CHAPTER - VIII:

STUDIES OF PHYSICAL VARIATIONS AND HISTOCHEMICAL CHANGES ON ALKALINE PHOSPHATASE IN ALLINO RAT AFTER TREATMENT OF THEVETIA NERIFOLIA JUSS EXTRACT
It was Lison (1936) who have identified and localised some chemically recognisable substances present in the cellular level and tissue of mammals with more perfectly and distinctly and described elaborately in his treatise. Cellular enzymes and some other constituents can be visualised under microscope by histochemical methods. Out of the enzymes which have been given particular attention in histochemical studies were alkaline phosphatase, cholinesterase, succinic dehydrogenase, cytochrome oxidase.

The development of histochemical methods for the demonstration of alkaline phosphatase has made possible the localisation of the enzyme in various tissues and has obtained facts and figures about their possible functions.

Cytochemically alkaline phosphatase were visualised by transferring them into a coloured precipitate at the site where hydrolysis of a suitable substrate has occurred. Gomori (1939) and Takamatsu (1939) independently first developed two methods for staining and visualisation of alkaline phosphatase histochemically. Wachstein (1946) and Danielli (1946) widely applied the first method in haematology. Menten et al. (1944), Gomori (1952), Manheimer (1948) after some modification second method was also applied for localisation of alkaline phosphatase.
Dixon (1949) showed that most important in vivo activity of alkaline phosphatase was occurred with the transfer of phosphate from one alcohol to another. Since there were a number of non-specific alkaline phosphatase, Moss (1964) pointed out that alkaline phosphatase from human tissues was distinct in electrophoretic and enzymatic properties and that there is several substrate specific alkaline phosphatase. Glick (1945), Dempsey (1949) and Friedenwald et al. (1950) reported that various alkaline phosphatase could be distinguished histochemically. The substrate specificity of alkaline phosphatase was shown elaborately by Dempsey et al. (1949).

Gomori (1949) used 19 different substrate in order to visualise distribution and localisation of alkaline phosphatase by histochemical means. Out of which he found with 18 of them no evidence in acetone-fixed paraffin embedded tissues. In 1950b he studied with 10 more substrates and observed intense reactions except one.

Novikoff (1958) stressed the pH activity range for substrate specific phosphatase in order to locate and demonstrate specific phosphatase histochemically in a given tissue.

The quantitative histochemical estimation of alkaline phosphatase in tissue sections was possible due to Gomori (1950b) and showed that for histochemical studies shorter
the incubations more accurate is the localisation. Doyle (1950) reported another method of Gomori's type for histochemical preparations.

Mechanism of calcification has almost invariably included a role in the variation of alkaline phosphatase. The alkaline phosphatase in fibroblasts, osteoblasts in tissues and in calcification of tuberculous lymph nodes were reported to be degenerative one (Henrichgen, 1956). Westmoreland et al. (1969) reported the abnormal degenerate state of epiphyseal plate cells from zn-deficient chicks. Buck (1953), Prichard (1952) and Mogg (1944) have reported the high alkaline phosphatase activity in extracellular matrix section. Moog et al. (1952) have shown histochemically that in embryonic chick bone, alkaline phosphatase activity was high. Teaford et al. (1964) showed in a study on osteogenesis in vitro using embryonic chick femora that the highest alkaline phosphatase activity related to cartilage cell degeneration. The presence of alkaline phosphatase in brain was first shown by Fleishhacker (1938) and reported its variation under unfavourable biological conditions.

Abolins (1948) have found small amounts of alkaline phosphatase in mammalian endocrines and he demonstrated that considerable quantity of this enzyme was present in the anterior pituitary lobe of the guinea pig. He also reported that
basophilic and chromophobic islet exhibit no or very slight enzymatic reaction. The presence of alkaline phosphatase was correlated with the occurrence of ribose nucleic acid and the presence of which in acidophilic cells of the guinea pig pituitary was shown by Desclin (1940).

The high alkaline phosphatase was found to be present in the cytoplasm of osteoblasts which was related to the activity of osteoblast in secreting materials which forms the bone matrix.

Liver normally contains very low concentration of alkaline phosphatase and hence larger portion of serum alkaline phosphatase would be formed extrahepatically. The polygonal hepatic cells also excrete it to the intestine through the bile canaliculi. Sherlock et al. (1947) have showed the liver phosphatase activity with histochemical pictures and thereby it was reported that in acute hepatitis, the alkaline phosphatase increased in hepatic cells and sinusoids. They also shown histochemically that in acute hepatitis the liver phosphatase was increased markedly due to its failure to excrete. They also showed that alkaline phosphatase in the hepatic cell rises and the liver enlarged in excreting phosphatase during bone disease.

