Selected Topics in Experimental Pharmacology

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KNOWLEDGE

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PADMAJA GARKAL

SELECTED TOPICS IN EXPERIMENTAL PHARMACOLOGY

PREFACE

The training program in experimental pharmacology for postgraduates in various departments of pharmacology is usually built up around the research interest of a particular department. In addition, the postgraduates are trained in certain basic experimental techniques which form a part of regular curriculum. While training our postgraduates over the last 15 years, we had keenly felt the necessity of having some text book which the postgraduates could use for regular reference for experimental techniques. A number of books and monograms on pharmacological screening methods are available, and recently two excellent monograms have been published from the Edinburgh University. A number of experimental techniques are also described in various pharmacological journals. Inspite of this, we felt that there was a need for some books specially written to meet with the needs of postgraduates which will provide all the necessary information for routine training at one place, obviating the need to refer to specialised books and journals from day to day. A large number of new medical colleges have been opened in last few years. The library facilities at these colleges are often not adequate, especially as far as availability of back-references in journals is concerned, thus making it difficult for postgraduates to get the required information. To meet with such needs, we thought it desirable to publish details of the training program followed at our department, with the hope that it will meet with the general requirements of postgraduates in experimental pharmacology. No attempt has been made to describe all the techniques and give all the information in experimental pharmacology, which would be a major undertaking.

We have described selected topics in experimental pharmacology, which would meet with the curricular requirements of postgraduates. There probably is some bias in selection towards the type of work carried out in this department. Techniques in biochemical pharmacology, use of radioactive isotopes, use of highly sophisticated techniques involving polygraphs, transducers and oscilloscopic recording are not included, as only few departments possess the necessary equipment for carrying out such techniques. We have limited our field to those techniques which could be carried out by postgraduates in most of the pharmacological departments of a medical college in our country for whom the book is primarily intended.

Each chapter covers a very brief theoretical background and gives details of experimental techniques. This is followed by practical exercises which the students can carry out and compare their results with those illustrated. This will help in establishing techniques with known drugs so that one can then handle problems using these techniques more confidently. For further detailed work references to original techniques have been indicated. The appendices at the end of the book cover subjects of anaesthesia in animals, elementary statistics, and certain important details about perfusing solutions, etc.

Majority of practical exercises have been worked out by my junior colleagues NKD and UGK giving practical instructions and some alterations on standard techniques based on their experience. Exercises on CNS and diuretics have been contributed by the members of the Pharmacological Research Unit of Council of Scientific & Industrial Research, which functions as the Drug Screening Unit at this department. NKD and UGK have been mainly responsible not only for the majority of scientific work, but also have contributed to the artistic side of this project. All the illustrations, lay-out of the exercises, lettering and cover design have been personally carried out by them without any help from professional artists. Without the enthusiasm and help of my these devoted, versatile co-authors, this book could never have been completed.

I am very thankful to our Dean, Dr. T. H. Rindani, MD, DSc, FAMS for his constant encouragement in this project

I am also thankful to Shri Vinoobhai Kothari of M/s Kothari Book Depot, our publishers, for his interest and co-operation in converting the raw manuscript into a full fledged text book.

UK Sheth

Bombay
August 1971

CONTENTS

It has long been an axiom of mine that the little things are infinitely the most important.

SIR ARTHUR CONAN DOYLE

Principles of Drug Action and Bioassay

Introduction

Pharmacology, the science of drugs involves a consideration of the effects of drugs on living organisms. The basic units of interaction are the drug molecules and the "receptor portion" of the organism as suggested by Paul Ehrlich and Langley. In recent times, the concept of receptors in relation to drug action has progressed a great deal as a result of the work of many distinguished scientists such as Ariens, Clark, Furchgott, and Paton.

It is commonly accepted today that drug action involves combination of drug molecules and receptor molecules leading to what is termed as drug "effect". The effects of drugs are expressed either in terms of physiological, biochemical or electrophysiological changes occurring in the organism as a whole, or in isolated tissues, or as a relief of symptoms or cure of a disease in a clinical situation. The effects may be of various types e.g. antispasmodic, hypotensive, analgesic, anticoagulant, hypoglycaemic and so on. To quantify these observations the effects must be studied in relation to different doses, termed as dose effect relationship, specificity of action or otherwise, effect in comparison to a known drug, mechanism of action, drug interactions, effects of repeated drug administration etc. Most of these drugs are governed by certain general principles of drug action and in this chapter experiments are designed to illustrate some of the principles of drug action.

In considering drug action we are concerned with the receptor and its immediate vicinity, the biophase; it is the presence of the drug in an optimum concentration in the biophase that will elicit a response. The response therefore depends on the dose of the drug, and the relationship between dose and response is a useful index to the pharmacologist, of conditions in the biophase.

In the simplest situation, a molecule of active drug combines with the receptor and sets off a reaction which ultimately manifests as a response; the magnitude of this response increases as the amount of drug increases until a maximum is reached. Expressed arithmetically, on a graph, the dose response curve is a hyperbolic curve with a plateau; when expressed in logarithms of dose, this graph gets straightened.

This relationship may be studied by plotting responses to graded doses of a drug on a convenient experimental preparation like the isolated guinea pig ileum

(Exercise 1). Another way of studying it, for example in an intact animal would be to compare pressor responses to graded doses of NE in a spinal cat where compensatory mechanisms are eliminated (Exercise 2).

For a more rapid and convenient way of studying dose response relationship, particularly in isolated tissues, a cumulative method may be used. Here, instead of washing out the drug after each response, it is added in increments such that at a given time the amount is double that at the previous response. This is continued till no further contraction occurs and subsequent addition of drug causes a small relaxation. This is the supramaximal dose. If a graph is plotted with log dose against response taking percentage maximal contraction instead of actual height, an S shaped curve will be obtained with the middle portion practically linear.

Consider the effect of two drugs present in the biophase; this can lead to three possibilities — there may be no effect of either drug on the other, there may be diminution in the action of one drug due to the presence of the other (antagonism) or there may be an increase in the action of one by the presence of the other (synergism). Synergism implies that the end result is greater than the arithmetic total of individual responses; it can occur either (a) due to protection of agonist from destruction, excretion or silent receptor occupation, or (b) sensitisation of receptors by depletion of the natural neurohumoral transmitter. This is shown in Exercise 4 where ephedrine, a sympathomimetic amine with dual action has been shown to have its own pressor action as well as cause potentiation of pressor response to NE implying thereby an acute depletion of NE as well.

Antagonism may be of a specific type or a non-specific type, apart from actual chemical neutralisations. Specific antagonism is caused by interaction of an agonist and antagonist with the same receptor, the antagonist being specific to the agonist. If there is a competition for the occupation of receptor, a molecule of antagonist being able to displace a molecule of agonist, it is called competitive, or surmountable, or reversible antagonism. If dose response curves are plotted with agonist in presence of different doses of antagonist, more and more of the agonist will be required to produce a given response. The maximal effect, however, will be reached even though a very large dose of agonist may be required. Hence log dose response curves will shift to the right, without change in the maxima (Exercise 5).

In non-competitive antagonism on the other hand, increasing doses of agonist will not be able to displace antagonist completely and a residual diminution in action will be apparent, which increases with antagonist concentration; also, since displacement of antagonist does not occur molecule for molecule, the relation in dose and response will change, thus altering the slopes of the log dose response curves. Hence in Exercise 6, a change in slope and in the maxima is apparent.

Non-specific antagonism however does not necessarily involve receptor occupation and may occur at any step from drug receptor interaction to the effector system which results in a response. For example, two drugs acting independently of each other to activate two different opposing effector systems, like epinephrine and acetylcholine, or papaverine and acetylcholine. The only difference between these two pairs is that the first pair occurs naturally in the body, subserving two essential opposing physiologic mechanisms and this antagonism is hence called physiological antagonism (Exercise 8).

In identifying two drugs which produce a similar response, for example, vasode-

pression by acetylcholine and histamine, advantage may be taken of the phenomenon of specific antagonism; a drug that antagonises only histamine e.g. chlorpheniramine would reduce or abolish the action of histamine alone, and leave the action of acetylcholine unaffected (Exercise 7). This would help to identify which drug is histamine.

When receptors are rapidly occupied by a large concentration of drug molecules, a drug effect is seen; if this is immediately followed by some more drug molecules, most of the receptors having already been occupied, only a small unoccupied fraction will combine to give a response. Hence, a progressively smaller response would be obtained in the presence of a persistent drug receptor combination. This phenomenon of diminished response is called tachyphylaxis (Exercise 9); another mechanism by which tachyphylaxis could occur is when a drug acts by the release of an endogenous substance e.g. NE with a slow turnover rate; as the drug is administered smaller and smaller amounts of norepinephrine would be released and smaller responses would be obtained.

An interesting corollary of the concept of receptor occupation in drug action is that if a large concentration of agonist is added to the receptors, addition of an antagonist will not affect responses plotted with doses of agonist. This is due to the phenomenon of receptor protection and is well seen in Exercise 10.

Bioassay

Bioassay, or the assay of a pharmacologically active substance by biological means is even today a sensitive and reliable method of assay, yielding valuable information particularly in the analysis of perfusate, effluents from various organs etc.

Various methods have been devised for the bioassay of substances, and the general principles of bioassay with the help of isolated tissues are discussed here. Methods of bioassay of specific substances are described in Chapter 6.

The simplest method would be to find out the response to a dose of a standard solution of known concentration (S) which would match the response obtained with a known volume of test solution (T). This is done by recording a dose response curve with standard, and with test solution diluted 1:1000, 1:100, 1:10 (not shown here). From the latter, a response (T) of about 6 cm height is chosen which would be in the linear portion of the standard's dose-response curve. Two doses of standard one larger and one smaller are chosen and repeated with T. The range between these two doses is then gradually reduced

e. g.:	0.2 μg	and 0.3 μg
	0.22 „	„ 0.28 „
	0.24 „	„ 0.26 „

each time repeating T so that any variation in sensitivity of the isolated tissue can be accounted for, and less inaccurate results obtained. A stage will be reached when T will be exactly matched by some dose of standard. In this exercise we see that T matches 0.24 μg.

If T was 0.4 ml of 1:10 diluted unknown

0.04	ml of unknown	contain	0.24 μg
0.4	„	„	2.4 μg
4.0	„	„	24 μg

1 ml = 6 μg

i.e. the unknown has a concentration of 6μg/ml. As this method involves repeating T inside a 'bracket' of standard doses it is called bioassay by bracketing or direct matching.

2 + 1 assay, 3 point assay (Exercise 12), owes its name to the fact that repetition of three doses, two of standard and one of unknown is done randomly to obtain a series of responses. A graded dose response curve is taken for the standard and for the unknowon solution suitably diluted. From these,

3 doses are chosen such that the response "T" to the test solution ('t') lies between the responses S1, S2 to two doses of the standard (s). These 2 doses should bear a known ratio to each other, preferably 1:2. These responses are repeated in the order shown and the means of three responses for each dose calculated. A graph is plotted which shows log dose on abscissa and response on ordinate. The distance between the points S1, S2 on the abscissa is equal to the log of the ratio i.e. log 2, or 0.301. The point T is extrapolated on the line joining S1, S2 and a perpendicular dropped onto the abscissa.

Now

$$\frac{s1}{s2} = 2$$

$$\log\left(\frac{s2}{s1}\right) = \log s2 - \log s1$$

$$= 0.301.$$

$$\text{Intercept obtained} = \log t - \log s1$$

$$= \log \frac{t}{s1}$$

$$\text{Antilog intercept} = \frac{t}{s1}$$

$$t = s1 \times \text{antilog intercept.}$$

Now $s1 = 0.2\ \mu g$ $t = 0.4$ ml test solution

$$s2 = 0.4\ \mu g$$

$$\text{Intercept} = 0.1$$

$$t = 0.2 \times \text{antilog } (0.1)$$

$$= 0.2 \times 1$$

$$= 0.2$$

From the formula

$$t = s1 \times \text{antilog} \frac{T - s1}{s2 - s1} \times \log - \left(\frac{s2}{s1}\right)$$

$$= 0.2 \times \text{antilog} \frac{35.75}{103.25} \times 0.301$$

$$= 0.2 \times \text{antilog } 0.1 = 0.2$$

$\therefore$ t = 0.4 ml test solution contains 0.2 histamine

$\therefore$ 1 ml test solution contains of $0.5\ \mu g$

This is a 1 : 100 diluted solution

$\therefore$ original solution contained 50 μg m.

Four point assay involves the addition of two doses of standard and two doses of test solution. In this assay therefore the critical point is the choice of doses. To begin with a dose response curve is plotted of the standard and the test solutions; from this the responses which show fairly linear relationship to log dose are selected. From this range of responses with both solutions, a pair is chosen such that the doses of each solution bear to each other the same ratio i.e.

$$\frac{S2}{S1} = \frac{t2}{t1} = 2$$

Preferably the response T1 should be between the responses S1 and S2 (Exercise 13).

Let the responses: S1, S2, T1 and T2 form the four points, A, B, C and D of the assay. They are repeated randomly and the means of four responses, to each dose, are calculated. A graph is plotted as directed in Exercise 14, and an intercept obtained. This intercept gives the logarithm of potency ratio. From this the strength of the unknown can be determined e.g. here D = 0.15

$$\text{potency ratio} = \text{antilog } (0.15)$$

$$= 1.413$$

$$\frac{t}{s1} = 1.413$$

$$t = s1 \times 1.413$$

From the formula

Potency ratio = antilog

$$\left[\frac{(T2 - S2) - (T1 - S1)}{(S2 - S1) - (T2 - T1)} \times \log\left(\frac{s2}{s1}\right)\right]$$

since S1 = 43.25 cm

S2 = 112.0

T1 = 25.75

T2 = 75.00

s1 = 0.2 ml std soln. s2 = 0.4 ml st—

t1 = 0.3 ml test solution t2 = 0.6 ml test solution

Potency ratio = antilog

$$\left[\frac{(11 - 75) - (43.25 - 25.75)}{(112 - 43.25) - (75 - 25.75)} \times \log 2\right]$$

$$= \frac{54.5}{118} \times 0.301$$

$$= \text{antilog } (0.139) = 1.377$$

The strength of t can be calculated knowing the potency ratio.

$\frac{t}{s} = \frac{S}{s}$ Potency ratio

Experimental Techniques.

Experiment 1.

The Isolated Guinea Pig Ileum. A guinea pig weighing about 450 gm is starved for 48 hours, being allowed water ad libitum. For the purpose of the experiment it is killed by stunning with a sharp blow on the head and cutting its throat to bleed it to death. The abdomen is quickly opened and the viscera inspected.

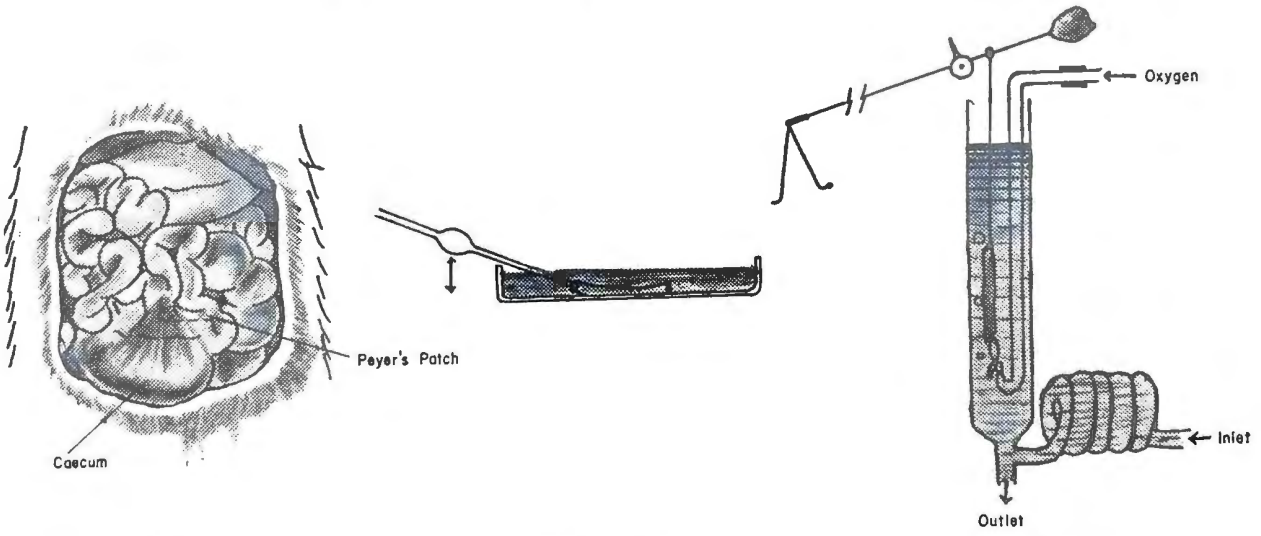
In the right lower quadrant of the abdomen is seen a greenish sac like structure, the caecum. This is gently turned outwards so as to expose its inner surface; this reveals a slender pinkish length of small intestine marked by a localised thickening in the wall — a Peyer's patch of lymphoid tissue.

Using this patch as a landmark, the lower-most 10 cm of ileum are removed from the abdomen and placed in a shallow dish containing warm Tyrode solution.

With the help of a 25 ml pipette, the lumen of the ileum is gently rinsed out, care being taken not to exert a hydrostatic pressure of more than 10 cm water. The ileum is then cut into segments of required length, generally 4 cm in the fully relaxed state, and sutures are taken with needle and thread through either end of a segment, care being taken to include only one point on the circumference without occluding the lumen.

With the lower suture the ileum is fixed on to the hooked end of a hollow oxygen tube while the upper thread is tied to the recording lever. In this case we use an isotonic lever with a frontal writing point balanced to provide a tension of 500 mg. The tissue is positioned in an organ bath in Tyrode solution at 37°C aerated with oxygen or carbogen mixture. The tissue generally takes about a half hour to stabilise; there should be no spontaneous contractions. The fluid bathing the tissue should be renewed every 10 min.

Fig. 1. 1 : The isolated guineapig ileum preparation.



1. 1 – a
Showing the position fo the Peyer's patch, which serves as the landmark for further dissection.

1.1 – b
The lumen of the ileum is rinsed with Tyrode, with minimal hydrostatic precssure, not exceeding 10 cm water.

1.1 – c
The final set up of the isolated preparation in Tyrode selution at 37°C.

Practical Points :

1. The most consistent responses are obtained with the segment of ileum above the last 6 cm.
2. The last few cm are inconsistent not only quantitatively, but sometimes also

qualitatively, e.g. epinephrine may cause a contraction by itself instead of inhibiting acetylcholine induced contraction.

3. It is generally recommended that the proximal end of the ileum should be the upper end in the bath, and the distal end the lower end.

Experiment 2.

Spinalisation of Cat. Posterior approach. A cat about 2.5 — 3.0 kg in weight is induced with ether anaesthesia and tied down to the dissection table. A midline incision is taken in the neck, the strap muscles separated and the trachea exposed. A loose ligature is placed under the trachea taking care not to damage the large anastomotic pretracheal vein. A small horizontal cut is made in the trachea, and a cannula inserted and tied in place with the loose ligature. The cat's chest is vigorously squeezed to expel any tracheobronchial secretions.

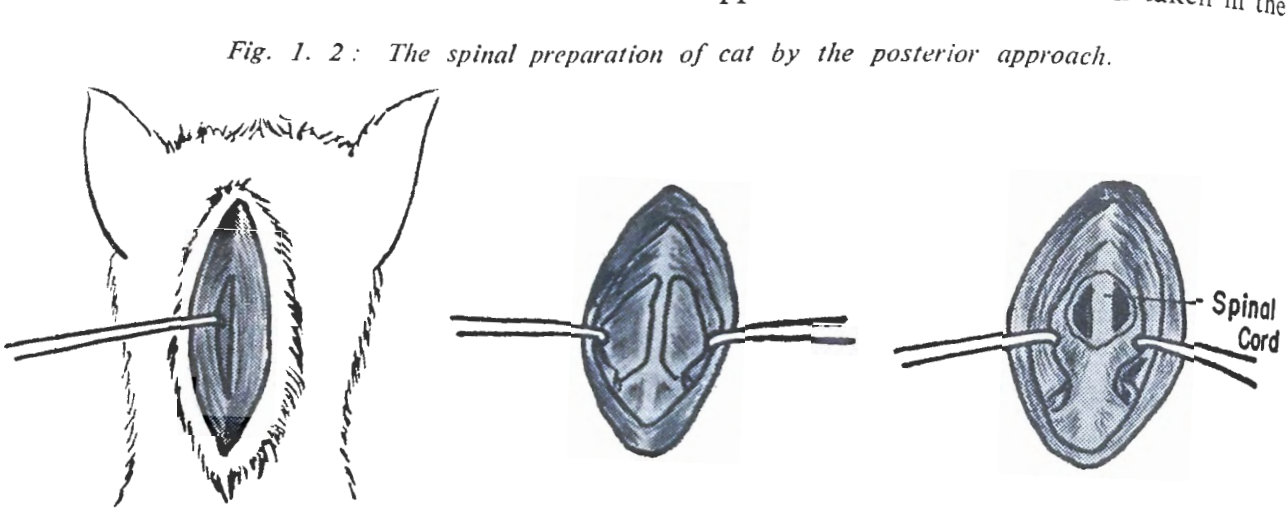
The tracheal cannula is then connected to a Wolff's bottle containing ether and the cat is maintained on deep ether, taking care to check heart beat and respiration regularly.

The common carotids on either side ar isolated and kept ready for subsequen clamping with the help of loose ligature as guides.

The cat is now placed in the prone posi tion and its head supported by an assist ant with the neck in full flexion. Palpate the vertebrae to locate the spine of the 1st cervical vertebra; for acute experiments high spinalisation is preferred, and for this the gap between 1st and 2nd cervical vertebrae is chosen. The fur is carefully clipped and a vertical incision taken in the midline with a scalpel. The skin, connective tissue and cervicalis muscles are carefully separated with good haemostasis. The muscular attachments to the spine are severed and the bone exposed. At this stage both common carotid arteries are clamped.

With the aid of a bone cutter the spine is cut away with a wrenching upward twist of the hand, the gap being widened with the help of a bone nibbler Bleeding is not profuse because of the clamped carotids. It is advisable to apply bone wax to the cut edges of the bone and warm saline compresses to soft tissues. The spinal cord can be seen a

Fig. 1. 2 : The spinal preparation of cat by the posterior approach.



1.2 - a
The skin and muscle overlying the spine of the 1st cervical vertebra are divided.

1.2 - b
The laminae of the cervical vertebra are exposed.

1.2 - c
The spinal cord is exposed in the vertebral canal.

Burn, J. H. (1952). 'Practical Pharmacology', p. 35. Blackwell Scientific Publications. Oxford.

a smooth white structure inside the vertebral canal. It is divided between ligatures; with the help of a blunt curved instrument inserted into the foramen magnum the brain is destroyed.

The cavity thus created is packed with cotton wicks and the muscles sutured over it. The skin is approximated with skin clips and the cat turned over in the supine position. The ether is disconnected and the tracheal cannula connected to an artificial respirator. Now the common carotids are unclamped.

The femoral vein is cannulated with a 3-way venous cannula. About 50 ml of 5 — 10% glucose in saline is then injected slowly to compensate for any blood loss that may have occurred. After $\frac{1}{2}$ — 1 hour, the blood pressure recording may be set up by cannulating the carotid artery. Generally, the blood pressure is about 80/70 mm Hg due to spinal shock.

Spinalisation of Cat. Anterior Approach: *In this method, the spinal cord is approached from the front of the neck, avoiding the procedure of turning the cat over after tracheostomy and occlusion of* both carotids. The trachea and oesophagus are divided between ligatures cephalad to the tracheostomy; the anterior spinal muscles are retracted and with the neck hyperextended, the dura over the space between the base of the skull and the 1st cervical vertebra is exposed. The excess spinal fluid is aspirated with a needle and the transection performed in this gap.

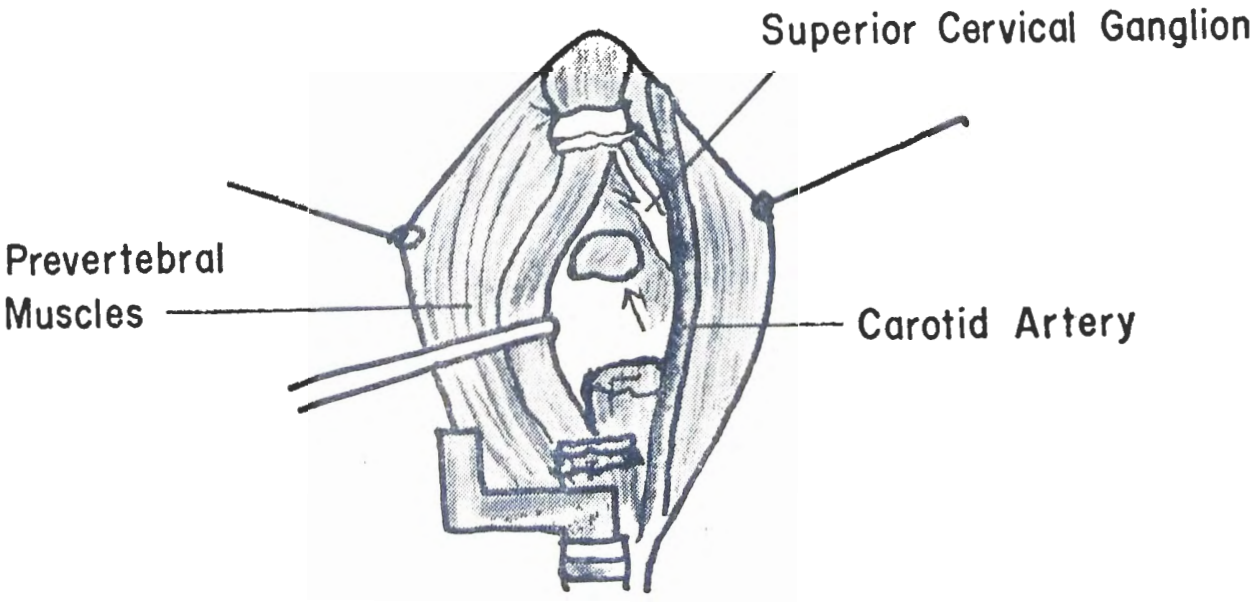
Experiment 3 :

Recording of blood pressure in dog. A dog weighing about 10 kg is anaesthetised with pentobarbital sodium, (35 mg/kg) given slowly into the saphenous vein. Pentobarbital sodium is prepared freshly in normal saline in a strength of 70 mg/ml.

Anaesthesia is achieved in about 60 — 90 seconds and the dog is then placed in the supine position on the dissection table and tied down. The femoral vein is cannulated as described below. The femoral artery is palpated carefully in the femoral triangle below the inguinal ligament. The vein lies immediately medial to it but lateral to the Adductor longus muscle.

Zarro, V. J. and Di Palma, J. R. (1964). J. Pharm. Pharmacol 16: 427 — 428.

Fig. 1. 3 : The spinal preparation of cat by the anterior approach.



The anterior aspect of the atlanto-occipital articulation is depicted as a window, with an arrow, in relation to adjacent landmarks.

A straight incision is taken in the skin overlying the vein and this is followed by careful blunt dissection which opens up the loose areolar tissue over the femoral triangle. The vein is identified as the bluish, thin walled non pulsatile structure in this space. A pair of loose ligatures of cotton thread is placed around it and the distal one is tied. Here it should be noted that tributaries must be below the distal ligature, if they cannot be avoided they must also be ligated. A bulldog clamp is applied above the proximal ligature and with a pair of fine pointed scissors, a cut is made in the vessel wall; the cut edge is lifted up with a cannula director or a silver probe. Under this, the venous end of a 3-way cannula is guided, the director removed, the bulldog clamp taken off and the cannula tied in place with the proximal ligature. The lumen of the cannula is then gently flushed with saline.

Next, the trachea is cannulated as described in the previous experiment and the tracheal cannula attached to an artificial respirator.

The carotid artery is next cannulated to set up blood pressure recordings. The carotid arteries are best approached between the strap muscles and the sternomastoid. The common carotid on either side is seen as a whitish, thick walled pulsatile structure with a sheath of loose connective tissue with an accompanying vein and a cord like nerve, the vagosympathetic trunk.

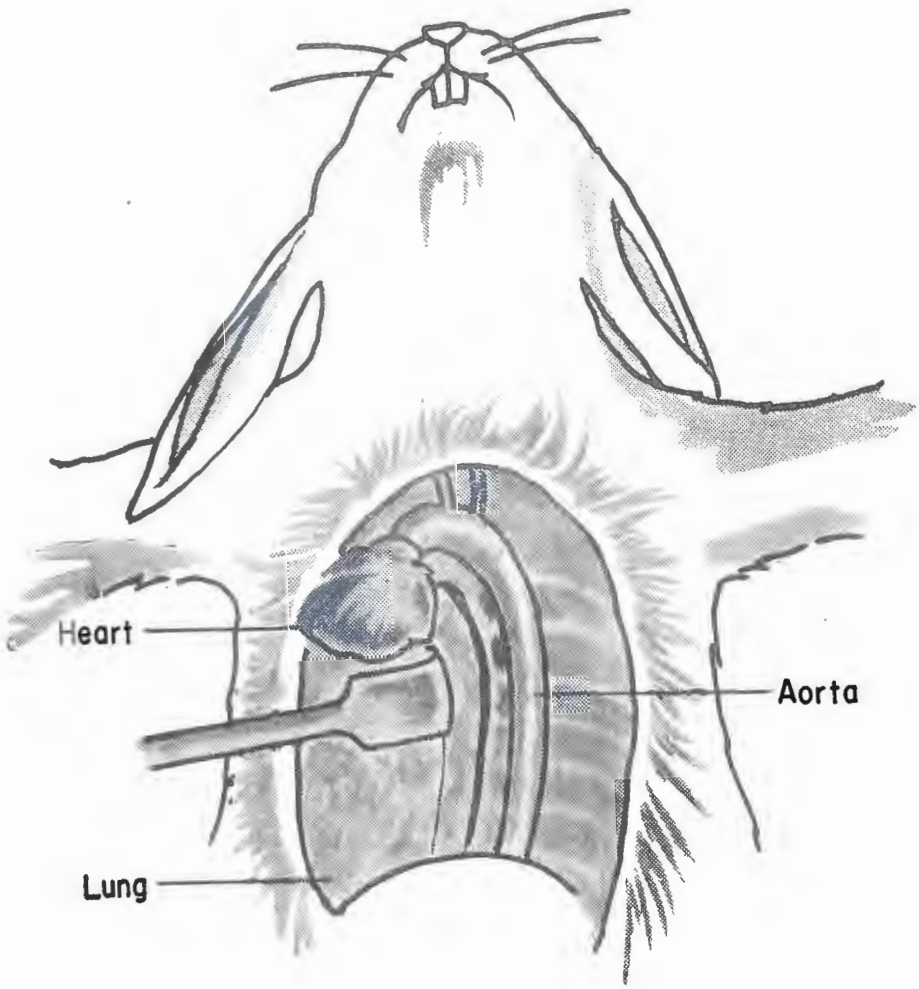
The carotid artery on one side is carefully cleared of these structures and a double ligature placed under it; the cephalad ligature is tied while a bulldog calmp is applied below the caudal one. the wall of the artery is carefully cut with a fine pointed pair of scissors directed down towards the heart. The arterial cannula is inserted and tied in place with the caudal ligature. The cannula is connected through citrate to a mercury manometer with a pointer. The pressure in this closed system is raised to about 100 120 mm Hg; about 0.6 ml of heparin (1000 units/ml) solution is injected into the side tubing of the arterial cannula and the bulldog clamp then released. The pointer on the mercury manometer is now adjusted so that it touches the drum in all its excursions.

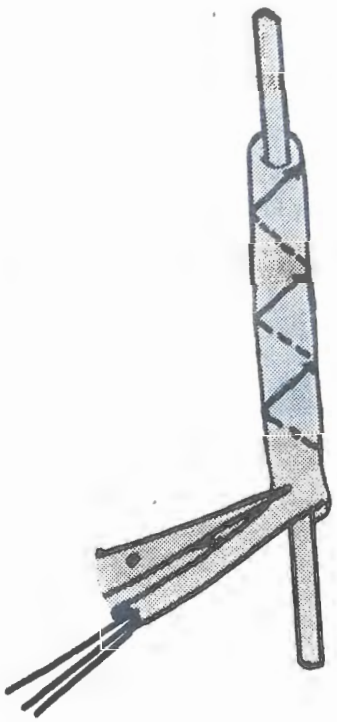
Experiment 4.

The isolated rabbit aortic strip. A rabbit weighing about 2 — 2.5 Kg is killed by stunning and cutting the throat. The thorax is opened by extending an abdominal incision upwards, cutting the sternocostal junctions ond separating the 2 halves of the thorax. The thoracic portion of the aorta is identified behind the heart, in front of the vertebral column. The aorta is cut as close to the heart and diaphragmatic aperture as possible, placed in Kreb's solution in a shallow dish and carefully cleaned of connective tissue until an opaque white glistening surface is exposed. The cut aorta is then slipped over a glass rod and cut helically as shown in the figure so as to obtain a strip about 3 mm wide.

Furchgott, R. F. and Bhadrakom, S. (1953). J Pharmacol 108: 129 — 143.

Fig. 1. 4 : The isolated aortic strip (spiral) of the rabbit.





1 4 - a
The aorta is identified in the posterior aspect of the thorax.

1. 4 - b
A strip is made, 2 mm wide by cutting in the direction of the arrows.

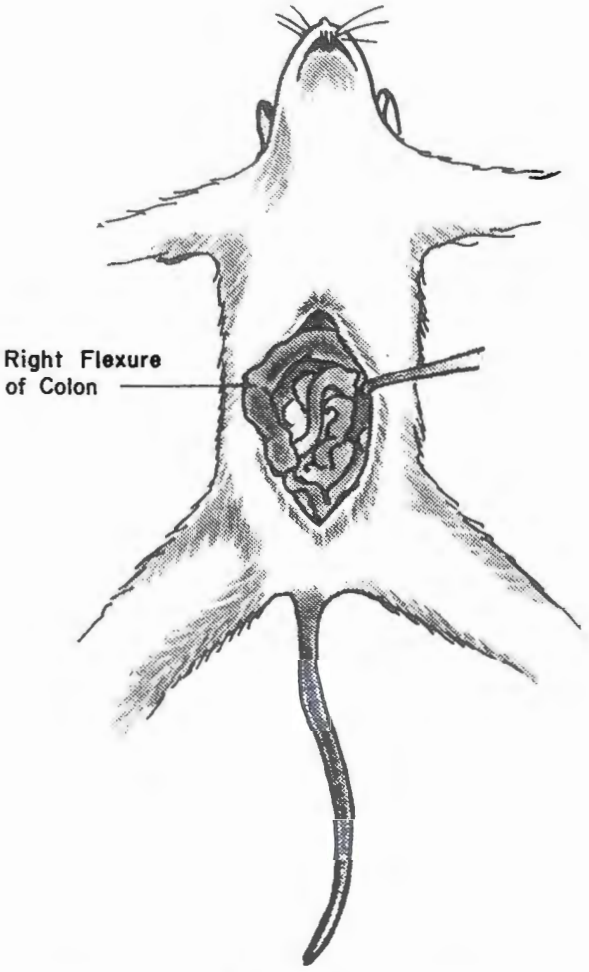
The desired length is then mounted in an organ bath in Kreb's solution at 37°C aerated with carbogen mixture. The upper end of the aorta is attached to a lever balanced with 1.5 — 2 gm and allowed to relax for 2 to 3 hours. The bathing fluid is renewed every 10 to 15 minutes.

Experiment 5:

The isolated rat colon. An average sized rat 150 — 200 gm is prepared for the experiment by starvation for 48 hours being allowed water ad libitum. This serves to provide an emptier, cleaner colon.

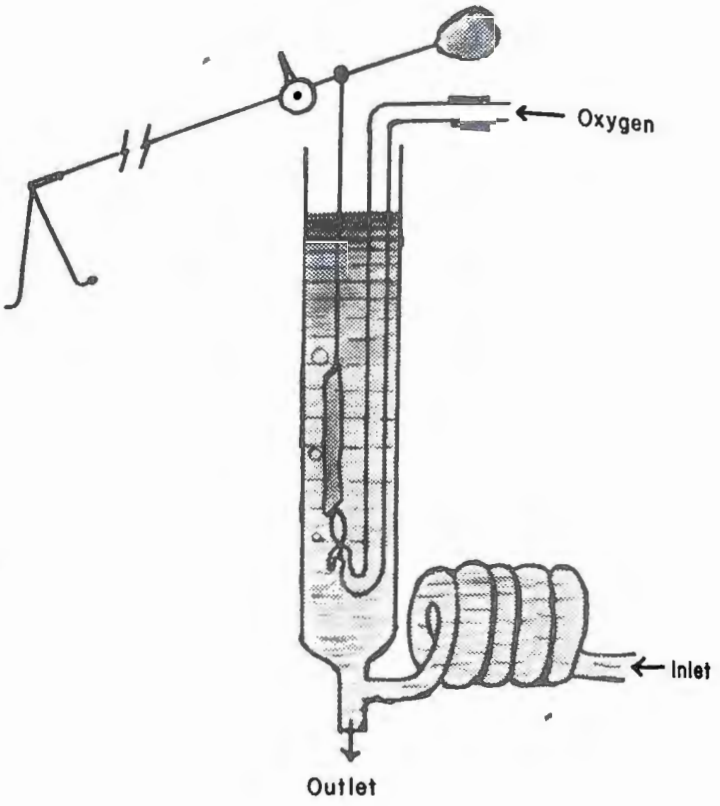
The rat is killed by stunning and cutting the throat; the abdomen is opened and the colon identified. The right flexure, i.e. the subhepatic region of the colon where the ascending colon turns to become the transverse colon, is cut out and placed in a shallow dish containing modified Ringer's solution. Its lumen is gently cleaned out and a segment about 4 cm long is mounted in modified Ringer's solution, in an isolated organ bath at room temperature, aerated with oxygen. Con-

Fig. 1. 5 : The isolated rat colon preparation.



1. 5 – a

The subhepatic portion of the colon (the right flexure) is removed for the experiment.



1. 5 – b

The final set up of the isolated rat colon in modified Ringer's solution at room temperature.

tractions are recorded with isotonic lever with frontal writing point balanced for a load of 500 mg. The tissue takes about $\frac{1}{2}$ to 1 hour to relax initially.

Practical Points :

a. Acetylcholine produces an irregular jerky response whereas Carbachol produces a smooth contraction.

b. Modified Ringer's solution contains much less calcium than Ringer's.

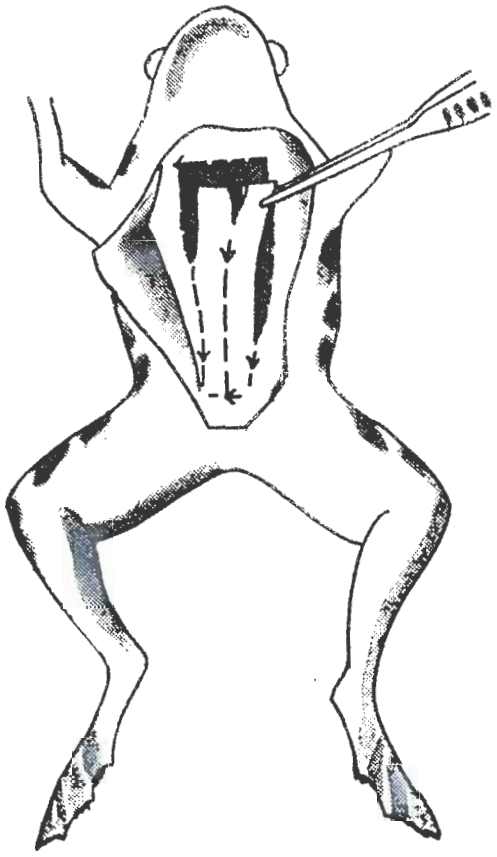
c. The low calcium content, and the room temperature both are required to diminish/suppress spontaneous contractions.

d. Though starvation of rats is generally recommended, we have found that unstarved rats give more stable responses.

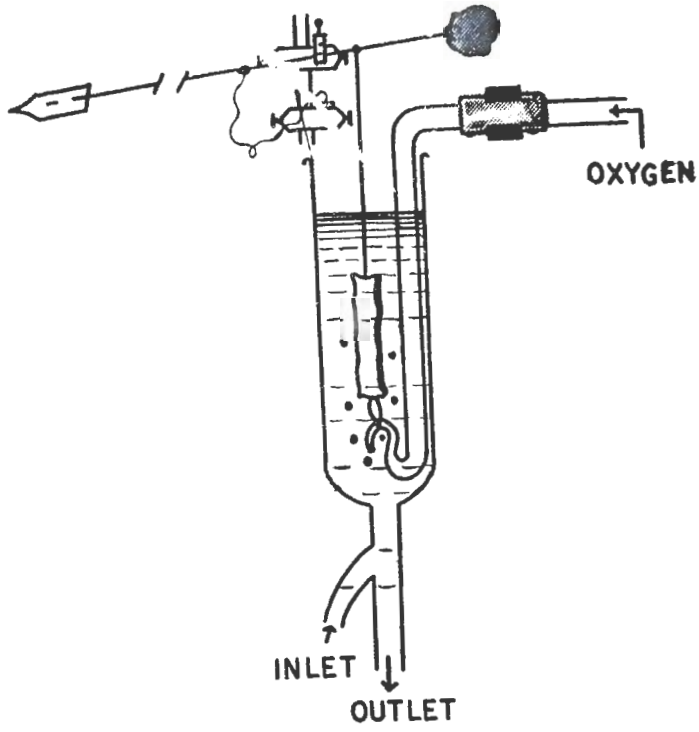
Experiment 6:

The isolated frog rectus abdominis. : A frog is pithed and laid out on the frog dissection board. The skin of the anterior abdominal wall is cut by a midline incision which is extended laterally onto the anterior aspects of the limbs. This exposes the flat whitish muscles of the anterior abdominal wall from their pubic origin to their sternal insertion. Cuts are placed as shown in the diagram, separating each

Fig. 1. 6 : The isolated frog's rectus abdominis muscle preparation.



1. 6 – a
The recti are dissected by placing cuts, as shown, along the aroows.



1. 6 – b
The final set up of the isolated rectus muscle in frog Ringer solution, at room temperature. Note the extra load of 1 g on the lever.

rectus abdominis from the surrounding musculature and from its fellow,

The two recti are removed and placed in frog Ringer solution in a shallow dish. They are carefully cleaned and one of them is trimmed to the desired size and mounted in an organ bath in frog Ringer's solution, at room temperature, aerated with oxygen. A very small bath of 5 ml capacity is sufficient but in this case solutions of drugs must be so made that when a dose is added, a change of more than 15% in bath fluid volume should not occur.

For recording purposes, an isotonic lever with a sideways writing point is used tangential to the smoked drum, balanced for a tension of 2.5 gm with an extra load of 1 gm on the long arm. The latter serves to bring back the lever to baseline, since when the rectus has contracted, it does not relax rapidly even on washing out of the drug.

Practical Points:

It is advantageous to add double the amount of glucose generally recommended.

Practical Exercises

Exercise 1.

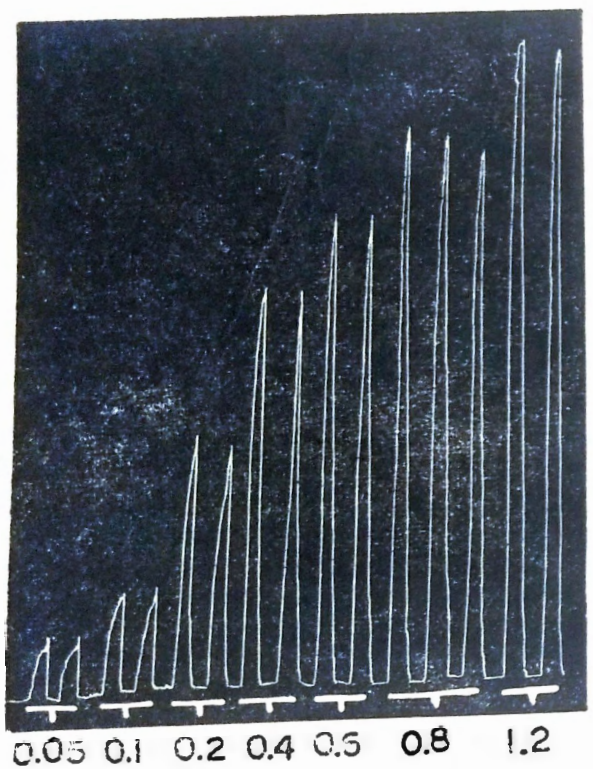
Objective : To study dose response relationship (guinea pig ileum).

1. Set up the isolated guinea pig ileum as described.
2. Prepare graded strengths of histamine acid phosphate solution e.g. 10 μg/ml. 1.0 μg/ml., 0.1 μg/ml. etc.
3. Start the drum.
4. After 30 seconds, add 0.1ml of the lowest strength of histamine solution to the bath fluid with a finely calibrated pipette, and start the stop watch.
5. After 30 seconds contact of the drug, stop the drum and drain out the bath, refill, twice.
6. Allow 3 minutes for recovery.
7. Depending on response obtained, select the subsequent doses of histamine and make appropriate dilutions.
8. Add these doses in the cycle mentioned above, each time allowing a drum movement of 30 seconds so that the responses are well spaced on the graph. For each dose take 2 responses.
9. Fix the graph.
10. Measure the heights of responses for each dose.
11. Plot a graph of response against dose and response against log dose.
12. Observe that conversion to log scale straightens out the curve.

DOSE RESPONSE CURVE

(HISTAMINE)

Isolated guinea-pig ileum



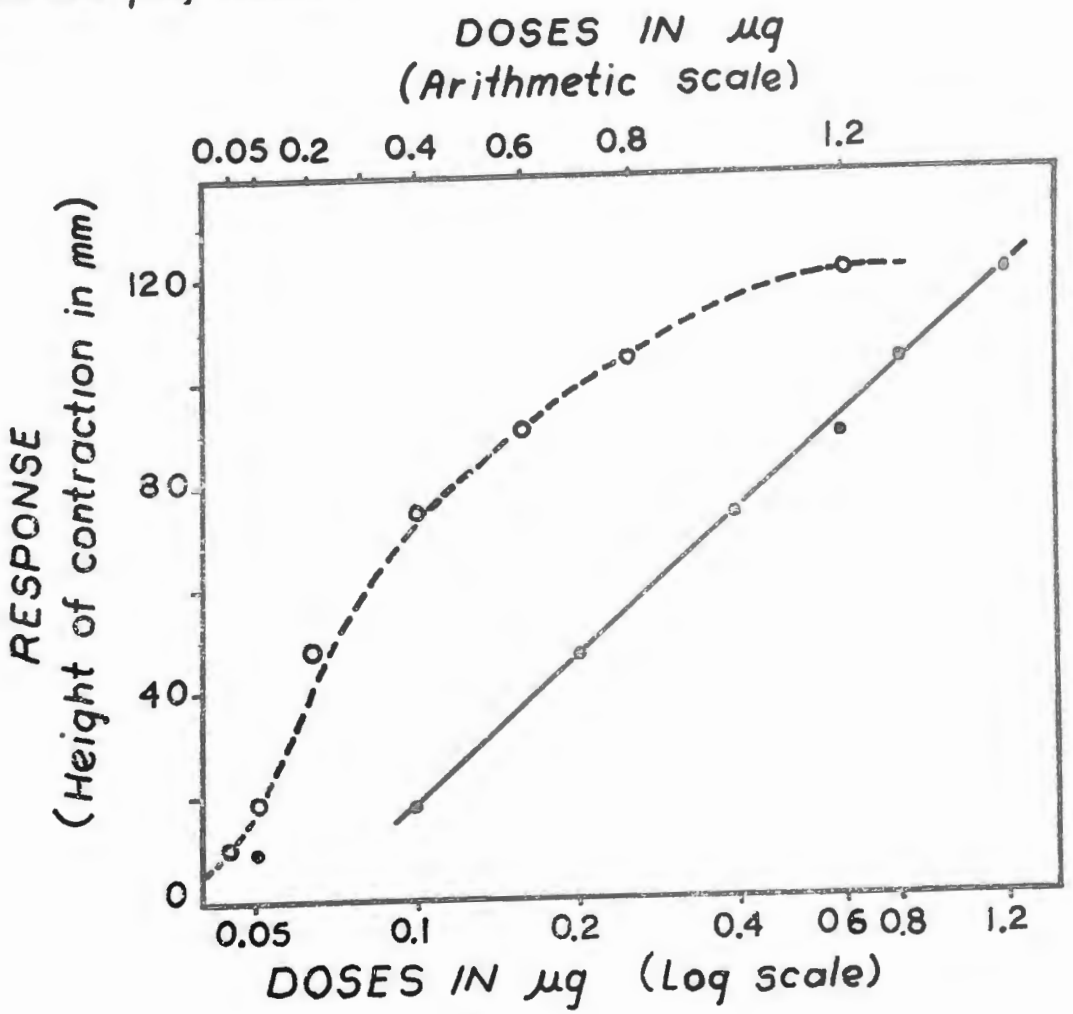


Fig. 1. 7

Exercise 2.

Objective : To study dose response relationship (spinal cat).

1. Set up the blood pressure recording of a spinalised cat as described and record a strip of tracing.
2. Stop the drum.
3. Inject 0.5 μg of norepinephrine via the femoral vein and flush the cannula with a fixed quantity of saline.
4. When pressor response has passed its peak and begun to decline, start the drum.
5. When the baseline is reached stop the drum.
6. Continue injecting graded doses of norepinephrine intravenously each time flushing the cannula with the same quantity of saline Record 2 responses for every dose.
7. Fix the graph.
8. Measure mean response for every dose.
9. Plot a graph of response against dose and response against log dose.

DOSE RESPONSE CURVE

(NOREPINEPHRINE)

SPINAL CAT : SECTION at C_2

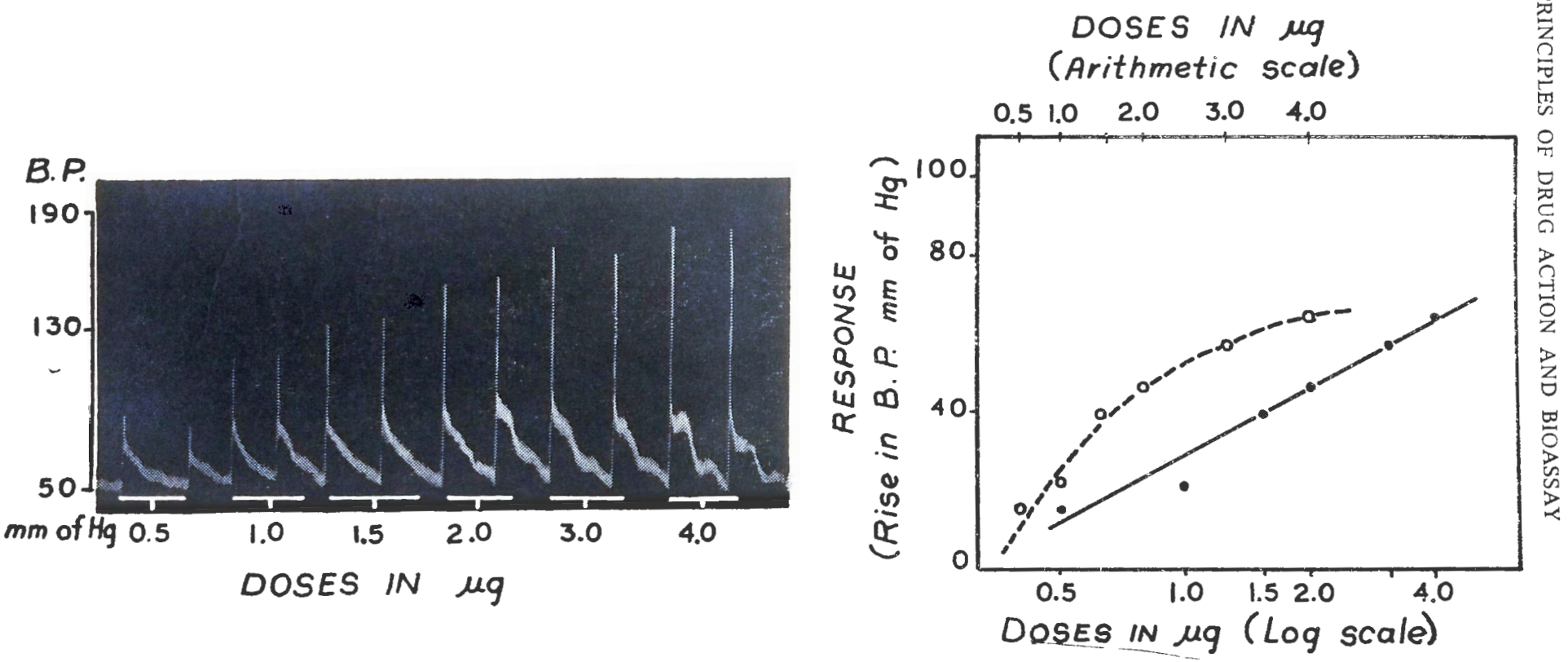


Fig. 1 – 8

Exercise 3.

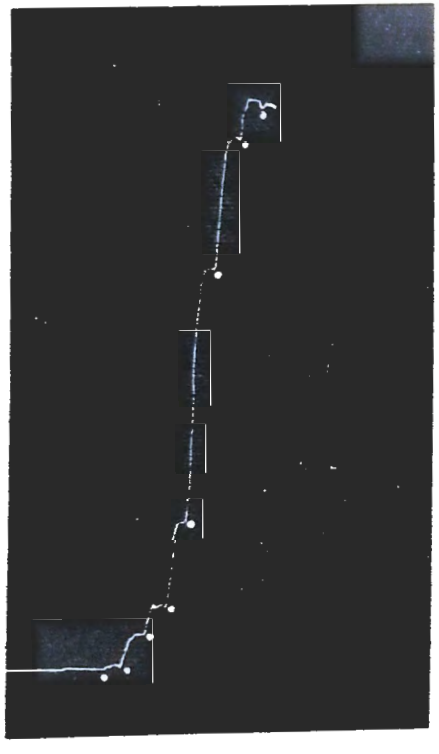
Objective : To demonstrate supramaximal effect by cumulative dose response study.

1. Set up isolated guinea pig ileum as described.
2. Prepare graded strengths of histamine acid phosphate solution.
3. Start the drum.
4. Add the lowest possible dose; start stop-watch.
5. After 30 seconds stop the drum.
6. Do not wash out the fluid but add another dose such that the total drug present is doubled; start the drum.
7. After 30 seconds stop the drum, add the next dose of the drug so as to double the amount present and start the drum. Continue in this fashion, till supramaximal effect is seen.
8. Fix the graph.
9. Measure the heights of the response at each addition from the baseline.
10. Calculate the percentage of the maximal contraction.
11. Plot a graph of % maximal contraction as response on Y axis, and dose on X axis.

CUMULATIVE DOSE RESPONSE CURVE

(HISTAMINE)

Isolated guinea pig ileum



Doses as shown in graph
BATH CAPACITY- 20 ml

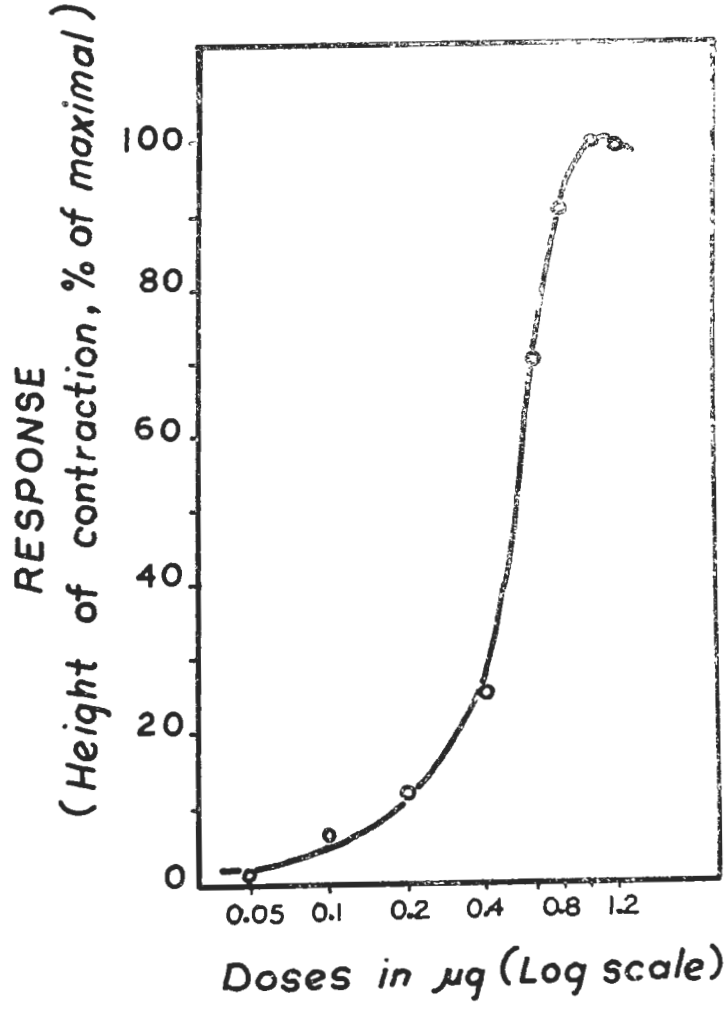


Fig. 1 – 9

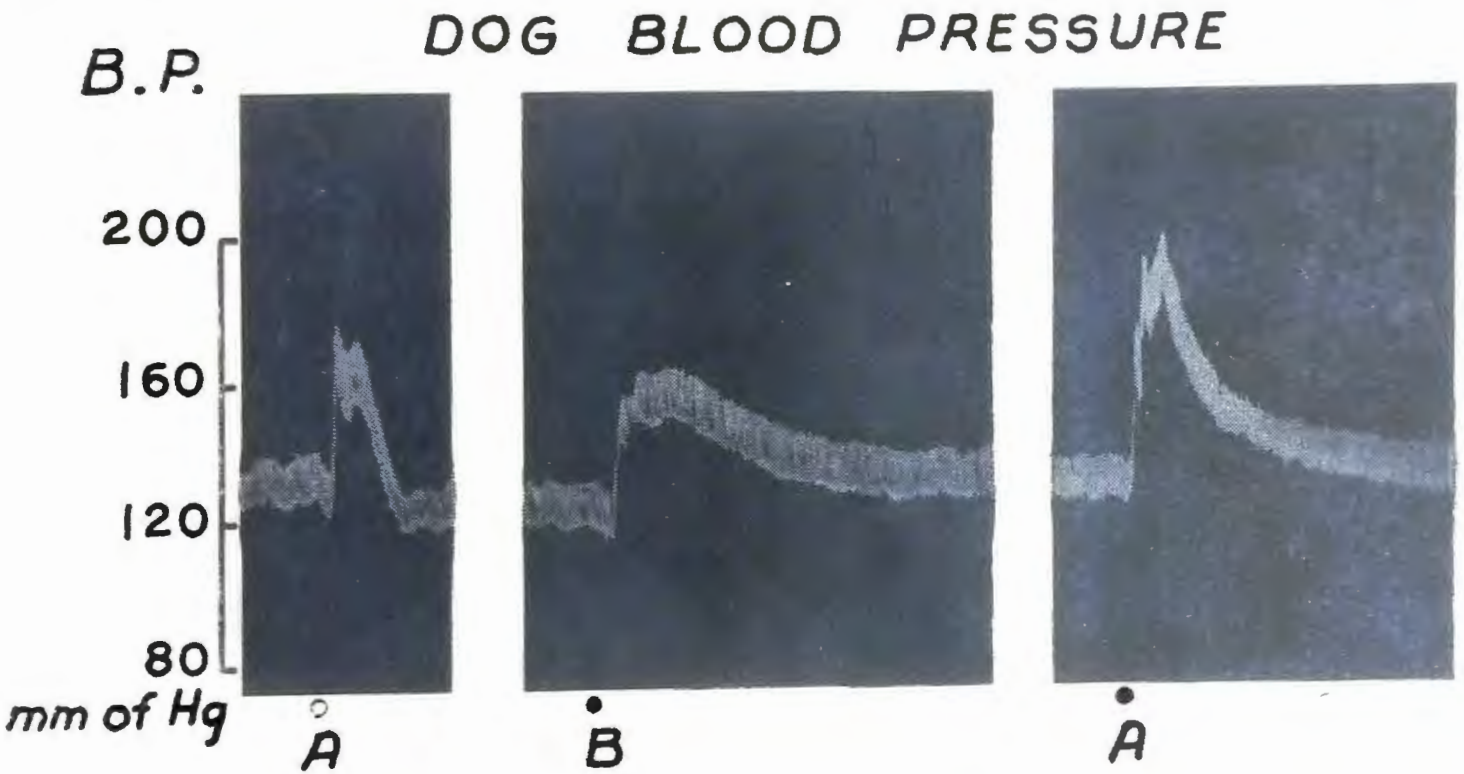
Exercise 4.

Objective : To demonstrate the potentiation of epinephrine pressor response by ephedrine.

1. Set up the carotid B. P. tracing in a dog as described. Start the drum.
2. Record an initial strip of tracing.
3. Inject epinephrine 2 µg/kg intravenously.
4. Record 2 such responses.
5. Inject ephedrine 0.2 mg/kg i.v.
6. When the pressor response ot ephedrine itself has returned to base-line inject epinephrine in the same dose.
7. Observe that a large response is now obtained.
8. Stop the drum.

DRUG INTERACTION

effect of ephedrine on epinephrine pressor response.



A: Epinephrine (2.0 μg/kg)

B: Ephedrine (0.2 mg/kg)

Fig. 1 - 10

Exercise 5.

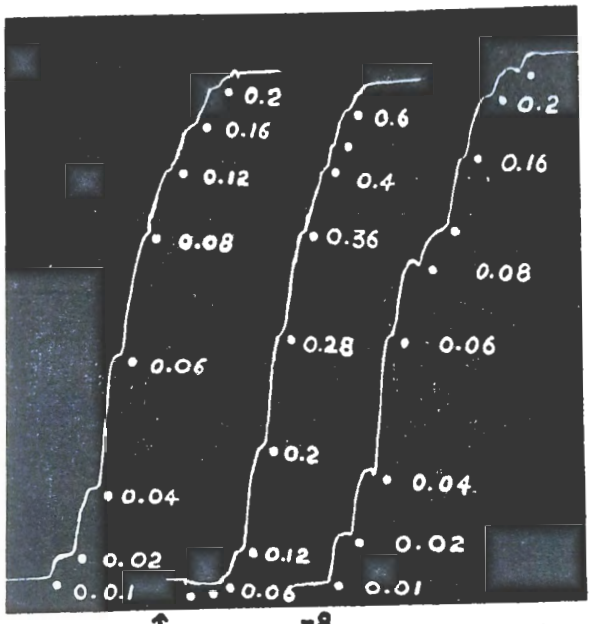
Objective : To study the properties of a competitive antagonist.

1. Set up the isolated rabbit aortic strip as described.
2. Prepare graded strenghts of norepinephrine and of an antagonist, like chlorpromazine; (we have used R-1361, a synthetic hypotensive agent).
3. Record a cumulative dose response curve with norepinephrine.
4. Wash out the bath fluid.
5. Add the antagonist in a small dose and take the response of the strip 5 minutes later to the lowest dose previously added.
6. Judging from this response either proceed to record a cumulative dose response graph, or if antagonist is inadequate, wash out and add a higher dose.
7. Proceed till the supramaximal effect is obtained.
8. Add the same doses as were added in the control tracing.
9. Wash out, and record a tracing showing recovery.
10. Proceed in this way for various strengths of antagonist.
11. Plot a graph of responses against log dose in presence of various strengths of antagonist
12. Observe that competitive antagonists cause a parallel shift of curves and have no effect on their maxima

COMPETITIVE ANTAGONISM

CUMULATIVE DOSE RESPONSE

Rabbit aortic strip



↑ 8.0×10^{-8} M OF R-1361

Numerals indicate doses of nor epinephrine (in µg)

BATH CAPACITY - 20 ml

Fig. 1-11-a

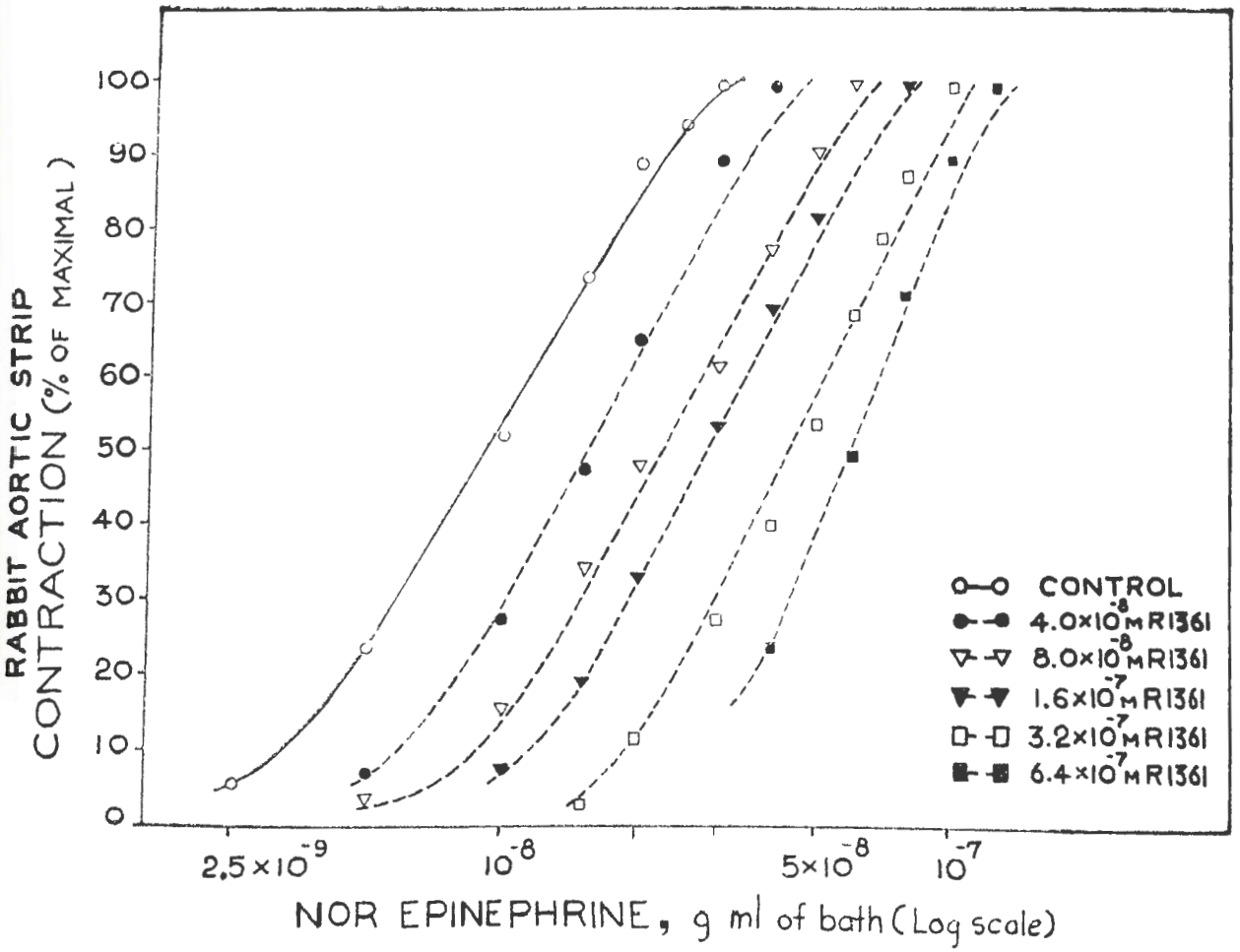


Fig. 1 - 11-b

Exercise 6.

Objective : To study the properties of a non competitive antagonist.

1. Set up the isolated guinea pig ileum as described.
2. Record cumulative dose response curves with histamine in the absence and presence of antagonist e.g. chlorpromazine in graded doses.
3. Between 2 doses of antagonist, record cumulative dose response curves with histamine alone and show recovery.
4. Plot log dose response graphs.
5. Observe that noncompetitive antagonists shift the curves in an unparallel manner; the maxima also progressively decline.

