Chapter - II

CHANGES IN BLOOD ACETYLCHOLINESTERASE ACTIVITY (IN VIVO AND IN VITRO) AND HEINZ BODY FORMATION AFTER PHYLHYDRAZINE HYDROCHLORIDE (PHH) AND THYROID HORMONE TREATMENTS IN JUVENILE RATS
Changes in blood acetylcholinesterase activity (in vivo and in vitro) and Heinz body formation after phenylhydrazine hydrochloride (PHH) and thyroid hormone treatments in juvenile rats

Introduction

Acetylcholinesterase (AChE; EC 3.1.1.7)

Key (1921) was the first to observe that the density of red cells from whole blood increases with the age of the cells in vivo. By exploiting this principle Sabine (1955) was able to separate red cells, by simple centrifugation, into two classes representing "younger" and "older" cells. He found that acetylcholinesterase (AChE; EC 3.1.1.7) was lower in the second group. Kadlubowski and Agutter (1977) have also concluded that AChE activity decreased with cell age but permits a more qualitative evaluation of the changes that occur.

In vertebrates two different enzymes hydrolyse acetylcholine. AChE terminates the action of acetylcholine at the post-synaptic membrane in the neuromuscular junction, the other enzyme hydrolyses acetylcholine as well as many other esters but has no physiological functions.

The presence of cholinergic enzymes, AChE on erythrocyte membrane, migrating neurocrest cells (Miki and Mizoguti, 1982; Cochard and Cotley, 1983) and early myotendinous junction (Wake, 1976) are both puzzling and intriguing. AChE exists as water soluble G4 form secreted by adrenal gland (Chubb and Smith, 1975), by nerve cell cultures (Lazar and Vigney, 1980), muscle cell culture (Wilson et al., 1973), by peripheral nerve cells in vivo or upon stimulation of nerve in hemidiaphragm preparation (Skau and Birimijoin, 1978).

The hydrophobic association of the enzyme with the membrane apparently accounts for the sensitivity of AChE activity in the intact cells to antihemolytic compounds such as acyl polyols and fatty acids (Aberlin Litman, 1979). Of the many enzymes recognized in the membrane of human red cell, alterations in activity associated with diseases are found
regularly only with AChE. Thus acetylcholinesterase activity is reduced in paroxysmal nocturnal hemoglobinuria, ABO hemolytic disease of newborn and occasionally in autoimmune hemolytic anemia (Hertz and Kaplan, 1974).

Erythrocyte ghosts are commonly used for studies of membrane properties. However, for understanding cell function and membrane properties of cells it is desirable to utilize a membranous enzyme which can be assayed in the intact cell and which signals changes in the membrane configuration. AChE appears to fulfil at least part of these requirements since it is a membrane-associated enzyme (Hertz, 1963; Branley et al., 1971) and its activity can be assayed in intact cell (Ellman et al., 1961). It was, therefore, of interest to evaluate changes in AChE activity as affected by various membrane alterations.

In our study an attempt has been made to demonstrate whether the blood acetylcholinesterase is sensitive to the toxic effects of PHH. A total blood acetylcholinesterase activity was measured in rats in vivo after varied degrees of PHH insult. As total blood acetylcholinesterase activity is consisted of its red cell membrane activity combined with plasma specific and non-specific cholinesterase activities, an in vitro study has also been undertaken parallelley to understand the effect of PHH on the red cell membrane AChE activity. Assessment of the RBC membrane enzyme activity will not only indicate the action of PHH on the enzyme activity but also reflect some clues about the membrane conformity of the RBC, after PHH insult.

In addition to the toxicity of PHH on the red blood cells expressed as a function of the AChE activity, efforts have been made to express the recovery of the PHH-induced toxic state by the simultaneous thyroid hormone treatment and monitoring the alterations in the dimensions of acetylcholinesterase activity in the same conditions.

