EXPERIMENTAL
1. Treatment of animals:

Male albino rats of body weight 140-150 grams were used in these experiments. Before experiments, the animals were kept for several days on the following stock diet: wheat 88%, milk powder 8%, yeast 2%, salt mixture 0.8% and cod liver oil 0.9%.

Different central nervous system stimulating drugs or agents viz. picrotoxin (10 mg./kg.), strychnine (5 mg./kg.) and tetanus toxin (5 ml./kg.) were injected intramuscularly. Control animal was given normal saline (1 ml./kg.) by intramuscular route. The effects of administration of strychnine, picrotoxin and tetanus toxin was noted as follows:-

**Picrotoxin**: Clonic type of continuous convulsion for 10-12 mins. were noted after 9-10 mins. of injection. Convulsions throughout the body specially the four limbs were continued for 5-6 mins. associated with jumping. Between 7th to 9th mins., vigorous muscular contraction together with protrusion of the head towards the belly were noted. Between 10th to 12th mins., the respiratory and heart rates significantly increased; sometimes death may occur due to respiratory failure.

**Strychnine**: Tonic type of continuous convulsion for only 90-120 secs. were noted after 12-15 mins. of administration of drug. Convulsions were tetanic in nature as both extensor
and flexor muscles were contracted simultaneously. Sometimes animal may die due to respiratory failure specially in case of strychnine poisoning.

**Tetanus toxin**: Convulsions started usually between 18th to 19th hrs. after administration of agent. Local tetani was noted at the region where the drug was injected. Effects were first noted at the sacral region, then gradually at the lumber, thoracic and lastly at the cervical region. The whole body became stiff. The animal did not like to move due to loss of balance. The head was dropping, the eyes were nearly closed, and saliva secretion was increased. Convulsion associated with jumping was noted for 1-2 mins. before the death of the animal.

2. **Preparations of tissue homogenate and tissue sections**:

The animals were sacrificed by decapitation. The cervical region of the spinal cord was immediately traced out, removed, blotted with filter paper to remove any surface blood, and fixed in freshly prepared Carnoy's fluid (absolute alcohol: chloroform: acetic acid; 6:3:1) for 1-1/2 hrs. or 10% neutral formalin for 24 hrs. for the preparation of tissue sections or placed known weight of spinal cord tissue in a chilled Potter Elvehjem type of homogenizer containing known volume of ice-cold 0.154 M KCl solution for the preparation of tissue homogenate.
Usually histologically tissue sections were made in two ways:

(i) Paraffin tissue sections.
(ii) Freezing tissue sections.

(i) Preparations of paraffine tissue sections: - The Carnoy's fixed tissues were dehydrated in absolute alcohol for 1-1/2 hrs. (three changes, each for 30 mins.). The tissues were cleared in benzene for 20 mins. and embedded in paraffin (m.p. 56-58°C) for 2 hrs. 6μ paraffin sections were cut from these paraffin blocks with the help of microtome. The paraffin sections were dried in incubator at 37°C for 24 hrs. and preserved at room temperature for histological studies.

(ii) Freezing tissue sections: - 10% Neutral formalin fixed tissues were used for the preparation of freezing tissue sections. The tissues were washed well with distilled water, cut in small pieces and placed on the platform of the freezing microtome. The tissues were frozen by carbon dioxide and was cut at 10μ for the study of different phosphatase activity.

I. METHODS EMPLOYED IN HISTOCHEMICAL STUDIES:

(i) Staining of 'Nissl granules'

(a) Thionin - Stain

"Nissl" granules staining was employed according to the modified method of Conn et al. (1).
Preparation of thionin dye solution:

(A) 25 Mg. of thionin was dissolved in 100 ml. of distilled water and filtered.

(B) 0.25 Ml. of glacial acetic acid in 100 ml. of 95% alcohol.

Method:

(1) 6μ Paraffin sections were deparaffinized in xylene, treated with alcohols to water and stained for 30 mins. in solution (A).

(2) The excess dye was rinsed off in 50% alcohol.

(3) The sections were transferred to the solution (B), the differentiating fluid. The differentiation was noted at frequent intervals (15-30 sec.) under a microscope and the correct timing for differentiation was ascertained.

(4) The sections were dehydrated in alcohol, cleared in xylene and mounted with Canada balsam. The stained sections were examined under the microscope.

(b) Galloccyanine Stain:

Nissl granules staining was studied according to the modified method of De Boer and Sarnakar (2).

Preparation of galloccyanine-chromalum dye solution:

600 Mg. of galloccyanine was suspended in 200 ml. distilled water by shaking for one minute. It was filtered and
the filtrate was discarded. The residue on the filter paper was transferred to 200 ml. of 5 percent, chromalum solution in distilled water. This solution was placed on water bath and boiled for 30 mins. After cooling the solution, allowed to filter and pH was adjusted to 1.6 with 1 percent HCl.

