Chapter - III

RADIOIMMUNOASSAY OF SERUM THYROID HORMONE STATUS: INFLUENCE OF PHENYLHYDRAZINE HYDROCHLORIDE
Radioimmunoassay of Serum Thyroid Hormone Status: Influence of Phenylhydrazine Hydrochloride

Introduction

The physiology and metabolism of thyroid hormones were areas of intense research investigation during the 1970s. Over the years, interest in the mechanism by which the thyroid hormone responses were brought about, has waxed and waned, but it has reached an unparalleled peak during the last decade or so. From the voluminous information that has resulted, two general hypotheses have emerged, one suggesting a single primary mode of action, a modification of gene expression, at the nuclear level, and the other suggesting independent actions at several sites within the cell, including the nucleus.

Radioimmunoassay techniques were applied to study the concentrations of iodothyronines in biologic fluids. These techniques proved to be highly sensitive, specific, and reproducible as well as rapid. The availability of specific radioimmunoassay, led to the detection of several iodothyronines in human biologic fluids, e.g., reverse triiodothyronine (rT₃), diiodothyronines, monoiodothyronines and acetic acid derivatives of thyroxin (T₄) and (T₃), which were previously either unknown or briefly considered but forgotten in the 1950s.

All the thyroid hormones are metabolised by two major mechanisms that include sequential monodeiodination and nondeiodination routes. The rates of degradation and production of the iodothyronines depend upon substrate available and the activity of the degradation process.

Sequential monodeiodination involves outer ring (3' or 5') deiodination (ORD) or inner ring (3 or 5) deiodination (IRD). Outer ring deiodination of T₄ results in T₃ and IRD of T₄ in rT₃ production. It has been estimated about 80% of daily produced T₃ and 94% of rT₃ is generated in peripheral tissues by nodeiodination of T₄ and that the remaining 20 and 6%, respectively, originates from thyroid secretion. (Chopra et al., 1978a, Engler and Burger, 1984).
Serum levels of total and free iodothyronines are the net result of hormone production and clearance. Hormone clearance and the distribution between the vascular and extravascular compartments may be affected by multiple factors, including binding to serum carrier proteins and extravascular sites, as well as transport between serum and tissues.

The biologic effects of thyroid hormones vary with different species, as well as with the stage of development within a species (fetal vs. adult life). The studies of thyroid hormone effects on erythropoiesis are especially interesting. These studies show that thyroid hormones influence the production of erythropoietin as well as its effect \textit{in vitro} on differentiation and growth of hematopoietic precursors (Golde et al., 1977). Certain metabolic effects of the thyroid hormones are initiated at the level of plasma membrane. The earliest evidence suggesting an action of thyroid hormones at the plasma membrane level came from the studies of Green and Matty in the early 1960s (Matty and Green, 1964). They demonstrated that T$_3$ and T$_4$, in concentrations of 10 nm and higher, produced a prompt increase in the movement of water and of isotopically labeled phosphate, sodium potassium, and chloride across the isolated skin and urinary bladder of \textit{Bufo bufo}.

Certain drugs inhibit thyroid function by antagonizing formation of thyroid hormones. These drugs are generally classified into those compounds that inhibit iodide transport into the thyroid gland and those that inhibit iodine incorporation into tyrosins. Phenytoin displaces T$_4$ from its binding site on TBG both \textit{in vitro} and \textit{in vivo} (Oppenheimer \textit{et al.}, 1967; Oppenheimer and Tavemetti, 1962). Phencobarbital influences cellular binding and disposal of thyroid hormone without affecting extra cellular binding (Cavaleiri and Pitt-Rivers, 1981). The anticoagulant heparin effects \textit{(in vivo)} the thyroid hormone binding to serum protein (Hollander \textit{et al.}, 1967). Lithium causes goiter and or hypothyroidism. Propranolol, inhibits the conversion of T$_4$ to T$_3$. Thiouracil and related compounds, particularly PTU have long been known to inhibit T$_4$ deiodination in extrathyroid tissues. In
humans, also, PTU inhibits $T_4$ deiodination \textit{in vivo} (Hershman, 1964).

The findings of the previous chapters of this thesis indicates strongly towards the involvement of thyroid hormone function in the PHH-mediated toxicity in the rat model. We were curious, eager and expectant to observe and probe further into the secrets of the thyroid hormone functions. And thus it only seemed worthwhile and logical to perform radioimmunoassay with the serum beholding secrets yet unrevealed.
Materials and Methods

A. Animals for study and its maintenance:
Juvenile male rats (body wt. - 40-50 g) were used for the study of radioimmunoassay and for thyroid gland morphology. The animals were maintained as described in Chapter I.

B. Materials for injection and treatment:

i) Phenylhydrazine hydrochloride (PHH):
As described in Chapter I.

ii) L-thyroxine - sodium salt (T4):
As described in Chapter I.

C. Reagents for radioimmunoassay:

i) RIA kit for L-triiodothyronine:
A kit (code No. RIAK 4) was purchased from BARC, Mumbai, India.

ii) RIA kit for L-thyroxine:
A kit (code No. RIAK 5) was purchased from BARC, Mumbai, India.

