Chapter 1

Use of the Rabbit Intestine in Smooth Muscle Pharmacology Experiments: A New Approach

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INTRODUCTION

The isolated intestinal smooth muscle preparation is one of the classical preparations in physiology and pharmacology for bioassays, or the study of drug action and autonomic control of motility. This preparation is included in many "in-house" laboratory manuals of various colleges and universities around North America, and in some commercially prepared manuals dealing with physiology and pharmacology (e.g. Nicpon-Marieb, 1981).

Basically, the method presented in this report is a modification of the original Finkleman preparation (Finkleman, 1930) for the study of the autonomic control of intestinal motility. What is different about our approach is the method of mounting the preparation and the method of stimulation of the sympathetic nerve. The classic way of mounting the piece of intestine is to suspend it vertically in a muscle bath between an aeration tube and a recording lever. The problems with this technique are (1) stress placed on the intestine when the chamber is emptied during the process of changing solutions, and (2) difficulty in stimulating the sympathetic nerve due to the fact that the preparation is totally submerged in a physiological saline solution. We have overcome these problems by mounting the segments of gut horizontally in a shallow muscle bath. As a result, less stress is placed on the intestine during changeover of solutions, and it is easier to manipulate and to stimulate the sympathetic nerve contained within the mesentery. Also students find it much easier to mount the preparation in the horizontal bath and are less likely to stretch the muscle preparation in the process.

With these modifications we have improved the student success rate from 50-60% to 90-100%. Large recordings of the muscle contraction, such as those shown in Appendix A, are easily obtainable using a kymograph and simple lever system. In fact, another advantage of this exercise is that it does not require expensive recording equipment.

Equipment and supplies:

A list of the equipment, supplies and solutions needed to operate this laboratory exercise is given in Appendix B. Instructions and dimensions for building the muscle bath, swivel assembly and stimulating electrodes are given in Appendix C.

We recommend using a simple isotonic lever system for recording the muscle contraction because of the ease with which students are able to comprehend and manipulate the recording system to obtain excellent tracings. Recordings of comparable quality, however, are also obtainable with an isometric transducer (e. g. a Physiograph F-60 force transducer).

Preparation of isolated segments of the rabbit intestine:

The rabbit should be starved for 24 hours prior to use. Food in the gut will result in a messy dissection and will necessitate flushing to remove the gut contents, a practice which could damage the intestine.

Before sacrificing the rabbit:

1. Prepare Tyrode's Ringer solution (see Appendix B) and place about 250 mL of this solution in a flask on ice.

2. Cut sections of thread (or 4-0 silk) into 5 cm lengths. One piece will be needed for each segment of gut.

3. You will need a pair of fine (iris) scissors, coarse scissors, and curved forceps. Hemostats may also be helpful in retracting the abdominal muscles.

4. A large Petri plate or other container for holding the segments of gut will also be necessary.

5. Wash all instruments and glassware before use. Also make sure your hands are clean before handling the intesting.

Sacrificing the rabbit and removing the intestinal segments:

1. Since anaesthetics can affect motility of the gut, the animal should be sacrificed by cervical dislocation, without use of anaesthetic.

2. Shave the abdomen and carefully vacuum the surface to remove any loose or cut fur.

3. Make a mid-line incision through the skin and abdominal muscle using a pair of coarse scissors.

4. Locate the ileum, a section located near the end of the small intestine. Note the intestinal arteries and veins coursing through the mesentery to and from the small intestine. If a segment of the ileum is picked up with both hands, the intestinal vessels are easily seen spaced about 2 to 3 cm apart (Fig. la). If you look very carefully, a fine white line following the intestinal artery and vein into each segment of the gut is visible. The white line is a branch of the sympathetic nerve arising from the prevertebral ganglion.

