Extraction of the venom:

Fresh venom was collected from scorpions of Buthus tamulus species by applying faradic current of 6 volts to the telson and the last but one segment of the scorpion (Fig. 5). Venom was weighed and diluted either with normal saline or distilled water to produce a dilution of 1 mg in 1 ml of the solvent. This solution of known strength was used in all the experiments. The solution thus prepared is stored in a refrigerator at 4°C. Scorpion venom is very stable and can be kept in solution under refrigeration for 3 to 6 months without any appreciable loss of potency.

Standardisation:

Dogs:

Dogs were anesthetised with alpha chloralose administered in a dose of 80 mg per kg body weight intravenously. The animals were secured to the operating table and a record of respiration and blood
Fig. 5
Collection of Venom
pressure put up (Fig. 6). Aqueous solution of scorpion venom was administered intravenously in doses ranging from 10 μg to 100 μg per kg body weight. The effect of the venom on blood pressure was observed. There was a fall in blood pressure followed by a return to normal within five minutes. However, heart rate was not affected with this dosage (Fig. 7).

In ten dogs, graded doses ranging from 1 to 4 mg per kg body weight of the venom were administered intravenously very slowly. There was a precipitous fall in blood pressure in all these animals to an extent of 50 mm to 100 mm of Hg associated with tachycardia. The increase in heart rate varied from 6 to 43 beats per minute and recovery depended on the dose. With a dose of 2 mg per kg body weight, the recovery of blood pressure was definite after 10 to 15 minutes artificial respiration was given and dogs kept alive for 3 to 4 hours. When the dose was ranging from 3 to 4 mg per kg, the fall in blood pressure
Fig. 6

Set up for recording dog's blood pressure.
Carotid artery cannulated for recording blood pressure.
Femoral vein cannulated for drug administration.
was sudden and tachycardia very marked. The animals succumbed within 5 to 10 minutes. With artificial respiration, these dogs were kept alive for 30 to 120 minutes. Dogs died as soon as the respirator was detached (Fig. 8).

In 6 dogs, the venom was administered in a dose ranging from 4 mg to 5.5 mg per kg body weight, and the blood pressure and heart rate were recorded. The hypotension was marked with high venom dose. There was an initial rise in heart rate followed by bradycardia and death occurred within 10 to 15 minutes (Fig. 9).

The minimal lethal dose was 3.2 mg per kg body weight in dogs.

Experiments on dogs:

Mongrel dogs of either sex weighing about 7 kg were used in our experiments. The dogs were kept under observation for one week to assess the healthy state of the animal. The animals were anaesthetised with alpha chloralose administered intravenously in
doses of 80 mg per kg body weight. The anaesthetised animals were secured to the operation table and blood pressure and respiration put up. Respiration was recorded by means of Marcy's tambour. During respiratory arrest the animals were ventilated artificially. Aqueous solution of the venom was administered through the femoral vein in doses ranging from 0.5 mg to 5 mg per kg body weight. The effect of the venom on blood pressure, and respiration were recorded (Fig. 10).

Bilateral vagotomy was carried out and the venom in doses of 2 mg and 4 mg per kg body weight was administered intravenously. The effect of venom following vagotomy was recorded (Fig. 11).

Heart rate was continuously monitored by electrocardiogram during drug administration.

Myocardioogram in dogs:

Mongrel dogs weighing about 5 kg were used in these experiments. The dogs were kept under observation for one week to assess the healthy state
RECORD OF DOG'S BLOOD PRESSURE & RESPIRATION
EFFECT OF SCORPION VENOM AFTER BILATERAL VAGOTOMY
of the animals. The animals were anaesthetised with alpha chloralose and secured to the operating table. Blood pressure was recorded in the usual manner and animal ventilated artificially. Left femoral vein was cannulated for administration of drugs. A midline incision was made on the thoracic wall extending from the clavicle down to xiphisternum. The skin and musculature were suitably retracted. The cartilages adjoining the ribs on the left side were cut and retracted in such a way as to expose the heart with pericardium. A midline incision was made over the pericardium and edges of pericardium were secured to the thoracic wall on either side in such a way as to form a cradle for the heart. The heart was always kept moist by the application of warm saline. The apex of the left ventricle was hooked and attached to a heart lever in such a way as to record the ventricular beats. The tip of the right auricle was hooked and connected to another heart lever so as to record the auricular contractions. After recording the normal
contractions of auricles, ventricles and blood pressure, solution of scorpion venom was administered intravenously in doses ranging from 0.5 mg to 4 mg per kg body weight and the effects recorded (Fig. 12).