*Heinz body formation:*

Heinz bodies are small, round, intrerythrocytic inclusions caused by oxidative
denaturation of hemoglobin. They can be demonstrated in drug-induced hemolytic anemias, defects in the intraerythrocytic reducing system (e.g., G-6-PD deficiency), unstable hemoglobins, excess α- and β chains in homozygous β and α thalassemia. In the process of denaturation of unstable hemoglobins, heme-depleted hemoglobin precipitates as Heinz bodies within the red cell. Because of the defect in the amino acid composition of the labile hemoglobins, globin is irreversibly denatured to Heinz bodies at the time the heme moiety is oxidised to the Met-Hb form. These bodies coalesce and attach themselves to the red cell membrane producing cation leaks. (Jacob, H.S, 1970).

Heinz body formation is a highly sensitive indicator of in vivo oxidative stress as they also contribute to anemia (Christopher et al., 1995) due to red blood cell membrane fragility and red cell lysis.
Materials and Methods

A. Animals for study and its maintenance:

Juvenile male rats (40-50 g) were used and maintained as described in Chapter I.

B. Materials for injection and treatment:

(i) Phenylhydrazine hydrochloride (PHH) solution was prepared as described in Chapter I.

(ii) L-thyroxine (Sodium salt, $T_4$):

It was a product of Sigma Chemical Company, USA. Solution at various concentrations of L-thyroxine-sodium salt ($T_4$) was prepared as described in Chapter I.

(iii) L-triiodothyronine (free acid, $T_3$):

It was a product of Sigma Chemical Company, USA. Calculated amounts were dissolved in minimum volume of 0.1M NaOH and required volume was made by 0.9% saline.

(iv) L-triiodothyroacetic acid (free acid, TRIAC):

It was a product of Sigma Chemical Company, USA. Calculated amounts were dissolved in minimum volume of 0.1M NaOH and required volume was made by 0.9% saline.

C. Treatment of animals:

Different groups of animals were treated with PHH (20 μg/g) alone, or with or without various doses of $T_4$ (1, 2, 4 and 8 μg per g) in three consecutive injections on day '0', day 1 and day 2. The control animals were injected with similar volume of 0.9% saline. The injected volumes did not exceed 50 μl.
D. Reagents for assay of acetylcholinesterase activity (EC 3.1.1.7) and Heinz body formation:

(i) **0.1M phosphate buffer (pH 8.0):**

1.40g of disodiumhydrogen phosphate (Na$_2$HPO$_4$) and 1.56g of sodium dihydrogen phosphate (NaH$_2$PO$_4$.2H$_2$O, E. Merck (India) Ltd.) were dissolved in minimum volume of double distilled water and 100ml volume was made. The pH was adjusted to 8.0 accordingly.

(ii) **5.0 mM phosphate buffer (pH 7.4):**

0.078g of disodium hydrogen phosphate (Na$_2$HPO$_4$) and 0.070g of sodium dihydrogen phosphate (NaH$_2$PO$_4$.2H$_2$O) were dissolved in minimum volume of double distilled water and volume was made upto 100ml. The pH was adjusted to 7.4 accordingly.

(iii) **0.01 M, 5,5- dithiobis 2-nitrobenzoic acid (DTNB) solution:**

39.6 mg of DTNB (Sisco Research Laboratory, India) and 15.0 mg of sodium bicarbonate (NaHCO$_3$, S.D. Fine Chem. Pvt. Ltd., India) were dissolved in a total volume of 10 ml with 0.1M phosphate buffer (pH 7.0). This solution was prepared freshly.

(iv) **0.075 M Acetylthiocholine iodide solution:**

Acetylthiocholine iodide (Sisco Research Laboratory, India) was freshly prepared by dissolving calculated amounts to double distilled water at a concentration of 21.67 mg/ml.

E. Assay procedure for acetylcholinesterase, *in vivo*:

Acetylcholinesterase (AChE) activity was assayed by the method of Ellman et al. (1961). The assay was carried out by preparing a fairly stable suspension of whole blood in phosphate buffer (pH 8.0, 0.1M) 1:600 dilution (i.e. 10 μl blood in 6 ml buffer). Exactly 3.0 ml of the suspension were pipetted into a cuvette and 25 μl of 0.01 M DTNB reagent added,
absorbance of the suspension was adjusted to zero. Changes in the absorbance at 412 nm was recorded for 6 min after the addition of 20 µl of substrate (Acetylthiocholine iodide, 0.075 M) at 37°C and found to be linear. Data were expressed as Δ OD/min/10 mg protein.