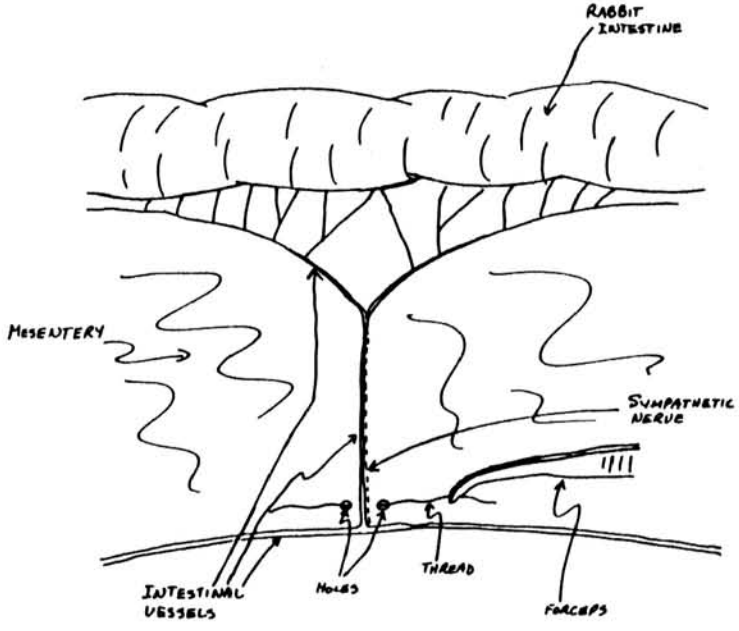


Fig. 1a

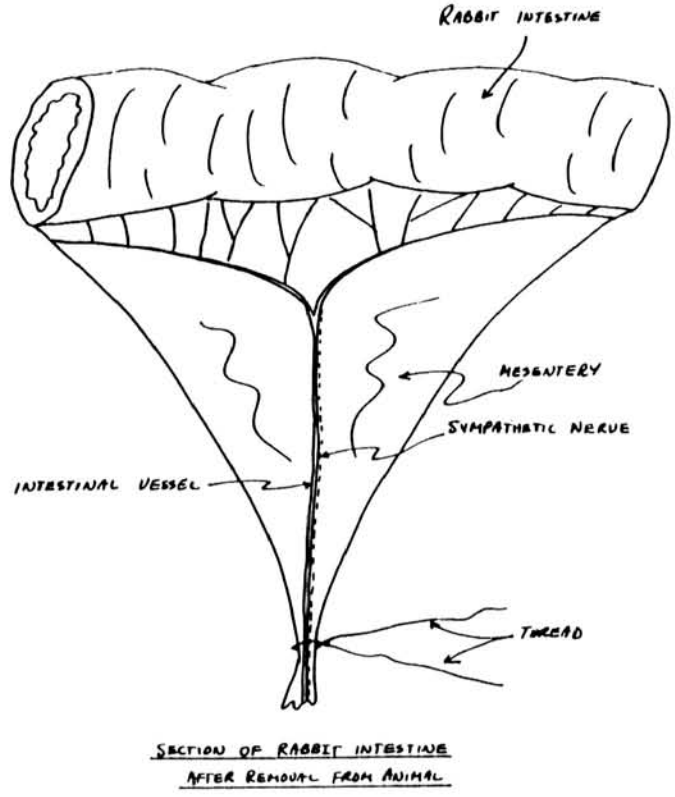


Fig. 1b

Fig. 1a and 1b Dissection of the rabbit intestine

5. Using a pair of fine curved forceps, carefully break through the mesentery on either side of the intestinal vessel-sympathetic nerve complex about 4 cm away from the gut (Fig. 1b). Insert a length of thread or silk suture through the hole and tie off the vessels and nerve.

6. Follow the vessels down to the gut. The vessels divide into many branches just above the surface of the intestine. Select a 3-cm segment of the ileum that is supplied by these vessels. Sever the gut and remove this segment along with the mesentery containing the vessels and nerve. Place in a Petri dish containing cold Tyrode's Ringer solution.

   N.B. If the rabbit has been starved for *24* hours, the gut segment should be free of chyme. However, if chyme is present, it can be removed by carefully flushing the segment with Tyrode's. A syringe filled with Tyrode's may be placed inside one end of the segment, and using very little pressure, the contents can be carefully forced out into a waste container. Caution must be exercised because high pressure will severely damage the tissue.

7. Repeat this procedure until enough segments have been removed to supply your laboratory needs for the day. If the extra pieces of ileum are kept in ice cold Tyrode's, they will stay viable for several hours. Do not store in warm Tyrode's.

Suggestions:

1. The ileal segments will shorten when placed into the cold Tyrode's solution and motility will cease. However, when mounted in the warm (37°C) Tyrode's solution inside the muscle bath, motility will return within 5-10 min.

2. As the muscle warms in the bath, it will relax and segmental and peristaltic waves will be evident.

3. It is essential that the muscle baths be at 37°C at the start of class. This means starting the circulation of warm water through the baths at least 1/2 hour before class. Also, it is essential that the extra Tyrode's solution for the muscle baths be at 37°C.

4. It is not necessary to warm the drug solutions used in the experiment since they will be added directly to the muscle bath in very small amounts (0.1 to 1.0 mL of drug/100 mL of Tyrode's).

5. One of the advantages in adding drug directly to the bath in concentrated form is that rapid changes in muscle tonus can be clearly recorded. We recommend that the Tyrode's solution be replaced between drug additions so as not to disturb the motility directly before the application of the drug. It may take 1-2 minutes for the rhythm to stabilize after replacing the solution in the muscle bath.

Student's Guideline

AUTONOMIC CONTROL OF THE VISCERAL SMOOTH MUSCLE OF THE RABBIT INTESTINE

OBJECT:

To show some of the autonomic properties of smooth muscle as it occurs in the rabbit's small intestine.

EXPLANATION:

All smooth muscle cells have one thing in common: the actin and myosin filaments of the sarcomeres are not organized into bands such as they are in skeletal muscle. Visceral smooth muscle, such as the type found in the digestive tract, is a syncytium of muscle cells where intercellular communication can occur via gap junctions. Electrical current is easily conducted from one cell to the next via the gap junctions; therefore, depolarization of one muscle cell can quickly lead to depolarization of neighboring cells.

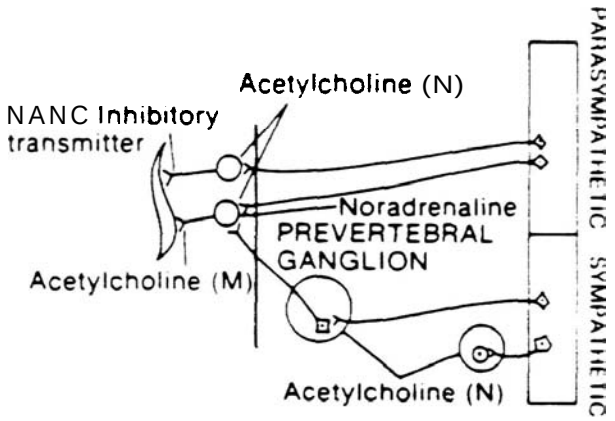
Visceral smooth muscle contraction occurs spontaneously, meaning that muscle cell action potentials are generated without input from either the motor or autonomic nervous system. The muscle cells undergo rhythmic oscillations in membrane potential which, occasionally, reach the threshold of an action potential, and, thus, generate a spike. The action potential spreads via gap junctions from muscle cell to muscle cell, initiating a wave of muscle contraction in its wake.

Visceral smooth muscle cells also exhibit muscle tonus, a state of long-term, steady contraction. The tonus is variable, depending on the number of muscle cells that participate.

The rhythmicity and tonus inherent in the intestinal smooth muscle may be enhanced or suppressed by two nerve plexuses found between the layers of muscle and mucosa (Auerbach's and Meissner's plexuses). Known as the enteric nervous system of the gut, the activity of the plexuses can be modified by the autonomic nervous system. When a piece of intestine is removed for study, the plexuses remain viable and can be stimulated or inhibited using parasympathomimetic or sympathomimetic drugs (mimicing the action of the parasympathetic and sympathetic nervous systems).

