CHAPTER II

MATERIALS AND METHODS

1. Drugs.

Drug, Ancistrotectorine (Ancis), was naphthalene-isoquinoline alkaloid from *Ancistrocladus tectorius* (Lour.) Merr.

Other drugs :-

- Barium chloride ($BaCl_2 \cdot 2H_2O$) (May & Baker)
- Calcium chloride ($CaCl_2 \cdot 2H_2O$) (Merck)
- 5-hydroxytryptamine creatinine sulphate (5-HT) (Sigma)
- Verapamil (Isoptin) (Knoll)
- Noradrenaline hydrochloride (NA) (Sigma)
- Potassium chloride (KCl) (BDH)

All chemicals used were of analytical grade. Distilled deionized water was used for the preparation of Krebs-Henseleit and drug solution. Ancistrotectorine was adjusted to form of chloride salt by adding HCl to pH 5-6.

The concentration of agonists and antagonists are expressed in molar of base in final bath concentration.

2. Preparation of the isolated rat vas deferens.

Wistar rats were killed by a sharp blow on the neck and cutting the throat. The abdominal wall was opened and testis were then pushed into the abdominal cavity by applying pressure to the scrotum. The vas deferen was cut just above the epididymus and also at the point where it joined the urethra (Fig. 5). The tissues were carefully divested of attachments, cleaned blood vessels and connective tissue and bisected into prostatic and epididymal halves about 12 to 14 mm long. A thread was attached at each end and the preparation was mounted in 20 ml organ chamber containing Krebs-Henseleit solution of the following composition (in g/l) NaCl 6.92, KCl 0.35, $CaCl_2$ 0.28, $MgSO_4 \cdot 7H_2O$ 0.29, KH_2PO_4 0.16, $NaHCO_3$ .1 and glucose 2.1 which was bubbled with a mixture of oxygen (95 %) and carbondioxide (5 %) maintained at 36 - 38° C by means of circulating water pump. One end of the vas deferen was fixed to a hook of the glass rod in the chamber with the other end attached to a force displacement transducer or lever transducer with a cotton.

The tissue was equilibrated for 60 min under a resting tension 0.5 g before being exposed to any drug. During equilibration period the Krebs-Henseleit solution was changed every 15 min. Isometric contractions was recorded by means of force displacement transducer (Washington Transducer Type D)and isotonic contraction (in some experiments) was recorded by mean of lever transducer (Washington Lever Transducer Type T_2), preamplifier (Biosciences Type FC 117) and Polygraph recorder (Washington 400 MD 2c).