Park (1939) revealed high alkaline phosphatase activity in the epiphyseal cartilage and in the osteoid tissues sub-
adjacent to the cartilage during the disease. Robinson (1932), Gutman et al. (1949) and Gutman (1959) have shown by histochemical and extraction process that the osteoblast cells secrete alkaline phosphatase into the extracellular space by a mechanism similar to osteogenesis and from this extracellular space the enzyme enters the plasma because of which serum alkaline phosphatase activity rises.

Wachstein (1946) and Plum (1950) have found that alkaline phosphatase activity in blood cells to be confined to the more mature granulocytes. They also determined the activity of alkaline phosphatase in chronic granulocytic leukaemia.

Kaplow (1955) used a similar method in order to localise alkaline phosphatase in blood cells and he reported positive response in this regard using azodye staining.

Wachstein et al. (1946) and Haight et al. (1950) reported that in the peripheral blood only the segmented and bend form neutrophils contains alkaline phosphatase. An adequate counter stain and a short incubation period made it apparent that alkaline phosphatase was demonstrable only in the cytoplasm of these peripheral blood cells and that, it does not appear in the nucleus. The increased activity of alkaline phosphatase of leukocytes in pyrogenic infection was observed by Wiltshow et al. (1955).
Kerppola (1951) reported that neutral cells contain both acid and alkaline phosphatase where acid cell contained acid and alkaline cell contained alkaline phosphatase and the distribution of phosphatase between the nuclei and the cytoplasm was also regular. In blood, phosphatase was found in red cells, chiefly in nuclei and leukocytes in the cytoplasm.

Phosphatase are commonly occurring enzymes important in enzyme producing metabolic processes and membrane transport. The distribution of energy activity is specific and the localisation have been related to the morphologically identifiable structures. Species differentiation is another factor in localisation of energy activity.

Gomori (1949a) have shown that phosphatase differ among themselves in three respects - i) substrate specificity, ii) organ specificity, iii) specific inhibition or activation effects. Alkaline phosphatase of different organ may be actually different which can be shown by their slightly different resistance to inhibitors or by different pH optima. Belfanti et al. (1935) and Hommerberg (1929) believed that bone phosphatase was different from renal or hepatic phosphatase. Cloeteus (1939) distinguished alkaline phosphatase on the basis of the degree of activation by
magnesium. Bodansky (1937) found that intestinal phosphatase could be distinguished from bone or renal enzyme.

Enzymes being an important constituents of the protoplasm of all cells functioning as biological catalysts in different biochemical reaction can be best understood by histochemical methods either in the normal or abnormal state.

In the present investigation, after the administration of aglycone extracted from *T.neriifolia* Juss have produced a change in behaviour, change in respiration rate etc. of the animal and a paralytic effect in the hinder part of the body. The effect was found high in the administrating part of the body. The motor activity of the animal reduced and coma state (Fig.21) was observed and finally at high dose death occur.

This behavioural and paralytic symptoms led us to investigate the effect of *Thevetia* extract on the alkaline phosphatase activity in the brain, liver and pituitary - by a histochemical method and thereby a correlation is being shown in this chapter.

**Behavioural and CNS effect of glycoside:**

The use of some cardiac glycosides like digitalis have produced some kind of side effects. The long standing use of
digitalis effect on the CNS was recognised by clinicians. Batterman et al. (1948) reported its additional CNS effect in man such as behaviour modifications, headache, paresthesias and other changes. The neural effect of digitalis on the respiratory centres, spinal cord, autonomic ganglia, peripheral nerves was reported by Frazer et al. (1925), Konzatt et al. (1952), Bircher et al. (1963), Gills (1969), Gills et al. (1972), Basu-Ray et al. (1972a,b) and others on the laboratory animals. The convulsive effect of digitalis like glycosides was also reported by Gold et al. (1947), Chen et al. (1938) and Buterbough et al. (1970).

Birks (1963) reported that digitalis have enhance release of acetylcholine in ganglia and in neuromuscular junction and thereby inhibition of normal mechanism of cellular system and nervous occurs. Similar action was reported to be shown by digitoxigenin. On the other hand, Okita (1969) reported that intravenous administration of ouabain exhibit similar effect of digitoxigenin. Fraser et al. (1925), Lewin (1970) and Petsche et al. (1970) have showed that the direct administration of ouabain to the brain or spinal cord induces convulsions or their electroencephalographic correlates. Repke (1963) showed that the aglycone portion of the glycoside produced the similar effect but the presence of one or more sugar units influence the penetrability into the CNS.
It was revealed by Chen et al. (1948) and Lage et al. (1966) that more polar compounds have less such effect on the nerve cells.