Effect of scorpion venom on blood pressure after pretreatment with drugs:

Dogs anaesthetised with alpha chloralose (80 mg per kg body weight) were secured to the operating table and a record of blood pressure put up. The effect of scorpion venom was observed in 6 animals after pretreatment of the animal with (1) Atropine; (2) Lytic cocktail (chlorpromazine, promethazine, pethidine); (3) Reserpine; and (4) Guanethidine. Atropine was administered in dose of 1 mg to 3 mg till there was no vasodepressor response to vagal stimulation or after the administration of 10 μg of acetyl choline (Fig. 13). Lytic cocktail was administered as a continuous drip, the rate of flow being 1 ml per minute. Each ml contains chlorpromazine = 0.1565 mg; Promethazine = 0.1565 mg; Pethidine = 0.3131 mg in 5% glucose distilled water.
Reserpine was administered in two doses of 1 mg per 5 kg body weight given, 24 hours and 12 hours before anaesthetising the animal (Fig. 14).

Guamethidine was administered in 2 doses of 10 mg per kg given 24 hours and 12 hours before the commencement of the experiment (Fig. 15).

Electrocardiogram in dogs:

Normal healthy dogs of mongrel brood which were kept in the laboratory for a week and were used in these experiments. The animals were anesthetised with alpha chloralose (80 mg per kg body weight) administered intravenously and secured to the operating table and a record of blood pressure was put up by cannulating the carotid artery; and the femoral vein was cannulated for drug administration.

The electrocardiogram was taken with Cardiart in the supine position. The normal ECG of a dog was established by recording 3 tracings taken at different times, in 3 dogs (Fig. 16). The body position of the dogs
particularly the chest position was carefully controlled
during the recording of ECG. The venom was given in a
dose of 2 mg per kg body weight, intravenously. The
study was repeated in 6 dogs. With serial 12 lead,
electrocardiograms were recorded after 15 minutes, 30
minutes, 1 hour, 2 hours on the same day and after 24
hours, after 48 hours and finally after the disappearance
of the effects of the venom (Fig. 17).

Two dogs were injected with 4 mg per kg body weight
of the venom and the ECG recorded till the animal
died.

In 6 dogs, lytic cocktail was administered as a
continuous drip, the rate of flow being 1 ml per minute
and ECG recorded before and after the administration
of scorpion venom (Fig. 18).

The venom from Palamnous species was also prepared
in a similar manner and diluted with normal saline and
stored in the refrigerator. The venom was so diluted
as to contain 1 mg in 1 ml of normal saline. Different
species of animals like dogs, cats and rabbits were exposed to the venom of Palamneus variety in a similar manner to assess the relative toxic effects observed with Buthus venom.

Enzyme studies:

Blood was drawn from dogs before and two hours after the injection of scorpion venom the dose being 2 mg per kg body weight. Serum was separated and estimated for LDH by King's method (1965), SGOT and SGPT by the methods described by S. Reitman and S. Frankel (1957) and serum amylase by Wooton (1974).

In another study, two dogs were injected with 4 mg per kg body weight and the same procedure repeated.

Electrolyte studies:

Blood was drawn before and after injecting 2 mg per kg body weight of scorpion venom into dogs and the serum estimated for electrolytes by flame photometry.