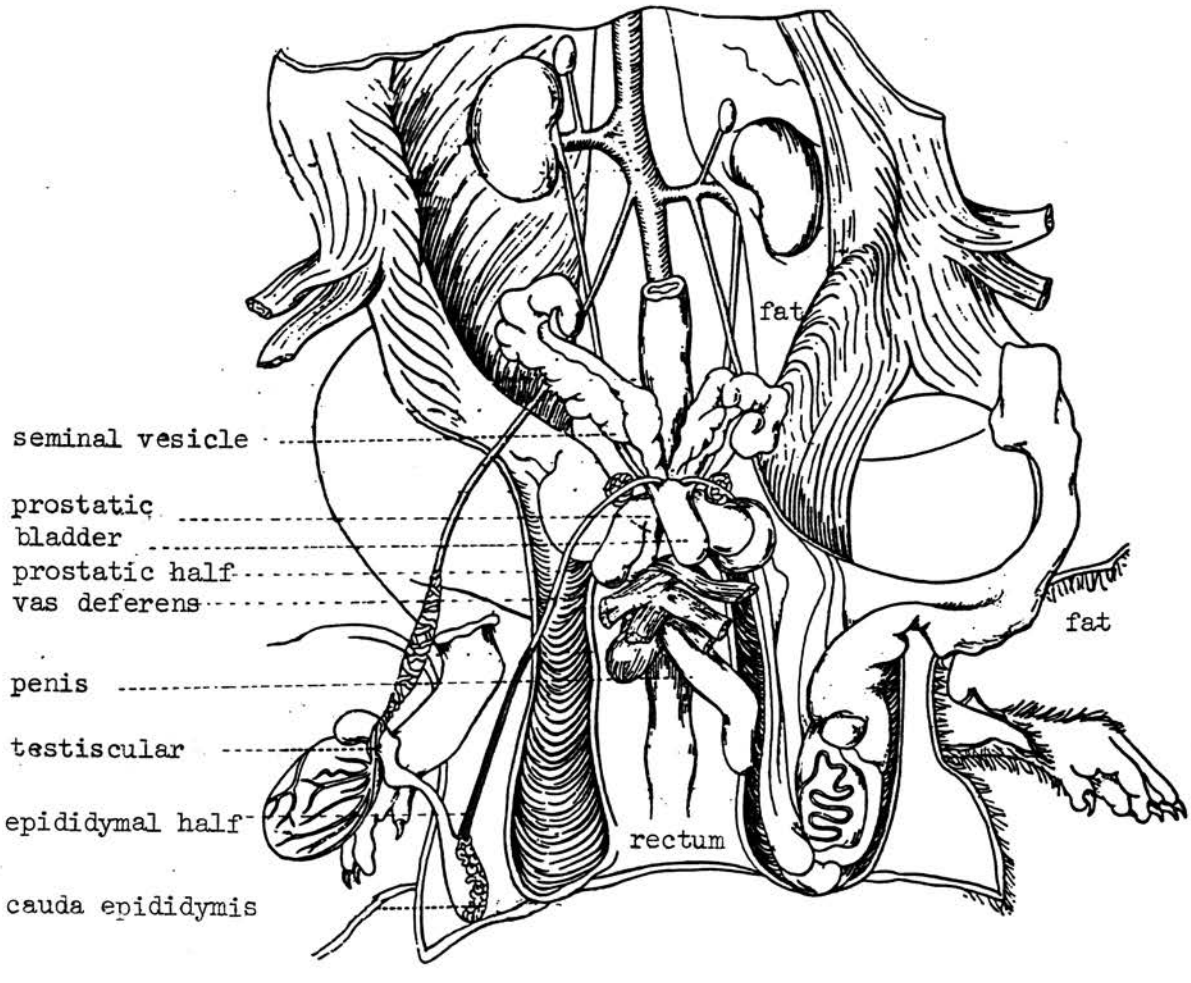

Figure 5. Male Urogenital System of rat

The fat has been removed from the right side

(from Anatomy of the rat, American Philosophical Society, 1975, pp.142).

3. The organ bath.

The organ bath used in isolated preparations (Fig. 6) was composed of two compartments, the inner chamber, capacity 20 ml, for tissue preparation being immersed in physiological fluid, and the outer jacket for flow-through circulation of 37° C prewarmed water so as to provide constant temperature to the inner compartment. The circulating water was supplied by a thermoregulating water pump (Churchill type). The tube leading from the solution reservoir passes through coil in the condenser tube, so that the solution is warmed to the desired temperature before entering the bath. This solution is allowed to flow freely from the coils, into the bottom of the tissue bath and out under another the tube of the bottom, whenever the bath solution is to be replaced with pure solution from the reservoir. The bath also had an oxygen inlet to oxygenate the inner chamber through a sintered glass opening. Usually 2 chambers were used in each experiment , to provide replication of the results.

4. Drug administration.

The isolated mammalian vas deferens has no spontaneous movement (Hay & Wadsworth, 1983), so in this experiment the tissues were induced to contract by either 5-HT (1.3×10^{-4}M), NA (3×10^{-5}M), KCl (1×10^{-1}M), $CaCl_2$ (1.2×10^{-1}M) or $BaCl_2$ (2×10^{-3}M). The isolated tissue was equilibrated for 60 min in the bathing medium which was changed every 15 min as a precaution against accumulation of interfering metabolites (Altura, 1970). The solution of KCl, $BaCl_2$, 5-HT and NA were administered to the bath fluid noncumulatively but $CaCl_2$ solution was applied in cumulative regimen, using either a microsyringe or an automatic micropipette.

