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An Inhibitor of the Binding of Thyroid Hormones to Serum Proteins is Present in Extrathyroidal Tissues

Abstract. *Extrathyroidal tissues of man and the rat contain a potent inhibitor of the binding of thyroid hormones to serum proteins and to an anion-exchange resin. The inhibitor is heat-labile and nondialyzable. It acts by reducing the binding affinity of thyroid hormones to serum proteins, not by reducing the number of binding sites. The tissue inhibitor is similar in several characteristics to an inhibitor described previously in the serum of some critically ill patients, suggesting that the tissue inhibitor may leak into the circulation in severe illnesses.*

Recent studies (*1, 2*) suggest that a substance that inhibits binding of thyroxine (T_4) and triiodothyronine (T_3) to serum proteins is present in the circulation of some critically ill patients with nonthyroid illnesses (NTI). The presence of the inhibitor is often associated with a reduction in serum concentration of T_4 (and T_3) in patients with NTI. This is so marked in some patients that a battery of carefully monitored biochemical tests is needed to avoid erroneously diagnosing hypothyroidism (*1, 2*). The low T_4 in patients with NTI carries an ominous prognostic significance (*3*), but no data are available to suggest that the prognosis improves by administering thyroid hormones as it does in hypothyroidism.

The dialyzable (free) fraction of serum T_4 is usually normal or high in patients with NTI. This increase is often far beyond what would be expected from the moderate reduction in the serum concentration of T_4-binding globulin that is common in NTI (*1*). Curiously, however, the resin uptake of T_3, a simple test used routinely in clinical laboratories for assessing thyroid binding proteins, frequently fails to demonstrate as severe a reduction in serum binding of thyroid hormones as is shown by equilibrium dialysis (*1*). The basis for the difference is not fully understood. It has been suggested that the difference may lie in the ability of the thyroid hormone binding inhibitor to inhibit the binding of T_3 not just to serum proteins but also to resin, charcoal, isolated rat hepatocytes (*2*), and possibly other solid matrices.

There is no clear information on the nature of thyroid hormone binding inhibitor or the mechanism by which it appears in the circulation of seriously ill patients. In this study we have tested the hypothesis that the inhibitor is a normal component of extrathyroidal tissues and that it leaks into the circulation when the integrity of tissues is compromised in severe illnesses. In this report we describe the characteristics of a thyroid hormone binding inhibitor in tissues of the normal rat and of humans who died from NTI.

The presence of the inhibitory activity in tissues was tested by measuring the dialyzable fraction of serum T_4 in samples of pooled normal human serum in the presence or absence of a homogenate of the tissues. The method of determination of dialyzable fraction of T_4 has been described in detail elsewhere (*1, 4*). In brief, a 0.1-ml sample of pooled normal human serum, diluted to 5 ml with 0.15*M* phosphate buffer (*p*H 7.4), was labeled with dialyzed ^{125}I-labeled T_4 (specific activity, ~ 100 mCi/mg) and placed on one (A) side of an equilibrium dialysis cell (Technilab Inc.); 5 ml of unmodified buffer was placed on the other (B) side of the cell. The cell was incubated at 37°C for 20 hours, at which time the buffer on B side of the cell was removed and carrier T_4 was added; a magnesium chloride solution (*p*H 9.3) was then added to precipitate radioactive and nonradioactive T_4. The precipitate was washed several times with a magnesium chloride wash solution (*p*H 8.6) and the radioactivity in the precipitate was determined with a precision of ± 2 percent by means of a Tracor-analytic gamma scintillation counter. The dialyzable fraction of T_4 in the serum of 25 normal subjects tested by this method averaged (mean ± standard error) 0.033 ± 0.01 percent. The dialyzable fraction of T_3, as determined by using radioactive T_3 instead of radioactive T_4 in the method described above, averaged 0.34 ± 0.16 percent (N = 16). The coefficient of variation of measurements in eight replicates of a serum in an assay was 13 percent for dialyzable T_4 and 9 percent for dialyzable T_3.

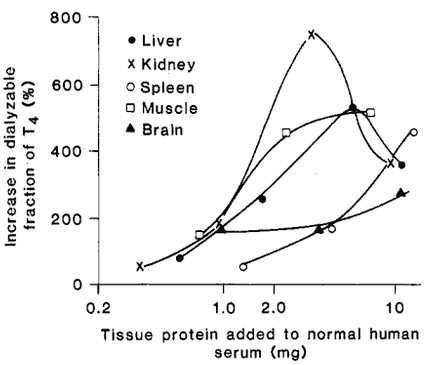

Fig. 1. Increases in the dialyzable fraction of T_4 caused by the addition of rat tissue homogenates to 0.1 ml of a pooled sample of normal human serum labeled with [^{125}I]T_4.

