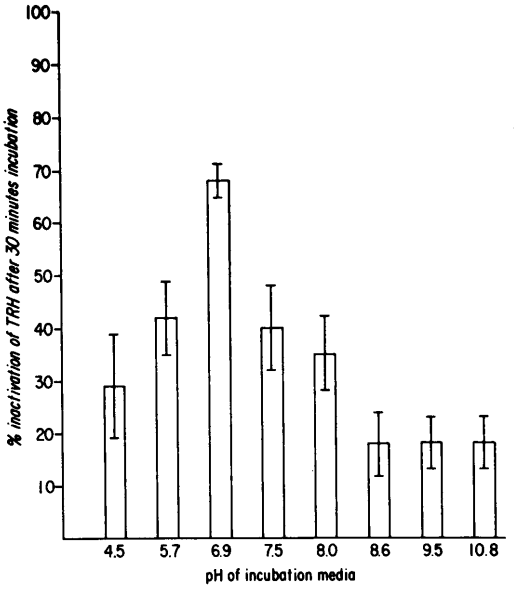

FIG. 5. Effect of pH on the enzymatic inactivation of TRH.

after boiling may be accounted for by the fact that a short boiling period of the tissues (1 min) may not be sufficient to completely abolish the biological activity of enzyme(s) responsible for the destruction of TRH.

The results of our study are best explained by the presence of an enzyme in plasma and various tissues which is capable of destroying or inactivating TRH. The action of this enzyme may be responsible for the difficulty in demonstrating TRH in peripheral and hypophyseal portal blood (14). Although there is circumstantial evidence to indicate that TRH may be present in peripheral blood, direct evidence of TRH in plasma has not been previously shown (15). Recent evidence obtained in our laboratory indicates that TRH may be found in peripheral blood only after certain physiological manipulations of the animal (16, 17). To detect TRH in blood, it has been necessary to increase the sensitivity of the bioassay for this substance.

The identification and purification of the enzyme responsible for inactivation of TRH may lead to further knowledge on the metabolism of TRH. This enzyme may play a physiologic role in the integrated hypothalamus–pituitary–thyroid axis. It is of interest to determine the effects of thyroid hormones and other physiological factors which may influence the levels or activity of this particular enzyme. Much of the future studies on the physiological role of TRH in the body must be related to all the known factors which modifiy its action. This has been particularly true in the case of the direct effect of the thyroid hormones in blocking the action of TRH at the pituitary level (18, 19).

Summary. Inactivation of porcine thyrotropin-releasing hormone (TRH) by plasma fractions *in vitro* was determined using mice maintained on a low iodine diet and pretreated with 5 μCi of ^{125}I and 0.085 μg of triiodothyronine. Incubation of TRH with porcine, bovine, or human serum caused a complete inactivation in 30 min. The optimum pH for the inactivation was about 7 and the optimal temperature was between 30 and 40°. The rate of inactivation of TRH was proportional to the enzyme concentration and time. Preheating rat plasma to 56° for 30 min greatly reduced this inactivation. When plasma fractions of porcine, bovine, and human origin were incubated with TRH in Krebs–Ringer bicarbonate, pH 7.4, at 37° for 30 min, alpha, beta, and gamma globulin fractions caused an 80–90% inactivation of added TRH. Albumin and fibrinogen caused a 40–50% reduction in TRH activity while the beta- lipoprotein fraction only induced a slight inactivation of TRH. Incubation of TRH with slices of rat liver, kidney, brain cortex, and skeletal muscle tissue also abolished TRF activity. Prior boiling of these tissues reduced the inactivation of TRH. These results are best explained by the presence of an enzyme in the plasma and various tissues which is capable of inactivating TRH.

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Studies on the Thyrotropin-Releasing Hormone (TRH)* Activity in Peripheral Blood† (33892)

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There is overwhelming evidence to support the concept that neurohormones secreted into the hypophyseal portal vessels control the release of anterior pituitary hormones (1–3). However, there is little data available on the presence of these neurohormones in the hypophyseal portal blood or in peripheral blood. Recently, Fink *et al.* (4) detected LRF in hypophyseal portal blood using a technique devised by Worthington (5). It seemed worthwhile to investigate TRH activity in the peripheral blood of rats under certain experimental conditions. The detection of this hormone in peripheral circulation has been difficult because of the relatively low sensitivity of the presently available assay systems. Furthermore, our laboratory demonstrated that blood rapidly inactivates TRH (6). Nevertheless, by subjecting the animals to certain physiological manipulations and by increasing the sensitivity of the TRH assay, we demonstrated a TRH-like activity in peripheral blood in response to the stimulus of mild cold. A study was also made of the inactivation of TRH in rats subjected to these various treatments in an attempt to shed some light on the conditions under which TRH can be detected in the peripheral circulation.

Materials and Methods. Female Sprague-Dawley weanling rats, weighing 75–90 g, were subjected to hypophysectomy to eliminate any effects due to endogenous TSH. After 30 days some rats were subjected to surgical thyroidectomy. Five days later they were separated into two groups of 5 to 6 animals. The experimental group was then exposed to cold at 5° for 2 hr while the control animals were maintained at room temperature. In an-

* The terminology proposed by Schally *et al.* (1) will be used in this paper.

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