The autonomic control of the gut is very complicated. Figures 2 and 3 illustrate the parasympathetic and sympathetic input to the enteric neurons of Auerbach's and Meissner's plexuses.

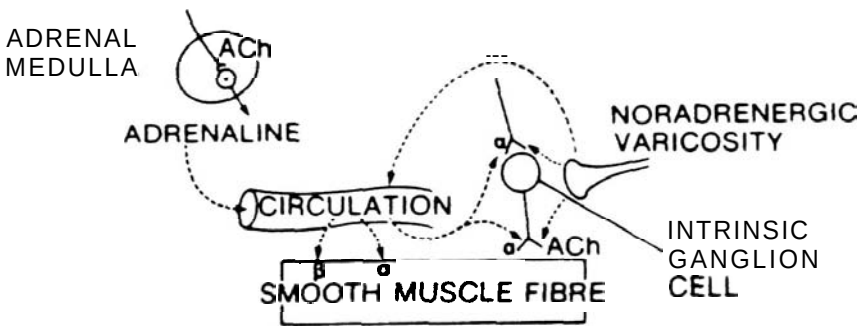
Figure 2*



Sympathetic stimuli subdue the inherent rhythmicity of the gut. Note that the sympathetic input is upon alpha-adrenergic receptors located on the axon terminals of the enteric neurons, or upon the axon terminals of the preganglionic parasympathetic fibers. Release of norepinephrine from the sympathetic postganglionic fibers stimulates the alpha receptors, which prevent release of acetylcholine from the enteric neurons or preganglionic parasympathetic fibers. This action is known as presynaptic inhibition.

There are alpha- and beta-adrenergic receptors on the smooth muscle cells which will respond to epinephrine and norepinephrine in the circulation, as shown in Figure 3. Both alpha and beta receptors inhibit muscle tone and rhythmicity.

Figure 3*.



The parasympathetic control of the gut is via cholinergic preganglionic fibers which synapse with enteric neurons. When activated, the parasympathetic fibers release acetylcholine at the enteric neurons, thus stimulating the enteric neurons to release acetylcholine at their neuro-muscular junctions. Enhanced smooth muscle tone and rhythmicity result.

*Reprinted from "Innervation of the Gastrointestinal Tract" in A Guide to Gastrointestinal Motility, Christensen and Wingate (eds.), with permission from John Wright Medical Publishers.

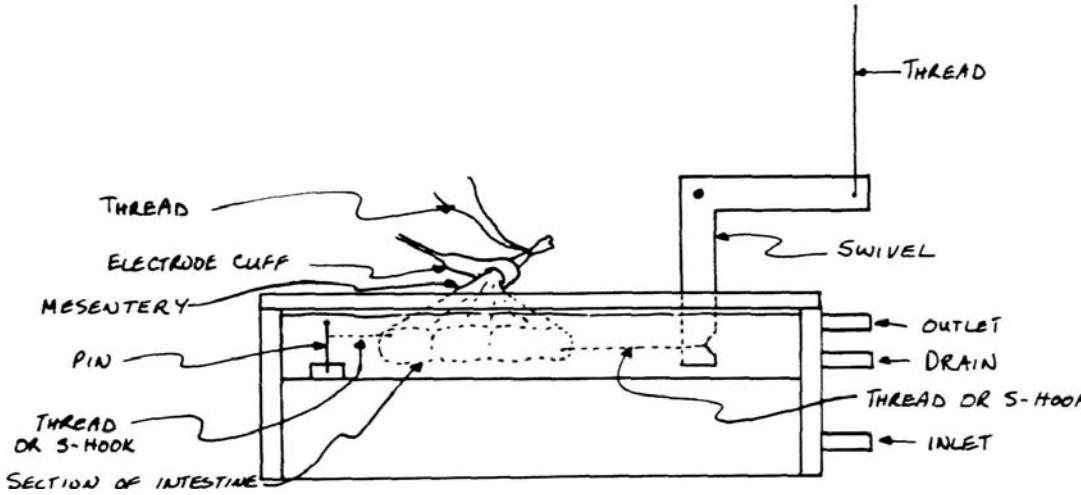
The receptors of the neuromuscular junction are muscarinic type cholinergic receptors. They can be blocked by the addition of atropine to the muscle preparation.

In the following laboratory exercise you will study the action of sympathetic nerve stimulation, and the effects of various pharmacological agents on the rhythmicity and tonus of the rabbit gut. The agents will include sympatho- or parasympathomimetic drugs, along with adrenergic and cholinergic blocking agents.

PROCEDURES:

The rhythmical activity of the piece of rabbit gut will be recorded using a lever system and kymograph. To maintain its activity, the gut will be suspended in a bath of Tyrode's solution at 37°C with adequate oxygen supply. (A mixture of 95% O_2 - 5% CO_2 will be used).

Wash your hands thoroughly with soap and water before handling the gut. Body salts, oil and dirt will kill the gut tissue.



SMOOTH MUSCLE BATH
(SIDE VIEW)

FIGURE 4. Arrangement of Intestine in Smooth Muscle Bath

Preparation:

The muscle chamber is set up as in Figure 4. Warm water is circulated through the outer bath to keep the muscle chamber at 37°C. One end of a section of intestine is attached to the anchor pin in the chamber using an S-hook. The other end is connected via an S-hook to a swivel which transposes horizontal contractions and relaxations of the gut into vertical movement of a recording lever. The movement of the recording lever are traced upon a kymograph drum, or chart recorder.

1. Attach two S-hooks to the gut, on opposite sides, at the opposing ends. During this operation, keep the gut in a Petri plate containing Tyrode's solution. Do not allow the tissue to dry.

2. Attach one hook to the anchor pin in the muscle chamber. Fasten the other hook to the swivel.

3. Fill the chamber with 100 mL of warm Tyrode's solution and bubble 95% O_2-5% CO_2 gas into the chamber fluid. There should be a fine stream of bubbles, rather than large bubbles which will disrupt your recordings.