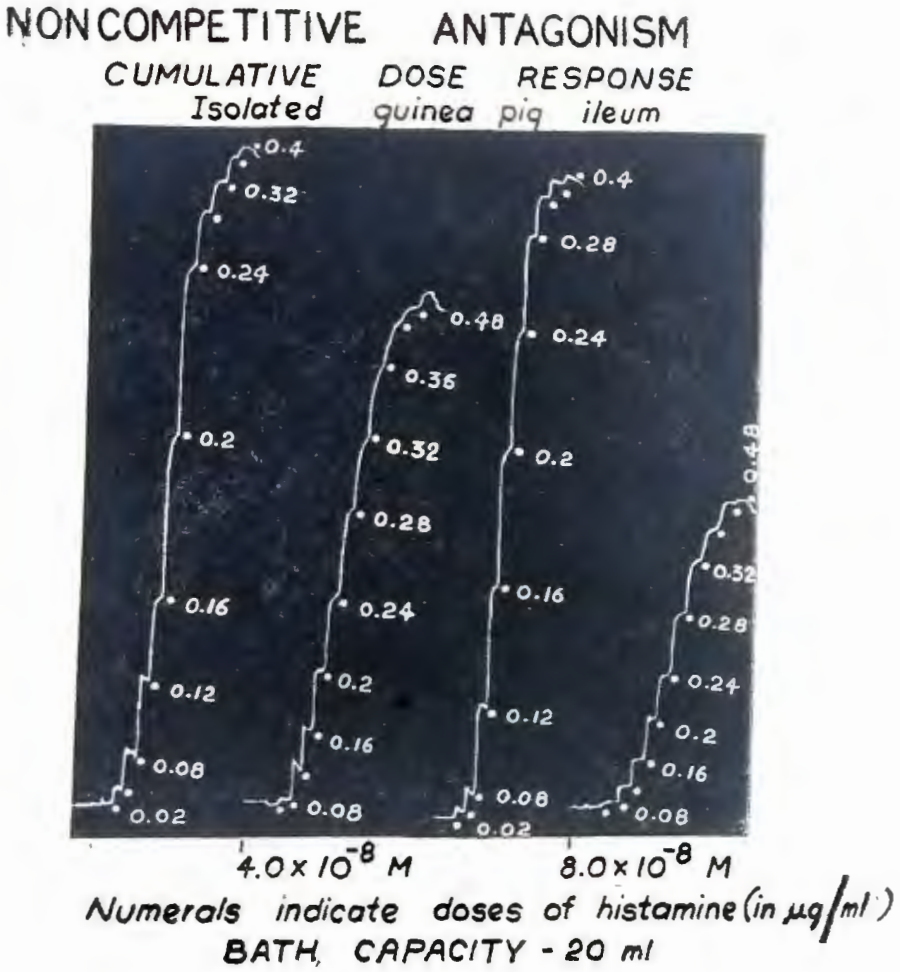


Fig. 1 – 12-a

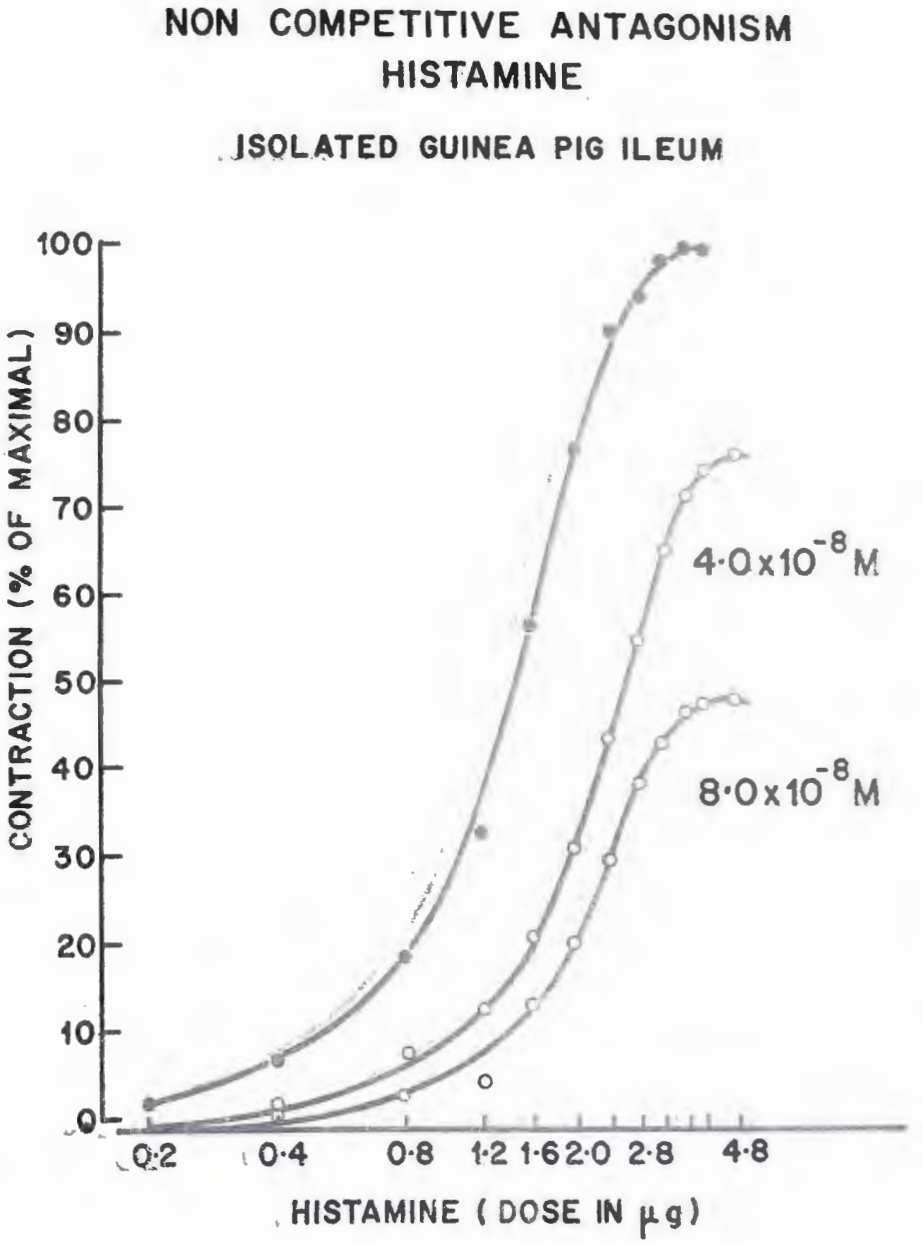


Fig. 1 — 12-b

Exercise 7.

Objective : To demonstrate specificity of antagonism.

1. Set up a recording of carotid blood pressure in dog.
2. Administer acetylcholine 3.0 µg/kg intravenously.
3. When B.P returns to normal, record the response to histamine 3.0 µg/kg.
4. Stop the drum.
5. Give chlorpheniramine maleate 5.0 mg/kg diluted in 10 ml saline slowly.
6. After 20 minutes record the responses to both acetylcholine and histamine in same dose as before.
7. Observe that the response to acetylcholine is unchanged, but the response to histamine is now only 30% of original, i.e. it is partially blocked by chlorpheniramine.

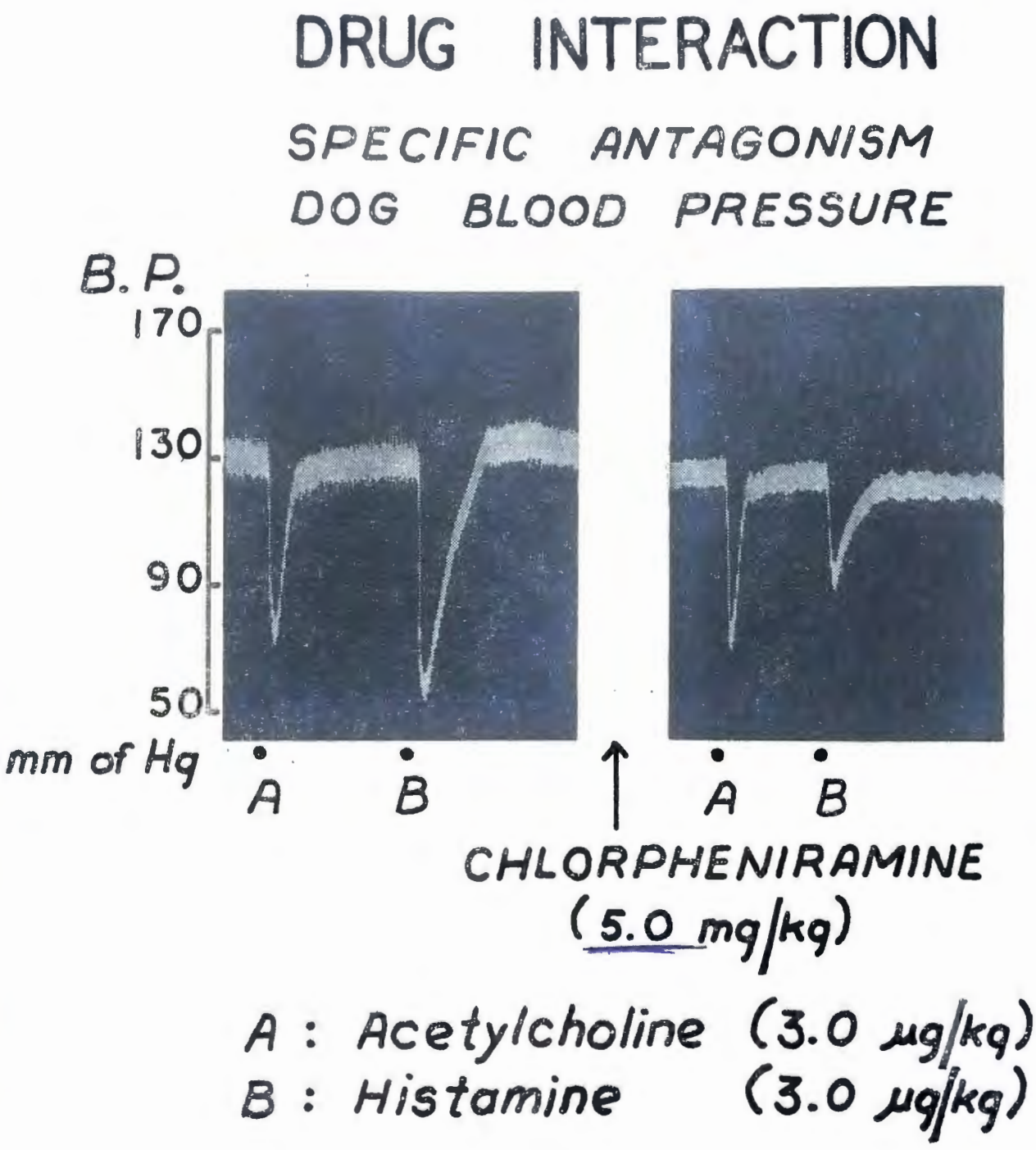

DRUG INTERACTION
SPECIFIC ANTAGONISM
DOG BLOOD PRESSURE
B. P.
170
130
90
50
mm of Hg
A
B
A
B
CHLORPHENIRAMINE
(5.0 mg/kg)
A : Acetylcholine (3.0 µg/kg)
B : Histamine (3.0 µg/kg)

Fig. 1 – 13

Exercise 8.

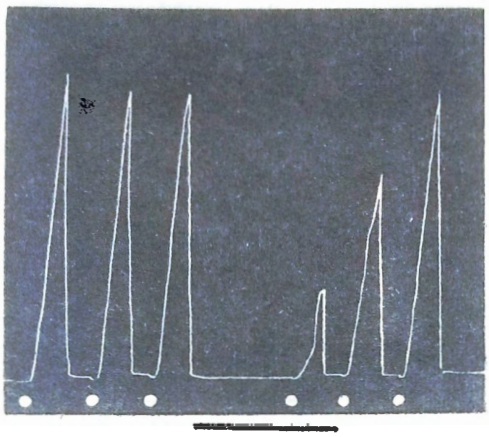
Objective : To demonstrate the non-specific antagonism between carbachol and papaverine, and carbachol and epinephrine.

1. Set up the isolated rat colon as described.
2. Record some control responses with carbachol (3.0 μg in this case) the period of contraction being 60 seconds and recovery time 5 to 10 minutes. Adjust the dose so as to obtain a response 6 cm in height.
3. Add papaverine 20 mg to the bath fluid and allow it to act for 2 minutes.
4. In the presence of papaverine record a response to carbachol in the same dose.
5. Wash out papaverine.
6. Record responses to carbachol and observe recovery of original height of response.
7. Repeat, using epinephrine instead of papaverine.

DRUG INTERACTION

PHYSIOLOGICAL ANTAGONISM

Isolated rat colon



At dots 3.0 µg carbachol were allowed to act for 60 sec.

Bar indicates presence of epinephrine 0.05 µg/ml for 3 min

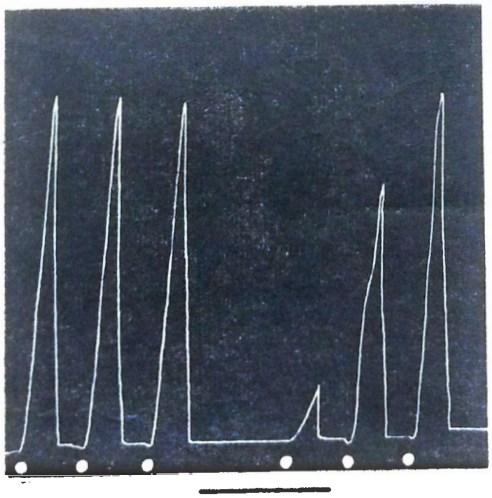
BATH CAPACITY : 20 ml

Fig. 1 – 14-a

DRUG INTERACTION

NON-SPECIFIC ANTAGONISM

Isolated rat colon



At dots 3.0 µg carbachol were allowed to act for 60 sec.

Bar indicates presence of papaverine 1.0 µg/ml for 3 min.

BATH CAPACITY : 20 ml

Fig. 1 – 14-b

Exercise 9.

Objective : To demonstrate the phenomenon of tachyphylaxis.

1. Set up a recording of carotid blood pressure in dog.
2. Inject amphetamine 500 μg/kg, or ephedrine 500 μg/kg, or tyramine 300 μg/kg.
3. Repeat the drug as soon as B.P. returns to normal.
4. Observe, after a few initial responses of equal height, the pressor response gradually declines, indicating tachyphylaxis.

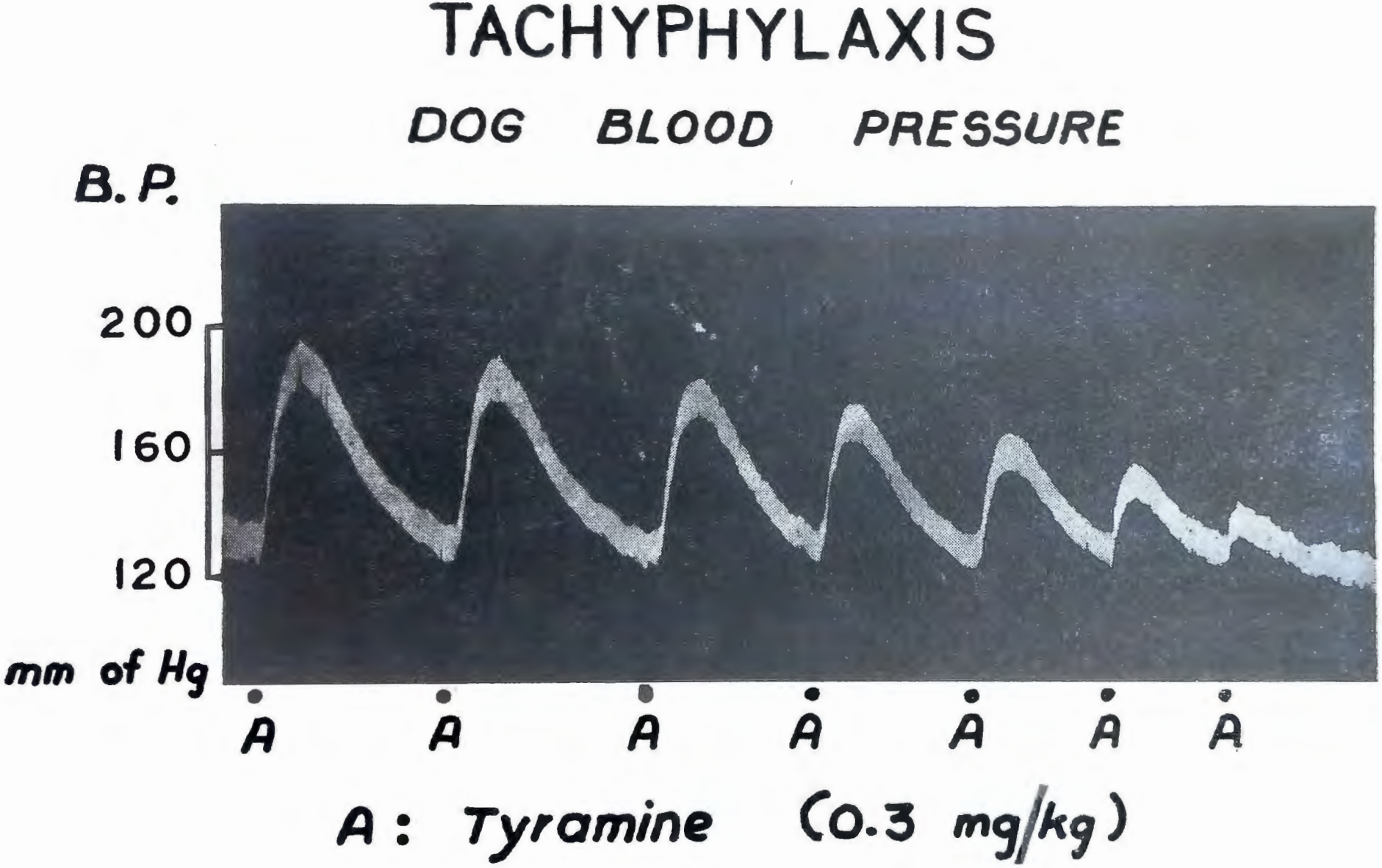

TACHYPHYLAXIS
DOG BLOOD PRESSURE
B.P.
200
160
120
mm of Hg
A
A
A
A
A
A
A
A : Tyramine (0.3 mg/kg)

Fig. 1 – 15

Exercise 10.

Objective : To demonstrate the phenomenon of receptor protection.

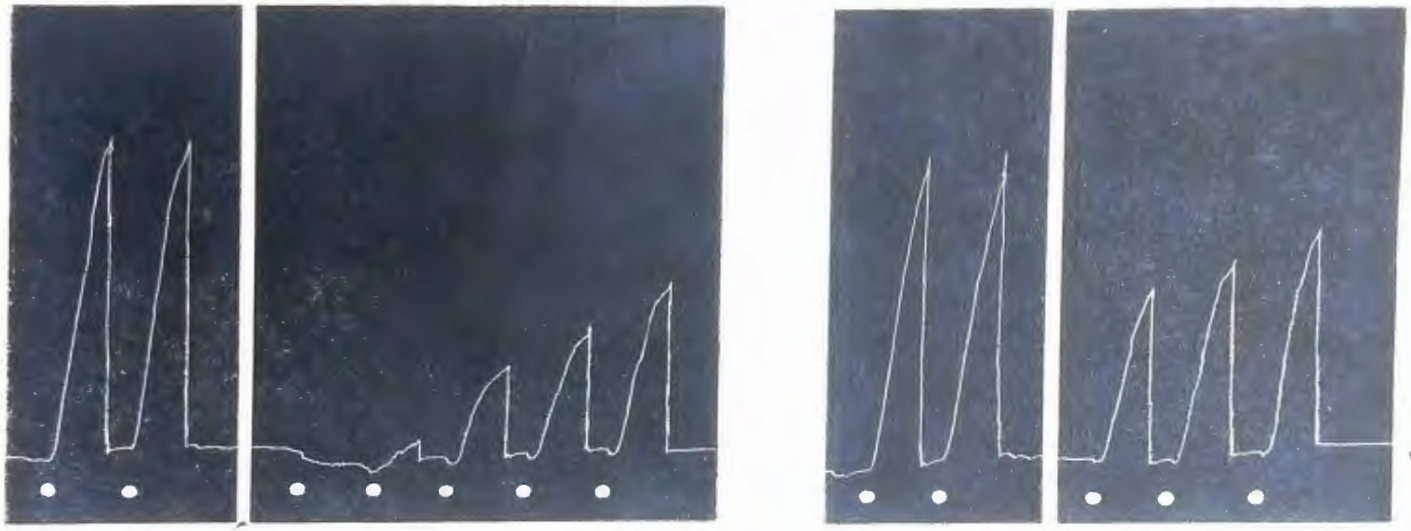
1. Set up a pair of isolated rabbit aortic strips from the same animal in 2 organ baths under identical experimental conditions (identical bath, lever, temperature, load, etc.).
2. In the perfusing fluid of one strip add norepinephrine so as to give a concentration of 70 μg/ml throughout the experiment.
3. Allow both strips to stabilise.
4. Elicit responses to norepinephrine with both strips.
5. Add an antagonist and observe that the inhibition of norepinephrine induced contraction is less in the strip bathed with NE i.e. this strip is 'protected'.

RECEPTOR PROTECTION

A paired rabbit aortic strip preparation from the same aorta

UNPROTECTED STRIP

PROTECTED STRIP



4×10^{-8} M R-1361

70 µg/ml Norepinephrine + 4.0×10^{-8} M R-1361

At dots norepinephrine was added in a dose of 0.02 µg/ml

R-1361: SYNTHETIC ADRENERGIC BLOCKING AGENT

Fig. 1 – 16

Exercise 11

Objective : To assay a histamine solution by bracketing.

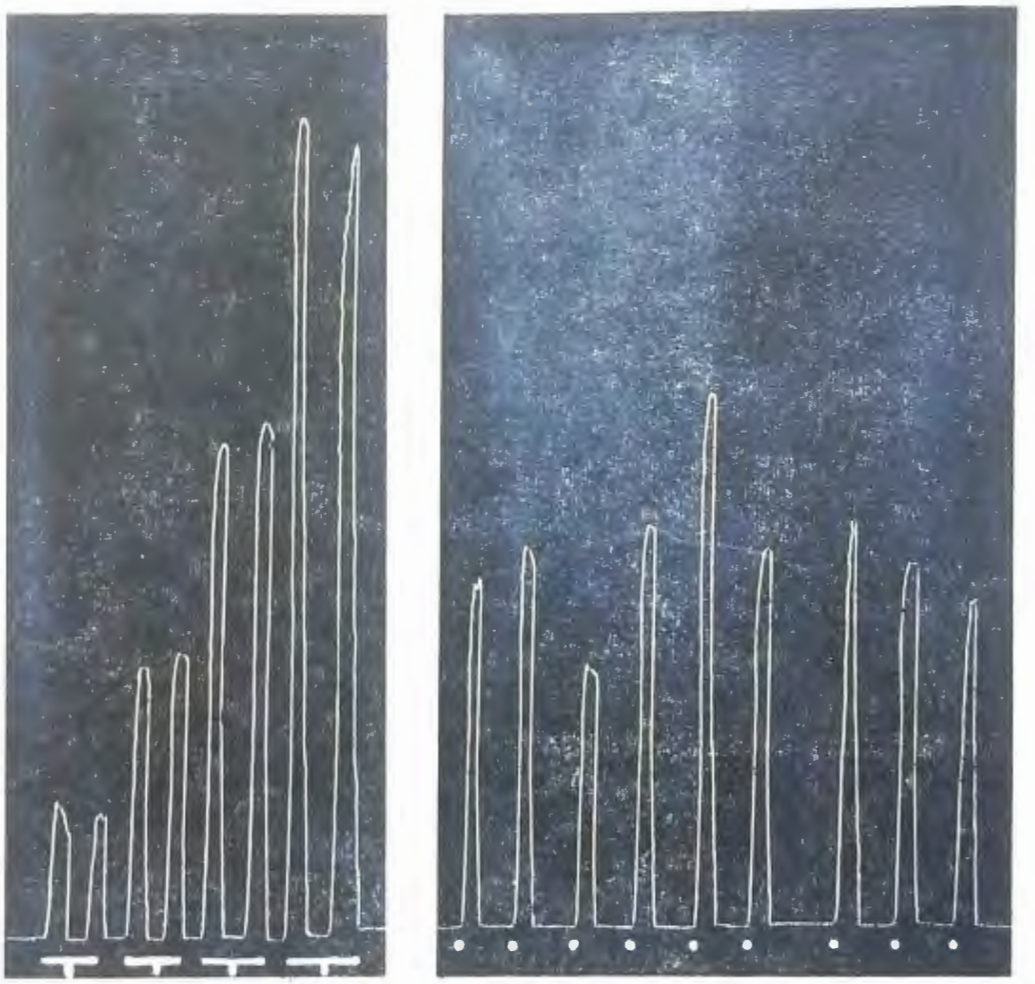
1. Set up the isolated guinea pig ileum as described.
2. Atropinise the preparation (0.5 — 1 µg/ml) so as to inhibit the spontaneity as well as render the preparation insensitive to acetylcholine as an impurity.
3. Prepare graded strengths of a known solution of histamine, and varying dilutions 1 : 10, 1 : 100, 1 : 1000 of the unknown.
4. Take a series of dose related responses with different doses of known and different volumes of appropriate dilution of unknown.
5. Choose a response 'T' to unknown that lies very close to a response to a known amount.
6. Record responses to this 'T' and known solutions, each time repeating 'T' after a known.
7. Narrow down the dosage of known, increasing and decreasing the smaller and larger dose respectively till an exact match is obtained.
8. Calculate the strength of unknown; If T (O.Y ml unknown) = O.X ml of known i.e. O.X µg, then 1 ml unknown = ? This amount is present in a dilution of the unknown. Therefore the concentration of unknown can be calculated.

BIOASSAY

(HISTAMINE)

Isolated guinea pig ileum

DOSE RESPONSE BRACKETING ASSAY



Doses in µg

BATH CAPACITY - 20 ml

Fig. 1–17

Exercise 12.

Objective : **To assay an unknown solution of histamine by a 3 point assay (2+1 assay).**

1. Set up the isolated guinea pig ileum as described: atropinise it.
2. Prepare graded strengths of a known solution, and dilute unknown 1 : 10, 1 : 100, 1 : 1000.
3. Record a series of dose related responses with different doses of standards and different volumes of unknown in appropriate dilutions.
4. Choose a response 'T' of unknown, that lies between a pair of responses S1, S2, of standard (where S1, S2 are elicited with doses 1 : 2).
5. Elicit responses to T, S1, S2 in a randomised fashion.
6. Fix the graph and determine mean heights of S1, S2, T.
7. Plot a graph of response against log dose. S1 and S2 are therefore separated along X axis by log 2 (s1 s2 = 2) i.e. 0.301.
8. Mark off T on the line joining S1 and S2 as shown in the figure.
9. From the perpendicular from T calculate the ratio of intercept to base of right-angled triangle: this gives the potency ratio between T and S1.
10. Hence the strength of the unknown solution can be calculated.
11. Also calculate the strength from the formula : Strength of unknown solution :

$$\left[\frac{s1}{t} \times \text{antilog} = \frac{T-S1}{S2-S1} \times \log \frac{(s2)}{(s1)}\right]$$

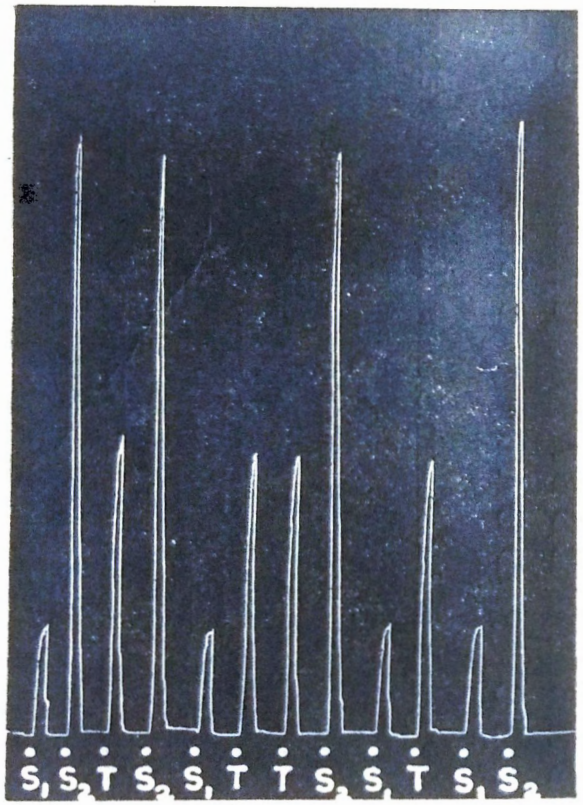
where s1, s2, t are amounts of solutions added.
S1, S2, T are responses recorded with s1, s2, t

BIOASSAY

(HISTAMINE)

Isolated quinea piq ileum

2+1 ASSAY



S_1 = 0.2 µg of Standard

S_2 = 0.4 µg of Standard

T = 0.4 ml of Test

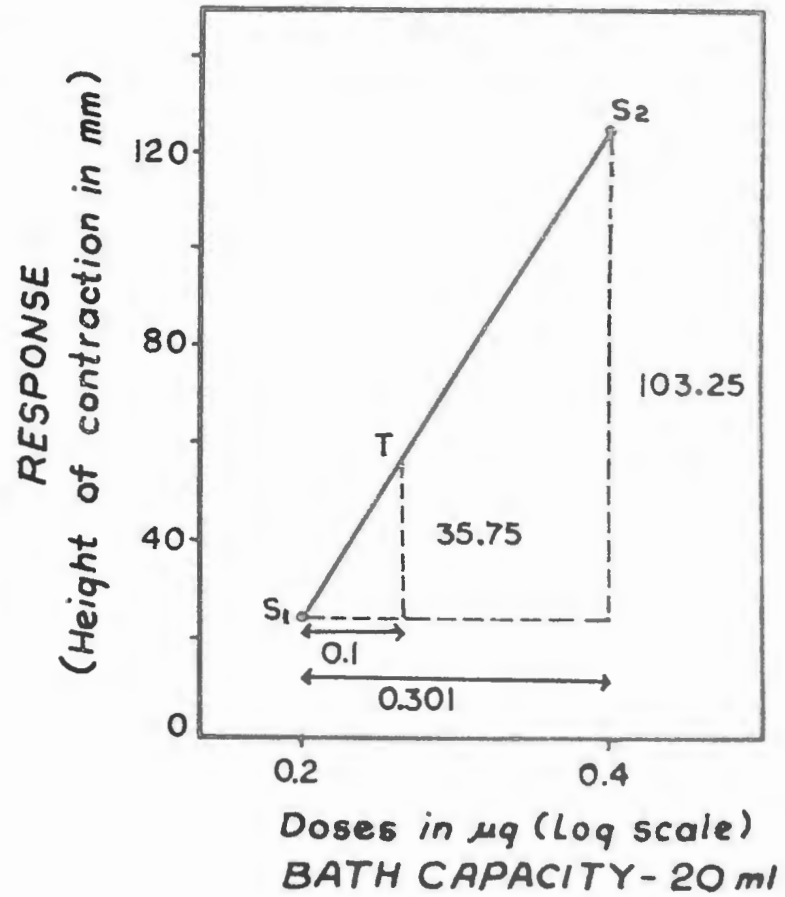


Fig. 1 – 18

Exercise 13.

Objective : To determine the choice of doses in a 4 point assay (2 + 2 assay).

1. Set up the isolated frog's rectus abdominis as described
2. Prepare graded strengths of known solutions of acetylcholine and graded dilutions of unknown.
3. Record a series of dose related responses with known and unknown. (The time period of contact = 90 seconds, of recovery, 5 minutes).
4. Select pairs of responses from each series that bear to themselves the same ratio i.e. $\frac{S_1}{S_2} = \frac{U_1}{U_2} = X$. and lie on the straight part of a log dose response curve.
5. These responses should not be identical. Preferably S1 > U1 > S2 > U2.
6. Record responses to these 4 doses using Latin squares to obtain good randomisation, getting 16 responses in all.
   Let S1, S2 be A, B and U1, U2 be C, D.

A B C D	A B C D	A B C D	A B C D
B C D A	B A D C	B D A C	B A D C
C D A B	C D B A	C A D B	C D A B
D A B C	D C A B	D C B A	D C B A

7. Fix the graph and determine mean heights of responses A, B and C. D. Also determine heights of dose related responses initially recorded. Plot dose response curves and confirm that the dose s1, u1, s2, u2 chosen with your judgement do lie on the straight segments. (Since doses are not known volumes of unknown solution added are plotted).

   Calculate strength of unknown from the formula.

$$\frac{(U1 - S1) + (U2 - S2)}{(S2 - S1) + (U2 - U1)} \times \log s_2 / s_1 = \text{Potency ratio}$$

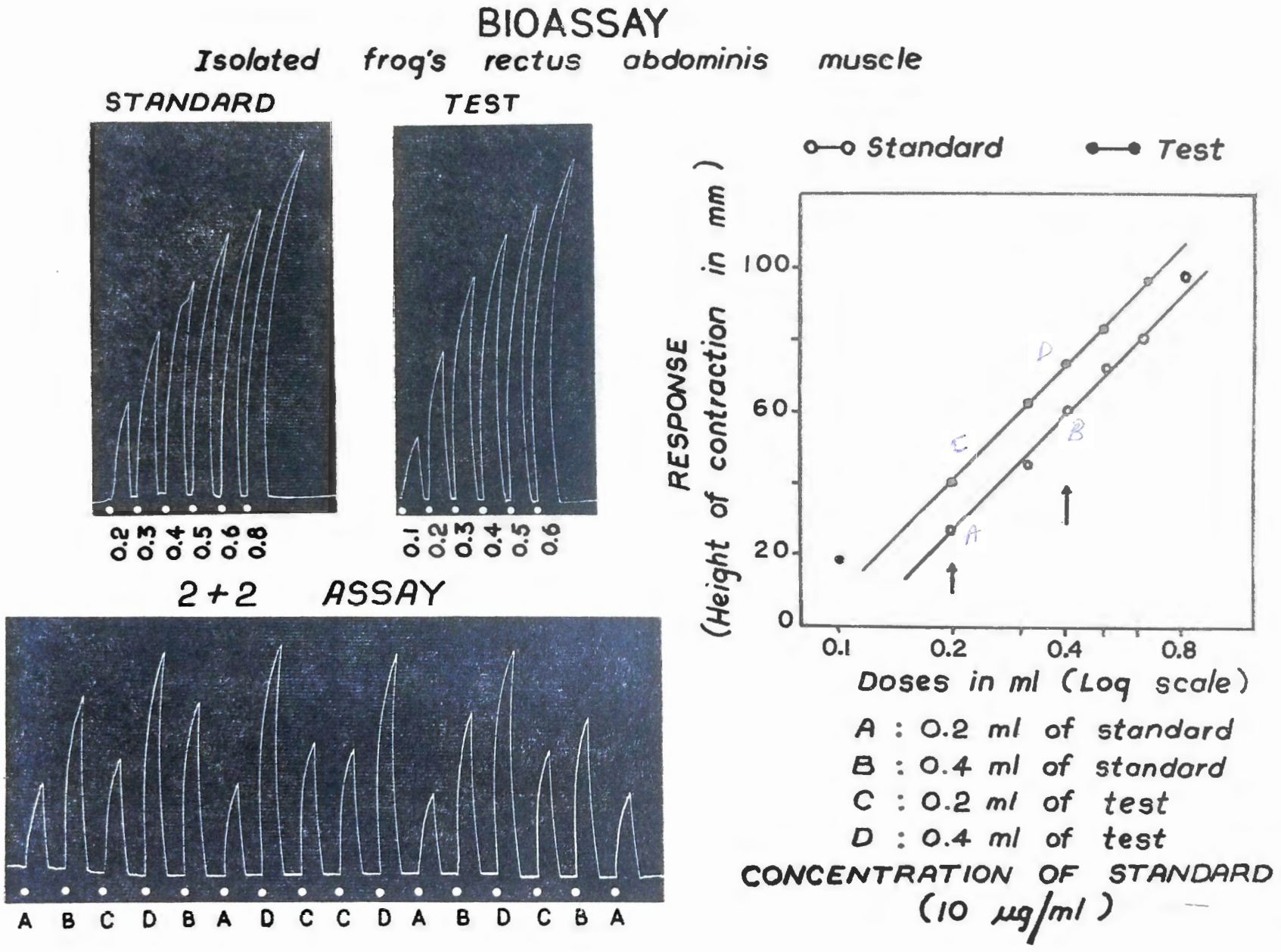

BIOASSAY
Isolated frog's rectus abdominis muscle
STANDARD
TEST
0.2 0.3 0.4 0.5 0.6 0.8
0.1 0.2 0.3 0.4 0.5 0.6
2+2 ASSAY
A B C D B A D C C D A B D C B A
Standard
Test
RESPONSE (Height of contraction in mm)
100
60
20
0
0.1
0.2
0.4
0.8
Doses in ml (Log scale)
A : 0.2 ml of standard
B : 0.4 ml of standard
C : 0.2 ml of test
D : 0.4 ml of test
CONCENTRATION OF STANDARD (10 µg/ml)

Fig. 1 — 19

Exercise 14

Objective : To perform a 4 point assay of histamine.

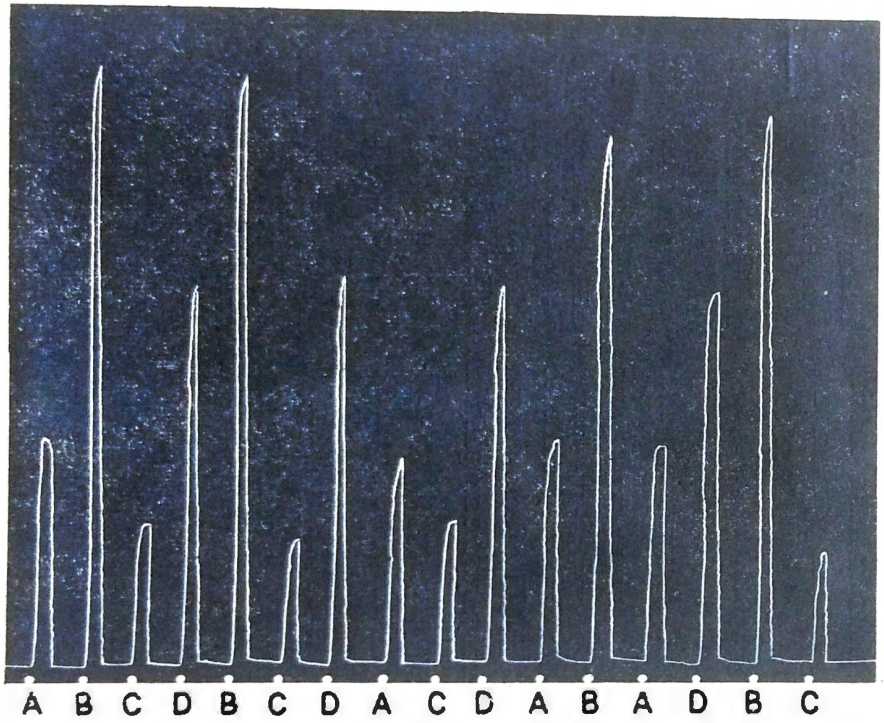
1. Set up an isolated guinea pig ileum preparation.
2. Prepare graded solutions of known, and varying dilutions of unknown.
3. Record dose response curves with both standard and test solutions.
4. Choose 4 doses, 2 of standard and 2 of test as directed in exercise 13.
5. Record 4 responses with each dose in a randomised fashion.
6. Fix the graph and measure mean heights of response to each dose.
7. Plot a graph of response against log dose since s1 : s2 = u1 : u2 = X (generally) the absolute dose need not be known. Plot the points at a distance of log X on the abscissa.
8. Note that the 2 lines need not always be parallel; draw a line passing exactly midway between them.
9. Join the mid points of S1, S2 and T1, T2; from the point of intersection of the 2 new lines thus obtained, draw a horizontal cutting S1, S2 and T1, T2. From these 2 points drop perpendiculars onto the abscissa.
10. The intercept 'D' thus obatined is the log potency ratio.
11. Hence potency ratio = antilog D.
12. Calculate strength of the unknown from the graph as well as the formula given in exercise 13.

BIOASSAY

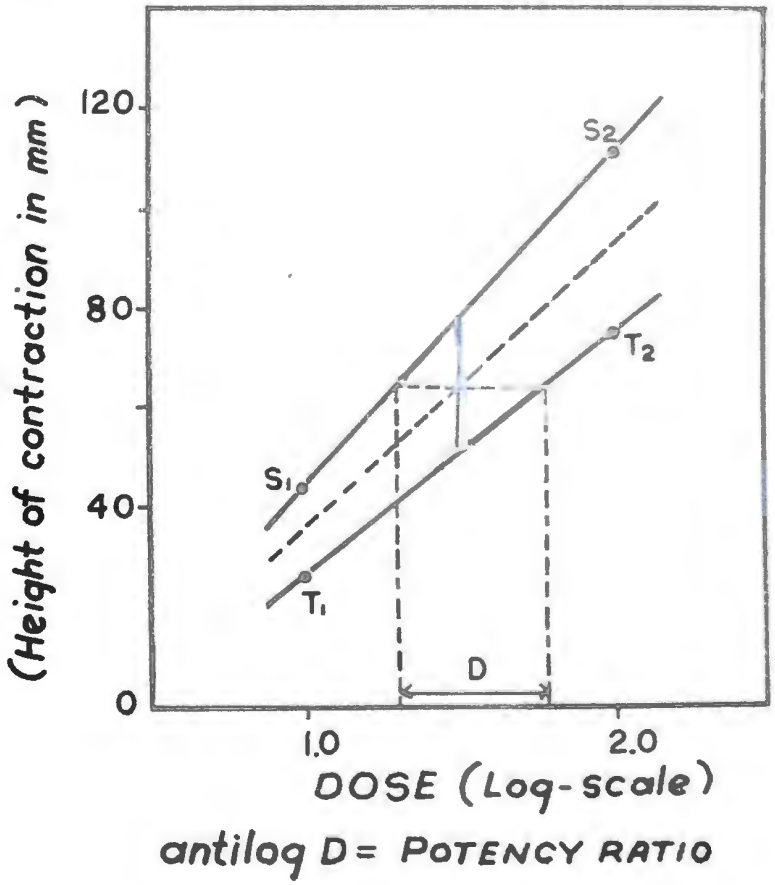
(HISTAMINE)

Isolated quinea pig ileum

2+2 ASSAY



A (S_1) = 0.2 ml of standard, B (S_2) = 0.4 ml of standard, C (T_1) = 0.3 ml of test, D (T_2) = 0.6 ml of test



antiloq D = POTENCY RATIO

Fig. 1 – 20

CHAPTER 2

Autonomic Pharmacology

'Begin at the beginning', the king said, gravely, 'and go on till you come to the end then stop!

ALICE IN WONDERLAND

Introduction

Autonomic pharmacology concerns the actions of drugs on the autonomic nervous system. This system is comprised of the sympathetic and the parasympathetic divisions. These divisions in turn consist of specific nerve fibres arising from the central nervous system, ganglia where they synapse, and post ganglionic fibres supplying various effector organs. The ganglia may be situated in accessible sites as an orderly chain remote from the organs supplied e.g. the sympathetic ganglia, or may be situated very close to or in the substance of the effector organ itself for e.g. the parasympathetic ganglia.

The actions of the two opposing divisions of the autonomic nervous system are mediated by the liberation (by nerve impulses or by drugs) of specific neurohumoral transmitter substances, being different at different sites. Acetylcholine acts as the transmitter at all ganglia, at post ganglionic parasympathetic nerve endings, at the neuromuscular junction, (Refer Chapter 3) and some rare instances of post ganglionic sympathetic nerve endings. The transmitter implicated in the sympathetic system is noradrenaline.

The adrenal medulla may be considered as a specialized ganglion being stimulated by acetylcholine to pour out its secretion of adrenaline and noradrenaline. These neurohumoral substances in turn act on specific receptors the nature of which depends on the end organ. Acetylcholine action involves activation of nicotinic receptors in the ganglia and neuromuscular junctions, and of muscarinic receptors in the other effector organs.

Norepinephrine on the other hand acts by mainly activating α receptors in the arteriolar wall, splenic capsule, etc. The actions of epinephrine are more complex and involve the activation of one more set of receptors called the β receptors found mainly in the heart, bronchial smooth muscle, skeletal muscle vasculature etc. The β receptors are mainly involved in myocardial excitation, bronchodilatation and peripheral vasodilatation. The action of epinephrine on α receptors is greater than the action of norepinephrine. The catecholamine isoprenaline activates mainly β receptors with negligible actions on α receptors.

The actions of the neurohumoral substances may be mimicked or antagonized by a large number of drugs respectively called mimetics, and blockers or lytics. The latter term is not favoured today.

We shall proceed to study the effects of drugs on each system separately.

The Adrenergic System.

Adrenergic drugs including the sympathomimetic amines. The effect of adrenergic drugs in vivo is best studied by the demonstration of their actions on three different systems namely the splenic volume blood pressure and respiration (Fig. 2-3)

Recording splenic volume in a closed, constant-pressure system would give us an idea of the contraction of the splenic capsule, an increase in volume denoting a contraction of the capsule (α effect) recorded as a downward deflection of the pointer of the Marey's tambour. Drugs with a greater α effect would produce a bigger splenic contraction (E > NE, Fig. 2-3).

Blood pressure recording gives us information about the α and β receptors of the cardiovascular system, the disadvantage being that in vivo the actions tend to be offset by the homeostatic mechanisms of the body like the baroreceptor reflexes.

A recording of respiration offers us information on the effect of drugs on both rate and amplitude of respiration. The effect on respiration is complex, being partly central and partly reflex from the effect on blood pressure. Pressor effects would be accompanied by bradypnoea (slowing) and depressor effects by tachypnoea (rapidity of respiration) as is demonstrated in Fig. 2-3 by the actions of norepinephrine and isoprenaline. Occasionally the classically described effect of epinephrine namely 'epinephrine apnoea' is produced which can also be seen in Fig. 2-3.

Recordings of blood pressure alone can give sufficient information as to the class of compound by judicious combination with agonist or antagonist. Drugs that act by release of norepinephrine from the nerve ending would cause an acute depletion, a form of 'chemical sympathectomy' and would potentiate the actions of exogenously administered epinephrine and norepinephrine. The effect of the drugs themselves on blood pressure would be a reflection of the relative rapidity of depletion: Fig. 2-5 shows the action of guanethidine a useful antihypertensive drug which acts partly by depleting norepinephrine from adrenergic nerve endings and reducing overall sympathetic tone. In doing this it produces a small pressor action of its own. Fig. 2-6 shows the action of tyramine an indirectly acting sympathomimetic amine which acts principally by release of norepinephrine.

Other drugs that can potentiate the action of NE would be those that can block cell membrane uptake of norepinephrine for e.g. cocaine (Fig. 2-4). Such a drug would not be expected to alter blood pressure as it does not release NE from intragranular stores, but prevents the uptake of exogenous and endogenously liberated NE, and thereby potentiates the action.

Turning our attention to agents that can block the action of epinephrine and norepinephrine, these would fall into two categories, the α blockers and the β blockers. A consequence of selective blockade of only one type of receptor in a system where both are present, would be the relative dominance of the unblocked receptor and this is the basis of what is termed as Dale's vasomotor-reversal phenomenon. This involves blockade of α receptors to unmask the β component of the action of epinephrine thus converting the pressor response to a depressor response (Fig. 2-7). The actions of norepinephrine would therefore be blocked but not reversed while that of isoprenaline would be unaffected. Isoprenaline can be selectively blocked by β blocking agents (Fig. 2-8). If in the presence of α blockade, a β blocking agent were administered, the α action of epinephrine would reappear and this is called

re-reversal (Fig. 2-9).

Another way of blocking sympathetic transmission would be to prevent the release of, or cause release and depletion of transmitter substance from adrenergic nerve endings. e.g. bretylium and guanethidine resply. This would best be demonstrated by failure of electrical stimulation of a sympathetic nerve to produce the usual response, for example, inhibition of normal pendular movements of jejunum will be inhibited by sympathetic stimulation and this effect will be blocked by adrenergic neurone blockers, (Fig. 2-10) The effect of exogenously added epinephrine will not be blocked serving to distinguish it from adrenergic blockers acting at receptor level.

The effects of adrenergic agonists and antagonists on the heart will be considered in Chapter 4 (Cardiovascular Pharmacology).

The actions of the sympathetic system are opposed by those of the **parasympathetic system.** The action of acetylcholine, the neurotransmitter of the latter system, is limited in duration because of its rapid destruction in the body by hydrolysis by cholinesterase enzymes (ChE). Acetylcholine causes a sharp transient fall in blood pressure. As a corollary to this, we would expect inhibition of the ChE enzyme to potentiate and prolong the action of acetylcholine (Figs. 2-11 and 2-12). The action of other cholinergic substances which are not substrates of ChE will not be prolonged or potentiated (Fig. 2-11). The ChE enzyme is present in blood (Fig. 2-11) and in tissues (Fig. 2-12). The presence in blood may be demonstrated by allowing fresh serum to act on acetylcholine before adding the latter to an isolated preparation, when hydrolytic destruction will lead to a diminution in response. Protection of this acetylcholine may be effected by adding an anti-ChE to the serum prior to its mixing with acetylcholine and if some of this anti-ChE acts on the enzyme in the frog rectus muscle itself, there may not only be failure of inhibition but actual potentiation of the response to acetylcholine (Fig. 2-13).

At the outset we had mentioned that acetylcholine acts on two types of receptors, nicotinic and muscarinic. The receptors in the vasculature are classified as muscarinic, and they mediate the depressor response. They must be blocked by atropine before the nicotinic action can be demonstrated (Fig. 2-14). It may also be noted that while atropine blocks the muscarinic actions of all cholinergic drugs administered, increase in the dose of pilocarpine or carbachol only results in an overcoming of this block, while acetylcholine alone produces a vasopressor action which is a nicotinic action comprised of two facets, ganglionic stimulation and liberation of adrenaline from the adrenal medulla. This action of acetylcholine may be directly compared to that of nicotine (Fig. 2-15).

The ganglion stimulant action of acetylcholine is mimicked by drugs like nicotine, DMPP (Fig. 2-16) and can be blocked by an excess of these drugs, or by a variety of other drugs including pentolinium (Fig. 2.15). These ganglion blocking agents do not block, and may enhance the pressor response to norepinephrine. An interesting application of ganglion blocking agents is to locate ganglia on the paths of autonomic nerves (Fig. 2-17). The effects of vagal stimulation and injected acetylcholine on blood pressure and heart rate (atrial beats) is studied before and after a short acting ganglion blocking agent. It may be seen that vagal stimulation is blocked while acetylcholine is unaffected thereby indicating the presence of ganglia between the vagus and SA node, probably in the myocardial substance.

Experimental Techniques

1. **Spleen volume, blood pressure and respiration of dog.** The carotid blood pressure recording is set up as described in

Chapter 1. The tracheal cannula is not connected to the artificial respirator as described but to a Marey's tambour.

The abdomen is opened by a midline incision in its upper half taking care to achieve good haemostasis. The spleen is lifted from its position under the left lobe of the diaphragm and carefully delivered into the incision: care is taken to handle the spleen with the fingers only as it is very friable and would tear or bleed if held with forceps. The splenic mesentery is cleared carefully and the spleen fitted into the lower half of a spleen oncometer lined with paraffin: care is taken to place the hilar blood vessels in the notch of the oncometer. The oncometer is then assembled and the edges of the two halves carefully sealed with paraffin. The entire instrument is placed in the abdominal cavity. The outlet tube is connected by means of rubber tubing to a Marey's tambour; the rubber diaphragm of this tambour is kept in a state of optimal tension by injecting a little air into the tubing. The pointers of the two tambours and the mercury manometer are all brought to lie along the same vertical line. A control strip of tracing is taken and the effect of drugs studied.

2. **Blood pressure and nictitating membrane preparation in cat.** Anaesthesia is induced with ether in a cat, and the femoral vein is rapidly cannulated. Maintenance anaesthesia is given with chloralose 80 mg/kg intravenously. This substance is soluble in warm propylene glycol, and is administered in this solvent 80 — 100 mg/ml, diluted with saline (Refer Appendix 1).

The lower lid of one eye of the cat is retracted downwards and sutured in this position to the skin over the mandible. The nictitating membrane is lifted up gently and a suture is taken through the cartilaginous portion, the thread passed round a light pulley (subtending an angle of 30° with the sagittal plane), and tied to an isotonic lever with gimbal, and a sideways writing point, previously balanced for a tension of 2 — 5 gm. Recordings are taken on a smoked drum.

3. **Open chest preparation of cat.** A cat is anaesthetised with ether and chloralose, and the level of anaesthesia allowed to stabilise for 45 minutes.

The cat is placed in the supine position and the fur over the sternum clipped. An incision is taken exposing the sternum and the sterno-costal junctions are divided with a bone cutter; bleeding from the bone is arrested with bonewax.

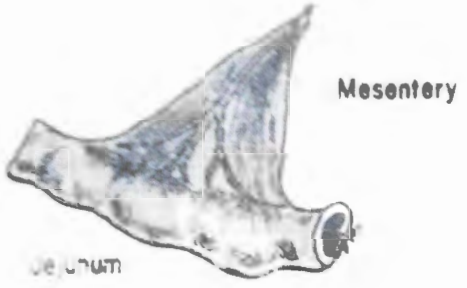
The cut edges of the chest wall are kept retracted and the heart in its pericardial sac exposed. The pericardium is gently picked up with a blunt rounded forceps, taking care not to touch the heart: it is slit open and the heart cleared of it as far as possible. The cut edges of the pericardium are kept retracted by taking stay sutures through it and through the retracted chest wall. A suture is taken through the muscular wall of the atrium on one side and the thread tied to a sensitive pressure transducer connected to the E & M Physiograph. Alternatively it may be attached to a Starling lever, recording on a kymograph.

The vagus is isolated in the neck as the thicker portion of the vago-sympathetic bundle adjacent to the carotid artery. The nerve is isolated and divided between ligatures, the peripheral cut end being stimulated with a square wave pulse generator.

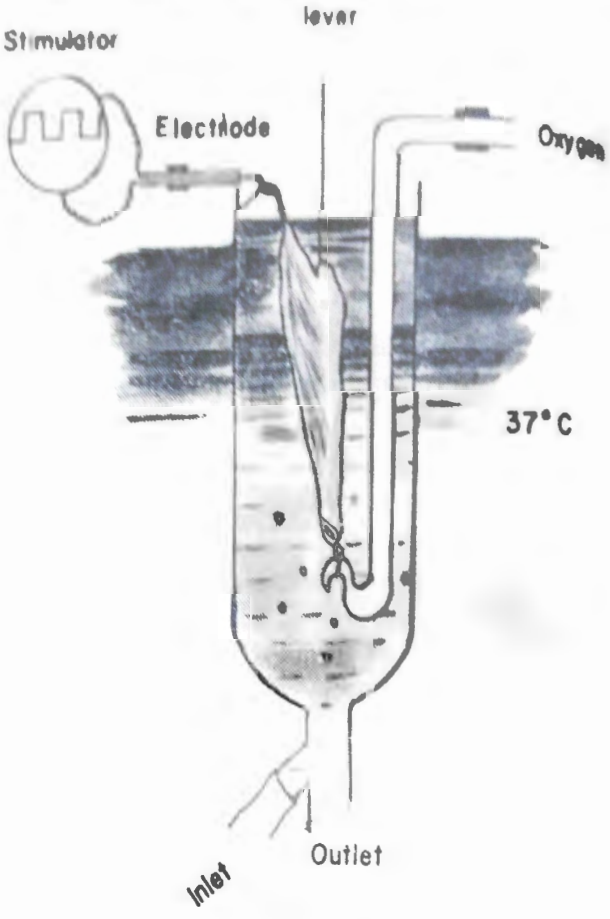
The carotid artery is cannulated and connected through heparinised saline to the blood pressure transducer of the E & M Physiograph, alternatively it is recorded by a mercury manometer on a smoked drum.

Responses to acetylcholine and vagal stimulation before and after pentolinium are recorded.

4. **Isolated innervated rabbit jejunum (Finkelman's preparation).** A rabbit is



2. 1-a
The selection of a segment of jejunum with an arteriole in the mesentery.



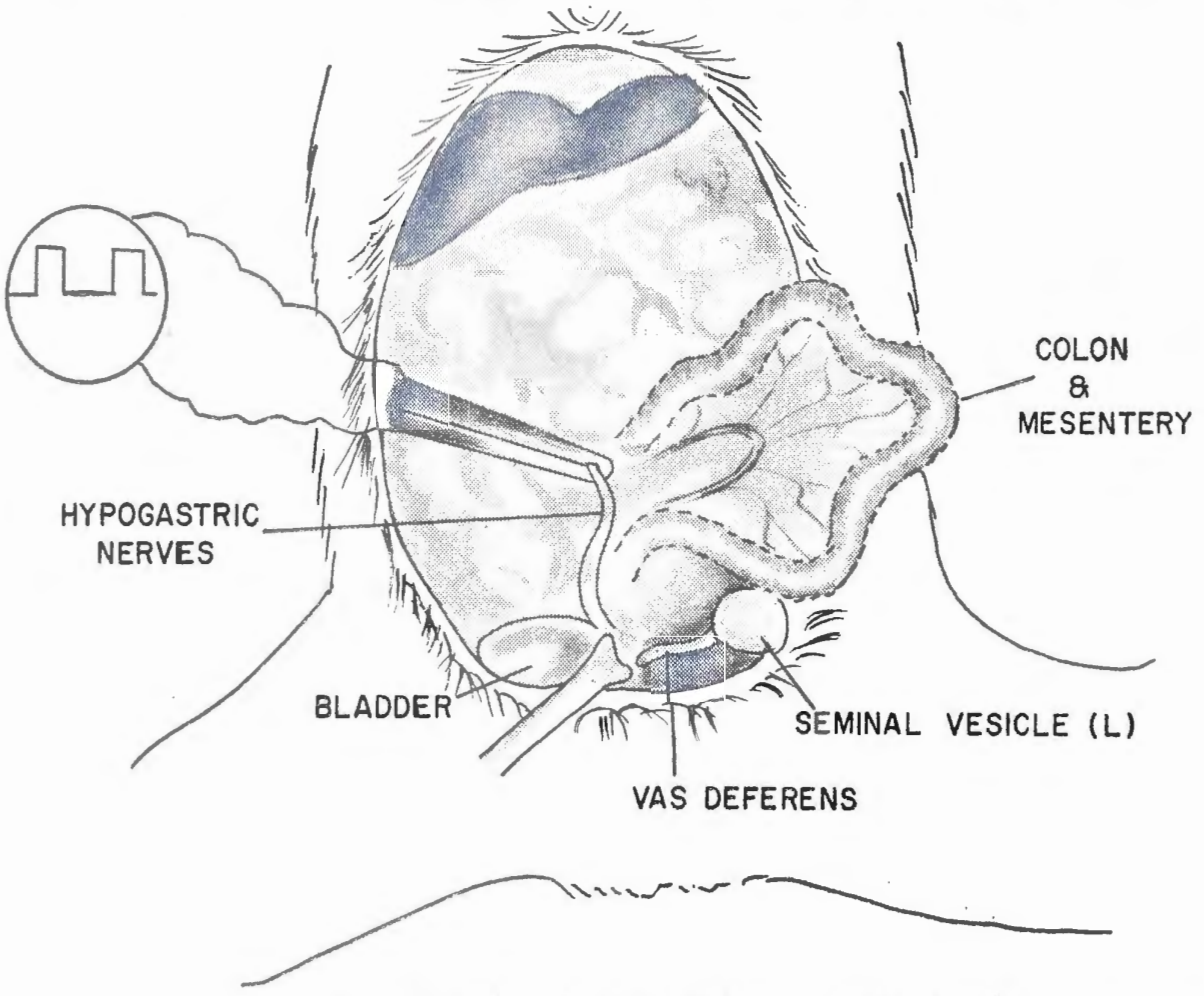
2. 1-b
The final set up of the innervated jejunum, in Tyrode solution at 37°C. The stimulating electrode must rest above the fluid level only during stimulation of the preparation.

ded in fat (which contains the accompanying adrenergic fibres). A segment of jejunum is chosen with an accompanying sector of mesentery and the embedded mesenteric arteriole. This is placed in a shallow dish in Tyrode's solution and tied onto an oxygen tube. A fine thread is tied at the apex of the mesentery and led onto a bipolar stimulating electrode. This entire assembly is with frontal writing point on a smoked drum. Stimulation of the mesentery leads to stimulation of periarterial sympathetic nerve fibres and the effect is seen as an inhibition of pendular movements.

5. **The innervated vas deferens preparation in vivo in the guinea pig.** A male guinea pig weighing 250—350 gm is anaesthetised with urethane (150mg/100 g) intraperitoneally. The internal

jugular vein and trachea are routinely cannulated. The abdomen is opened and the pelvic viscera exposed. Immediately behind the pubic symphysis the bladder is visible. Two cord like structures can be felt entering the base of the bladder, one on either side. These are the vasa deferentia. Select the one more convenient to use, slip a ligature around it and sever its connection with the bladder. Clear it of connective tissue for a length of about 1/cm so as to allow free movement during contractions. These are recorded by a ligature tied to a light transducer of a polygraph or a light lever recording on a smoked drum. Locate the descending colon and its mesentery, and gently spread it out for inspection. Two shining white slender structures may be seen at the root of the mesentery, travelling downwards into fatty tissue around the vas deferens. These are the hypogastric nerves which should be gently cleared for the placement of a bipolar stimulating electrode. It is not necessary to trace them through the fat because in this region they divide and ramify and dissection would damage the branches. Electrical stimulation of the nerve should elicit a brisk contraction of the vas deferens, which fades rapidly on cessation of stimulation.

Fig. 2. 2 : The hypogastric nerve – vas deferens preparation in the quintea pig.



The preparation is shown in situ in relation to adjacent landmarks

Epinephrine and norepinephrine may be administered through the cannula in the jugular vein to elicit a typical slow, sustained response. Adrenergic neurone blocking agents would block stimulation induced contractions, but not epinephrine induced one. (This method can be appropriately modified for in vitro studies).

Practical Exercises

Exercise 1 :

Objective : To study the effects of adrenergic drugs on B.P., spleen volume and respiration.

1. Set up a recording of spleen volume, blood pressure and respiration in a dog as described, and take a control strip of tracing.
2. Inject epinephrine 2.0 μg/Kg, and observe the effect.
3. Allow the pointers to return to their original base lines and administer norepinephrine 2.0 μg/kg.
4. Similarly, inject isoprenaline 1.0 μg/kg.
5. Observe that the pressor effect of epinephrine is slightly smaller than that of an equal dose of norepinephrine. The effect on spleen volume however is larger.
6. Observe the respiratory changes produced : epinephrine produces apnoea, which is not seen with norepinephrine; isoprenaline by producing a fall in B.P. causes a reflex tachypnoea.

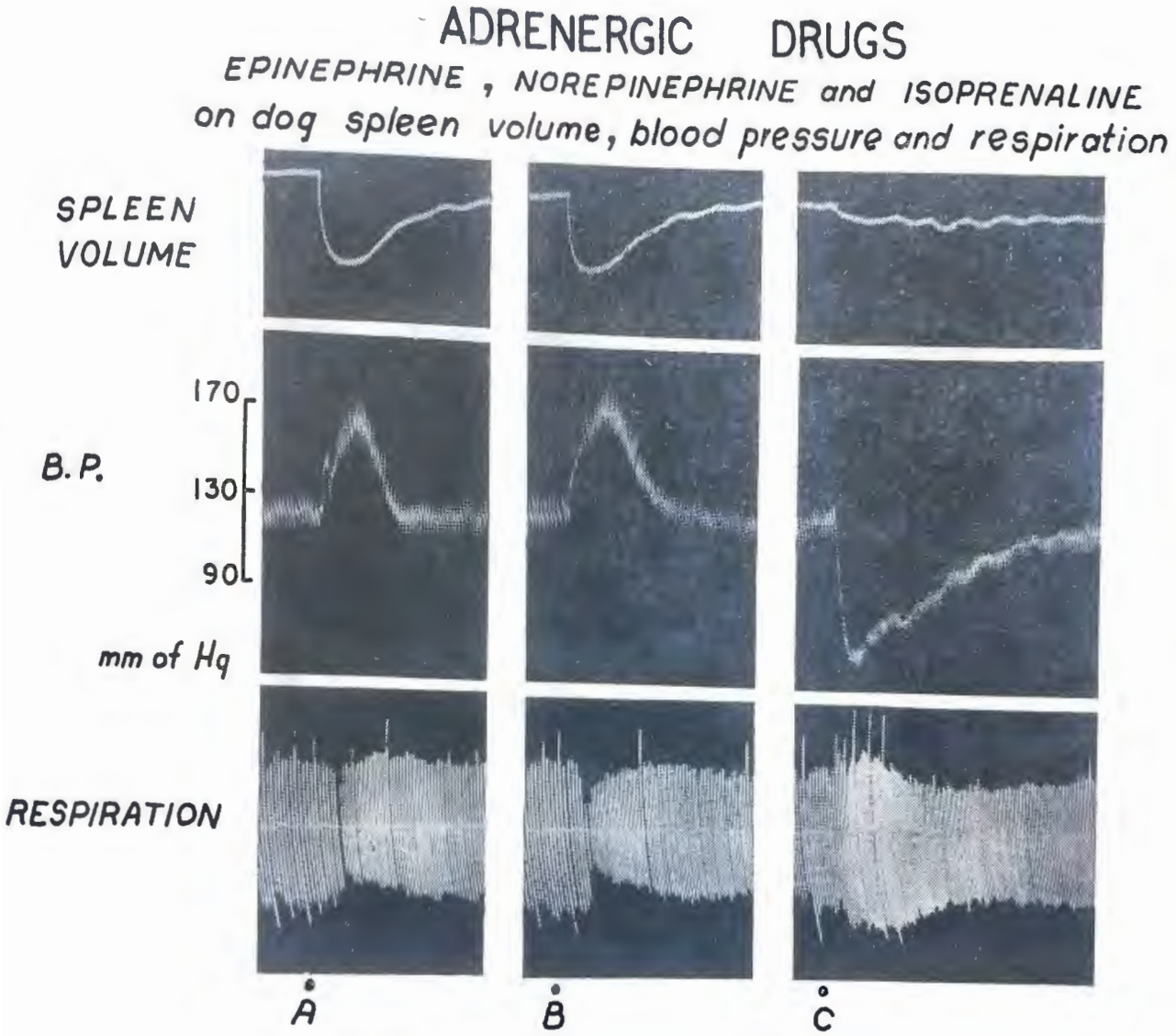

ADRENERGIC DRUGS
EPINEPHRINE , NOREPINEPHRINE and ISOPRENALINE
on dog spleen volume, blood pressure and respiration
SPLEEN VOLUME
B. P.
170
130
90
mm of Hg
RESPIRATION
A
B
C
A : epinephrine (2.0 μg/kg)
B : norepinephrine (2.0 μg/kg)
C : isoprenaline (1.0 μg/kg)

Fig. 2-3

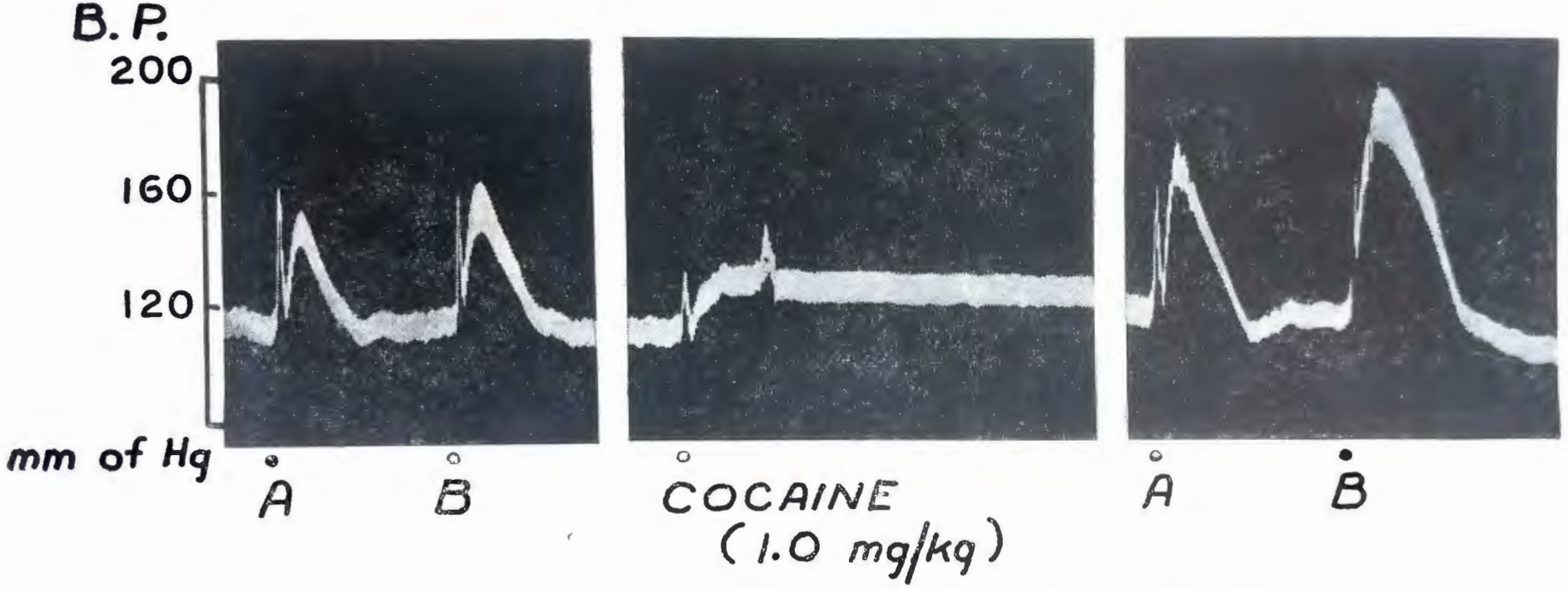
Exercise 2 :

Objective : To study the action of cocaine on blood pressure and epinephrine and norepinephrine pressor responses.