F. Assay procedure for acetylcholinesterase activity, in vitro:

Acetylcholinesterase (AChE) activity was assayed by the method of Ellman et al. (1961). The assay was carried out by incubating a suspension of whole blood in phosphate buffer (pH - 7.4, 0.1 M at 1:600 dilution i.e, 10 µl blood in 6 ml of buffer). The blood suspension was pre-incubated for 1 hr with various concentrations (0.1, 1.0, 10.0 and 100 nM) of T₃, T₄ and TRIAC at 37°C with gentle shaking. After preincubation, PHH was added at a final concentration of 2 mM and again subjected to incubation at 37°C, for 1 hr with mild shaking.

A set of control (i.e. only blood suspension), blood suspension containing PHH (2 mM) or various concentrations (0.1, 1.10 and 100 nM) of T₃ or T₄ or TRIAC were parallelly incubated. After incubation, all the samples were washed twice with phosphate buffer (pH-8.0, 0.1 M) and finally resuspended in 6 ml of the same buffer.

Exactly 3ml of the suspension were pipetted into a cuvette and 25 µl of 0.01 M DTNB reagent added, and the photometric absorbance (at 412 nm) of the suspension was adjusted to zero. Changes in the absorbance at 412 nm was recorded for 6 min after the addition of 20 µl of substrate (Acetylthiocholine iodide, 0.075 M) at 37°C and found to be linear. Data were expressed as ΔOD/min/10 mg Hb.

Standardization of incubation time and concentration of PHH:

To establish the optimum time of incubation the blood samples were incubated for 15, 30, 45, 60, 75 and 90 minutes in PHH (1 or 2 mM) containing medium and the AChE activity of the red cell membrane was measured accordingly. Maximum inhibitory effect of
PHH on the red cell membrane AChE activity was obtained after 60 minutes of incubation in the PHH-containing medium. Therefore, 60 minutes were accepted as the optimum incubation time for the blood samples.

To evaluate the concentration of PHH, necessary in the incubation medium that elicits maximum effect on the red cell membrane AChE activity, blood samples were incubated at different concentrations (0.25 mM, 0.5 mM, 1 mM, 2 mM, 4 mM and 8 mM) of PHH for 1 hr. After incubations, red cell membrane AChE activity was measured. PHH at 4 mM maximally inhibited (70%) the AChE activity of the red cell membrane. A 44% inhibition over the control AChE activity was found with PHH at 2 mM concentration. PHH at 8 mM concentration showed similar effect, like PHH at 4 mM, on the red cell membrane AChE activity. Lower concentrations of PHH at 1 mM and 0.5 mM demonstrated an inhibition of 20% and 15% respectively, on the control enzyme activity. The lower concentration of PHH (0.25 mM) was without any significant effect. Therefore, we have used PHH at 2 mM concentration (44% inhibitory) in the successive experiments.

G. Assay procedure for Heinz body formation, in vivo:

Heinz body formed in terms of turbidity was determined by the method of Winterbourn (1979). Briefly, 0.1 ml of whole blood was lysed in 3.0 ml of 5.0 mM Sodium phosphate buffer (pH 7.4) and the turbidity was read at 700 nm in Shimadzu, model uv-160 spectrophotometer. The turbidity of each sample was expressed relative to its hemoglobin content according to the relationship (Diana and Winterbourn, 1983):

\[
\text{Turbidity at } A_{700} = \frac{A_{700} \text{ (Day '3')} \times \text{Hb (Day '0')}}{\text{Hb (Day '3')}}
\]

H. Estimation of blood protein:

Blood protein was assayed with the help of Microprotein determination kit (Cat NO.
610-A) obtained from Sigma Chemical Company, U.S.A. 10 μl of the diluted blood (1:600 dilution in 0.1mM phosphate buffer, pH 7.4) was used for the protein assay. The standard protein solution (provided in the kit) at 1.5 μg, 3.0 μg, 4.5 μg, 6.0μg and 7.5 μg were used for making the standard curve. Absorbancy of colour was read at 595 m in a Shimadzu uv-160 spectrophotometer.

