CHAPTER 3

MATERIALS AND METHODS
3.1. MATERIALS

3.1.1. Experimental animals.

The experimental animals used for the present investigation were rats (150 to 200g), mice (15 to 25g) and guinea-pigs (300 to 600 g) of either sex and frogs and leeches. Albino rats (Haffkin's strain), albino mice (Haffkin's strain) and guinea-pigs (Haffkin's strain) were inbred in nature and were obtained from the animal house of the institute. Leeches (Hirudo medicinalis) and frogs (Rana tigrina) were obtained from the local animal suppliers. Rats, mice and guinea-pigs were fed with Hindleve feeds for laboratory animals and were given water ad libitum. Leeches were fed with liver of rats and guinea-pigs.

3.1.2. Drugs

The drugs used were: acetylcholine chloride (E. Merck), apomorphine hydrochloride (Sandoz), arecoline hydrochloride (BDH), atropine sulphate (E. Merck), benztropine mesylate (BDH), biperiden hydrochloride (Knoll), cycrimine hydrochloride (Lilly), dopamine hydrochloride (Koch-Light), harmine hydrochloride (Sigma), hemicholinium-3 (Aldrich), heparin (Biological Evans), hexamethonium bromide (Koch-Light), muscarine chloride (Sigma), nicotine sulphate (Sigma), oxotremorine sesquifumarate (Aldrich), physostigmine (eserine), sulphate (E. Merck), pimozide (McNeil Lab.), prostaglandin E₂ (Upjohn Co.), (+)-tubocurarine chloride (Sigma) and urethane (Merck). Decamethylene bis(hydroxyethyl) dimethylammonium bromide (C₄Dichol) was generously supplied by Prof. R.B. Barlow of University of Edinburgh, Scotland as gift. Dr. Josef Fried of the University
of Chicago, Illinois supplied 7-Oxa-13-prostynoic acid as gift. Indomethacin was supplied by Dr. Cooper of MSD as gift.

Indomethacin, 7-Oxa-13-prostynoic acid and prostaglandin E₂ were dissolved in absolute alcohol and a 1 mg/ml solution was prepared. Further dilutions were made with physiological solution. Pimozide was dissolved in tartaric acid solution (10% w/v). A 8 mM solution of pimozide was made in tartaric acid. Further dilution was carried out with physiological solution. All other drugs were dissolved either in physiological solution or in distilled water.

Doses and concentrations of the drugs refer to appropriate salts.

3.2. METHODS

3.2.1. IN VITRO EXPERIMENTS

3.2.1.1. Isolated rat phrenic nerve diaphragm preparation

These were made from albino rats (150 to 200 g) of either sex according to the method of Hill-Tring (1946). The rats were stunned by a blow on the head and killed by exsanguination. The left phrenic nerve along with a triangular portion of the left hemidiaphragm surrounding the entry of the nerve was dissected out. The rib end of this piece of diaphragm was securely tied to the tissue holder and a thread was tied to the tendinous end of the piece of diaphragm. The phrenic nerve was passed through a bipolar platinum electrode. The preparation was suspended in a 40 ml organ bath containing physiological solution of the following composition (mM): NaCl 118, KCl 4.7, CaCl₂ 1.9, NaHCO₃ 25, dextrose 11, KH₂PO₄ 1.2 and MgSO₄ 1.2. The tissue was aerated with 5% CO₂ in oxygen and
maintained at a temperature of 29-30°C. The thread tied to the tendinous end of the diaphragm was tied either to a loaded (1 g) simple straw lever having 8 to 10 fold magnification for recording isotonic muscle contractions or to a force displacement transducer (Encardio-Rite model ET 3; tension range 10 mg to 10 kg) for recording isometric muscle contractions on a multichannel recorder (Encardio-Rite) at 0.25 mm/sec chart speed employing a differential preamplifier (Encardio-Rite 532; f.r. D.C-100 Hz).

Supramaximal square-wave pulses of 0.2 ms duration at a frequency of 0.2 Hz were used to stimulate the phrenic nerve as well as the diaphragm.

3.2.1.2. Pharmacological denervation in rat diaphragm.

This was performed according to the method described by Chaudhuri and Ganguly (1974). The rat phrenic nerve diaphragm preparation was set up as described earlier. At a time when the muscle contractions to nerve stimulation were completely abolished with hemicholinium-3 (0.42 mM), the muscle was stimulated directly through two platinum wires attached to the diaphragmatic muscle and the effect of agents on these responses was studied. This process excluded any role of acetylcholine in the muscle contraction to direct stimulation. Since hemicholinium also impaired responses to direct stimulation under such conditions, presumably reflecting current spread to nerve terminals, a higher voltage (40 to 60 v) was invariably needed to elicit a sizeable direct response in its presence.
3.2.1.3. The dorsal muscle of leech:

These were made according to the method described by McLeod and the staff of the department of pharmacology, University of Edinburgh (1970).