1. Set up a recording of carotid blood pressure in a dog.
2. Elicit responses to epinephrine and norepinephrine.
3. Inject cocaine 1.0 mg/kg.
4. After an interval of 10 to 20 minutes again elicit the responses to epinephrine and norepinephrine.
5. Observe that by itself cocaine has a small (nondescript) pressor effect.
6. Cocaine potentiates the pressor responses obtained with epinephrine and norepinephrine.

ADRENERGIC DRUGS

DOG BLOOD PRESSURE



A : Epinephrine (2.0 μg/kg)
B : Norepinephrine (1.5 μg/kg)

Fig. 2–4

Exercise 3 :

Objective : To study the action of guanethidine on blood pressure and on pressor effects of epinephrine and norepinephrine.

1. Set up a recording of carotid blood pressure in a dog.
2. Record pressor responses to epinephrine and norepinephrine.
3. Administer guanethidine 3.0 mg/kg intravenously.
4. After an interval of 10 minutes record the pressor responses to epinephrine and norepinephrine.
5. Observe that guanethidine produces biphasic responses by itself, an initial depressor and a later pressor response.
6. Guanethidine potentiates the action of exogenously administered catecholamines.

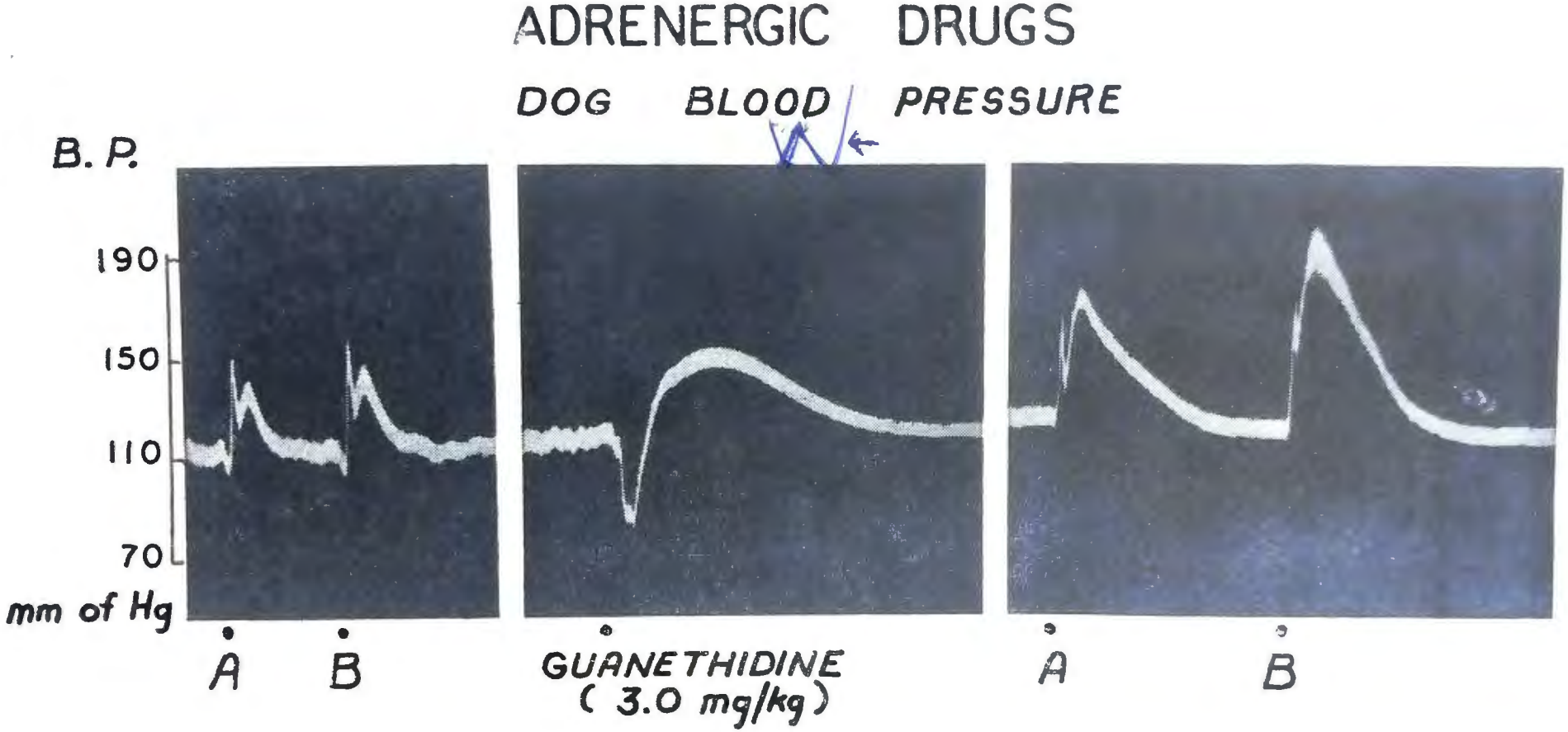

ADRENERGIC DRUGS
DOG BLOOD PRESSURE
B. P.
190
150
110
70
mm of Hg
A
B
GUANETHIDINE
(3.0 mg/kg)
A
B
A : Epinephrine (2.5 μg/kg)
B : Norepinephrine (2.0 μg/kg)

Fig. 2–5

Exercise 4 :

Objective : To study the action of tyramine on blood pressure and pressor actions of epinephrine and norepinephrine.

1. Set up a recording of carotid blood pressure in a dog.
2. Elicit responses to epinephrine and norepinephrine.
3. Inject tyramine 300 μ g/kg.
4. After 10 minutes interval, inject epinephrine and norepinephrine.
5. Observe that tyramine has a dramatic pressor action by itself.
6. Tyramine potentiates the actions of exogenous catecholamines.

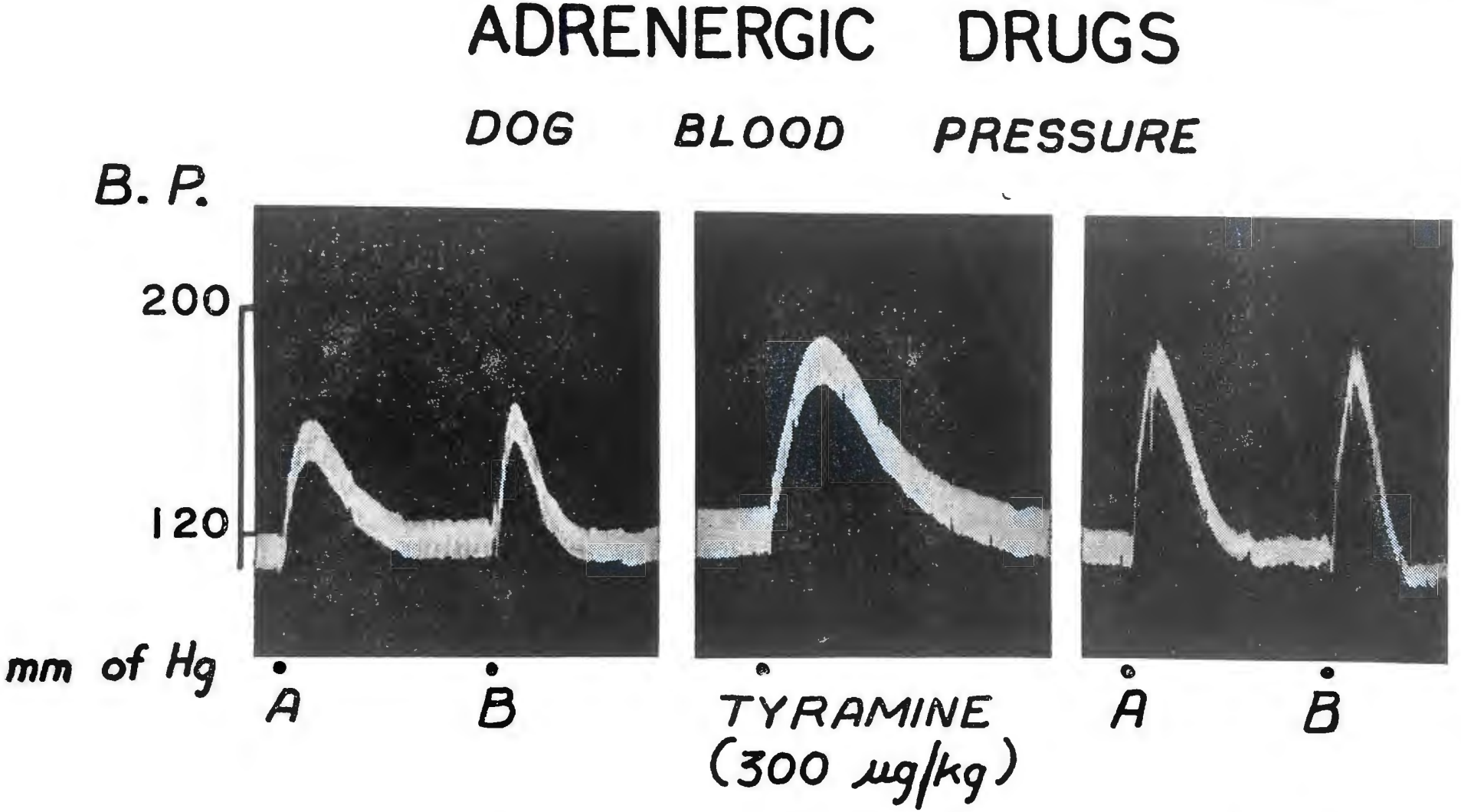

ADRENERGIC DRUGS
DOG BLOOD PRESSURE
B. P.
200
120
mm of Hg
A
B
TYRAMINE
(300 μg/kg)
A
B
A : Norepinephrine (2.0 μg/kg)
B : Epinephrine (2.5 μg/kg)

Fig. 2-6

Exercise 5 :

Objective : To study the effect of ∝ blockade and demonstrate Dale's vasomotor reversal phenomenon.

1. Set up a recording of carotid blood pressure in a dog.
2. Record responses to epinephrine, norepinephrine and isoprenaline, all in doses of 2 μ g/kg.
3. Administer the ∝ blocking agent tolazoline (Priscol) in a dose of 5 mg/kg and allow the system to stabilise, for a half hour.
4. Repeat epinephrine, norepinephrine and isoprenaline in the same doses as before.
5. Observe that the pressor effect of epinephrine has been blocked (∝ blockade) and replaced by a depressor effect due to unmasking of its β action (Dale's vasomotor reversal).
6. The action of norepinephrine has also been reversed but to a much smaller extent.
7. The action of isoprenaline is unaffected.

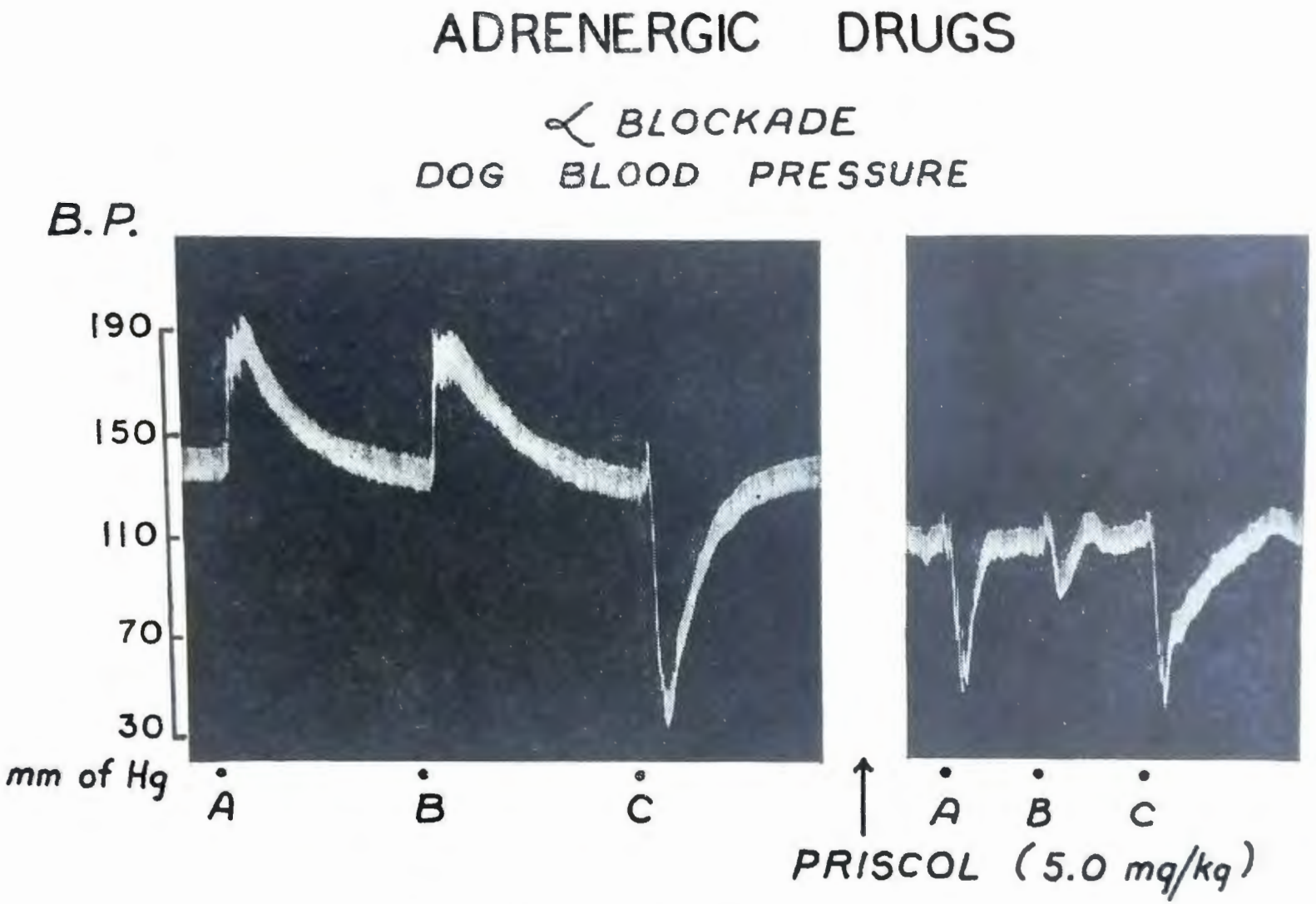

ADRENERGIC DRUGS
∠ BLOCKADE
DOG BLOOD PRESSURE
B.P.
190
150
110
70
30
mm of Hg
A
B
C
A
B
C
PRISCOL (5.0 mg/kg)
A : Epinephrine (2.0 μg/kg)
B : Norepinephrine (2.0 μg/kg)
C : Isoprenaline (2.0 μg/kg)

Fig. 2-7

Exercise 6 :

Objective : To demonstrate blockade of β receptors.

1. Set up a recording of carotid blood pressure in a dog.
2. Elicit responses with epinephrine, norepinephrine and isoprenaline in the doses indicated.
3. Administer propranolol (0.1 mg/kg) and wait for an interval of 20 minutes.
4. Repeat the administration of epinephrine, norepinephrine and isoprenaline.
5. Observe that the vasodepressor effect of isoprenaline has been blocked completely.

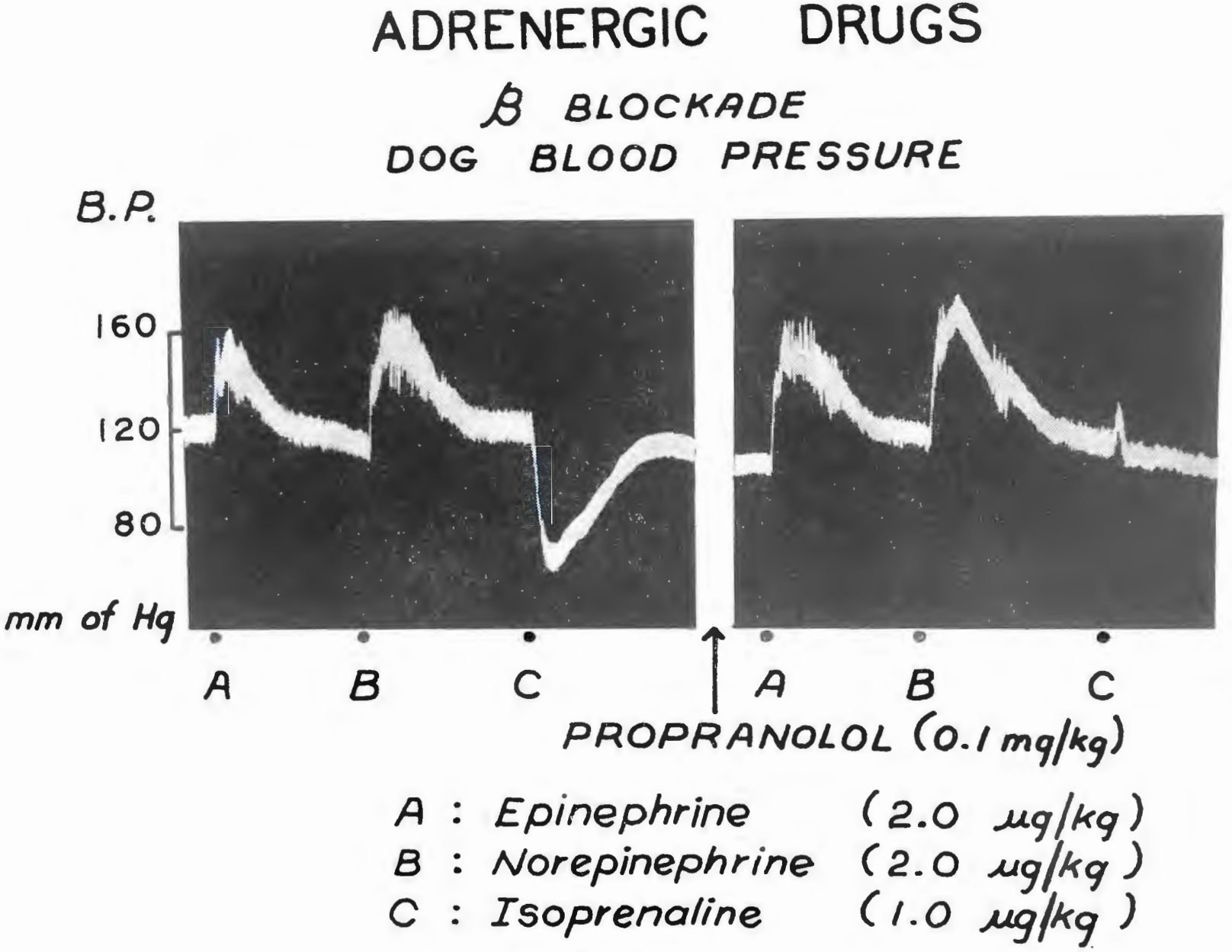

ADRENERGIC DRUGS
β BLOCKADE
DOG BLOOD PRESSURE
B.P.
160
120
80
mm of Hg
A
B
C
A
B
C
PROPRANOLOL (0.1 mg/kg)
A : Epinephrine (2.0 μg/kg)
B : Norepinephrine (2.0 μg/kg)
C : Isoprenaline (1.0 μg/kg)

Fig. 2-8

Exercise 7 :

Objective : To demonstrate α blockade and the reversal of α blockade by a β blocking agent.

1. Set up a recording of carotid blood pressure in a dog.
2. Elicit responses to epinephrine, norepinephrine and isoprenaline.
3. Administer phenoxybenzamine 5.0 mg/kg slowly intravenously well diluted in 20 ml of saline.
4. Twenty minutes later, repeat epinephrine, norepinephrine and isoprenaline.
5. Administer propranolol (0.1 mg/kg) slowly intravenously.
6. After 10 minutes repeat epinephrine, norepinephrine and isoprenaline.
7. Observe that when phenoxybenzamine is administered the vasopressor action of epinephrine is reversed, that of norepinephrine considerably blocked and the small pressor component of isoprenaline action blocked. (Refer Fig. 2-7 where β blockade has unmasked this small α component of isoprenaline action).
8. Observe that the administration of propranolol causes a reappearance of the pressor actions previously blocked by phenoxybenzamine but blocks the vasodepressor action of isoprenaline.

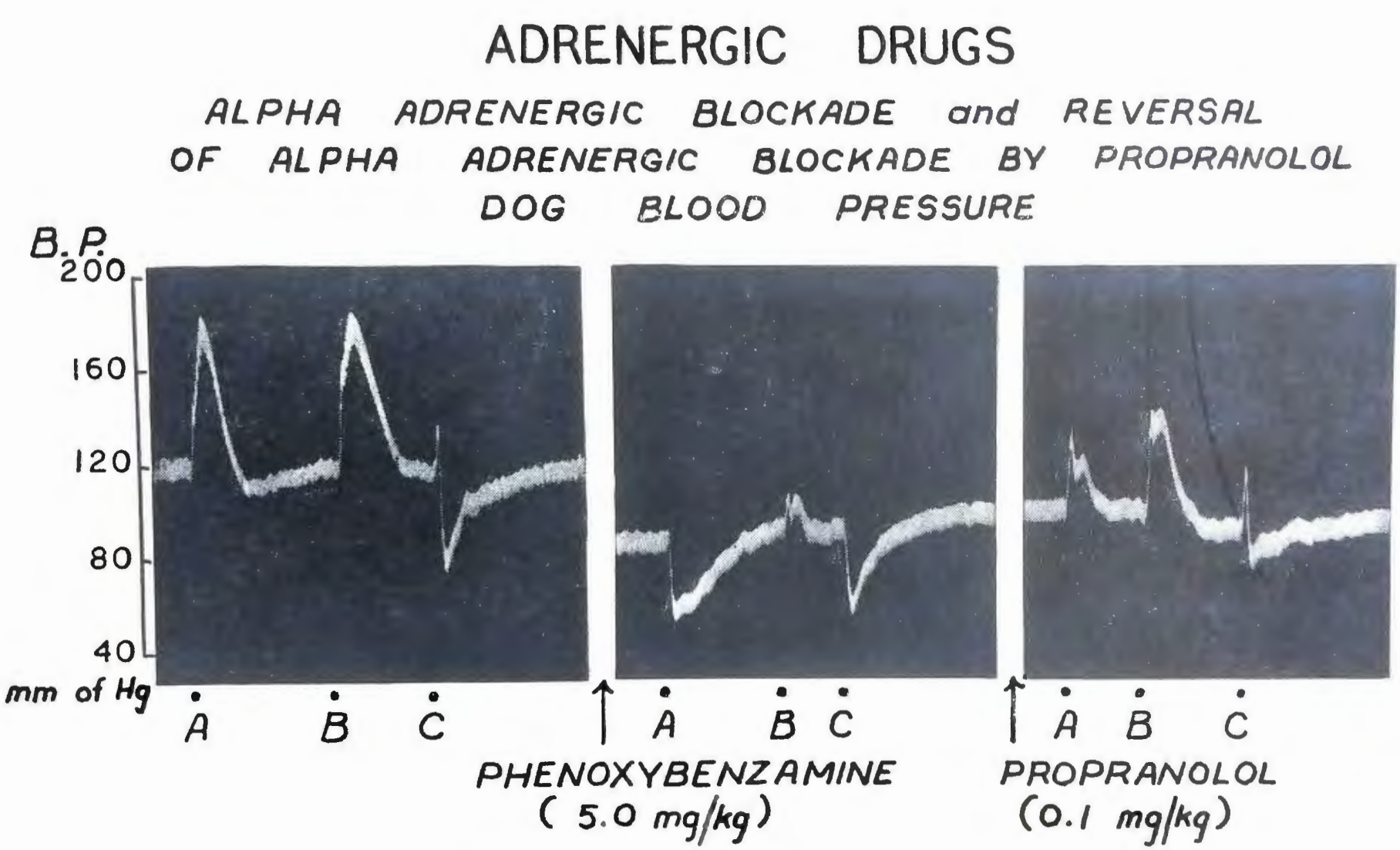

ADRENERGIC DRUGS
ALPHA ADRENERGIC BLOCKADE and REVERSAL
OF ALPHA ADRENERGIC BLOCKADE BY PROPRANOLOL
DOG BLOOD PRESSURE
B.P.
200
160
120
80
40
mm of Hg
A B C
A B C
A B C
PHENOXYBENZAMINE
(5.0 mg/kg)
PROPRANOLOL
(0.1 mg/kg)
A : Epinephrine (3.0 μg/kg)
B : Norepinephrine (2.5 μg/kg)
C : Isoprenaline (1.0 μg/kg)

Fig. 2–9

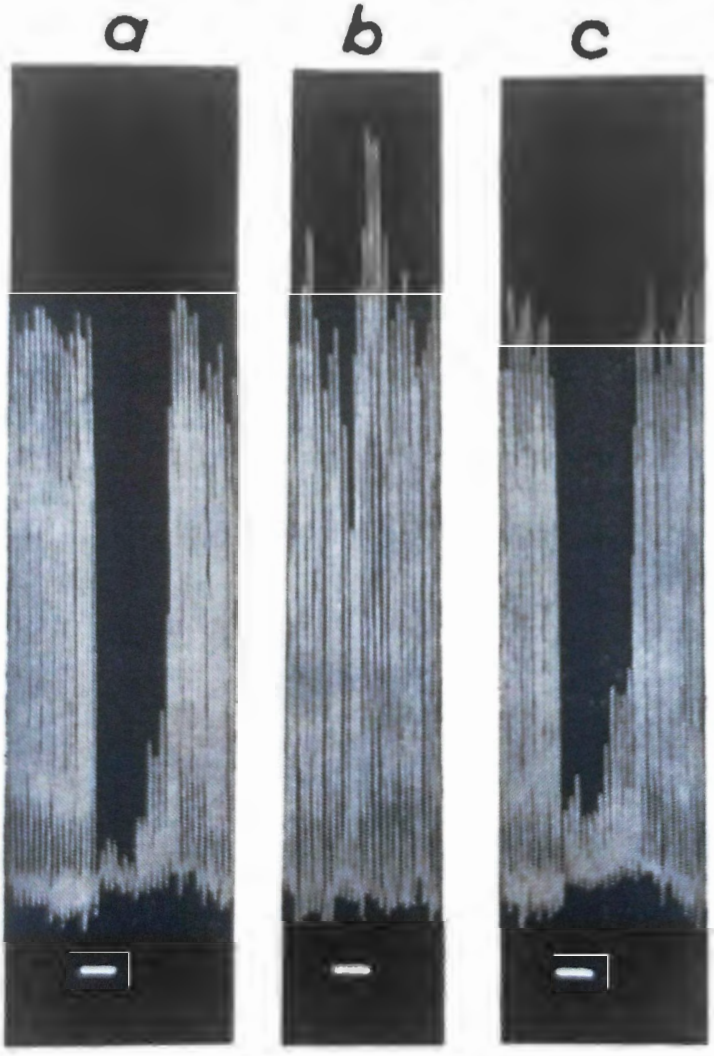
Exercise 8 :

Objective : To demonstrate the action of adrenergic neurone blocking agents on responses elicited by nerve stimulation.

1. Set up the isolated innervated preparation of rabbit jejunum as described above.
2. Record a control strip of tracing of pendular movements of the jejunum and the effect of electrical stimulation on these movements.
3. Add an adrenergic neurone blocking agent like bretylium to the bath fluid (1.0 μ g/ml) and allow a period of about 5 to 10 minutes to elapse.
4. Repeat electrical stimulation and observe that the inhibition of pendular movements is not produced i.e. the effect of nerve stimulation has been blocked.
5. Keep stimulating the preparation every 10 minutes and observe recovery of response.

ADRENERGIC DRUGS

Isolated innervated rabbit jejunum (Finkelman preparation)



a- Control : b- In the presence of bretylium (1.0 µg/ml): C- Recovery

Bars indicate stimulation of mesentery for 30 sec.

(Volts :- 10, Duration :- 0.5 msec

Rate :- 50 shocks/sec)

Fig. 2-10

Exercise 9 :

Objective : To demonstrate the potentiation of acetylcholine by an anticholinesterase agent.

1. Set up a recording of carotid B.P. in a dog.
2. Administer carbachol, pilocarpine and 2 doses of acetylcholine.
3. Inject physostigmine (0.1 mg/kg) slowly intravenously.
4. Twenty minutes later inject the smaller of the 2 doses of acetylcholine, pilocarpine and carbachol.
5. Observe that all 3 drugs produce a fall in blood pressure, the fall due to acetylcholine appears dose dependent.
6. Administration of an anticholinesterase agent potentiates the action of acetylcholine so that the effect of the smaller dose is now much larger than the effect of the large dose elicited in absence of ChE.
7. The actions of carbachol and pilocarpine which are not the substrates of ChE, are not potentiated.

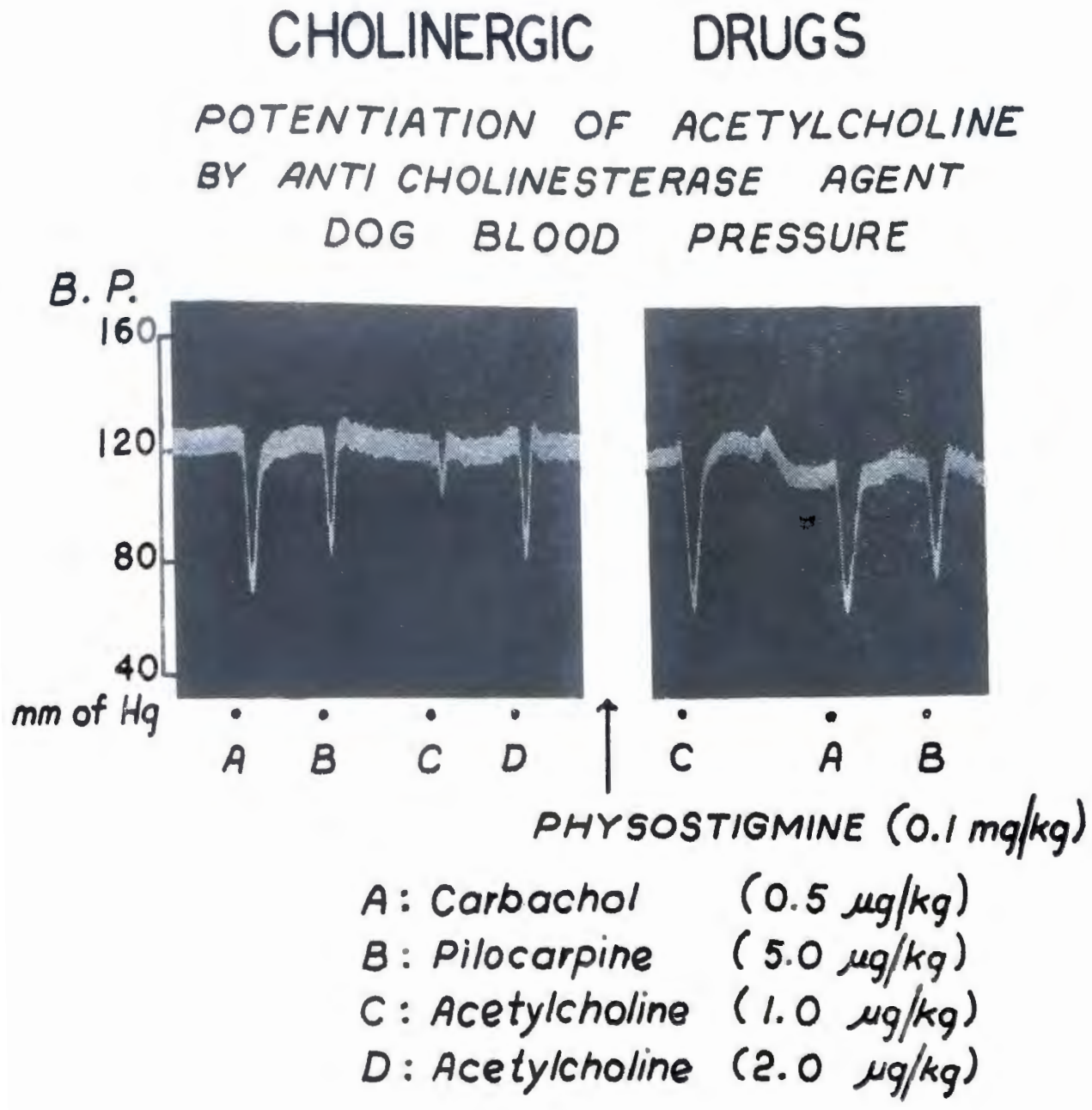

CHOLINERGIC DRUGS
POTENTIATION OF ACETYLCHOLINE
BY ANTI CHOLINESTERASE AGENT
DOG BLOOD PRESSURE
B. P.
160
120
80
40
mm of Hg
A B C D
C A B
PHYSOSTIGMINE (0.1 mg/kg)
A : Carbachol (0.5 μg/kg)
B : Pilocarpine (5.0 μg/kg)
C : Acetylcholine (1.0 μg/kg)
D : Acetylcholine (2.0 μg/kg)

Fig. 2-11

Exercise 10 :

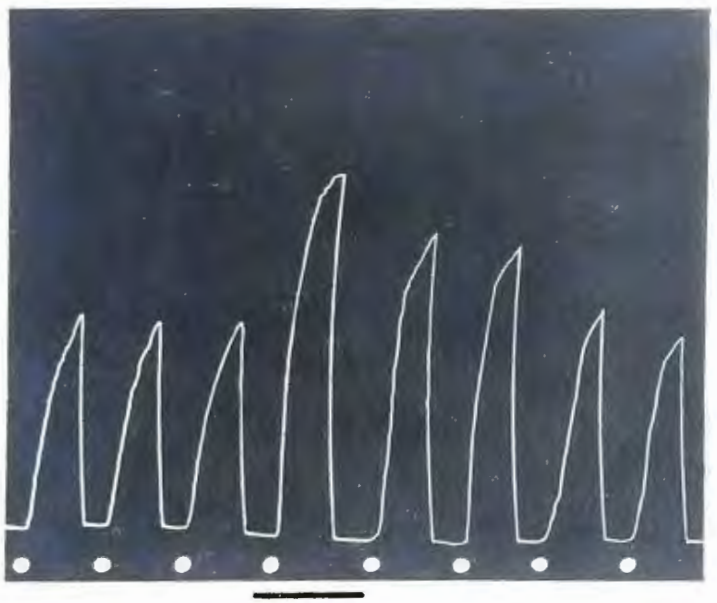
Objective : To demonstrate the potentiation of acetylcholine action by an anticholinesterase agent.

1. Set up an isolated frog's rectus abdominis preparation.
2. Elicit 3 responses to acetylcholine 2.0 μ g.
3. Add physostigmine 0.5 μ g, and allow to act for 3 minutes.
4. In the presence of physostigmine elicit a response to the same dose of acetylcholine.
5. Wash out the fluid and refill the bath.
6. Elicit responses to the same dose of acetylcholine till recovery of original height of contractions occurs.
7. Observe that physostigmine potentiates the action of acetylcholine and this potentiation is reversible.

CHOLINERGIC DRUGS

ISOLATED FROG'S RECTUS ABDOMINIS MUSCLE

Potentiation of ACh by anti cholinesterase agent



At dots 2.0 µg acetylcholine were allowed to act for 90 sec.
Bar indicates presence of physostigmine 0.5 µg/ml for 3 min
BATH CAPACITY : 20 ml

Fig. 2-12

Exercise 11 :

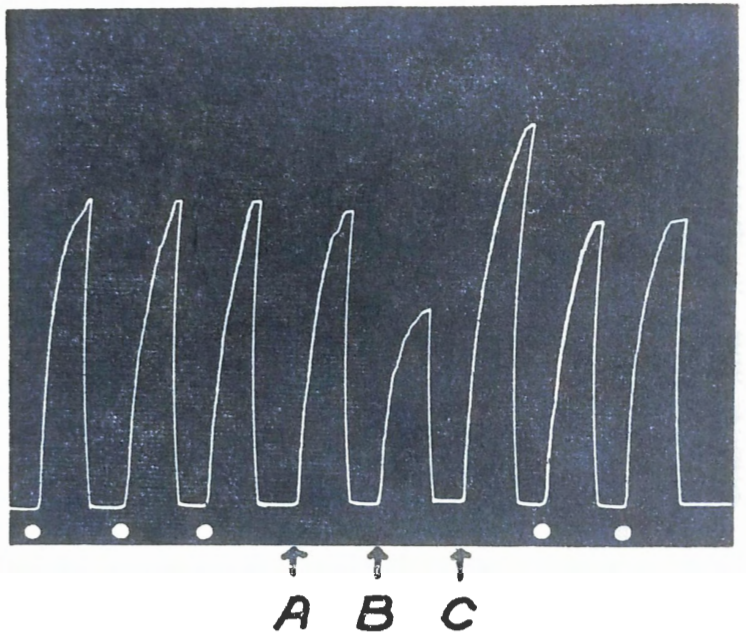
Objective : To demonstrate hydrolysis of acetylcholine by ser cholinesterase.

1. Set up an isolated frog's rectus abdominis preparation
2. Elicit 3 responses to 3.0 μg of acetylcholine.
3. Add 0.5 ml of 1 : 5 diluted fresh serum to 3.0 μg ACh.
4. Immediately add this mixture to the bath fluid, record the respor
5. To 3.0 μg. ACh add 0.5 ml serum and allow it to act for 10 minu
6. Now add this mixture to the bath and record the response.
7. To 0.5 ml serum add 4.0 μg physostigmine and 3.0 μg ACh a allow to stand for 10 minutes.
8. Record a response with the mixture thus obtained.
9. Repeat acetylcholine as above until recovery is seen.
10. Observe that (a) the addition of serum just before eliciting a r ponse does not alter the height of contraction; (b) allowing t serum to act for 10 minutes diminishes the effect of acetylcholi thereby indicating partial hydrolysis; (c) addition of physostigm potentiates the action of acetylcholine because not only is ser ChE inhibited, preventing hydrolysis of ACh, but muscle ChE also inhibited resulting in actual enhancement.

CHOLINERGIC DRUGS

ISOLATED FROG'S RECTUS ABDOMINIS MUSCLE

Hydrolysis of ACh by serum cholinesterase



At dots 3.0 µg acetylcholine were allowed to act for 90 sec.

A : 0.5 ml serum + 3.0 µg ACh added immediately.

B : 0.5 ml serum + 3.0 µg ACh, 10 min after mixing

C : 0.5 ml serum + 4.0 µg physostigmine + 3.0 µg ACh

Fig. 2–13

Exercise 12 :

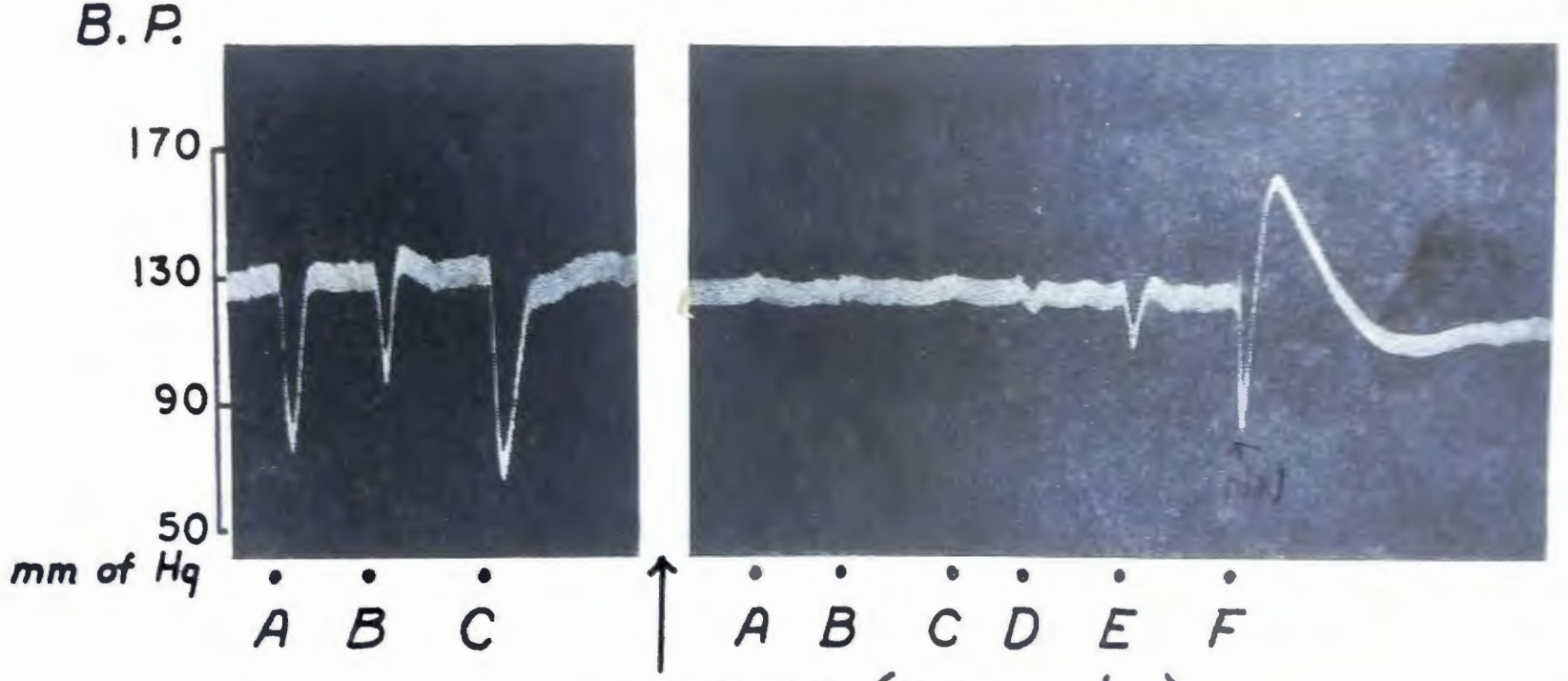
Objective : To demonstrate the nicotinic action of acetylcholine.

1. Set up a recording of carotid blood pressure in dog.
2. Administer carbachol, pilocarpine and acetylcholine in doses 0.5 μg/kg, 5.0 μg/kg and 4.0 μg/kg respectively.
3. Inject atropine (3.0 mg/kg) slowly i.e. well diluted in 20 ml saline.
4. Twenty minutes later repeat carbachol, pilocarpine and acetylcholine in the same doses as before.
5. Administer carbachol, pilocarpine and acetylcholine in larger doses 0.5 mg/kg, 1.0 mg/kg and 1.0 mg/kg respectively.
6. Observe that atropine blocks the vasodepressor effect of carbachol, pilocarpine and acetylcholine.
7. This blockade is surmountable because very large doses of carbachol and pilocarpine show small depressor effects.
8. Acetylcholine alone possesses a pressor effect in large doses, in presence of atropine, which is its nicotinic action.

CHOLINERGIC DRUGS

NICOTINIC ACTION OF ACETYLCHOLINE

DOG BLOOD PRESSURE



A: Carbachol (0.5 μg/kg)
B: Pilocarpine (5.0 μg/kg)
C: Acetylcholine (4.0 μg/kg)
D: Carbachol (0.5 mg/kg)
E: Pilocarpine (1.0 mg/kg)
F: Acetylcholine (1.0 mg/kg)

Fig. 2-14

Exercise 13 :

Objective : To demonstrate muscarinic blockade.

1. Set up a recording of carotid blood pressure in a cat.
2. Elicit responses to acetylcholine 3 mg/kg and nicotine 0.1 mg/kg.
3. Inject atropine 2.0 mg/kg well diluted in saline, slowly i.v.
4. Twenty minutes later repeat acetylcholine and nicotine in the same dose.
5. Observe that acetylcholine produces a depressor response; nicotine produces a biphasic response with wide fluctuations in B.P. at the peak of pressor response due to respiratory stimulation.
6. Observe complete blockade of action of acetylcholine, and blockade of depressor component of nicotine action.

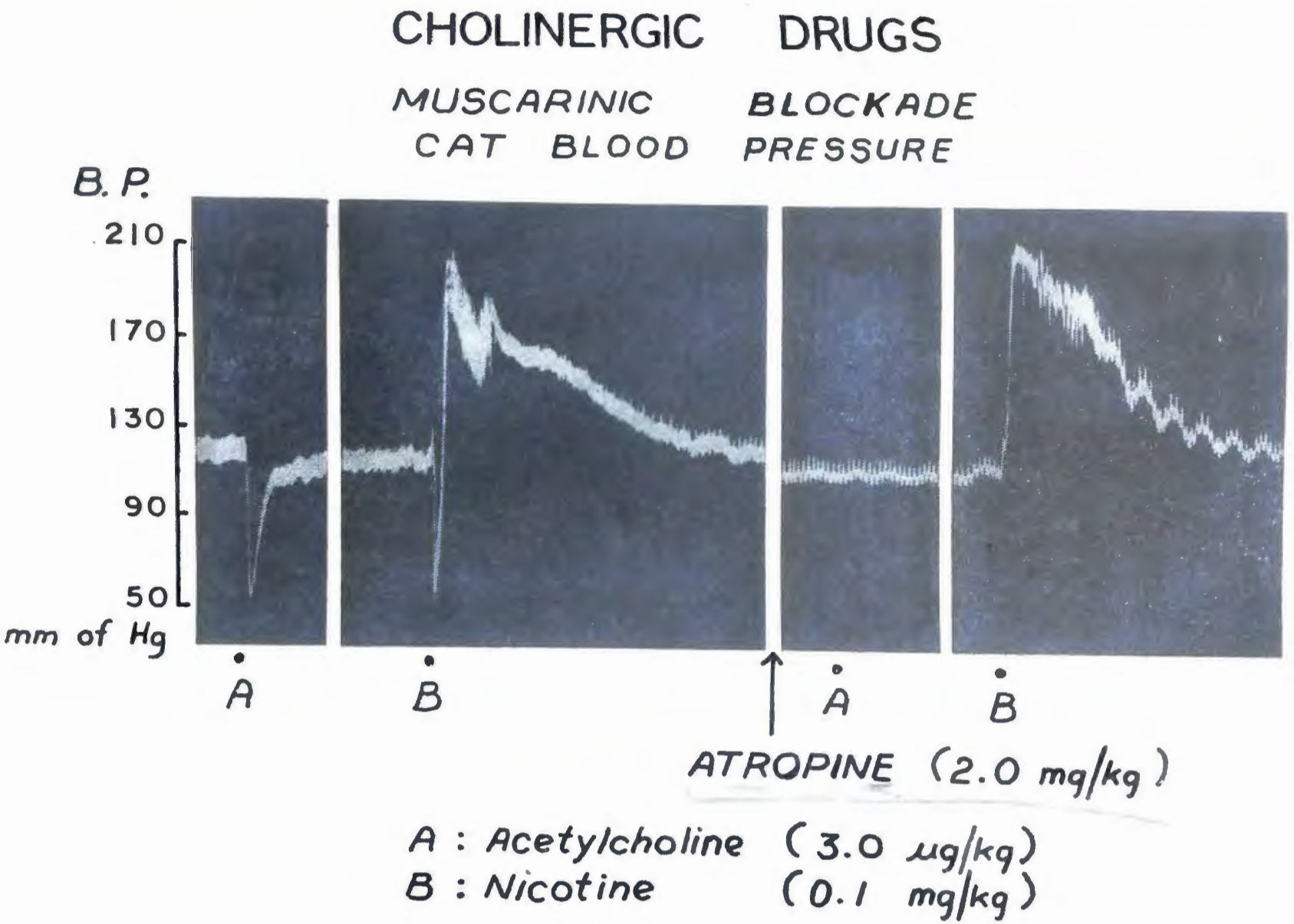

CHOLINERGIC DRUGS
MUSCARINIC BLOCKADE
CAT BLOOD PRESSURE
B. P.
210
170
130
90
50
mm of Hg
A
B
A
B
ATROPINE (2.0 mg/kg)
A : Acetylcholine (3.0 μg/kg)
B : Nicotine (0.1 mg/kg)

Fig. 2–15

Exercise 14 :

Objective : To demonstrate ganglion stimulation and blockade.

1. Set up a recording of carotid blood pressure and nictitating membrane in a cat as described.
2. Inject epinephrine (E) 1.5 μ g/kg and DMPP (50 μ g/kg)
3. Administer pentolinium tartrate (2.0 mg/kg) slowly i.v.
4. Twenty minutes later, repeat epinephrine and DMPP
5. Observe the initial pressor and nictitating membrane stimulating actions of E and DMPP (catecholamine and ganglion stimulant).
6. Observe that pentolinium blocks the action of DMPP on blood pressure and nictitating membrane without any action on effect of E on the nictitating membrane; it potentiates the pressor effect of E

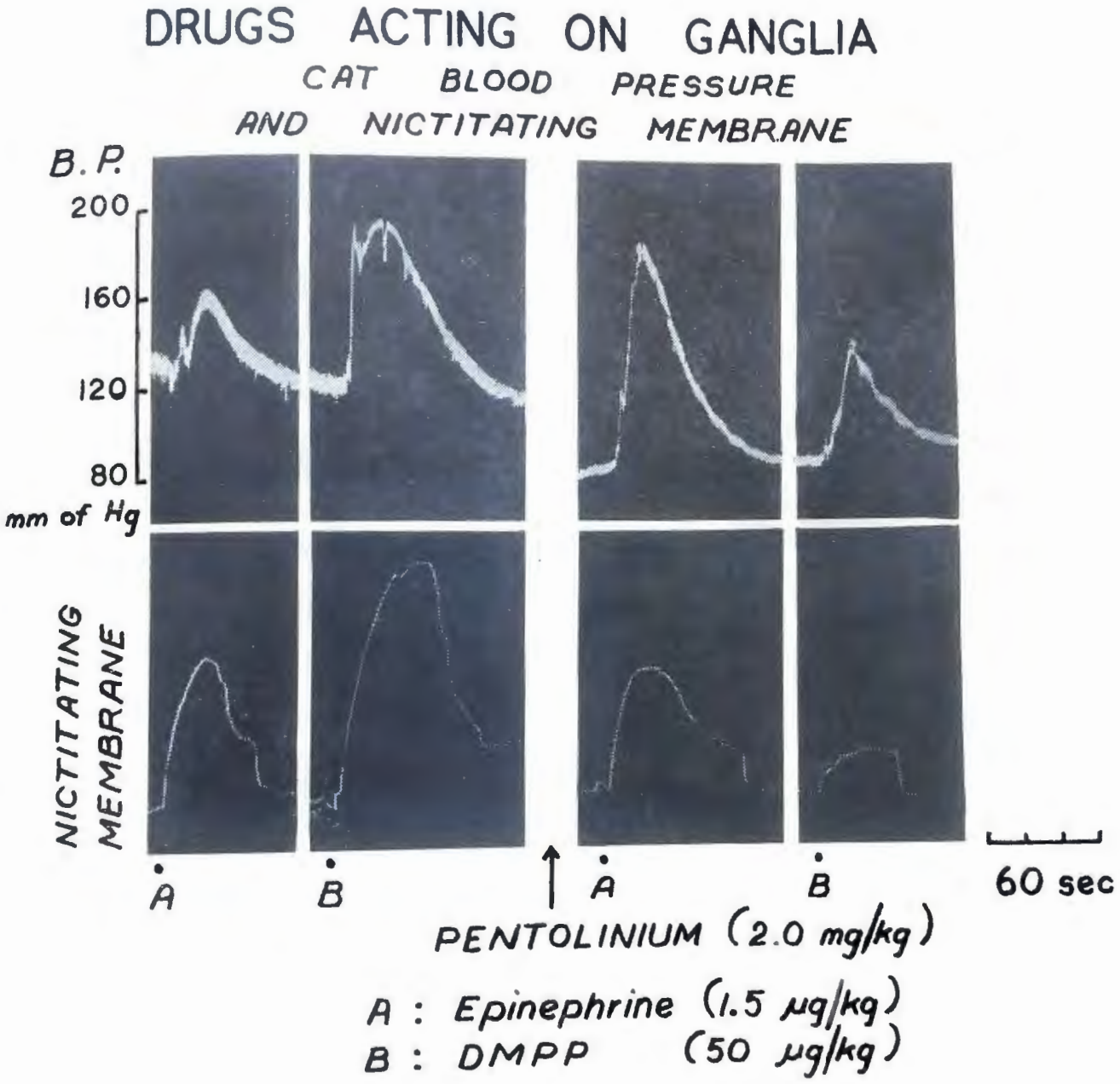

DRUGS ACTING ON GANGLIA
CAT BLOOD PRESSURE
AND NICTITATING MEMBRANE
B.P.
200
160
120
80
mm of Hg
NICTITATING MEMBRANE
A
B
A
B
60 sec
PENTOLINIUM (2.0 mg/kg)
A : Epinephrine (1.5 μg/kg)
B : DMPP (50 μg/kg)

Fig. 2–16

Exercise 15 :

Objective : To demonstrate ganglion blockade in relation to the vagus nerve.

1. Set up carotid blood pressure and atrial beat recordings as described.
2. Elicit control responses to vagal stimulation and acetylcholine injection.
3. Administer pentolinium tartrate 3.0 mg/kg.
4. Repeat vagal stimulation and acetylcholine injections 10 to 20 minutes later.
5. Observe that both vagal stimulation and acetylcholine injection lower the blood pressure and depress atrial contractility.
6. Pentolinium blocks the action of vagal stimulation on B.P. and atrial beats, without effect on acetylcholine injection.
7. This blockade is reversible as shown by reappearance of vagal action at 120 minutes.

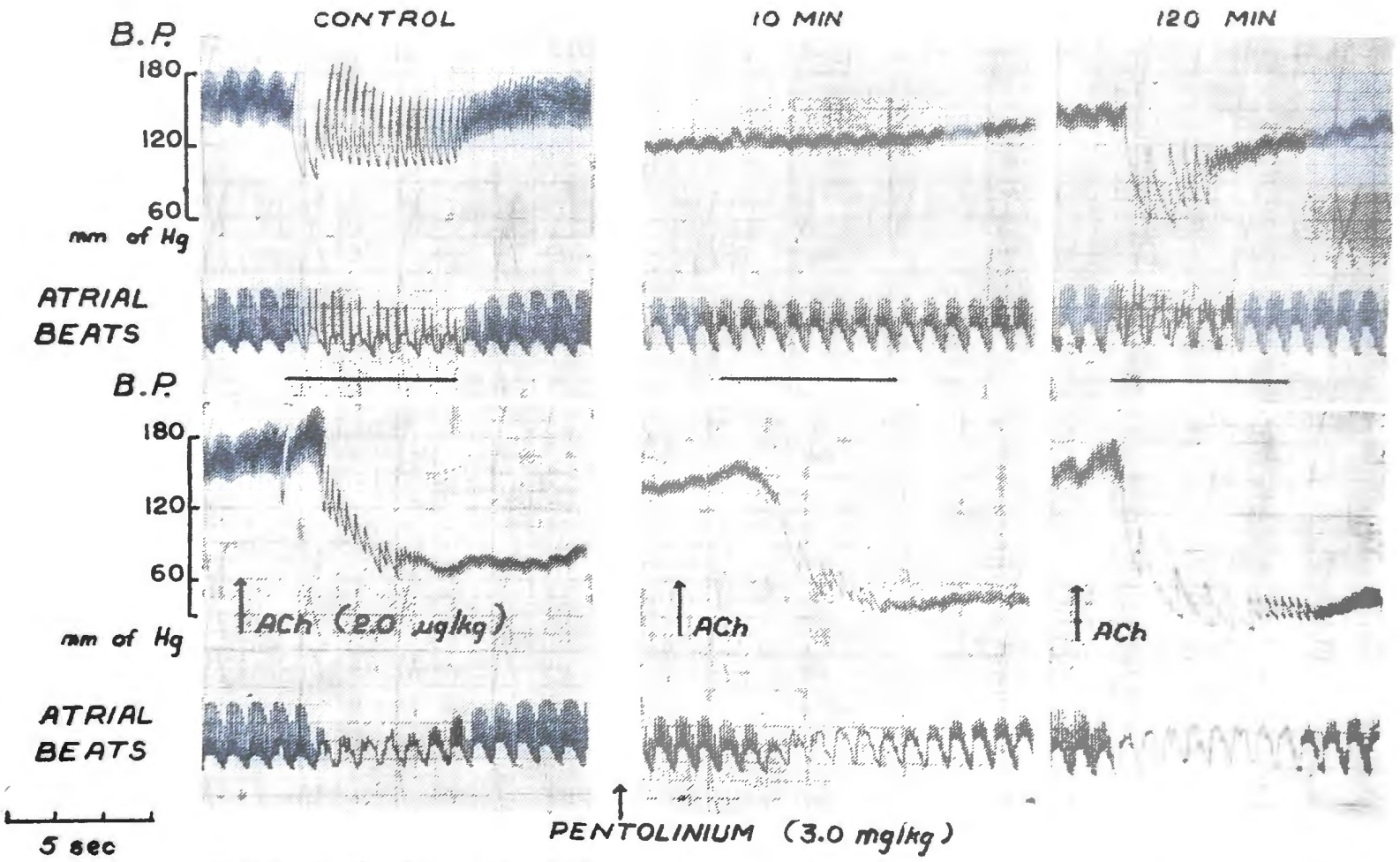

GANGLION BLOCKING DRUGS
OPEN CHEST PREPARATION OF CAT
CONTROL
10 MIN
120 MIN
B.P.
180
120
60
mm of Hg
ATRIAL BEATS
B.P.
180
120
60
mm of Hg
ACh (2.0 µg/kg)
ACh
ACh
ATRIAL BEATS
5 sec
PENTOLINIUM (3.0 mg/kg)
Bars indicate stimulation of peripheral cut end of vagus
(20 shocks/sec : 6.0 volts)

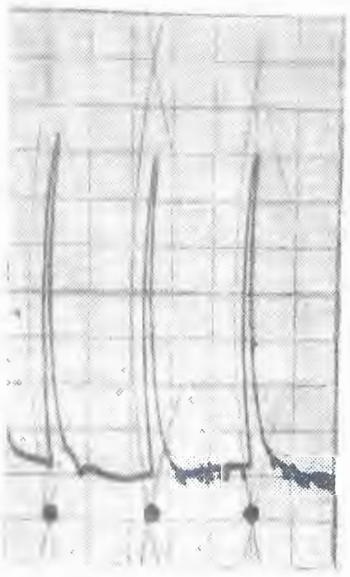
Fig 2-17

Exercise 16 :

Objective : To demonstrate adrenergic neurone blocking activity in vivo.

1. Set up a hypogastric nerve vas deferens preparation as described.
2. Elicit responses to electrical stimulation.
3. Administer norepinephrine intravenously and record the response.
4. Inject guanethidine 1 — 2 mg/kg i.v.
5. After a period of fifteen minutes re-elicit the response to electrical stimulation with the same parameters.
6. Observe partial to complete blockade of contraction.
7. Inject norepinephrine in the same dose and observe that the response is practically unchanged.

INNERVATED VAS DEFERENS OF GUINEA-PIG (IN VIVO)



AT DOTS SINGLE STIMULI WERE APPLIED TO THE HYPOGASTRIC N.

PARAMETERS OF STIMULATION

FREQUENCY = 80/sec
PULSE WIDTH = 0.5 msec
AMPLITUDE = 4.0 V
INTERVAL 3.0 min

Fig. 2-18

The work of Science is to substitute facts for appearances, and demonstrations for impressions.

CLAUDE BERNARD

Skeletal Muscle Relaxants

Introduction

Voluntary movements performed by skeletal muscle are under the control of central mechanisms of a complex nature. Impulses originate in the cerebral cortex and pass down the pyramidal tracts into the spinal cord. Here in the anterior horn, the tracts synapse with a new set of nerve fibres, the motor neurones, which directly reach the skeletal muscles that they supply. This junction of nerve and skeletal muscles is a specialised junction, the myoneural junction or the end plate region.

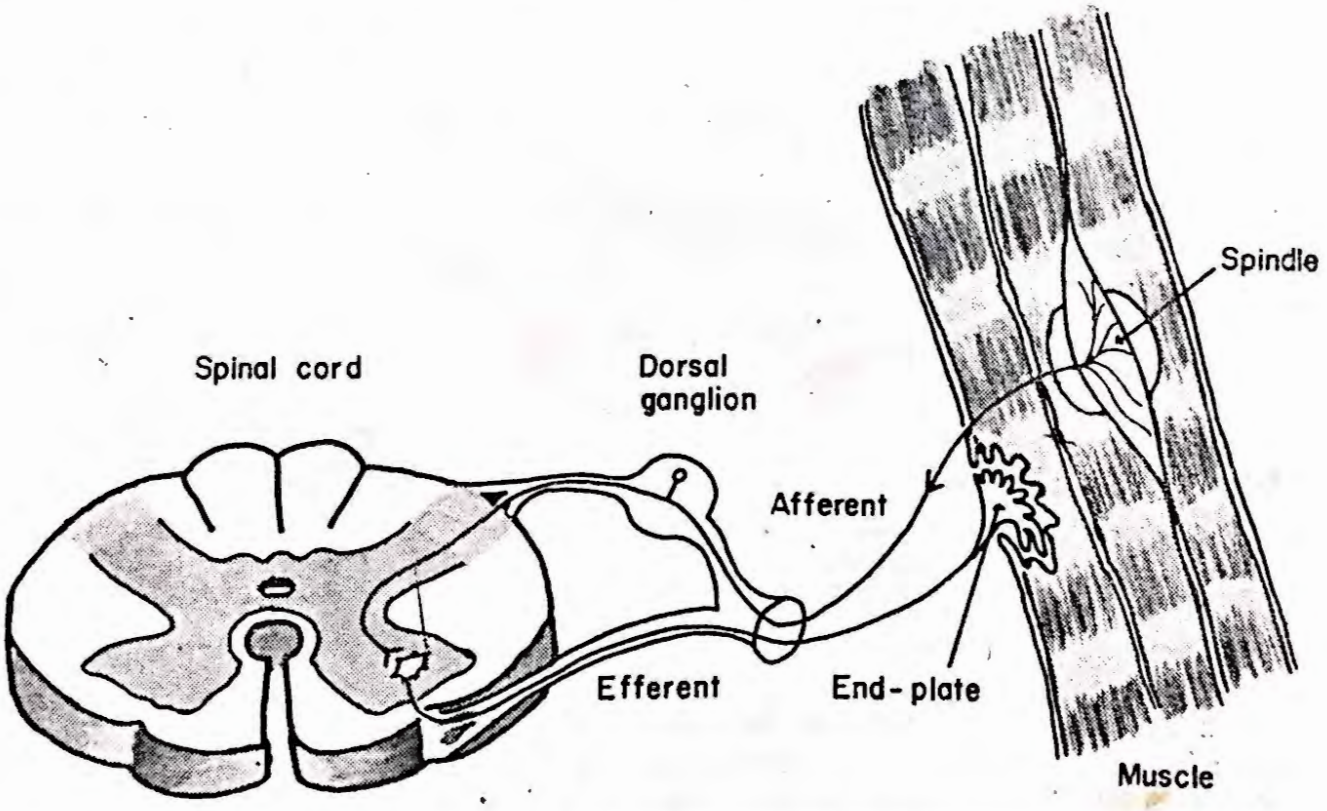
The fine control of voluntary movements is brought about by the 'extra pyramidal system', which is not under conscious control. This system exerts its influence through a vast network of neurones collectively called the reticular formation of the brain, extending upwards into the thalamus & subcortical regions, and downwards into the grey matter of the spinal cord. Such influences are felt at the anterior horn synapse in the spinal cord, and hence any drug acting on these synapses would affect control of skeletal muscles.

For movements of skeletal muscle to occur, the muscle must possess a basal degree of 'tone'. (This tone is something akin to the tautness of a violin string). This muscle tone is normally maintained at a physiological level by the monosynaptic reflex arc, i.e. a sensory fibre, a spinal synapse and the motoneurone. This reflex arc is also influenced by central regulating mechanisms, which may be facilitatory or inhibitory. The best example of this monosynaptic reflex is the patellar reflex or knee jerk. When the patellar tendon is tapped, a slight stretching of the muscle belly occurs. This causes a stretching not only of the skeletal muscle fibres themselves, but also of certain associated structures called muscle spindles (refer Fig. 3.1). When these spindles are stretched, the nerve endings surrounding the central bag like structure are stimulated, and impulses travel along these afferent nerves (α afferents) to the spinal cord. Here a synapse occurs with the motoneurones innervating the muscle fibres and a co-ordinated contraction of the entire muscle is seen as the knee jerk. The entire process occurs in fractions of a second. Fine control of muscle tone occurs by alteration in the state of the muscle spindle itself. This is achieved through the efferents from the spinal cord, which innervate the myofibrils of the muscle spindles. These fibrils, being inserted into the nuclear bag, cause changes in the tone of this bag, and thus the degree of stretch is affected, in

turn affecting the entire monosynaptic reflex.

At the neuromuscular junction, a nerve impulse liberates acetylcholine from the nerve ending into the cleft between muscle and nerve fibre. This acetylcholine causes a depolarization of the muscle fibre which in turn sets off a muscle action potential, and contraction of the muscle fibre. The peculiarity of mammalian muscle is that each muscle fibre has a single end-plate and single nerve fibre innervating it. Hence, the contraction obtained by nerve stimulation is a quick, brisk twitch. This is seen in the response of rat phrenic nerve hemidiaphragm preparation and in the contraction of the cat's Tibialis muscle nerve preparation. The muscle fibres of lower species like frog are multiply innervated and hence nerve stimulation causes persistent depolarization and a prolonged, slow contracture of the muscle.

Fig. 3. 1 : The monosynaptic reflex pathway.



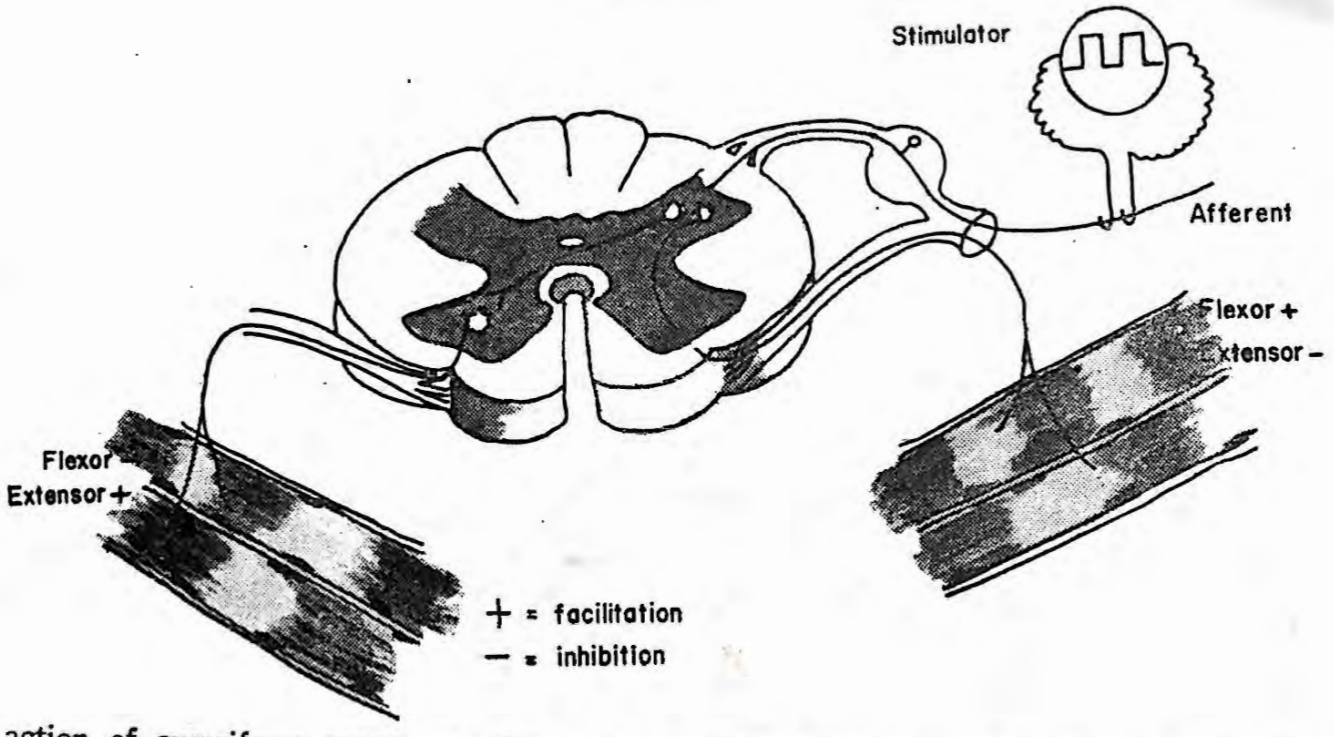
On the basis of this concept of muscle function, the actions of various drugs may be considered. At the periphery the neuromuscular junction is susceptible to the action of peripherally acting skeletal muscle relaxants. These are so named, because, by interfering with neuromuscular transmission, they cause a relaxation of muscle by loss of muscle tone. They are of two types — curariform, which prevent depolarization by competitive antagonism, and the depolarizing type, like succinylcholine or decamethonium (C 10). This latter group are partial agonists of acetylcholine. By themselves, they cause depolarization and would therefore, (i) facilitate neuromuscular transmission in small doses; (ii) cause a contracture of denervated muscle. In larger amounts, by causing persistent depolarization they prevent the development of the propagated action potential essential for muscle activation, resulting in loss of muscle tone and relaxation. These actions are well demonstrated by muscle nerve preparations in vitro e.g. rat phrenic nerve diaphragm, or in vivo as in the cat, Tibialis anterior muscle nerve, or gastrocnemius

sciatic nerve preparations. The twitches obtained by stimulation of the nerve would provide a good index of neuromuscular transmission and at the same time, direct muscle stimulation could be done to pinpoint site of action of the drug. Blockade of transmission would manifest as a reduction in height of twitch elicited indirectly, but not that obtained directly.