4. Attach a thread from the muscle lever to the recording lever using plasticine. Remove slack from the thread by adding plasticene to the recording lever to act as a counter-balance. Keep the recording lever horizontal.

Experiment:

During the following experiment, check that your tracings are labelled clearly. Be sure that all drug concentrations, washes and solution changes are labelled so as not to confuse a drug response with an artifact. The "normal" muscle rhythmicity and tonus (i.e. prior to drug application) will vary throughout the experiment. Compare the drug effect to the "normal" muscle contraction in Tyrode's just preceding the addition of the drug.

A. Acetylcholine and Norepinephrine:

These two compounds act as postganglionic neurotransmitters of the parasympathetic and sympathetic nervous systems, respectively. Apply the drugs in the order listed below. After you have noted the effect of the drug, drein the muscle bath, and refill with 100 mL of fresh, warm Tyrode's solution. After the muscle contractions have returned to the normal rhythmicity and tonus, you may add the next drug. Add drops to the bath away from the gut; mixing occurs quickly by diffusion. Check bath temperature frequently.

1. 10^{-7} M acetylcholine: add 0.1 mL of 10^{-4} M acetylcholine to the bath.
2. 10^{-6} M acetylcholine: add 1.0 mL of 10^{-4} M acetylcholine to the bath.
3. 10^{-7} M norepinephrine: add 0.1 mL of 10^{-4} **M** norepinephrine to the bath.
4. 10^{-6} M norepinephrine: add 1.0 mL of 10^{-4} M norepinephrine to the bath.

B. Alpha- and Beta-Adrenergic Agonists:

1. Drain and refill the chamber with fresh, warm Tyrode's. Apply 1.0 mL of 10^{-4} M phenylephrine and note the response. Phenylephrine is an alpha-adrenergic agonist (stimulant).

2. Drain the chamber and wash the gut with Tyrode's. Refill the chamber and add 1.0 mL of 10^{-4} M isoproterenol, a beta-adrenergic agonist.

What can you conclude about the type of adrenergic receptors present in the smooth muscle of the gut?

C. Sympathetic Nerve Stimulation:

1. Mount the stimulating electrode on the side of the muscle chamber. Carefully slide the mesentery containing the sympathetic nerve into the electrode sleeve via the thread attached to the mesentery.

2. Stimulate the nerve with 30V (0.5 msec duration, 15 pps) for 10-20 seconds. If you do not see a response try 40V. Note the change in tonus and rhythmicity.

D. Alpha and Beta Adrenergic Blockade

1. Place 1.0 mL of 10^{-4} M phentolamine in the chamber. Wait one or two minutes, then try stimulating the sympathetic nerve.

2. Add 1.0 mL of 10^{-4} M phenylephrine.

   If the alpha receptors are blocked, phenylephrine should have no effect.

3. Drain and refill the chamber with warm, fresh Tyrode's solution. Add 1.0 mL of 10^{-4} M propranolol.

   Wait two to three minutes, then try stimulating the sympathetic nerve.

4. Add 1.0 mL of 10^{-4} M isoproterenol to the chamber.

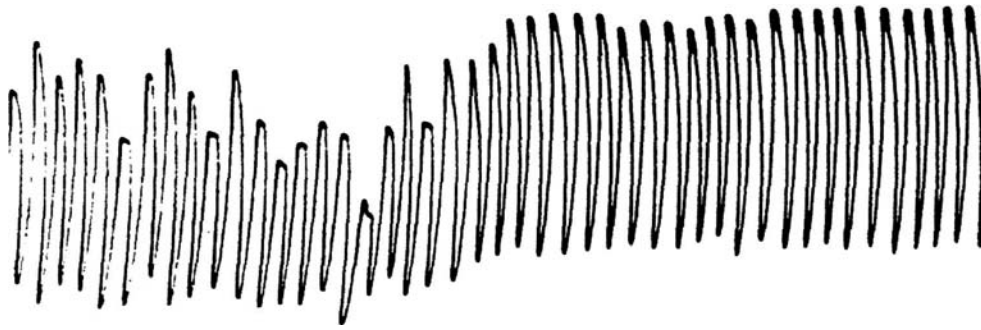
   If the beta receptors are blocked, isoproterenol should have no effect.

E. Atropine - a cholinergic blocking agent

Drain and refill the bath with warm, fresh Tyrode's solution. Add 1.0 mL of 10^{-4} M atropine. Atropine blocks the action of acetylcholine and is, therefore, a parasympatholytic drug. Wait two to three minutes and then add 1.0 mL of 10^{-4} M acetylcholine to the bath. Does the atropine successfully block the action of ,acetylcholine?