Human tissues obtained at autopsy or fresh tissues obtained from male Sprague-Dawley rats were homogenized in 3 volumes of 0.15*M* phosphate buffer, *p*H 7.4, with a Polytron (Brinkmann Instruments). Homogenates were filtered through four layers of gauze. The protein concentration of the homogenates was measured by the method of Bradford (*5*) with kits obtained from Bio-Rad Laboratories.

Addition of 0.2 to 1.7 mg of protein from a homogenate of human liver or kidney to normal human serum increased the dialyzable fraction of T_4 by 20 to 480 percent in a dose-dependent manner; addition of higher amounts of tissue protein was associated with less than maximum increases in dialyzable T_4, apparently because of an increasing effect of T_4-binding proteins in the homogenates on the assay for dialyzable T_4. Since there was a possibility of an interference resulting from postmortem autolysis of tissues or by the various drugs administered to ill patients prior to death, we examined the effect of fresh homogenates of various rat tissues on the dialyzable fraction of serum T_4. Even small amounts of tissue proteins substantially reduced the serum binding of T_4, as shown by the marked increases in dialyzable fraction of T_4 in normal human

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serum (Fig. 1). The amount of rat tissue protein that caused a 300 percent increase in the dialyzable fraction of T_4 of normal serum was 1.2 mg for kidney, 1.4 mg for muscle, 1.9 mg for liver, 7.5 mg for spleen, and 14 mg for brain (Fig. 1). For convenience, we used homogenates of rat liver for further studies of the binding inhibitory activity.

The question of whether the binding inhibitory activity is caused by a small or a large molecule was examined by prolonged (4 days) dialysis of a liver homogenate against 400 times the volume of 0.15*M* phosphate buffer at 4°C. Rather than a reduction in the binding-inhibiting activity in the dialyzed homogenate we found an increase in the activity: the control tissue (4.5 mg of protein) and the dialyzed tissue (4.5 mg of protein) increased the dialyzable fraction of T_4 in human serum by 270 percent and 394 percent, respectively. Similarly, addition of up to 100 units of Aprotinin (Sigma), a protease inhibitor, did not decrease the binding inhibitory activity of 3.0 mg of protein of rat liver homogenate; the increase in the dialyzable fraction of T_4 in normal serum was 215 percent in the absence of Aprotinin and 259 percent in its presence. The binding inhibitor was heat-labile. Its effect was reduced minimally (207 percent compared to 191 percent) by heating at 56°C for 30 minutes but was reduced substantially (207 percent compared to 40 percent) by heating at 90°C for 30 minutes. We examined the possibility that the thyroid hormone binding inhibitory activity in liver homogenate is due to T_4 or a metabolite of T_4 by studying the effect of T_4 on the dialyzable fraction of the hormone in human serum; T_4-binding globulin in human serum has greater affinity for T_4 than any other thyroid analog (*6*). We found no change (< 10 percent) in the dialyzable fraction when T_4 was added to human serum in quantities up to seven times greater than those measured in liver homogenate (which increased the dialyzable fraction of T_4 in serum by 340 percent). When T_4 was added in concentrations 22, 66, and 198 times greater, it caused a 10, 100, and 125 percent increase in the dialyzable fraction, respectively.

The possibility that the thyroid hormone binding inhibitor in liver homogenate is a substance of small molecular weight bound noncovalently to tissue proteins was evaluated by examining the inhibitory activity in evaporated ethanol extracts of liver homogenate. The serum dialyzable fraction of T_4 was not affected by dried ethanol extract of homogenate (protein, ~ 4.5 mg), but was increased

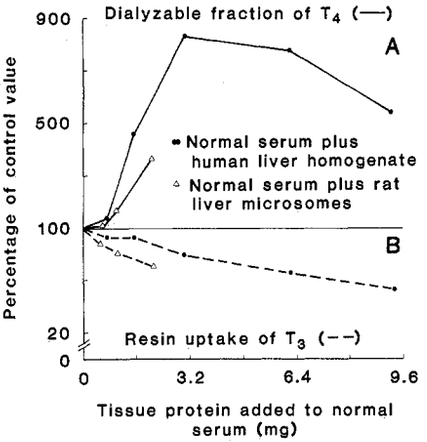

Fig. 2. Divergent effects of hepatic T_4 binding inhibitor on (A) the dialyzable fraction of T_4 and (B) the resin uptake of T_3 in the pooled sample of normal human serum. The dialyzable fraction of T_4 was determined as described in the text. The resin uptake T_3 was determined using RES-O-Mat kits available commercially from Mallinckrodt Inc., St. Louis, Missouri.