Thus the effect of digitalis or digitalis like glycosides or aglycone have profound effect in the behaviour of nerve cells and thereby a total effect distributed in the living body.

Osterberg et al. (1973) have shown the effect of digitalis on the CNS and thereby seizure the activity of the nerve cell, brain cell and parallel profound side effects were noted.

MATERIALS AND METHOD:

Materials:
The material used in this investigation were brain, liver and pituitary of the albino rats and collected in two groups.

Group I: Brain, liver and pituitary of the albino rat were collected after one hour of administration of 0.1 ml. absolute alcohol as control group.

Group II: Brain, liver and pituitary of the albino rat were collected after one hour of administration of Thevetia extract as test group.

Method:
Detection of alkaline phosphatase by Gomori (1939) method:
Principle:
The principle of the method is to convert the site of alkaline phosphatase into a visible range. Initially, the site of enzyme activity is determined by using calcium ions and the phosphate ions liberated from the ester due to the activity of alkaline phosphatase is then converted into visible black precipitate of cobalt salt. The distribution of this specific enzyme in the fresh tissue was identified.

Reagents required:
The substrate was prepared by the following reagents:

i) 2% aqueous sodium glycerophosphate - 25 ml.
   2% aqueous sodium barbitol - 25 ml.
   2% aqueous magnesium sulphate - 5 ml.
   Distilled water - 50 ml.
   and a few drops of chloroform was added.
   The pH of this substrate media was 9.4.

ii) 2% aqueous cobalt nitrate solution.

iii) 1 ml. ammonium sulphide solution and 100 ml.
    distilled water.

iv) Paraffin wax B.P. 40°to 50°C.

Procedure:
1) The tissue sections of the control and treated groups were taken out immediately from the killed animals and were fixed in absolute acetone and kept for 24 hours.
Dehydrated at room temperature in changes of absolute alcohol for 12 hours.

Fluid was rapidly drained off and placed in two changes of benzene for 30 minutes each.

Embedded in paraffin at 50°C for 2 hours and then cut into thin sections and floated in warm water and mounted.

Slides were then dried, placed in oven for 10 mins. to melt the paraffin and then deparaffinized and brought up to water in the usual manner.

The sections were incubated for 2 hours in the substrate media at 37°C.

Rinsed in distilled water and immersed in cobalt nitrate solution for 5 minutes.

Placed in diluted ammonium sulphide solution for 2 minutes. Washed well in distilled water.

Dehydrated quickly in graded alcohols, cleaned in acetone and then mounted in D.P.X.

The slides were then visualised and photographs were taken with the help of olympus (Japan) microphotographic apparatus.

A section of the liver both of the control and treated animals were also developed for histopathological observations by the usual procedure in order to locate the cell structure.
Effect of Thevetia extract on rate of respiration in albino rat:

During this total investigation the rate of respiration of the control and treated groups were noted in three groups:

Group I: Control group of albino rats.  
Group II: Administered 2 mg/ml. of the Thevetia extract as test group.  
Group III: Administered 4 mg/ml. of the Thevetia extract as test group.

The rate of respiration per minute of the animals were noted after 15 minutes, 30 minutes and 45 minutes of administration of the glycoside at least for five times in the test groups using stop watch. The respiration rate of the control groups were noted randomly.

The motor and other physical activity of the animals were also noted both in the control and test groups.

Observations and Results:

The elevation of serum alkaline phosphatase was shown by histochemical analysis in Fig.22, 23, 24 and Table XXXVI. The histochemical analysis was done with the tissue slices before (control) and after treatment of 4 mg/ml. (treated) of the Thevetia extract.
Photomicrograph showing histopathological changes in liver.

Fig. 22: Photomicrographs showing histochemical changes of alkaline phosphatase activity in the hind brain of control and treated.
Fig. 23: Photomicrographs showing histochemical changes of alkaline phosphatase activity in the liver of control and treated (4 mg/ml.) albino rats (x 400).
Fig. 24: Photomicrographs showing histochemical changes of alkaline phosphatase activity in the pituitary of control and treated (4 mg/ml.) albino rats (x 400).
The brain cell has shown a distinctly high activity (++++) of alkaline phosphatase in broad band after the treatment of the Thevetia extract (Fig.22). The distribution and activity of alkaline phosphatase in the control was not as high to the treated one (++) (Fig.22, Table XXXVI).

