THYROID HORMONE-BINDING INTERACTIONS IN CYTOSOL OF RAT ANTERIOR PITUITARY

By

Valerie Anne Galton

ABSTRACT

Thyroxine ($T_4$) and triiodothyronine ($T_3$)-binding interactions in preparations of rat anterior pituitary gland have been studied. $T_4$ is bound primarily to extranuclear binding sites located in the cytosol fraction of the cell. These sites have a medium affinity for $T_4$: $K_a = 2.5 \times 10^8 \text{ l/mol}$ and a maximum binding capacity (MBC) of 1.15 pmol/mg tissue (wet weight). Binding of $T_3$ to these sites is minimal. The extent of binding of $T_4$ is influenced by the pH of the system and the temperature of incubation. The relative effectiveness of $T_4$ analogues in displacing bound $T_4$ is tetrac > $T_4$ > triac > $D-T_4$ > $T_3$. Similar $T_4$-binding sites are present in other rat tissues, but in all except serum, binding activity is lower than in the pituitary. $T_4$-binding by serum containing the pituitary preparations contributed only partially to the total activity observed. Concomitant assessment of $T_4$-binding activity and $T_4$ metabolism in pituitary homogenates prepared at different pH values indicated an inverse relationship between the two processes. The possible role of thyroid hormone binding in cytosol in influencing the intracellular distribution of thyroid hormones is discussed.

Several investigators have convincingly demonstrated the presence of high affinity, low capacity binding sites for the thyroid hormones in the nuclear fraction of various rat tissues including anterior pituitary gland (Schadlow et al. 1972), liver (Surks et al. 1973; Oppenheimer et al. 1974; Koerner et al.)

This investigation was supported in part by USPHS Grant No. AM 16172 from the National Institute of Arthritis and Metabolic Diseases, Bethesda, Maryland, USA.
1974; DeGroot et al. 1974; Samuels et al. 1974a,b; Docter et al. 1976), kidney (Oppenheimer et al. 1972; Samuels et al. 1974a) and GH₁ cells of a rat pituitary tumour line (Samuels et al. 1973, 1974a,b; Samuels & Tsai 1973). It is generally accepted that these sites are specific thyroid hormone receptors associated with a primary action of thyroid hormone at the nuclear level.

The nucleus is not, however, the only locus of intracellular binding sites for thyroid hormone. Thyroxine (T₄) and 3,5,3'-triiodothyronine (T₃) bind to proteins in the cytosol fraction of rat liver and kidney (Dillman et al. 1974; Sterling et al. 1974; Visser et al. 1976), dog liver and kidney (Davis et al. 1974) and porcine anterior pituitary (Sufi et al. 1973).

The role of these cytosol binding sites is not known. Their hormone-binding properties are not characteristic of specific hormone receptors (Dillman et al. 1974; Visser et al. 1976) and they are apparently not essential for the translocation of the hormones to the nuclear receptors (Surks et al. 1975; Docter et al. 1976). Nevertheless, it is very likely that they exert some influence on the intracellular distribution and metabolism of the thyroid hormones.

Observations relevant to this latter point are described herein. A study was made of the thyroid hormone-binding interactions of preparations of rat anterior pituitary gland, including whole pituitary, pituitary homogenate and subcellular fractions of pituitary tissue. The relation between T₄-binding and metabolism was assessed. This work is part of an investigation into the action and metabolism of thyroid hormone in this organ.

METHODS AND MATERIALS

All experiments were carried out on tissues obtained from male Sprague-Dawley rats (120–150 g) maintained on Purina Labina rat chow and water ad libitum unless stated otherwise. Rats were killed by decapitation and the pituitaries were removed and placed in ice-cold Krebs-Ringer-phosphate buffer, pH 7.4 (KRP). In some experiments, pieces of other tissues were similarly treated. Homogenates (generally 1:100 w/v) were prepared in different media: KRP, pH 7.4 or 6.5; sodium phosphate buffer, 0.1 mol/l, pH 9.0 and MgCl₂, 1.5 × 10⁻³ mol/l, pH 5.9. Cytosol was prepared in the following manner. Homogenates were centrifuged for 5 min at 700 g and the supernatant was then centrifuged at 100 000 g for 1 h. The second supernatant was considered the cytosol fraction. The two particulate fractions were combined and suspended in the original volume of medium. The presence of nuclei in this fraction was observed microscopically. Binding studies were carried out in glass tubes (5 ml capacity) with 1 ml homogenate, 1 ml cytosol (second supernatant), 1 ml re-suspended particulate fraction, or 10 mg whole pituitary in 1 ml buffer. [¹²⁵I]T₄¹) and [¹²⁵I]T₃ were diluted in 0.1 % human serum albumin (HSA) to yield stock solutions 5 × 10⁻⁸ mol/l. Ten μl aliquots of these solutions were added to the tissue preparations (1 ml) to yield a