I. Estimation of hemoglobin:
   Hemoglobin of blood was estimated as described before in Chapter I.

J. Statistical analysis of the data:
   As described in Chapter I.
(a) Acetylcholinesterase activity (in vivo) (Table 10):

The values of the total acetylcholinesterase (AChE) activity in different control and treatment groups on day '0' before the first injection of saline or other substances (PHH and T4), were statistically similar. Also the AChE activities between the day '0' control and day 3 control groups did not show any statistical variation. Therefore, the values of AChE activity after injections of various materials obtained on day 3 were compared with the control and other groups on the same day.

Injections of PHH (20 μg/g) caused a 48.21% decrease (P<0.001) in the activity of the AChE on day 3 in comparison to the control value obtained on day 3. Injections of T4 (1, 2 and 4 μg per g) did not show any effect on the blood AChE activity in comparison to the control on the 3rd day.

A 45.19% rise (P<0.001) in the activity of AChE in blood was observed after injections of the T4 at higher dose of 8 μg/g in comparison to the control on day 3. T4 (1 μg/g) was effective when injected with PHH (20 μg/g) to the juvenile rats, to recover 27.58% (P<0.02) of the AChE activity as compared to only PHH (20 μg/g) treated rats on 3rd day.

Injection of T4 (2 μg/g) along with PHH (20 μg/g) demonstrated a recovery of 55.17% (P<0.001) in the AChE activity in comparison to the only PHH (20 μg/g) treated animals. A higher dose of T4 at 4 μg/g when injected with PHH 20 μg/g, showed similar nature of effect as found in the T4 (2 μg/g) plus PHH (20 μg/g) injected animals. Although injections of T4 at 1, 2 and 4 μg per g with PHH (20 μg/g) dose showed partial recovery from the toxic effect of only PHH (20 μg/g) injections, the values were still 19.64% to 23.21% less (P<0.001) than the control values found on day 3 and failed to show a total recovery in the AChE activity of blood from the inhibitory effect of PHH (20 μg/g).

Interestingly, when higher dose of T4 (8 μg/g) were injected with PHH (20 μg/g) the
Table 10

Effect of three consecutive injections of PHH (20 µg/g), T₄ (1, 2, 4 and 8 µg per g) and PHH (20 µg/g) Plus T₄ (1, 2, 4 and 8 µg per g) on acetylcholinesterase activity in the blood of juvenile rats. The injections were given on day '0', 1 and 2. First injection of PHH was given on day '0' post blood sampling. Each group contained 25 animals. The data (Mean±SEM) are expressed as AOD/min/10 mg protein.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Control</th>
<th>PHH (20 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) + T₄ (1 µg/g)</th>
<th>PHH (20 µg/g) + T₄ (2 µg/g)</th>
<th>PHH (20 µg/g) + T₄ (4 µg/g)</th>
<th>PHH (20 µg/g) + T₄ (8 µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day '0'</td>
<td></td>
<td>0.243</td>
<td>± 0.012</td>
<td>0.250</td>
<td>± 0.015</td>
<td>0.239</td>
<td>± 0.007</td>
<td>± 0.025</td>
<td>± 0.027</td>
<td>± 0.011</td>
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<tr>
<td>Day '3'</td>
<td></td>
<td>0.280</td>
<td>± 0.015</td>
<td>0.145</td>
<td>± 0.011</td>
<td>0.262</td>
<td>± 0.033</td>
<td>± 0.014</td>
<td>± 0.029</td>
<td>± 0.015</td>
</tr>
</tbody>
</table>

Contd.
1. No statistical difference was found in the data compared between the control and experimental groups on day 0, and between day 0 and day 3 controls.

2. Data of AChE activity as found between control and T₄ (1, 2 and 4 μg per g) were statistically insignificant on day 3.

3. Data compared between day 3 control and T₄ (8 μg/g) on the same day was statistically significant at P < 0.001.

4. Data compared between day 3 control and PHH (20 μg/g) plus T₄ (1, 2, 4 and 8 μg per g) were statistically significant at P < 0.02 to P < 0.001 on the same day.

5. Data compared between PHH (20 μg/g) + T₄ (1 μg/g) and PHH (20 μg/g) + T₄ (2 μg/g) were significant at P < 0.001, between PHH (20 μg/g) + T₄ (2 μg/g) and PHH (20 μg/g) + T₄ (4 μg/g) were not significant, between PHH (20 μg/g) + T₄ (4 μg/g) and PHH (20 μg/g) + T₄ (8 μg/g) were significant at P < 0.001.