Hematological studies:

Blood was drawn from the dogs before and after injecting scorpion venom. The clotting time, prothrombin time and kaolin cephalin time and platelet count were estimated.
Autopsy studies:

The following organs were removed after 7 to 8 hours of envenomation and histological changes observed and were compared with the normal viscera:

<table>
<thead>
<tr>
<th>Heart</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lungs</td>
<td>Cerebellum</td>
</tr>
<tr>
<td>Pancreas</td>
<td>Midbrain</td>
</tr>
<tr>
<td>Small intestines</td>
<td>Pons</td>
</tr>
<tr>
<td>Adrenal gland</td>
<td>Medulla</td>
</tr>
<tr>
<td>Kidney</td>
<td>Cerebrum</td>
</tr>
</tbody>
</table>

Determination of prothrombin time (Quick's method - one stage):

Blood was drawn into test tube containing sodium citrate and shaken well. It was centrifuged without delay at 1200 revolutions for 15 minutes. The supernatant plasma was then removed and kept in a clean glass tube. If the test was not carried out at once the sample was stored at 4°C and it will remain stable for several hours. Haemolysod specimens were discarded.

0.1 ml of plasma was delivered into the bottom of 100 x 10 mm tube placed in a water bath at 37°C and 0.1 ml of brain suspension added to it. After a delay
of about one minute, 0.1 ml of prewarmed 0.025 M. CaCl₂ was added and the contents of the tube were carefully mixed. A stop watch was started and the tube was held with its lower end submerged. The tube was continuously but gently inclined from the vertical position so that the contents could be observed for the first sign of clotting. Fibrin clot developing within a second marked the end point. The test was repeated at least once for each specimen and the mean time recorded. At least one normal control plasma sample should be included in each batch of tests.

It had been repeatedly demonstrated that the normal values obtained in any laboratory depended upon exactly how the test was carried out. In particular, the values observed with normal and pathological plasma samples depend greatly on the source and type of brain thromboplastin used.

Reagents:

0.035 M. Calcium Chloride solution. It was made by dissolving 2.77 g. of anhydrous CaCl₂ in one litre of distilled water.
Trisodium citrate solution: 31.3 g. of trisodium citrate dihydrate were dissolved in 1 litre of water.

Brain Thromboplastin: Human brain removed fresh from post mortem room was stripped of its covering membrane and blood vessels. It was cut and macerated in a teflon homogenizer with acetone. After the acetone had been changed several times a non-adhesive granular material was obtained. This was the crude "acetone brain". This was dried in a vacuum desiccator and after drying 0.3 gm amounts were placed in 100 x 10 mm tubos and stored at 4°C.

This material was suspended in 5 ml of 0.9% saline and warmed at 37°C for 15 to 30 minutes with occasional shaking. The saline suspension was stable for a couple of days at 4°C or longer at -20°C.

Prothrombin time test is a non-specific indicator of the extrinsic blood coagulation mechanism. Deficiencies of prothrombin and Factor V, VII and X give rise to an increased prothrombin time as well as the presence of heparin and hypofibrinogenemia.
Kaolin cephalin clotting time (J. Margolis (1961)):

Principle: Whole blood is diluted with citrated distilled water. The haemolysate obtained is recalcified at 37°C after brief activation with Kaolin. The observed clotting time is then compared with that of known normal controls of identical haemoglobin concentration.

The method may be used for venous or capillary blood. There is a relationship between the clotting time and the haemoglobin level, the haemoglobin concentration of the sample to be tested must be recorded. Venous blood was collected with 3.8% citrate solution in the proportion of 9:1. One volume of citrated blood was then haemolysed with 3.5 volumes of 0.1% trisodium citrate in water kept at room temperature for 1 to 2 hours before testing. This was referred to as "Haemolysate".