The depolarizing type of muscle relaxants also possess an interesting property, namely, a dual mechanism of blockade. This consists of two phases; in the first phase the type of blockade is non competitive, while in the second phase it is competitive. It is more easily seen in 'fast' muscles like Gastrocnemius and Soleus than in 'slow' muscles like Tibialis anterior. From these facts it can be appreciated that the actions of curariform and depolarizing agents are mutually antagonistic. Furthermore, the action of curariform agents can be overcome by excess of acetylcholine as provided by anticholinesterase treatment. The overaction of depolarizing type of agents cannot be overcome by any measures, and only eventual repolarization of muscle fibres and endplate region will restore neuromuscular transmission. The nature of post synaptic blockade, whether curariform or otherwise may be confirmed on the isolated frog's rectus abdominis muscle preparation.

As mentioned before, muscle tone maintained by the monosynaptic reflex arc, is regulated by supraspinal influences. These reach the spinal cord by descending fibres which synapse on spinal interneurones. These neurones serve as a relay for the impulse before it reaches the spinal motoneurone. Thus, for an impulse to reach the spinal reflex arc it has to traverse more than one synapse, and these mechanisms are therefore termed polysynaptic. They are evidence of a more advanced form of transmission and are more susceptible to drug action than monosynaptic mechanisms.

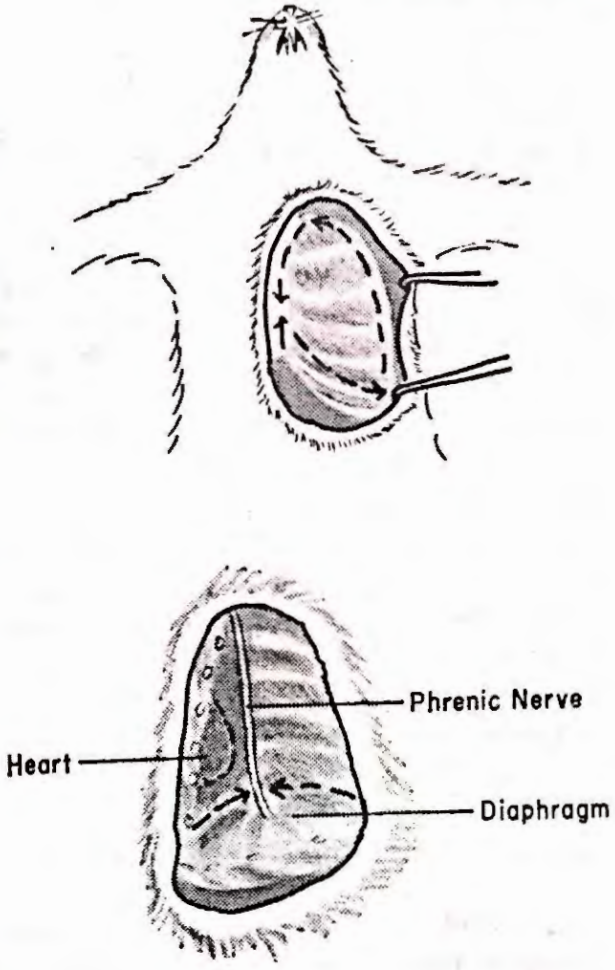
Fig. 3.2 : The polysynaptic pathways in the spinal cord, and the mechanisms of reciprocal inhibition or facilitation.



Muscle tone can therefore be influenced by drugs that act centrally by disrupting supraspinal facilitatory or inhibitory influences (via polysynaptic pathways) on the basic monosynaptic reflex arc. Such drugs will leave the patellar tap unaffected.

The phenomenon of clonus and decerebrate rigidity represent disinhibition of spinal neurones from supraspinal influences, with facilitatory influences dominating. These reflex or linguomandibular reflex; (b) facilitation or inhibition of knee jerk by stimulation of reticular formation in the brain stem in appropriate areas; (c) patellar

Fig. 3. 3 : The isolated rat phrenic nerve - hemidiaphragm preparation.

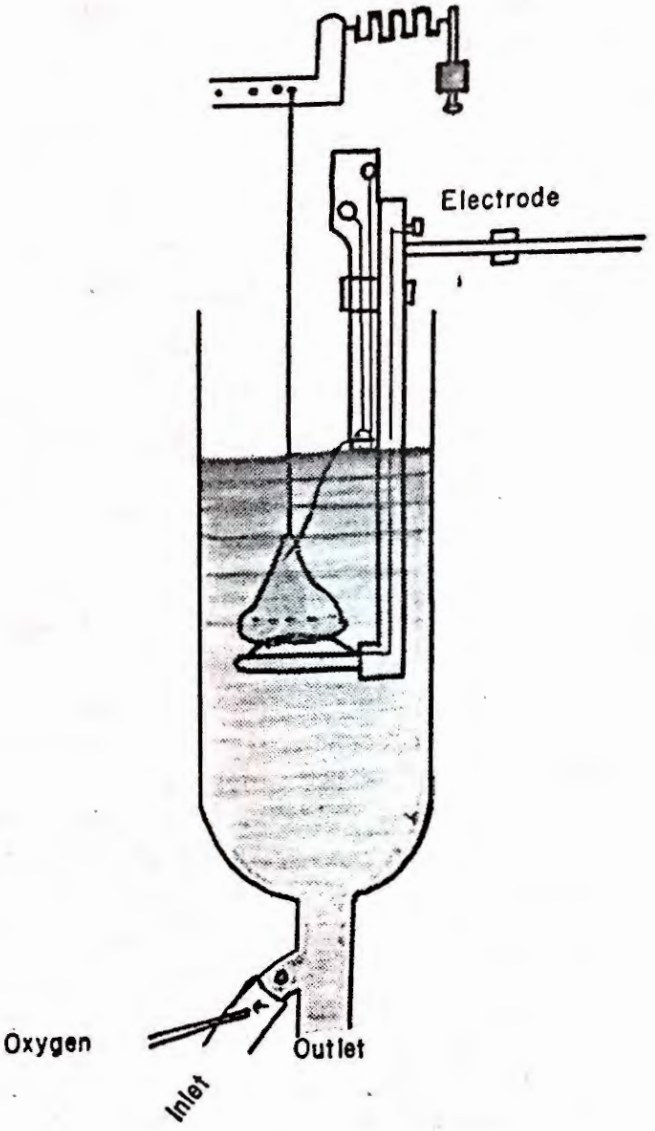


3. 3 - a

The thoracic cage is opened in the direction of the arrows.

3.3 - b

The phrenic nerve is identified by the side of the heart, travelling downwards to its corresponding hemidiaphragm.



3. 3 - c

The final set up of the preparation in krebs solution at 37 °C. During stimulation the fluid level should be lower than the nerve stimulating terminal of the 3-way electrode. The muscle stimulating terminal serves as the support for the preparation.

will accordingly be ameliorated by centrally acting muscle relaxants. Therefore such agents may be investigated by studying (a) polysynaptic reflexes like flexor clonus on decerebration.

The linguomandibular reflex is an example of a polysynaptic reflex. It originates in the sensory fibres of the 5th cranial

(Trigeminal) nerve, is integrated in the spinal cord and medulla, and the afferent pathway is subserved by the facial nerve. It is elicited by electrical stimulation of the root of the tongue and manifests as a jaw — jerk. The action of drugs that produce relaxation of skeletal muscle must therefore be studied on these polysynaptic mechanisms to arrive at a possible central mechanism.

The selectivity of drugs acting on polysynaptic motor pathways, as opposed to other polysynaptic pathways may be studied by simultaneously recording one such response namely the carotid occlusion response or spinal compression vasomotor response, (S.C.V.R.). Failure of a drug to affect this polysynaptic autonomic response implies selective action on motor pathways.

Experimental Techniques

Peripherally acting muscle relaxants.

The isolated frogs' rectus abdominis muscle preparation. The technique has been described in Chapter 1, experiment 6.

The isolated rat phrenic nerve diaphragm preparation.* A rat weighing 150 — 200 gm is killed by stunning and cutting the throat. The skin overlying the xiphoid process of the sternum is picked up and carefully cut upwards, exposing the thoracic cage, and downwards into the abdomen. The thoracic wall is gently separated from the thoracic cage on both sides. Cuts are placed on either side of the sternum as shown in the diagram, proceeding from above downwards, care being taken not to damage the sternal attachment of the diaphragm. From the ends of these cuts, lateral extensions are made so as to expose the contents of the thorax. The diaphragm is identified as the flat pinkish brown structure completely separating the thorax from the abdomen.

The phrenic nerves are seen as thin tough white strands embedded in connective tissue, one on either side of the heart, going up into the neck and down to the respective half of the diaphragm which it supplies. The nerve is gently cleared of connective tissue, care being taken to prevent drying by gently dripping Krebs solution from time to time on the nerve.

A single lobe of the diaphragm is chosen for the experiment, and is cut in the direction of its fibres. This gives a triangular segment, the apex being the tendinous portion and the base a strip of muscle attached to the ribs (which are cut along with it). The phrenic nerve is cut in the neck and cleared to the point where it enters the muscle substance near the tendinous apex. This preparation now consists of a nerve and hemidiaphragm.

It is tied onto a perspex electrode with three terminals which can be connected as described. The strip of ribs is tied onto the horizontal bar of the electrode while the nerve is gently placed on the sliding terminal. This preparation mounted on the electrode is now set up in a large organ bath (capacity about 80 ml) in Kreb's solution, maintained at 37°C and aerated with oxygen.

A suture through the tendinous apex connects this preparation to a Straub's lever with minimal inertia, recording the muscle twitches on a smoked drum. These twitches are elicited either by direct stimulation of the muscle or by indirect stimulation through the nerve, each requiring correct connection of the appropriate terminal. Stimuli are obtained from a square wave pulse generator, the voltage, pulse width and frequency being adjusted optimally for each preparation. The response elicited by both direct and indirect stimulation is a quick sharp downward twitch, with a rebound flyback upwards due to the spring of the lever.

Drugs under consideration are added to the bath fluid and their actions on the

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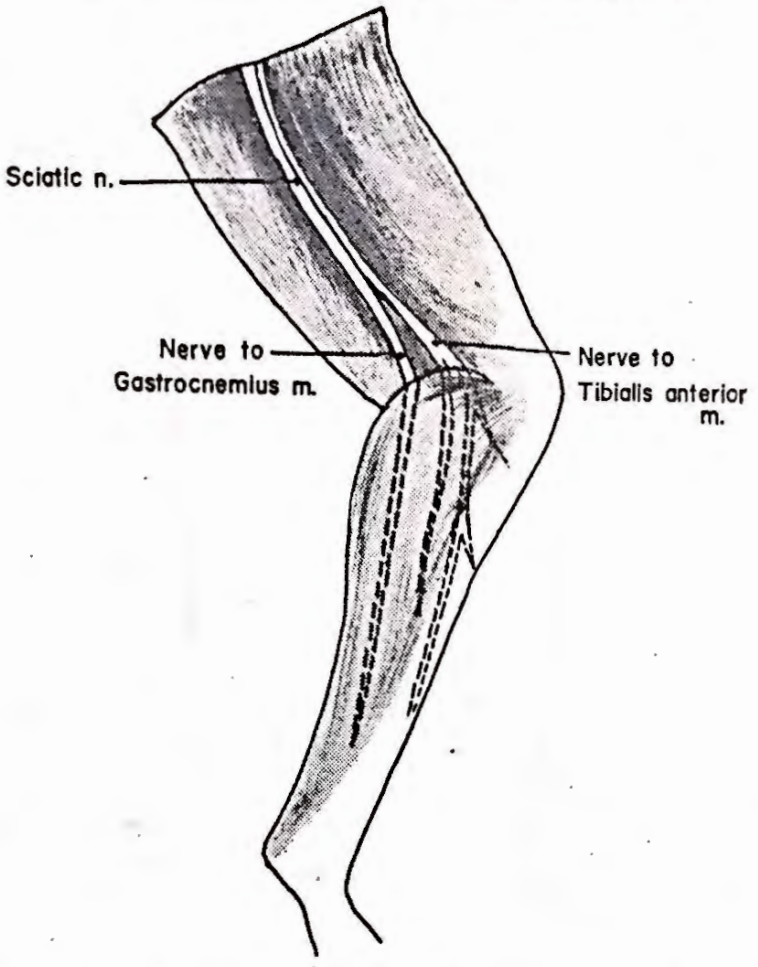
Bulbring, Edith, (1946) Br. J. Pharmac Chemother. 1. 38.

muscle twitches elicited directly and indirectly are studied.

The tibialis anterior muscle nerve preparation of cat. A cat weighing about 3.0 kg is anaesthetised with ether and maintained on chloralose as described in chapter 2. (Also refer Appendix I).

After the level of anaesthesia has been allowed to stabilise for about 40 min, the dissection may be commenced. The fur on the dorsal aspect of the thigh and all over the lower leg is removed by close clipping. A vertical incision is taken in the skin over the dorsal aspect along its length so as to expose subcutaneous fat and muscle. All the vessels are carefully ligated and the gap between the two major groups of muscles is opened up gently to expose the sciatic nerve in the floor of the gap. This nerve comprises two trunks, a thicker medial, and a thinner lateral supplying the Tibialis anterior muscle. This nerve is carefully isolated, and a loose ligature placed under it, the entire gap being filled with liquid paraffin to prevent drying of the nerve.

Fig. 3.4 : Simplified anatomy of the hindleg of cat.

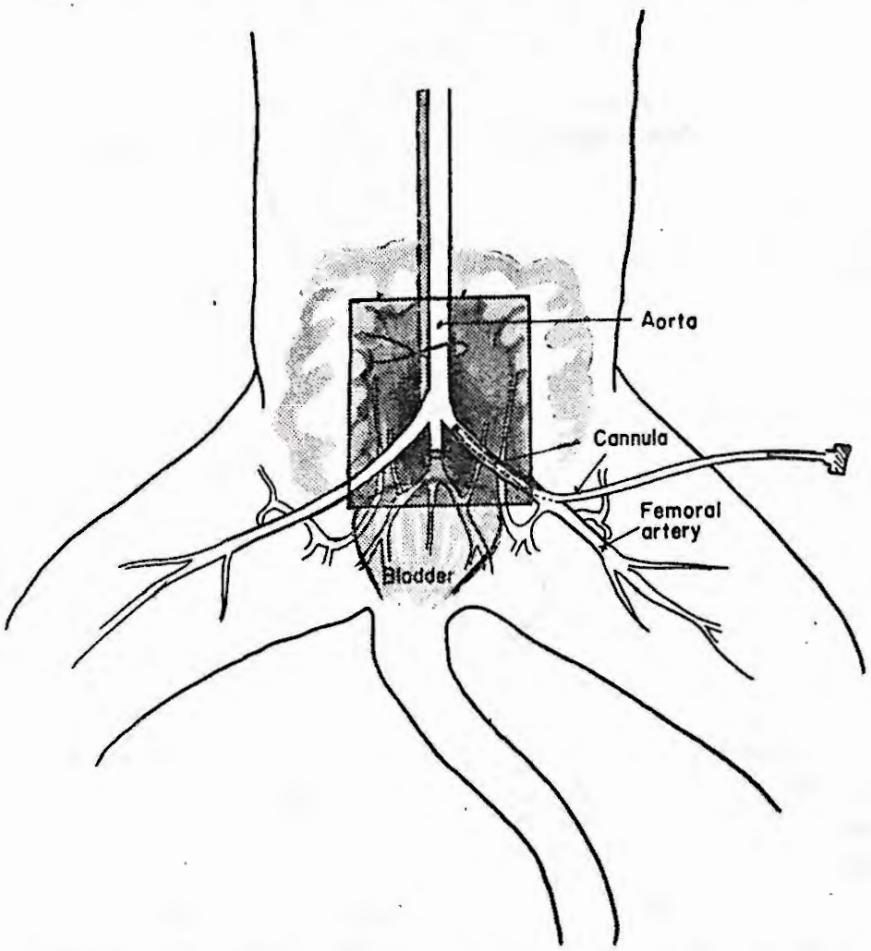


A steel pin is now driven through the lower end of the femur to fix the knee joint. (When a muscle contraction has to be recorded, one end must be fixed so that the entire contraction is transmitted to the recording lever. In this case, the Tibialis anterior under study takes its origin from the lower end of the femur which is therefore kept fixed). The entire muscle is freed of its surrounding structures through a longitudinal skin incision, and the tendon of insertion located on the anterior aspect of the ankle. Here it is in relation to a large vein which must be carefully ligated and cut. The tendon may be identified as the large, flat shining white structure just

beneath the vein. The tendon is transfixed, ligated and cut, the suture being led over a pulley to be attached to the bar of a Palmer isometric lever.

For the purpose of close intra-arterial injection of drugs, the contralateral femoral artery is cannulated through an inguinal incision, and the tip of the cannula guided through an abdominal laparotomy to the bifurcation of the aorta. The cannula is tied securely in place and the abdomen closed in layers. Injections made into this cannula will be pushed by aortic blood flow into the ipsilateral femoral artery without the disadvantage of occluding the blood supply to the limb.

Fig. 3.5 : Close intra-arterial injection in the femoral artery of cat.



The cannula is introduced into the contralateral femoral artery and guided to the bifurcation of the aorta.

For the purpose of the experiment a bipolar electrode is placed on the nerve to the *Tibialis* anterior and stimuli delivered to the muscle nerve preparation from a square wave pulse generator, the parameters of the stimuli being adjusted optimally for each experiment. Isometric twitches are recorded on a smoked drum, and the effects of the drugs studied.

4. **The gastrocnemius-sciatic muscle nerve preparation of cat.** A cat of about 3.0 kg weight is anaesthetised with ether and chloralose, and the level of anaesthesia allowed to stabilise for about 40 min.

The nerve supply of the gastrocnemius arises above the level of bifurcation of the sciatic nerve, and hence, this nerve is isolated on the posterior aspect of the hip joint rather than in the thigh. It is found in the floor of an easily palpable gap between two bony prominences on the posterior aspect of the hip joint. After isolation

a loose ligature is placed under it for further manipulations. It is preferred to tie an additional ligature on the nerve itself cephalad to the point of stimulation so that stimuli do not reach the spinal cord by antidromic (retrograde) conduction.

The gastrocnemius muscle is the fleshy muscle or the calf muscle on the posterior aspect of the leg, and the twitches are recorded from the tendon of this muscle in a manner similiar to experiment 3.

Centrally acting muscle relaxants.

5. **Monosynaptic reflexes in cat.** A cat weighing about 3.0 kg is anaesthetised with ether chloralose. The trachea is cannulated routinely.

A pin is driven through the lower end of the femur and the knee joint maintained at 90° flexion, with free movement. The position of the cat is adjusted such that the hip is also at 90° flexion. A suture is taken through the Tendoachilles, and connected to the recording system, either a Palmer isometric lever writing on a kymograph, or a heavy pressure transducer, and a polygraph. A patellar hammer driven electrically is fixed over the knee joint such that the hammer lightly taps the tendon

Fig. 3.6 : Simplified anatomy of the cat's hindleg..

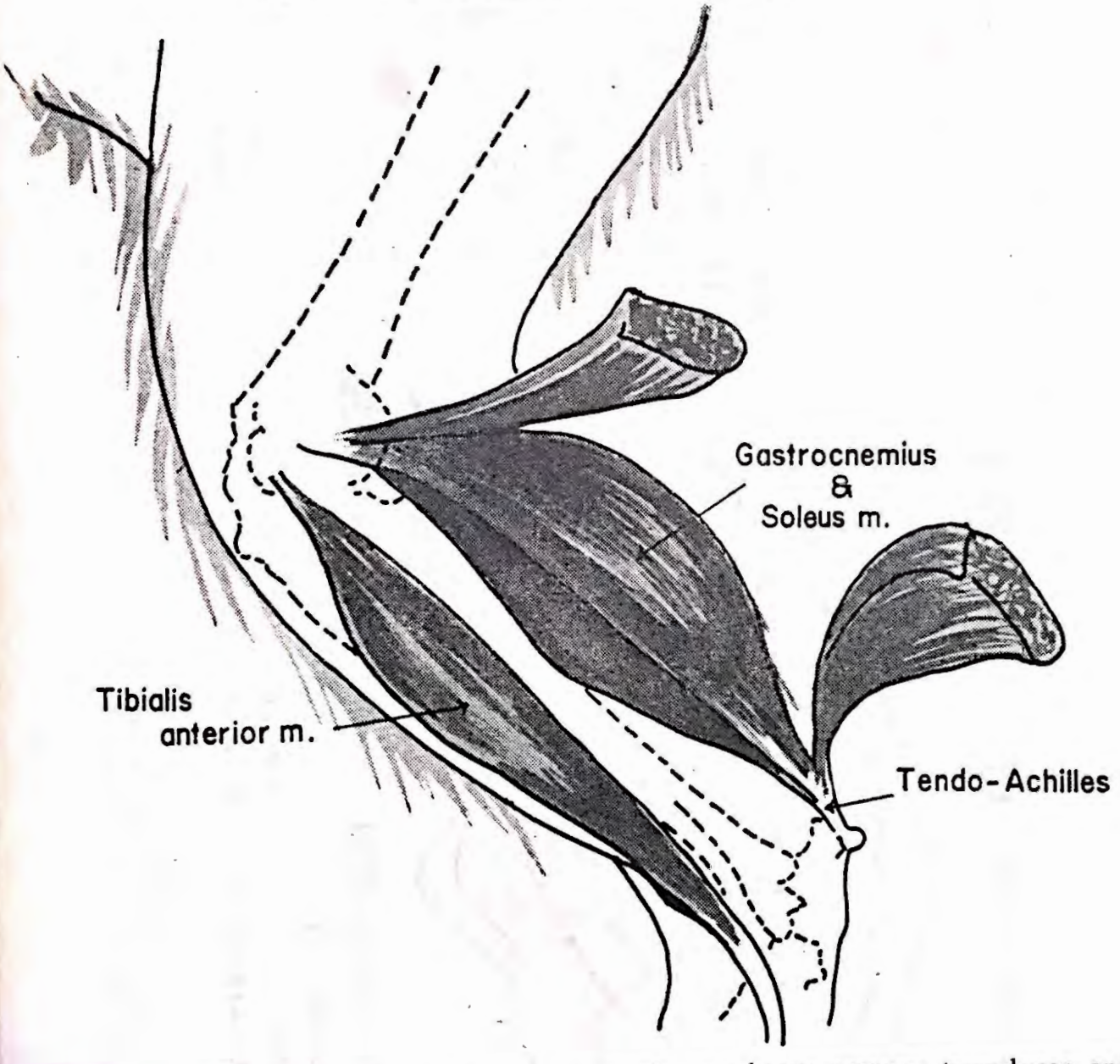
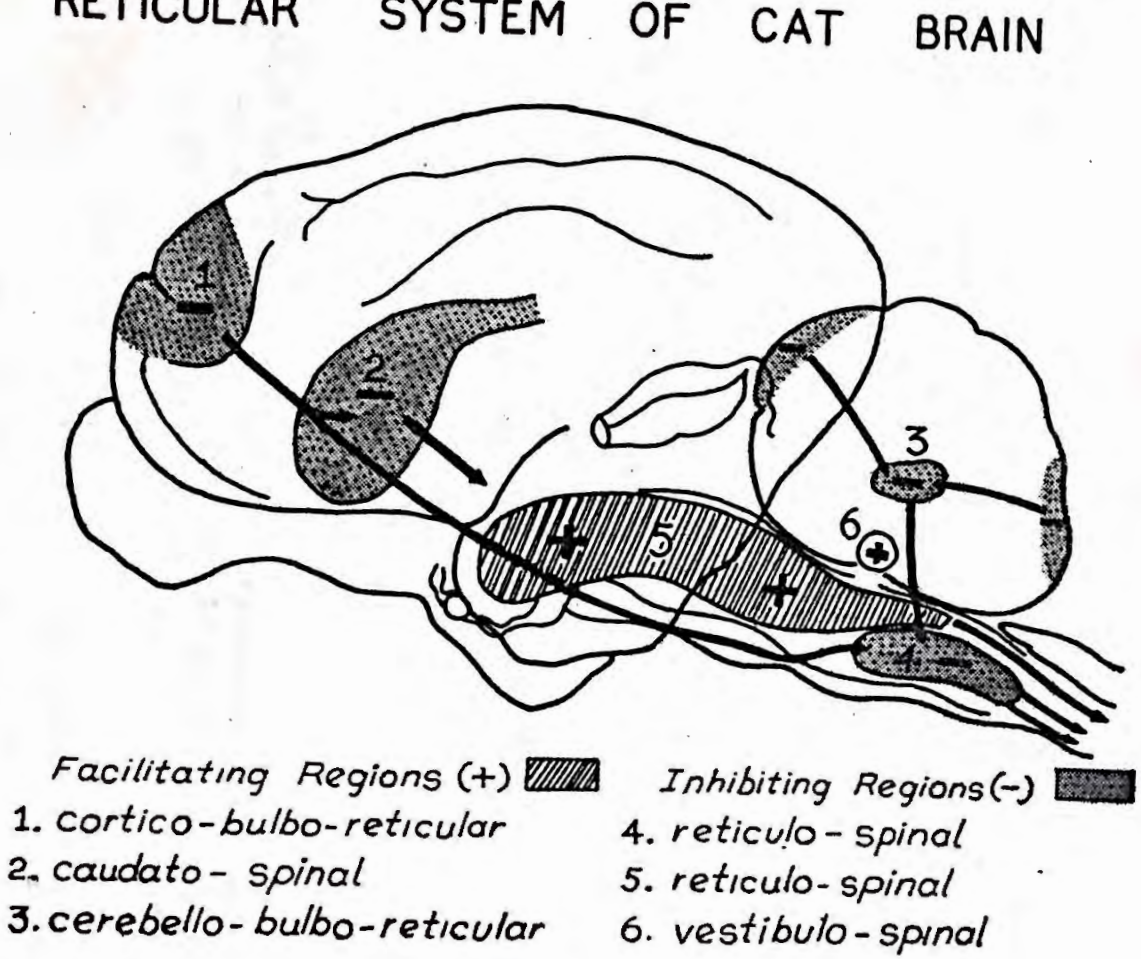
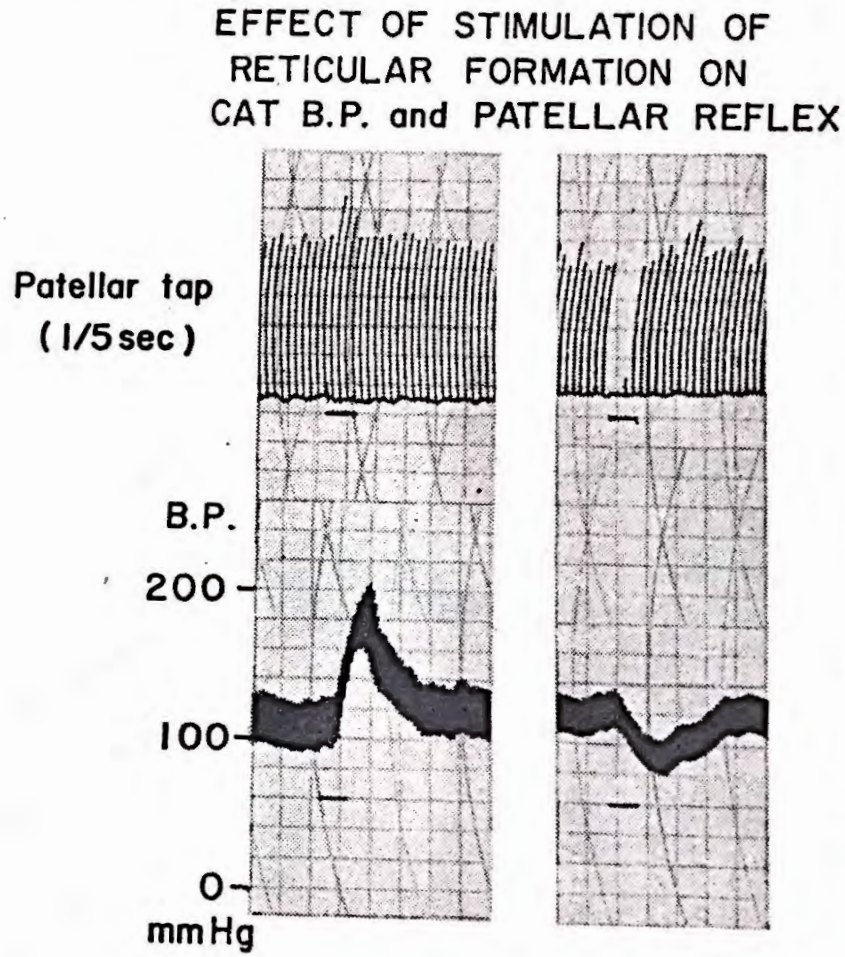


Fig. 3.7 : Stimulation of reticular system of cat brain.



3. 7 – a

The anatomical basis for the experiment, depicting the relevant tracts and the areas to stimulate them.



3. 7 – b

The pattern of the recording obtained by stimulation of the facilitatory area (1st panel) and the inhibitory area (2nd panel).

of the Quadriceps femoris, and elicits a knee jerk. The frequency of this tap is adjusted as desired, and a record of the knee-jerk is obtained.

6. **Polysynaptic reflexes.** The simplest method is to elicit the flexor reflex by stimulating the central end of the posterior tibial nerve, and recording the contractions of the Tibialis anterior muscle. Alternatively the central cut end of the sciatic nerve distal to the origin of the nerve supply of the Tibialis muscle may be stimulated.

7. **Linguomandibular reflex.** Needle electrodes inserted into the root of the tongue deliver rectangular pulses, eliciting the jaw-jerk as described above.

8. **Effect of stimulation of Reticular** formation. A cat weighing between 2.8 and 3.5 kg is anaesthetised with chloralose and positioned in a stereotaxic instrument (Horsley Clarke). The skin and muscle over the occipital region is divided and the bone exposed. A flap of this bone is removed and the reticular formation in the floor of the IVth ventricle is stimulated by using the appropriate co-ordinates defined by Alexander. The effect of such stimulation on the patellar reflex and blood pressure recorded from the femoral artery, is studied, before and after administration of drug.

9. **Decerebrate Cat.** A cat is anaesthetised with chloralose, and the trachea cannulated. It is then placed in the prone position, with the head maintained in the fully flexed position by an assistant. The skin and muscles overlying the parietal region are divided to expose the bone.

A trephine hole is made on an imaginary line joining the ears, with a minimum distance of 0.65 cm from the midline. The circular disc of bone is then removed and the dura mater carefully slit open. A pliable strip of plastic, or a flat strip of metal curved at the end with a radius of curvature of approximately 5.0 cm, is then inserted into this opening and moved as described. It is thrust forwards until it hits the opposite parietal bone, then swept in an arc backwards until one edge touches the bony part of the tentorium cerebelli. Now it is rotated through 120°, so that the upper surface becomes the lower surface, resting on the tentorium. It is then slid forwards and downwards along the tentorium, and a mid-collicular section of the brain stem thus effected. Care should be taken not to point the handle downwards and backwards as this would damage the medulla.

Practical Points:

1. Venous bleeding may be controlled by simple elevation of the head.
2. Bony bleeding may be arrested by application of Horsley's bone wax or plasticine.
3. Arterial bleeding arises from branches of the vertebral arteries and may be stopped by application of pressure on the artery against the laminae of the atlas vertebrae.
4. Oozing from muscle and skin can be checked by warm (60°C) saline compresses which have been squeezed dry before application.

The patellar reflex of this cat is recorded 60 min after the decerebration procedure and the effect of putative centrally acting muscle relaxants investigated.

Practical Exercises

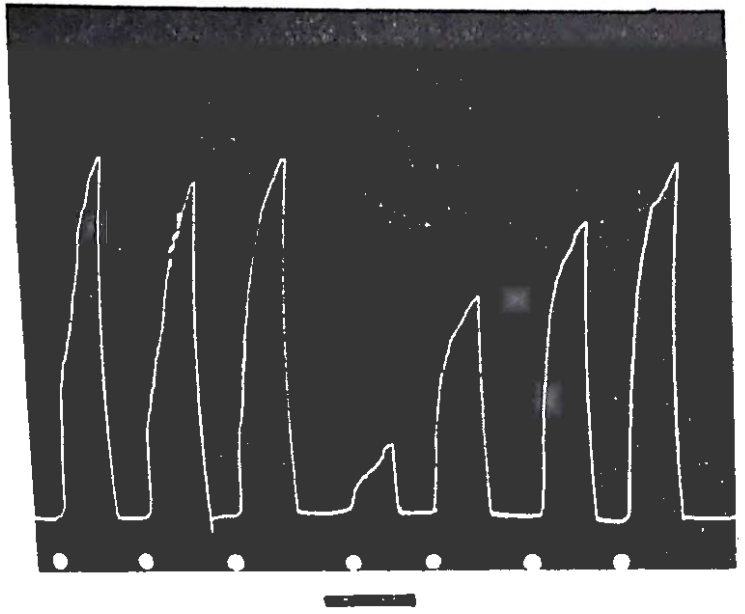
Exercise 1.

Objective : To study the effect of d-tubocurarine on acetylcholine induced muscle contraction.

1. Set up an isolated preparation of Frog's rectus abdominis muscle.
2. Elicit responses to acetylcholine as shown in the figure.
3. Add d-Tubocurarine in an equivalent strength and allow it to act for 3 minutes.
4. In the presence of d-Tc elicit a response to the same dose of acetylcholine as before.
5. Observe the diminution in magnitude of response.
6. Wash out the bath fluid repeatedly.
7. Record responses to acetylcholine.
8. Observe that the blockade produced by d-Tc is reversible.

SKELETAL MUSCLE RELAXANTS

Isolated frog's rectus abdominis muscle



At dots : 4.0 µg acetylcholine were allowed to act for 90 sec.
Horizontal bar indicates presence of d-TC (10 µg) allowed to act for 3 min.
BATH CAPACITY - 20 ml

Fig. 3-8

Exercise 2:

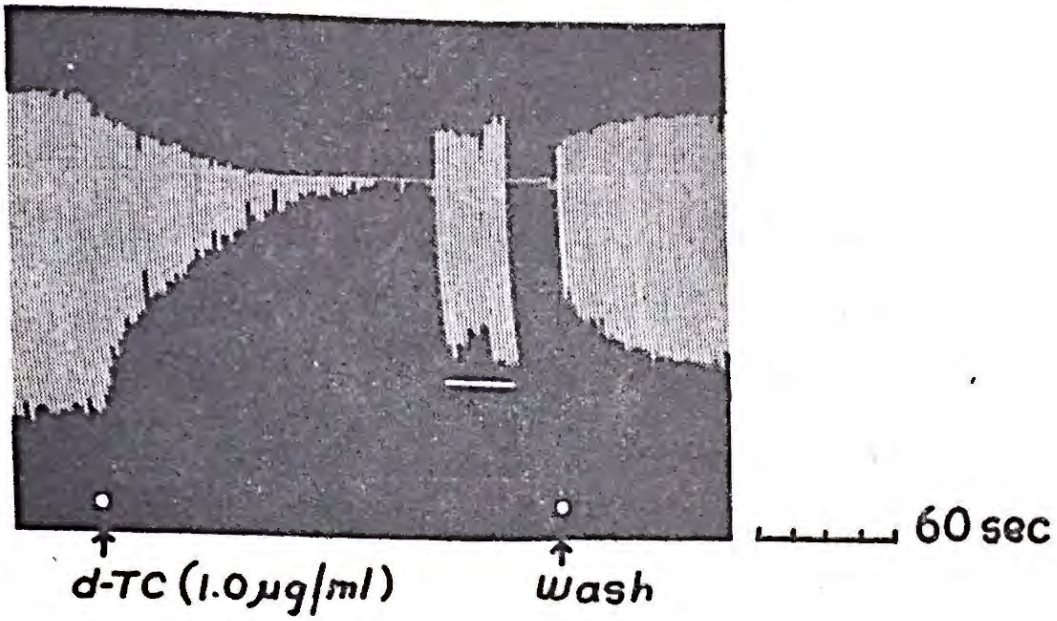
Objective: To study the effect of d-tubocurarine on neuromuscular transmission.

1. Set up an isolated preparation of rat phrenic nerve-hemidiaphragm as described.
2. Obtain a recording of muscle twitches by electrical stimulation of the nerve.
3. Add d-Tc to the bath fluid.
4. Observe the gradual diminution in height of twitches obtained by nerve stimulation.
5. Stimulate the muscle directly.
6. Observe that d-Tc does not affect direct muscle stimulation.
7. Wash out the bath.
8. Note that the twitches reappear immediately indicating the reversibility of action of d-Tc.
9. Repeat the addition of d-Tc.
10. In presence of d-Tc, add neostigmine in the dose indicated.
11. Observe that the action of d-Tc can be partly overcome by excess of acetylcholine provided by neostigmine treatment.
12. This experiment has therefore demonstrated that d-Tc;

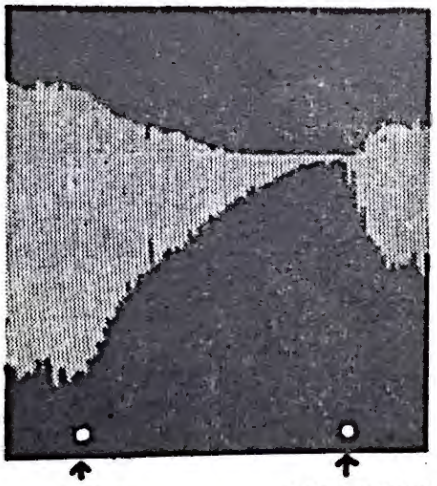
    (i) blocks neuromuscular transmission because indirect, but not direct, stimulation of muscle twitch is blocked.

    (ii) shows competitive antagonism to acetylcholine.

Isolated rat's phrenic nerve diaphragm



White bar indicates direct stimulation of muscle



BATH CAPACITY - 80 ml

PARAMETERS OF STIMULATION

VOLTAGE - 2-4 Volts : DURATION - 5 msec

FREQUENCY - 1/10 sec

For direct stimulation - 8-10 Volts

Fig. 3-9

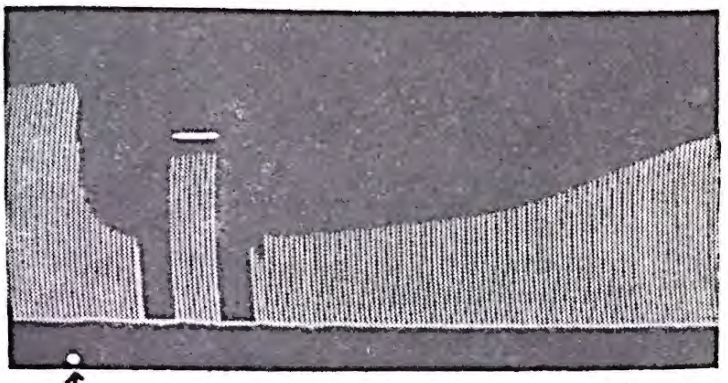
Exercise 3 :

Objective : To study the effects of peripherally acting skeletal muscle relaxants on the neuromuscular junction.

1. Set up a Tibialis anterior muscle nerve preparation as described in an intact cat.
2. Inject d-Tc (150 μg) intra-arterially.
3. Observe reduction in the height of twitches.
4. Directly stimulate muscle and observe failure of blockade by d-Tc.
5. After the twitches have regained the original height, repeat the injection of d-Tc (150 μg).
6. Immediately inject neostigmine (100 μg) and observe the dramatic recovery in this case, as opposed to gradual recovery in absence of neostigmine.
7. Repeat d-Tc, and inject succinylcholine 50 μg immediately.
8. Observe rapid recovery of twitches, though not as rapid as with neostigmine.
9. It may be concluded that succinylcholine has a partial agonistic action at the neuromuscular junction, thus being able to antagonize d-Tc, though less active than neostigmine.

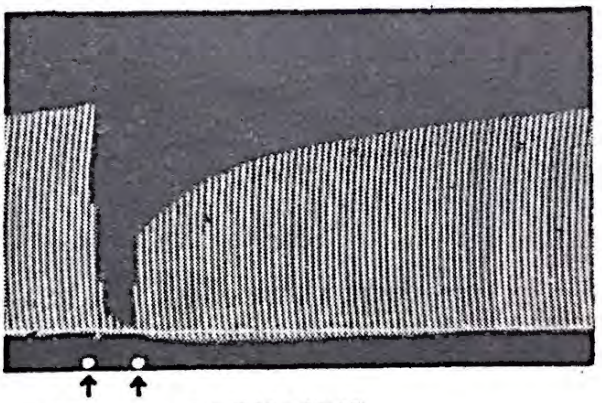
SKELETAL MUSCLE RELAXANTS (PERIPHERAL)

Cat tibialis anterior muscle nerve preparation



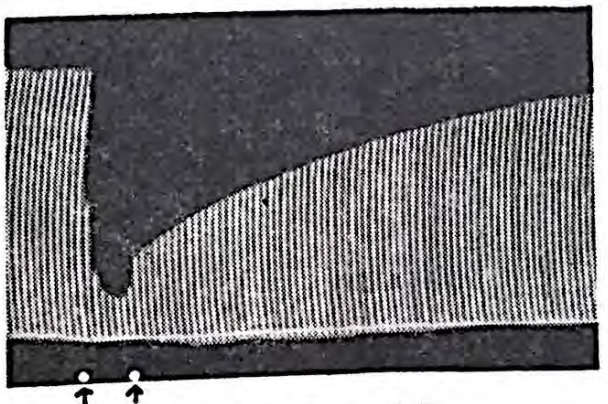
d-TC (150 μg)

White bar indicates direct stimulation of muscle



60 sec

d-TC (200 μg) NEOSTIGMINE (100 μg)



d-TC (200 μg) SUCCINYLCHOLINE (50 μg)

All injections made intra-iliac

PARAMETERS OF STIMULATION

VOLTAGE – 4-6 Volts DURATION – 5 msec
FREQUENCY – 1/10 sec

For direct stimulation voltage was 8-10 volts

Fig. 3-10

Exercise 4:

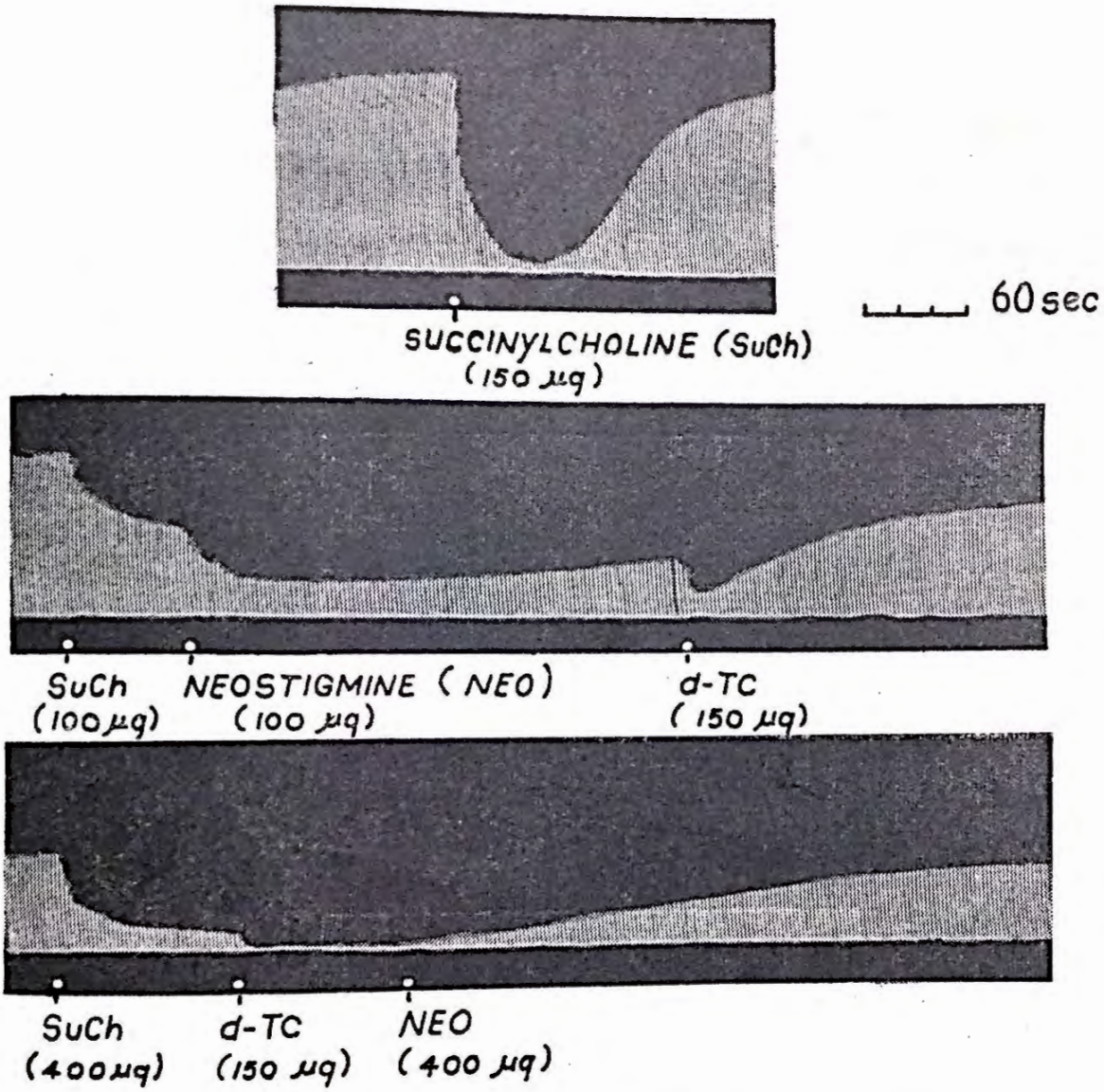
Objective: To demonstrate the phenomenon of dual blockade.

1. Set up a preparation of Gastrocnemius muscle — sciatic nerve in intact cat.
2. Inject succinylcholine (150 μg) intra-arterially and observe the degree of blockade of neuromuscular transmission.
3. Choose the appropriate dose of succinylcholine (100 μg) so as to get only partial blockade.
4. Inject neostigmine (100 μg) and note the worsening of the blockade.
5. Inject d-Tc and observe a partial recovery of twitches to nerve stimulation. This is phase I of dual block.
6. Repeat succinylcholine carefully until tachyphylaxis sets in, i.e. until twitch heights are no longer decreased by succinylcholine in the same dose.
7. Now inject a larger dose of succinylcholine, and observe reappearance of neuromuscular blockade.
8. Inject d-Tc and observe a worsening of the effect.
9. Inject neostigmine and observe recovery of twitches to their original height.

This is phase II, or the competitive phase, of dual blockade by succinylcholine.

SKELETAL MUSCLE RELAXANTS (PERIPHERAL)

Cat gastrocnemius muscle nerve preparation



A: shows spontaneous recovery after succinylcholine.

B: shows phase I of dual block.

C: shows phase II of dual block.

PARAMETERS OF STIMULATION

VOLTAGE : 6-10 Volts

DURATION : 5 msec

FREQUENCY: 1/10 sec

Fig. 3-11

Exercise 5 :

Objective : To study the effect of centrally acting muscle relaxants on somatic reflexes in the cat.

1. Set up a recording of the linguomandibular, flexor and patellar reflexes in a chloralose anaesthetised cat.
2. With the parameters of recording kept constant, administer mephenesin (25 mg/kg) i.v. slowly.
3. Observe selective depression of the polysynaptic reflexes viz. linguomandibular and flexor reflexes, while the patellar reflex is unaffected.

SKELETAL MUSCLE RELAXANTS (CENTRAL)

SOMATIC REFLEXES IN CAT

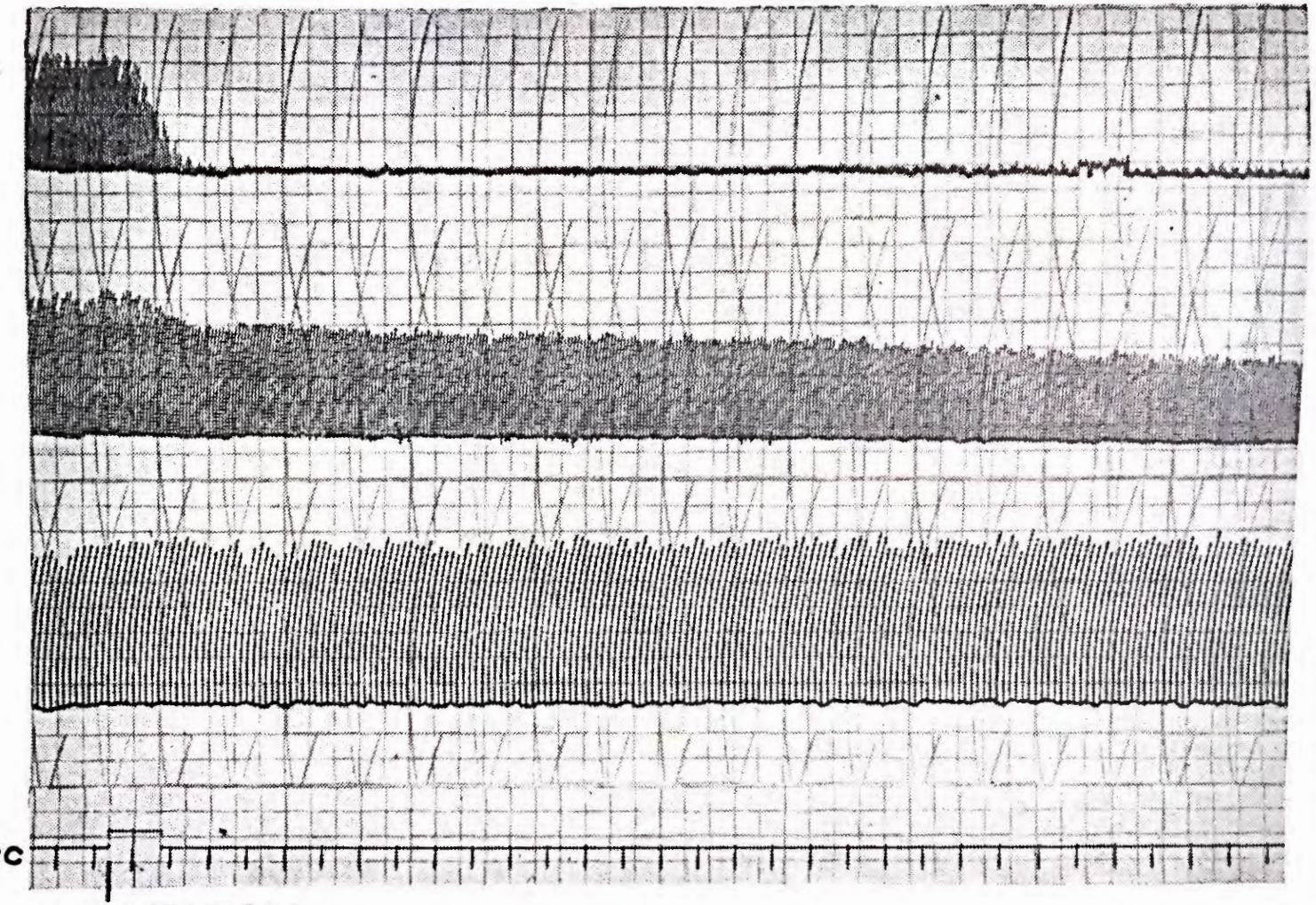


Fig. 3-12

Exercise 6 :

Objective : To study the effect of centrally acting muscle relaxants in decerebrate cat.

1. Perform decerebration of an ether anaesthetised cat as described before.
2. Set up a recording of patellar reflex.
3. Observe that instead of a single sharp, jerk, a series of oscillations or clonus is obtained with every tap.
4. Inject a centrally acting muscle relaxant like mephenesin.
5. Observe that the clonus is markedly damped with only a few small oscillations with every tap.

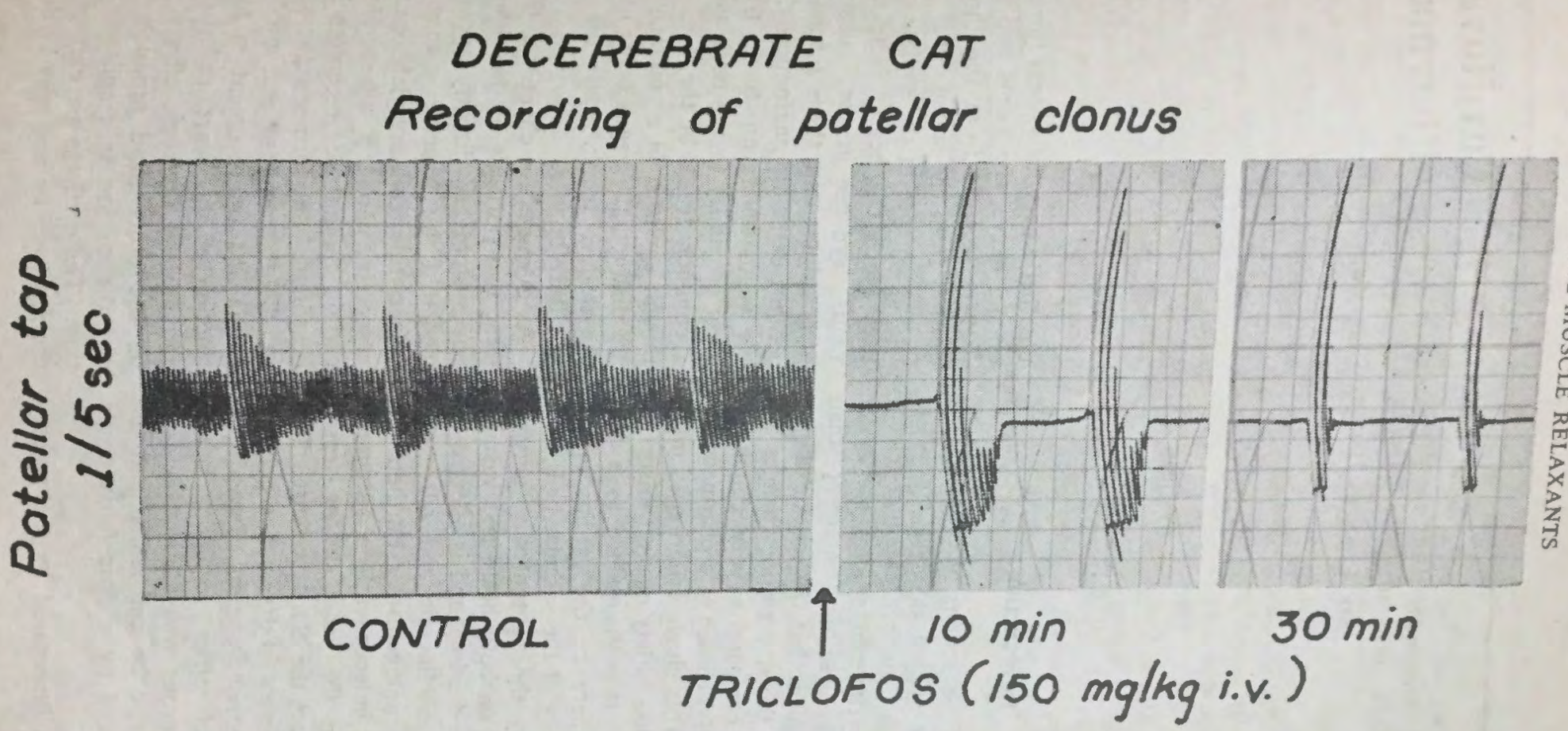

DECEREBRATE CAT
Recording of patellar clonus
Patellar tap
1/5 sec
CONTROL
10 min
30 min
TRICLOFOS (150 mg/kg i.v.)

Fig. 3–13

Cardiovascular Pharmacology

Our road does not lie on a level, but ascends and descends, first ascending to axioms, then descending to works.

SIR FRANCIS BACON

Introduction

A study of the actions of drugs on the cardiovascular system involves consideration of a large number of inter-related factors. Experimental studies are easier to perform, analyse and interpret than clinical assessment, but the criteria chosen are similar. Blood pressure changes are an index of comparison very commonly (if not routinely) chosen in a pharmacology laboratory. This single criterion is influenced by a wide variety of mechanisms. In the first place, it is maintained by a delicate balance between the cardiac output and the capacity of the vascular bed. It is controlled by the autonomic nervous system acting on both heart and vasculature as the need arises, and is affected accordingly by cholinergic and adrenergic agonists and antagonists. Furthermore, drugs that act independently of autonomic innervation on the heart and/or vessels would also affect the blood pressure. This index of assessment of drug action is hence qualitatively extremely useful. In addition, the interplay of other regulating factors like central nervous system, electrolyte intake etc. can also be understood and the changes produced by a drug fairly easily quantified.

The heart is innervated by the parasympathetic nervous system through the 10th cranial nerve, the vagus and by the sympathetic system from the thoraco-lumbar outflow from the spinal cord. These latter nerves synapse in the stellate ganglion and then reach the heart. The fibres of the vagus synapse in the substance of the myocardium and these intramural ganglia are not definable anatomically. Generally, the vagus and sympathetic trunks are in close proximity in the neck and may form a common trunk in the dog. In the cat, though enclosed in a common sheath they are distinct, the vagus being identifiable as the fleshier trunk, while the sympathetic is a finer bundle.

As discussed in chapter 2, the actions of these two portions of the autonomic nervous system are directly opposite and are mediated by specific transmitters, norepinephrine and acetylcholine. They act respectively on β adrenergic and muscarinic cholinergic receptors in the myocardium. The former subserves the "fight or flight" response and produces tachycardia and improvement in force of contraction of the heart, called chronotropic and positive inotropic effects. The vagus on the other hand produces bradycardia and diminution in amplitude of contraction. These effects are exerted directly on the myocardium and may be duplicated on

isolated preparations of this muscle such as rabbit atria (Exercises 1—3).

The myocardium derives its energy requirements from coronary arterial blood supply, and any alteration in this arterial system would hamper myocardial performance, as illustrated in the pathological condition of coronary heart disease. This disease calls for drugs that can dilate the narrowed lumen of the coronary arteries selectively, without deleterious effect on the myocardium. A simple method of studying both myocardial performance and coronary arterial calibre as reflected by coronary flow, is the isolated perfused rabbit heart preparation of Langendorff. However, it must be remembered that drugs that increase myocardial work can secondarily improve coronary flow in absence of significant arterial disease as may be noticed with epinephrine in exercise 4. Extrapolation of results from such normal hearts to clinical situations is therefore unwarranted. Further, the removal of extraneous factors such as innervation, effect of venous return, peripheral resistance of arterial bed etc causes the emergence of an over-simplified picture. There is as yet no selective coronary vasodilator of therapeutic efficacy. The coronary vasodilator action of sodium nitrite (Exercise 4) is not the basis of its efficacy in angina pectoris; a venodilatation and reduction in venous return causes a diminution in cardiac output and reduces the work load on the heart which secondarily relieves anginal pain.

Another method of studying a direct vasodilator action which has the advantage of being uninfluenced by cardiac performance is the method of perfusion of isolated regions of the body for example rabbit ears, cat hindquarters (Exercise 5) etc. As explained in the method later in this chapter, the perfusion of hindquarters is a closed circuit with a constant pressure maintained by a Sigmamotor pump. A change in calibre of the blood vessels would alter the capacity of the vascular bed, and cause a fall in the perfusion pressure being recorded. Such a preparation is useful for the study of vascular changes in absence of reflex adjustments or myocardial action of drugs. It is necessary to pinpoint the site of action of drugs producing hypotension in routine screening experiments. Having looked for direct cardiac and vascular effects, the action of the drug on adrenergic transmission (which is the sole autonomic regulator of blood pressure) is investigated. This may be conveniently done by means of the cat's superior cervical ganglion-nictitating membrane preparation (Exercises 6a, b and c). Generally this is accompanied by a blood pressure recording with the help of which specific agonist-antagonist interactions can be pinpointed as for example, if a drug is producing hypotension by α -adrenergic blockade, the pressor response to exogenous epinephrine and norepinephrine will be selectively blocked. The superior cervical ganglion is a sympathetic ganglion with easily accessible pre- and post-ganglionic nerve trunks. The end point of its stimulation is a contraction of the smooth muscle of the nictitating membrane of the eye, which can be easily recorded on a smoked drum/polygraph.

A hypotensive drug which blocks ganglionic transmission will leave responses to a post-ganglionic stimulation and injected epinephrine unaffected while transmission of pre-ganglionic stimuli will be blocked. This blockade will also occur with adrenergic neuron blockade but the character is somewhat different. The effect of prolonged stimulation e.g. for 90 seconds easily differentiates the two, for example exercises 6a and 6b. A well sustained contraction of the nictitating membrane and a plateau-shaped tracing are found with neuron blockade. An α -receptor blocking agent would block the effects of nerve stimulation as well as injected epinephrine. A useful scheme for localising the site of action of a hypotensive drug is

included in the Appendix.

Maintenance of blood pressure involves the property of contractility of the myocardium; the other properties of this tissue for example conduction, impulse generation etc. are of importance when one considers the organism as a whole. The rhythm of the heart beat is a sum of all these actions, and plays a major role in normal cardiovascular functioning. Alterations in this rhythm in disease states or following drug therapy, have therefore to be set right with the help of antiarrhythmic drugs. The actions of such drugs are often needed in arrhythmias induced by digitalis therapy, and hence the induction of experimental arrhythmias with digoxin serves a dual purpose as a model for antiarrhythmic agents in general and those useful in digitalis arrhythmias in particular (Exercise 7).

Experimental Techniques

1. **The isolated perfused heart of rabbit or the preparation of Langendorff.** A rabbit weighing about 2.0 kg is killed by stunning and cutting its throat to exsanguinate it. The thorax is opened quickly, cutting the sternocostal junctions with a pair of large scissors. The heart is seen behind the sternum, beating in its pericardial covering. This fine membranous covering is carefully picked up and opened out, exposing the heart and the great vessels. The heart and half inch of aorta are removed by cutting all these attachments and placed in a shallow dish containing warm Ringer Locke solution through which oxygen is bubbled. The heart is gently squeezed to remove blood from the cavities of atria and ventricles, and to prevent clotting inside the coronary arteries.

The heart is then cleared of extraneous connective tissue and the aorta tied in position over the tip of the glass cannula in the Langendorff apparatus. A suture is taken through the apex of the ventricle and the thread is led over the system of pulleys to the bar of the Starling lever which records the contractions on a smoked drum.

The coronary flow is measured in terms of cardiac output per minute because in this preparation, the fluid enters the aorta, the coronaries, the right atrium, right ventricle and leaves by the cut ends of the pulmonary arteries. Drugs to be studied for their action on coronary flow and rate and amplitude of heart beat, are injected into the side limb of the cannula in the aorta and the actions noted.

Practical points: It is generally advisable to adjust the rate of flow of perfusion fluid in the Langendorff apparatus and maintain the temperature at about 37° C. The aerating mixture used is oxygen. The solution to be perfused (Ringer Locke) should be filtered through a cinter-glass funnel by vacuum filtration so as to remove all particulate matter.

2. **The isolated rabbit atria.** A rabbit weighing between 1.5 and 2.0 kg is killed by stunning and cutting its throat and the heart removed as described in experiment 1. It is placed in Ringer Locke solution in a shallow dish and the excess fat is carefully but rapidly trimmed off.

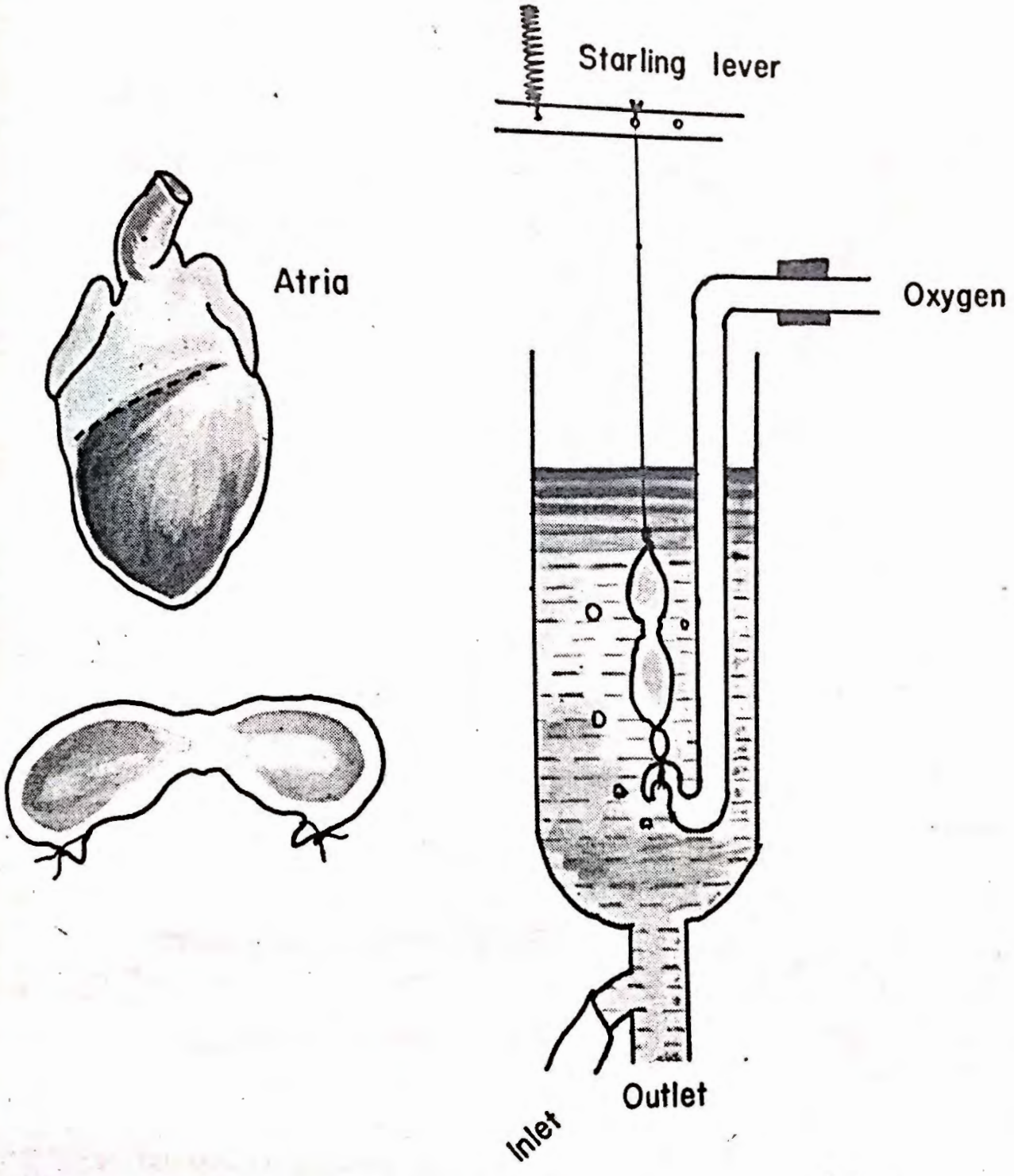
With a pair of fine pointed scissors, the heart is cut along the atrioventricular junction and the dark brownish ventricles discarded. The atria are left behind as a rapidly beating pale pink leafy pair of structures. They are carefully opened out and threads tied onto either end. The atria are then mounted onto an oxygen tube in a large bath in Ringer Locke solution aerated with oxygen and their contractions recorded by a Starling lever on a smoked drum.

Drugs under study are added to the bathing fluid. Practical points: (1) This preparation does not need a bath temperature of 37° C. (2) The automaticity of

the node in the right atrium may be increased by using Feigan's solution as the perfusion medium (Refer Appendix 4).

(3) In experiments where the spontaneous pacemaker is not required or is to be excluded, the left atrium alone may be

Fig. 4. 1: The isolated atrial preparation of the rabbit.

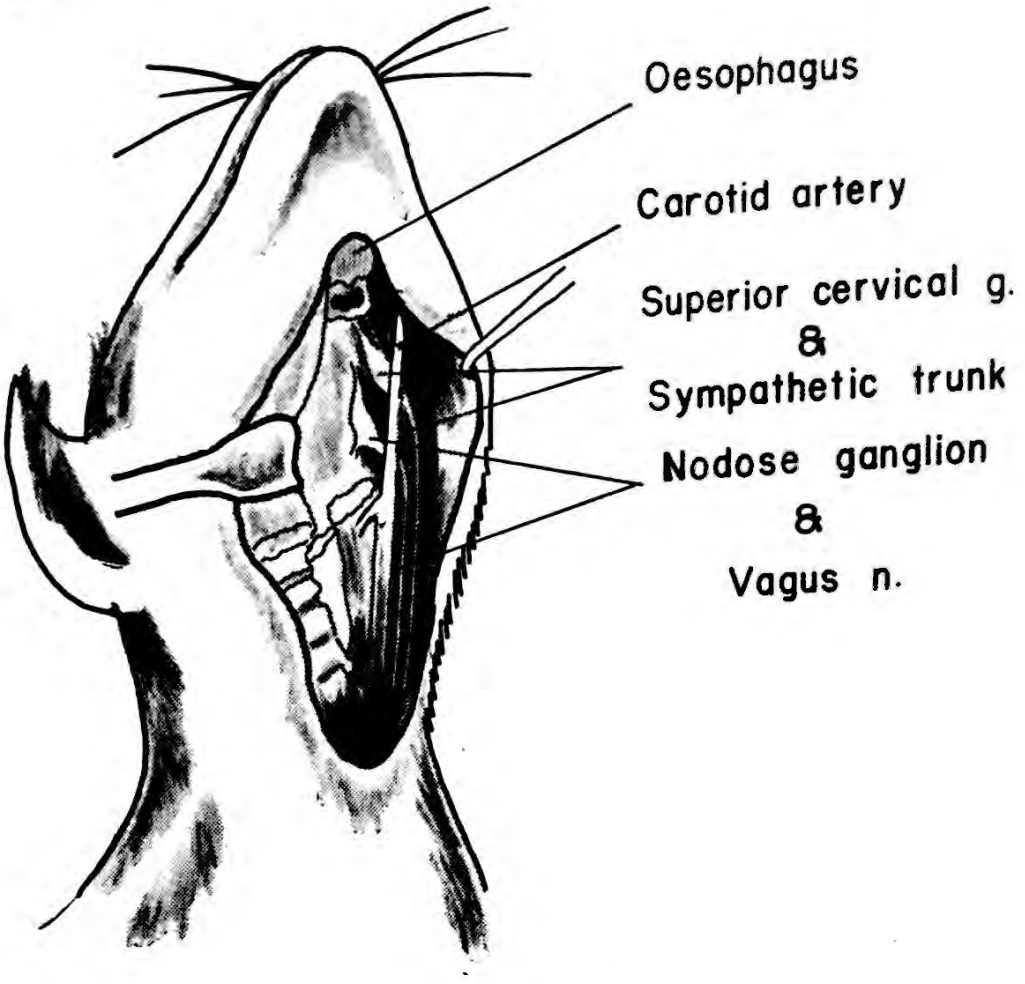


4. 1 – a
The distinction between atria and ventricles is made by the separating groove and the difference in colour.