D. Radioimmunoassay of T4 and T3:
The protocols of T4 and T3 were assayed as described in the RIA kit. Briefly, the method was as follows:
Reagents of the RIA kit and samples were kept at room temperature prior to assay and mixed thoroughly. The non-specific binding tubes contained buffer and hormone-free serum with zero hormone concentration. The specific binding assay tubes contained buffer, hormone-free serum and antiserum with zero hormone concentration. Aliquots of various standard solution, as provided in the kit (0.15, 0.3, 0.6, 1.2 and 2.4 ng/ml for T3 and 2.5,
5.0, 10.0 and 20.0 μg/dl for T₄), assay buffers, respective antiserum(sample)rat serum for assay were carefully taken in different RIA tubes. ¹²⁵I-T₄ and ¹²⁵I-T₃ were added to each tube for assay of T₄ and T₃ respectively and mixed well. The assay mixtures were incubated at room temperature for 75 minutes and 180 minutes in case of T₄ and T₃ respectively. After incubation, the precipitating agent (PEG) was mixed thoroughly to precipitate the antigen antibody complex. All the samples were analysed in duplicate. The antigen-antibody complex was then centrifuged at 3000 x g for 20 minutes. The supernatant from the tubes were decanted completely and carefully so that the precipitate was left undisturbed, with the exception of the tubes containing a known aliquot of ¹²⁵I-T₄ and ¹²⁵I-T₃ for the purpose of total count. The counts of the pellets were recorded in a Beckman gamma counter (Model 5500) for one minute (counts per minute, cpm). The experiment was repeated thrice.

**Validity of RIA:**

The sensitivity of the T₄ and T₃ assay was 0.5 μg/dl and 0.24 ng/ml respectively of the sample based on 90% B/B₀ intercept where B was the corrected average counts of standard/sample and B₀ was the corrected average counts of zero standard. The T₄ assay kit showed 5% and 0.1% cross reactivity with T₃ and T₂ respectively. The T₃-assay kit demonstrated a cross reactivity at 0.1% with both T₄ and T₂. The RIA kit reagents also contained 8 anilinonaphthosulphonic acid which made T₄ and T₃ free from the protein bound form. About 80-85% of the added hormones were recovered with the RIA kits.

**D. Statistical analysis of the data:**

As described in Chapter I.
Results

Effect of injections of PHH, T4 and PHH+T4 (Table 15, Figs. 13-18)

Three consecutive injections of different doses (10, 20 and 30 μg per g) of PHH demonstrated alterations in the serum T4 and T3 contents of the juvenile rats. Injections of 10 μg/g of PHH showed an increase in the serum levels of T4 (21.35%, P<0.001) and T3 (3.9 fold, P<0.001) over the control value obtained on day 3. Whereas, injections of higher doses of PHH at 20 and 30 μg per g effected to fall in the serum levels of T4 (48% and 68.26% respectively, P<0.001) and T3 (47% and 77.41% respectively, P<0.001) in comparison to those found in control animals.

Injections of different doses of T4 (1, 2, 4 and 8 μg per g) uniformly raised (4.7 to 5.4 fold, P < 0.001) the serum T4 content in the animals, in comparison to the control levels observed on the day 3. However, a dose-dependent rise in the serum T3 contents (6.5- to 18.0-fold, P<0.001) were observed in the animals after injections of the different doses of T4, in comparison to the T3 levels found in control animals.

The serum T4 levels were also increased (6.1 to 8.6-fold, P<0.001) compared to the control values, when various doses (1, 2, 4 and 8 μg/g) of T4 were injected in combination with PHH (20 μg/g). Such injections of T4 (at various doses) plus PHH (20 μg/g) also showed a maintenance of higher levels of T4 (30.0 to 62.85% higher, P<0.001) in comparison to the corresponding doses of only T4-injected groups. A dose-dependent increase in the T4 levels was found between T4 (2 μg/g) and T4 (4 μg/g), and between T4 (4 μg/g) and T4 (8 μg/g) in combination with PHH (20 μg/g) injected animals.

As usual, injections of different doses of T4 in combination with PHH showed a rise (6.7- to 9.7-fold, P<0.001) in the serum T3 contents of the animals in comparison to the control values. However, comparatively lower levels of T3 (10.40 to 46.42%, P<0.001) were found in serum of PHH (20 μg/g) + T4 (2, 4 and 8 μg per g) injected animals in comparison to the corresponding doses of only T4-injected groups.
Table - 15

Effect of three consecutive injections of various doses of PHH (10, 20 and 30 µg per g), T₄ (1, 2, 4 and 8 µg/g) and PHH (20 µg/g) + T₄ (1, 2, 4 and 8 µg/g) on the serum T₄ and T₃ contents of juvenile rats. Injections were given on day 0, day 1 and day 2, and blood samples were collected on day 3. Each group contained 15-20 animals. Data were expressed as ng/ml T₄ or T₃, Mean ± SEM