400 percent by the untreated homogenate. We also tested the possibility that liver homogenate induces, under the conditions of our assay for the T_4 dialyzable fraction, a breakdown of T_4 into more readily dialyzable products, but this possibility was not supported by the data (*7*).

We examined the distribution of the binding inhibitory activity in various subcellular fractions of a rat liver homogenate. For this purpose, about 2.0 mg of protein from each of the fractions obtained by ultracentrifugation (*8*) were added to 0.1-ml portions of pooled normal human serum and the dialyzable fraction of T_4 was measured. The dialyzable fraction increased 184 percent in the presence of the homogenate, 10 percent with the nuclear fraction, 170 percent with the mitochondria, 193 percent with the microsomes, and 42 percent with the cytosol. These data suggested that the inhibitory activity is associated mainly with particulate fractions of the liver homogenate. The binding inhibitor could be solubilized in rat liver mitochondria and microsomes by using 1 percent sodium cholate. Column chromatography of solubilized microsomes on G-150 Sephadex processed with 20 m*M* tris buffer, *p*H 7.4, revealed that the inhibitor elutes mainly in the first protein peak.

Presence of the inhibitor of thyroid hormone binding in the serum of patients with NTI affects the binding of T_4 to serum proteins to a much greater extent than that of T_3 (*1*). The binding inhibitor in rat liver homogenate proved to be similar to that in serum in its relative effects on the dialyzable fractions of T_4 and T_3. Thus, 2.0- and 6.0-mg portions of liver homogenate protein that increased the dialyzable fraction of T_4 in a pooled normal human serum by 132 and 448 percent, respectively, increased the dialyzable fraction of T_3 by only 7 and 82 percent, respectively. Similarly, 2.0 mg of a microsomal protein increased the T_4 dialyzable fraction of normal serum by 284 percent, whereas it increased the T_3 dialyzable fraction by only 33 percent.

Like the inhibitory factor in the serum, the inhibitory factor in tissue affects the dialyzable fraction of T_4 to a much greater extent than it affects resin uptake of T_3. At all concentrations of tissue protein tested, the T_4 dialyzable fraction of normal serum was increased whereas the resin uptake of T_3 was either unaffected or actually decreased (Fig. 2). To explore the reasons for this difference we examined the effect of rat liver homogenate and human serum albumin (HSA) on the resin uptake of T_4 and T_3 in the absence of serum. A 25 percent reduction in the resin uptake of T_4 was caused by 16 mg of HSA but only 5 mg of liver homogenate protein. Similarly, liver homogenate was three to four times more potent than HSA in inhibiting the resin uptake of T_3. In different experiments, using equilibrium dialysis, we found that the liver homogenate binds T_4 with a potency that approximates 15 to 34 percent that of HSA (*9*). The data thus suggested that the pronounced inhibition of the resin uptake of T_4 by liver homogenate was due not to the homogenate having a greater avidity for T_4 compared to HSA, but to its ability to directly inhibit the resin binding of T_4.

To study the mechanism of inhibition of thyroid hormone binding to serum proteins, especially the role of T_4-binding globulin, we used the technique of Murphy and co-workers (*10, 11*) to examine the binding of radioactive T_4 to serum diluted 1/32 in 0.075*M* barbital buffer in the absence and presence of different quantities of nonradioactive T_4. The data we obtained were subjected to Scatchard analysis (*12*). The slope of the binding of T_4 to globulin in the serum of three normal subjects (-0.34 ± 0.036 as compared to -0.04 ± 0.003, $P < .001$) was significantly reduced in the presence of 2 mg of tissue homogenate, whereas the maximum T_4 binding capacity of the globulin changed little or increased. These data suggested that the thyroid hormone binding inhibitor acts mainly by reducing the affinity of the globulin for T_4.