The alkaline phosphatase of liver cells after treatment of 4 mg/ml. of the extract raised the activity slightly to a high level (+++) to the control liver cells (+) (Fig.23, Table XXXVI).

The marked and significant increased activity of alkaline phosphatase was also found in pituitary cells (+++) after the treatment of the Thevetia extract (Fig.24, Table XXXVI).

The effect of the Thevetia extract on the respiration rate was shown in Table XXXVII. The mean respiration rate in the control group was found to be 161.40 ± 4.99 times/min. with a range of 152 to 168 ± 4.99 times/min.

The respiration rate after the administration of 2 mg/ml. (Group II) and 4 mg/ml. (Group III) of extract were measured at the end of 15 minutes, 30 minutes and 45 minutes.

In Group II, the respiration rate at the end of 15 minutes was found to fall to 112.10 ± 3.63 times/min. At the end of 30 minutes it slightly rises to 130.80 ± 4.37 times/min. and
Fig. 25: Showing the rate of respiration in control and treated (2 mg/ml. and 4 mg/ml.) groups of albino rats.
again at the end of 45 minutes the rate falls to 122.70 ± 3.09 times/min. and the range of variations were 107 to 140 times/min., 125 to 140 times/min., 118 to 128 times/min. respectively. The co-efficient of variation were 3.24, 3.34 and 2.52 respectively (Table XXXVII).

The administration of 4 mg/ml. (Group III) the respiration rate after 15 minutes fall to 95.80 ± 10.96 times/min. But at the end of 30 minutes the rate again rises to 108.10 ± 17.47 times/min. and again at the end of 45 minutes it slightly rises to 119.30 ± 13.96 times/min. (Table XXXVII). The range of variations were 85 to 116 times/min. 78 to 132 times/min. 100 to 145 times/min. with co-efficient of variation 11.44, 16.16 and 11.70 respectively (Table XXXVII).

Results of the variation of rate of respiration were represented in Fig.25.

In the physical observations it has also been found that the motor activity of the animals decreases significantly i.e. control group (++++) to Group III (+) and at the same time the restlessness of the animals were found highly significant (Table XXXVIII). At the same time the drowsyness of the animals were also observed.
TABLE XXXVI:

Histochemical demonstration of alkaline phosphatase activity of Brain, liver and Pituitary before (control) and after administration of Thevetia extract in white albino rat.

<table>
<thead>
<tr>
<th>Tissue sections</th>
<th>Control (Group I)</th>
<th>Treated (Group II)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>++</td>
<td>++++</td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Pituitary</td>
<td>+</td>
<td>+++</td>
</tr>
</tbody>
</table>

+ = minimum activity.

+++ = maximum activity.
<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Control (Gr.I)</th>
<th>Treated 2mg/ml (Gr.II)</th>
<th>Treated 4mg/ml. (Gr.III)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respiration rate/min.</td>
<td>Respiration rate/min.</td>
<td>Respiration rate/min.</td>
</tr>
<tr>
<td></td>
<td>At the end of 15 min.</td>
<td>At the end of 30 min.</td>
<td>At the end of 45 min.</td>
</tr>
<tr>
<td>1</td>
<td>152</td>
<td>107</td>
<td>118</td>
</tr>
<tr>
<td>2</td>
<td>159</td>
<td>114</td>
<td>112</td>
</tr>
<tr>
<td>3</td>
<td>160</td>
<td>109</td>
<td>122</td>
</tr>
<tr>
<td>4</td>
<td>162</td>
<td>112</td>
<td>129</td>
</tr>
<tr>
<td>5</td>
<td>155</td>
<td>118</td>
<td>140</td>
</tr>
<tr>
<td>6</td>
<td>168</td>
<td>115</td>
<td>133</td>
</tr>
<tr>
<td>7</td>
<td>163</td>
<td>112</td>
<td>130</td>
</tr>
<tr>
<td>8</td>
<td>165</td>
<td>110</td>
<td>127</td>
</tr>
<tr>
<td>9</td>
<td>166</td>
<td>108</td>
<td>128</td>
</tr>
<tr>
<td>10</td>
<td>164</td>
<td>116</td>
<td>129</td>
</tr>
</tbody>
</table>

Mean: 161.40  112.10  130.80  122.70  95.80  108.10  119.30
Cv:    3.09    3.24    3.34    2.52    11.44   16.16   11.70
**TABLE XXXVIII:**

Physical observations of the albino rats in control and test groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Motor activity</th>
<th>Restlessness</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>+++</td>
<td>Nil</td>
<td>NIL</td>
</tr>
<tr>
<td>(control)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group II</td>
<td>++</td>
<td>++</td>
<td>slight nose mucous</td>
</tr>
<tr>
<td>(2 mg/ml)</td>
<td></td>
<td></td>
<td>appears with drowsiness.</td>
</tr>
<tr>
<td>Group III</td>
<td>+</td>
<td>becomes almost senseless</td>
<td>excess nose mucous appears with almost completely drowsiness.</td>
</tr>
<tr>
<td>(4 mg/ml)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

+ = minimum activity.