(1) 6μ Paraffin sections were prepared, deparaffinised and hydrated to water.

(2) The sections were stained for 24 hrs. in gallo-cynine-chromalum.

(3) Rinsed for 1 minute in distilled water at pH 1.6 (acidified with HCl).

(4) Washed well in water until no further dye could be removed.

(5) The sections were dehydrated in absolute alcohol, cleared with xylene and mounted with Canada balsam.

(ii) Localisation of acid phosphatase and alkaline phosphatase activity:

(A) Acid phosphatase activity in spinal cord tissue sections was studied according to the modified technique of Gomori (3) :- The cervical region of the spinal cord was fixed in 4% cold neutral formalin for 16 hr. immediately after killing the animal. The tissue was washed well in cold distilled water and 10μ frozen sections were prepared and were allowed to incubate at 37° for different periods of time (5 minutes to 2 hrs.
in 0.01 M sodium β-glycerophosphate in 0.05 M acetate buffer (pH 5.0), containing 0.004 M lead nitrate. After incubation, the sections were washed for a short time in distilled water and mounted on the clean glass slide, immersed in dilute yellow ammonium sulphide for 1-2 minutes. The sections were again washed well in distilled water and mounted in glycerine jelly, and examined under the microscope. In order to check any free inorganic phosphate in the substrate Na-β-glycerophosphate which interfere with the results, tissues were allowed to incubate for 0 minute in the cold incubating mixture; the enzyme activity had been measured by following the same procedure as already mentioned.

(B) Localization of alkaline phosphatase was carried out according to Gomori (4):-

Preparation of reagents: Substrate solution was prepared by mixing the following ingredients:

- 3% Sodium β-glycerophosphate ... 10 ml.
- 2% Sodium diethyl barbiturate ... 10 ml.
- 2% Calcium chloride ... 20 ml.
- 5% Magnesium sulphate ... 1 ml.
- Distilled water ... 5 ml.

The pH of the solution was adjusted to 9.0-9.5 with 0.1 N HCl or 0.1 M sodium barbitone.
Method:

1. Small pieces of nerve tissues taken immediately after killing the animal were fixed in 4% neutral formol for 24 hrs.

2. After fixation, the tissues were washed several times with cold distilled water. 10μ Frozen sections were prepared and mounted on the clean slides without any adhesive. The sections were allowed to dry at room temperature (30°-32°) for one hr.

3. The sections were then incubated at 37° in substrate medium previously raised to that temperature for different periods of time (5 minutes to 2 hrs.).

4. After incubation, the sections were washed in several changes of water; then treated with 2% cobalt nitrate for 3-5 minutes.

5. Washed thoroughly in tap water followed by distilled water.

6. The sections were then dipped in yellow ammonium sulphide solution (a few drops in 50 ml. of distilled water) for a few minutes (3-5 minutes).

7. Washed thoroughly in water, dehydrated in alcohol, cleared in xylene and mounted in Canada balsam.

Alkaline phosphatase activity was indicated by black or brownish black staining.
(iii) Localization of 5'-nucleotidase of the nerve tissue:

5'-Nucleotidase activity was studied according to the method of Pearse and Reis (5):

Preparation of reagents: - Substrate solution was prepared by mixing the following ingredients.

- 5 Vol. barbiturate buffer (pH 7.5).
  - (Sodium diethyl barbiturate, 0.1 M, 3 vol.;
    HCl, 0.1 N, 2 vol.; distilled water 1 vol.)
- 1 Vol. 12% (w/v) Ca (NO₃)₂.
- 1 Vol. 2% (w/v) MgCl₂.
- 1 Vol. 0.04 M adenylic acid (adenosine - 5' phosphate).
  The pH of the solution was adjusted to 7.5.

Method:

(1) 10μ Formalin - fixed frozen sections were prepared as the methods described for alkaline phosphatase.

(2) Sections were incubated with 0.01 M Na β-glycerophosphate) as for alkaline phosphatase at pH 9.2, another similarly at pH 7.5, a third in the above medium at pH 7.5 and a fourth in the same medium containing water instead of substrate, all for 5 mins. to 2 hrs. at 37°.

(3) The sections after incubation, washed with 2 percent Ca (NO₃)₂ of pH about 8.0 and then in distilled water.

(4) Then treated with 2 percent cobalt nitrate for 3-5 minutes and rinsed well in distilled water.
(5) The sections were then dipped in yellow ammonium sulphide solution for a few minutes (3-5 minutes).