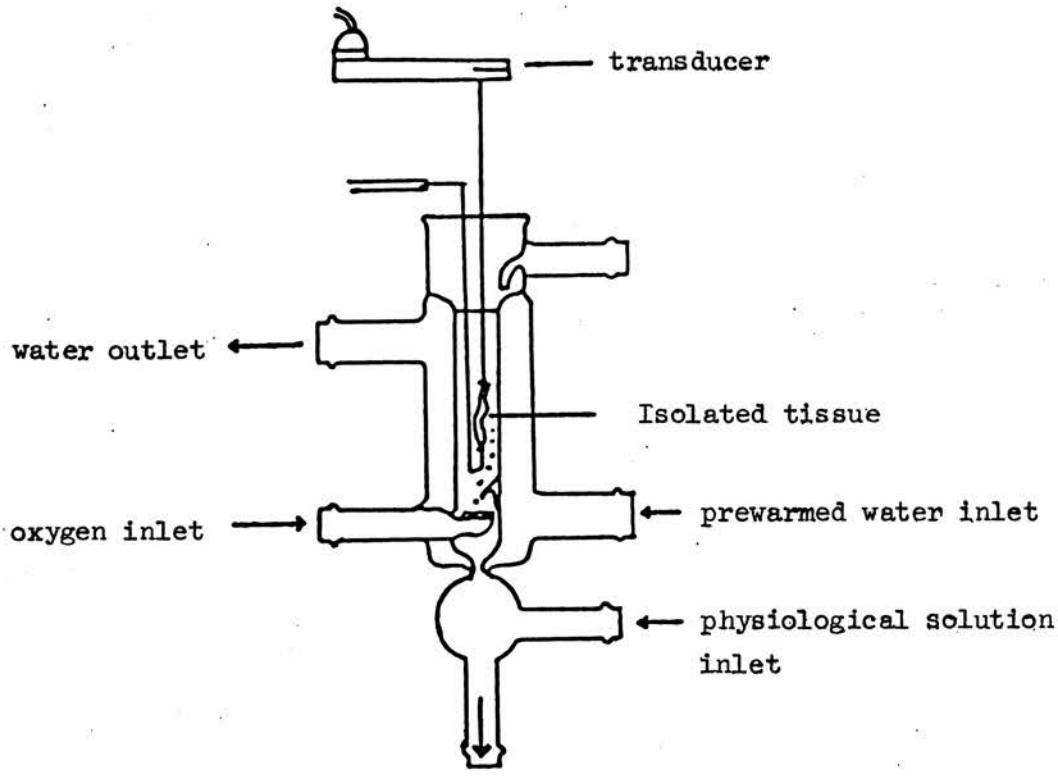

Figure 6. Organ Bath

Tachyphylaxis was a frequent complication of dose-response measurement with 5-HT and NA on the vas deferens (Nishino *et al.*, 1970; Hay & Wadsworth, 1974). It was found that tachyphylaxis could be avoided by the use of long dose cycles or by frequent changes of the bath fluid, usually six times between each dose of 5-HT and NA.

Two control applications of agonist were usually made before the addition of antagonist. Each concentration of antagonist was allowed to equilibrate 15 min before addition of the agonist.

Calcium chloride concentration-response curves were obtained by adding calcium chloride cumulatively (each increase in concentration being left in contact until tension stabilized) using a depolarizing solution of the following composition (in g/l) : NaCl 1.2, KCl 7.72, $MgCl_2$ 0.24, glucose 2.1, HEPES (biological buffer) 1.2 and EDTA .01 (Hay & Wadsworth, 1982a). After completion of each concentration-response curve, the vas deferen was relaxed using Ca^{2+} free Krebs-Henseleit containing 0.03 g/l EDTA. Following 30 min in Ca^{2+} free Krebs-Henseleit, the tissue was washed 3 times at 1 min intervals with depolarizing solution and then allowed to relax before addition of $CaCl_2$ for the next concentration-response curve.

5. Analysis of data.

For statistical evaluation of data, the significance of the difference between control and drug treated means were determined using Student's t test. Values of P which were less than 0.05 were taken to implicate statistical significance. Mean values were given together with S.E.M. : n is the number of estimation.