4. 1 – b
The atria are dissected free and the ends tied for purposes of mounting.

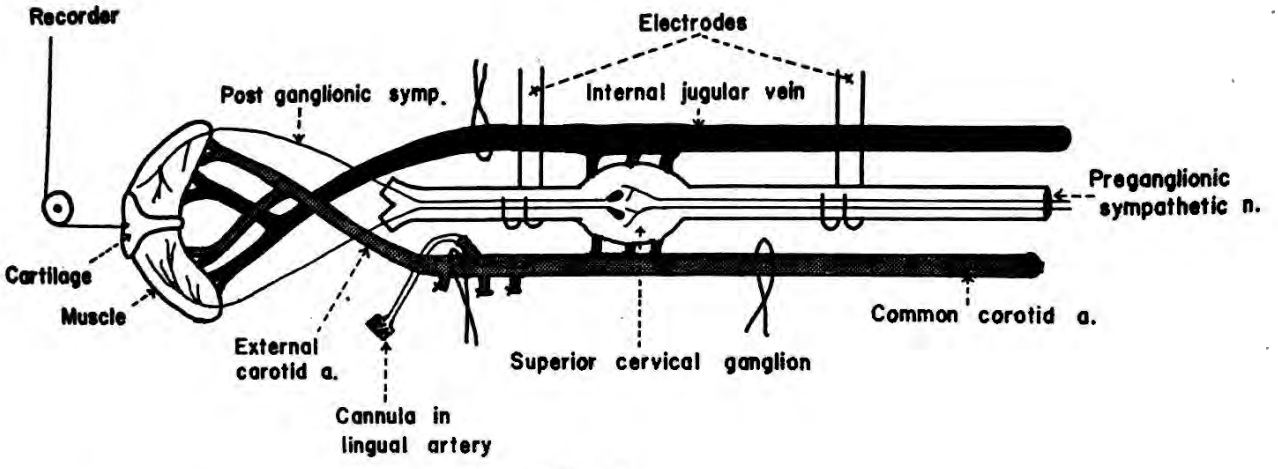
4. 1 – c
The final set up of the isolated atria in Ringer Locke solution at room temperature.

Fig. 4. 2: The superior cervical ganglion - nictitating membrane preparation of cat.



4. 2 – a

The approach to the superior cervical ganglion, from the anterior aspect of the neck. Adjacent landmarks are identified, and redundant structures removed or divided between ligatures for easy access.



4. 2 – b

A diagrammatic representation of the preparation. (Additional features include details for provisional experiments to cannulate the lingual artery)

mounted.

3. **The superior cervical ganglion and nictitating membrane of cat.*** A cat weighing about 3.0 kg is anaesthetised with ether chloralose (refer Appendix I) and the depth of anaesthesia allowed to stabilise for a period of one hour.

An incision is taken in the midline of the neck and the paratracheal intermuscular gap around the neurovascular bundle in the neck is gently opened up on one side, i.e. the side on which the carotid is not to be cannulated. The lateral branches of the carotid are tied off. Similarly, the carotid artery on the opposite side is cleared of connective tissue and kept prepared for cannulation with the placement of two loose ligatures.

Further dissection of the superior cervical ganglion is facilitated by severing the oesophagus and trachea at this stage and retracting their cut ends. This exposes the flat muscles inserted into the anterior surface of the vertebral column and the base of the skull. In a shallow depression immediately lateral to these muscles is seen a fleshy pinkish nodular structure which is the superior cervical ganglion. From this ganglion a nerve trunk can be traced into the base of the skull for a short distance of about 5-6 mm, the superficial pinkish fibres of which constitute the post-ganglionic part of the sympathetic supply destined for the eye. A bipolar electrode is carefully manipulated into position under this post-ganglionic nerve. It may be emphasized at this stage that the ganglion derives its blood supply from multiple small arterioles arriving from its lateral aspect which must therefore be untouched throughout the experiment. The preganglionic sympathetic nerve is readily accessible in the sympathetic trunk in the neck, accompanying the carotid artery, and an electrode is placed around it here.

The nictitating membrane consists of a cartilaginous portion and a muscular portion, the contraction of which is to be recorded. The lower eyelid is sutured in a retracted position and the nictitating membrane freed of its conjunctival attachment. A suture through the cartilaginous portion is led over a pulley to an isotonic lever, balanced for a tension of 3-5 g, with a sideways writing point. The carotid artery on the other side is now cannulated for the purpose of direct blood pressure recording.

Stimuli are obtained from a square wave pulse generator, the amplitude, width and frequency being adjusted optimally for every experiment.

The preganglionic and post-ganglionic nerves are stimulated at varying voltage and the supramaximal strength of current is chosen for the experiment.

4. **Hindquarters perfusion in the intact anaesthetised cat.** A cat 2.5 — 3.0 kg in weight is starved for 12 hours before the experiment.

It is anaesthetised with ether chloralose and the depth of anaesthesia allowed to stabilise for half an hour. The carotid artery is cannulated for direct blood pressure recording.

The abdomen is opened and the coils of small intestine retracted gently to one side to expose the peritoneum on the anterior surface of the vertebral column. This peritoneum is gently opened and the aorta located as the pulsatile, shining, thick-walled structure in the midline, accompanied by a large vein. The aorta is carefully cleared for a distance of about six inches from its bifurcation. Three loose ligatures are placed under it in readiness for cannulation as follows: a central one, one just above the bifurcation and one as high in the abdomen as possible.

The apparatus used for perfusion (in this case a Sigmamotor pump) is prepared for

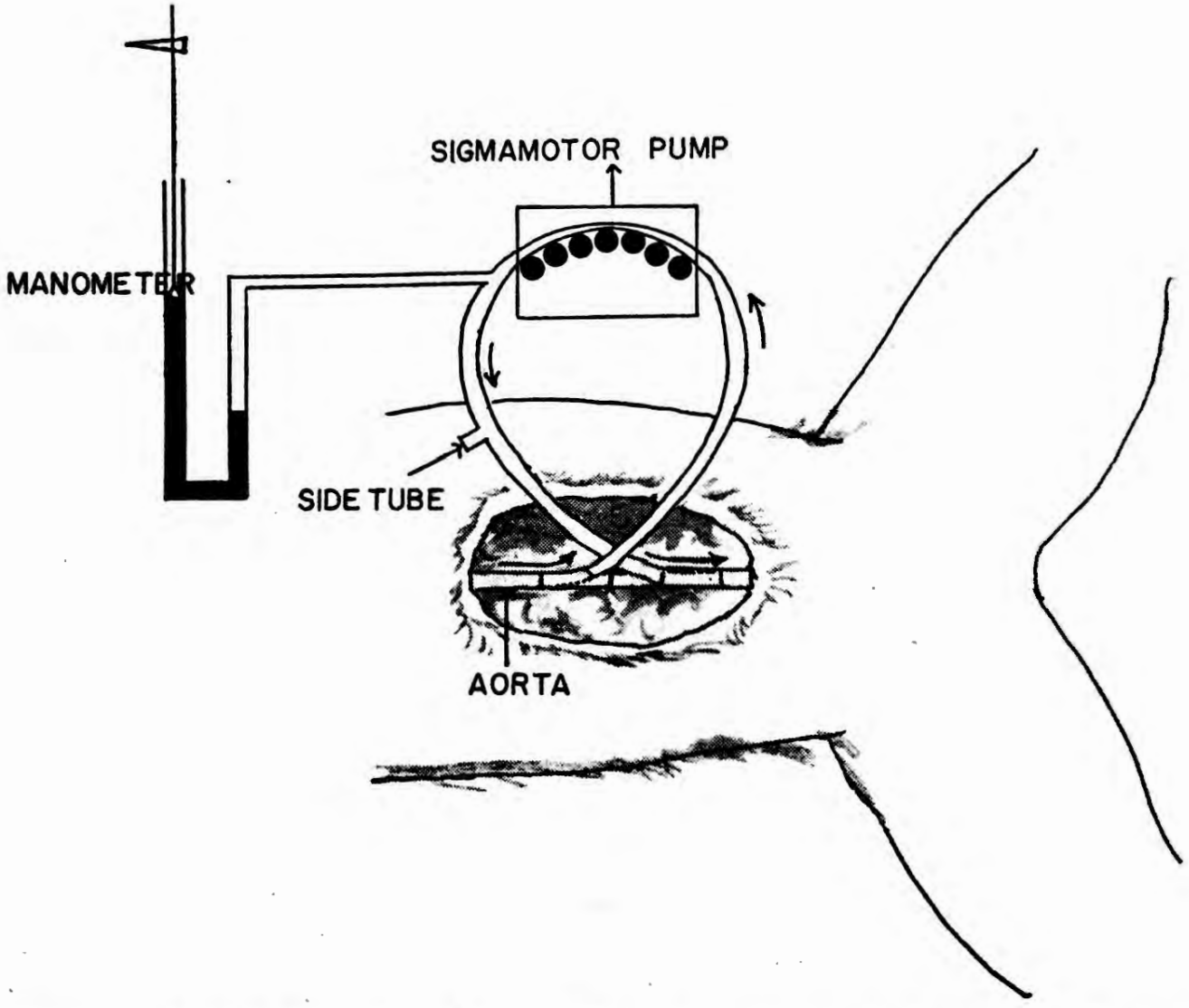
* Kharkevich D. A. "Ganglion-blocking and ganglion stimulating agents" Pergamon Press, Oxford, 1967.

the experiment by filling the entire system with heparinised saline (10 units/ml), and connecting it to a mercury manometer. The central ligature on the aorta is then tied and the two ends of the perfusion cannula inserted above and below this ligature and secured firmly in place with the other 2 ligatures (refer Fig. 4.3). The rate of perfusion of the hindquarters is adjusted so as to maintain a uniform pressure head very similar to that recorded from the carotid artery in this animal.

If a drug under consideration when injected into the side tubing of the perfusion system causes a fall in perfusion pressure, it has done so by increasing the volume of a closed, constant pressure circuit and it is thereby interpreted as being a vasodilator. Conversely, a rise in perfusion pressure would mean a vasoconstrictor effect.

These changes will occur only in perfusion pressure, and the systemic blood pressure recorded from the carotid will not be

Fig. 4. 3 : Hindquarters' perfusion in the cat.



Heparinised blood is circulated by a Sigmamotor pump from above to below an aortic ligature. A mercury manometer placed in the circuit, distal to the pump records perfusion pressure. Drugs are injected into a side-tubing in the same limb of the apparatus.

changed significantly.

5. **Digoxin induced arrhythmias.*** A dog weighing between 10.0 and 14.0 kg is anaesthetised with pentobarbital sodium 35 mg/kg i.v. in saline. The limb leads of an electrocardiograph (or a Polygraph connected through the appropriate amplifier and lead selector) are placed in position with subcutaneous needle recording electrodes. A standard II lead is recorded, preferably more than twice, at 10 minute intervals prior to induction of arrhythmias.

Digoxin is then infused slowly intravenously in a dose of 40 μg/kg over a period of 20 minutes. The ECG is scanned for the appearance of the characteristic irregularities. Supplements of 20 μg/kg after 30 minutes and 10 μg/kg after 60 minutes may be required to establish typical arrhythmias. After the appearance of such ECG changes as prolongation of P-R interval, or depression of ST segment, or inversion of T waves, the drug to be tested for antiarrhythmic properties is given intravenously. Alternatively, if the dose of digoxin required for induction of arrhythmia has been standardized within a fixed range, the change in this dose by pretreatment with the antiarrhythmic may be assessed.

6. **Recording of atrial and ventricular beats in an open chest preparation.** The open chest preparation of an anaesthetised cat is set up as described in chapter 2. In addition to the suture placed in the ventricular wall, an additional one is taken through the atrial appendage. A recording of each beat, atrial and ventricular, is obtained by separate displacement transducers on the Polygraph. The effects of drugs to alter atrioventricular conduction, or preferentially depress ventricular function, or to cause atrioventricular dissociation is thus amenable to investigation.

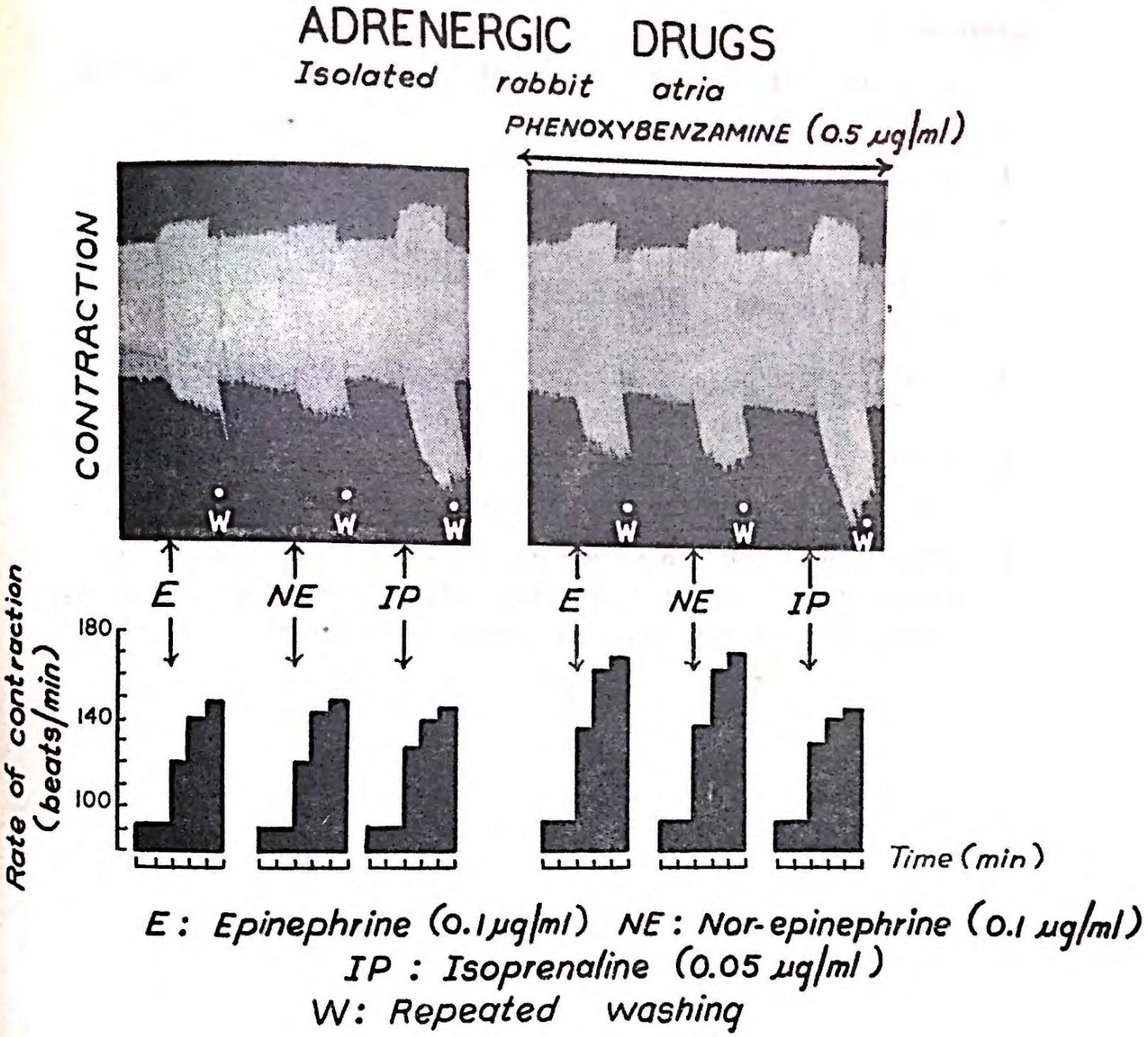
* Burger A. (Ed.): "Selected Pharmacological Testing Methods". Medical Research Series, Vol. 3, Publ: M. Dekker Inc., New York, 1968.

Practical Exercises

Exercise 1:

Objective: To study the effects of adrenergic drugs on cardiac muscle.

1. Set up a preparation of isolated rabbit atria as described.
2. Allow the preparation to stabilise for 15-30 minutes.
3. Add epinephrine in a dose to obtain a concentration of 0.1 μg/ml for a period of 180 seconds.
4. Wash out the drug and allow a recovery period of 2 minutes.
5. Add norepinephrine in the same dose and for the same period of time.
6. Follow this by isoprenaline in an identical dose and timing sequence. Note that all 3 catecholamines enhance force and rate of contraction in the order Iso > E > NE
7. Add phenoxybenzamine to the reservoir fluid so as to obtain a concentration of 0.5 μg/ml.
8. Repeat the sequential additions of epinephrine, norepinephrine and isoprenaline in the presence of phenoxybenzamine.
9. Observe failure of blockade and actual slight enhancement of the action of these catecholamines, indicating that mainly β receptors are involved in the process of myocardial stimulation by these catecholamines.



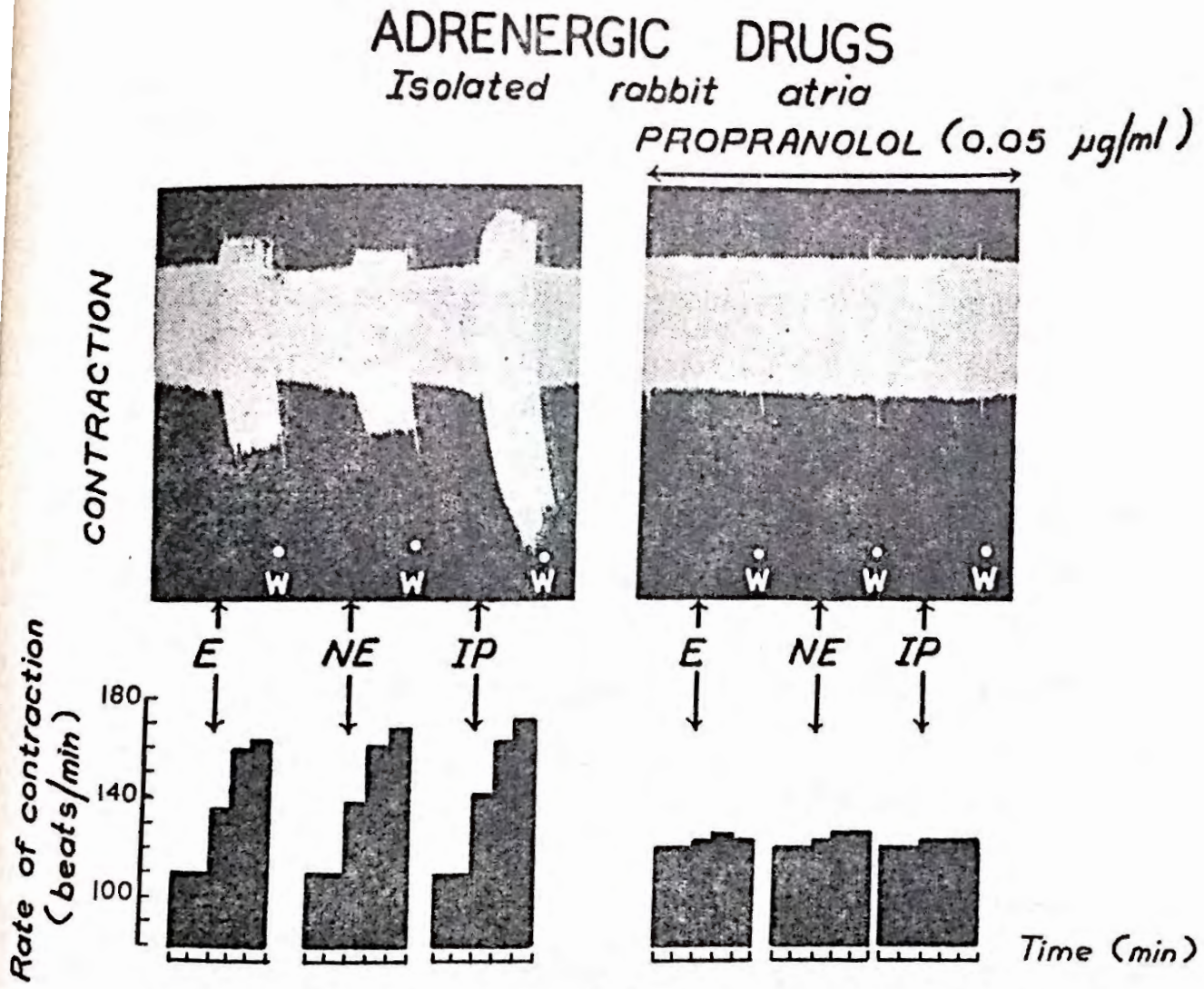
E: Epinephrine (0.1 μg/ml) NE: Nor-epinephrine (0.1 μg/ml)
IP: Isoprenaline (0.05 μg/ml)
W: Repeated washing

Fig. 4-4

Exercise 2:

Objective: To study the nature of the cholinergic receptor in the heart.

1. Set up an isolated preparation of rabbit's atria.
2. Allow a stabilization period of about 30 minutes.
3. Add epinephrine and isoprenaline to a concentration of 0.1 μg/ml in the bath fluid as in exercise 1.
4. Add propranolol (a β receptor blocking agent) to the reservoir fluid to achieve a concentration of 0.05 μg/ml.
5. In the presence of propranolol, add epinephrine, norepinephrine and isoprenaline in the same amounts as before.
6. Observe a complete blockade of the actions of epinephrine, norephrine and isoprenaline, confirming the involvement of β receptors in the stimulant action of these catecholamines on the myocardium.



E : Epinephrine (0.1 μg/ml) NE : Norepinephrine (0.1 μg/ml)
IP : Isoprenaline (0.05 μg/ml)
W : Repeated washing

Fig. 4-5

Exercise 3(a)

Objective : To study the nature of the cholinergic receptor in the myocardium.

1. Set up an isolated preparation of rabbit's atria.
2. After an initial stabilising period, add acetylcholine to a concentration of 0.1 μg/ml in the bath fluid. Elicit two such responses. Note the diminution in force and rate of contraction of the atria.
3. Add atropine to the bath fluid (1.0 μg/ml).
4. In the presence of atropine, re-elicit the response to acetylcholine.
5. Note that atropine has completely blocked the response to acetylcholine, confirming the muscarinic nature of the myocardial cholinergic receptor.

Exercise 3(b)

Objective : To study the effect of cholinergic drugs on the myocardium.

1. Set up an isolated perfused preparation of rabbit heart.
2. Inject carbachol (0.1 mg) into the side tubing of the Langendorff perfusion apparatus.
3. Observe the depression of contractility (in this case, actual cardiac arrest) produced by carbachol.
4. Inject atropine (0.1 mg) and note the immediate recovery of the heart.

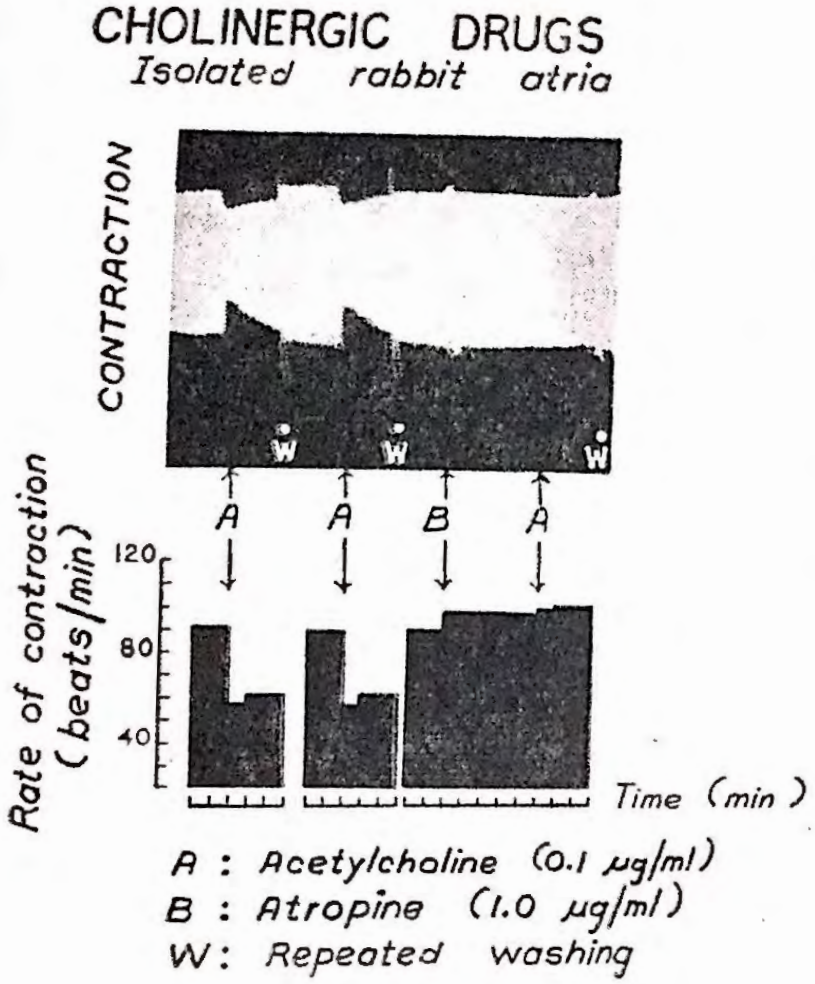


Fig. 4.6-a

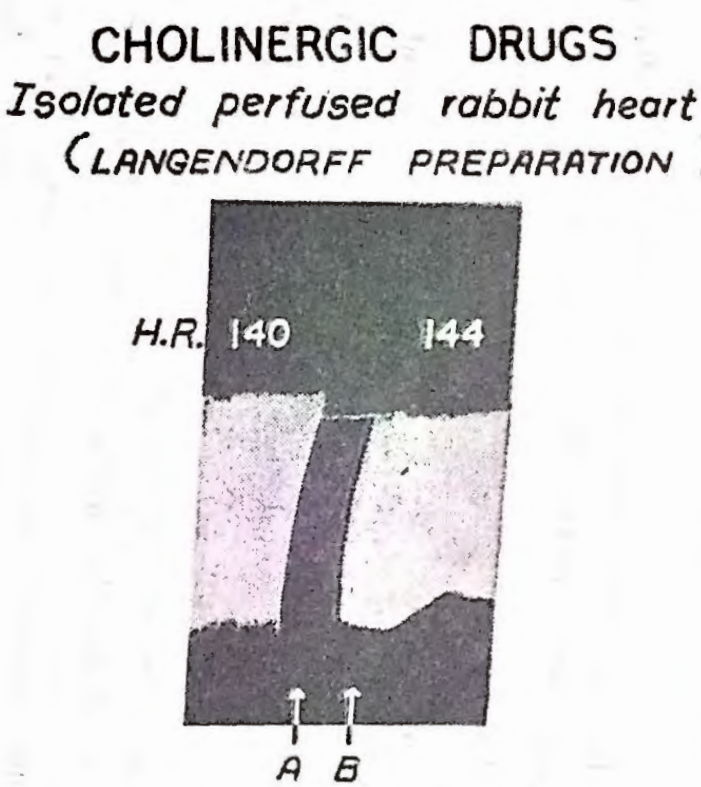


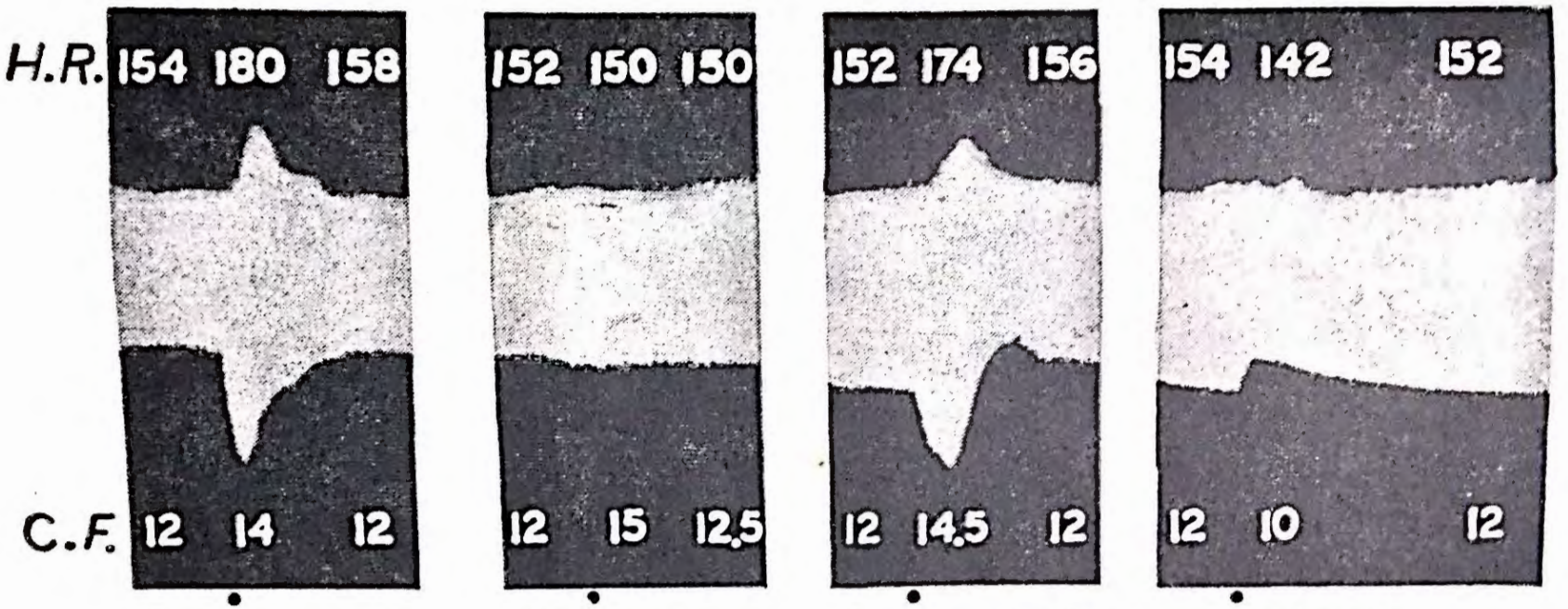
Fig. 4 6-b

Exercise 4:

Objective: To study the effect of drugs on myocardial performance and coronary flow.

1. Set up an isolated, perfused preparation of rabbit heart.
2. Record the amplitude of heart beat on a smoked drum, simultaneously note the heart rate, and the coronary outflow in ml/min.
3. Inject epinephrine (2.0 μg) into the tubing of the aortic cannula and observe the increase in force and rate of heart beat and the increase in coronary flow.
4. Inject sodium nitrite (0.5 mg) and observe absence of significant effect on myocardial performance, but increase in coronary outflow.
5. Inject aminophylline (1.0 mg) and note the improvement in force of heart beat, tachycardia and increase in outflow through the coronaries.
6. Inject oxytocin (2.5 I.U.) and note a slight myocardial depression and diminution in coronary flow.

ISOLATED PERFUSED RABBIT HEART
(Langendorff preparation)
Effect of drugs on rate and amplitude of heart beat and on coronary flow.



Epinephrine 2.0 µg

Sodium-nitrite 0.5 mg

Aminophylline 1.0 mg

Oxytocin 2.5 I.U.

H.R.- Heart rate (beats/min)

C.F.- Coronary flow (ml/min)

Fig. 4-7

Exercise 5:

Objective: To study the effect of a drug on blood vessels.

1. Set up a hindquarters perfusion experiment as described, with simultaneous recording of systemic blood pressure from a common carotid artery.
2. Inject graded doses of a vasodilator drug into the side tubing of the perfusion cannula.
3. Observe the fall in perfusion pressure unaccompanied by any changes in systemic pressure, which confirms a direct vasodilator effect.

(In our experiments we used a synthetic piperazine R-1361; however, acetylcholine and histamine would yield the same results. In addition it would be possible to study the interaction with specific antagonists).

EFFECT OF DRUGS ON BLOOD VESSELS

HINDQUARTERS' PERFUSION OF CAT

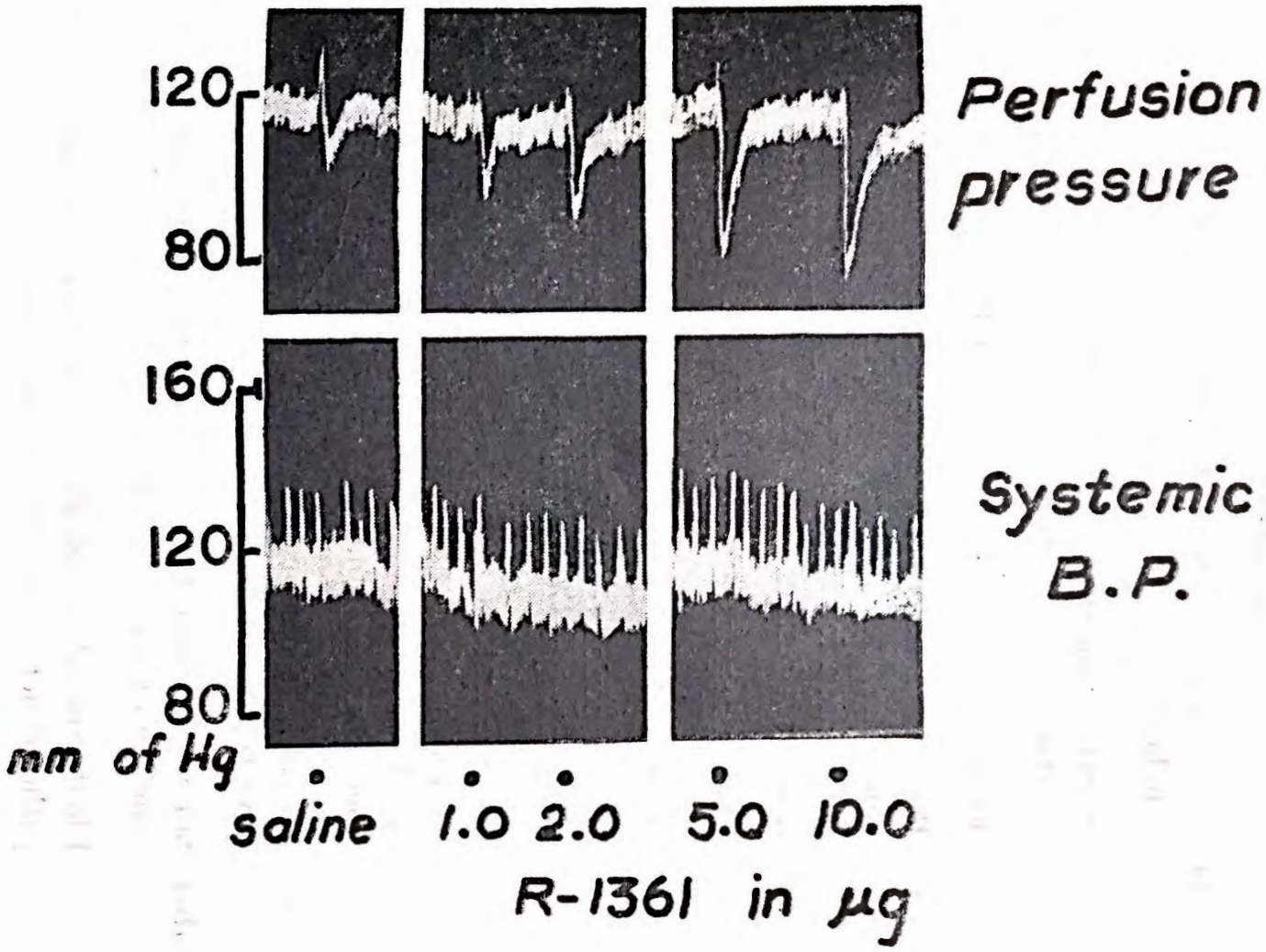


Fig. 4-8

Exercise 6 :

Objective : To compare the modes of action of hypotensive drugs on (a) ganglion, (b) adrenergic neuron and (c) adrenergic receptor.

(a)1. Set up a preparation of nictitating membrane — superior cervical ganglion in the cat as described.

2. Select stimulation parameters for preganglionic and post-ganglionic by gradually increasing voltage until a maximal response is obtained.

3. Keep these parameters constant throughout the experiment.

4. Record the responses of the nictitating membrane to preganglionic stimulation for periods of 30 and 90 seconds and post-ganglionic stimulation for 30 seconds. Elicit two responses of each to ensure consistent results.

5. Elicit a response to epinephrine injected i.v. 2.0 μg/kg.

6. Administer pentolinium tartrate in a dose of 1.0 mg/kg.

7. After a period of 10-15 minutes repeat stimulation of pre- and post-ganglionic sympathetic nerves.

8. Observe that the response to preganglionic stimulation is diminished for both 30 and 90 second periods. The response to post-ganglionic nerve stimulation is unaffected, as also the response to injected epinephrine.

9. Elicit these responses after 60-120 minutes and observe that they have recovered from drug action completely.

(b)1. Set up a preparation of nictitating membrane — superior cervical ganglion of cat as described.

2. Elicit control responses to stimulation of the preganglionic sympathetic nerve for 30 and 90 second periods and of the post-ganglionic sympathetic nerve for 30 seconds.

3. Record a response to injected epinephrine (3.0 μg/kg).

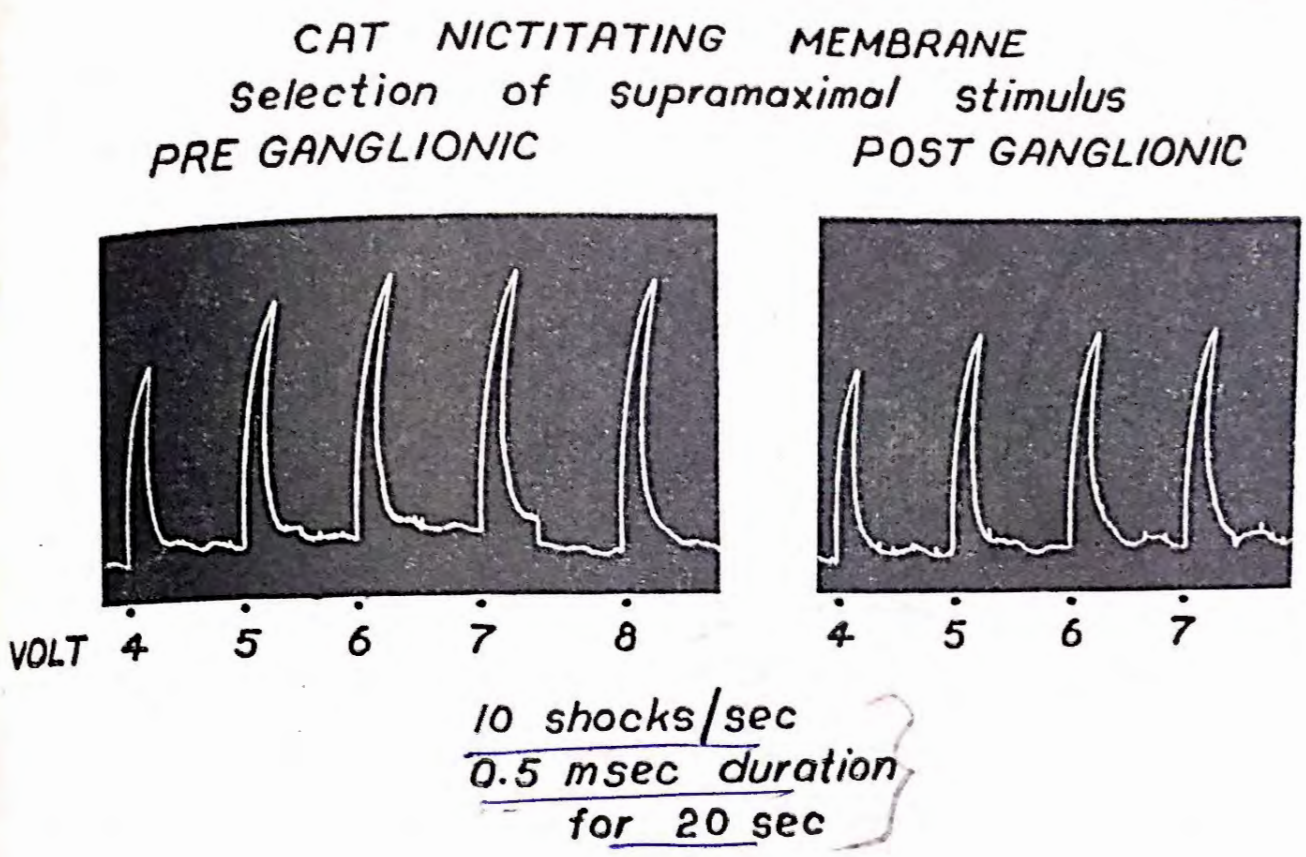


Fig. 4.9–a

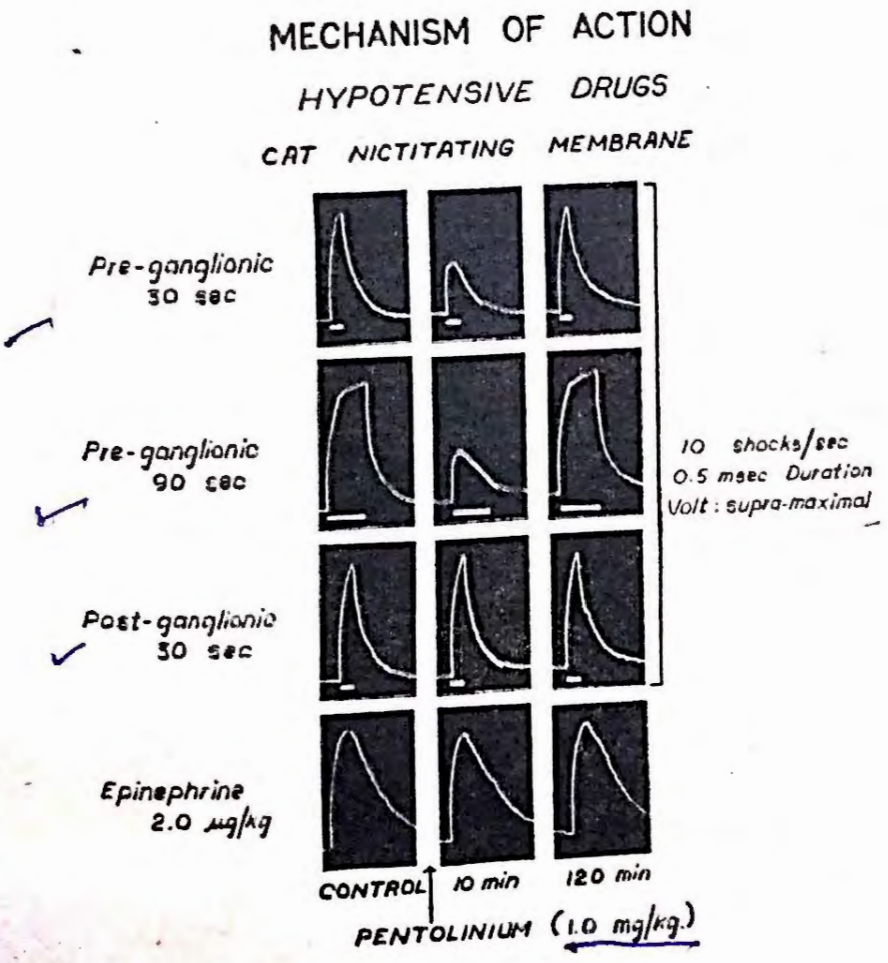


Fig. 4.9-b

4. Administer bretylium tosylate (2.0 mg/kg).

5. After a period of 10 minutes re-elicit the responses recorded previously.

6. Observe that the response to post-ganglionic stimulation is unaltered, while the response to preganglionic stimulation for both 30 and 90 seconds is diminished.

7. Contrast the latter response (to 90 sec stimulation) to that obtained after pentolinium tartrate and note that whereas the ganglion blocker caused a gradual loss of tone in the stimulated nictitating membrane, the neuron blocker maintains this tone thus causing a plateau shaped response to continuous stimulation.

8. The response to injected epinephrine is unaffected.

9. Recovery is seen after 120 minutes.

(c)1. Set up a preparation of superior cervical ganglion-nictitating membrane in a cat.

2. Elicit control responses to stimulation of pre- and post-ganglionic sympathetic nerves, and to injected epinephrine (2.0 μg/kg).

3. Administer phenoxybenzamine (3.0 mg/kg).

4. Re-elicit the responses previously recorded.

5. Observe that responses to nerve stimulation as well as injected epinephrine are blocked.

6. Recovery is seen after 120-180 minutes.

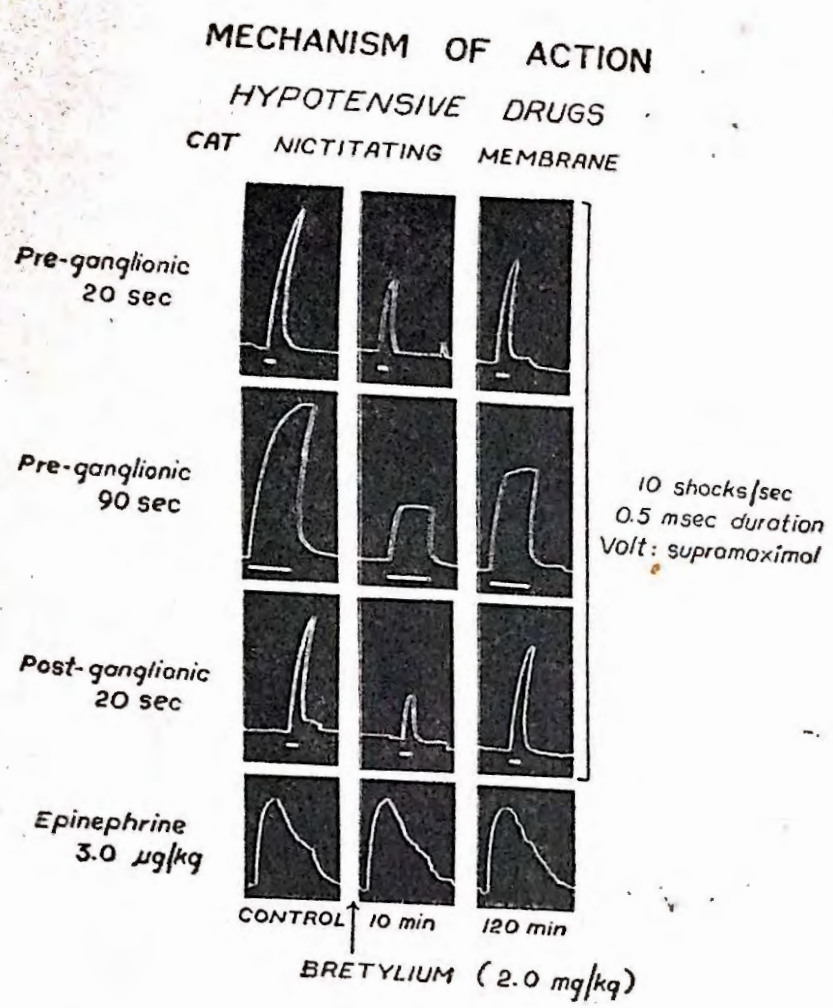


Fig. 4.9–c

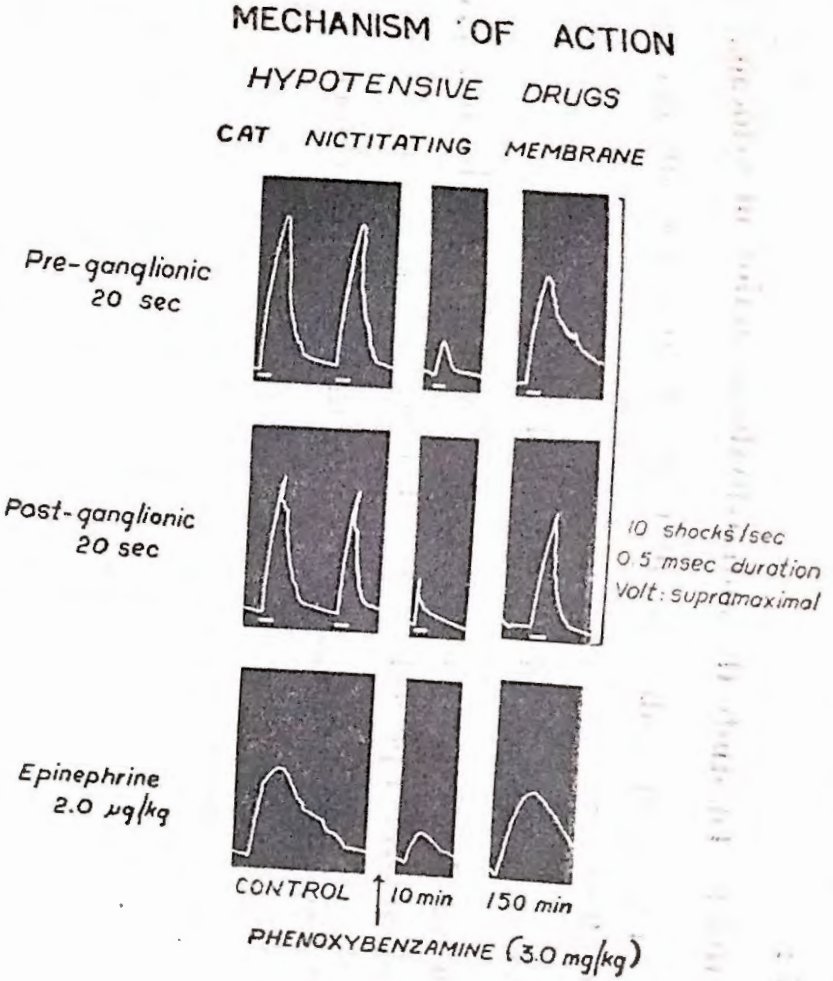


Fig. 4.9-d

Exercise 7 :

Objective : To study the antiarrhythmic action of xylocaine.

1. Start an infusion of digoxin 40.0 μg/kg/20 min in an anaesthetised dog as described.
2. Observe the onset of characteristic ECG changes.
3. Begin another intravenous infusion containing xylocaine (3.0 mg/kg) given over a period of 10 minutes.
4. Note a complete reversal of digoxin induced arrhythmias.

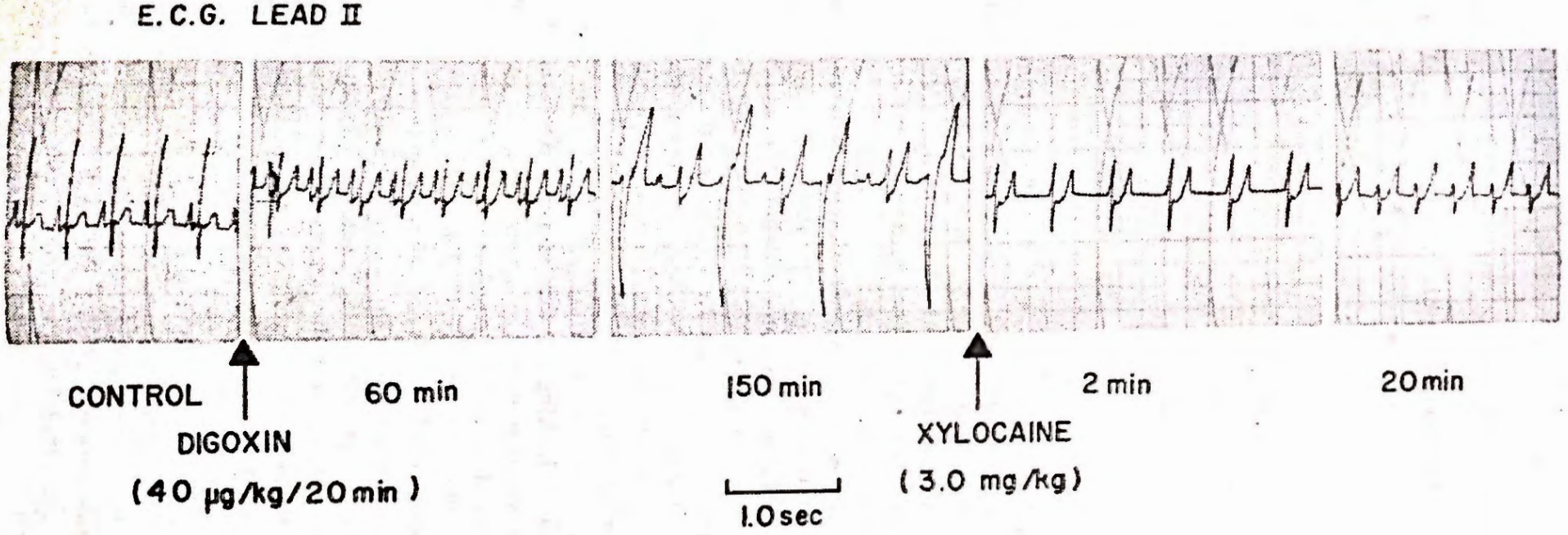

E.C.G. LEAD II
CONTROL
DIGOXIN
(40 µg/kg/20 min)
60 min
150 min
XYLOCAINE
(3.0 mg/kg)
2 min
20 min
1.0 sec

Fig. 4–10

Though this be madness, yet there is method in't.

HAMLET W. SHAKESPEARE

Drugs Acting on the Central Nervous System

Introduction

It is practically impossible to cover in a single chapter an account of methods used for evaluating the effects of drugs on the central nervous system (CNS). The functions of the CNS are so complex and widespread, that at best one can give a very sketchy account of the techniques used to evaluate effects of drugs on some of the functions. Broadly speaking, the drugs may either decrease or increase a particular function or functions of CNS. Depending upon the dosage used, one could get a variety of degree of alterations in functions. The interpretation of the test will depend on what one is looking for as the final objective. A CNS depresant may produce a mild sedation, decrease in aggressiveness, hypnotic effect or general anaesthesia. In this chapter we have given a general plan of screening drugs acting on CNS, and have included a few specific tests for specialised activities. A general discussion will be followed by a series of practical exercises which will illustrate the discussion. Selection of the exercises has been based on the simplicity, reproducibility and ease with which they can be carried out in most of the pharmacology laboratories in teaching institutions. Highly sophisticated techniques involving the use of precision electronic equipment have not been mentioned, since these will be carried out only by a few specialised departments. A bibliography of selected readings will cover the references to these techniques.

General Screening Procedures

For successful testing, it is necessary to define the approximate dosage on which the tests will be carried out, and also if possible information on onset, peak effect and duration of action. When a new chemical is made available, primary screening for CNS activity is carried out in mice or rats. Some of the compounds are also screened in cats. A variety of information can be made available from these screening procedures by simple observations. These tests though developed as tests for central action of drugs, not only detect sedatives, hypnotics, tranquillizers, CNS stimulants, muscle relaxants, analgesics, and anticonvulsants, but also detect neuromuscular blocking agents, atropine like drugs, sympathomimetics, peripheral vasoconstrictors and vasodilators, diuretics, acetylcholine like compounds, etc. The screening test in mice is carried out in male albino mice. The test compound is injected intraperitoneally in doses of 50, 100, 200, 400 and 800 mg/kg. Three

animals are used per dose. The mice are then observed and tested in various ways to measure onset, peak, duration and type of activity. The approximate LD_{50} of the drug can also be estimated. From these approximate LD_{50} values, accurate LD_{50} determinations can be planned more effectively. The various changes observed could be quantified by assigning scores. Four may be assigned for a characteristic which is normal, which can be increased to 8 for increase in that particular activity and decreased to O for decrease of that activity. Signs normally absent begin at O and are graded upwards upto 8. Thus, all the effects are scored in a scale varying from 0 to 8. The effects observed are motor activity, posture, closure of the eyelids, size of the pupils, motor incoordination, muscle tone, reflexes, awareness of the surroundings, autonomic and miscellaneous functions. Each of these can be subdivided: under reflexes include pinnal, corneal, scratch and righting reflex; under awareness, one can observe the animal's alertness, orientation, struggle response, aggressiveness, stupor, etc. A typical chart of the observations recorded, is given in Fig. 5-1a, 5-1b and 5-1c. The observations are made before the administration of the drug and repeatedly at periodic intervals for a period of 4 hours and at 24 hours. The animals are further observed for 4 days to detect any toxic effects. During these observations, measurements can be made of body temperature, analgesic effect to painful stimulants, etc. Some of the mice are always injected with vehicle to act as a control group. This screening procedure gives an overall profile for the activity of the drug, approximate LD_{50} and some idea about its potency, onset of action, duration of action, etc. There are many limitations to this screening procedure. Since the procedure is observational, it is liable to be biased. However, this can be minimised by using a blind procedure, use of saline controls and use of standard drugs which will prove the reproducibility of the results.

Drugs which show interesting CNS activity by these tests can also be screened for behavioural effects in cats. Various parameters of observations can be noted. The details of the recording chart are given in Fig. 5-1-c. Detailed specific tests are carried out usually at 1/5th or 1/10th the LD_{50} dose as judged by primary screening procedure in mice.

Specific Tests

Tranquillizers and Sedatives: The methods available for the assessment of tranquillizers are numerous, though their interpretation is sometimes difficult. The major problem of obtaining an animal model resembling human psychosis and neurosis remains still unsolved. However, many experimental techniques which have been proposed for the evaluation of tranquillizers do provide a reasonable working foundation for further investigation. The methods may be considered under the following headings as suggested by Riley and Spinks (1958).

1. Methods of measuring general sedative action.
2. Behavioural methods (not involving conditioning).
3. Neurophysiological techniques.
4. Antagonism to psychotomimetic drugs.
5. Conditioning methods.

Methods for measuring sedation. A number of methods are used for measuring sedation. Their value depends on the extent to which one expects sedation to be the property of a drug one is looking at.

Fall-Time Methods: Fall-time has been used to assess the ability, of control and treated mice, to maintain equilibrium and muscle control. The methods fall into two groups; those using inclined planes, and those using rotating rods. The angle of the slope of the inclined plane, or the rate

PRIMARY SCREENING

DATE ANIMAL: MOUSE

SAMPLE SOLVENT pH ROUTE

	STIMULATION									DEPRESSION							AUTONOMIC EFFECT										
Time	Dose mg/kg	Hyperactivity	Piloerection	Twitching	Rigidity	Irritability	Jumping	Clonic convulsion	Tonic convulsion	Ptosis	Sedation	Sleep (Loss of R.R.)	Loss of Traction	Loss of Pinna Reflex	Loss of Pl. Reflex	Catatonia	Ataxia	Loss of Muscle Tone	Analgesia	Straub Tail	Laboured Resp	Cyanosis	Blanching	Reddening	Abnormal Secretion	REMARKS
30 min.																										
1 hour																										
2 hours																										
4 hours																										
24 hours																										

COMMENTS

ONSET OF EFFECT APPROX LD-50 DURATION OF ACTION

EFFECTIVE DOSE

NATURE OF ACTIVITY PEAK ACTION SIGNATURE

O=NORMAL
+=MILD OR MODE RATE EFFECT
++=MARKED EFFECT

Approximately LD_{50} _____ mg/kg i.p. mice Cause of death _____

PRIMARY SCREENING

Code — = no effect
+ + = strong effect + = mild effect

GROSS OBSERVATIONS (MICE)	DOSE MG/KG	REF-			
		—	+	+	+
Stimulant					
Hyperactivity					
Irritability					
Tremor					
Convulsions (tonic)					
Convulsions (clonic)					
Piloerection ...					
Depressant					
Ptosis					
Sedation					
Anaesthesia					
Ataxia					
Loss of righting reflex					
Catatonia					
Analgesia (tail clip)					
Straub's tail phenomenon					
Rotating rod test					
Loss of placing reflex					
Loss of pinna reflex					
Autonomic Effects					
Laboured Respiration					
Cyanosis					
Blanching					
Reddening					
Hypothermia					
Abnormal Secretions					
Diarrhoea					
Constipation					
Distress					
Motor Activity (mice)					
Increased					
Decreased					
Anti-Convulsant test (mice)					
Analgesic test					
b) Chemical					
a) Electrical (M.E.S.)					
Barbital Sodium Hypnosis					
Anti-reserpine test.					

CARDIOVASCULAR (DOGS OR CATS)	—	+	+ +
Adrenergic			
Adrenergic Blockade			
Cholinergic			
Anticholinergic			
Ganglionic Blockade			
Central Blockade			
Antihistaminic			
Hypertensive			
Hypotensive			
Tachycardia			
Bradycardia			
Diarrhoea			
Emesis			
Smooth Muscle (g. pig ileum)			
Antiacetylcholine			
Antiserotonin			
Antinicotine			
Antihistaminic			
Anti Barium-Chloride Contraction			
Antipyresis (rats)			
Diuresis (Rats)			
Anti inflammatory test			
Carrageenin Oedema (Rats)			
Cats :			
Hyperactivity			
Restlessness			
Decreased activity			
Nic-Mem relax			
Vomiting			
Diarrhoea			
Salivation			
Aggressiveness			
Sleep			
Motor tone			
Gait			
Appetite			
Tremors			
Convulsions			
Respiration			

PRIMARY SCREENING

DATE :

ADMINISTRATION ROUTE OF DOSE :—

ANIMAL : CAT
SEX :
Wt.

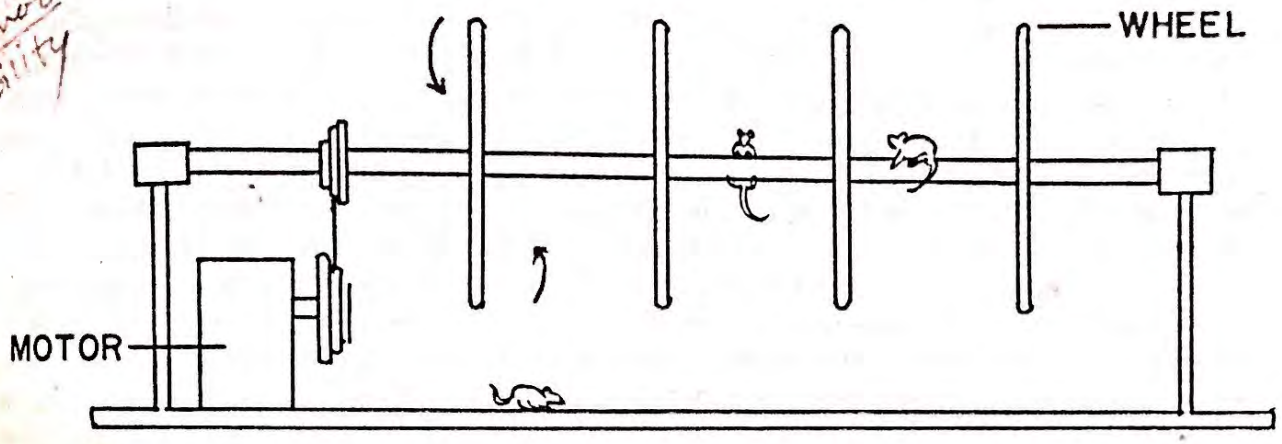
Solvent :—

	Basal	½ hour	1 hour	1½ hour	2 hours	3 hours	4 hours	24 hours
Convulsion								
Tremors								
Hyperactivity								
Restlessness								
Piloerection								
Decreased activity								
Sleep								
Motor tone								
Gait								
Aggressiveness								
Nictitating membrane								
Pupil dilatation								
Pupil-constriction								
Salivation								
Vomiting								
Urination								
Diarrhoea								
Respiration								
Appetite								

of revolution of the rod is adjusted so that normal mice remain on the apparatus for a certain fixed time, and the mice dosed with the type of drug to be studied, fall off either due to excessive sedation, or loss of muscle tone. The results give an indication of onset, the peak action and the duration of the action of the drug. The results are calculated in terms of percentage of mice falling off at various intervals (Exercise no. 1). The classes of substances which are shown to be active by these tests include sedatives, tranquillizers, hypnotics, alcohol and also convulsants, curare and other muscle relaxant drugs. The effect measured in fact is neurotoxicity and the test might be most valuable to discard rather than select potential tranquillizers.

culations on drug responses. Rotarod performance of mice treated with CNS depressants could be recorded as either a per cent decrease in performance time on the rod or per cent of animals falling off the rod at a predetermined time. A more consistent dose response effect is obtained from chlorpromazine when the performance time method of recording is used. If an animal scores "x" seconds on the rod under a low dose and a less time under a higher dose, a satisfactory dose response curve would be achieved under the time response procedure. The fall out time method would represent a 100 per cent depression for both the doses. It is also argued that rotarod does not just pick up neurotoxicity but also gives some indication of other behavioural effect which could be called tranquillizing or anti-anxiety. In the standard rotarod procedure the flat table on which the mice fall is substituted by an electrified grid. There is a marked difference in effective doses or ED_{50} between shock and non-shock experiments with drugs like chlordiazepoxido and diazepam, whereas there is no significant difference in animals treated with meprobamate and mephenesin. Chlordiazepoxide probably alters the motivation to stay on the rod to avoid the shock situation. It is argued that the rodents may

Fig. 5. 2 : The rotating-rod (rotarod) set up for fall time of mice.

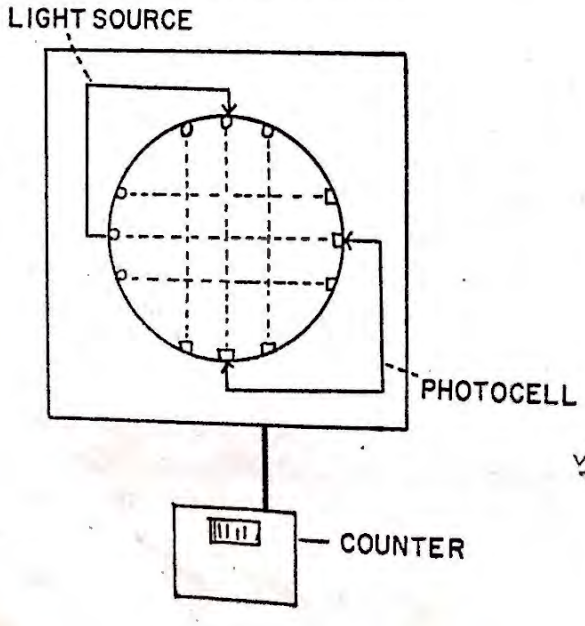


There has been recently some renewed thinking about the importance of rotarod method (Kinnard and Watzman, 1966). Instead of just noting 'fall off' time, more detailed observations should be made. It is important to observe the animal while it is walking on the rotating rod to see how many times the animal takes 'free rides'. A free ride is defined as a revolution of the rod during which the animal hold on to the rod, rather than walks on it. These free rides could affect the cal-

fall off the rod with some drugs because of lack of motivation and not necessarily due to ataxia. It seems with the initial depression that the dose of a compound as measured in the observational procedure, correlates quite well with ED_{50} from the rotarod test without shock. The initial ataxic dose seems to correlate well with ED_{50} obtained with shock. These observations need further confirmation.

Reduction of Spontaneous Activity. One of the most obvious signs of sedation in animals is a reduction in their motor activity. The animals used are either mice or rats. Movements are measured in various types of activity cages. These activity cages may be those which revolve or may move up and down (jiggle cage) due to movements of animals, or those with photocells which are activated when the rays of light falling on photocells are cut off by animals crossing the path of the light beam (actophotometer). The revolving wheel records the running activity of an animal. The jiggle cages record the overall activity of the animal, whereas actophotometers record the active exploratory or forced movements of the animals.

Fig. 5. 3 : Diagram of the construction of an acto-Photometer.



The activity of mice as measured in a photoelectric activity box (actophotometer) is one of the commonest methods used. A group of 6 mice is placed in the actophotometer and the movements are recorded for a period of 10 minutes at a time. Each time a mouse cuts through a beam of light the photocell is activated and a cumulative count is recorded. The movements are recorded at 0 hours and at the end of 1, 2 and 4 hours after drug administration. A control group receiving only the vehicle is always included Cutting (1959) described a variation of this method designated as orientation hyperactivity. Mice are allowed food and water till the beginning of the test. The total activity for 20 minutes is recorded. The recorded activity is compared to solvent treated controls and expressed as percentage inhibition or increase. In a new environment it is expected that the animals will be curious and explore the surroundings for a short period of time. Another variation of this test is to stimulate the spontaneous motor activity by injecting amphetamine (4 mg/kg s c.). The effect of the test compound on this amphetamine induced hyperactivity is then recorded, and expressed in terms of percentage of amphetamine treated group.