(6) Washed thoroughly in water, dehydrated in alcohol, cleared in xylene and mounted in Canada balsam.

Black staining of the tissue indicating 5'-nucleotidase activity (the glycerophosphate control at pH 7.5 is subtracted from the total result given by adenylic acid at pH 7.5 to obtain a true estimate of 5'-nucleotidase. This control is negative and the corresponding substrate control is also negative.

(iv) Localization of adenosine triphosphatase activity:

Adenosine triphosphatase activity was studied according to the modified method of Padykula and Herman (6, 7):

Following was the incubating medium:

This solution was freshly prepared whenever required.

0.1 M Barbiturate buffer (pH 8.6) ... 2.0 ml.
0.18 M CaCl₂ (1.998 g/100 ml.) ... 1.0 ml.
Distilled water ... 3.0 ml.
Adenosine triphosphate (disodium salt) ... 15.2 mg.
Final concentration of ATP ... 0.005 M.

As soon as the ATP was dissolved, the pH was adjusted to 8.6 and made up to 10.0 ml. with distilled water.
Method:

(1) Immediately after killing the animal, the nerve tissues were fixed in 4\% cold neutral formalin for 2 hrs., 10\% thicked frozen tissues were prepared after washing the tissues in cold distilled water; sections were directly transferred into the incubating mixture for 15 to 90 mins.

(2) After incubation, the sections were washed in three changes of 1 percent CaCl\(_2\).

(3) These were all transferred to 2 per cent CoCl\(_2\) for 3 mins.

(4) Washed well in distilled water, and mounted in glass slides without any adhesive.

(5) The sections were then treated with dilute yellow ammonium sulphide for 2-3 mins.

(6) Washed thoroughly in water, dehydrated in alcohol, cleared in xylene and mounted in Canada balsam.

The substrate, ATP, may contain free inorganic phosphate which may interfere the localization of ATPase activity. To avoid this, a zero-time experiment was done. Frozen tissues were allowed to incubate in the medium for zero-time, and immediately inhibited by cold. The remaining procedure was same as mentioned previously.
Due to the softness of nerve tissue, fresh frozen tissue section was not possible to cut below 25μ, which was very thick for localization of ATPase. To avoid this difficulty, 10μ frozen sections were cut after using 4% cold formalin fixative.

METHODS EMPLOYED IN BIOCHEMICAL STUDIES:

(i) Acid and alkaline phosphatase activity:

Acid and alkaline phosphatase activity was measured according to the method of Lowry et al. (8).

1. The incubating mixture containing 0.3 ml. 10% tissue homogenate, 0.05 ml. 0.1 M Na β-glycerophosphate; 0.5 ml. 0.01 M acetate veronal buffer (pH 9.8 or 5.4); 0.15 ml. distilled water. The reaction mixture was incubated for 5 mins. to 1 hr. at 37°.

2. After incubation, the enzyme activity was inhibited by cold 25% trichloroacetic acid (final concentration 5%), allowed to keep in cold for 1/2 hr. for complete precipitation of protein, nucleic acid etc.

3. The precipitation was removed by centrifugation of the sample for 10 mins. at 250 r.p.m.

4. The supernatant contain inorganic phosphate, liberated by the hydrolysis of Na β-glycerophosphate was
estimated by the method of Lowry et al. (8) as follows: -

(a) 0.5 Ml. of the supernatant was taken and made up to 2 ml. volume by acetate buffer.

(b) To this was added 2 ml. of ammonium molybdate-ascorbic acid reagent which was prepared by mixing 1 ml. 2.5% ammonium molybdate, 23 ml. acetate buffer and 1 ml. of 1% ascorbic acid. The blue colour obtained after waiting for 15 to 45 minutes. The reading was noted with the help of Coleman (Junior) at 660 m\(\mu\).

The results were expressed in terms of \(\mu g\) P liberated per hour per 10 mg. wet weight of the spinal cord tissue.

(ii) Estimation of 5'-nucleotidase activity of spinal cord tissue of rats:

Nucleotidase activity was measured according to the method of Reis (9, 10).

The incubating mixture containing the following ingredients:

10% Tissue homogenate ... 0.3 ml.

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\begin{cases}
\text{either } 0.05 \text{ M adenosine - 5'}-\text{monophosphate} \\
\text{or } 0.05 \text{ M guanosine - 5'}-\text{monophosphate} \\
\text{or } 0.05 \text{ M uridine - 5'}-\text{monophosphate} \\
\text{or } 0.05 \text{ M cytosine - 5'}-\text{monophosphate} \\
0.05 \text{ M diethyl barbiturate buffer (pH 7.6) } 0.5 \text{ ml.}
\end{cases}
\]
The total volume of the incubating mixture was made to 1 ml. by the addition of distilled water. It was allowed to incubate for 5 to 60 mins.