The leech was pinned on its back through the mouth and tail-suckers on to a cork board. A cut was made with scissors along the two pale lateral lines, from the mouth nearly to the other end and the internal organs were removed. The dorsal muscle lying below the lateral line was separated longitudinally, threads were tied 3 to 4 cm apart and the piece of dorsal muscle was cut off. The muscle was suspended at room temperature (26 to 30°C) in a 2 ml bath containing physiological solution of the following composition (mM: NaCl 119.8, KCl 4.4, CaCl₂ 1.2, NaHCO₃ 1.4 and physostigmine sulphate 8 M). The organ bath was continuously bubbled with a mixture of 95% O₂ and 5% CO₂. One end of the muscle was tied to the bottom of the bath and the other end was tied to a loaded (2 to 2.5 g) straw lever having a 10 fold magnification. Before use the preparation was allowed to relax for 2h with continuous washing every 15 min. The contractions of the muscle were recorded on a smoked rotating kymograph. The contact time for acetylcholine was 2 min and each cycle was for 15 min.

3.2.1.4. Collection and bioassay of released acetylcholine from the rat phrenic nerve

The rat phrenic nerve diaphragm preparation dissected according to the method described earlier was suspended in a 4 ml organ bath containing Krebs solution (mM : NaCl 118, KCl 4.7, CaCl₂ 1.9,
NaHCO₃ 25, dextrose 11, KH₂PO₄ 1.2 and MgSO₄ 1.2) plus physostigmine sulphate 8 M and was bubbled with a mixture of 95% oxygen and 5% carbon-dioxide. Supramaximal square wave pulses of 0.2 ms duration of a frequency of 1 Hz were used to stimulate the phrenic nerve.

The schedule of collection of samples was exactly according to the procedure of Bowman and Hemsworth (1965a) except that only two samples were collected from a single diaphragm and that the samples were assayed immediately after collection on the dorsal muscle of the leech.

At the start of each experiment the phrenic nerve was stimulated continuously for 1 hour at 1 Hz, after which stimulation was stopped and the tissue was washed with Krebs solution containing physostigmine. The bath was then filled with the same solution and the nerve was stimulated for 15 min at a frequency of 1 Hz. Stimulation was then stopped for 2 min to allow the acetylcholine to diffuse out of the tissue (Krnjevic and Mitchell, 1961). This period of 15 min stimulation followed by 2 min rest was constant throughout all experiments and is subsequently referred to as the 'Collection period'. At the end of the collection period the bath fluid was removed by suction and was immediately assayed. The bath was refilled with Krebs solution containing physostigmine and the procedure was repeated at hourly intervals. During the 43 min between each collection period the preparation was stimulated at 1 Hz and the bath fluid was replaced with fresh Krebs solution containing physostigmine every 20 min to avoid the accumulation of muscle metabolites in the small organ bath (Bowman and Hemsworth, 1965a).
The effect of drugs on resting (without nerve stimulation) release of acetylcholine was determined by use of the same schedule of collection of samples. The tissue was kept in Krebs solution alone or in Krebs solution with added drugs for 15 min followed by collection of the bathing fluid.

All collected samples were immediately bioassayed on the dorsal muscle of leech, suspended in a 2 ml bath containing physiological solution of the following composition (mM: NaCl 119.8, KCl 4.4, CaCl$_2$ 1.2, NaHCO$_3$ 1.4 and physostigmine sulphate 8 M).