There are several similarities between the physicochemical characteristics (for example, thermolability and elution from

a Sephadex G-150 column) and functional characteristics (for example, more marked effects on the dialyzable fraction of T_4 than on DFT_3 or RT_3U) of the thyroid hormone binding inhibitory activity in extrathyroidal tissues and that (*1*, *2*) in the serum of patients with NTI. However, further study is needed to determine whether these factors are identical. The available data support the thesis that the inhibitory factor may leak from a tissue into the circulation in severe illness. The data also offer a possible explanation of the common clinical phenomenon of abnormal thyroid hormone levels in NTI (*1–3*). The use in our studies of a ratio of liver protein to total serum protein of about 1:7 to document a three- to fourfold increase in the T_4 dialyzable fraction of serum is of some concern, however. It may imply that there is actually an abundance of tissue proteins in the circulation of patients with NTI, or it may indicate that there are other factors that enhance the activity of the binding inhibitor in tissues in vivo.

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11. A sample (0.9 ml) of pooled serum of normal subjects (diluted 1/32 in 0.075*M* barbital buffer, *p*H 8.6) and [^{125}I]T_4 (30,000 to 40,000 count/min; 100 mCi/mg) were incubated with different amounts (0.45 to 20 ng) of nonradioactive T_4 for 10 minutes at 22°C in the absence or presence of 2.0 mg of rat liver homogenate protein; the final volume of reaction mixture was 1.1 ml. The separation of unbound [^{125}I]T_4 from radioactivity bound to serum proteins (predominantly T_4-binding globulin under these conditions) was accomplished by adding an excess (~ 400 mg) of an anion exchange resin (Amberlite, IRA-400). The methods of preparation of resin and its use in the assay were the same as those described by Murphy and Jachan (*10*).
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Recombinant Inversion Chromosomes in Phenotypically Normal Chickens

Abstract. *Some progeny resulting from interbreeding of individuals heterozygous for a pericentric inversion of chromosome 1 in the chicken have the two complementary types of recombinant chromosomes arising from a single crossing-over within the inverted segment. These individuals are capable of reproduction. Their progeny can have one or the other of the two recombinant chromosomes or, if crossing-over occurs, either a normal or an inversion chromosome.*

A fertile individual heterozygous for a pericentric inversion can produce gametes that have one of two morphologically distinct recombinant chromosomes as a result of crossing-over within the inverted segment. Individuals with such a recombinant chromosome occur in many species (*1*, *2*). Progeny with one of these recombinant chromosomes are usually phenotypically abnormal because of the absence of portions of the chromosome containing the inversion. We do not know of any previous reports of viable vertebrates having both types of complementary recombinant chromosomes.

When individuals heterozygous for a pericentric inversion in chromosome 1 [inv(1)] (*3*) of the chicken (*Gallus domesticus*) were mated, some progeny—designated rec(1)—had both recombinant chromosomes. These two types of chromosomes are apparently completely complementary, since the carriers are phenotypically normal and fertile when sexually mature. Furthermore, the interbreeding of rec(1) carriers may give rise to normal, inversion, or either of the recombinant types of chromosomes, because of crossing-over during meiosis.

The original inversion was induced by injection of ethyl methanesulfonate into a mature male (*4*). A daughter of this male identified as having the inversion was outcrossed, for development of the rearrangement line, to a male with standard chromosomes. Progeny were examined for their chromosome complement by use of the feather pulp technique (*5*). Some of the progeny from subsequent interbreeding had the two complementary types of duplication and deficiency recombinant chromosomes that had arisen from a single crossing-over within the inverted segment. Trypsin-Giemsa banding (G banding) (*6*) (Fig. 1) indicated that the longer chromosome was duplicated for approximately two-thirds of the p arm, and this was attached to the distal end of the q arm. The shorter chromosome did not have this same segment. A small portion of the terminal region of the q arm, designated F in Figs. 2 and 3, appears to be duplicated on the p arm of the shorter (1p−q+) chromosome and missing on the longer (1p+q−) chromosome.

Two different meiotic configurations occurred in inv(1) males, in about equal frequencies (Fig. 1). Interpretations of these configurations are given in Fig. 3, a and b. The open-loop diakinesis configuration (Fig. 1, lower left) suggests that the entire inverted segment is unpaired, preventing any crossing-over in this region. Only inv(1) and normal chromosomes can be recovered from this configuration. The figure-eight diakinesis configuration (Fig. 1, lower right) shows an apparent reversed chiasma such as that described in the lily (*7*), where homolo-

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