<table>
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<th></th>
<th>Control</th>
<th>PHH (10 µg/g)</th>
<th>PHH (20 µg/g)</th>
<th>PHH (30 µg/g)</th>
<th>T₄ (1 µg/g)</th>
<th>T₄ (2 µg/g)</th>
<th>T₄ (4 µg/g)</th>
<th>T₄ (8 µg/g)</th>
<th>PHH (20 µg/g)</th>
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<td>282.00</td>
<td>320.00</td>
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<td>390.00</td>
<td>363.00</td>
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<tr>
<td>T₃</td>
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<td>6.00</td>
<td>0.82</td>
<td>0.35</td>
<td>10.00</td>
<td>16.00</td>
<td>22.00</td>
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<td>±</td>
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<td>± 0.30</td>
<td>± 0.10</td>
<td>± 0.12</td>
<td>± 0.43</td>
<td>± 0.52</td>
<td>± 0.83</td>
<td>± 0.65</td>
<td>± 0.75</td>
<td>± 0.43</td>
<td>± 0.62</td>
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Contd.
1. Data compared between control and PHH (10, 20 or 30 µg per g) for T₄ and T₃ were significant at P<0.001.

2. Data compared within the different doses (10, 20 or 30 µg per g) of PHH for T₄ and T₃ content were significant at P<0.02 to P<0.001.

3. Data compared between control and different doses of T₄ (1, 2, 4 or 8 µg per g) for T₄ and T₃ content were significant at P<0.001.

4. Data compared within the different doses (1, 2, 4 or 8 µg per g) of T₄ groups for T₄ content showed no statistically significant variation.

5. Data compared within the different doses (1, 2, 4 or 8 µg per g) of T₄ groups for T₃ content were significant at P<0.001.

6. Data compared between control and PHH (20 µg/g) + different doses (1, 2, 4 or 8 µg per g) of T₄ for T₄ and T₃ content were significant at P<0.001.

7. For T₄ content, data compared between PHH (20 µg/g) + T₄ (1 µg/g) and PHH (20 µg/g) + T₄ (2 µg/g) did not show any significant variation, between PHH (20 µg/g) + T₄ (2 µg/g) and PHH (20 µg/g) + T₄ (4 µg/g) were significant at P<0.02, between PHH (20 µg/g) + T₄ (4 µg/g) and PHH (20 µg/g) + T₄ (8 µg/g) were significant at P<0.001.

8. For T₃ content, significant differences (P<0.001) were found in the data between groups PHH (20 µg/g) + T₄ (4 µg/g) and PHH (20 µg/g) + T₄ (8 µg/g), and between PHH (20 µg/g) + T₄ (1 µg/g) and PHH (20 µg/g) + T₄ (8 µg/g).
Fig. 13 Effect of three consecutive injections of various doses of PHH (10, 20 and 30 µg/g) on the serum T₄ content of juvenile rats. First injection of PHH was given on day '0'. Each group contained 15-20 animals. The data are expressed as Mean±SEM. The vertical bars denote the concentration of T₄ (ng/ml) and the vertical lines over the bars are the standard error of mean.
Fig. 14  Effect of three consecutive injections of various doses of PHH (10, 20 and 30 μg/g) on the serum T₃ content of juvenile rats. First injection of PHH was given on day '0'. Each group contained 15-20 animals. The data are expressed as Mean±SEM. The vertical bars denote the concentration of T₃ (ng/ml) and the vertical lines over the bars are the standard error of mean.
Fig. 15 Effect of three consecutive injections of $T_4$ (1, 2, 4 and 8 µg/g) on the serum $T_4$ content of juvenile rats. First injection of $T_4$ was given on day '0'. Each group contained 15-20 animals. The data are expressed as Mean±SEM. The vertical bars denote the concentration of $T_4$ (ng/ml) and the vertical lines over the bars are the standard error of mean.
Fig. 16 Effect of three consecutive injections of $T_4$ (1, 2, 4 and 8 μg/g) on the serum $T_3$ content of juvenile rats. First injection of $T_4$ was given on day '0'. Each group contained 15-20 animals. The data are expressed as Mean±SEM. The vertical bars denote the concentration of $T_3$ (ng/ml) and the vertical lines over the bars are the standard error of mean.
Fig. 17  Effect of three consecutive injections of PHH (20 µg/g) alone, with T₄ (1, 2, 4 and 8 µg/g) on the serum T₄ content of juvenile rats. First injection of PHH was given on day '0'. Each group contained 15-20 animals. The data are expressed as Mean±SEM. The vertical bars denote the concentration of T₄ (ng/ml) and the vertical lines over the bars are the standard error of mean.
Doses of PHH & thyroxine

Fig. 18 Effect of three consecutive injections of PHH (20 μg/g) alone with T4 (1, 2, 4 and 8 μg/g) on the serum T₃ content of juvenile rats. First injection of PHH was given on day '0'. Each group contained 15-20 animals. The data are expressed as Mean±SEM. The vertical bars denote the concentration of T₃ (ng/ml) and the vertical lines over the bars are the standard error of mean.