I. D. P. N. (β/β iminodipropionitrile) when injected in mice produces a hyperactive state which lasts for months (Azime et al, 1956). Mice so treated show a continuous circling activity. Chlorpromazine, reserpine and hydroxyzine stop the agitation and circling of the IDPN mice, and normalise their response to noxious stimuli. Hypnotics like barbiturates arrest circling activity only at narcotic doses.

Amphetamine Aggregation Toxicity. Amphetamine when injected in mice causes excitement which is much more pronounced if the mice are kept together in one cage rather than separately, one per cage. Incre-

ased stimulation occuring in grouped mice increases the toxicity of amphetamine by nearly 10 times (amphetamine 14 mg/kg i. p.). Protection against the toxicity of amphetamine in grouped mice can be obtained by treatment with tranquillizers. Chlorpromazine and reserpine afford protection in this test whereas meprobamate and benactyzine are inactive.

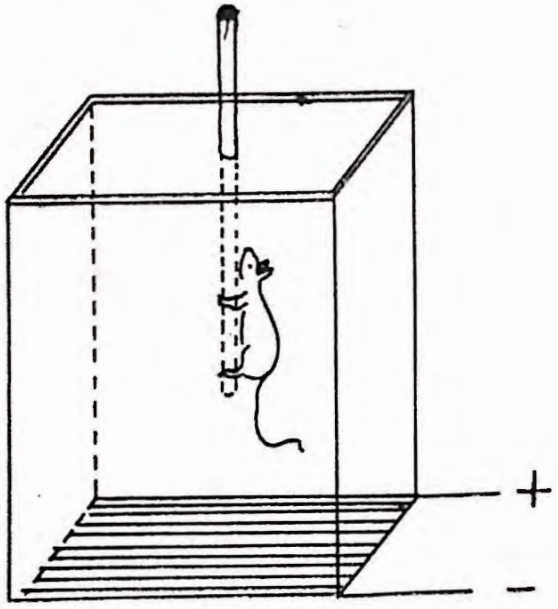
Oxygen Consumption in Mice (Cutting et al, 1959). This experiment is carried out by putting a mouse in an airtight bottle, which contains calcium oxide to absorb moisture and carbon dioxide. The bottle is closed with a tight fitting 2 holed cork through which pass a glass tube of uniform diameter open at both ends and a thermometer to record the room temperature. The glass tube is marked in c.cm. from its upper end. Soap solution is used to make a thin film on the upper open end of the glass tube. As the animal uses up oxygen, the volume inside the bottle decreases and the soap film moves down. The movement of the soap film depends upon the amount of oxygen consumed by the mouse which in turn depends upon the activity of the animal. Time is recorded in seconds for the soap film to travel 2 cm. distance. The animals are starved overnight. The drugs and the vehicle are injected intraperitoneally. Observations are also recorded of the activity status of the animal as seen through the glass.

Potentiated Narcosis.—This test measures the influence of the new drug on the duration of sleep induced by a standard hypnotic in mice (Winter, 1948). The hypnotic frequently used is hexobarbital. The parameter for recording sleeping time is the loss and recovery of the righting reflex in the animal. The test is preferably carried out at a constant temperature between 34° and 36°C. Sleeping time is prolonged by all the known tranquillizers. Unfortunately, a large variety of other substances which are not even CNS depressants prolong hexobarbital sleeping time. This is due to the fact that there are several possible ways in which drugs may prolong barbiturate action. Some substances may increase the susceptibility of the brain to the action of barbiturate or may increase or decrease vascularity altering the rate of entry of barbiturate in the brain. The short duration of action of hexobarbital is partly due to its rapid inactivation by liver microsomal enzymes. These enzyme systems can be affected by many drugs, and thus, prolong or shorten the hexobarbital sleeping time inspite of the drugs lacking CNS activity. Barbital sodium is excreted unchanged by the kidney because it is not biotransformed by the liver enzymes. Potentiation of barbital sodium hypnosis by a test drug is more likely due to its depressant action on CNS provided inhibition of renal excretion can be excluded. A screening method for CNS depressants utilising potentiation of barbital sodium hypnosis has been described (Sethy et al, 1967):

Conditioned Avoidance Response : The effects of drugs on conditioned responses in experimental animals has been used as a screening procedure. Cook and Weidley in 1957 described the use of conditioned avoidance response in rats as a screening procedure for major tranquillizers. the test is carried out in rats in the pole climbing apparatus described with an electrified grid and a buzzer arrangement. The pole is attached to the lid which can be removed after the rat has climbed onto the pole. A group of rats are trained in this apparatus. During training, the buzzer and shock are simultaneously applied. This differs from the classical conditioning methods where the unconditioned stimulus follows the conditioned stimulus after a certain interval. The shock (80 volts, 5 pulses/sec.) is applied through a stimulator to the floor grid. The rats are shocked intermittently for variable lengths of time,

each time the shock lasting for not more than a maximum of 30 seconds. The rats are trained initially 3 times a day. After a week's trial only the buzzer is tried and the shock is applied only when the rats do not climb the pole on hearing the buzzer.

Fig. 5. 4 : Diagrammatic representation of Cook's pole-climbing apparatus, with electrified grid (The buzzer has not been shown).



The rats usually learn to climb the pole within 3 seconds of the buzzer in about 2 weeks. Reinforcement runs have to be repeated 2 or 3 times a week. During the experiment the rats receive the drugs and are tested at intervals of 30 minutes. Blockade of the conditioned response (CR) i.e. failure of the rat to climb with buzzer sounding is considered as the main parameter. The CR is taken as completely blocked if the rat does not climb within 30 seconds with the buzzer on. At the end of 30 seconds, shock is applied to find out the presence of unconditioned response (UR). Blocking of UR is considered as an indication either of motor paralysis or severe toxic effect of the drug. A dose response relationship in a time recovery curve should be plotted. CR is blocked effectively by major tranquillizers (Chittal and Sheth, 1963).

M. A. O. Inhibition Test : Hydrazine and non-hydrazine antidepressants exhibit an intensity of MAO inhibition which parallels to some extent their clinical effectiveness as anti-depressant drugs. This led to the introduction of MAO inhibition as a parameter to screen possible anti-depressant compounds. Activity on MAO in vitro is usually measured manometrically, using tyramine or tryptamine as substrate. Reserpine and tetrabenazine induce CNS depression, ptosis and miosis, a fall in body temperature, prolongation of hexobarbital sleeping time and blocking of CR. They also reduce 5-HT and NE content in the brain These effects can be blocked or reversed by iproniazid and other MAO inhibitors (Pletscher et al, 1960). MAO inhibitors can only prevent the reserpine effects, but cannot reverse them once they are fully established. In rodents imipramine and amitriptyline antagonise the effects of tetrabenazine and reserpine (Sulser et al, 1962). The reversal of reserpine induced hypothermia has sometimes been used to assess the anti-depressant activity in rabbits and rats. Other tests for antidepressant screening procedures have been well discussed by Graham Chen in 'Evaluation of drug activities', Pharmacometrics, Vol. 1 (1964).

Anticonvulsants: These drugs are intended for the treatment of convulsive disorders in man. Convulsions may arise as a result of many causes. Most of the animal techniques used for screening anticonvulsant drugs are aimed at evaluating anti-epileptic drugs. Human epilepsy is not a single disease, but a syndrome with a variety of manifestations. The principal seizure types are determined largely by a focus of abnormal activity in the brain. The extent and accessibility of the areas secondarily invaded, the characteristic changes in the electrical activity as seen by EEG recording and the external manifestation of functional hyperactivity differ

in different types of epileptic disorders. Differences in these factors are sometimes accompanied by considerable differences in clinical drug response. Hence, a battery of tests is essential to evaluate the total profile of the test drug.

The general principle is to produce seizures by electrical stimulation, or systemic administration of convulsant chemical agents eg. pentylenetetrazole, or production of a chronic, focal epileptogenic lesion. The parameter measured may be either a change of threshold to the stimulus applied, a change in the pattern of convulsive seizure or a change in the EEG pattern. The effectiveness of a drug is expressed in terms of the ratio of the dose causing some undesired toxic effect (TD_{50}) to the dose having desired anticonvulsant effect (ED_{50}).

By using electrical stimulation of the brain, a variety of seizures can be produced, the principal one being (a) supramaximal electric shock (MES); (b) 'psychomotor' electric shock; and (c) minimal electric shock threshold. The test animals used are generally mice or rats.

Supramaximal Electric Shock: Male mice weighing about 25 gms. are stimulated through corneal electrodes (150 mA alternating current, 0.2 sec. stimulus duration) (Swinyard, 1949). The resultant seizure in normal mice shows a tonic phase of limb flexion of roughly 1.5 sec., followed by full tonic extension of roughly 10 sec. and a few clonic jerks thereafter. The number of post-tonic asphyxial deaths are noted. Drugs which are clinically accepted as effective in grand mal, e.g. diphenylhydantoin, can abolish all of the tonic phase without causing neurologic impairment, and this is the basis of our standard screening test for evaluating drugs against grand mal. Dose response lines for standard test drugs are determined by giving groups of 4 or more animals various doses of the drug and subjecting them to MES at the time of peak activity of the drug. Peak activity of the drug and the neurotoxicity is determined by using rotarod test as mentioned before. At least 3 dose levels are established between the limits of 100% protection or toxicity. The results obtained then are plotted on logarithmic probability paper and regression lines are fitted to the plotted points by the method of maximum likelihood. The dose of the drug producing the desired endpoint (protection or neurotoxicity) in 50% of animals (ED_{50} or TD_{50}) are computed, the regression lines are tested for parallelism by the chi-square method (Finney, 1952), and protective indices (P. I. $= TD_{50}/ED_{50}$) are calculated. Duration of anticonvulsant effect is determined from a time response graph from the data obtained by testing groups of 4 or more animals by the MES test at various intervals after the administration of ED_{50} of the drug.

Analgesics. The problem of ascertaining whether a chemical compound has the ability to relieve pain or not in animals, has been a perplexing one for the experimental pharmacologist. The variety and number of experimental procedures and designs that have been developed over the past three decades, are reflections of the difficulties inherent in the problem. A potentially noxious stimulus in the animal will evoke reflex response when the pain threshold has been reached and many of these reflexes have been used as indicators of the character of stimulus. Most of the reflex activity initiated by a potentially noxious stimulus is of an 'escape' nature. The tail flick, skin twitches, limb flexion, etc. are all movements which would tend to remove the animal or the affected part away from the stimulus. Such reflexes have formed the basis for determining the "pain threshold" in animals and analgesic drugs are screened by testing their ability to raise this threshold. Animal experiments do away with many problems of patient suggestibility, placebo reactions.

patient and observer bias. Bias of the investigator can be removed by a proper choice of an experimental design.

Methods :

Pressure on tails :

Haffner (1929) and Eddy (1932) described methods using pressure on tails of animals but they were hampered by lack of precision and very often by injury to the area used for pressure.

The criteria of having attained the nociceptive threshold is usually vocalisation. In rats this takes the form of a brief, but just audible squeak and hence one uses the term "squeak threshold". The neural mechanisms involved in vocalization are different from those in the heat induced tail flick. The latter can be elicited in spinal rats, whereas vocalization disappears after mid-brain section. It does not necessarily follow that a drug eliminating the squeak response must act on higher centres. Eagle and Carlson (1950) and Green (1955) have used metal rods with sharp or blunt tip to produce pressure at the base or tip of the tail in rats. This was connected to a scale graduated in gms. of pressure at 10 gms. interval. As the end point, the squeak or any other evidence of discomfort was used e.g. struggle. Often a mean of two thresholds, squeak and struggle was used. Way et al (1953) and Winder et al (1959) have used modifications of this method. It is generally agreed that this method lacks precision unless young rats 4 to 5 weeks old of the weight range about 80 gms. are used. Cut off pressure is important, if the animal is to be used repeatedly but different investigators have used different parameters varying from 25 to 500 gms. elevation above pre-drug treatment levels. Exact details of the pressure actually applied at the point of contact on the rats' tails and the rate at which the pressure is increased are often missing. Generally, pre-drug treatment and post-drug treatment values in same rats are determined, but Green (1955) has stated that when rats were tested five times at 20 minute intervals, variance between trials were not significantly greater between rats. Hence there is no advantage in using a rat as his own control.

Repeated use of the animal leads to decrease in threshold due to conditioning. However, if no more than two pre-injection and two post-injection determinations are made (e.g. 30 to 60 minutes after injection) the threshold remains sufficiently stable. Winder (1959) has put major emphasis on results with the squeak threshold in rats.

Collier et al (1961) applied artery clips to each toe of guinea pigs or rats and counted the squeaks thus elicited. The doses of narcotic drugs to reduce the squeaks by 50% were compared to those required by other methods of assessment.

Randall and Selitto (1957) have introduced an interesting modification. They applied the pressure to the hind paw of the rat instead of the tail. The sensitivity of the foot was increased by injection of 0.1 ml. of 20% suspension of brewers' yeast. Threshold in inflamed feet was increased not only by narcotic drugs but also by sodium salicylate and phenylbutazone. This method was further analysed by Gilfoil (1963) who showed a lack of strict correlation between the amount of edema and the degree of hyperaesthesia in the inflamed foot. Aspirin in doses having no significant effect on foot volume markedly raised the reaction threshold for squeak response.

Writhing Syndrome: Vander-Wende and Margolia (1956) and Siegmund et al (1957) introduced this method. Writhing is induced in mice or rats by intraperitoneal injection of acetic acid or 2-phenyl-l, 4-benzoquine (0.25 ml. of 0.02%). The syndrome was characterised by intermittent contraction of abdominal muscles, with extension of the hind limbs and twisting of the trunk. Most mice show this syndrome

only occasionally, and with enormous variations in frequency. Non-narcotic analgesics block this reaction but so do local anaesthetics, anti-histaminics and a large number of compounds of various pharmacological classes. LSD is effective in rats but not in mice. The dose of aminopyrine and of morphine, effective in mice is ineffective in rats. The relationship of the "writhing syndrome" to nociceptive reactions requiring analgesic drugs for their relief is questionable. The use of bradykinin as the writhe inducing agent has also not been helpful to make the test useful.

Thermal Methods : Goldscheider (1894) introduced heat as a means of evoking pain experimentally. In the beginning, heat was transmitted by contact either through hot water, or hot objects applied to the skin. Contact methods however, include touch and pressure in addition to heat or cold. Alrutz, in 1897, suggested the use of radiant heat. Oppel and Hardy, in 1937 showed that heat radiation technique could be applied to study quantitatively, temperature sense in man. The extensive work of Hardy, Wolff and Goodell on the radiant heat method for producing experimental pain stems from this. They used human subjects, but the general principle of applying sharply localised heat to elicit a response from animals, has been more widely adopted as for example in various dolorimeters.

Radiant heat method, fixed duration, variable intensity. Light or heat from a 500 to 1000 watt projection lamp is focussed for precisely 3 sec. on an area of 3.5 cms. of blackened skin — usually the forehead of the subject. The current delivered to the filament lamp is the only variable. Increased current delivers increased heat to the subject till he experiences a sharp jab of pain, the "threshold pain", at exactly the end of 3 second exposure. The period between exposures is one minute. It is explained to the subject, that the procedure is not the test of his ability to endure pain, but rather of his capacity to detect the first trace of pain.

Radiant heat method, fixed intensity, variable duration. D'Amour and Smith, in 1941, introduced a modification of the method of Hardy, Wolff and Goodell, by letting a fixed intensity of heat to act for a variable time, until the "threshold response" is obtained. They focussed the light rays from a light bulb on the tip of rat's tail and the intensity was adjusted so that a sudden flick of the tail occurred in about 5 seconds. The criteria for the end point was failure of the animal to move the tail. A cut-off time of 10 seconds was introduced and any animal not responding in 10 seconds was judged to have complete analgesia. Cut-off time is necessary to prevent tissue damage. Bavin et al (1952) have advocated 12 seconds and Davies et al (1946) 15 seconds as cut-off time.

Davies et al (1946) further modified the procedure in 1946 by using a red hot nichrome wire 1/8 inch below the tail as the source of heat instead of the lamp. Winter and Flataker (1953) have suggested a cut-off time of 10 seconds (with pre-drug reaction time of 4-5 sec.). Bonnycastle and Ipsen (1950) determined the pre-drug reaction time and set a cut-off time 2.3 standard deviation above that. But this did not add any significant advantage. The D'Amour-Smith (1941) method has also been used in mice with a normal reaction time of 3.4 ± 0.7 seconds and cut-off time 3 times the pre-drug reading for each animal. Winter et al (1953) reported that consecutive observations on the same rat with 3 successive readings about 1 hour apart showed a variation of only 0.7 second of the average for the group in 90% of cases.

Conducted Heat methods: Ben-Basant et al (1959) and Janssen et al (1963) introduc-

ed a modification in thermal method in mice and rats. This consisted in dipping the tail in hot water and timing the withdrawal reaction. The method has not been widely used, but appears to be sensitive.

Another method of measuring analgesic drug reaction by noting the response to thermal stimuli is called the "Mouse hot plate method" introduced by Wolff and Macdonald (1944). Mice were placed in a cylinder on a metal plate maintained at a fixed temperature. The animal's first sign of discomfort was to sit on its hind paws and lick and blow on its fore paws. A few seconds later, the animals would kick with their hind paws or attempt to jump out. Only the hind paw reaction is used for evaluation, since the front paw is often used for grooming etc.

This method was used by Eddy in 1950 to screen a large number of analgesic drugs. The temperature of the plate was maintained at 55.5°C by a light bulb and later on by use of constant temperature obtained by boiling a mixture of ethyl formate and acetone as described by Chen and Beckman in 1951. Reaction time is determined 5, 10, 20, 30, 45 and 60 minutes after subcutaneous injection of the drug and area of time effect curve is plotted. The percentage of animals, exhibiting plotted areas in excess of twice the standard deviation, above the control group is determined for several dose levels and ED_{50} is calculated by probit analysis.

The use of thermal stimuli permit quantitative determination of threshold values. The amount of energy delivered by the apparatus to produce heat can be readily measured and easily varied, but the actual stimulus which is the energy absorbed by the mechanism, initiating the response of the organism, cannot be measured. The relation of energy delivered, by the apparatus to the surface of the skin, and that reaching the reactive site will vary depending on many factors such as (1) Haemostatic mechanisms of the area, (2) absolute temperature attained, (3) degree of rise of temperature above the resting level, (4) rate of temperature changes, (5) the temperature gradient across the skin to the level of receptors and (6) the temperature difference between adjacent areas.

Electrical Methods. More than 100 years ago Von Helmholtz (1851) used faradic current to produce pain. The modern use of electrical methods for producing experimental pain started nearly 60 years ago with the work of Gordenoff who used this method in rabbits. Man was the subject of choice and galvanic, faradic, high frequency currents and condenser discharges have been used. A liquid finger electrode was used by Martin et al in 1914. Knowlton and Gross, in 1943 used dogs, using widening of palpebral fissure as the sign of "pain". Goetzel et al (1943) used a stimulator arranged to give peaks of induced current to man's and dog's teeth through amalgam fillings. They have highly recommended the use of tooth pulp stimulation as an ideal method for screening analgesics. But the data presented by them and many other workers, using similar methods is not uniform or convincing as satisfactory.

Thorp (1946) used an electronic stimulator with electrodes on scrotal sacs of rats, but the contact resistance of the electrodes proved to be too variable.

A number of workers have used electrical stimulation of tail of mice described by Grewal (1952). Shocks were applied once each second and the number of shocks counted, which caused the mouse to squeak. If a drugged mouse required more than 3 shocks above the pre-drug number, it was rated as having analgesia. Large number of mice are needed per dose (about 60) and the method is not very sensitive. Nilsen (1961) inserted electrodes in the mouse's tail beneath the skin, to decrease the variability, but could not get

desired results and commented on extreme individual variation. Significant threshold differences are seen associated with age, sex, strain and pre-drug threshold of the mouse. The use of electric shock for producing pain in man and nociceptive responses in animals has given rise to many difficulties and inconsistencies and as yet none of these electrical methods have proved satisfactory.

Behaviour test. The use of operant behavioural techniques for testing analgesic drugs is relatively recent. An element of behaviour response may be present in many testing procedures. The "flinch-jump", method introduced by Evan (1961) relies upon behavioural observations. Several procedures based upon Skinnerian operant behaviour have recently yielded interesting information.

In general, this approach consists of administering a nociceptive stimulus to the animal which can be graded in intensity from subthreshold levels to a level which becomes aversive. The animal can then reduce the intensity by an appropriate behavioural response, such as pressing a lever, to a non-aversive level. Intensity is then again gradually increased until the threshold is reached again, and the cycle is repeated. Thus by recording the intensity of stimulus and the response of the animal, a record is obtained of the "aversive threshold".

In contrast to more widely used "avoidance schedule", in "aversive threshold" determination there is a warning signal, and the shock level reaches an aversive level more or less insidiously. Once it is reached it can be escaped by lever pressing. Generally the schedule is arranged so that a number of presses (about 20) are required. The stimulus can be escaped but not avoided. Stimuli can be applied by electrodes implanted in various subcortical areas.

At present the principle usefulness of behavioural techniques in the study of analgesic drugs appears to be as a tool for investigating physiological mechanisms, rather than as a primary screen for evaluating unknown compounds. Clinical and experimental methods of evaluation of analgesics are covered in greater detail in a review by Sheth and Sethy (1967).

Antifatigue tests: Various methods of screening have been used to study the stimulant effect of a drug which can increase physical endurance and increase the duration of some well-coordinated organised physical activity, e.g. swimming, or running ability in animals. The simplest ones described have been a variety of swimming tests in rats, where the rats are made to swim over certain distances, with or without increasing amount of loads attached to their tails and the time noted to cover a particular distance, without the rat getting drowned. A simple apparatus which can measure a rat's ability to run in an organised fashion for varying periods of time, till it is exhausted is described below.

The apparatus consists of a revolving circular plastic cage (diameter $10\frac{1}{2}$") closed on two sides, and connected over the circumference by plastic strips placed a small distance apart. A part of the circumference can be opened to serve as a door to place the rat inside the cage. The cage is submerged in a rectangular plastic tub in which it can rotate freely. This tub is filled with water (20°C) so that the cage is partly submerged.

When a rat is placed in the cage, it tries to climb, to avoid being submerged in water. This causes the cage to rotate and the rat continues to climb up the rotating cage, which in turn maintains the rotation of the cage, till the rat is tired. The end point is the time when the animal is fatigued and begins to drown. At this stage the animal is removed from the cage, dried and returned to its own cage. The total period of the stay of the animal in the rotating cage is recorded in minutes (swimming time).

Practical Exercises

Exercise 1.

Rota-Rod Test

Objective: To determine the effect of drugs on muscle tone and balance in mice.

1. Select mice of either sex weighing 20-25 gms.
2. Train the mice to walk on a rotating rod 2 cms in diameter, rotated at 12 rpm by an electric motor, till the mice can balance for at least 2 minutes (2 to 4 trials per day for 2 days).
3. For further test, select only those mice which complete three successful trials per day for two days.
4. Administer the test compound (e.g. chlorpromazine 1, 2, 4, and 8 mg/kg i.p.; chlordiazepoxide 5, 10, 20, 40 & 80 mg/kg i.p. and meprobamate 50,100, 150, 200 and 400 mg/kg) intraperitoneally. Use 10 mice per group per dose. Include one control group treated with saline only.
5. Place the mice on the rota-rod at 15, 30, 60, 90, 120, 180 and 240 minutes after the treatment.
6. Note the number of mice falling off within 120 seconds at each time interval from each group.
7. Plot the percentage mice falling against time interval to determine peak drug effect at any dose level. Note the onset of action and recovery from drug effect.
8. Calculate ED_{50} from time of peak effect and percentage fall with different doses and compare the different drugs for potency.

TABLE 5.1

Results: Rota rod test in mice.

Drug	ED_{50} (mg/kg) (95% confidence limits)
Chlorpromazine	2.6 (2.0 — 3.4)
Librium	45.0 (33.5 —60.0)
Meprobamate	88.0 (67.0 — 117)

Exercise 2.

Motor activity in mice

Objective: To study the effect of drugs on spontaneous motor activity in mice as measured by Actophotometer.

1. Select male mice (body weight 20-25 gms) into groups of 6 mice each.
2. Inject one group i.p. with saline or 2% starch (vehicle).
3. Inject one group each with chlorpromazine in different doses — e.g. 0.5, 1, 2, 4 mg/kg i.p. in 2% starch solution.
4. Inject one group each with meprobamate in different doses — e.g. 50, 100, 150, 200 and 400 mg/kg i.p. in 2% starch solution.
5. Measure the activity of each group by placing them in an Actophotometer for 10 minutes, at the interval of $\frac{1}{2}$ hr, 1 hr, $1\frac{1}{2}$ hrs, 2 hrs and 3 hrs, after drug treatment.
6. Calculate the percentage decrease in activity considering saline control activity as 100.
7. Determine the time at which each drug shows maximum activity.
8. To calculate ED_{50} value, take 6 groups of 6 mice at each dose level. Record as above the motor activity of each treated group for 10 minutes at the time of peak drug action. Calculate ED_{50} from the results obtained.

TABLE 5.2

Results : Spontaneous motor activity in mice.

Drug	ED_{50} (mg/kg) (95% confidence limits)
Chlorpromazine	1.45 (0.81 — 2.60)
Meprobamate	125 (96 — 162)

Exercise 3.

Effects of drugs on motor activity in mice

Objective: To study the effects of CNS depressant drugs on amphetamine induced hyperactivity in mice.

1. Select 3 groups of 6 male mice each (weight 20-25 gms).
2. To one group administer the test drug (1/10th LD_{50} dose) i.p.
3. To other groups inject saline and vehicle i.p.
4. Inject amphetamine (4.5 mg/kg) i.p. to one group treated with saline and one group treated with test drug, one hour after the administration of test drug.
5. After 30 minutes record the motor activity of each group for 10 minutes.
6. Calculate the percentage inhibition of amphetamine-induced hyperactivity with the test drug.

TABLE 5.3

Effect of tranquillizers on Amphetamine-induced Hyperactivity in mice (Actophotometer studies)

Treatment	Dose mg/kg i.p.	Activity Count for 10 min. (mean S.E.)	Percentage inhibition
Saline	1 ml/100 g	862 ± 25.0	—
Saline + Amphetamine	1 ml/100 g 4.5	1015 ± 31.0	77.0
Chlorpromazine + Amphetamine	2.0 + 4.5	1532 31.0	—

Exercise 4.

Aggregation Toxicity Tests

Objective: To study the protective effects of drugs on amphetamine induced toxicity in grouped mice.

1. Select 2 groups of 10 male mice per each group (weight 20-25 gms).
2. Place each group in a covered circular cage (40 x 25 x 25 cms) in a quiet room.
3. Administer the test drug i.p. to one group (1/10th LD_{50} dose).
4. Administer saline or vehicle to the other group.
5. Inject both groups with challenging dose of d-amphetamine (14.5 mg/kg i.p.) one hour after administration of test compound.
6. Observe both groups for motor activity, and mortality. Record mortality at the end of 24 hours in each group.
7. Observe if test compound has protected mice against amphetamine induced mortality.

Exercise 5.

Prolongation of Barbiturate induced sleeping time

Objective: To study the effects of drugs on sleep (loss of righting reflex) produced by Barbiturates.

(a) Barbital Sodium Hypnosis

1. Select two group of 10 mice each of either sex (body weight 20-25 gms).
2. Inject saline or vehicle i.p. in one group.
3. Inject test drug i.p. (1/10th LD_{50} dose) in another group.
4. One hour after treatment, inject Barbital Sodium (180 mg/kg — i.p.) to both groups.
5. Place the mice on their backs, repeatedly, till they fail to regain their normal posture of resting on their ventral side (loss of righting reflex). With barbital sodium, the loss of righting reflex lasts for 5 to 6 hours. So instead of noting the duration of loss of righting reflex, only count the number of mice which lose their righting reflex.
6. In the control group only 3 out of 10 will lose the righting reflex.
7. With CNS depressants — 7 or more will lose the righting reflex, whereas with CNS stimulants, none may sleep (results between 3 and 7 are not significant).

TABLE 5.4

Results : Barbital sodium hypnosis

Barbital Sodium + Test Compound	Dose mg/kg	Route	No. lost R. R. / No. observed
Reserpine	0.25	i.p.	9/10
Chlorpromazine	20	i.p.	10/10
Meprobamate	200	i.p.	10/10
Nikethamide	125	i.p.	0/10
Amphetamine	5	i.p.	0/10

(b) Hexobarbital sleeping time

1. Select mice of body weight 20-25 gms — in two groups each of 12 mice (6 males and 6 females).
2. Use one group as control, inject saline or vehicle i.p.
3. Inject intraperitoneally the test compound (see table) in the second group.
4. Inject both groups with Hexobarbital sodium 125 mg/kg (solution strength 12.5 mg/2ml) dissolved in distilled water i.p. in a volume of 2ml/100gms of mouse body weight.
5. Note the sleeping time — which is the interval between the loss of righting reflex after the injection and regaining of the righting reflex as measured by the stop-watch. Calculate the mean time of the group and express in minutes with SD and SE.
6. Calculate at what level of significance ('t' test) the treated group differs from the control group.

TABLE 5.5

Hexobarbital Sleeping Time (Mice)

	Hexobarb alone	Hexobarb + Drug	Results	Significance
Solvents				
Tween 80 (12.5%)	74 ± 18	179 ± 66	Increase	0.001
Propylene glycol (50%)	44 ± 10	185 ± 56	Increase	0.001
Serpasil solvent (50%)	77 ± 25	137 ± 28	Increase	0.001
Librium solvent (50%)	80 ± 21	102 ± 27	Increase	0.05
Depressants				
Librium (10 mg/kg)	57 ± 27	102 ± 25	Increase	0.001
Methaqualone HCl (20 mg/kg)	85 ± 32	139 ± 81	Increase	0.001
Antazoline (50 mg/kg)	76 ± 17	94 ± 24	Increase	0.05
Stimulants				
Caffeine et sod. benzoate (25mg/kg)	105 ± 37	70 ± 31	Decrease	0.02
Methedrine (15 mg/kg)	94 ± 19	75 ± 21	Decrease	0.50
Nikethamide (1.25%)	89 ± 23	178 ± 39	Increase	0.001
Nikethamide (5%)	75 ± 14	212 ± 32	Increase	0.001
Picrotoxin (10 mg/kg)	99 ± 38	42 ± 15	Decrease	0.001
Strychnine sulphate (1 mg/kg)	85 ± 18	69 ± 16	Decrease	0.05
Nicotine salicylate (2 mg/kg)	88 ± 24	109 ± 19	Increase	0.01
Miscellaneous				
Hydergine (2.3 mg/kg)	86 ± 20	127 ± 30	Increase	0.001
Calcium gluconate (5%)	97 ± 20	117 ± 24	Increase	0.05
Sodium salicylate (250 mg/kg)	74 ± 19	55 ± 16	Decrease	0.05

Exercise 6.

Conditioned Avoidance Response (CAR)

Objective: To establish CAR in rats and study the effects of drugs on CAR.

1. Select a group of rats of either sex weighing 80 to 120 gms.
2. Place a single rat in the chamber of Cook's pole climbing apparatus.
3. Deliver a series of foot shocks (80 volts — 5 pulse/sec) through the grid floor, concurrently with activation of buzzer.
4. Continue shock and buzzer for 30 seconds or until the rat manages to climb the pole where it does not get the shock. Stop the stimuli immediately. In the beginning the rat will show random escape movements, but in a few exposures to shock and buzzer, the rat learns to escape onto the pole.
5. Train the rats 3 times a day for 3 days.
6. On the fourth day, expose the rat to buzzer only (shock switch closed). If the rat responds to buzzer only and climbs the pole, CAR is considered to be established. If the rat does not respond to the buzzer, deliver foot shock and repeat till CAR is established. Now the rat should escape on the pole within 3 seconds of sound (buzzer) stimulus.
7. Periodic shock and buzzer reinforcement may be necessary to maintain CAR for longer periods.
8. Effects of drugs.
   In groups of trained rats, inject the following in different groups:
   a) Chlorpromazine — 2, 4, 6 and 8 mg/kg — i.p.
   b) Chlordiazepoxide — 20, 40, 60 and 80 mg/kg — i.p.
   c) Meprobamate — 100, 150, 200 and 250 mg/kg — i.p.
   d)—Normal saline for control group.
9. Test for CAR 30 minutes and 60 minutes after injection.
10. For those rats which do not respond to sound stimulus within 30 seconds (CAR blocked) first deliver foot shock and note if they now climb the pole.

With major tranquillisers, CAR is blocked but escape response to electrical stimulus persists. With sedatives like barbiturates and other CNS depressants, rats do not escape either with sound stimulus or foot shock, when the dose of the drug is large enough to produce ataxia and incoordination of motor functions.

Amnestic agents → both responses blocked

Exercise 7.

Antireserpine Test

Objective: **To study MAO inhibitor type of drugs in mice using reversal of reserpine activity.**

1. Select mice of either sex (body weight 20-25 gms) in groups of 3 each.
2. Inject in one group saline or 2% starch vehicle i.p.
3. Inject in one group each nialamide in doses of 10 and 20 mg/kg i.p.
4. Two hours after the injection, administer reserpine to all groups (2.5 mg/kg i.p.)
5. Observe the animals for presence or absence of sedation, ptosis or excitation for the next 6 hours. Compare the results obtained in nialamide group with that of reserpine.

Dr. Rajani, Renuka, Uma Girish

Behavioral studies

Elevated plus maze

Complex maze

Discrimination 60×

2-chambered -"-

Cook's pole

To test depend. potential.

Diff. other types of mazes

Anxiety

Memory

RP thesis

Antipsych' sed hyp.

RP thesis.

Anx. PTZ ind. conv → RP RK Thesis

Anx memory → RP Thesis

Exercise: 8.

Objective: To screen anticonvulsant activity in mice by electro. convulsion method (MES Test).

1. Select mice of either sex weighing about 25 to 30 gms.
2. On the previous day of testing, choose the mice by giving electrical shock through corneal electrodes (30 MA duration 0.2 sec.) which should produce extension of hind limbs. Select only those mice which show positive results.
3. Next day, divide into 2 groups of 6 mice each.
4. Administer one group with test drug orally (e.g. Dilantin sodium 40 mg/kg, or phenobarbital sodium 50 mg/kg), and use another group as control treated with saline or vehicle.
5. Shock the mice after 60 minutes after drug administration.
6. Note the percentage of mice which do not show hind limb extension (clonic convulsion may occur). Effective anticonvulsants abolish the extensor response (tonic phase).

ED-50 values of Std. drugs for screening tests.

mg./kg (I.P.)

Anticonvulsant

a. MEST

Dilantin → 16.2 (12.1 – 21.7)

phenobarb. Na → 25.0 (16.6 – 38.0)

b. Pentylene tetrazole induced seizure (mice) mg/kg ip.

phenobarb. Na → 20.0 (14.3 – 28.0)

30 mA, duration 0.2 sec.

Exercise: 9.

Objective: **To screen anticonvulsant activity in mice against Leptazole (pentylenetetrazole) induced convulsions in mice.**

1. Select mice of either sex weighing about 25 to 30 gms.
2. On the previous day, inject s.c. (in the scruff of the neck) Leptazole in a dose of 70 mg/kg (7 mg/ml solution). Observe for clonic convulsions for next 15 minutes. Select those mice which show clonic convulsions.
3. Next day, divide into 2 groups of 6 mice each.
4. Administer one group with test drug orally, and use another group as control, treated with saline or vehicle.
5. An hour after the drug administration, inject Leptazole as above.
6. Observe the mice for 15 minutes and note the percentage of mice protected from clonic convulsions. Drugs effective in petit mal like Tridione, block 100% of clonic convulsions.

Exercise: 10.

Objective: To study the analgesic activity of a test compound in mice.

Albino mice of either sex weighing 20-30 gms are used for this experiment. Select the mice which will react to clip, electro-shock and hot plate methods before administering the drugs in order to choose only those animals which react to the noxious stimuli.

(A) Clip method

1. Apply a bull-dog clip to the base of the tail of the mouse.
2. Note down the reaction time in seconds from the moment the clip is applied to the moment when the animal tries to remove the clip by biting.
3. Discard the animals not responding within 5 seconds.

(B) Electric shock method

1. Apply electric shocks (Voltage 20, pulse width 20 ms. frequency 1/sec.) to the tail of the mouse, using a square wave stimulator.
2. Select only those animals which respond with a squeak at this particular voltage.
3. Discard the animals which require more than 20 volts to squeak.

(C) Hot Plate method

1. Place a mouse on a hot plate, maintained at 55.5 $\pm$ 0.5°C
2. Note the time required for each mouse either to lick its hind paws or to jump in an attempt to escape. Normal mice react to this stimulus within 4 seconds.
3. If the reaction time is more than 4 seconds, discard the animal.

After selection, divide the animals in groups of 10 for each dose. Administer the test drug intraperitoneally (3 different doses one per group) in the volume of 1 ml/100 gm. Test the animals by clip, electro-shock and thermal method 15, 30, 45 and 60 minutes after the administration of drug.

The test compound is considered to have an analgesic effect, if: (a) reaction time is prolonged by 10 seconds for the clip method; (b) animal requires more than 20 volts to sqeak for the electro-shock method; and (c) reaction time increased by 7 second for the thermal method.

For each dose carry out all these three methods at the same time.

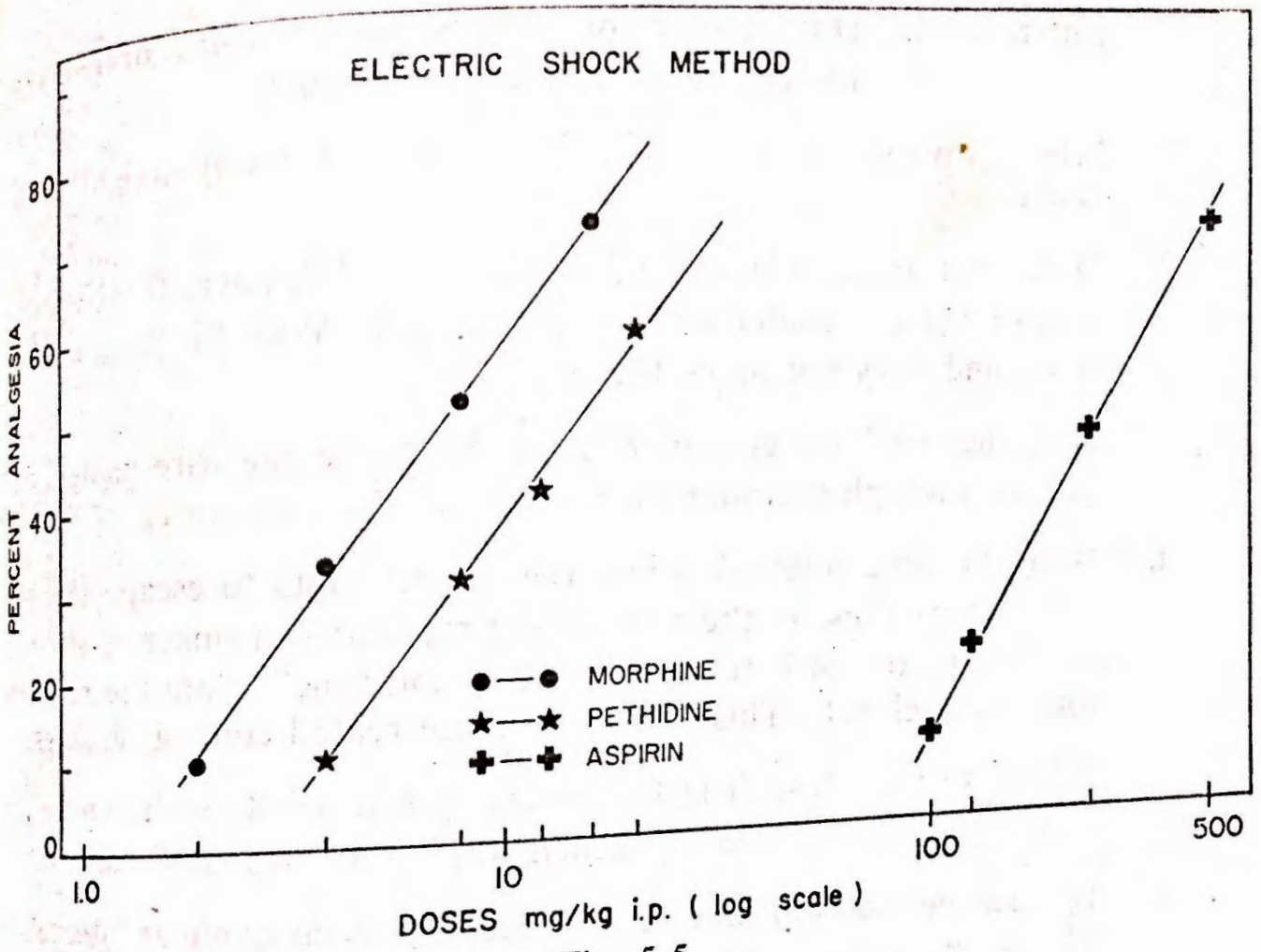

ELECTRIC SHOCK METHOD
PERCENT ANALGESIA
0
20
40
60
80
MORPHINE
PETHIDINE
ASPIRIN
1.0
10
100
500
DOSES mg/kg i.p. (log scale)

Fig. 5.5

(D) Tail flick method

(Instrument: Dolorimeter or analgesiometer using heated Nichrome wire as source of stimulus).

1. Select two groups of rats each of either sex (body weight 80-100 gms).
2. Hold a rat on the instrument, so that the tail lies over the Nichrome wire of analgesiometer without touching it. Wait till the rat settles down and does not move the tail.
3. Start the "on" switch which heats the Nichrome wire using 5 mA current through galvanometer and start the stop watch.
4. Note the time interval, when the rat attempts to escape the heat stimulus by flicking the tail. The time between onset of stimulus and flicking of tail is termed as "Reaction time". Note the reaction time for each rat. This will give the untreated control readings.
5. Administer the test drug to one group intraperitoneally and saline or vehicle to another group which will act as untreated control.
6. Record the reaction time of each rat from each group at intervals of 15 and 30 minutes after the administration of drug. If a rat fails to flick the tail do not continue beyond 8 seconds to avoid injury to tail.
7. Compare the results of treated group with control group.
8. To calculate ED_{50}, take one group each at 3 different dosages. (e.g. Morphine 6, 8, 12 and 16 mg/kg; Pethidine 6, 8, 12 and 24 mg/kg).

TABLE 5.6

ED_{50} (19/20 confidence limits) of morphine sulphate, meperidine hydrochloride and metamizole in mg/kg by various methods.

Drug	Clip Method	Hot Plate Method	Electric Shock Method
Morphine sulphate	8.0 (4.6 — 13.6)	5.9 (2.7 — 12.4)	6.6 (4.7 — 9.3)
Meperidine hydrochloride	8.3 (4.3 — 15.0)	17.0 (8.3 — 34.0)	12.0 (8.4 — 17.2)
Metamizole	540 (295 — 930)	1920 (987 — 2240)	780 (565.5 — 1030)

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Isolated Tissue Experiments

You do one experiment in medicine to convince yourself, then 99 more to convince others.

ALPHONSE RAYMOND DOCHEZ

Introduction

The isolated tissue preparation and the method of bioassay may well be considered the trademark of the pharmacologist. With greater advances in the field of neurohumoral mechanisms, and better insights into human biochemical pharmacology, it is yet difficult to better the bioassay by chemical assay. This is particularly true with studies of mixtures of substances in perfusates/effluents or in the isolation of an intermediate with less biological but almost identical chemical activity as the end product in biosynthesis. Bioassay or the assay of a substance by biological means, takes diverse forms of animal experiments in general, of three types.

Measurement of an all or non-phenomenon, either as 1) an endpoint in each animal e.g. head drop by curare, cardiac arrest by digitalis, or as 2) a percentage response in a group of animals e.g. cornification rat's vaginal epithelium after oestrogens, development of hypoglycemic convulsions in mice after insulin injection, etc.

Measurement of a graded phenomenon e.g. the dose related response of an isolated tissue (also refer Chapter 1).

This chapter will deal with the usefulness of particular isolated tissues for a given substance and for further details of bioassay method or isolated tissue preparations the reader is recommended references cited below.

The guinea pig's isolated ileum is a tissue suitable for bioassay of histamine. The responses are generally quite consistent and the relation to dose fairly predictable. Discrimination between doses is good enough for purposes of bioassay. Acetylcholine can also be assayed by this method, but this again has the disadvantage of promoting the development of spontaneous contractions. The sensitivity of the tissue is not marked.

Practical points

The tissue should be perfectly clean, because accumulation of mucus in the lumen sets off spontaneous contractions. This should be ensured by starving the animal for 24-48 hours before the experiment and repeated washing of the tissue.

The frog's isolated rectus abdominis muscle is an extremely convenient prepara-

"Pharmacological Experiments on isolated preparations" by the Staff, Department of Pharmacology, University of Edinburgh, E. & S. Livingstone Ltd., London & Edinburgh, 1968.

tion for the bioassay of acetylcholine and d-tubocurarine. It has the advantage of ease of mounting, convenience to use and free availability. It does not need temperature regulation and does not pose problems of spontaneity of contraction. The sensitivity of the method is sufficiently high for all practical purposes and may be increased by addition of physostigmine (0.5 μg/ml) or alcohol to the perfusion fluid. Storing the tissue at 4°C overnight in Frog Ringer saturated with oxygen increases its sensitivity. The main disadvantage of the method is the lengthiness of the entire experiment, as each contraction takes 90 seconds for completion and 15 minutes for relaxation.

The isolated rat colon is a convenient preparation for the bioassay of epinephrine. The method followed is that of De Jalon who originally used the rat uterus. Graded doses of epinephrine are added to produce inhibition of a standard carbachol induced contraction. The degree of inhibition is considered as the response to epinephrine. The method is easy, convenient and fairly sensitive. Generally a bracketing assay is done. The usual spontaneity seen is damped by utilising a modified Ringer solution poor in calcium as the perfusion fluid (refer Appendix 4).

The isolated rabbit jejunum is another method of bioassay of epinephrine utilising the dose dependent inhibition of pendular movements by epinephrine. This inhibition is accompanied by actual relaxation, also dose related. The method is only fairly sensitive. The tension on the tissue is extremely critical and can produce waxing and waning of pendular movements, rendering the preparation unsuitable for bioassay purposes. Thus, though easy to set up, the method is tedious to continue.

The isolated aortic strip of the rabbit is yet another means of assaying catecholamines (in this case, norepinephrine). It is easy to set up, fairly specific for norepinephrine and extremely consistent in its response. The main disadvantage is the slowness of contraction which can be overcome by using the cumulative dose response curve as described in Chapter 1. It is not very discriminating as far as small increments in doses are concerned and is best suited for the study of α adrenergic antagonists to decide the nature of the antagonism. Cumulative dose response curves are obtained in the absence and presence of graded amounts of the antagonist, each time attempting to elicit supramaximal contraction. The doses of agonist and antagonist are converted to molar amounts and curves are plotted using percentage of maximal response obtained against the log of the corresponding dose. In other words, if a response is 40 mm high and the maximal height of the cumulative curve obtained is 160 mm high, the response is considered as 40/160 = 25%, and this value is plotted on the 'Y' axis. The nature of the curves obtained in presence of antagonist indicates the type of antagonism. Competitive type is shown by a parallel shift of the curves without change in maxima, while noncompetitive antagonism is reflected by a non-parallel shift combined with decline in maximal heights.

The isolated rat uterus is utilised for bioassay of epinephrine by De Jalon's method (as with rat colon) and of 5-hydroxytryptamine. The latter produces graded responses to nanogram doses. The method is very sensitive and discriminating. The disadvantage of spontaneity is overcome by using De Jalon's solution which is poor in calcium, and by eliciting responses at room temperature instead of at 37°C.

Another preparation useful for bioassay of 5-HT is **the isolated rat fundic strip.** This tissue is also sensitive, and discriminating but occasionally instead of a

smooth contraction, a series of contraction-relaxation movements are obtained, producing a zig-zag record. This tissue can be converted for bioassay of catecholamines which relax it, by producing a steady tonus in the tissue by addition of 5-HT in the perfusing fluid. In the presence of this 5-HT, epinephrine produces a well-graded relaxation.

A similar principle is utilised in **the isolated tracheal chain of guinea pig**. Tonic contraction due to pilocarpine is utilized to study epinephrine dose dependent relaxation. The effect of agonists like 5-HT, histamine and acetylcholine which produce contraction are easier to elicit. The nature of adrenergic receptors in the tracheobronchial tree (of practical interest in bronchial asthma) may also be studied utilizing appropriate agonists and antagonists.

Isolated Cat Spleen : This preparation is of greater academic than practical importance and is useful to study the nature of the adrenergic receptor in the spleen **in vitro.** It is not commonly used for bioassay of epinephrine.

Experimental Techniques

1. **The isolated guinea pig ileum**. This preparation has been described in Chapter 1.

2. **The isolated rabbit jejunum.** This preparation has been described in Chapter 2.

3. **The isolated frog rectus abdominis muscle.** Refer Chapter 1 for a description of this preparation.

4. **Isolated rat colon preparation** is described in Chapter 1.

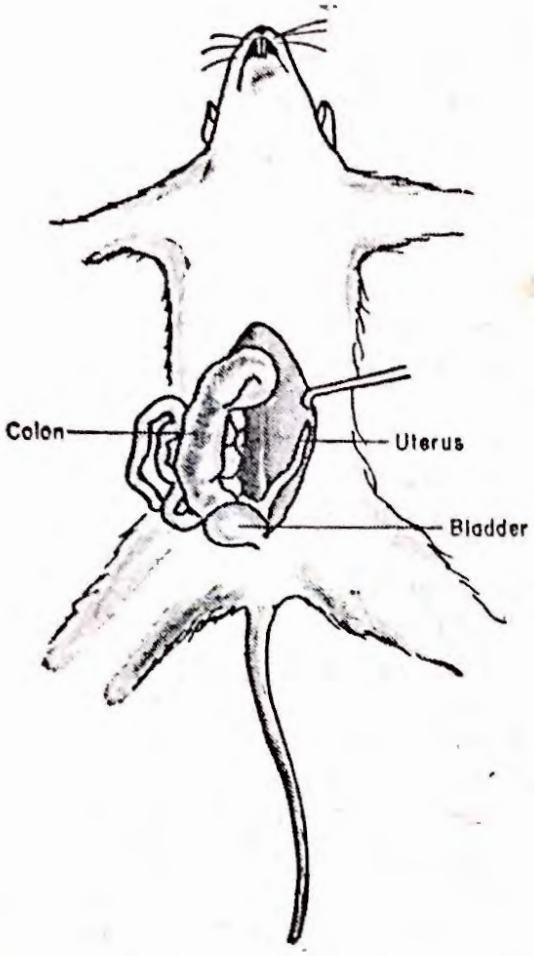
5. **Isolated rabbit aortic spiral/strip** is set up as described in Chapter 1.

6. **The isolated rat uterus**. A young female rat is injected with stilboestrol (0.1 mg/kg) in arachis oil i.m. 24 hours prior to the experiment. It is killed by stunning and the abdomen quickly opened. The bicornuate uterus is identified behind the coils of small intestine, one horn lying in each flank, with large amounts of fat along its inferior border. It is carefully removed and placed in a shallow dish containing De Jalon's solution and dissected free of the fat, ovaries etc. Care is taken not to stretch the preparation. One horn is then mounted in an isolated organ both in De Jalon's solution at 37°C aerated with oxygen.

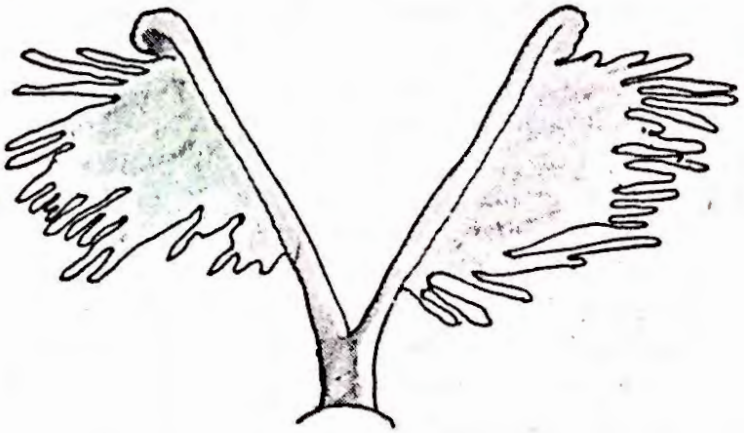
A period of about half an hour is allowed for stabilisation and the experiment then begun. Responses to 5-HT are recorded, using a contact period of 30 seconds, and a recovery period of 3-5 minutes in between successive doses, by means of an isotonic lever loaded for a tension of 0.5 g.

7. **The isolated rat fundic strip.** This is a preparation of the fundic portion of the stomach of rat. After a period of 24-48 hours starvation a rat is killed by stunning and the abdomen opened to reveal the viscera. The stomach is identified as a hollow viscus just beneath the diaphragm. It is removed and placed in a shallow dish containing Krebs solution. On close inspection it may be found to consist of an upper greyish and a lower pinkish portion representing respectively the fundus and the pylorus of the stomach. The fundus and a strip of the pyloric part are cut out and slit open as shown in the figure. With a series of parallel cuts placed alternately from either margin the fundus is cut in the direction of its fibres to form a strip 3 mm in width (the bits of pylorus may now be removed having served their purpose as a landmark). This fundic strip is mounted in Krebs' solution at 37°C and responses recorded with an isotonic lever loaded for a tension of 1-2 g. The tissue is allowed to relax for 3/4 — 1 hour and this may be further enhanced by an extra 0.5 g load on the lever. Reponses to graded doses of 5-HT are recorded with a contact period of 90 seconds and a recovery period of about 10-15 minutes.

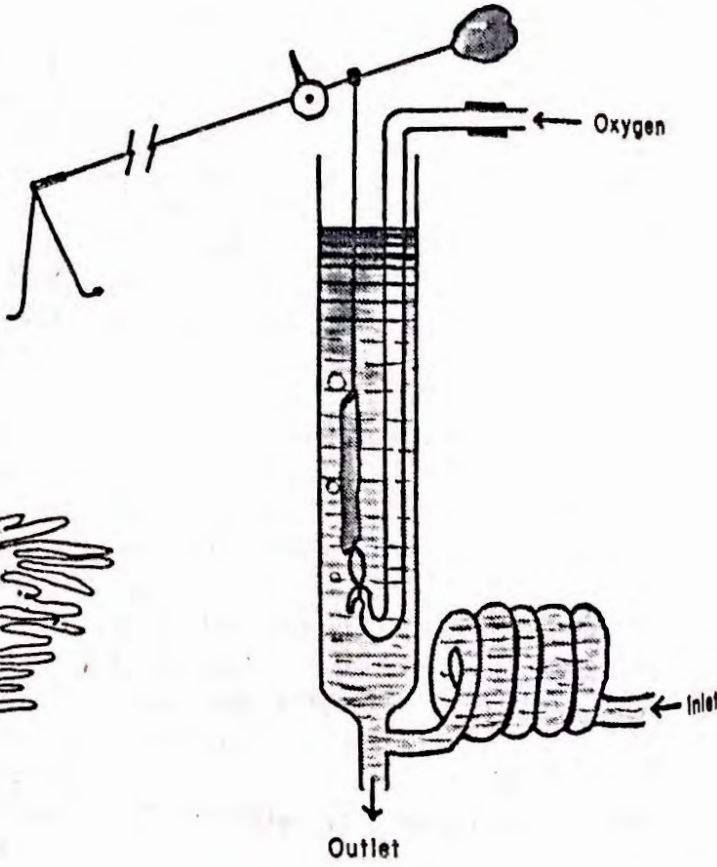
Fig. 6. 1: The isolated rat uterus preparation.



6. 1-a
The uterus is identified as a bicornuate structure behind the other abdominal viscera.

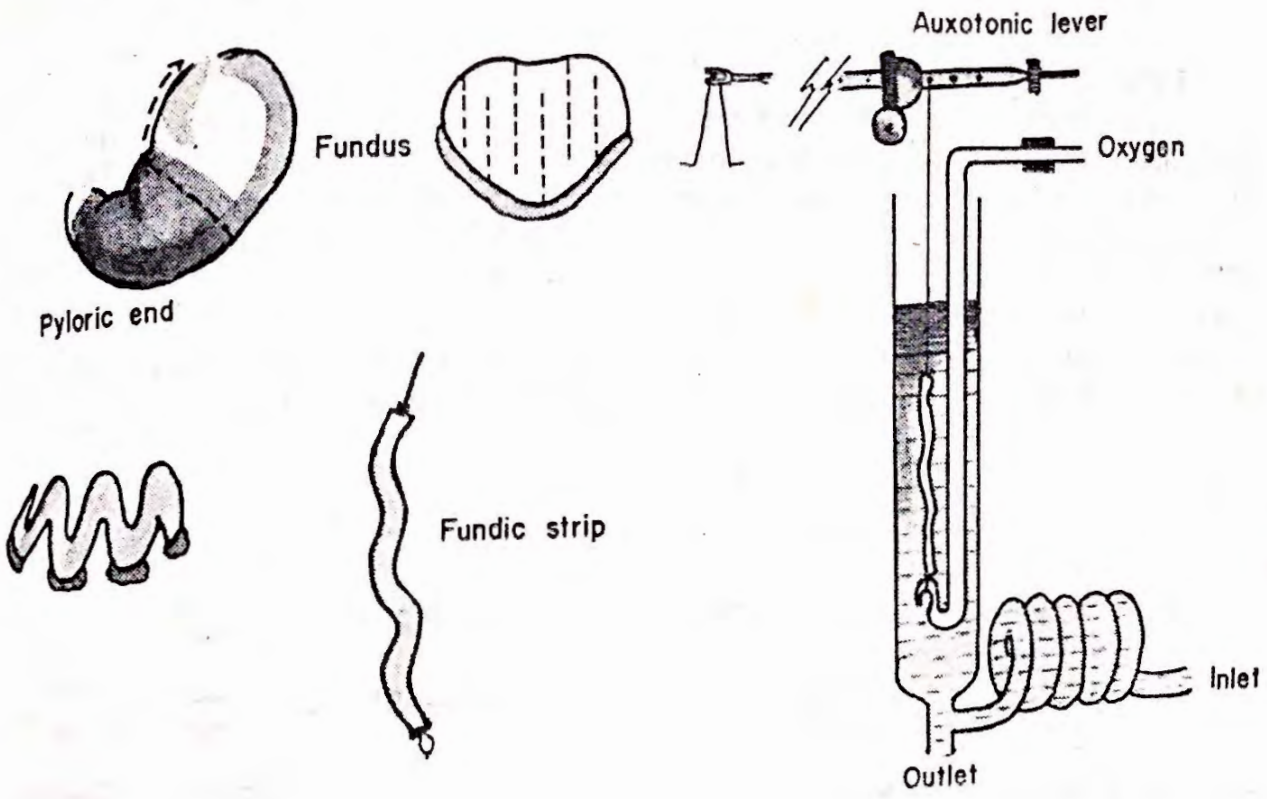


6. 1-b
The uterus is removed, and the fat seen along the inferior borders is trimmed. One horn is used for the experiment.



6. 1-c
The final set up of the isolated rat uterus in De-Jalon's solution at room temperature.

Fig. 6. 2. The isolated fundic strip of rat.



6. 2-a

The fundus of the stomach is identified, removed along with a strip of pylorus as shown by the arrows, and cut open.

6. 2-b

Cuts are placed along the vertical lines in (a) and the strip thus obtained opened out.

6. 2-c

The final set up of the isolated fundic strip in Krebs' solution at 37°C.

8. **The isolated tracheal chain of the guinea pig.** A guinea pig of about 450 g weight is killed by stunning and cutting the throat as close to the mandible as possible. The skin and muscles of the neck are divided and the trachea isolated and removed. It is placed in a dish containing Krebs solution and cleared of connective tissue until a glistening white cartilaginous surface is revealed. This also includes a strip of smooth muscle on the posterior aspect. The cartilaginous portion is cut vertically in the midline and the trachea opened out. It is then cut into transverse strips each of which contains a central segment of smooth muscle with cartilage at the ends. These strips are joined end to end, as shown in the diagram and the tracheal chain thus obtained is mounted in an organ bath in Krebs' solution at 37°C. The recording lever is balanced for a tension of 0.5 g and the tissue allowed to relax for about 1 — 1½ hours. Responses to drugs are elicited using a contact period of 90 seconds and recovery period of 15-20 minutes.

9. **The isolated phrenic nerve — hemidiaphragm preparation of rat** (refer Chapter 3).

10. **Isolated atria of rabbit** (refer Chapter 4).

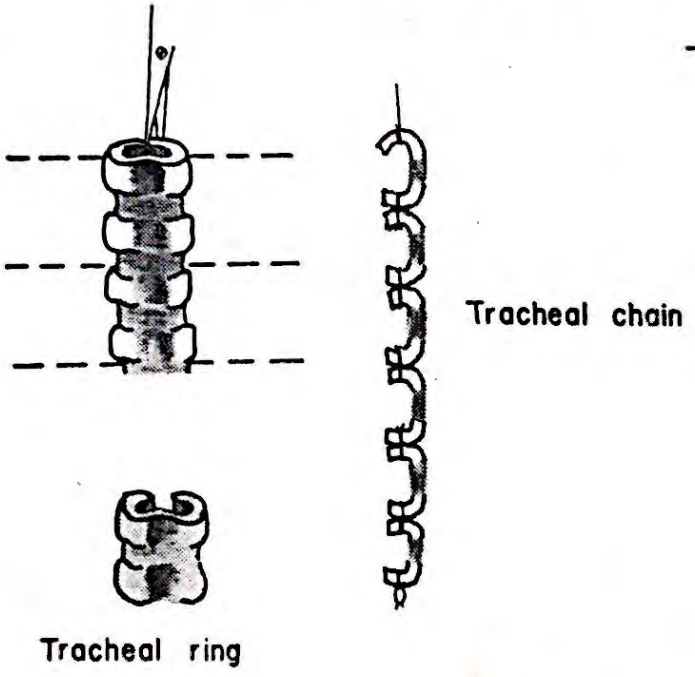
11. **Isolated, perfused rabbit heart** or Langendorff's preparation (refer Chapter 4).

12. **Isolated cat spleen.** The spleen from an anaesthetised cat is removed prior to administration of any drug in the course of a routine experiment. It may be preserved overnight at 4°C in Tyrode solution saturated with oxygen.

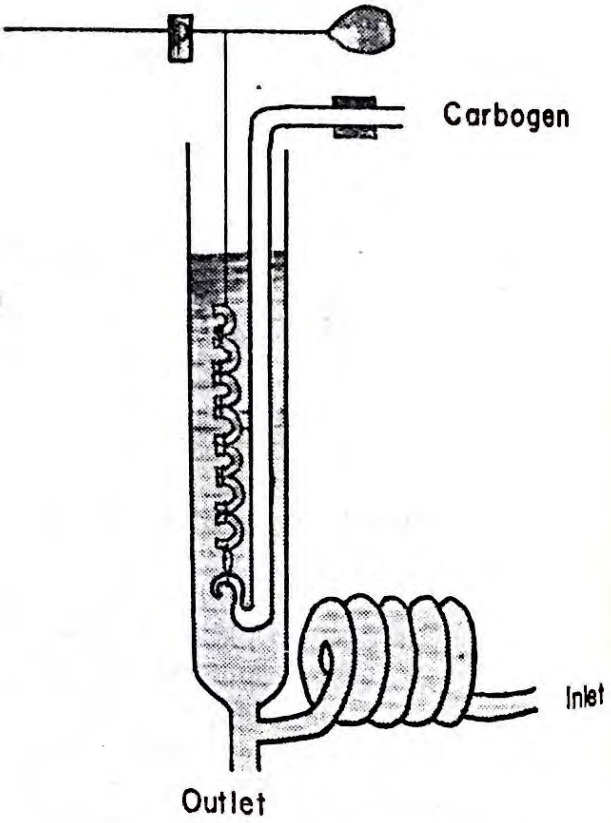
For the purpose of this experiment it is allowed to regain room temperature and a slice is cut as shown in the figure so as to obtain a complete ring of capsule around the slice. It is mounted in an organ bath at 37°C in Tyrode, with a recording lever balanced for a tension of 0.5 g. Relaxation is allowed to occur for about 1 hour and the experiment then begun. Responses to graded doses of an α agonist like epinephrine are recorded, using a contact period of 30 seconds and a recovery period of 10 — 15 minutes.

13. **The isolated, innervated rabbit jejunum** is described in Chapter 2.

Fig. 6. 3. The isolated tracheal chain preparation of guinea pig.

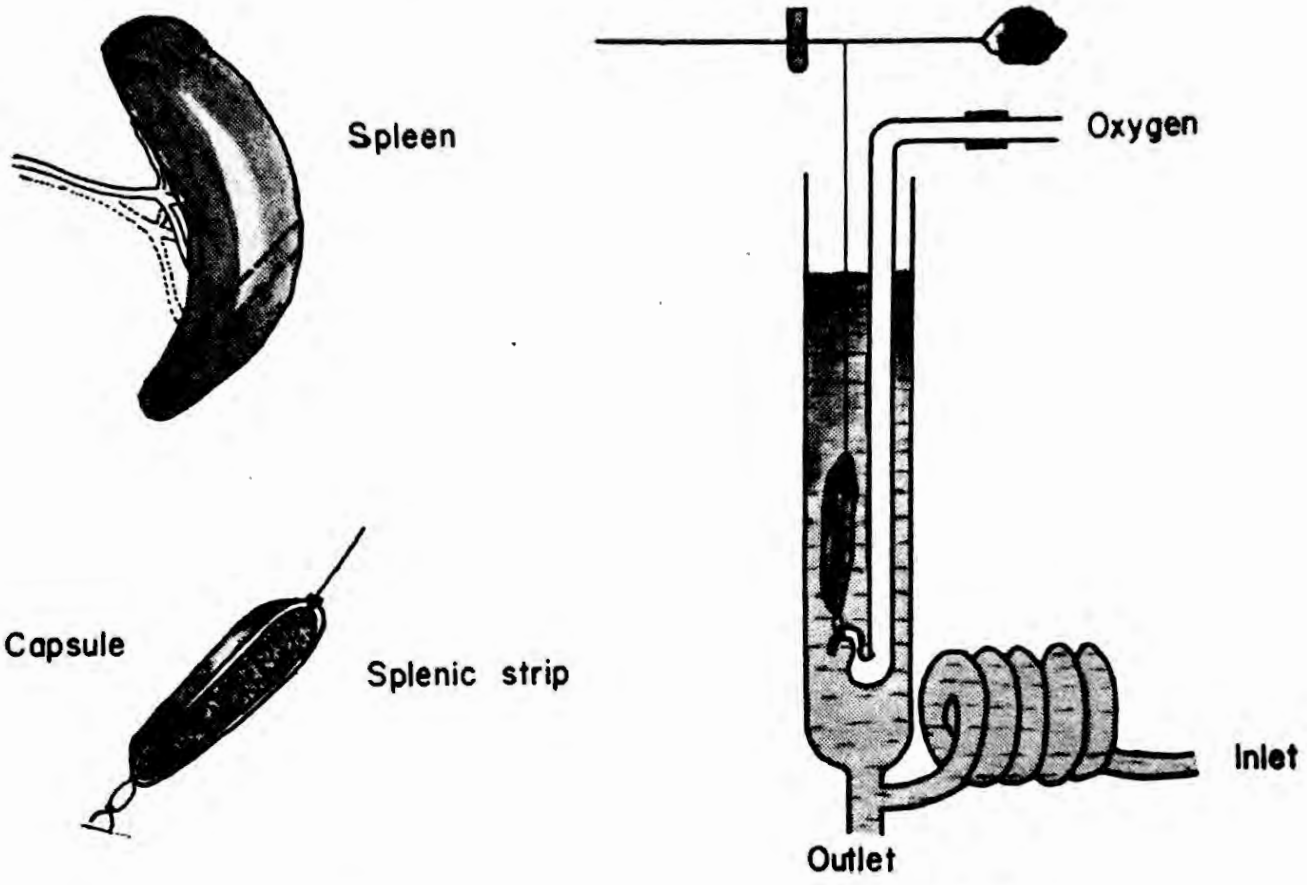


6. 3-a
The trachea is removed, cut as shown and the semi circle thus obtained joined together end to end.



6. 3-b
The final set up of the tracheal chain in Krebs' solution at 37°C aerated with carbogen 95° oxygen, 5% carbondioxide).

Fig. 6.4. The isolated cat spleen preparation.



6. 4-a

The spleen slice removed as shown so as to obtain a complete ring of splenic capsule around it.

6. 4-b

The final set up of the splenic strip in Tyrode solution at 37°C

Practical Exercises

Exercise 1

Objective: To assay an unknown solution of histamine.

1. Set up an isolated preparation of guinea pig's ileum.
2. Elicit dose-related responses to a standard solution of histamine.
3. Similarly, elicit responses to graded amounts of a suitable dilution of the unknown solution.
4. Choose 2 doses of unknown and two of standard and perform a 4 point assay.

Exercise 2

Objective : To assay an unknown solution of acetylcholine.

1. Set up a preparation of isolated frog's rectus abdominis muscle.
2. Elicit dose related responses to a standard solution of acetylcholine.
3. Elicit responses to graded amounts of a suitable dilution of the unknown solution.
4. Select 2 doses of unknown and 2 doses of standard and perform a 4 point assay as described in Chapter 1.