---

¹) [¹²⁵I]T₄ and [¹²⁵I]T₃ were purchased from Industrial Nuclear, St. Louis, Missouri and Amersham Scarle, Arlington Heights, Illinois.
final concentration of $5 \times 10^{-10}$ mol/l (no correction was made for endogenous T₄ in the tissue). In substrate inhibition and analogue competition experiments, compounds were dissolved in 25 % p-propylene glycol and diluted at least 100-fold in 0.1 % HSA to yield stock solutions. 6-Propyl-2-thiouracil (PTU), methimazole, NaClO₄, and TRH were prepared in the appropriate buffer. All compounds were added in a volume of 10 μl. Incubations were carried out at 4, 22 or 37°C for various lengths of time. The percentage of the labelled hormone bound to tissue components was determined using the following Dextran-coated charcoal absorption technique: aliquots (100 μl) of charcoal solution (1.0 g Norit Neutral 2) and 0.1 g Dextran 60 in 100 ml MgCl₂, $1.5 \times 10^{-3}$ mol/l) were placed in 1.5 ml plastic tubes (Eppendorf) and chilled to 4°C. An aliquot of incubation mixture (100 μl) was added to each tube. The mixture was shaken on a Vortex agitator for 5 seconds, held at 4°C for 60 min (unless stated otherwise), and then centrifuged at 4°C in an Eppendorf centriuge (Model 3200) for 2 min. The supernatant was mixed with an equal volume of human plasma and the proportion of organic to inorganic $^{125}$I present determined by electrophoresing 30 μl samples on paper for 90 min in glycine acetate buffer, 0.05 mol/l, pH 8.6, using a constant current of 12 mA. NaI was added as a marker. Under these conditions, the iodide, located by staining with palladium chloride, moved several cm from the origin; the iodothyronines remained at the origin. The appropriate sections of the strips were cut out and the radioactivity determined in an automatic dual-channel gamma counter (Searle Analytic Inc., Model 1195). The total counts and their distribution between organic and inorganic iodide in the incubation mixture were determined by re-vortexing a 1:1 mixture of charcoal solution and incubation mixture after holding at 4°C for 60 min and the mixing equal volumes of total suspension and human plasma. This latter preparation was analyzed as described above. In many experiments, the nature of the labelled compounds was assessed by paper chromatography using two solvent systems: butanol-dioxane-NH₄OH (4:1:5); tertiary amyl alcohol-NH₄OH (1:1). These methods have been published elsewhere (Galton 1972).

In some experiments, pituitary activity with regard to TSH production and secretion was altered by pre-treatment of rats for at least 3 weeks with either PTU (approximately 10 mg PTU/day, administrated in the diet) or T₄ (50 μg T₄/day injected SC in 0.25 ml 25 % p-propylene glycol). Controls were injected with the vehicle. T₄-binding activity was assessed as described above using individual 1:100 homogenates prepared in phosphate buffer, 0.1 mol/l, pH 9.0.

To determine the volume of serum trapped in the pituitary and other tissues employed in these studies, experiments were performed using $[^{131}$I]serum albumin. Rats were given an iv injection of $[^{125}$I]T₄ (10 μCi). Three hours later they received $[^{131}$I]albumin (1 μCi, iv) and 15 min later they were killed either by exsanguination under ether or by decapitation. In the latter case, blood was collected directly from the neck. Tissues were removed and pieces were weighed and counted with the counter set to count $^{131}$I and $^{125}$I simultaneously. Aliquots of serum were also counted. Standards prepared of each labelled compound were counted separately to determine the degree of overlap in the dual channel counting. Appropriate corrections were made to the values obtained in the tissue samples. The volume of serum in a tissue was calculated from the concentration of $^{131}$I in serum and tissue. The contribution of serum T₄ to the T₄ count in the tissues was then calculated.

All experiments were performed a minimum of three times.