Student's t - distribution, P values:
AChE activity in blood showed an abrupt rise (P<0.001) of 124.13% over the values obtained in only PHH (20 μg/g) treated animals and 16.07% over the control values as obtained on day 3.

(b) Acetylcholinesterase (AChE) activity of red cell membrane, in vitro (Tables 11-13):

In vitro system mostly offers such an environment that a number of compounds may be tested for a particular effect without any difficulty or hindrance that are faced in vivo. In our experiment, we had a free access to demonstrate the effects of T₄, T₃ and thyroid hormone analog, like L-triiodothyroacetic acid (TRIAC), on the alterations of the PHH-induced toxic effects in the red cells.

In vitro incubation of the red cells in PHH (2 mM)-containing medium showed an inhibition (44%, P<0.001) of the AChE activity in comparison to the control cells. T₄ at different concentrations (0.1 nM, 1 nM, 10 nM and 100 nM) did not show any alterations in the AChE activity. Incubations of the red cells with T₄ (1 nM) plus PHH (2 mM) caused 25.30% (P<0.001) recovery of the AChE activity (rise) in comparison to the enzyme activity found with only PHH (2 mM)-treated red cells. Higher concentration of T₄ (10 nM) with PHH (2 mM) showed similar level of recovery of the AChE activity like T₄ (1 nM) plus PHH (2 mM)-treated cells. But addition of T₄ at 100 nM in the PHH (2 mM) containing medium effected to 43.37% (P<0.001) recovery (rise) of the red cell membrane AChE activity in comparison to the enzyme activity found in only PHH (2 mM)-treated red cells.

When the action of T₃ was examined it appeared to be more potent than T₄, in elevating the PHH-induced inhibited AChE activity of the red cell membrane in a dose-dependent manner, when added along with PHH in the medium. A significant rise (P<0.001) of 21.01% over the PHH (2 mM)-induced activity of the AChE was found when the red cells were incubated in PHH (2 mM) plus T₃ (0.1 nM)-containing medium. When the concentration of T₃ was raised to 1 nM with PHH (2 mM), the incubated red cells showed
Effect of PHH (2 mM), T4 (0.1 nM, 1 nM, 10 nM and 100 nM) and PHH (2 mM) + T4 (0.1 to 100 nM) on the red cell membrane acetylcholinesterase activity of juvenile rats measured in vitro. Blood samples from 15-20 juvenile animals were assayed in triplicates. The data (Mean±SEM) are expressed as ΔOD/min/10 mg Hb.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PHH (2 mM)</th>
<th>T4 (0.1 nM)</th>
<th>T4 (1 nM)</th>
<th>T4 (10 nM)</th>
<th>T4 (100 nM)</th>
<th>PHH (2 mM) + T4 (0.1 nM)</th>
<th>PHH (2 mM) + T4 (1 nM)</th>
<th>PHH (2 mM) + T4 (10 nM)</th>
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<tr>
<td></td>
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<td>0.166</td>
<td>0.289</td>
<td>0.291</td>
<td>0.284</td>
<td>0.302</td>
<td>0.172</td>
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<td>(F)</td>
<td>(G)</td>
<td>(H)</td>
<td>(I)</td>
<td>(J)</td>
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</tbody>
</table>

Student's t-distribution P values:

A-B = P < 0.001
A-C = NS
A-D = NS
A-E = NS
B-G = NS
B-H = P < 0.01
B-I = P < 0.001
H-I = P < NS
Table 12

Effect of PHH (2 nM), T3 (0.1 nM, 1 nM, 10 nM and 100 nM) and PHH (2 mM) + T3 (0.1 to 100 nM) on the red cell membrane acetylcholinesterase activity of juvenile rats measured in vitro. Blood samples from 15-20 juvenile animals in each group were assayed in triplicates. The data (Mean±SEM) are expressed as ΔOD/min/10 mg Hb.