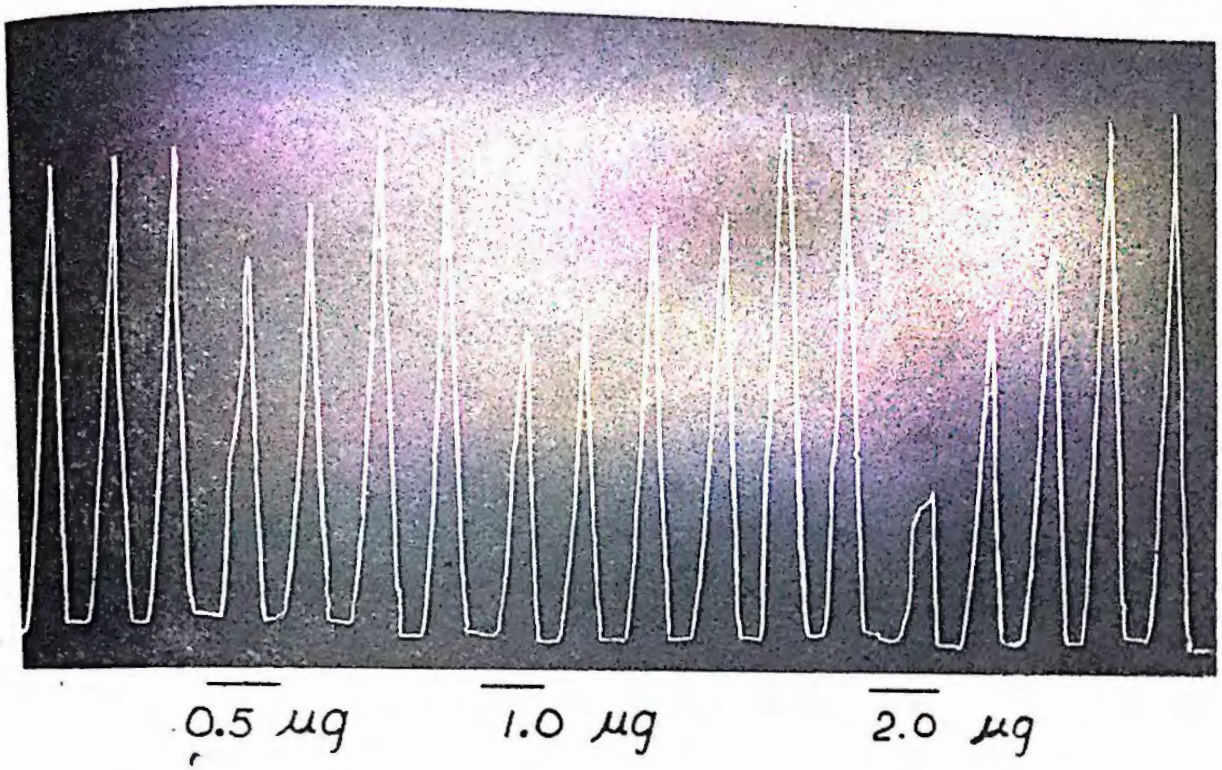
Exercise 3

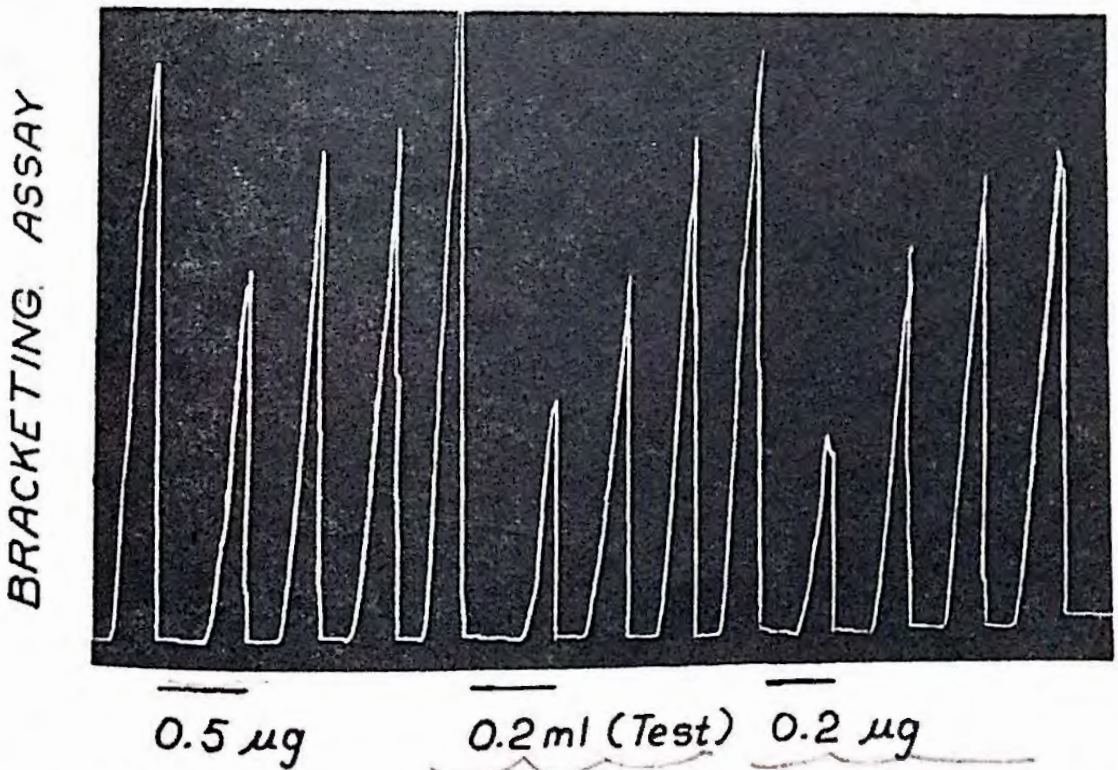
Objective: To assay an unknown solution of epinephrine (De Jalon's method).

1. Set up an isolated preparation of rat colon as described.
2. Elicit responses to carbachol (10 μ g/ml) such that the height of the contraction is about 6 cm.
3. Add 0.5 μ g epinephrine and in its presence elicit a response to carbachol in the same dose as before.
4. Wash out the drug and record recovery of the response to carbachol.
5. Similarly, elicit responses to carbachol in the presence of 1.0, 1.5, 2.0 μ g epinephrine.
6. Observe that the degree of inhibition of the carbachol response is related to the dose of epinephrine.
7. With a suitable dilution of unknown solution of epinephrine, perform a bracketing assay, matching the inhibition of the carbachol responses produced with standard and unknown.

ISOLATED RAT COLON

Estimation of epinephrine by De Jalons method





All responses recorded with 4.0 µg of carbachol. Bars indicate presence of

Exercise 4 (a)

Objective: To assay an unknown solution of epinephrine.

1. Set up an isolated preparation of rabbit jejunum.
2. Allow the tissue to stabilise.
3. Add graded doses of a standard solution of epinephrine.
4. Observe the dose related relaxation of the jejunum.
5. Proceed to perform a bracketing assay with the unknown solution.

Exercise 4 (b)

Objective : To study the sensitivity of rabbit aortic strip to various agonists.

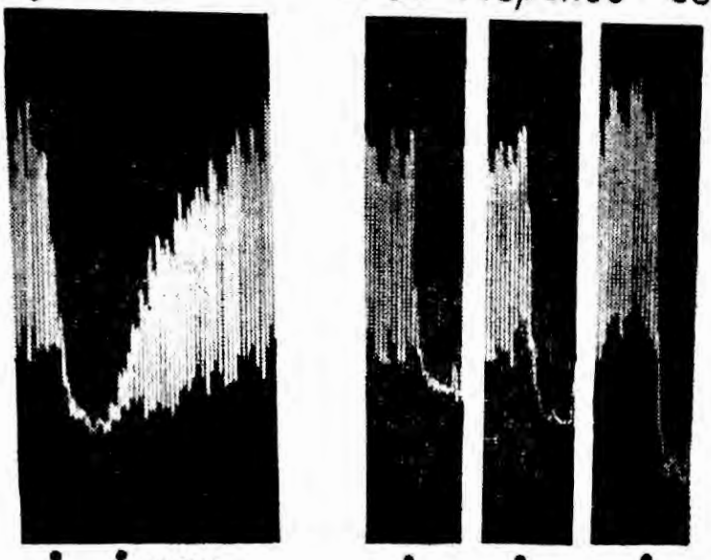
1. Set up an isolated aortic strip of rabbit.
2. After stabilization of the tissue, elicit responses to the agonists as follows : Epinephrine and norepinephrine in micrograms, 5-HT and histamine in microgram doses larger than those of E,NE and acetylcholine in very large doses (100 μ g).

BIOASSAY (EPINEPHRINE)

ISOLATED RABBIT JEJUNUM

Effect of epinephrine

Single dose Dose response curve



(DOSES IN μg)

BATH CAPACITY: 20 ml

Fig. 6.6–a

ISOLATED RABBIT AORTIC STRIP

Sensitivity to various agonists

Exercise 5

Objective: (a) To assay an unknown solution of 5-HT.

1. Set up an isolated fundic strip from the stomach of rat.
2. Elicit responses to graded doses of 5-HT (10, 15, 20, 25 nanograms).
3. Observe the good discrimination between doses and the high sensitivity of the tissue which make it suitable for bioassay purposes.

Objective: (b) To assay an unknown solution of epinephrine.

1. Set up an isolated rat fundic strip.
2. Maintain a degree of tonus in the tissue by addition of 5-HT to the perfusing fluid (10 μ g/litre).
3. Balance the lever after the tissue has reached a stable tone.
4. Add graded doses of epinephrine and observe the dose related response.
5. This tissue has a sensitivity in the region of nanogram amounts of epinephrine and is suitable for assay of this substance in small amounts.

CATECHOLAMINE ASSAY
Isolated rat fundus strip

Fig. 6.7-a

BIOASSAY (5-HT)
ISOLATED RAT FUNDUS STRIP

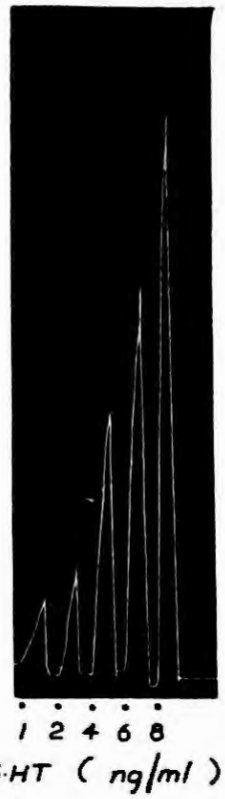


Fig. 6.7-b

Exercise 6

Objective: To assay an unknown solution of 5-HT.

1. Set up an isolated preparation of rat uterus.
2. Add graded doses of 5-HT in nanograms (10, 15, 20, 25).
3. Observe the sensitivity of the preparation which makes it suitable for bioassay of small amounts of 5-HT.

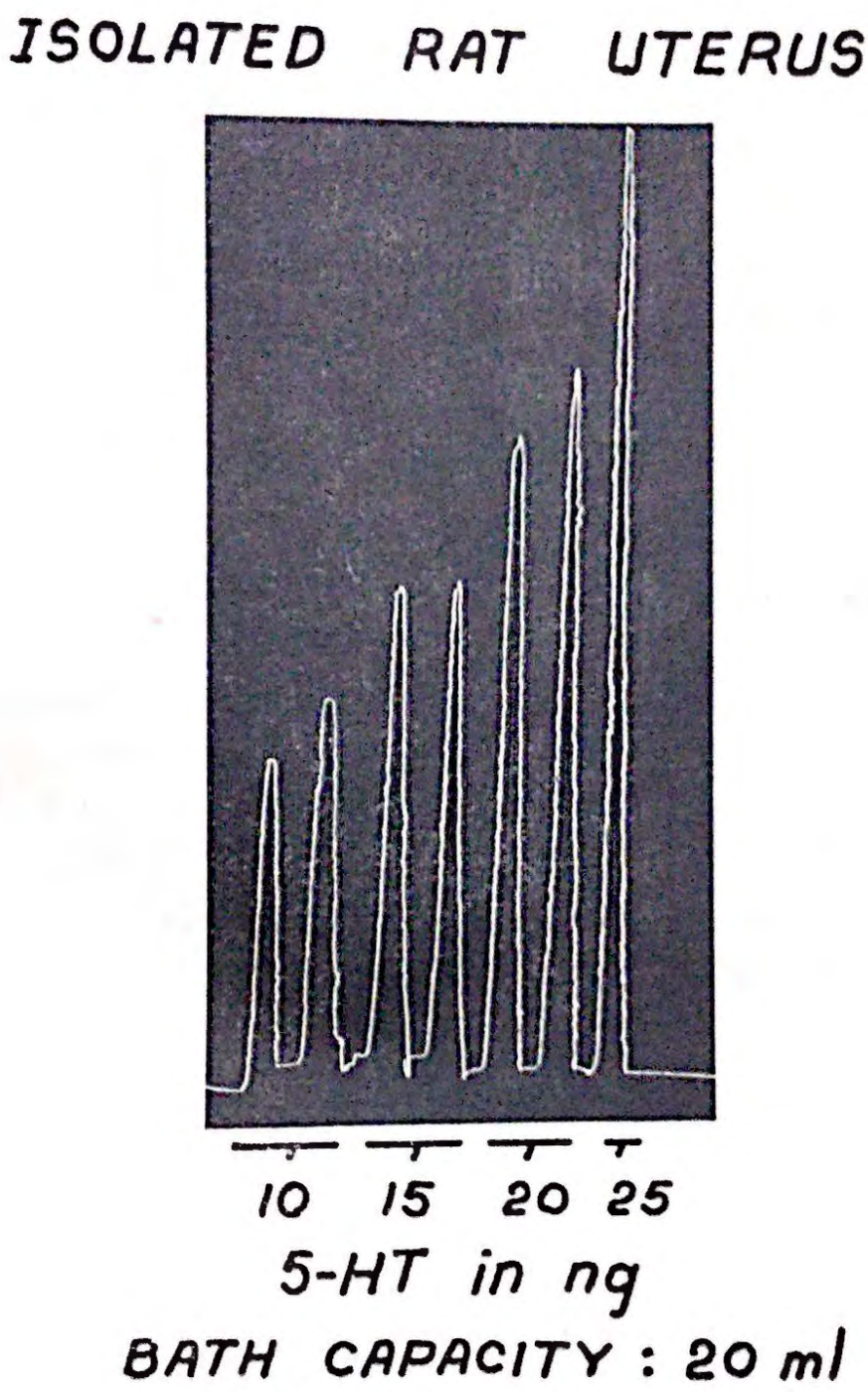


Fig. 6.8

Exercise 7

Objective: (a) To assay an unknown solution of isoprenaline.

1. Set up an isolated preparation of guinea pig's tracheal chain.
2. Maintain a steady state of tone in the tissue by addition of carbachol 50 μg/litre in the perfusion fluid and adjust the balancing of the lever.
3. Add graded amounts of isoprenaline and observe that the relaxation elicited is proportional to dose.
4. Observe the sensitivity of the tissue in nanogram quantities.
5. Proceed to perform a bracketing assay with an unknown solution of isoprenaline.

Objective: (b) To study the sensitivity of the guinea pig tracheal chain to various agonists.

1. Set up an isolated preparation of guinea pig tracheal chain as described.
2. Elicit responses to graded doses of histamine, acetylcholine and 5-HT.
3. Observe that the sensitivity is in microgram quantities as opposed to the nanogram doses of isoprenaline recorded in part (a).

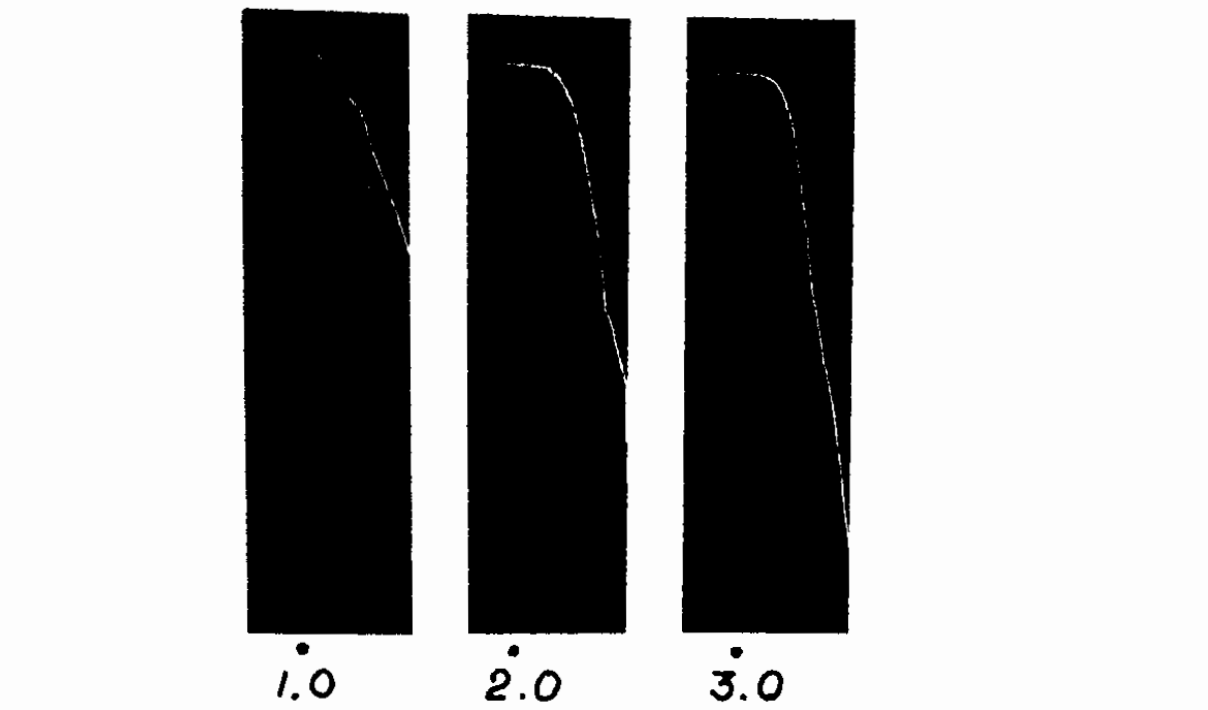


Fig. 6.9 - a

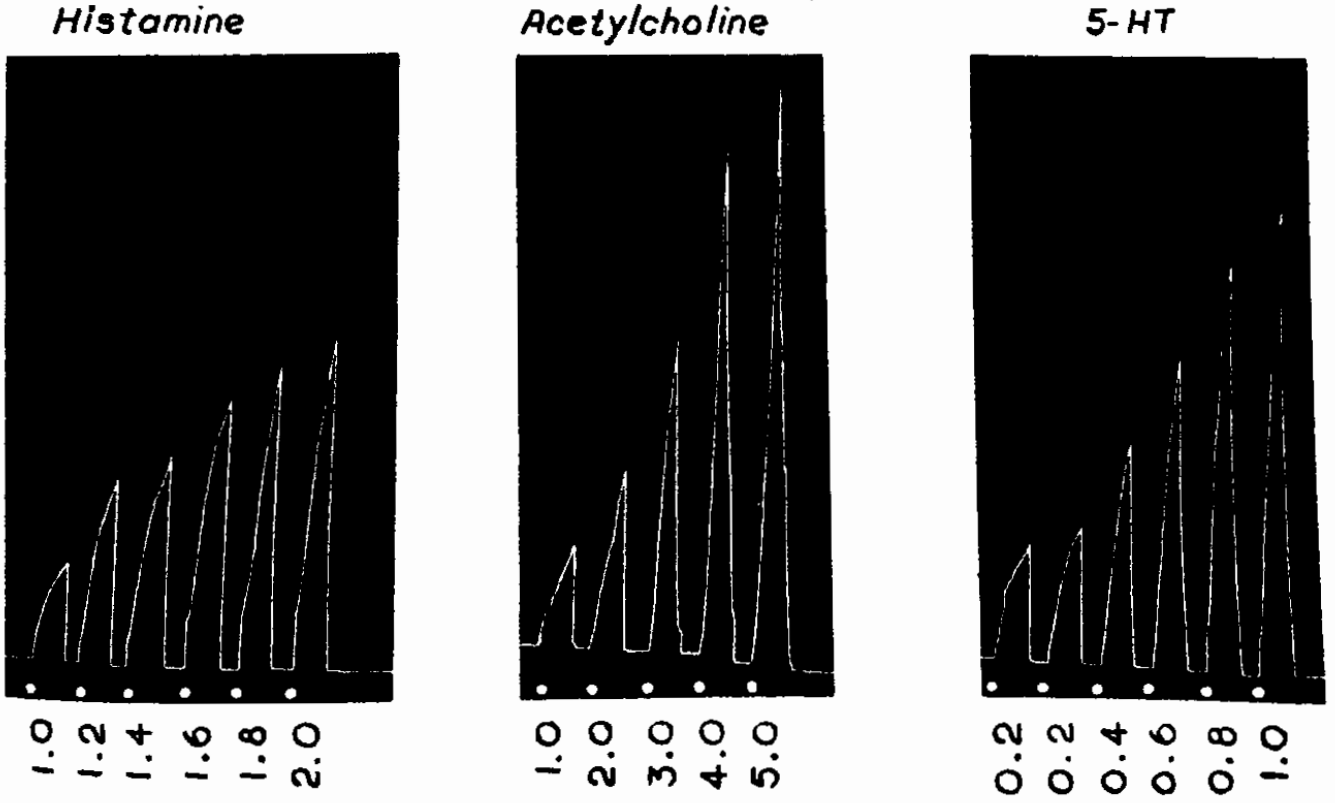


Fig. 6.9 - b

Objective: (c) To study the nature of the adrenergic receptors in the guinea pig tracheal chain.

1. Set up an isolated preparation of guinea pig tracheal chain.
2. Elicit responses to isoprenaline in presence of carbachol in the perfusion fluid.
3. Add phenoxybenzamine to the bath 10 μ g/ml and elicit response to isoprenaline.
4. Observe no potentiation/blockade of isoprenaline (β agonist) by phenoxybenzamine (α antagonist).
5. Elicit responses to epinephrine in absence and presence of phenoxybenzamine.
6. Observe that the relaxant (β) effect of epinephrine (α and β agonist) is potentiated by phenoxybenzamine by blockade of the α receptors i.e. this shows that apart from the predominant β receptors, certain α receptors must be present.
7. Similarly, elicit isoprenaline responses before and after D.C.I. and observe blockade of the response.

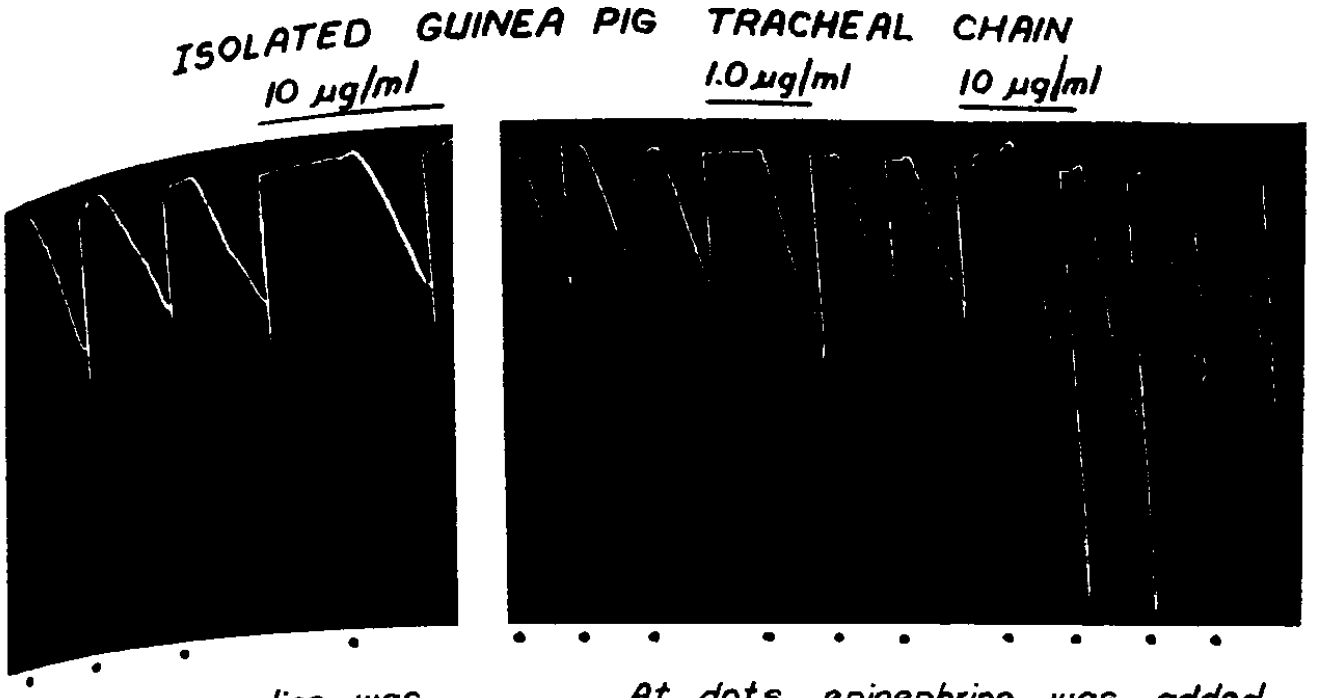
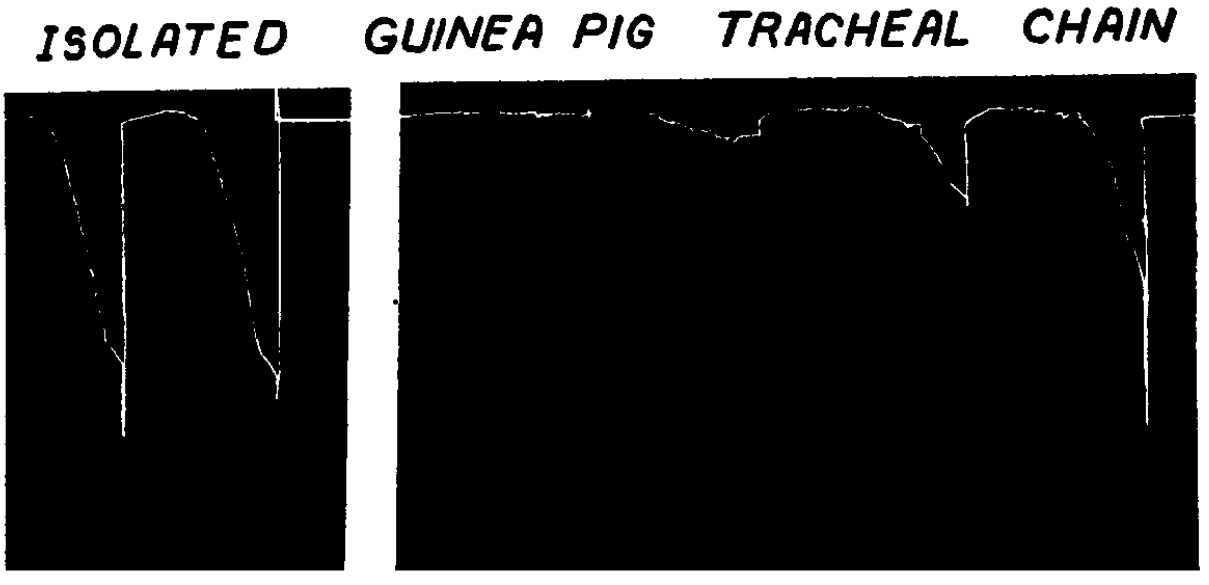

ISOLATED GUINEA PIG TRACHEAL CHAIN
10 µg/ml
1.0 µg/ml
10 µg/ml
At dots isoprenaline was added in a dose of 2 ng/ml
At dots epinephrine was added in a dose of 10 ng/ml
BARS INDICATE PRESENCE OF PHENOXYBENZAMINE IN DOSES MENTIONED
Carbachol was present in the concentration of 50 µg/ml of bath fluid

Fig. 6.9-c


ISOLATED GUINEA PIG TRACHEAL CHAIN

Experiment 8

Objective: To assay an unknown solution of d-tubocurarine.

1. Set up a preparation of rat phrenic nerve-hemidiaphragm as described.
2. Add d-tubocurarine in graded amounts and observe dose related inhibition of contractions.
3. Utilise this for a bracketing assay of d-Tc (Alternatively, a frog's rectus abdominis muscle preparation may be used, utilising De Jalon's principle).

Experiment 9

Objective: To study the effect of drugs on the myocardium in vitro (Refer Chapter 4).

Experiment 10

Objective: To study the effect of drugs on myocardium and coronary vessels in vitro (Refer Chapter 4).

Experiment 11

Objective: To study the suitability of the cat spleen as an adrenergic system in vitro.

1. Set up a preparation of cats isolated spleen slice as described.
2. Elicit responses to graded doses of epinephrine and norepinephrine.
3. Observe that the sensitivity is low and the responses to the pure α agonist norepinephrine are smaller than the responses to the α and β agonist epinephrine.

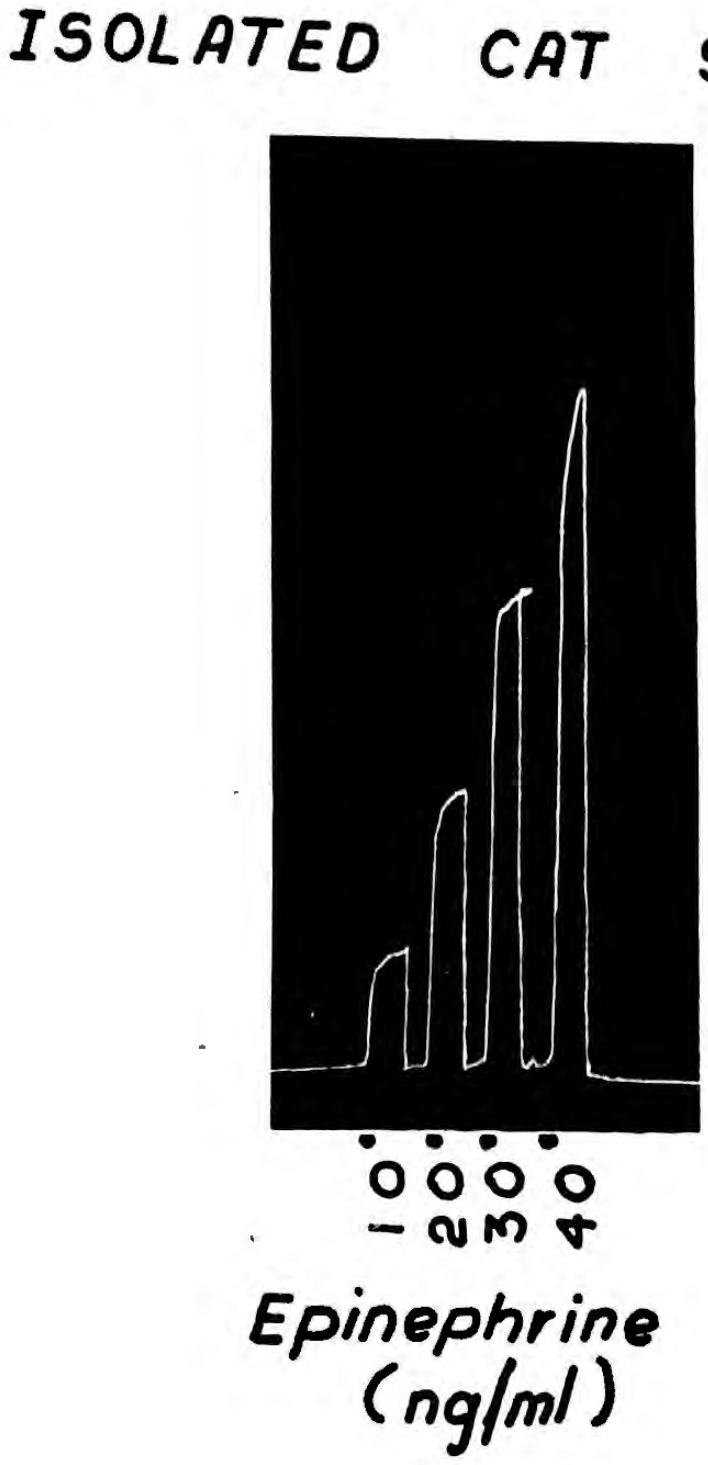


Fig 6.10

Experiment 12

Objective: To study the site of adrenergic blockade produced by an unknown drug in vitro.

1. Set up an isolated Finkelman preparation as described.
2. Elicit control responses to electrical stimulation and to epinephrine added to the bath fluid.
3. Add the test drug to the bath fluid.
4. Elicit responses to electrical stimulation and added epinephrine.
5. Observe that an adrenergic neuron blocker inhibits the response to electrical stimulation but not that to added epinephrine, while an adrenergic receptor blocker will block both.

Isolated innervated
rabbit jejunum
FINKELMAN PREPARATION

Bar indicates stimulation
of mesentery for 30 sec.
(Volts-10, Duration-0.5 msec,
Rate-50 shocks/sec.)

Fig. 6.11

Miscellaneous Topics

Somehow, problems get into my blood and they don't give me peace, they torture me. I have to get them out of my system, and there is but one way to get them out, — by solving them. A problem solved is no problem at all, it just disappears.

ALBERT SZENT-GYORGYI — PERSPECTUS IN BIOLOGY & MEDICINE, 5, 173, 1962

In this chapter, we have described a few miscellaneous topics, like screening of diuretics, anti-inflammatory drugs and bronchodilators. Each one of them by themselves, could form a separate chapter, with extensive theoretical and practical details. Such details would only interest a few workers, who are actively engaged in these fields. We have described here only a few of these techniques which are being carried out in our laboratory and could be of interest to all the postgraduates in pharmacology as exercises in a general training program. Most of these practical exercises could be carried out without the need for specialised equipment.

Diuretics

The screening of diuretics for clinical utility is a matter of practical importance. Promising laboratory results at times are not supported by subsequent clinical trials. This may be due to species difference in response to drugs. Diuretics are most commonly screened in rats and dogs.

A method using rats for estimation of antidiuretic potency was described by Burn in 1931. This method or a modification of it has been used for diuretic assays by most of the subsequent workers. Lepchitz*. et al (1943) described a method which was a modification of Burn's method for diuretic assay using several commonly used diuretics. Next to the rat the most commonly used animal for assessing diuretic activity is the dog. The technical details vary considerably with different workers. Dogs used are either anaesthetised or unanaestheti.ed. Generally, unanesthetised trained female dogs are used. A catheter is passed in the bladder and urine is collected over a fixed period of time. Sometimes the female dogs are prepared before hand by some preoperative procedure to allow easy catheterisation. However, in practice, with a little training, self-retaining catheter can be easily passed in a female dog. Animals are either hydrated with saline or with distilled water, or not hydrated at all. It is best to assay a new diuretic in both dogs and rats, since some of the drugs may be active in one species and not in the other.

Diuretic studies in the rat

Male albino rats weighing in group of 4 between 750 to 850 g (average 800 g) are selected. Prior to the experiment the rats are allowed free access to food and water. During the experiment no food or water is allowed. Some workers hydrate the animals prior to the experiment. Each

* Lepchitz W. L., Maddian, Z. and Kerpecsor, A. (1943) J. Pharm Exptl. Therap 79 : 97.

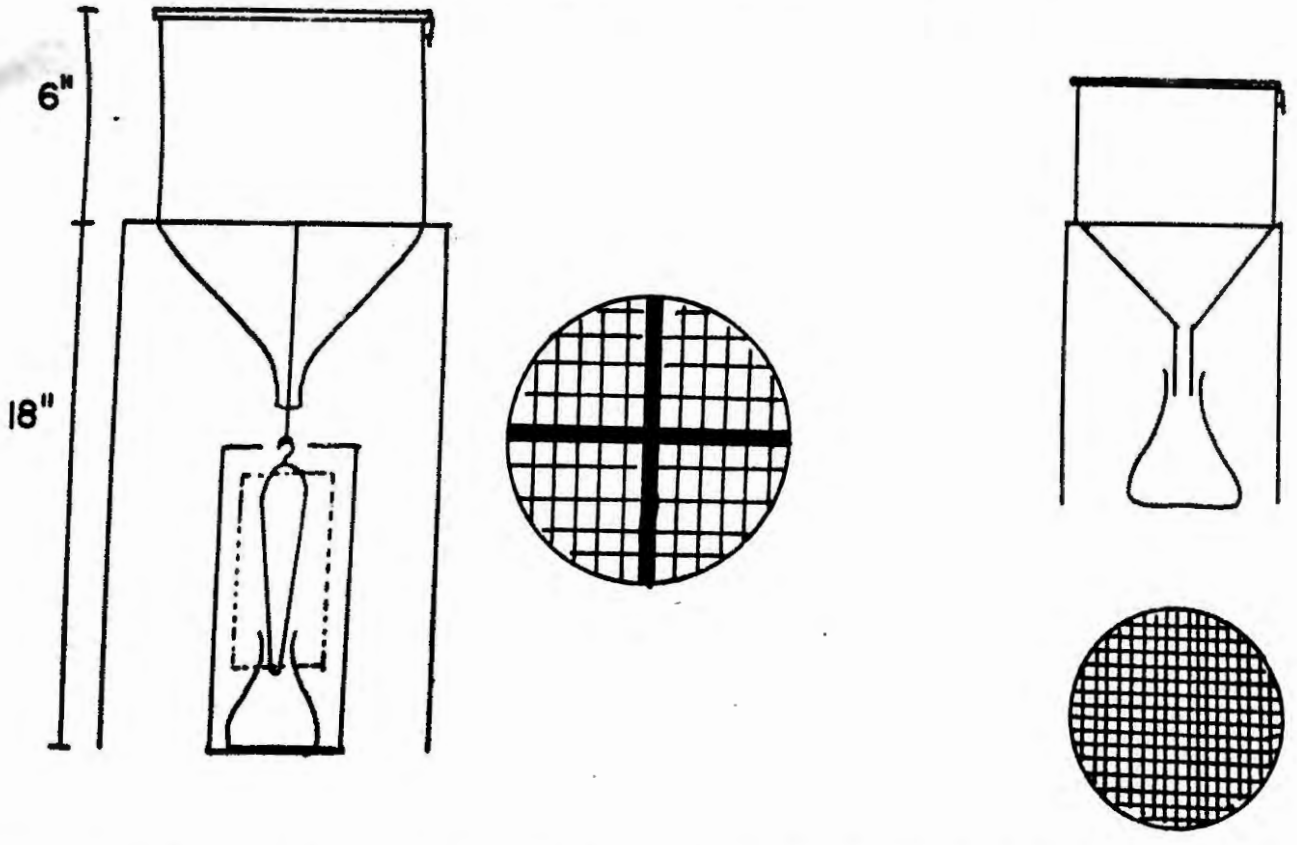
rat is primed by administration of 25 mg/kg of 0.9% saline intraperitoneally. Cummings* et al (1960) advocated use of non-hydrated animals. The period of urine collection varies from 5 hours to 24 hours. Most of the workers have used 5 hours as the scheduled period. Modi et al (1963a,** 1963b,*** 1963c****) advocated use of 24 hours collection period. Drugs are administered either orally (0.5 ml 2% starch solution as vehicle for the drug), or intraperitoneally. Control group receives only the vehicle, another group treated with hydrochlorthiazide (2.5mg/kg) serve as the standard treated control. Urine volume is recorded. Excretion of Na, K and chloride is estimated and recorded in mEq/litre.

Alternately the same set of 4 is used for control and treated observation with a minimum period of 48 hours rest in between two experiments. A specially designed metabolic cage is used which houses 4 rats (Refer Fig. 7.5). In this cage urine can be collected free from faeces. Urine volume is recorded at the end of 5 and 24 hours. Sodium, potassium and chloride excretion during observation period is estimated. Some prefer to use smaller cages which house only one rat.

Diuretic studies in dogs

Dogs under anaesthesia. Either male or female dogs can be used. Anaesthesia is induced by pentobarbitone 35 mg/kg given intraperitoneally. The dog is hydrated by

Fig. 7. 1: Metabolic cage design for diuretic screening in rats.



The designs include a cage for a group of four rats, and a cage for a single rat.

* Cummings, J. R., Mayres, J. D., Lipchuck, L. M. and Rosenberg, M.A. (1960) J. Pharm. Exptl. Therap., **128** : 414.

** Modi, K. N., Vartak, M. N. and Sheth, U. K. (1963a) Arch. Int. Pharmacodyn. 142 : 539

*** Modi K. N., Vartak, M. N., Shah, N. N., Lotlikar, K. and Sheth U. K. (1963b) Arch. Int. Pharmacodyn. **144** : 51.

**** Modi, K. N. Shah, N. N., and Sheth, U. K. (1963c) Arch Int. Pharmacodyn. **144** : 61.

giving 0.9% saline intravenously, in a dose of 20 ml/kg given by drip at the rate of 10 ml/kg per hour.

The urinary bladder of the animal is exposed by a median incision over lower part of abdomen. Both ureters are identified, cleared and cannulated about an inch above the urinary bladder with polythene tubing. Urine is collected and volume recorded every 15 minutes. Control observations are recorded for about 1 to 2 hours after the saline drip is over. The test drug is then injected, and the urine volume is recorded every 15 minutes for a further period of 5 hours. Each sample is estimated for sodium, potassium and chloride (mEq/litre). The results obtained in the period of drug treatment are compared with those of the control period.

Unanaesthetized dogs. Female dogs weighing from 12 to 14 kg are selected. The dogs are kept in animal house on a diet of milk, meat and bread for a week before starting the experiment. Water is freely allowed.

After an overnight fast — each dog is first used for untreated control studies. The dog is catheterised with a self-retaining catheter and the bladder is emptied. The dog is weighed and kept in the standing position in a dog-stand. The urine is collected hourly for 5 hours. The output per hour is recorded and the samples analysed for electrolytes. This gives the normal pattern of fluid and electrolyte excretion from the kidney, for that particular animal. Some workers prefer to hydrate the animal by feeding 500 ml of water orally prior to the start of the experiment.

The same dog is used after 2 days for studying the response to a standard diuretic like hydrochlorthiazide. After one hour's urine collection without drug, the dog receives orally hydrochlorthiazide 2.5 mg/kg. The urine is collected for the next 4 hours and the results noted in terms of urine volume and electrolyte excretions.

After 4 days rest the same dog is used for studying any new compound. The results with the new compound are compared to those obtained without any drug treatment and also to those obtained with hydrochlorthiazide. Every new compound should be tried at different doses to find out the maximum effective dose. A sequential plan as suggested by Modi et al (1963b) is useful in screening compounds with potent diuretic activity.

In order to know more about the mechanism of action of a diuretic various techniques may be used, such as the effectiveness of the compound in acidosis and alkalosis, clearance studies to assess the effect on the glomerular filtration rate and renal plasma flow, stop flow techniques and micropuncture studies. These are described in detail in various monograms on diuretics. The effect under alkalosis or acidosis and clearance studies may be carried out in dogs, without elaborate laboratory facilities.

Metabolic Acidosis (Beyer*, 1958). Metabolic acidosis is produced by giving ammonium chloride 100 mEq/day (5.3 gms) for five days mixed with the daily diet. On the sixth day, the dog is tested for diuretic activity with the test compound. A week later, when the animal is not under the previous effect of metabolic acidosis, control readings are repeated.

Metabolic Alkalosis. Metabolic alkalosis is produced by giving sodium bicarbonate 100 mEq/day (8.4gms) for five days mixed with the daily diet. On the sixth day the diuretic effect of the test compound is observed in presence of the metabolic alkalosis. Control readings are repeated as mentioned above.

* Beyer, K. H. (1958) Ann. New York Acad. Sc. 71 : 363.

Clearance studies in dogs. Prior to the experiment the dog is given 3.0 gms of sodium chloride for three days, mixed with normal diet. The dog is starved overnight, but free access to water is allowed till the beginning of the experiment.

On the day of the experiment, the dog is weighed and anaesthetized with pentobarbitone sodium (30 mg/kg i.p.). The trachea is opened and cannulated. The femoral vein and artery one on each side are exposed for continuous infusion and collection of blood samples respectively. Carotid arterial pressure is recorded by a mercury manometer. A self-retaining catheter is passed in the bladder.

Sixty and thirty minutes prior to the collection of control samples, 500 ml of wash is given each time orally by a stomach tube. Immediately after this a continuous intravenous infusion at a rate of 7 ml/minute is started. The contents of this infusion are creatinine 0.7 g, paramino hippuric acid (PAH) 0.1 g, Mannitol 8.0 g in 30 ml of distilled water. (Intravenous continuous infusion, creatinine 0.2%, PAH 30 mg%, 0.023M. Phosphate buffer containing 0.23 gm of Mannitol at pH 7.4 — at a rate of 7 ml/minute). Three control samples of blood and urine are collected at ten minute intervals. Blood samples are collected at the mid point of each time interval. Immediately after the collection of the third urine sample, the test compound is injected in the infusion. The drug is allowed to act for ten minutes and then a further three samples of blood and urine are collected at intervals of ten minutes. The test compound may be given as a single dose, or as a priming dose followed by more doses.

Urine and blood samples are analysed for creatinine, PAH, electroytes; pH and titrable acidity of the urine samples is also estimated (Beyer et al, 1958). GFR and RPF are estimated from the rate of excretion of creatinine and PAH.

Anti-inflammatory Screening techniques

Recent advances in the use of nonsteroidal anti-inflammatory drugs in the treatment of arthritis, has created interest in laboratory techniques which are used in screening new drugs for anti-inflammatory activities. Most of the anti-inflammatory drugs owe their action to their pharmacological property of inhibiting tissue reaction to a variety of agents which produce inflammation and tissue reaction injury. In animal tests, a tissue reaction is provoked by chemical, thermal or radiation injury and this reaction is inhibited by anti-inflammatory drugs.

These reactions may be of the nature of acute oedema of rat paw induced by subplantar injection of irritants like carrageenin, formalin, mustard, yeast, egg white, silver nitrate, bradykinin etc. Erythema may be produced in guinea pigs by exposure to thermal stimuli. Chronic inflammation is produced by use of implantation of foreign bodies like cotton pellets in rats, or injection of irritants in joints to produce arthritis, e.g. silver nitrate, or sodium urate crystals, or adjuvant induced arthritis. The effects of drugs on such inflamed joints, for e.g., suppression of inflammation, decreased pain (squeak response on moving joints in rats) and increased mobility of joints etc. could be studied. Inflammation produces increased capillary permeability. Inhibitory effects of anti-inflammatory drugs on increased capillary permeability induced by irritants can be studied in rabbits, guinea pigs or mice. (Brown & Robson*, 1964). Biochemical studies on the composition of inflammatory tissue exudate, and involvement of various enzymes,

* Brown, D. M. and Robson, R. D. (1964). Nature, 202, 812

mediating these changes, and effects of anti-inflammatory drugs on these biochemical parameters have been extensively reported by *Garattini & Dukes (1964).

Carrageenin induced oedema. Carrageenin is used as a phlogistic agent to produce acute oedema in rat's hind paw. Drugs to be screened are fed orally, usually an hour before the injection of carrageenin. This time interval may vary depending upon the rate of absorption and excretion of the test drug. The degree of inhibition of oedema is assessed by measuring rat's paw volume of both control group and treated group at the end of three hours, after carrageenin injection. The rat's paw volume is measured in a plethysmograph by noting the displacement of mercury, when the paw is dipped in mercury column to a pre-determined mark on the paw (Fig. 7.2a).

Cotton pellet granuloma. A chronic inflammatory granuloma is produced by implanting sterilised, weighed cotton pellets subcutaneously in rats; a period of seven days is required for the formation of a granuloma. From the first day of implantation, one group receives the test drug orally for the duration of the experiment. On the eighth day, under ether anaesthesia, the cotton pellets, covered with inflammatory tissues, which has now formed a granuloma is dissected free. The difference in the wet and dry weight of granuloma, from control group and treated group gives an estimate of the inhibitory activity of the test compound.

Adjuvant arthritis. Chronic arthritis and connective tissue lesions are induced in rats by injecting adjuvant, which consists of methanol dried tubercle bacilli, emulsified in liquid paraffin. Swellings of the joints appear within 14 days. In the next two weeks nodular swellings appear on the tail and ears. Drugs can be screened either to suppress the initial phase of arthritis, if administered from the beginning of the experiment and continued for 2 weeks, or to suppress the late phase of connective tissue involvement, (as indicated by nodular swellings), by starting the treatment 2 weeks after the injection of adjuvant and continuing it for two weeks.

Screening for Antihistaminic Activity :

Since the introduction of compounds with antihistaminic activity by Fourneau and Bovet in 1933, there has been a continued interest in developing new antihistaminic drugs. A number of methods for screening compounds for antihistaminic activity have been described. Most of these methods demonstrate antagonism by test compounds to histamine either in vivo or vitro. Antihistamines also have shown other interesting activities not related to their property of blocking histamine. These activities include topical local anaesthetic activity, antitussive activity, anti-emetic activity, anti-arrhythmic activity, etc. These compounds are also effective in treatment of parkinsonism and motion sickness.

Antihistaminic activity can be demonstrated in isolated preparations, e.g. guinea pig ileum on which competitive antagonism to histamine can be demonstrated. In vivo, antihistaminic activity can be demonstrated in various animals like rabbit, cat, or guinea pig. The most favoured animal is guinea pig, since it is very sensitive to histamine. A number of methods are used to counteract bronchospasm and asphyxia induced in guinea pigs by histamine administered as an aerosol (Armitage,** et al. 1961) or by systemic route. Intravenously administered histamine causes death of the animal. By pretreat-

* Garattini, S. and Dukes, M. N. G. Ed. Symposium on 'Non-steroidal anti inflammatory drugs', Excerpta Medica Foundation No. 82, Milan, 1964.

** Armitage, A. K., Boswood, J. and Large B. J. (1961) Brit. J. Pharmacol. 16, 59.

ment with antihistaminics given in various doses, ED_{50} or PD_{50}, the dose of antihistamine protective to 50% of animals can be calculated by interpolation on a probit-log dose curve: (Lisch,* et al. 1960). Micro shock method using rabbits as test animals has been described by Herxhimer** (1952). Micro-shock in rabbits can also be produced by sensitising the animals to foreign protein (egg albumin). A challenging dose of the same protein given after an interval of 3 weeks will lead to anaphylactic shock due to release of histamine and other autacoids. Antihistamines can be tested for protective effect by pretreating the animal before the challenging dose of foreign protein is administered (Herxhimer *** and Stresemann, 1960). In anaesthetised cats or rabbits, histamine produces a fall in blood pressure which can be prevented or decreased by pretreatment with antihistamines. (Preziosi,**** 1958).

The Konzett-Rossler method of screening for bronchodilator activity.

A guineapig of either sex weighing 250-350 gms. is anaesthetised with urethane (150 mg/100 g) intraperitoneally. A midline incision on the anterior aspect of the neck exposes the trachea. In the intermuscular gap lateral to the trachea the internal jugular vein may be located embedded in fatty tissue. This vein is carefully cannulated with a venous cannula. The trachea is cannulated by means of a glass cannula with 3 arms — two of these are connected to a Palmer respirator for small animals. The third arm is connected to the apparatus for recording as shown in the diagram. The respirator pushes air into the tracheobronchial tree against a constant resistance. A recording of a constant height and frequency identical to that of the respirator is thus obtained. A change in the tracheobronchial resistance would alter the height of the tracing in the following manner. Bronchoconstriction would increase the resistance offered to air flow, and consequently the pressure transmitted to the recording water manometer would be increased. Conversely, bronchodilatation would decrease the resistance and the pressure recorded would fall. For observation of maximal changes, the basal tone of bronchial musculature should be inversely related i.e. to observe bronchoconstriction there must be good dilation initially and vice versa. These requirements are avoided by studying the effect of a drug on histamine-induced bronchoconstriction.

This method has the advantage of being adjustable, short-lived and reversible. Control responses to histamine administered intravenously are elicited. The drug to be tested is then administered, and its effects on the bronchial resistance if any noted. Histamine is then administered as before, and the responses compared to the control. The ability of the drug to antagonize histamine induced bronchoconstriction indicates mainly a bronchodilator action, and to some extent may be also present in antihistaminic drugs.

(The method may be modified to screen antitussive agents).

Domenjoz method of screening for antitussive activity.

(Superior laryngeal nerve stimulation induced cough in cats).

A cat of either sex weighing between

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* Lisch, P. M., Albert J. R. Peters, E. L. and Allen, L. E. (1960) Arch. Int Pharmacodyn. 16, 77.

** Herxhimer, H. (1952) J. Physiol. (London) 117, 251.

*** Herxhimer H. and Stresemann, E. (1960) Arch. Int. Pharmacodyn. 125, 265.

**** Preziosi, P. (1958) Arch. Int. Pharmacodyn. 115, 62.

2.8 and 3.5 kg is anaesthetised with pentobarbital sodium (25-30 mg/kg). The choice of drug and its dose are of importance because anaesthesia in general, obtunds, respiratory reflexes and augments the activity of antitussives. Barbiturates in smaller doses are believed to heighten respiratory reflexes elicited by peripheral stimuli, as distinct from their direct depressant effect on the respiratory centre.

The femoral vein is cannulated for purposes of drug administration. The trachea is cannulated and connected to a Marey's tambour. The vago-sympathetic trunk is identified adjacent to the carotid, and the superior laryngeal nerve located supplying the laryngeal muscle. This nerve is carefully isolated and its laryngeal end ligated securely to avoid retrograde stimulation. The nerve forms the afferent limb of the cough reflex arc and stimuli applied to the nerve should proceed centrally to the brain. The coughs elicited by stimulation are recorded as brisk increases in respiratory excursions of the recording lever of Marey's tambour.

Practical Exercises

Exercise 1

Objective: To study the effect of diuretic drugs in unanaesthetized female dogs.

1. Select female dogs weighing 12 kg to 14 kg.
2. Keep the dogs on normal diet (milk, meat, bread) which has adequate salt content, for 4 days.
3. After fasting overnight, next day morning start the control studies.
4. Catheterise the dog with a self-retaining catheter (child size).
5. Empty the bladder; wash 3 or 4 times with water and ensure that it is completely empty by inflation and deflation with air.
6. Make the dog stand in a special stand, with broad canvas sling supporting the dog.
7. Collect urine in a bottle containing toluene. Measure volumes, 1 hourly for 5 hours. Estimate pH and electrolyte concentration of each sample. (Na and K is estimated by a flamephotometer). Chloride is estimated as sodium chloride gms/litre by titration with standard silver nitrate solution (2.906 g/litre) using a drop of potassium chromate (5%) as an indicator. All values are finally expressed as total mEq/litre in unit of time.
8. After 2 days of rest, the entire experiment is repeated in the same dog, but this time the dog receives orally hydrochlorthiazide 2.5 mg/kg orally in a fine suspension in a minimum quantity of water, prior to collection of urine samples.
9. Collections and estimations are carried out similar to step (7).
10. Effect of hydrochlorthiazide is expressed as percent increase, taking control values as 100.

Note: (i) Some workers prefer to hydrate the dog with 500 ml of tap water fed orally by a stomach tube prior to the beginning of the experiment.

(ii) The first hours sample may be considered as control and the drug could be given orally, or intravenously; the next 4 samples collected 1 hourly may then be taken as due to drug effect and results calculated accordingly.

Exercise 2

Objective: To study the diuretic activity of a compound in rats.

1. Select male albino rats weighing about 150 to 180 g (total weight in a group of four between 750-850 g).
2. Allow free access to water and food till start of experiments.
3. Divide the rats in groups of four each and place them in metabolic cages.
4. To one group administer orally 0.9% sodium chloride solution in a dose of 5% of their body weight. Also give orally 2% starch solution in a dose of 5 ml/kg, as vehicle.
5. Hydrate similarly other 3 groups of 4 rats each, and give them orally varying doses of hydrochlorthiazide in 2% starch solution in a volume of 5 ml/kg (1.2 mg: 2.5 mg: 5 mg per kg of body weight). Place each group separately in metabolic cages.
6. Collect urine from each at the end of 5 hours. Note volume and take a sample for electrolyte and pH estimations.
7. Collect urine samples at the end of 24 hours. Note volume and take samples for electrolyte and pH estimations.
8. Estimate Na and K by using flame photometer.
9. Chlorides are estimated by titration.
10. Estimate percent increase in urine volume and electrolyte excretion, taking control values as 100%. Plot a dose effect relationship by plotting % increase against log dose.

Exercise 3

Objective: To study the effect of anti-inflammatory drugs on cotton pellet granuloma in rats.

1. Select rats of either sex weighing 80 — 120 g.
2. Prepare cotton pellets of 10 mg each from 5 mm section of cotton rolls (dental rolls).
3. Sterilize the pellets in an autoclave for 30 — 45 min under 15 lb pressure.
4. Insert subcutaneously four pellets in the ventral region in the groin area, two on either side in each rat under light ether anaesthesia.
5. Administer the test drug orally suspended in 1% w/v C.M.C. (Carboxymethylcellulose) 1 ml/100 g daily for seven days. Control group is treated with either normal saline or vehicle used for the test drug for number of days.
6. Sacrifice the rats on the 8th day and remove cotton pellet. Clean the cotton pellet of extraneous tissue and weigh on chemical balance. This is recorded as wet weight. Dry the pellets in an hot air oven at 60° — 70° and weigh it on balance; repeat drying till a constant weight is obtained.
7. Calculate percentage inhibition of granuloma comparing dry weight of control group to the treated group. Construct a dose response curve by plotting log dose against percentage inhibition. Calculate ED_{50}.

Table 7.1

Effect of drugs on cotton pellet granuloma in rats.

Drug	Dose mg/kg	Dry Weight (mg) Mean ± S.E.	Percentage inhibition
Control	1 ml/100 g	128 ± 2.8	—
Phenylbutazone	50	95 ± 1.8	25
	100	72 ± 2.1	47
	150	52 ± 1.2	56
Indomethacin	2	90 ± 1.8	30
	4	68 ± 2.1	44
	6	46 ± 1.7	64

Exercise 4.

Objective: To study the effect of anti-inflammatory drugs on carrageenin oedema in rats.

1. Select rats of either sex weighing 80-100 g. Make a mark on the hind paw just beyond tibio-tarsal junction, so as to fix a constant level upto which the rat's paw must be dipped in the mercury in the plethysmograph.

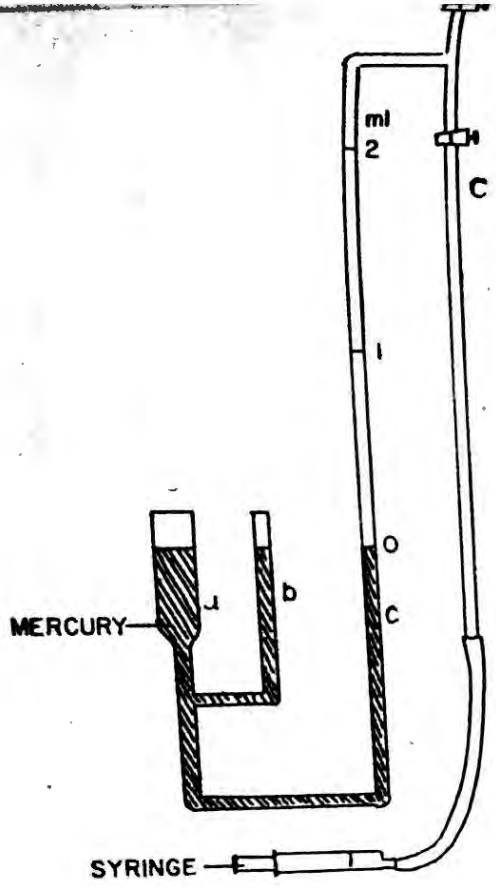
2. Measure the initial volume of hind paws on the plethysmograph apparatus. To begin with the mercury levels in the column in A and B are adjusted to the same level, with the mercury level in C at zero. The paw is dipped in the reservoir A to the predetermined mark. This will raise the level of mercury in the column B. With the syringe attached to column C withdraw air till the raised level of mercury in column B comes back to the initial level. This procedure raises level of mercury in the column C. This rise of mercury in the column C is measured by reading divisions marked on the column C and this represents the volume of the foot.

3. Divide the rats into two groups. One group will serve as control and receive normal saline or vehicle. In the other group administer the test drug orally suspended in 1% w/v C.M.C. (1 ml/100 g).

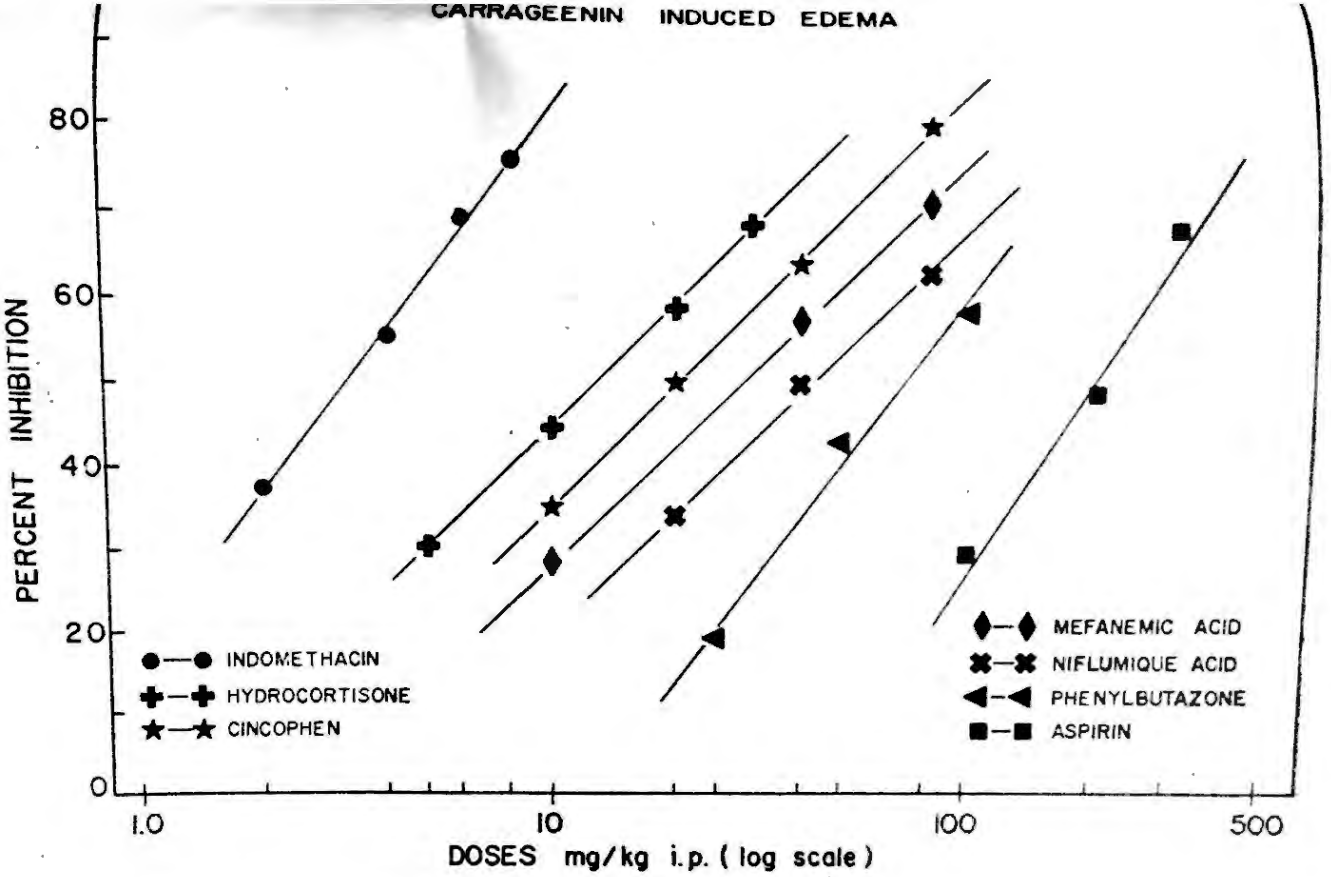
4. After 60 minutes inject carrageenin suspension in C.M.C. 1% w/v 0.1 ml subcutaneously into the plantar region of each hind paw.

5. Record the hind paw volume 3 hours after carrageenin injection. The difference in the initial and final volume indicates the volume of inflammation. Calculate percentage inhibition produced by the drug by taking volume of inflammation in the control group as 100%.

6. Using three different dosages (one dose per group) construct a dose response curve by plotting percentage inhibition against log dose. Calculate ED_{50}.



7. 2-a
The plethysmographic apparatus used.



7. 2-b
The expression of the results as dose against % inhibition of oedema (response).

Table 7.2
HIND PAW OEDEMA

Animals ..... Wts ..... Drug .....Dose .....
Carrageenin 1% in 0.5% C.M.C. Dose ... 0.1 ml/Paw Route .....

No. of Animal	Initial Readings		Readings 3 hrs after carrageenin		Oedema	%oedema	deviation	d^2	% Inhibition	deviation	d^2
	1st paw	2nd paw	1st paw	2nd paw							

Mean
S. D.
S. E.

t =
p=

Total
Mean

S. D. =
S. E. =

Exercise 5.

Objective : To study the antihistaminic property of a drug by its protection of guinea pig against histamine aerosol.

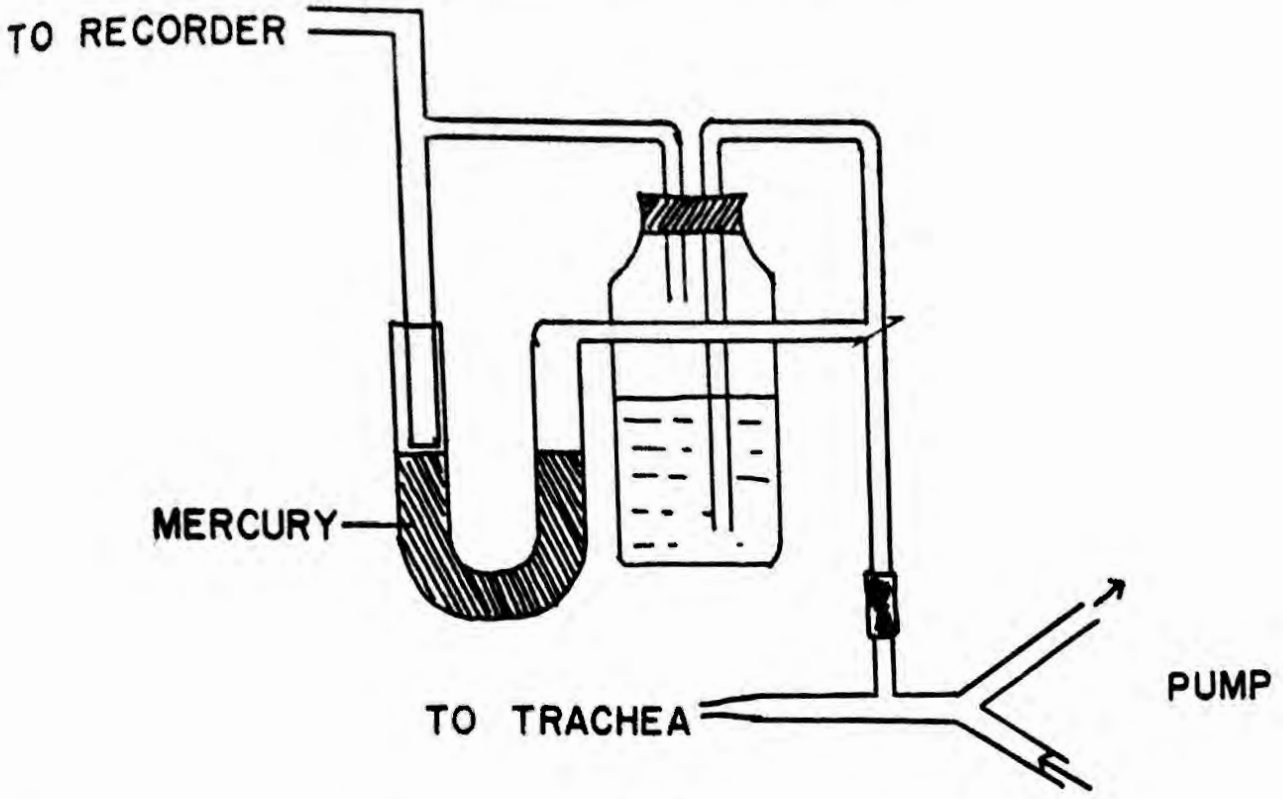
1. Select guinea pigs of either sex (weight 250-350 g) and starve overnight.
2. In one animal inject 1 ml vehicle (saline or 2% starch) intraperitoneally — to serve as control.
3. In another animal inject test drug (e.g. Chlorpheniramine) 1/5th LD_{50} intraperitoneally.
4. One hour after the injection confine the animals to the twin chamber, one in each compartment.
5. With the help of a compressor, spray a finely atomized mist of 5% histamine-diphosphate from a nebulizer in both the compartments (use compressed air at constant pressure of 100 mm/Hg to operate the nebulizer).
6. Observe both the animals for signs of respiratory distress like difficulty and cessation of breathing and asphyxial convulsion (saline control will show this).
7. Observe if the test drug protects the animal from respiratory distress.

Exercise 6.