2) Norit Neutral was obtained from Fisher Scientific Company.
RESULTS

Preliminary experiments were performed with human serum albumin (HSA) to assess the value of the charcoal binding technique for determining high affinity binding activity in a preparation. Under the conditions employed herein, the charcoal absorbed 90% of the [125I]T4 (5 x 10^{-10} mol/l) present in a 0.5% solution of HSA in 60 min, 92% in 120 and 180 min, respectively. In another experiment, pituitary homogenate (1:100 w/v) prepared in phosphate buffer, 0.1 mol/l, pH 9.0 was incubated with [125I]T4, either 5 x 10^{-10} mol/l or 10^{-7} mol/l for 60 min. Aliquots of the incubation mixtures were then exposed to charcoal for different lengths of time. At both concentrations of T4, charcoal absorption was for all practical purposes complete in 60 min. (Thus, in subsequent experiments, this time period was employed). At the low concentration of T4, only 20–40% of the radioactivity was taken up by the charcoal. However, when the T4 concentration was 10^{-7} mol/l, more than 80% was bound to the charcoal. The charcoal technique thus provides an excellent means of distinguishing free and weakly bound T4 from T4 that is bound to relatively high affinity sites.

These findings indicate the presence of T4-binding sites which are saturated in the presence of T4 at 10^{-7} mol/l. The binding sites have a higher affinity for T4 than T3, and the extent of binding of T4 is greatly dependent on the pH of the homogenizing medium. As shown in Fig. 1, at pH 9.0, more than 80% of [125I]T4 (5 x 10^{-10} mol/l) is bound to tissue components, and only 5%
Binding of \([^{125}I]T_4\) (5 \times 10^{-10} \text{ mol/l}) to saturable sites in different fractions of pituitary tissue prepared at pH 9.0, 7.4 and 5.9. The saturating concentration of \(T_4\) employed was 10^{-7} \text{ mol/l}. The pellet contained nuclei and all the particulate matter which sedimented at 100 000 g. The remaining supernatant fraction was termed cell “cytosol”. Bars indicate the mean of three closely agreeing values obtained in a single typical experiment.

of \([^{125}I]T_3\). The saturability of the \(T_4\)-binding sites at 3 \times 10^{-8} \text{ mol/l} is evident at both pH's.

The \(T_4\)-binding sites appear to be located primarily in the cytosol fraction of the cell. \(T_4\)-binding activity was compared in whole homogenate, cytosol fraction (100 000 g supernatant) and particulate fraction re-suspended in medium. (At pH 7.4, this fraction contained many intact nuclei). Typical results are shown in Fig. 2. In the three media studied (pH 9.0, 7.4 and 5.9), the \(T_4\)-binding sites were located primarily in the supernatant or cytosol fraction. Binding by the fraction containing nuclei, etc., was less than 20 % and these sites were not as readily saturable. Values obtained in supernatant and homogenate were not significantly different and hence, most subsequent experiments were performed with unfractinonated homogenate.

The rate of association of \(T_4\) with pituitary binding sites was greatly dependent on temperature. At 37°C, association reached a steady state in less than 10 min. At 22°C it was evident within 30 min. At 4°C the rate was greatly slowed and even after 120 min less than 40 % of \([^{125}I]T_4\) was bound.

\([^{125}I]T_4\) bound to high affinity sites in pituitary homogenates is readily dis-
Displacement of bound $[^{125}\text{I}]T_4$ (5 x 10^{-10} \text{ mol/l}) by addition of stable $T_4$ (10^{-7} \text{ mol/l}) in rat pituitary homogenate incubated at 22°C. Each point is the mean of duplicate values obtained in a single typical experiment.

Scatchard analysis of $T_4$ binding in rat pituitary homogenate. The maximum binding capacity, given by the intercept with the X-axis is 1.15 pmol/mg tissue (wet weight). Each point is the mean of closely agreeing triplicate values obtained in a single typical experiment.
placed by addition of cold T₄ (10⁻⁷ mol/l). As shown in Fig. 3, at 22°C complete displacement was achieved in approximately 120 min. A Scatchard plot of data from a typical displacement experiment in pituitary cytosol is shown in Fig. 4. The Ka was 2.5 × 10⁸ l/mol, and the maximum binding capacity (MBC) was 1.15 pmol/mg tissue (wet weight).

These binding sites were evident in intact cell preparations. Binding activity was compared in whole pituitaries incubated in KRP, pH 7.4 with [¹²⁵I]T₄ (5 × 10⁻¹⁰ and 10⁻⁷ mol/l) and in pituitary homogenate prepared in the same buffer. After incubation, all preparations were chilled and the whole pituitaries were homogenized in their own medium prior to charcoal analysis. Saturable binding sites for T₄ but not T₃ were invariably observed in both preparations. The percentage of the [¹²⁵I]T₄ (5 × 10⁻¹⁰ mol/l) bound was generally 5–10% less in the intact cells than in the homogenate preparation.