<table>
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<tr>
<th>Groups</th>
<th>PHH (2 mM)</th>
<th>T3 (0.1 nM)</th>
<th>T3 (1 nM)</th>
<th>T3 (10 nM)</th>
<th>T3 (100 nM)</th>
<th>PHH (2 mM) + T3 (0.1 nM)</th>
<th>PHH (2 mM) + T3 (1 nM)</th>
<th>PHH (2 mM) + T3 (10 nM)</th>
<th>PHH (2 mM) + T3 (100 nM)</th>
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<tbody>
<tr>
<td>Control</td>
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<td>0.314</td>
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<td>0.303</td>
<td>0.301</td>
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<td>Student’s t-distribution P values:</td>
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<td></td>
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<tr>
<td>A-B</td>
<td>P &lt; 0.001</td>
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<tr>
<td>A-C</td>
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<tr>
<td>A-D</td>
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<tr>
<td>B-G</td>
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Note: P < NS indicates a non-significant difference.
Table 13

Effect of PHH (2 mM), TRIAC (0.1 nM, 1 nM, 10 nM and 100 nM) and PHH (2 mM) + TRIAC (0.1 to 100nM) on the red cell membrane acetylcholinesterase activity of juvenile rats measured *in vitro*. Blood samples from 15-20 juvenile animals were assayed in triplicates. The data (Mean±SEM) are expressed as AOD/min/10 mg Hb.

<table>
<thead>
<tr>
<th>Control</th>
<th>PHH (2 mM)</th>
<th>TRIAC (0.1 nM)</th>
<th>TRIAC (1 nM)</th>
<th>TRIAC (10 nM)</th>
<th>TRIAC (100 nM)</th>
<th>PHH (2 mM) + TRIAC (0.1 nM)</th>
<th>PHH (2 mM) + TRIAC (1 nM)</th>
<th>PHH (2 mM) + TRIAC (10 nM)</th>
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<tr>
<td>0.296</td>
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<td>0.287</td>
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<td>(H)</td>
<td>(I)</td>
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</tr>
</tbody>
</table>

Student's t-distribution P values:

A-B = P < 0.001  B-G = NS
A-C = NS  B-H = NS
A-D = NS  B-I = NS
A-E = NS  B-J = P < 0.02
A-F = NS

acetylcholinesterase activity of juvenile rats measured *in vitro*. Blood samples from 15-20 juvenile animals were assayed in triplicates. The data (Mean±SEM) are expressed as AOD/min/10 mg Hb.
58.02% (P<0.001) increase in the AChE activity over the PHH (2 mM)-treated cells. A maximum rise of 81.32% (P<0.001) of the AChE activity was found in the red cell membrane incubated with T₃ (10 nM) plus PHH (2 mM)-containing medium in comparison to the only PHH (2 mM)-treated red cells. The AChE activity found in red cells in T₃ (10 nM) plus PHH (2 mM)-containing medium was very similar to the enzyme activity found in control (untreated) red cells. T₃ (100 nM) with PHH (2 mM) showed similar effect on the red cell AChE activity as found with T₃ (10 nM) plus PHH (2 mM)-containing medium.

L-triiodothyroacetic acid (TRIAC), a thyroid hormone analog was examined for its hormonal potency to recover the PHH-induced inhibited AChE activity, in vitro.

The red cells, when incubated in only TRIAC (0.1, 1, 10 and 100 nM)-containing medium did no show any effect on the membrane AChE activity in comparison to the control red cell AChE activity. Only high concentration of TRIAC (100 nM) with PHH (2 mM) was able to recover (raise, P<0.001) 21.68% of the PHH (2 mM)-induced inhibited red cell membrane AChE activity. Other concentrations of TRIAC (0.1, 1 and 10 nM) tested with PHH (2 mM) did not show any significant alteration in the level of red cell membrane AChE activity in comparison to that found with only PHH (2 mM)-treated red cells.