Preparation of standard acetylcholine solution for bioassay

A 1 mg/ml solution of acetylcholine was prepared. From this 0.1 ml was pipetted out into a 100 ml measuring cylinder and the volume was made upto 100 ml with a mixture of Leech and Krebs solution in the ratio of 2 : 1. The cylinder was shaken well and 0.64 ml of the solution was pipetted out from it into a 50 ml beaker. To this 19.36 ml of the Leech - Krebs mixture was added and mixed well to make a 32 ng/ml solution of acetylcholine. From this 10 ml was pipetted out into another 50 ml beaker and the volume was made upto 20 ml to give a 16 ng/ml solution of acetylcholine. This procedure of double dilution was continued to obtain 8 ng, 4 ng, 2 ng, 1 ng, 0.5 ng, 0.25 ng, 0.125 ng, 0.06 ng, 0.03 ng, and 0.015 ng acetylcholine/ml. During addition of acetylcholine to the leech muscle, the bath fluid was drained out and the standard acetylcholine solution was poured into the bath as such. The contraction of the leech muscle was recorded on a rotating kymograph using a loaded (2 g) straw lever having 8-10 fold magnification.
A bracketing-cum-matching assay was employed for estimation of the released acetylcholine. A cross-over design was employed for both collection and bioassay of the control and treated samples in all experiments. Those leech muscles which were not sensitive to standard acetylcholine in the range of 0.03 to 0.125 ng/ml were discarded. Prior to assay all samples were diluted at least 3 times in leech solution to minimize any change in sensitivity due to difference in composition of the two physiological fluids.

3.2.1.5. Auerbach's plexus of guinea-pig ileum

These were made from guinea-pigs (300 to 600 g) of either sex according to the method of Paton and Abo Zar (1968). Guinea-pigs were stunned by a blow on the head and bled out. The abdomen was opened and a length of small intestine was removed discarding about 8 to 12 cm of the terminal ileum. The lumen of the ileum was flushed with 25 ml of Kreb's solution. A piece of the ileum, approximately 10 cm long, was stretched on a glass rod of 7 mm diameter and the mesentery was cut away. To obtain the longitudinal strips, the method described by Rang (1964) was followed. By stroking tangentially at one end of the strip away from the mesenteric attachment with a wisp of cotton wool, the longitudinal muscle layer was separated at this point from the underlying circular muscle, and was tied with a thread. Afterwards by gentle traction of the thread the longitudinal muscle layer was stripped for the whole length of the piece of ileum. Care was taken to keep the strip moist throughout this procedure.
A small strip so obtained was placed in a petri dish containing Krebs solution and was examined under a binocular microscope at low power. With suitable external illumination Auerbach's plexus could be readily visible to the naked eye without vital staining. Before starting the experiment a small strip of longitudinal muscle was always examined under the microscope to ascertain that the nerve plexus is retained.

To obtain better release a double folded strip of longitudinal muscle was taken. The strip was mounted in a 10 ml organ bath containing Kreb's solution (mM: NaCl 118, KCl 4.7, CaCl$_2$ 1.9, NaHCO$_3$ 25, dextrose 11, KH$_2$PO$_4$ 1.2 and MgSO$_4$ 1.2) with 1.6 M physostigmine solution maintained at 37°C. The solution was bubbled with a mixture of 95% oxygen and 5% carbon-di-oxide. Supramaximal square wave pulses of 1 ms duration at a frequency of 1 Hz were used to stimulate the strip through two circular platinum electrodes (3 cm apart) placed at the top and bottom of the organ bath.

Collection and bioassay of released acetylcholine from the Auerbach's plexus of guinea-pig ileum.

When the acetylcholine output from the Auerbach's plexus was to be determined, the Krebs solution always contained 1.6M physostigmine solution. Since some time is required for physostigmine to develop its full action, a period of preliminary incubation of 1 h was allowed during which the preparation was washed at 15 min intervals with physostigminised Krebs solution. Four 15 min stimulations were given to each preparation with 30 min interval in between. During the stimulation period and the 30 min interval the bath fluid was replaced every 15 min with Kreb's solution containing physostigmine.
Samples collected after the first and second stimulation periods were discarded. The samples collected after the third and fourth stimulation periods were assayed for acetylcholine. Samples collected immediately before the third and fourth stimulation periods were assayed for resting release of acetylcholine. Drugs were added immediately before the third and fourth stimulation periods.

At the end of the collection periods the fluid was removed by suction and was immediately bio-assayed on the dorsal muscle of leech, suspended in a 2 ml bath containing physiological solution of the following composition (mM: NaCl 119.8, KCl 4.4, CaCl$_2$ 1.2, NaHCO$_3$ 1.4 and physostigmine sulphate 8 M).