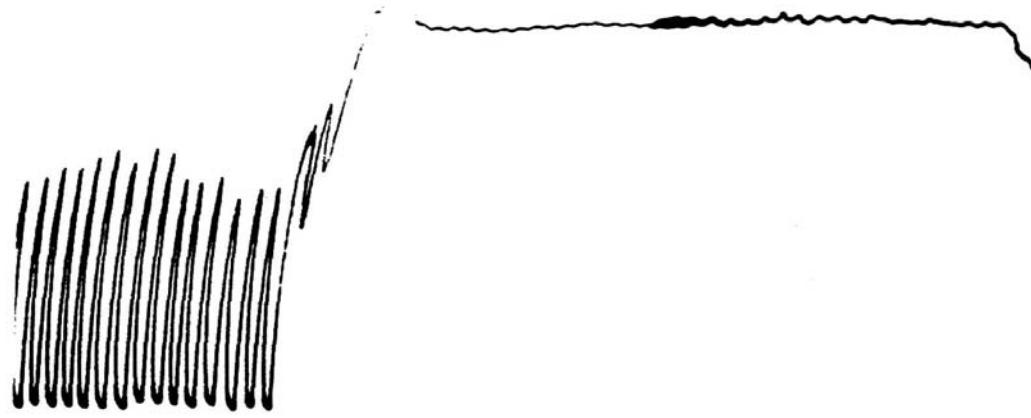
APPENDIX A

The chart recordings of the motility of an isolated segment of rabbit ileum, shown below and on the following pages, were collected by a pair of students using a kymograph and ink-recording lever system. The intestine was mounted horizontally in a muscle bath containing Tyrode's solution at 37°C. The preparation was aerated with a mixture of 5% CO_2-95% O_2. Small volumes of concentrated drug solutions were added directly to the muscle bath at the points indicated in each record. The Tyrode's solution was replaced between drug additions and the motility was allowed to stabilize before the next addition of drug. Stimulation of the sympathetic nerve was performed at 15 pulses/sec, with a pulse duration of 0.5 msec and strength of 30–40 volts (A.C.), for 20 seconds. See text of the exercise for other details.



Tyrode's Solution t Added 10-7 M Acetylcholine

paper speed = 1 mm/sec



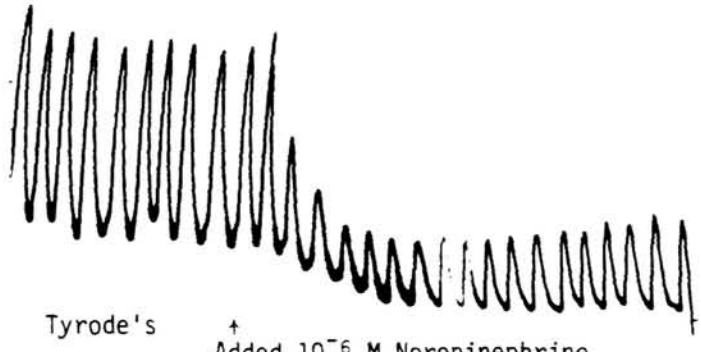
Tyrode's solution t Added 10-6 M Acetylcholine

paper speed = 1 mm/sec

Tyrode's

Added 10^{-7} M Noropinephrine

paper speed = 1 mm/sec



Tyrode's

Added 10^{-6} M Norepinephrine

paper speed = 1 mm/sec

30 V Stimulation

stopped

30 V Stimulation

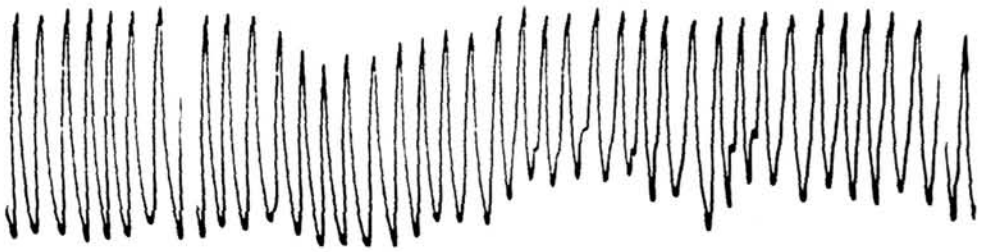
stopped

40 V

stopped

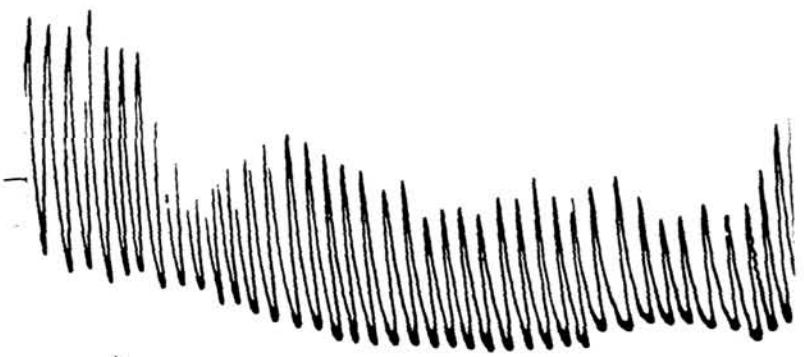
paper speed = 1 mm/sec

Duration = 0.5 mm/sec, 15 pps for 20 sec.



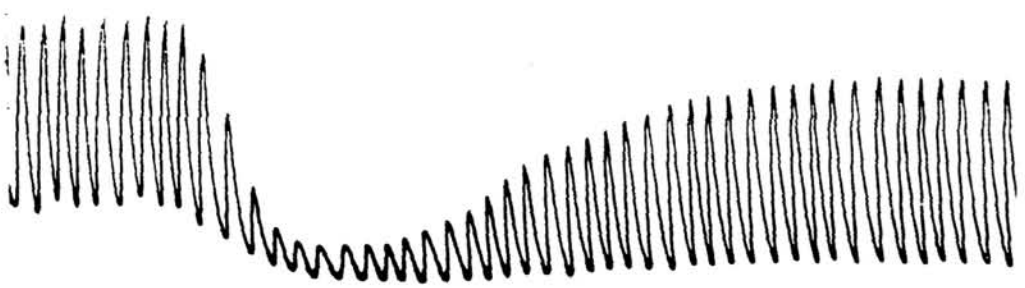
Tyrode's Added 10^{-6} M Phenylephrine

paper speed = 1 mm/sec



Tyrode's Added 10^{-6} M Isoproterenol

paper speed = 1 mm/sec



Added 10^{-6} M Atropine Added 10^{-6} M Acetylcholine

paper speed = 1 mm/sec

APPENDIX B

List of equipment/group of students:

muscle bath and swivel assembly
clamp for muscle chamber drain tube
thermometer
spool of thread
2-"S" hooks
wax pencil
7 30-mL beakers
250-mL beaker
Petri dish
100-mL graduated cylinder
pipet bulb or Pi–Pump
0.1-mL pipet
1-mL pipet
Pasteur pipet and bulb
platinum wire electrodes
stimulator (capable of delivering 10–100 volts A.C.)
recording device (e.g. kymograph or Physiograph)
stand for holding transducer or recording lever
plasticene

Materials at supply bench:

extra plasticene
recording paper for chart mover or kymograph
extra Pasteur pipets and bulbs
recording ink

water bath at 37ºC containing 2–L bottles of Tyrode's Ringer solution

carboy with extra Tyrode's solution (make up 2 L/group of students; we run through about 100 L/50 groups of students).

the following drugs made up in Tyrode's solution: (make fresh prior to each lab*, and protect from light)

10^{-4} M norepinephrine	10^{-4} M atropine
10^{-4} M acetylcholine	10^{-4} M propranolol
10^{-4} M phenylephrine	10^{-4} M phentolamine
10^{-4} M isoproterenol	

* see p.18 concerning preparation of these dilutions.