The remaining procedure was the same as that of acid and alkaline phosphatase activity (8).

Following Reis (10), the results were reported in terms of μg P liberated from 0.0025 M 5'-nucleotide/hr./10 mg. wet weight of the tissue.

(iii) Estimation of adenosine triphosphatase activity of spinal cord tissues of rats:

ATPase activity of the spinal cord tissues were estimated according to the method of DuBois and Potter (11).

The incubating mixture containing the following ingredients:

3% Tissue homogenate ... 0.3 ml.
0.025 M adenosine triphosphate ... 0.05 ml.
0.05 M diethyl barbiturate buffer (pH 7.2) ... 0.5 ml.

The total volume of the incubating mixture was made up to 1 ml. by the addition of distilled water. The reaction mixture was allowed to incubate for 1 min. to 15 min. at 37°C.
The remaining procedure was the same as mentioned in the case of acid and alkaline phosphatase activity (8). The results were expressed in terms of \( \mu g \) P liberated from \( 0.0025 \ M \) ATP/hr./mg. wet weight of the tissues.

(iv) **Estimation of acid soluble nucleotides**:

The release of acid soluble nucleotides from the Carnoy's fixed tissue sections were studied according to the modified method of Jonsson and Lagerstedt (12) and Lagerstedt (13). Tissues were fixed in Carnoy's fluid for 3 hrs., dehydrated in absolute alcohol for 1-1/2 hrs. followed by clearing in benzene for 20 mins. The cleared tissues were embedded in paraffin (m.p. 45\(^\circ\)) for 1-1/2 hrs. 50 \( \mu \) thick sections were taken in centrifuge tubes, and deparaffinized in petroleum ether, centrifuged, further washed with ether, and allowed to dry at room temperature (30-32\(^\circ\)). Each tube containing 4 mg. dry weight equivalent of tissue sections were incubated with 1 mg. of pure yeast RNA and without RNA, 2 c.c. of McIlvaine's buffer at pH 7.0 for 2 hrs. at 37\(^\circ\)C. For studying the effect of various agents viz., 0.5 M NaCl, 0.5 M MgCl\(_2\), 0.4 M NH\(_4\)Cl, 4.0 M urea, 0.1 M EDTA on the release of acid-soluble nucleotides and ribonuclease activity, one of these agents was added in the incubating mixture containing 4 mg. dry weight equivalent of tissue sections, 1 mg. of pure yeast RNA or without RNA,
2 c.c. of McIlvaine's buffer at pH 7.0. The reaction was stopped by addition of 2 c.c. of ice-cold 10% perchloric acid containing 0.25% uranyl acetate and allowed to wait at 2°C for approximately 1 hr. After centrifugation the supernatants were analysed spectrophotometrically for the estimation of acid-soluble nucleotides by using Beckman DU Spectrophotometer at 260 μ. The concentration of acid-soluble nucleotides was determined on the basis of optical density value of 33 at 260 μ of a solution containing 1 mg. of completely hydrolyzed yeast RNA per ml. of 0.1 M McIlvaine's buffer, pH 7.0.

**MATERIALS**

**Drugs used:**

Picrotoxin used was of British Drug Houses Ltd., England. Strychnine sulphate used was obtained from Carnegie Chemicals (Welwyn) Ltd., Welwyn Garden City. Hertfordshire, England. Tetanus toxin (Batch No. 5404 E) was supplied by Bengal Immunity & Co. Ltd., Calcutta. The test dose of the toxin was such that 0.022 c.c. of the toxin used against 0.1 unit of standard antitoxin caused death to mice of body weight 18-22 g. within 48-60 hrs.

**Substrates used:**

RNA, ATP, AMP, CMP, UMP were purchased from Schwartz Laboratories Inc. or from Sigma Chemical Co., U.S.A. Ascorbic
acid, Na β-glycerophosphate were obtained from British Drug Houses, England.

Dye:
Thionin was supplied by Dr. G. Grabble & Co., Leipzin. Gallocyanine used was made of the British Drug Houses Ltd., London.

Buffer:
Na-diethylbarbiturate, Na or K phosphate, Na-acetate etc. were of E. Merck, Darmstadt.

Solvents:
Xylene, petroleum ether, benzene, acetone were supplied by International Chemical Industries, Calcutta. Glacial acetic acid and formic acid were obtained from E. Merck AG. Darmstadt.

Common laboratory chemicals:
Common laboratory chemicals of analytical quality used in the present experiments were obtained either from E. Merck, British Drug Houses, Riedel or Rhodia. Ethylenediamine tetraacetic acid (Na-salt) was obtained from E. Merck, Darmstadt.
REFERENCES