Results of analogue competition experiments indicated that tetrac (TA₄) is the most effective inhibitor of [¹²⁵I]T₄ binding. At a concentration of 10⁻⁸ mol/l, D-T₄ and T₃ were ineffective. Some inhibition by these latter compounds occurred at 10⁻⁷ mol/l. At 10⁻⁶ mol/l all four compounds inhibited the saturable binding activity. In another study, additional compounds were tested. At 10⁻⁷ mol/l, the effectiveness of the following compounds in inhibiting [¹²⁵I]T₄ binding was: TA > T₄ > triac > triiodothyropropionic acid > D-T₄ > T₃. 3,5-Diiodothyronine and thyronine were ineffective at all concentrations tested.

T₄-binding activity was not affected when pituitary activity with regard to TSH production was altered. T₄-binding was not changed in pituitary homogenates or whole pituitaries prepared from rats pre-treated with large doses of T₄ or PTU. It was not clear why partial saturation of the binding sites was not observed. Information concerning the amount of exogenous T₄ that entered the pituitary would have been relevant in this experiment.

In previous studies from this laboratory, it was shown that preparations of pituitary, both intact cells and homogenates, deiodinate T₄. The rate of metabolism is dependent on the pH of the medium and the only labelled product formed is iodide; no evidence of monodeiodination of T₄ to T₃ was obtained. Moreover, the preparation did not deiodinate T₃ (Galton 1975). An experiment was performed in which the extent of both T₄ binding to tissue compounds and T₄ deiodination was compared in pituitary homogenates prepared in buffers of different pH. At pH 9.0 when binding was maximal, deiodination was minimal. At pH 6.5 deiodination was greatest and binding minimal. At pH 7.4, both binding and metabolism were evident, albeit the latter was relatively slow.

High affinity binding sites were found in tissues other than pituitary, and in serum (Fig. 5). Under comparable conditions (1:100 homogenates, T₄, 5 × 10⁻¹⁰ mol/l and 1 h incubation at 22°C), pituitary and serum bound 70–80% of the total [¹²⁵I]T₄, kidney, heart, liver, and spleen bound 30–40%, and thyroid, hypothalamus, testes and brain approximately 10%. In all tissue,
Fig. 5.
Relative affinity and saturability of different rat tissues for T4. Bars represent the mean of closely agreeing triplicate values obtained in a single experiment.

the 9% [125I]T4 bound was greatly reduced in the presence of T4 at 10^-7 mol/l.

Since the T4-binding activity of serum was comparable to that in pituitary homogenate, it was important to assess the volume of serum trapped in the pituitary in order to determine the extent to which T4-binding by this serum contributed to the total T4-binding activity observed in pituitary homogenate. This was determined by iv administration of both [125I]T4 and [131I]albumin. From the 131I counts in serum and tissue the volume of trapped serum was calculated in pituitary, liver and kidney. The mean range of values obtained were 9.5 (7.3–10.2), 8.1 (6.0–9.1), 9.7 (7.7–12.6) μl/100 mg wet tissue, respectively. In brain and hypothalamus, the values ranged from 1.0–2.1. From these calculated values and the 125I present in serum tissues, the fraction of total tissue [125I]T4 present in the trapped serum was determined. Following in vivo injection of T4, this proved to be approximately 25% of the total T4 counted in the pituitary preparation. In liver and kidney, it was 10–15%.

DISCUSSION

The primary purpose of this investigation was to assess T4-binding interactions as they might influence studies of hormone action and metabolism in preparations of rat pituitary tissue. The results indicate that T4 is bound primarily
to extranuclear binding sites. These sites are located primarily in the cytosol fraction of the cell; under the conditions employed, results obtained with cytosol were not significantly different from those obtained with whole homogenate. The activity remaining in the pellet was due in part to contamination with cytosol since it could be removed by washing.

The cytosol-binding sites are saturable and have greater affinity for T4 than for T3. As indicated by direct interaction studies, binding of T3 to cytosol proteins was minimal. However, when present in sufficiently high concentrations, T3 could displace [125I]T4 from the sites, indicating some degree of competition.

Similar T4-binding sites were detected in other rat tissue, but with the exception of serum, binding activity was lower than in pituitary. The characteristics of the cytosol and serum T4-binding proteins appeared identical, including their relative affinities for T4 analogues. However, it was demonstrated experimentally that the activity in pituitary tissue was only attributable in part to T4-binding by trapped serum.