Common Apparatus:

Warm water must be recirculated to each muscle bath. We use one water bath to supply two muscle baths. Water is pumped from the central water bath to each unit and back. Because of the temperature drop en route, we set the central bath thermostat to 40-45ºC, depending on the distance the water must travel and the velocity of flow.

If you wish to construct such a system, a sturdy submersible pump and Tygon tubing are necessary.

There are commercial organ baths available that contain built-in heating elements and a vertical muscle chamber.

A system for delivering a 5% C02-95% 0, gas mixture is necessary. We use one 200 cu. ft. tank of gas to supply 10 stations. This provides enough gas for five 3-hr laboratory sessions (with a 1/4 tank reserve). The gas stream to each muscle bath need only be enough to provide a steady stream of small bubbles.

Tygon tubing is used to deliver the gas to each station.

The gut segments survive much longer and the contractions are more regular using the 5% $C0_2$-95% 0_2 mixture than with air or pure 0_2.

Tyrode's Ringer Solution:

NaCl	8.0 g
KCl	0.2 g
$NaHCO_3$	1.0 g
$MgCl_2 \cdot 6\ H_20$	0.1 g
$CaCl_2 \cdot 2\ H_20$*	0.1 g
$NaH_2PO_4 \cdot H_2O$	0.05 g
Dextrose	1.0 g

* dissolve the calcium chloride separately and add last

Bring up to 1 L with distilled water.

Adrenergic and Cholinergic Drugs:

All the drugs mentioned in "Materials at supply bench" are available from Sigma Chemical Co., with the exception of phentolamine, which usually can be purchased through a local pharmacy. The names and Sigma catalogue numbers are shown below:

drug name	Sigma cat. No.
(-) norepinephrine (arterenol hydrochloride)	A 7381
L-phenylephrine hydrochloride	P 6126
(±) isoproterenol hydrochloride	I 5627
acetylcholine chloride	A 6625
DL-propranolol hydrochloride	P 0884
atropine	A 0132

Preparation of 10^{-4} M dilutions of each drug

To facilitate ease and accuracy in preparation, 100 mL of a 10^{-3} M stock solution of each drug is prepared at the beginning of the week and stored in a refrigerator. Acidify with 2-3 drops of 2.0 M HCl. Just prior to each laboratory session, a 1:10 dilution of the stock solution is made, using Tyrode's, to obtain 10^{-4} M.

APPENDIX C

Constructing the Muscle Bath

Figure 5 illustrates the muscle bath used in the measurement of the motility of the rabbit intestine. As shown in the diagram, the bath is constructed of 1/4-inch and 1/8-inch acrylic plastic. A water jacket surrounds the muscle chamber and allows warm water to be circulated on three sides of the chamber to keep the gut at 37°C. An aeration tube, made from P.E. 90 polyethylene tubing, is glued around the bottom of the chamber. The tube is perforated with small holes over its entire length to provide maximum aeration and mixing of the Tyrode's solution with a fine stream of bubbles. The shank of a 20 gauge needle is attached to the end of the aeration tube as a means of connection with an air line from a tank of compressed 5% CO_2-95% O_2 gas. A small platform attached to one end of the chamber contains a pin hole. The platform serves as a holder for a push pin, which is used to anchor one end of the preparation in position. The gut segment is mounted horizontally and attached to a swivel and the push pin via "S" hooks (see Fig. 4). When attached to a writing stylus or a transducer lever with thread, the swivel serves to convert horizontal movements of the gut into verticle movements of the stylus or transducer lever. A drain is attached near the bottom of the muscle chamber. Except for the aeration tube, which is epoxyed to the bottom of the chamber, the entire unit is glued together with dichloromethane.

Constructing the Swivel Assembly

Figures 6a and 6b show the dimensions and placement of the swivel assembly which attaches to one side of the water jacket. The assembly is constructed from pieces of 3/4, 1/2, 1/4 and 1/8-inch acrylic plastic. There are three parts to the assembly; the swivel, the part that holds the swivel, and a track which is glued to the side of the water jacket.