Screening test:

0.2 ml of haemolysate is taken in a 10 x 100 mm tube along with 0.1 ml of kaolin suspension. (Freshly prepared 5 mg of kaolin pulvis per ml of 0.9% saline). The
mixture is kept in a waterbath at 37\(^\circ\)C. The mixture is recalcified with 0.025m. \(\text{CaCl}_2\) solution. A stop watch is started at once. The tubes are agitated carefully at regular intervals of 5 seconds. Noted the time at which the clot appeared. The end point is very sharp and can be easily read. This test is performed in triplicate and the average taken as the result. It is very important to keep the temperature of the waterbath at 37\(^\circ\)C. Otherwise, the clotting time is shortened by about 5% for each rise of one degree centigrade. A common error is due to insufficient immersion in the waterbath. This leads to cooling of the sample in contact with the upper wall of the tube and again results in inaccurate determination of the clotting time. During the first 30 minutes after haemolysis the clotting time often decreases on incubation. It then remains almost constant for 2 hours after collection. The clotting time increases with haemoglobin concentration. In the presence of haemolysed red cells the system is relatively
insensitive to variations in platelet numbers. For practical purposes, the platelet count may therefore be ignored except for gross platelet deficiency when an appropriate correction would have to be introduced in cases of co-existing borderline abnormalities.

The time taken by the animals (dogs) to die after an intravenous injection of the venom is as follows:

Buthus Tamulus - 0 hours 10 minutes with a
dose of 3.2 mg per kg body weight.

Palamneus - 1 hour 10 minutes with a
dose of 3.2 mg per kg body weight.

In the case of adult species of Buthus Tamulus, the minimal lethal dose is 3.2 mg per kg killing the animal in 10 minutes and with the venom obtained from younger species of Buthus, the minimum lethal dosage is 12.9 mg per kg body weight, killing the animal in 2 hours 30 minutes.
Experiments on frogs:

Adult species weighing 20 to 25 gms were used in these experiments. The venom was injected into the anterior lymph sac through the floor of the mouth.

Gastrocnemius - Sciatic preparation:

Pithed frog was cut across about 1 cm anterior to the sacro-iliac joints and the skin was cut between the legs and peeled downwards towards the toes. The urostyle was grasped with forceps and cut away from the spinal column. The frog was turned on to the dorsal surface, and the sciatic nerve was seen clearly. Spinal column was bisected longitudinally, carefully, by means of scissors, without injuring the nerves. The piece of bone was held with forceps and the nerve dissected in the thigh region by cutting the muscles upto the region of the knee. A ligature was tied round the tendo Achillis and cut on the distal side and the connective tissue was separated; the leg bone just below the knee and one inch of bone in the thigh were cut, carefully keeping the
gastrocnemius muscle and the sciatic nerve intact. The tissues were kept moist, in saline throughout the experiment. The preparation was fastened to the myograph stand with a femur clamp and the thread from tendon was tied to the isotonic lever balanced with a 10 gm weight and the pointer adjusted to record on a smoked paper on a Sherrington-Startling drum. The drum was connected to an inductorium regulating the current passing on to the electrodes (Fig. 19).

Scorpion venom and drugs were added by means of a dropper and the actions recorded on a slow moving drum.

The preparation was kept moister with 0.6% saline and 1% solution of the venom of either Buthus or Palamneus was added to record the effects. The observations were made on the following lines:

1. General physiological response of the muscle and nerve over a period of one hour.

2. Electrical stimulation with break shocks applied to the motor nerve muscle and the suralised and atropinised preparation.
Fig. 19.

Myogram
Gastrocnemius sciatic preparation
Mounted in Luca's Trough
Hyoglossus - Hypoglossal preparation:

The preparation may be used instead of the sciatic gastrocnemius, especially when the action of drugs is to be investigated; the muscle lies enclosed in lymph space below the mucous membrane, and the drug can be introduced by means of a syringe, so that the muscle is bathed in it.

The pithed frog was fixed on its back to the board, skin removed from the lower jaw and shoulder girdle, and a thin muscle running transversely across the jaw, cut and the central hypoglossal nerve was dissected as it runs from the angle of the jaw. The lower jaw was pulled forward and cut so that the jaw and hyoid plate could be removed. The hyoid plate was pinned to the myograph stand and a ligature was tied round the tip of the tongue and attached to the lever.

Venom was added through a syringe or filler and the effects observed. Muscle was washed with frog's Ringer solution and the drugs - curare, atropine, physostigmine, (escapine) - were added separately (Fig. 20).
Fig. 20

Myogram
Hyoglossus muscle preparation
**Effect of the venom on the frog's heart:**

Frog's heart was exposed, a thread passed under the sinus venosus and a small slit made a little above the entry of vena e cavae into the sinus.