(c) Heinz body formation, in vivo (Table 14):

Heinz body formed in the blood samples were measured as the turbidity observed at A700, as described in the 'Materials and Methods' section in this chapter. On day 0, the animals in control and different experimental groups showed no statistically significant variation in turbidity of blood samples collected before the commencement of hormone or drug treatments. Even the control values of the turbidity measured on day 0 and day 3 remained statistically unchanged. This showed the identical condition of blood turbidity in the different groups of animals prior to treatment on day 0, and in the controls animals on day 0 and day 3.
Table 14

Effect of three consecutive injections of PHH (20 μg/g), T4 (1, 2, 4 and 8 μg/g) and PHH (20 μg/g) plus T4 (1, 2, 4 and 8 μg per g) on the Heinz body formation in blood of juvenile rats. Injections were given on day 0, 1 and 2. The first injection was given on day '0' post blood sampling. Each group contained 15-20 animals. Heinz body formation is expressed as turbidity of the blood samples at A700, Mean±SEM.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PHH (20 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>
<th>PHH (20 μg/g)</th>
<th>PHH (20 μg/g)</th>
<th>PHH (20 μg/g)</th>
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<tr>
<td>Day 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.155</td>
<td>0.158</td>
<td>0.160</td>
<td>0.156</td>
<td>0.161</td>
<td>0.152</td>
<td>0.163</td>
<td>0.160</td>
<td>0.155</td>
<td>0.164</td>
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<tr>
<td></td>
<td>± 0.010</td>
<td>± 0.012</td>
<td>± 0.009</td>
<td>± 0.010</td>
<td>± 0.007</td>
<td>± 0.012</td>
<td>± 0.005</td>
<td>± 0.011</td>
<td>± 0.015</td>
<td>± 0.014</td>
</tr>
<tr>
<td></td>
<td>(A)</td>
<td>(B)</td>
<td>(C)</td>
<td>(D)</td>
<td>(E)</td>
<td>(F)</td>
<td>(G)</td>
<td>(H)</td>
<td>(I)</td>
<td>(J)</td>
</tr>
<tr>
<td>Day 3</td>
<td>0.161</td>
<td>0.742</td>
<td>0.159</td>
<td>0.163</td>
<td>0.161</td>
<td>0.157</td>
<td>0.620</td>
<td>0.412</td>
<td>0.387</td>
<td>0.962</td>
</tr>
<tr>
<td></td>
<td>± 0.011</td>
<td>± 0.015</td>
<td>± 0.009</td>
<td>± 0.010</td>
<td>± 0.008</td>
<td>± 0.011</td>
<td>± 0.008</td>
<td>± 0.010</td>
<td>± 0.012</td>
<td>± 0.014</td>
</tr>
<tr>
<td></td>
<td>(A₁)</td>
<td>(B₁)</td>
<td>(C₁)</td>
<td>(D₁)</td>
<td>(E₁)</td>
<td>(F₁)</td>
<td>(G₁)</td>
<td>(H₁)</td>
<td>(I₁)</td>
<td>(J₁)</td>
</tr>
</tbody>
</table>

Student's t-distribution, P values:

1. No statistical difference was found in the data compared between the control and experimental groups on day 0 and between day 0 and day 3 controls.
2. Data compared between control and T4 (1, 2, 4 and 8 μg per g) - treated groups were found to be statistically identical.
3. The data compared between groups A₁ - B₁, B₁ - G₁, B₁ - H₁, G₁ - H₁ and B₁ - J₁ on day 3, were significant at P < 0.001.
4. The data compared between groups H₁ - I₁ on day 3 did not show any statistically significant variation.
PHH (20 μg/g) injections to the animals significantly increased (4.6-fold) the blood turbidity in comparison to the control on day 3. Injections of various doses of T4 (1, 2, 4 and 8 μg per g) alone did not alter the turbidity of blood on day 3, in comparison to the control data obtained on the same day.

T4 (1 μg/g) when injected with PHH (20 μg/g), caused 16.44% decrease (P<0.001) in turbidity over the value obtained with injection of PHH (20 μg/g) only, on day 3. T4 at 2 μg/g injected with PHH (20 μg/g) further decreased (44.47%, P<0.001) the turbidity of blood found only after injections of PHH (20 μg/g) alone. A higher dose of T4 (8 μg/g) injected with PHH (20 μg/g) showed aggravation of the turbidity formation (29.64%, P<0.001) compared to that of PHH (20 μg/g) treated group. No statistically significant difference in the degree of inhibition of turbidity formation in blood was found after injections of T4 at 2 μg/g and 4 μg/g in combination with PHH (20 μg/g).