The tension developed by the phasic and tonic responses and the phasic and rhythmic responses were measured separately. Responses to NA, 5-HT, KCl and $BaCl_2$ were determined after 60 min initial incubation in Krebs-Henseleit solution. The phasic response was determined by measurement of maximal amplitude of contracture (Fig. 7). The maximum tension of phasic response occured within 1 min after application of drug and were followed by rhythmic or tonic response. The stabilizing tonic response was measured and the mean frequency of the rhythmic contraction was evaluated by the contraction occuring during five min of drug contact period.

The phasic and tonic component were expressed as percentage inhibition relative to the control response before addition of the antagonist. The rhythmic response was expressed as a percentage increasing or decreasing of the control values.

In cumulative $CaCl_2$ application a dose-response curve was constructed, employing about fourteen doses. Subsequently the dose-response curves were obtained in the presence of the antagonists.

The affinity of non-competitive antagonist for its receptor, pD_2', is the negative logarithm of the molar concentration of the antagonist that produces 50 % reduction of the maximum response obtained with an agonist. pD_2' values were calculated by the equation.

$$pD_2' = pD_x' + \log \left(\frac{E_{am}}{E_{abm}} - 1\right)$$

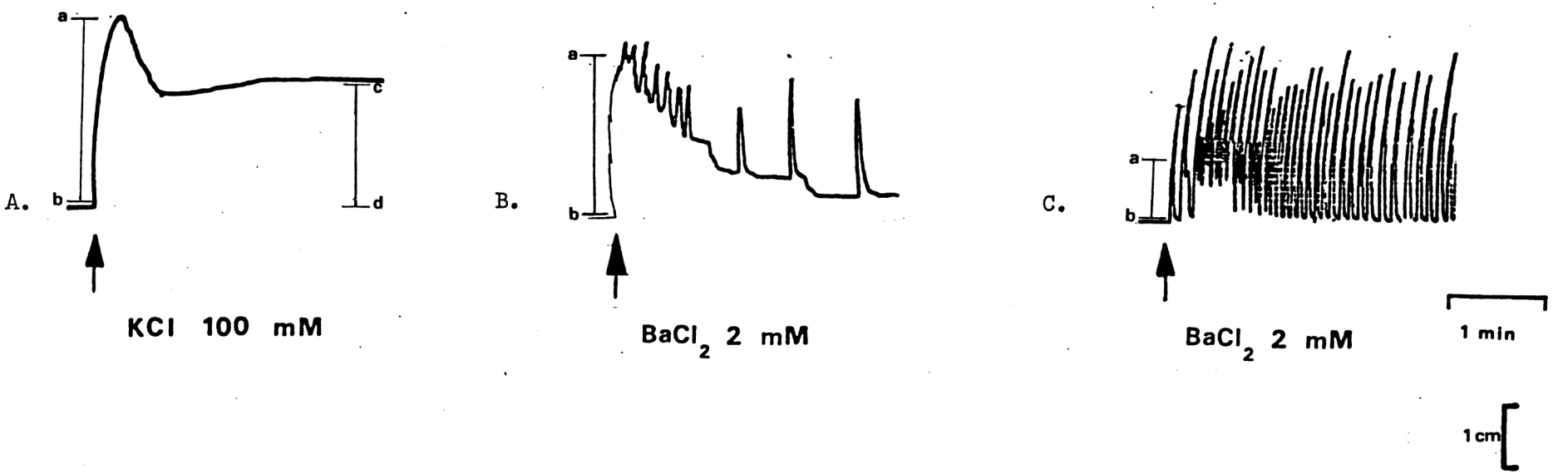

Fig. 7. Sample traces of the measurement of contractile response as recorded by isotonic transducer of rat vas deferens : the amplitude of phasic responses of KCl (A) and $BaCl_2$ (B and C) induced contraction are the distance between a to b. The distance between e and d is the amplitude of tonic response. The frequency of rhythmic response was evaluated by the rate of contraction occuring during five min of drug contact periord.

pD'_x is the negative logarithm of the molar concentration of the antagonist in the presence of which the maximum response of the preparation to the agonist is E_{abm}. E_{am} is the maximum control contraction to the agonist (Van Den Broucke *et al* , 1982).