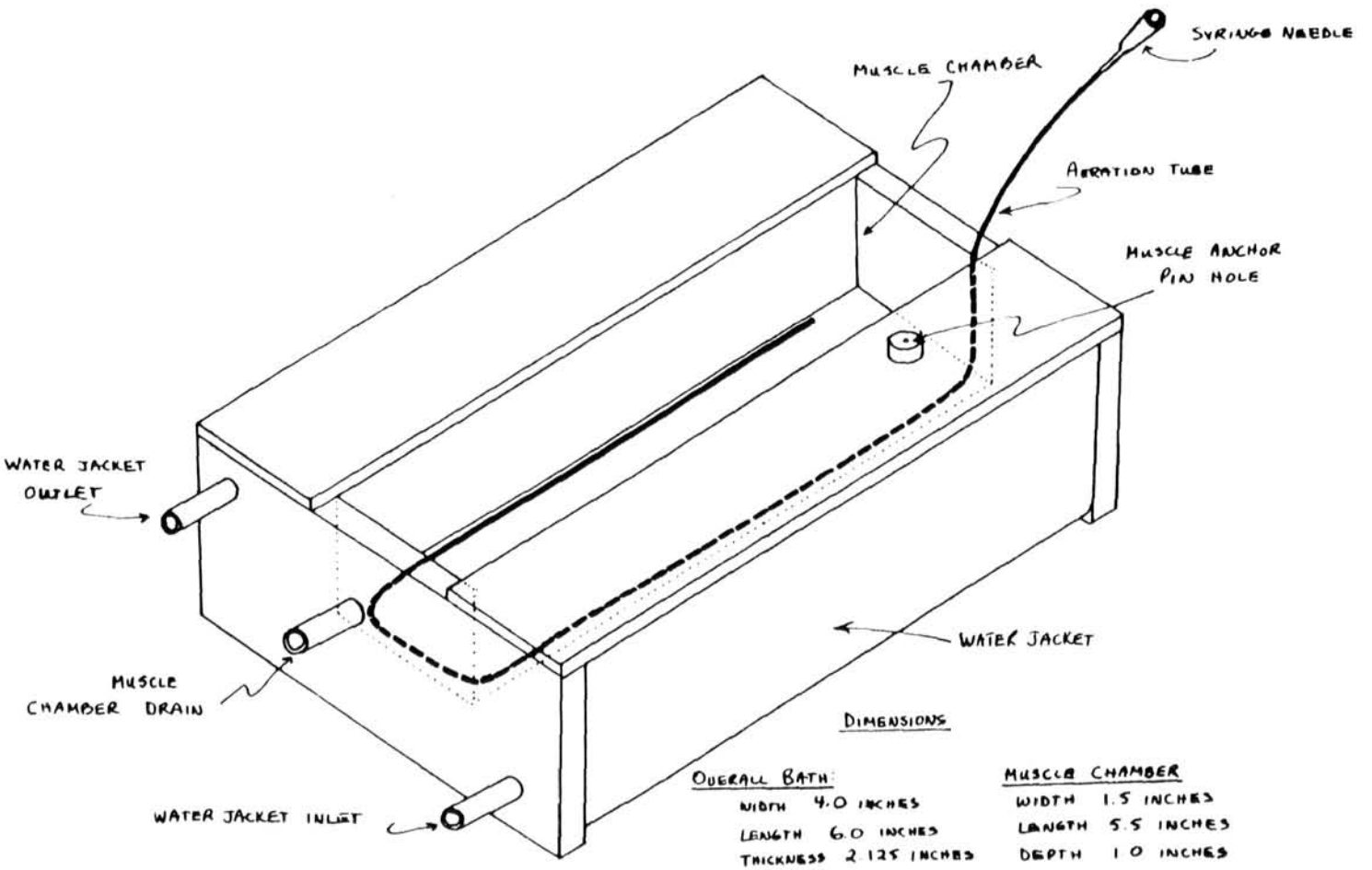
The swivel is made from 1/8-inch plastic. The arms, which form a right angle, should be 5/16-inch wide and 2 inches on a side. A small hole is drilled at the end of each arm for attachment of the hooks or thread to the muscle and recording lever. Alternatively, the ends can be notched. A 3/32-inch hole is drilled at the intersection of the two arms to act as a pivot. When placed in the holder, the swivel is held in place by a 1/16-inch rod which acts as an axle, as shown in Figure 6b. Refer to Figure 4 for a side view of the swivel.

As shown in detail in Figure 6a, the holder for the swivel is constructed from pieces of 1/4 and 3/4-inch plastic. The swivel is mounted in a 5/8 x 1/4-inch groove as shown, with the axle extending through holes which hold it snugly in place. A bolt two inches long secures the holder onto a track (Fig. 6b) which is glued to the side of the water jacket.

The track is made from a piece of 1/2-inch plastic which extends the full length of the water jacket. A 3/16-inch slot is cut into the plastic as shown in Figure 6b. When held in the slot by the bolt, the holder for the swivel can be positioned to accommodate the length of the intestinal segment.

Constructing the Electrode

The stimulating electrode consists of a pair of platinum wires which are encased in an epoxy cuff (see Fig. 7). Two 2-cm pieces of 28-30 gauge platinum wire are soldered to two 1-meter lengths of 20 gauge steel wire, which have been braided together. The solder joints are insulated with a layer of epoxy to keep them from making a short circuit. The platinum wires are then looped to form a circle about 3 mm in diameter. The inside curvature of the loop is filled with wax and then the terminal 2-3 cm of the electrode is dipped in epoxy several times to form a protective layer around the patinum wires. When the epoxy has hardened, the wax layer on the inside curvature of the electrodes is scraped or dissolved away to expose the bare platinum wire.



DIMENSIONS

OVERALL BATH		MUSCLE CHAMBER	
WIDTH	4.0 INCHES	WIDTH	1.5 INCHES
LENGTH	6.0 INCHES	LENGTH	5.5 INCHES
THICKNESS	2.125 INCHES	DEPTH	1.0 INCHES