++++ = maximum activity.
DISCUSSION:

The histochemical analysis of brain, liver and pituitary shows a change in the activity of the alkaline phosphatase in the treated groups. The activity of alkaline phosphatase in brain cells were found to be much higher than pituitary and liver cells as represented in Table XXXVI and Fig. 22, 23 and 24 respectively. In Chapter VII, in the treated groups the elevation of serum alkaline phosphatase activity was highly significant (P < 0.0005).

The primary source of serum alkaline phosphatase was reported (Robinson, 1934) to be osteoid tissue and different organs. In times of unfavourable biological conditions the level of serum alkaline phosphatase activity in different organs rises to a high level.

The highly significant alkaline phosphatase activity in the brain cells of the treated animals may indicate an increase production of alkaline phosphatase by the brain cells or the higher alkaline phosphatase activity observed in the brain cell may be an effect of the increase in calcium availability in the brain cells of the treated groups. It has been discussed in the Chapter VII that the serum calcium level in the treated groups was significantly high (P < 0.0005).

The mild and moderate increase of alkaline phosphatase
Plate 21: Photographs showing the postures of albino rats in control and after administration of 4 mg/ml. of Thevetia glycoside.
activity in the liver and pituitary as observed in the histochemical analysis is thought to be due to their lower alkaline phosphatase activity than the brain.

In both the treated groups receiving 2 mg. and 4 mg. of Thevetia extract the rate of respiration was lowered significantly. The effect is more pronounced with higher doses of extract. In both the groups there is an initial phase of rapid fall in the rate of respiration up to 15 minutes followed by gradual increase in the rate of respiration up to 45 minutes without achieving the initial rate (Fig. 25). In the group receiving 4 mg. the gradual increase after the initial depression is more uniform than in the group receiving 2 mg. which has a tendency of secondary decrease in the rate of respiration after 30 minutes.

The effect of the aglycone on respiration may be tried to explain in the light of Birks (1963) on the direct glycosidic effect on the CNS, ganglion and neuromuscular transmission mechanism of the animals. Birks (1963) reported that digitalis have such action which was found specific on the ganglion of the CNS. This was again supported by Batterman et al. (1948) that digitalis have similar actions on CNS, brain, spinal cord and autonomic ganglion of man.

Because of much similarity of the Thevetia glycosides with
digitalis in its chemical composition and structure the similar action of Thevetia glycoside on different sites of the nervous system can be interpreted.

Serum calcium is a factor which enhance the action of glycosides on the tissues and contraction of striated muscle. The elevated serum calcium level in the treated groups of animals may also contribute in this effect.

After administration of the extract the animals assumed a typical posture characterised by stiffness of the limbs, more pronounced in the hind limbs with generalised rigidity of the whole animal and frothing from the nastriles (Fig.26). This postural and muscular changes may also indicate the effect of the aglycone on the different sites of the nervous systems.

Considering all these parameters it may be assumed that the Thevetia glycoside has a direct action on the nervous system which may be enhanced by the elevated serum calcium level and produces a significant rise in the alkaline phosphatase activity in different tissues more pronounced in brain.
SUMMARY:

1. The histochemical analysis of the brain, liver and pituitary have shown a high activity of alkaline phosphatase.

2. The activity of alkaline phosphatase in brain, liver and pituitary can be shown in the following way:

   brain → Pituitary → Liver.

3. A histopathological investigation of the liver indicated the ineffectiveness of the aglycone to change the cell structure.

4. The respiration rate of the animals were varied significantly. The mean value of the Group I was $161.40 \pm 4.99$ times/minutes. The mean value of Group II and Group III after 15 minutes, 30 minutes and 45 minutes were $112.10 \pm 3.63$, $130.80 \pm 4.37$, $122.70 \pm 3.09$ times/minutes and $95.80 \pm 10.96$, $108.10 \pm 17.47$, $119.30 \pm 13.99$ times/minutes respectively.

5. The motor activity and other physical abnormalities were noted in the treated and control groups as shown in Table XXXVIII.