3.2.1.6. Frog Rectus Abdominis muscle

These were made according to the method described by Burn (1952). The frog was pithed and was laid on its back on a cork covered board to which it was pinned. The abdominal skin was cut away and the rectus muscle of one side was dissected from the pelvic girdle to its insertion in the cartilage of the pectoral girdle. The muscle was then pinned to the cork table and a narrow strip of muscle 6-8 cm in length and 1 cm in thickness was cut off and threads were tied to both the ends. One thread was tied to a tissue holder and the muscle was suspended in a 10 ml organ bath containing Ringer solution of the following composition (mM): NaCl 111, KCl 4.7, CaCl$_2$ 0.88, NaHCO$_3$ 2.4, glucose, 5.5 and KH$_2$PO$_4$ 0.074. The bath fluid was kept at room temperature and aerated with 95% oxygen and 5% carbon-di-oxide. The other thread was attached to a straw lever having 6-8 fold magnification. A load of 0.5-1 g
was placed on the lever. As the muscle does not relax rapidly after contraction, an additional weight (2 g) was attached to the lever and was raised and lowered as necessary. A rest period of at least 2 h was allowed to provide maximum stretch of the muscle. A 10 min time cycle was usually maintained and the drugs were allowed to act for 2 min to record the effect. The contractions were recorded on a smoked rotating drum.

3.2.2. **IN VIVO EXPERIMENTS**

3.2.2.1. **Rat blood pressure**

The experiments were performed in the conventional way as described by McLeod *et al.* (1970). Albino rats of either sex were anaesthetised with urethan (1.4 g/kg s.c.). The anaesthetised animal was placed in the supine position on the operation table with the legs fastened and the head pinned down. A midline incision was made on the skin of the neck starting from the lower end of larynx up to the upper end of thorax. Trachea was exposed by retracting the pretracheal muscles and a transverse cut was made in between two rings so as to make an opening for the introduction of the tracheal cannula. The cannula was introduced into the opening pointing towards the lungs and held firmly in position with the help of twine ligature. The tracheal cannula was connected to a respiratory pump (INCO) for artificial respiration. The left femoral vein was exposed by an incision in the inguinal region and was then tied as distally as possible. A bull dog clamp was placed near the proximal end of the exposed vein and a thread was passed round the vein. A cut was made on the vein close to the first
ligature with the help of a sharp curved scissor so as to make a small opening through which a fine polythene cannula was inserted so that it was directed towards the heart and firmly secured with the help of the ligature already in position. The drugs were injected through this cannula followed by a constant volume (0.2 ml) of normal saline. The left carotid artery was cleaned from the accompanying structures for a sufficient length with the help of a blunt dissector. It was then tied as near the head end as possible, a bull dog clamp was placed about 3 cm nearer the heart and a thread was passed round the artery. A cut was made carefully on the artery close to the first ligature through which a glass carotid cannula filled with the anticoagulant fluid, was inserted so that it was directed towards the heart and firmly secured with the help of the ligature already in position. The carotid cannula was in turn connected to a mercury manometer (Condon type) filled with heparinised saline. The pressure in the manometer was then increased to about 120 mm Hg and the bull dog clamp was removed so that the manometer then remained in communication with the cannula. Blood pressure was recorded on a smoked kymograph through a floating stylus of the manometer. Before the experiment 0.05 to 0.1 ml (5000 I.U/ml) of heparin was administered through the venous cannula to prevent clotting.

3.2.2.2. Measurement of tremor in mice

For assessing tremor a simple subjective scoring method was employed, since it was earlier found to be in agreement with an objective scoring device (Ganguly, 1976). Tremor scores were assessed
visually in mice of 5 in a group by the method of Spencer (1965). Adult mice of either sex weighing between 15-25 g were used for this purpose. The mice were allowed free access to pellet food and drinking water until the commencement of the experiment. The experiments were performed in an air-conditioned room and the temperature was maintained at 25° - 29°C. Score values were determined, 5,10,20,40 and 80 min after administration of the tremorogenic agents as : 0 = no tremor, 1 = mild and occasional tremor, 2 = moderate and intermittent tremor, 3 = continuous tremor of moderate intensity, 4 = violent and continuous tremor. The mean individual score was determined for each group of mice and was termed as tremor index (Spencer, 1965). \( C_{10} \)Dichol (0.5 to 4 mg/kg i.p.) was injected 2 h prior to the administration of the tremorogenic agents. The control groups of mice received saline pretreatment and were tested with each of the experimental group. The protection afforded by the agent was characterised by reduction of the tremor index.

3.2.2.3. Physostigmine induced lethality in mice

Protection against physostigmine induced lethality was assessed by the method of Nose and Kojima (1970). The experiments were performed on male albino mice weighing from 18 to 22 g. An intraperitoneal dose of 2 mg/kg of physostigmine caused tremor with salivation, chronic convulsions and death for all mice within 20 min after administration. \( C_{10} \)Dichol was injected 2 h before physostigmine administration and its anti-physostigmine activity was expressed as \( \text{ED}_{50} \) which was determined from the number of mice which survived more than 60 min.