Heart was flushed with Ringer's solution and the nozzle of syne's cannula introduced and tied in position.

**Ringer's solution:**
- Sodium chloride : 0.65%
- Potassium chloride : 0.012%
- Calcium chloride : 0.014%
- Sodium bicarbonate : 0.02%

The heart was removed along with the cannula and connected to the perfusion funnel fixed to a stand and filled with Ringer's solution. A bent hook was passed through the apex of the heart for recording ventricular beat. Heart beats were recorded on a smoked drum (Fig. 21).

A. Venom was periodically perfused through the cannula in graded doses and the effects recorded before and after exposure to the venom.

B. Atropine sulphate in doses of 100 µg per ml was perfused into the amphibian heart followed by the administration of scorpion venom solution in dose...
Fig. 21
Myocardio gram
Record of contractions of the intact heart of frog.
ranging from 500 μg to 2 mg. The effects observed were recorded.

C. The effect of the venom on amphibian heart was observed after pretreatment with propranolol administered in doses of 100 μg to 1 mg per ml.

Experiments on cats:

Domestic cats weighing about 2 kg were used in these experiments. The animals were anesthetised with alpha chloralose administered intramuscularly. The anesthetised animals were secured to the operation table and blood pressure and respiration were recorded. The animals were kept under artificial respiration to study the effect of the venom on the cardiovascular system. Aqueous solution of venom was administered through the femoral vein in doses ranging from 0.25 mg to 5 mg per kg body weight. The effect of the venom on blood pressure and heart rate was studied. The minimum lethal dose was determined. Dead animals were autopsied for pathological study.
Fig. 21-A
Ventriculogram
Record of isolated frog's heart preparation.
Experiments on rats:

White rats were used in these experiments. The rats were bred in the department over a period of ten years and the same strain was used in all experiments. Rats of either sex weighing about 100 g were used. The venom was injected as an aqueous solution subcutaneously in doses ranging from 0.5 to 3 mg per 100 body weight and the effects observed over a period of ten hours. Autopsy was done in all the animals at the end of this period and the viscera was subjected to pathological study.

Experiments on rabbits:

White rabbits weighing about 1 kg were used in the experiments. These rabbits were used from the Colony maintained over a period of ten years. Aqueous solution of the venom in doses of 0.5 to 3 mg per kg body weight was administered subcutaneously in some animals and intravenously in 5 rabbits and the effects observed. The minimum lethal dose was determined. Autopsy was done on dead animals and the viscera were subjected to pathological study.
Effect of the venom on isolated guinea pig-ileal preparation:

Male guinea pigs weighing 300 to 400 gms were used in these experiments. The animal was stunned by a sharp blow on the head and the abdominal cavity quickly opened. A distal loop of ileum was gently pulled out, freed of mesentric attachment and excised. The lumen of the cut loop was flushed with warm Tyrode's solution. A loop of 2" length was cut and mounted in an organ bath containing Tyrode's solution (Fig. 22). The bath was aerated with oxygen and the temperature maintained at 35°C. The movements of the ileum were recorded on a slow moving Kymograph. The tissue was exposed to scorpion venom in dose ranging from 2 to 8 μg per ml and the effects observed.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Tyrode's solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium chloride</td>
<td>8.00 gm</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Calcium chloride</td>
<td>0.2 gm</td>
</tr>
<tr>
<td>Magnesium chloride</td>
<td>0.01 gm</td>
</tr>
<tr>
<td>Sodium acid phosphate</td>
<td>0.05 gm</td>
</tr>
<tr>
<td>Sodium bicarbonate</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Dextrose</td>
<td>1.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

pH adjusted to 7.3 to 7.4
Fig. 22

Set up for recording the response of isolated ileal preparation.
Separation of toxic components from scorpion venom:

Electrophoresis of
serum proteins:

Serum proteins were separated by paper electrophoresis on Whatman paper No.1 as described by Henry et al. (1957).