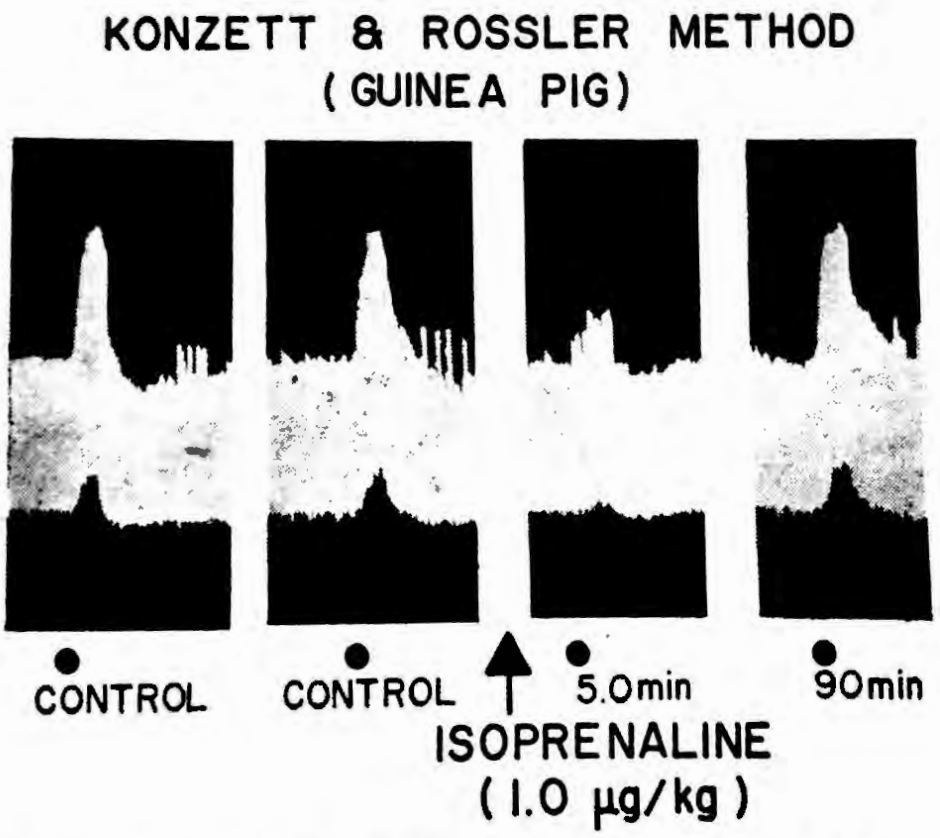
Objective : To study the bronchodilator action of a test drug.

1. Set up a Konzett and Rossler preparation as described.
2. Record a control strip of tracing.
3. Elicit responses to intravenous histamine.
4. Administer the test drug.
5. Observe the effects if any on bronchial resistance.
6. Re-elicit responses to histamine.
7. Observe antagonism to histamine indicative mainly of a bronchodilator effect.
8. Rule out antihistaminic activity by other experiments.

Fig. 7.3: The Konzett Rossler method of screening for bronchodilators.



7. 3-a
The experimental set up used for the method.



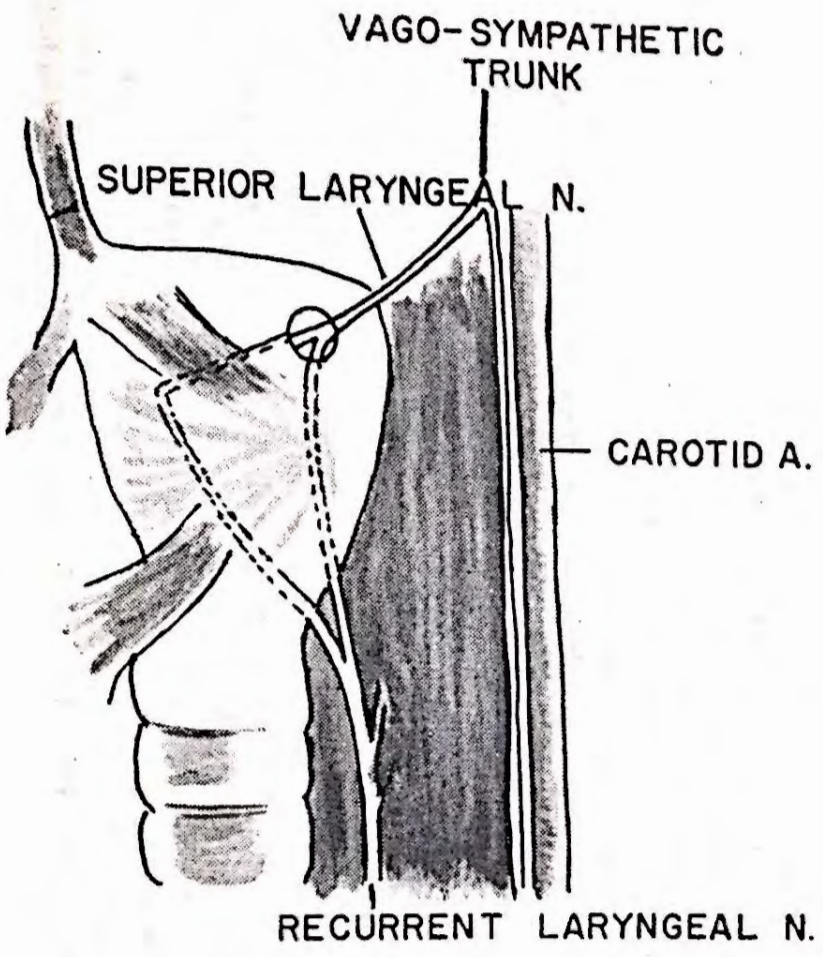
7. 3-b
The recording obtained by the method.

Exercise 7.

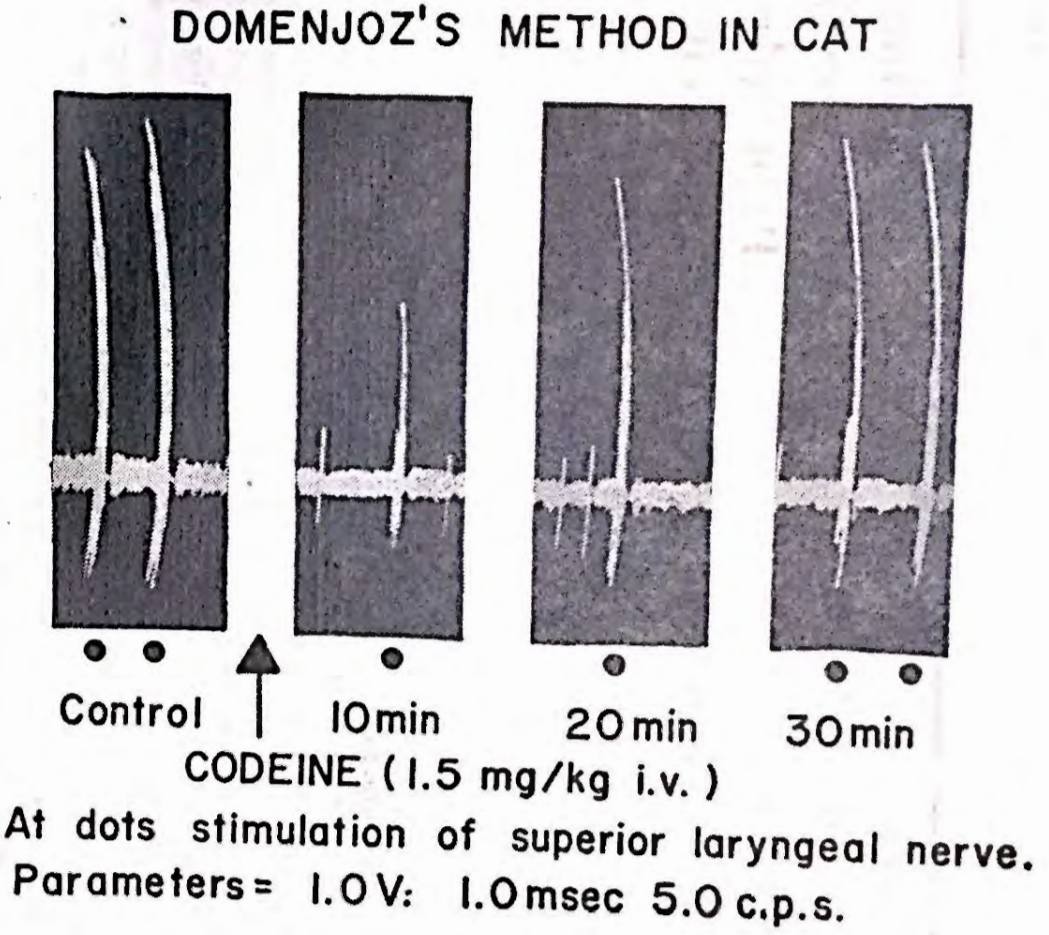
Objective : To study the antitussive activity of a test drug.

1. Set up a superior laryngeal nerve stimulated preparation as described by the method of Domenjoz.
2. Elicit responses to electrical stimulation of the nerve for 10 seconds at 3 min intervals.
3. Administer the test drug intravenously.
4. After 5-10 minutes re-elicit the responses to electrical stimulation of the nerve with the same parameter as before.
5. Observe the blocking effect, if any, of the drug.
6. Follow the course of recovery of responses to determine the duration of action of the drug.

Fig. 7.4: Domenjoz's method for anti-tussive screening.



7. 4-a
The pathway subserving the cough reflex, elicited by stimulating the central end of the superior laryngeal nerve.



7. 4-b
The recording obtained by this method.

Laboratory Anaesthesia: Principles and Techniques

And the Lord God caused a deep sleep to fall upon Adam and he slept, and he took one of his ribs and closed up the flesh instead there of.

BIBLE GENESIS 2:21

Introduction

For human experimentation, the use of anaesthesia, either local or general, is a foregone conclusion. The wide use of experimental techniques in drug evaluation today has compelled biomedical workers to study the effects of anaesthesia in animals more carefully. Anaesthetic agents are not pharmacologically inert and their presence in the body alters physiological homeostatic mechanisms. It has to be yet clarified whether, and to what extent, these alterations themselves affect drug actions studied in the experimental animal. Their effects may vary from an extremely subtle unnoticeable change, to a gross alteration in physiological functions. The choice of anaesthetics and their routes of administration must therefore be done carefully in the light of their role in basic design and results of research.

At the very outset, while planning his experiment, the careful research worker will decide whether or not he needs an anaesthetic. If he decides in favour of one, he must carefully evaluate the possible changes this might necessitate in his experimental design. Having decided in favour of anaesthesia, he will then choose the particular agent such as will affect his experiment minimally. This will then lead to a consideration of route and technique of administration of the agent, and for this a great amount of skill and care, and gentle but efficient handling of animals is a must.

It can be appreciated that two sets of complications can arise when anaesthesia has been produced. The first is the effect on homeostasis and the second is the effect on specific organ systems concerning a particular experiment for example myocardial depression and direct vasodilatation should be guarded against in experiments particularly designed to study cardiovascular pharmacology in vivo, renal blood flow while studying diuretics, synaptic transmission while considering autonomic agents and so on. The effect of different anaesthetic agents on these systems is different.

Another important fact to be considered is that following general anaesthesia an impairment of respiratory functions definitely occurs. This would have two implications: firstly, it necessitates assistance of respiration; secondly, there will be a tendency for the carbon dioxide tension in the blood to rise and oxygen tension to fall, in turn altering blood pH, and possibly affecting drug action through ionisation effects. CO_2 itself is a central depressant. A natural and binding corol-

lary of this would be that assistance in respiration should also not cause hyperventilation.

Another wise precaution would be monitoring of body temperature. Most animals show hypothermia body temperature would also depend on the thickness of the fur and the administration of drugs like atropine. The level of body temperature might conceivably alter the actions, or manifestations of drug actions.

It is therefore clear that anaesthesia acts as a form of stress, with accompanying endocrine changes and unless these alterations in physiological function are taken into account, meaningful and reliable conclusions cannot be drawn from a research experiment.

Techniques of Anaesthesia

(A) **Inhalational anaesthesia** though not the method of choice in a laboratory animal has the advantage of being fairly safe and most easily reversible. Many methods of administration of volatile anaesthetics are available.

Open drop method: A small tin for large animals, or a metal cap of a bottle for small animals is perforated and the bottom packed with gauze soaked in anaesthetic. The "mask" thus made is placed over the muzzle of the animal and when it breathes air through the perforations, the anaesthetic is also inhaled along with it. If properly performed it is very useful for short surgical procedures in small animals for example adrenalectomies in mice, placement of cotton pellets under the skin of rats, etc. The following points must be taken care of—

i) The anaesthetic concentration is variable and the animal may go "under" too deeply and quickly.

ii) Reflex apnoea may occur preventing further inhalation.

iii) Artificial respiration by positive pressure would be impossible.

Nonrebreathing techniques: This requires the introduction of a tracheal cannula either by open tracheostomy or by endotracheal intubation. It has the advantage of maintaining a constant alveolar tension of anaesthetic gas and complete elimination of exhaled carbon dioxide.

In its simplest form the apparatus consists of a small metal Y tube, one limb of which is connected to the anaesthetic mixture either directly or through a reservoir, one limb is open to the atmosphere and the stem of the Y is connected to the tracheal cannula. Elaborations of this non-rebreathing system can be devised with the help of several types of non-rebreathing valves. Valve control is also utilised in the closed (complete rebreathing) and semiclosed (partial rebreathing) systems. The advantage of such complex systems is the prevention of loss of moistures and heat in exhaled air, while a disadvantage would be a tendency towards carbon dioxide retention if elimination of carbon dioxide by absorption into soda lime is incomplete.

Endotracheal intubation is necessitated because of the danger of the tongue falling back over the epiglottis in an anaesthetised animal and the accumulation of secretions in the pharynx. Intubation also serves to reduce dead space and decrease resistance to breathing. (In this connection it would be relevant to mention that the resistance to flow is inversely proportional to the fifth power of the internal radius of the tube and directly proportional to the length).

Endotracheal intubation may be done orally for survival experiments. In small animals where even minimal leakage constitutes a significant proportion of tidal volume, a tracheostomy is mandatory.

(B) **Non-inhalational Anaesthesia**

1. **Intravenous:** This is the most widely

employed method of administering general anaesthesia particularly in large co-operative animals with accessible veins. It would also depend on the nature of the anaesthetic agent required, for example barbiturates are best given by this route. These barbiturates may be (i) of the ultrashort-acting type like thiopental, used for induction and followed by maintenance on inhalational anaesthesia, or (ii) a mixture of an ultrashort and a long-acting barbiturate for example thiopental and phenobarbital, or (iii) an intermediate acting barbiturate like pentobarbital.

2. **Intramuscular and intraperitoneal** are employed in unmanageable animals like cats (pentobarbital) or in small animals like guinea pigs (urethane).

3. **Other routes:** Regional anaesthesia for short-lasting minor procedures like introduction of an intracerebroventricular cannula in rabbits. These routes are not commonly employed as they require well trained co-operative animals.

Rectal route of administration though safe and simple is rendered unreliable by the presence of faecal material.

Choice of Anaesthetic Agents for Different Species

(1) **Anaesthesia for the dog.**

Injectable Anaesthetics barbiturates

(i) Pentobarbital: This is the most commonly used anaesthetic in the dog. It is administered dissolved in saline in a dose of 35 mg/kg. For convenience, it is prepared as a 70 mg/ml solution and the amount required diluted to about 10 ml and injected slowly intravenously. Anaesthesia is complete in about 60-90 seconds and is maintained for about 1½ — 2 hours. At this stage, supplementation with 5-10 mg/kg may be needed.

(ii) Thiopental: Freshly prepared solutions are given in doses of 15-25 mg/kg and produce anaesthesia in about 30 seconds, lasting for 20-25 minutes. It requires repeated supplementation and may therefore be given as a (iii) Thiopental-Phenobarb mixture.

Here maintenance is achived automatically by the administration of phenobarb 35 mg/kg or barbital 250 mg/kg.

If phenobarbital is given alone (50 mg/kg) it is some what slower acting but anaesthesia is stable and quite prolonged.

The barbiturates in general are depressant to cardiovascular reflexes like carotid occlusion reflex and to spinal cord reflexes. Tryptamine induced reflexes are also depressed, and myocardial contractility decidedly diminished. Another disadvantage is that muscular relaxation is inadequate.

1. **Morphine.** 30 mg/kg is effective in anaesthetising dogs with the singular disadvantage of severe vomiting. An advantage claimed for morphine anaesthesia is its reversal by n-allyl-normorphine.

2. **Chloralose,** dissolved in propylene glycol as a 10% solution, heated and cooled and diluted with saline is given in doses of 80-100 mg/kg. This anaesthesia is adequate for cats, while in dogs it causes a hyperexcited state that necessitates morphine pretreatment 2 mg/kg. Chloralose anaesthesia is useful for the study of drugs affecting ganglionic transmission and cardiovascular reflexes. Neuromuscular reflexes are also heightened.

3. **Morphine urethane :** This is not a convenient combination as anaesthesia is induced erratically. Morphine 2 mg/kg is given subcutaneously and urethane 0.5 — 1.5 g/kg intraperitoneally.

4. Allobarbital: Consisting of the primary ingredient diallyl barbituric acid 0.1 g/ml, urethane 0.4 g/ml, monoethyl urea 0.4 g/ml. The dose of this mixture is 0.4 — 0.6 ml/kg intraperitoneal and is not convenient to use.

Inhalational Anaesthetics: These have declined in popularity since the availability of the barbiturates. Inhalational anaesthetics necessitate skilful handling, require expensive equipment and those like ether demand heavy atropinisation to dry respiratory secretions. Atropine would itself interfere with many experimental designs.

Route of anaesthesia for the dog: The intravenous route is relatively safe, easy and decidedly humane.

Accessible veins.

The cephalic vein in the forelimb as it crosses the anteromedial aspect of the carpus and runs along the anterior aspect of the foreleg. The elbow of the sitting dog is grasped in the palm of the hand, with the thumb across the extensor surface of the joint: the thumb is then rotated laterally, tensing the skin and making the vein more prominent.

The saphenous vein as it crosses the lateral aspect of the tarsus obliquely in the hindleg behind the gastrocnemius muscle.

The femoral vein lying in the femoral triangle generally requires a cut down but is routinely cannulated for administration of drugs throughout the experiment.

The external jugular vein as visualised in the neck superficial to the sternomastoid muscles.

The lingual, the marginal ear vein and visceral veins have also been used for supplementation of anaesthesia.

Other routes

Intrapleural: With the dog in standing position, low in the right 9th intercostal space. Requires extreme caution not to enter lung parenchyma.

Intramuscular injection is best made into the thigh muscles avoiding the sciatic nerve. Barbiturates are irritating by this route.

Intraperitoneal injections are made just lateral to the umbilicus: care is taken to withdraw the piston of the syringe to ensure that the needle is not in a viscus before the injection is made.

Epidural anaesthesia in dogs. This may be done by injecting into the slight depression palpable just caudal to the spine of the 7th lumbar vertebra. This may be identified as the prominence felt in the midline on the imaginary line joining the wings of the hipbone. A 22 gauge needle is used, passing it downwards and slightly forward until it is felt to pierce the ligament.

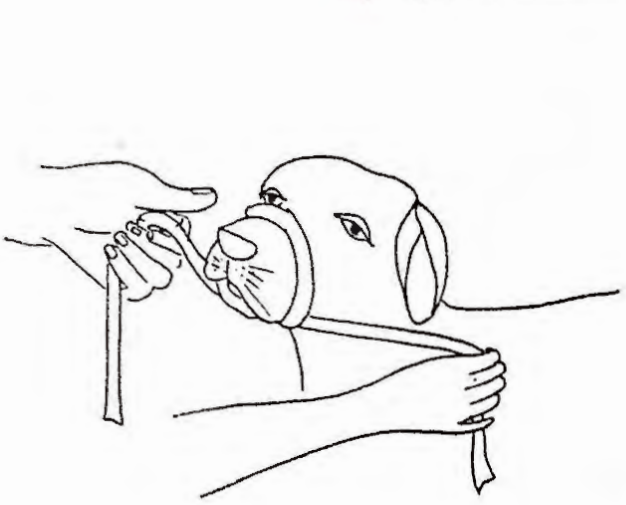
Artificial Ventilation in the dog. The amount of air reaching the alveoli is of greater importance rather than the amount of air inhaled. The dead space of the dog is 30-80 ml, the physiological dead space being 40-200 ml. The tidal volume of dogs weighing 16-30 kg is in the region 300-450 ml air. Accordingly, the artificial respirator is set to maintain as natural a gaseous exchange as possible.

(2) Feline Anaesthesia

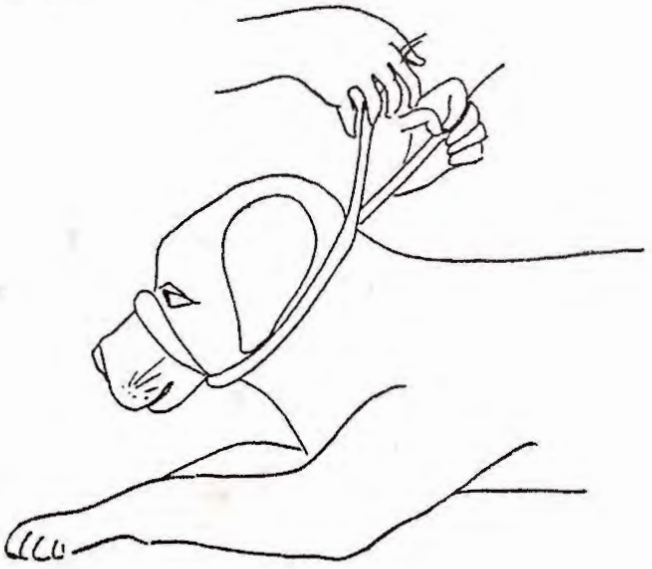
The cat is the only laboratory animal which metabolizes drugs at a slower rate than man, the other species having faster metabolic rates.

Pre-anaesthetic medication. Pethidine hydrochloride is tolerated i.m. or s.c. in doses upto a critical level of 11 mg/kg. Larger doses cause CNS stimulation with convulsions.

Fig. App. 1: Intravenous anaesthesia in the dog.



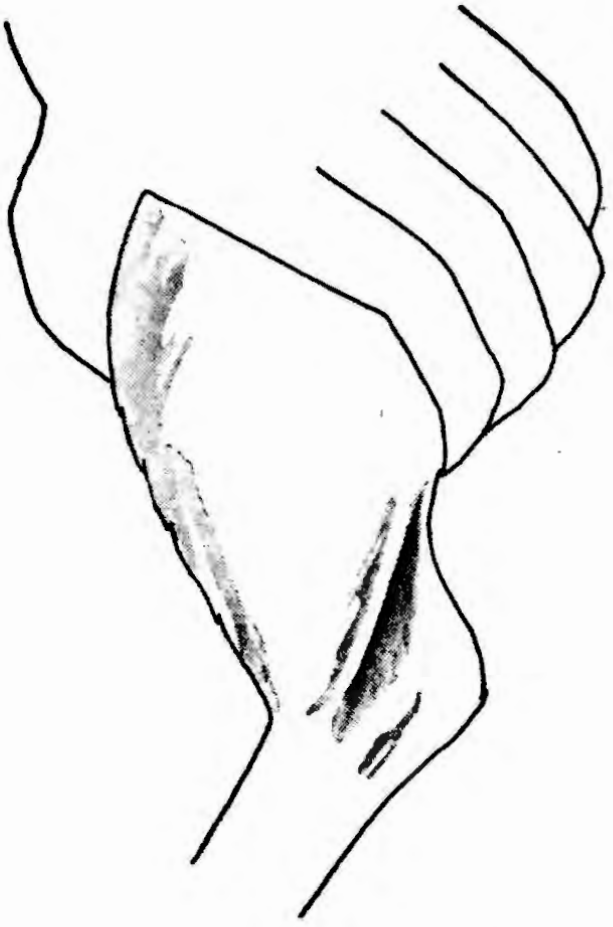
App. 1-a
Tying the muzzle, first knot.



App. 1-b
Tying the muzzle, second knot.

App. 1-c
Positioning the animal, for injection into the saphenous vein.

Atropine: The usual dose in cats is 0.06 mg/kg. The highest tolerated dose is about 40 mg/kg.



App. 1-d.
Bringing the dog's saphenous vein in prominence.

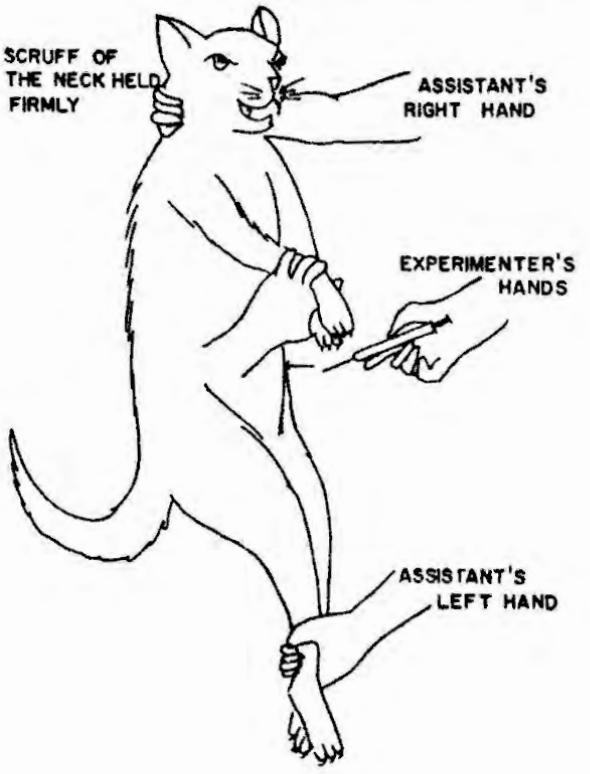
Succinylcholine for muscle relaxation particularly for endotracheal intubation, small doses of about 0.1 mg/kg are adequate. Muscle paralysis requires about 1 mg/kg. The administration of succinylcholine necessitates pretreatment with atropine.

General Anaesthesia in Cats

The short-acting barbiturates, volatile anaesthetics like ether or halothane, chloralose and urethane have been used for general anaesthesia in cats. The latter two have the distinct advantage of not obtunding certain reflexes e.g. neuromuscular and cardiovascular.

Criteria of anaesthesia. The palpebral, corneal and ear whisker reflexes are reliable indicators of surgical anaesthesia in the cat.

Fig. App. 2 : Intraperitoneal injection in the cat.



App-2
This method of immobilisation of untamed cats requires the help of a single assistant.

Barbiturates. These anaesthetics are administered intraperitoneally or intravenously. The former is an easier route though somewhat less reliable than the latter. Generally pentobarbital sodium is administered in a dose of 35-40 mg/kg.

Chloralose. This agent is administered as a 10% solution in propylene glycol, diluted in saline, intravenously.

Intravenous administration to untamed stray cats generally necessitates induction with ether in an etherbox, a rapid venesection and administration of the anaesthetic agent through an indwelling cannula. For this reason such anaesthetic procedures are employed only for acute experiments.

For chronic experiments, it is advisable to inject directly intravenously, or use the intraperitoneal route with sterile precautions.

(3) Anaesthesia in the rabbit

Rabbits, though easy to handle, are very apprehensive and therefore more difficult to anaesthetise. They are immobilised in a rabbit box specially constructed to leave head and ears accessible to handling. The marginal ear vein of the rabbit is fairly prominent and can be rendered more so by swabbing the ear with xylene. Intravenous anaesthesia in the form of pentobarbital sodium is most commonly administered by this vein in a dose of 25-40 mg/kg. The best criteria of surgical anaesthesia in the rabbit are the rate and depth of respiration and the toe-pinch reflex. The toe reflex is elicited by pinching between the 1st and the 2nd toe of either front or hind foot. This anaesthesia lasts for 30-45 minutes and may be supplemented as desired.

For prolonged anaesthesia, urethane is an excellent agent, in a dose of 1.6 gm/kg i.p., or 1-1.5 gm/kg with morphine 2 mg/kg. The onset of action is slow, but duration is prolonged upto 3-4 hours. It has a wider margin of safety than the barbiturates.

Paraldehyde orally in a dose of 1.5 ml/kg mixed with tap water 1:7 is also satisfactory anaesthesia. It may be noted that the rabbit has certain peculiarities worth mentioning :

(a) The response to histamine is very poor and hence it is not suitable to demonstrate the action of antihistamines.

(b) The gastric emptying time in the rabbit is extremely prolonged and may contain food when fasted for as long as 5 days. Hence it is not the animal of choice for investigations on the absorption of orally administered drugs.

(4) Anaesthesia in the Guinea Pig

The usual intravascular routes are extremely fragile and are rarely used. The simplest approach is the intraperitoneal route. Pentobarbital sodium 20-30 mg/kg or urethane (150 mg/100 g) may be used. Criteria of anaesthesia are rate and depth of respiration, flaccidity of skeletal muscles and disappearance of reflex responses.

The guinea pig is particularly well suited to the testing of antihistamines, as it responds with a marked bronchoconstriction to histamine.

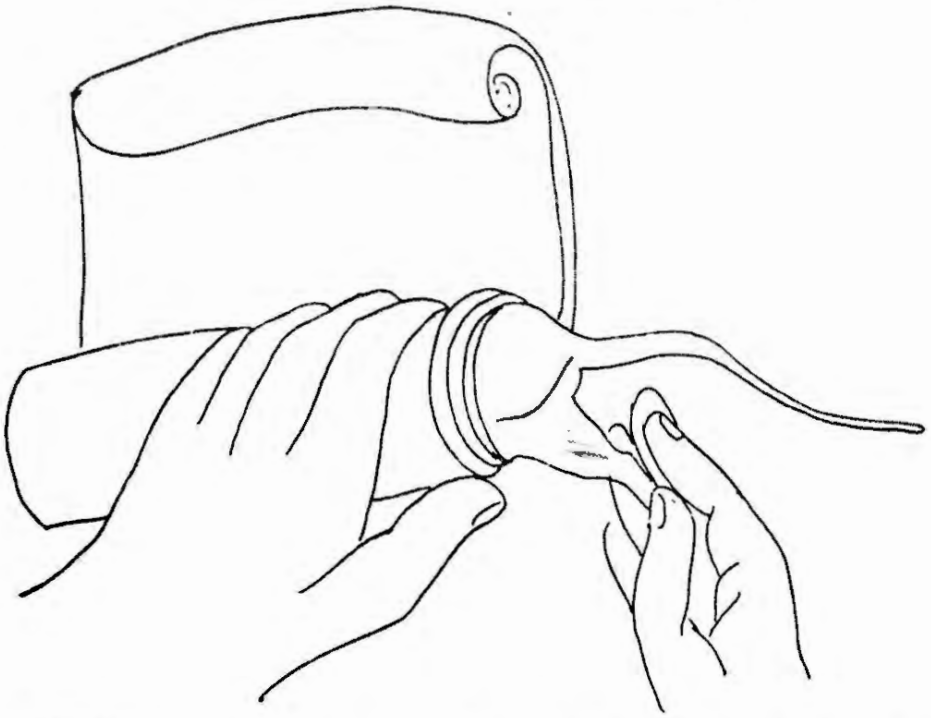
(5) Anaesthesia in the rat

The intravascular anaesthetics are most easily administered via the tail vein of the rat. Subcutaneous injections are made by pinching up the skin and injecting between skin and underlying muscle. Intraperitoneal injections are fairly easy to make and may be given single handed. Pentobarbital in a dose of 30-40 mg/kg dissolved in saline so as to inject 0.1 ml/100 g body weight is commonly used intraperitoneally.

For short-lasting procedures like nephrectomy, adrenalectomy, recording of blood pressure, inhalational anaesthesia with ether may be safely administered. The simplest way to do this is to place the animal on a perforated platform. Beneath this is placed cotton wool saturated with ether. The jar is closed and the rat gets anaesthetised. During the experiment a small cone with ether soaked cotton may be placed near the muzzle, for maintenance of anaesthesia. Guides to depth of anaesthesia are the respiration, muscle tone and colour of the ears, paws and tail.

Resuscitation from overdosage of ether: A wide-bore rubber tube is held over the nostrils and the operator breathes gently through this at a fast rate i.e. the normal respiratory rate of rat. Gentle chest massage may also be done.

Fig. App. 3 : Intravenous injection in the rat.



The femoral vein may be easily exposed, with the rat rolled in a towel as shown.

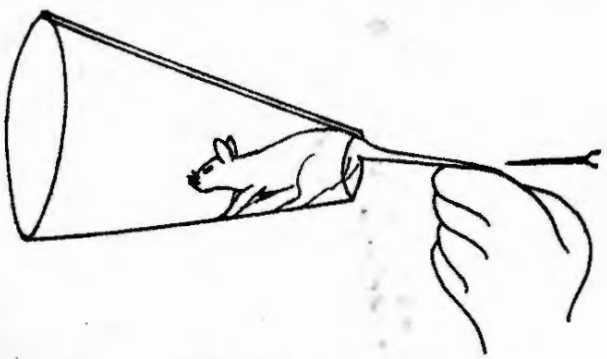
Note: The rat is very resistant to tropine requiring upto 20 mg/kg for blockade of respiratory secretions.

(6) Anaesthesia in the mouse

The choice of anaesthetics is governed by the duration of anaesthesia required for short-lasting procedures; ether is the agent of choice, administered by a small cone as for the rat, care being taken of respiration and general pink colour of the ears, muzzle paws and tail. Onset of surgical anaesthesia is indicated by the toe reflex or the tail, pinch reflex.

The most commonly used parenteral anaesthetic in mice is pentobarbital sodium intraperitoneally, sometimes intravenously via the tail vein, in a dose of 40-70 mg/kg. For purposes of intravenous injection the mouse may be placed in a mouse holder.

Fig. App. 4 : Intravenous injection in the mouse.



App-4
The tail veins are accessible, with the mouse placed in a mouse holder.

(7) Anaesthesia in Monkeys

There is no one universally satisfactory general anaesthetic in monkeys due to wide variations with species and age. Intravascular anaesthetics are the simplest and most convenient to administer. Pentobarbital sodium in dose of 20 mg/kg and 30 mg/kg will produce surgical anaesthesia lasting 30 and 180 minutes respectively. For short-lasting procedures, sodium thiopental 25 mg/ml, given 1 ml/kg is a useful anaesthetic. The onset of surgical anaesthesia is judged by disappearance of corneal reflex. Inhalational anaesthesia is also practicable in well equipped laboratories.

Miller, E. V. (1969) : Fed. Proc 28 (4) 1373-1586,

Some solved examples in Statistics :

Investigators seem to have settled for what is measurable instead of measuring why they would really like to know.

EDMINO D. PELLEGRINO — CLINICAL RESEARCH 12, 421, 1964

1) Calculation of Standard Deviation (S.D.)

If there are "n" number of observations $x_1, x_2, x_3, x_4, \ldots\ldots x_{n-1}, x_n$, the following steps are done if $n < 30$

a. Square the individual observations and find the sum Σx^2

b. Square the sum of the individual observations and divide by n

$$\frac{(\Sigma x)^2}{n}$$

c. Subtract (b) from (a)

i. e. $\Sigma x^2 - \frac{(\Sigma x)^2}{n}$

d. Divide (c) by (n-1)

i. e $\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}$

e. Find the square root of (d)

i. e. S. D. $= \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$

2) Standard Error of the mean S.E.

$$= \frac{\text{S. D.}}{\sqrt{n}}$$

3) To find the statistical significance of the difference of the means of two sets of observations.

If $\bar{x}_1$ and $\bar{x}_2$ are the means of two sets of n_1 and n_2 number of observations, and SE_1 and SE_2 the respective standard error of the means,

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sqrt{(SE_1)^2 + (SE_2)^2}}$$

Degree of freedom $N = n_1 + n_2 - 2$

A table of 't' is consulted, and the value obtained read off against the corresponding degree of freedom. The level of probability is thus obtained. This denotes the probability of the difference being a chance occurrence. The lower the probability level, the less the likelihood of the difference being due to chance, i.e. the more the significance statistically. The most widely accepted level is 5%, which indicates that the difference is likely to be significant.

Solved example:

Latent period of reaction to a given stimulus were recorded in groups of 10 mice, of which one group had been treated with a test drug:

Group A	Group B
3.8	13.0
3.2	14.2
4.6	13.2
5.0	15.8
4.8	16.2
3.0	14.0
2.4	17.0
4.8	12.0
3.6	17.0
4.8	17.0
40.2	149.4
Mean = 4.02	Mean = 14.94
S.D. = ± 1.26	S.D. = ± 1.88
S.E. = 0.3987	S.E. = 0.595

$$= \sqrt{\frac{\left((13.0)^2 + (14.2)^2 + (13.2)^2 + \cdots\cdots \right) - \frac{(149.4)^2}{10}}{9}}$$

$$t = \frac{14.94 - 4.02}{\sqrt{(0.595)^2 + (0.398)^2}}$$

Reading off the calculated value of t from the table, the value of p obtained is less than 0.002. This means that the level of probability is 0.2%. The difference is therefore highly significant.

(41 The Chi Square test χ^2

This method is based on the null hypothesis. It is applicable if two sets of observations have to be compared in the form of ratios. The assumption is made that there should be no difference between the two observed sets (0). On the basis of this assumption, an expected set (E) of observations is calculated.

$$\chi^2 = \text{the sum of all values} \left(\frac{O - E}{E} \right)^2$$

If the difference is great, χ^2 will be high

and conversly a high value of Chi² indicates that the null hypothesis is wrong. Consider the effect of a new antiviral agent in protecting against influenza. One set of patients is treated with the drug, while the other is not.

	Infected	Not infected	Row Total
Treated	8	112	120
Not treated	33	372	405
Column total	41	484	525

Out of 525 subjects, 41 developed influenza. If the antiviral drug is useless (null hypothesis) the infection rate should be the same in both groups of subjects. The proportion of treated subjects is 120/525 in the entire sample. Therefore if the drug is useless, the treated group should have the same proportion out of the total influenza cases.

(a) Therefore expected value of treated and infected cases

$$= \frac{120}{525} \times 41 = 9.3$$

The remainder, i.e. 41 — 9.3 = 31.7 should be the number of influenza cases in the untreated group.

In the first case, observed number of treated but infected was 8 (instead of expected value 9.3) and in the latter case 33 instead of 31.7. Similarly, out of 484 subjects who did not get influenza, we expect $\frac{120}{525} \times 484 = 110.6$ to be in the treated group and the rest, i.e. 484 — 110.6 = 373.4 to be in the untreated group.

These are the expected values.

(b) Find the difference D between observed and expected values.

(c) The square of D is then determined.

(d) Divide each value of D^2 by its corresponding value of E

(e) The sum of these values of D^2/E is equal to χ^2

O	$E = \frac{R \times C}{N}$	(O - E)=D	D^2	$\frac{D^2}{E}$
8	9.3	— 1.3	1.69	0.17
33	31.7	+ 1.3	1.69	0.05
112	110.6	+ 1.4	2.56	0.02
372	373.4	— 1.4	2.56	0.01

$\chi^2 = 0.25$

The degrees of freedom (d.o.f) for, (a) a single set of numbers with c classes in a row, d.o.f. = c — 1, and (b) 2 or more sets of numbers consisting of c vertical columns and r horizontal rows degrees of freedom = (c-1) (r-1).

In our example there are three classes and hence degrees of freedom are 2. Reading off the value of 0.25, in a table we find the probability to be > 10%. This means that the probability of our null assumption being true is greater than 10%, or that the antiviral drug did not cause any significant difference in incidence of influenza between treated and untreated subjects.

(5) The Litchfield and Wilcoxon* method of regression analysis:

In considering data such as the activity of a drug in a given percentage of animals in a group, it has been often observed that day-to-day variations occur which make the comparison of ED_{50} values difficult. This necessitated the development of other methods for comparison of activities of 2 drugs; one of the most useful and widely accepted method is that of Litchfield and Wilcoxon.

The following are the important transformations to which data are subjected:

Consider the effects of two analgesic drugs A and B on the responses to noxious stimuli as determined by three methods: (a) Having ascertained the percentage responses at each of at least four doses of each drug, graphs of dose against percentage response are plotted on log-probit paper on the basis of closest-fit as judged visually. This procedure automatically straightens out the expected cur-

* Litchfield J. T. Wilcoxon F. (1949) J. Pharmacol. Exptl Ther. 96: 99-113.

vilinear graph. (b) From the graph, ascestain the ED_{16}, ED_{50} and ED_{84} of both A and B. (Obviously, the doses should have been so chosen as to yield responses ranging from 10-90% but **not** 0 and 100%). (c) From these values of ED_{16}, ED_{50} and ED_{84}, determine "s" where

$$= \left(\frac{ED_{84}}{ED_{50}} + \frac{ED_{50}}{ED_{16}} \right) \div 2.$$

(d) From the graph find the number N of animals responding to doses lying between ED_{16} and ED_{84}. Take into consideration all the points that have been plotted, including those that do not actually lie on the graph.

(e) Find the value of

$$ED_{50} = (S)^{\frac{2.77}{\sqrt{n}}}$$

(f)' Alternatively, from the nomogram (No. 2) of Litchfield and Wilcoxon, determine fED_{50} from the value of S as "base" and $\left(\frac{2.77}{\sqrt{n}}\right)$ as "exponent", reading off the fED_{50} on the line connecting the 2 points where it intersects the "result" column.

(g) Calculate the potency ratio P. R. between A and B by

$$\text{P. R.} = \frac{ED_{50A}}{ED_{50B}} \quad \text{where } ED_{50A} > ED_{50}$$

(h) From the values of fED_{50} for both A and B, from nomogram No. 4, find the value of fPR as follows: Take fED_{50} of A as f_1, fED_{50} of B f_2. connect them and read off the intersected point on f1/2 as being the value of fPR.

(i) Compare the values of fPR and P.R. If P.R. is greater than fPR, the difference between the drugs A and B is statistically significant at the 5% probability level.

solved example. Analgesic study.

Drug A

Method	ED_{16}	ED_{50}	ED_{84}	S
Clip	8.8	25.0	76.0	2.92
Electroshock	62.0	82.0	102.0	1.25
Thermal	37.0	80.0	170.0	2.11

Drug B

Method	ED_{16}	ED_{50}	ED_{84}	S
Clip	11.5	54.0	245.0	4.61
Electroshock	37.5	190.0	180.0	4.4
Thermal	31.0	78.0	170.0	2.47

Method	N		fED_{50}		fPR	P. R
	A	B	A	B		
Clip	30	30	1.7	2.2	2.57	2.16
Electroshock	30	20	1.12	2.2	2.25	2.31
Thermal	30	30	1.45	1.6	1.82	1.02

The potency ratio PR exceeds fPR for the electroshock method, and a significant difference exists between these 2 drugs by this method at the 5% level.

(j) To find the 95% confidence limits of the ED_{50} values

$ED_{50} \times fED_{50}$ is the upper limit

$ED_{50} \div fED_{50}$ is the lower limit

(6) Regression Analysis

This procedure is of use in ascertaining how far a particular function regresses on another for example, the functions of time of administration on the response to a drug. Consider a set of data, x_1, x_2, x_3, x_4 and x_5 in ascending order, with corresponding values y_1, y_2, y_3, y_4 and y_5 to be analysed for regression.

(a) $\frac{x_1 + x_2 + x_3 + x_4 + x_5}{5} = \bar{x}$, i.e. mean of the x values.

(b) Similarly, obtain $\bar{y}$

(c) For each value of x, find $x - \bar{x}$, and retain the algebraic sign of (+) or(-).

(d) Find (x-x) for each value of x, and determine their sum $\Sigma(x-\bar{x})^2$

(e) Find $(y - \bar{y})$ for each value of y, with the appropriate algebraic sign.

(f) Determine the product $(x - \bar{x})$ $(y-\bar{y})$ for each set of x and y.

(g) Add all the products thus obtaining

$\Sigma(x-\bar{x})(y-\bar{y})$

(h) Calculate $b = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})^2}$

(i) The regression equation is $y = \bar{y} + b(x - \bar{x})$

(j) With the values of $\bar{y}$, b, and $\bar{x}$, write out the equation for y.

(k) For each value of x, find the expected value of y from the equation.

(l) Plot a graph of this expected value of y, i.e. y' against x.

(m) Confirm that the function x regresses on y by the linearity of this graph.

7) Correlation coefficient 'r'

(a) Make a table of $(y - \bar{y})^2$.

(b) Calculate $r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\sqrt{\Sigma(x-\bar{x})^2 \, \Sigma(y-\bar{y})^2}}$

c) Interpret the results as follows: When 'r' approaches — 1.0, there is a negative correlation, when it approaches + 1.0, there is a positive correlation, and when it is close to, or equal to, zero, there is no correlation.

Solved example

The blood levels of a drug A were determined at varying intervals after intravenous administration to determine whether they were dependent on time. A set of values was obtained as follows:

Time in minutes (x)	Blood level (y)
1	1.70
5	3.40
10	1.28
20	0.87
40	0.82

This was found to be a curvilinear graph. Conversion to logarithms ... a mean of these fore and log-time values taken as x ... ot be exactly

Sr. No.	x	y	$x - \bar{x}$	$(x - \bar{x})^2$
1.	0.30	1.70	— 0.68	0.4624
2.	0.70	3.40	— 0.28	0.0784
3.	1.00	1.30	+ 0.02	0.0004
4.	1.30	0.90	+ 0.32	0.1024
5.	1.60	0.80	+ 0.62	0.3844
	$\Sigma x = 4.9$	$\Sigma y = 8.1$		$\Sigma (x - \bar{x})_2 = 0.7280$
	$\bar{x} = 0.98$	$\bar{y} = 1.62$		

	$y - \bar{y}$	$(x - \bar{x})(y - \bar{y})$
1	+ 0.08	— 0.0542
2	+ 1.78	— 0.5000
3	— 0.32	— 0.0064
4	— 0.72	— 0.2300
5	— 0.82	— 0.5100

$$\Sigma (x - \bar{x})(y - \bar{y}) = -1.3006$$

$$b = \frac{\Sigma (x - \bar{x})(y - \bar{y})}{\Sigma (x - \bar{x})^2}$$

$$= \frac{-1.3}{0.73} = -1.78$$

$$\therefore \quad y = \bar{y} + b(x - \bar{x})$$

$$= (1.62) + (-1.78)(x - 0.98)$$

For each value of x, y can be obtained. In this case for x = 0.7, 1.0 and 1.3, y was found to be 2.12, 1.584, and 1.05. These were found to lie on a straight line.

The choice of statistical method for various types of measurements is discussed lucidly by Langley* (1968), while the statistical basis of the calculations of a 4-point assay are elucidated in the treatise by the staff of the University of Edinburgh.**

* 1) Langley, R. "Practical Statistics". Pan Books, London. 1968.
**2) Staff Univ. Edinburgh: "Pharmacological experiments on isolated preparations. Publ.: E & S Livingstone, London, 1970.

The potency rat
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Principles of General Pharmacology

The pAx, pDx and pD'x scales.

In Chapter 1, the use of cumulative log dose response curves to elucidate the nature of antagonism between two drugs was described. Having ascertained the nature of this antagonism, it might be necessary to compare the activity of the antagonist to a known reference compound. For purposes of such comparison the pAx, pDx and pD'x scales are used. The pAx scale is useful for the quantitative comparison of competitive antagonists. By pAx we mean the negative logarithm (to base 10) of that molar concentration of the antagonist that is required to reduce the effect of 'x' times the dose of agonist to that of a single dose (in absence of antagonist). The more active the antagonist the smaller the dose required and therefore, the larger the pAx value. Some specific examples of pAx values are the pA_2 and the pA_{10}

1. Confirmation of nature of antagonism. The Ariens theory may be employed to arrive at the final deduction that $pA_2 - pA_{10} = 0.95$ for competive antagonists. In general, the values obtained are only approximately 0.95. If the value obtained is 0.5 or less, the type of antagonism is most certainly not competitive.

2. Non-competitive antagonism is indicated by non-parallel shift of the log dose response curves as indicated in Chapter I. Such a non-parallel shift would also be accompanied by a change in pAx with different doses of agonist indicates a competitive type of antagonism.

3. The pAx value is actually a property of the receptor and not of the antagonist. Thus, the determination of pAx values for a specified agonist-antagonist combination on different isolated issues would serve to clarify the identity of the receptors involved. This would also be of help to find out whether the same receptor is involved in the actions of a number of agonists on the same isolated tissues, if a common antagonist can be found. The activities of non-competitive antagonists can be compared with the pD'x scales. This is the negative logarithm (to base 10) of the molar concentration of the antagonist which is required to reduce the maximum effect of the agonist to 1/X of that which it exerts in the absence of antagonist. The pD'x is the measure of choice because the concentration of a drug required to produce half its maximum effect is inversely related to its affinity. The greater the activity the smaller the

amount of antagonist required and therefore the larger the pD'_2 value.

The pD_2' value should be independent of the dose of agonist if the antagonism is truly non-competitive. If, on increasing the dose of agonist, the pD'_2 falls, the antagonism has some competitive component, because the higher dose of agoinst has overcome the effect of antagonist. The **pAx** and **pD'x** values are therefore affected in opposing directions by an increase in dose of agonist, and in different manner for competitive and non-competitive antagonists. The **PAx** is unaffected for competitive antagonists, while it increases for non-competitive ones; the **pD'x** decreases for competitive antagonists and is unaffected for non-competitive ones. The **pAx** is unaffected for competitive antagonist can be utilised to find out whether a non-specific antagonist like papaverine has a common mechanism of action against a number of agonists.

Mathematical Basis of the 4-Point Assay

In Chapter 1, the manner of performing a 4-point (or 2 + 2) assay has been described. If S1, S2 are the responses to volume X1, X2 of standard solution (or S1, S2 doses), aud U1, U2 the responses to the same volumes of the unknown solution, since we have chosen the points to lie on the linear part of a hypothetical LDR curve, the two lines obtained with these 4 points will be assumed parallel. The horizontal distance 'H' between the 2 curves (if not parallel, this is taken at their respective mid-points), represents the logarithm of the ratio of the concentration of active material in the standard and unknown solutions.

If the slope of the lines is 'S' $H = \frac{v}{S}$

where v is the vertical distance between the 2 lines. Now, v should be U1 — S1 = U2 — S2.

The best value for v is a mean of these two because the lines may not be exactly parallel.

Hence $v = \frac{(U1 - S1) + (U2 - S2)}{2}$

The slope $S = \frac{S2 - S1}{d}$ where d = log X2 − log X1

$= \frac{U2 - U1}{d}$

$s = \frac{(S2 - S1) + (U2 - U1)}{2d}$ for a more accurate estimate

Hence, $H = \frac{v}{S}$

$= \frac{(U1 - S1) + (U2 - S2)}{\frac{(S2 - S1) + (U2 - U1)}{d}}$

$= \frac{(U1 - S1) + (U2 - S2)}{(S2 - S1) + (U2 - U1)} \times d$

where d is $\log \left(\frac{X1}{X2}\right) = \log 2$ in the experiment described.

The Choice of recording levers

The response obtained from an isolated preparation requires appropriate translation into a tracing by correct choice of levers.

The most commonly used lever for isolated smooth muscle is the frontal writing lever. The writing point of such a lever touches the drum surface end-on, and traces a linear contraction. Friction can be reduced to a minimum, and due to linearity of recorded response, comparison of heights of responses is easier.

Another commonly used lever is the sideways writing lever. This makes contact tangential to the drug surface and the responses are recorded in a curved

manner (Ref. experiments with frog rectus, Chap. 1). The drum must therefore be absolutely vertical, and the lever must maintain contact with the drum throughout its movement. To ensure this latter requirement, the sideways writing lever has been mounted in gimbals so that the writing arm always falls onto the paper. The curvilinearity of the tracing imposes a distortion error because it converts a linear contraction of muscle into a curved response which is not directly proportional to it. The levers thus far described are isotonic in their performance i.e. they record changes in length against unchanging tone in the contracting muscle.

A lever was devised by Paton to investigate the phenomenon of drug action with the help of isolated tissues. This lever is called an auxotonic lever, because as the contraction proceeds, the tension in the muscle also increases. It is claimed that this lever produces tracings which are not unacceptably steeper than predicted theoretically and presents a more accurate picture of the changes occurring in tissues. This lever also demonstrates the renowned "fade" phenomenon.

As contrasted to these levers, the recording of changes in tone against a background of unaltered length is done by isometric levers. These are used particularly in recording responses of mammalian skeletal muscles of large species like the cat (Ref. Chapter 3).

Lastly, the recording of rapid twitch-like responses of isolated skeletal muscle (e.g. the rat's hemidiaphragm) or the rapid spontaneous beating of cardiac muscle is best done with the help of spring levers. These have minimal inertia and the spring ensures rapid return of the recording arm to the baseline in time for the next response. This spring however causes a rebound flyback in the tracing in the opposite direction to the original contraction.

Some Basic Tenets of Drug Receptor Interaction

The basic formulation of a drug receptor interaction is as follows:

$$\text{Drug (D)} + \text{Receptor (R)} \underset{K2}{\overset{K1}{\rightleftharpoons}} \text{Drug receptor complex (DR)}$$

Hence $\frac{d(DR)}{dt} = K1\,(D)\,(R) - K2\,(DR)$,

i.e. rate of occupation = K1 (D) (R) — K2 (DR) of receptor by drug. Generalising further Clark derived the following:

$$\frac{d(DR)}{dt} = K1\,(D)\,(R)t - K1\,(D) + K2\,(DR)$$

where (R) t = total number of receptors.

Receptor occupancy in the presence of agonist A and antagonist B is given by

$$Y^1 = \frac{(A)}{(D)^1 + K2/K1\ (1 + B'/KB)}$$

K2/K1 = KA which is the dissociation constant for the agonist. In absence of antagonist

$$Y = \frac{(A)}{(D) + K2/K1}$$

when Y = Y′, we rearrange to get

$$\frac{(A')}{(D)} - 1 = \text{dose ratio} - 1$$

$$= \frac{(B^1)}{KB}$$

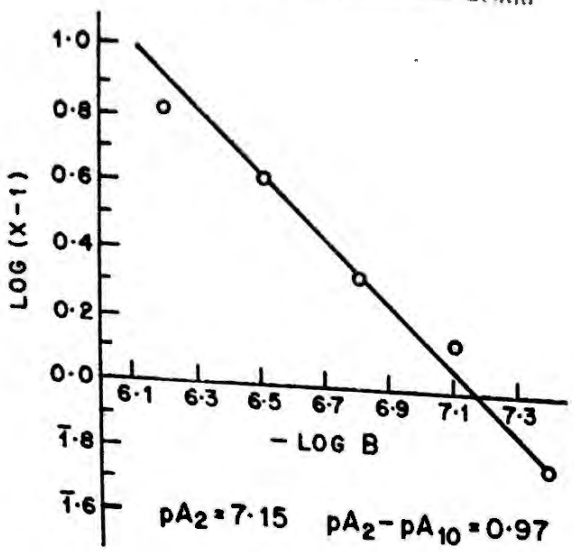
$$KB = \frac{(B)^1}{(\text{dose ratio} - 1)}$$

B′, D′ etc. denote the respective Concentrations in presence of antagonist.

Arunlakshana and Schild converted this equation to log (dose ratio — 1) = log (B)′ — log KB.

KB, and hence the pA_2 may be obtained directly from the intercept with the log (B)′ axis of the plot of log (dose ratio—1) against log (B)′. The slope should be unity.

Fig. App 5: Derivation of pA_2 and $pA_2 - pA_{10}$ by the method of Arunlakshana and Schild



Determination of Dissociation Equilibrium Constant of an agonist:

In the presence of an irreversible competitive antagonist, an agonist will lead to a receptor occupancy

$$y' = \frac{(D)'}{(D)' + K2/K1} \times (1 - Y')$$

where Y^1 is receptor pool fraction blocked by antagonist. Equating with receptor occupancy before blockade, we get

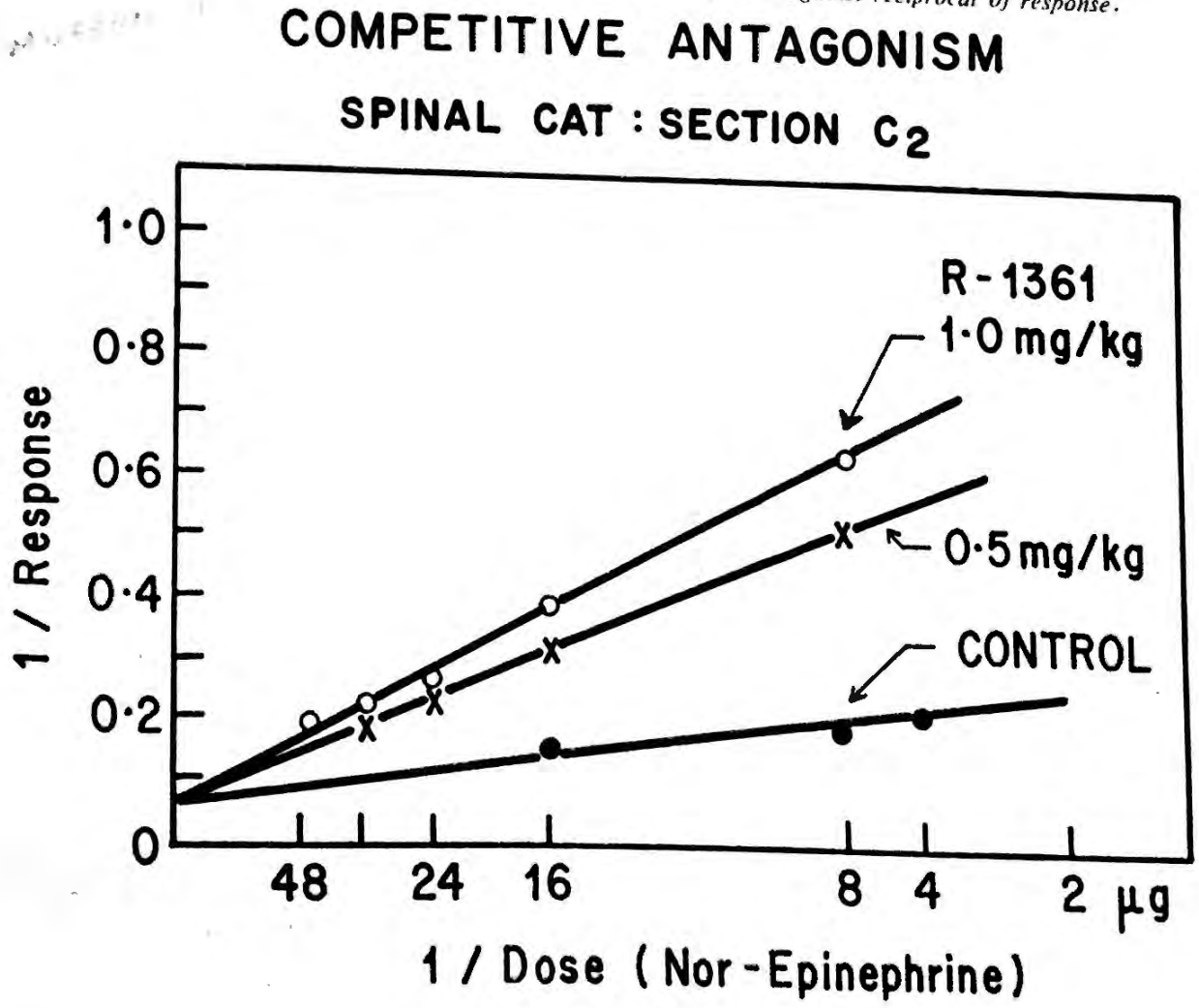
$$\frac{1}{(D)} = \frac{Y'}{1 - Y'} \frac{K1}{K2} + \frac{1}{1 - Y'} \frac{1}{(D)'}$$

Hence a plot of the reciprocal of the control dose for a given response against the reciprocal of the dose required to give the same response after antagonist blockade will give the K1/K2 of agonist as

$$\frac{K1}{K2} = \frac{\text{Intercept on 1/D axis}}{\text{slope} - 1}$$

Such a plot is called the Lineweaver-Burke plot or a double reciprocal plot. The equation of Arunlakshana and Schild can

Fig. App. 6: The Lineweaver-Burke plot of reciprocal of dose against reciprocal of response.



be used in another way for competitive reversible antagonists. A plot of $\frac{1}{(D)}$ against $\frac{1}{(D)'}$ from the above equation will go through the origin and the slope will be the equilibrium dose ratio. Equilibrium dose ratios may thus be obtained for use in this equation.

The use of pD'_2 values for comparing potencies of non-competitive antagonists is limited by the nature of the dose-effect relationship. From the equation from $\frac{1}{(D)}$ the slope of the relation between $\frac{1}{(D)}$ and $\frac{1}{(D)'}$ works out to $\frac{1}{(1 + y')}$. Thus the receptor occlusion by the non-competitive antagonist is obtained directly as

$$y' = \frac{\text{slope} - 1}{\text{slope}}$$

B′ D′ etc. denote the respective concentrations in presence of antagonist.

TABLE 1: Identification of an unknown hypotensive, using an adrenergic system.

Drug	Carotid Occlusion response	Blood Pressure			Nictitating Membrane			
		Direct	E	NE	Direct	Pre G	Post G	E
∝ Receptor Blockers								
Phenoxybenzamine	↓	↓	↓	↓	—	↓	↓	↓
Tolazoline	↓	↑, ↓	↓	↓	↑	↓	↓	↓
Adrenergic neurone blockers								
Bretylium	↓	↑	↑	↓	—	↓	↓	–, ↑
Guanethidine	↓	↑, ↓	↑	↓	—	↓	↓	–, ↑
Reserpine	↓	↓	↑	↑	—	↓	↓	↑
Uptake inhibitors								
Cocaine	↑	—	↑	↑	—	↑	↑	↑
DMI								
Ganglion blockers								
Pentolinium, C6	↑	—	↑	↑	—	↑	↑	↑
Ganglion stimulants								
DMPP	–, ↓	↑	↑	↑	↑	–, ↓	—	—
Nicotine	–, ↓	↓, ↑	↑	↑	↑	–, ↓	—	—
S. M. Amines								
Ephedrine amphetamine tyramine	—	↑*	↑	↑	↑	—	—	↑

* shows tachyphylaxis
↑ = Action or increase.
↓ = Decrease

TABLE 2: Choice of Isolated Preparation for Bioassay.

Preparation	Speed of Assay	Technique	Threshold dose
Bioassay of Cholinergic Agents			
Leech dorsal muscle	Slow	Organ bath	6×10^{-9} mole ($0.001\ \mu g/ml$)
G. pig ileum	Rapid	" "	1.5×10^{-8} mole ($0.0025\ \mu g/ml$)
Frog rectus	Adequate	" "	6×10^{-8} mole ($0.01\ \mu/ml$).
Cat B. P.	Rapid	In vivo	$0.002\ \mu g/kg$
G. pig ileum	Very rapid	Superfused	$0.0025\ \mu g/dose$
Frog rectus	More rapid	"	$0.01\ \mu g/inj.$
Bioassay of Adrenergic Agents			
Rat uterus	Adequate	Organ bath	2×10^{-8} mole ISO 1×10^{-7} mole E 2×10^{-6} mole NE
Rabbit aortic strip	Slow	" "	2×10^{-7} mole ISO 5×10^{-9} mole E 5×10^{-9} mole NE
G. pig tracheal chain	Slow	" "	5×10^{-8} mole ISO 1×10^{-7} mole E 1×10^{-7} mole NE
G. pig vas deferens	Adequate	" "	3×10^{-5} mole NE
Rat colon	Adequate	" "	2×10^{-7} mole E
Bioassay of Histaminergic Agents			
Rabbit aortic strip	Slow	Organ bath	5×10^{-8} mole
G. pig. tracheal chain	Slow	" "	5×10^{-7} mole
Bioassay of 5HT			
Rat fundus	Adequate	Organ bath	1×10^{-9} mole
Rabbit aortic strip	Slow	" "	5×10^{-8} mole

TABLE 3 : Showing experimental set up with various isolated preparations.

Preparation	Initial Tension Previous Treatment if any	Perfusing Solution	Temperature C	Drug Sensitivity and uses
Frog rectus	0.5 — 1.0 gm Eserinise 1.5 x 10-6 mole/ml	Frog Ringer	R.T.	Ach. 6 x 10^{-8} mole or 0.01 μ/ml.
Leech dorsal m.	nil	Frog Ringer or Locke	R.T.	0.001 μ/ml. Ach.
Rat phrenic n. diaphragm	nil	Tyrode with double glucose or Krebs	37	
G. pig ileum	0.3 — 0.5 gm.	Tyrode	37	Ach. 0.0025 μ/ml.
Finkelman	0.5 gm	Tyrode	37	Qualitative study of adrenergic agents.
Rat fundus	1 gm	Krebs	37	0.4 - 0.8 mg/ml.
Rat uterus	0.1 mg/kg, sc Stilboestrol 24 hrs prior 0.5 gm	De Jalon	30-32	5HT, β -adrenergic
Rabbit aortic strip	3 — 4 gms	Krebs	37	NE, E, 10^{-9} mole adrenergic
G. pig tracheal chain	0.2 — 0.5 gm	Krebs	37	E, NE, 10^{-7} mole Isopren 10^{-8} mole
G. pig vas deferens	0.75 — 1.0 gm	Krebs	32	Useful for study of adrenergic agents.
Langendorff	Nil	Ringer Locke	37	Qualitative
Atria	Nil	Feigans, Ringer-Locke	R.T.	
Isolated cat spleen	0.5 gm	Tyrode	37	E 10 - 15 μg/ml
Rat colon	0.3 — 0.5 gm	Modified Ringer	R.T.	E 10 - 15 μg/ml

TABLE 4: Showing time intervals for various isolated tissues.

Isolated preparation	Initial time of relaxation	Time of drug contact in seconds	Time of recovery in minutes
Frog Rectus	30 mins	90	5 — 10
Guinea pig ileum	30 mins	30	3
Leech dorsal m.	3 hours	90	20
Rat phrenic n. diaphragm	Not reqd.	Peak effect	On washing Out
Finkelman	30 mins	60	On washing Out
Rat fundus strip	30 min	60 — 90	6 — 10
Rat uterus	30 min	30 — 60	2 — 5
Rabbit aortic strip	60-120 mins	90	15 — 30
G. pig tracheal chain	30 — 60 mins	90	15 — 30
G. pig vas deferens	Not reqd.	60	Immediate
Rat colon	15 — 30 mins	60	5 — 15
Isolated cat spleen	2 — 3 hours	30 — 60	5 — 10

TABLE 5 : Table showing compositions of commonly used physiological salt solutions (in g/litre)

Solutions	Glucose	NaCl	KCl	$CaCl_2$	$MgCl_2$	NaH_2PO_4	$NaHCO_3$	Aeration
Frog Ringer	2.0	6.5	0.14	0.12	—	0.005	0.4	Oxygen
Ringer	1.0	9.0	0.42	0.12	—	—	0.5	Oxygen
Tyrode	1.0	8.0	0.2	0.2	$MgSo_4$ $7H_2o$ 0.26	0.05	1.0	Carbogen
Krebs	2.0	6.9	0.35	0.28	0.14	KH_2PO_4 0.16 or $Na\ H_2PO_4$ 0.11	2.1	Carbogen
De Jalon	0.5	9.0	0.42	0.03	—	—	0.5	Carbogen
Feigan	1.0	9.0	0.42	0.62	—	—	0.6	Carbogen
Locke Ringer	2.0	9.0	0.42	0.24	—	—	0.3	Oxygen
Modified Ringer	1.00	9.0	0.40	0.03	—	—	0.15	Oxygen

TABLE 6: Blood Pressure Recording Chart

Expt. No. .....

Species ..... Sex ..... Weight ..... Date .....

Anaesthetic ..... Dose ..... Route .....

Time Surgery began ..... Time Surgery ended .....

Drug under study ..... Expt. Terminated—

A. Time .....

B. Manner .....

Time	Drug or procedure	Dose mg/kg	Route of Adm.	Onset of effect	Duration of effect	Blood pressure			Remarks
						Basal	Max effect	Diff.	

Respiration

Basal		Expt.		Spleen Vol.	Nictitating membrane
Rate	Ampl.	Rate	Ampl.		

TABLE 7 : The Average Normal Temperature and Pulse Rate of Some Common Laboratory Animals

Animal	Rectal Temperature °C	Rectal Temperature °F	Rates Per Minute	
			Pulse	Respiration
Cat	38.7	101.6	130	24
Dog	38.6	101.4	95	14
Guinea Pig	38.6	101.48	150	80
Mouse	37.4	99.3	600	163
Monkey, Rhesus	38.4	101.1	100	19
Rabbit	38.7	101.6	135	55
Rat	37.5	99.5	300	210
Hamster	36-38	98-101	450	74

TABLE 8 : Some data on the reproductive cycles of common laboratory animals.

Species	Mating Age	Breeding Season	Oestrus		Length of Pregnancy	Recurrence of Oestrus
			Duration	Interval		
Cat	10 months	Jan. — Aug.	7-14 days	14-21 days	63 days	End of lactation
Guinea Pig	5-6 months	No definite season	6-12 hours	14-16 days	66 days	Post partum then regular cycle
Hamster	8-12 weeks	No definite season	7	4 days	16 days	End of lactation
Mouse	6-8 weeks	No definite season	10-20 hours	5 days	19 days	Post partum then regular cycle
Rabbit	6-9 months	No definite season	Prolonged in absence of male	.....	28 days	End of lactation
Rat	90-100 days	No definite season	10-20 hours	5 days	21 days	Post partum then regular cycle.

NOTE: Figures given above are subject to slight variation.