THICKNESS OF WALLS:
0.25 INCHES EXCEPT ON TOP OF THE WATER JACKET WHICH IS 0.125 INCHES

WATER INLET/OUTLETS:
OD 0.25 INCHES
ID 0.125 INCHES

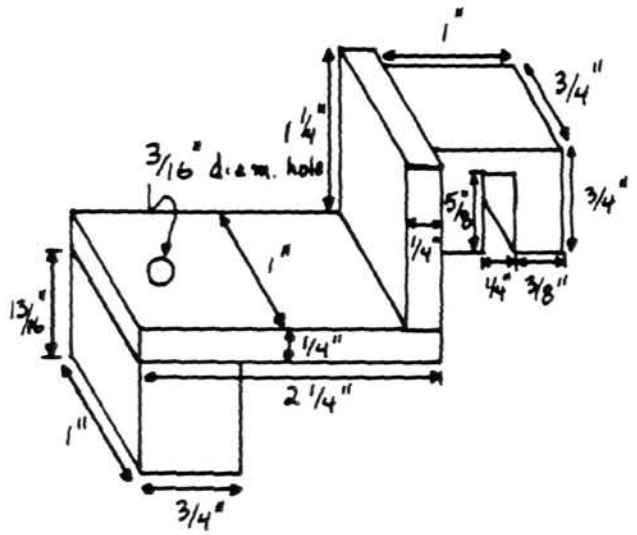


Fig. 6a Holder for Swivel

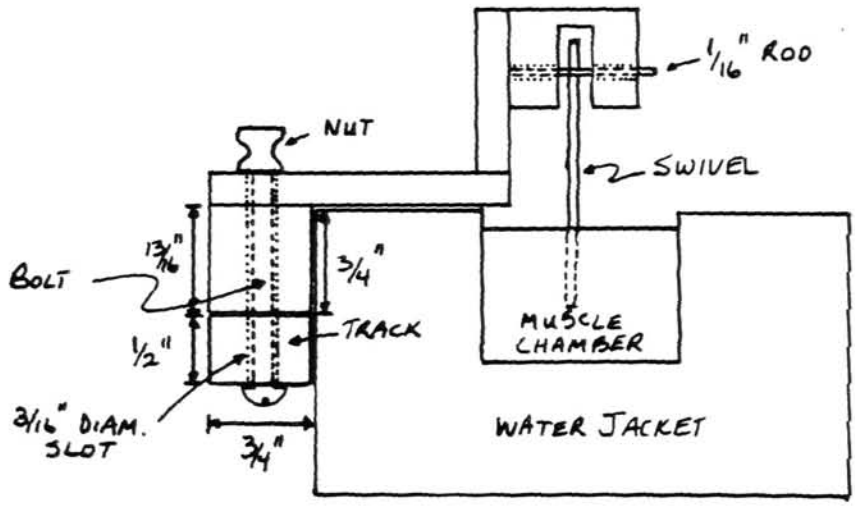


Fig. 6b Side View of Swivel Assembly and Track

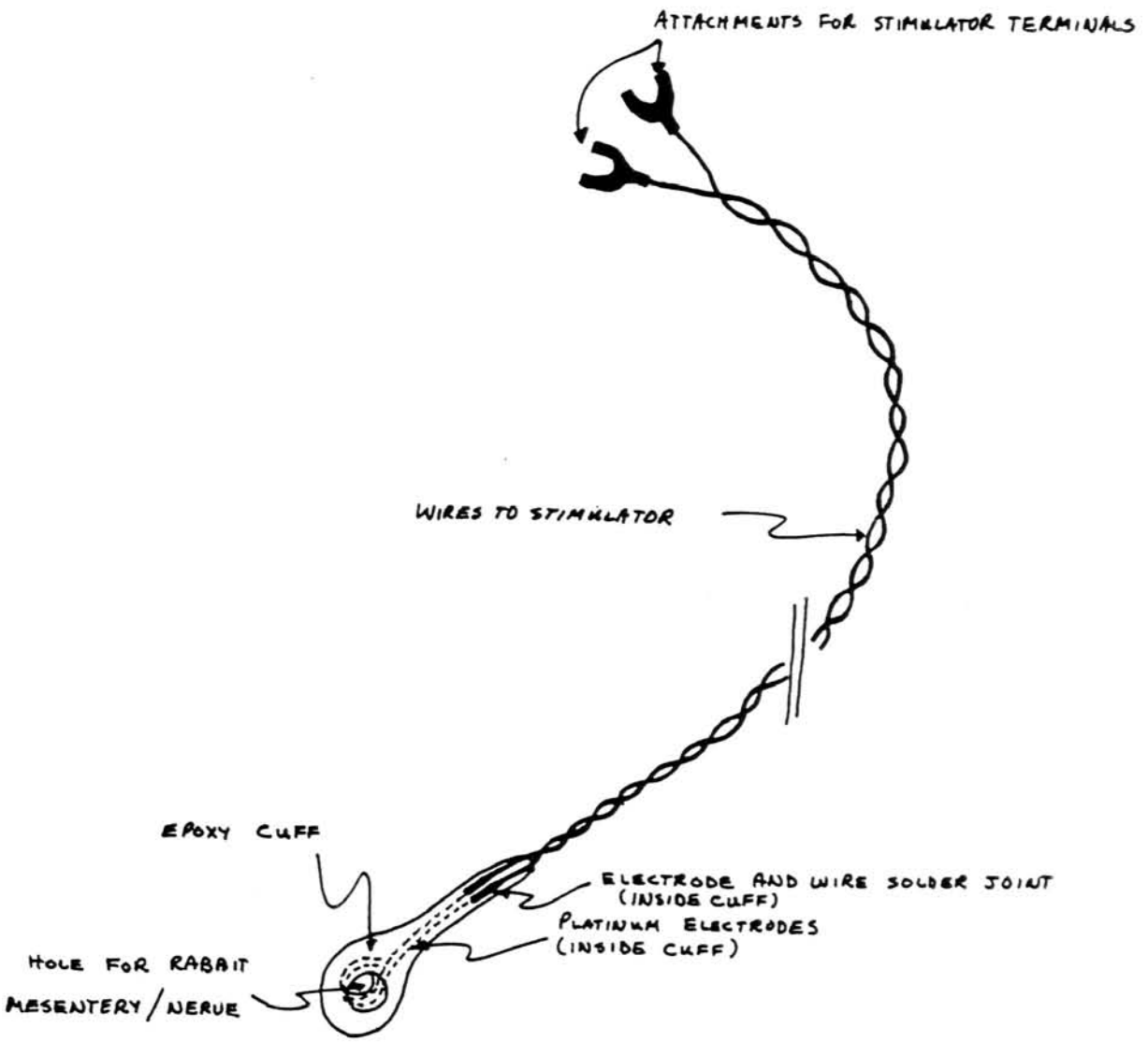


Fig. 7 Stimulating Electrodes

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