The binding sites described herein appear to be physiological entities; T4-binding activity with similar characteristics was observed when whole pituitaries incubated in a physiological medium were allowed to interact with T4.

Under the conditions employed in these experiments, binding of thyroid hormone to nuclei was minimal and the phenomenon was not explored further. However, using methods described by Oppenheimer et al. (1974), the presence of high affinity, low capacity binding sites in rat liver nuclei has been confirmed in this laboratory.

Contemporary studies of hormone binding interactions have led to the view that certain criteria must be met before a set of binding sites can be considered as possible hormone receptors. The binding entities should have relatively high affinity for the hormone, and be saturable at low hormone concentrations. Moreover, the relative affinity of a receptor for hormone analogues should bear some resemblance to the relative biological potencies of the analogues.

The association constant (Ka) obtained in the present study for the binding interaction of T4 with pituitary cytosol was 2.5 x 10^8 l/mol, indicating only a moderate affinity of the sites for the hormone. Association constants for the interaction between thyroid hormone and the nuclear or GH1 receptor sites are considerably higher than this (Oppenheimer et al. 1974; Docter et al. 1976; Samuels et al. 1973). However, the usefulness of this measurement in vitro as an indicator of affinity has been questioned. Oppenheimer et al. (1974) found that the Ka for the T3-nuclear receptor interaction was lower when the interaction occurred in vitro than when it was assessed by an in vivo technique (Koerner et al. 1974). Oppenheimer et al. (1974) attributed this to a lack of optimal binding conditions in vitro. This same argument could be applied to
the Ka values determined in the present study. However, other observations are also inconsistent with the view that the cytosol T₄-binding sites described here are specific hormone receptors. The amounts of hormone required to saturate these sites are considerable, and the relative effectiveness of T₄ analogues in displacing [¹²⁵I]T₄ bound to the cytosol sites bears little relationship to the hormonal potencies of the analogues.

The binding sites in rat pituitary cytosol are to some extent similar to the sites described by others for liver, kidney and porcine cytosols. Thus, the Ka values are comparable and, although the sites are saturable, the values for MBC appear relatively high. Of perhaps greatest significance is the observation that, in contrast to the nuclear receptor sites, the affinity of the cytosol binding sites for T₄ is greater than for T₃ (Sufi et al. 1973; Davis et al. 1974; Visser et al. 1976).

It is now generally accepted that the thyroid hormones probably exert their physiological activity following their interaction with high affinity, limited capacity binding sites (receptors) in the cell nucleus. However, the physiological role of the cytosol binding sites is unknown. The available evidence indicates that they are not specific hormone receptors concerned directly with the hormonal action. Moreover, in contrast to the situation with the steroid hormones (Baulieu 1973), cytosol binding interactions are not prerequisite for the association of thyroid hormones with the nuclear receptors (Surks et al. 1975; Docter et al. 1976).

On the other hand, it has been reported that binding of T₄ and T₃ to liver nuclei is decreased in the presence of cytosol (Docter et al. 1976), and as shown, in the present study, the rate of deiodination of T₄ is also decreased under conditions which increase the extent of hormone binding in cytosol. These in vitro observations are consistent with the possibility that the intracellular distribution, translocation to the nuclear receptor, and metabolism of thyroid hormone is influenced in vivo by specific binding moieties in cytosol. The role played by these cytosol sites may be comparable to that played by serum proteins; indeed, the binding sites in rat serum and pituitary cytosol appear to be very similar.

The differences in the relative affinities of nuclear and cytosol binding components for T₄ analogues could permit a differential intracellular distribution of T₄ and T₃. For example, in pituitary, preferential transfer of T₃ to the nucleus should be facilitated by the fact that the nuclear receptors have a greater affinity for T₃ than for T₄, while the reverse is true for the cytosol binding sites. In addition, T₃, unlike T₄ is metabolized slowly if at all in this tissue (Galton 1975). If these observations obtain also in vivo, then it becomes easier to understand why T₃, although it generally constitutes the minor fraction of the total thyroid hormone in serum and tissues, appear to be metabolically more important than T₄. One might speculate teleologically as to whether such
conditions have developed because $T_3$ is the most effective hormone or whether it has become so because of these conditions.

Nevertheless, at present a complete understanding of the role of cytosol binding of thyroid hormone must await further investigation.

ACKNOWLEDGMENT

The excellent technical assistance of Miss Mary Jane Levesque is gratefully acknowledged.

REFERENCES


Received on August 23rd, 1976.