Reagents:

i) Barbitone buffer (pH 8.6 and μ 0.05). This was prepared by dissolving 1.84 g. of barbitone and 10.3 g. of barbitone sodium in 1 litre of distilled water.

ii) Staining solution: 0.1 g of bromophenol blue was dissolved in 25 ml of 95% ethanol and made up to 1 litre with 5% acetic acid containing 50 g. zinc sulphate (ZnSO₄·7H₂O).

iii) Wash solution: Acetic acid 5% (V/V).

iv) Fixative solution: This was prepared by dissolving 3.0 g. of sodium acetate (CH₃COONa 3 H₂O) in 1 litre of 5% acetic acid.
Procedure:

The buffer compartments were filled with buffer so that the levels were equal. Whatman No.1 paper strips of 30 cm x 4 cm were soaked in buffer and then placed in the tank. After equilibrium, 10 μl of serum was applied along with a pencil line in the middle of the paper. The electrophoresis was run for 5 hours at 180 V. and 2 mA per two strips. At the end of the run, the current was switched off and the paper dried in an oven at 100°C for 10 minutes. They were placed in the staining solution overnight. The strips were then washed with successive changes of 5% acetic acid and fixed in the fixative solution.

The intensity of each of the separated protein bands was quantitated with Joyce-Loebel densitometer and the percentage distribution was calculated.

Polyacrylamide gel electrophoresis:

The use of polyacrylamide gels as a medium for electrophoresis had been suggested by Raymond and Weintraub (1959). Later, the method was developed by Davis (1964) and Ornstein (1964). Since its introduction,
polyacrylamide gel electrophoresis has been widely used in the separation of proteins, nucleic acid etc. Extensive literature pertaining to the application of polyacrylamide gel electrophoresis is available. Ornstein (1964) and Davis (1964) carried out acrylamide gel electrophoresis in cylindrical glass tubes or rigid plastic tubes, a process which they called the "disc electrophoresis". Polyacrylamide gel has the advantages over other gels in that it is optically clear, and suitably stained bands can be determined by scanning procedures. There is also sharper separation of the resulting protein bands. It is easy to construct gels of different pore sizes suitable for the separation of specific groups of macromolecules on the basis of the size. In this way separation may be effected in a controlled manner not only by electrophoretic (principles) but also by molecular sieving principles (Fig. 24).

Many modifications of the original Davis method are also in use at the present time and the one employed in the present study is essentially that of Dietz et al (1972).
Reagents:

1) Buffered - TEMED: 48 ml of 1 N HCl, 36.3 g of TRIS and 0.23 ml of N, N, N', N' - tetramethylene - diamine were dissolved in distilled water and made upto 100 ml. The solution was filtered and kept in a brown bottle at 4°C.

ii) Ammonium persulphate. 1.4 mg per ml. An aqueous solution contained 140 mg of ammonium persulphate in 100 ml was prepared fresh at the end of every week.

iii) Acrylamide, 30 g per 100 ml. 30 g of acrylamide and 0.8 g N, N' - methylenebis acrylamide were dissolved in water and made upto 100 ml with water and filtered. The solution was kept at 4°C in a brown bottle away from light.

iv) Electrophoresis buffer (pH 8.2 - 8.4). 6.0 g of TRIS and 28.8 g of glycine were dissolved in water, made upto 1 litre and stored at 4°C. Before use, 1 part was diluted with 9 parts of water.

v) Tracing solution: 10 mg of bromophenol blue dissolved in 1 litre of water.

vi) Acetic acid. 7.5 (V/V) in water.

vii) 40% sucrose solution in water.
Intraventricular administration of the venom in anaesthetised dog:

In two dogs, a burr hole was made on the cranium, 3.6 cm from the occipital protruberance and 0.4 cm away from the midline. Dura was incised and the canula fixed over the vault of the skull. Venom was administered into the lateral ventricle through this canula, in doses of 2 mg per kg body weight and the effects observed on respiration and blood pressure.