EXPERIMENTS IN PHYSIOLOGY AND BIOCHEMISTRY

Edited by G. A. KERKUT

Department of Physiology and Biochemistry, University of Southampton, Southampton, England

Volume 1

ACADEMIC PRESS
London and New York
1968

14

A. The Use of the Kymograph

B. The Clam-heart Technique of Bioassay for Acetylcholine

C. Preparation of Nerve Extracts for Bioassay of Bound and Free Acetylcholine

D. The Function of the Cardioregulator Nerves in the Crayfish

E. Pharmacology of the Crayfish Heart

F. Spontaneous Activity of the Crayfish Hindgut and its Control by Drugs

ERNST FLOREY

Department of Zoology, University of Washington, Seattle, Washington, U.S.A.

A. The Use of the Kymograph

In this age of mechanoelectric transducers and electronic recording instruments, the use of mechanical recording devices has been more and more neglected and many principles of the proper use of the kymograph and of its accessories have been forgotten. Consequently, the conviction is spreading that kymographs are not very suitable as recording devices, and the word "old-fashioned" has become a strong weapon in an unjust endeavor to abandon their use altogether and to ban kymographs from the physiology laboratory.

The following instructions are offered in the hope that they will help to restore the lost confidence in the classical tool of the physiologist, and to help the beginner to see the enormous potential of the kymograph as a universal recording device.

Types of Kymographs

The main feature of a kymograph is a metal cylinder mounted on a rotatable spindle. This is driven by a motor. Some kymographs have spring wound motors, others have electric motors.

Cheap spring wound motors tend to slow down as the spring unwinds. Their speed is governed by an adjustable brake. This consists often of external,

exchangeable, rotating vanes of different sizes. The velocity of their rotation is limited by their air-resistance. The better spring wound motors are provided with several reducing gears. Constant speed is maintained by a governor similar to that used in spring wound watch motors. Electric kymographs have always constant speed because of the inherent frequency control provided by the a.c. line current. Electric kymographs, like their better mechanical counterparts, are provided with gears to allow several speeds of rotation of the drum.

The drum is to be covered with suitable paper on which the records are then inscribed by the stylus of the recording lever.

Two types of inscribing are commonly used: (1) soot writing, and (2) ink writing.

1. The Soot Writing Technique

Preparation of the drum for recording: the application of the kymograph paper

Glossy white paper must be selected for this purpose. The drum, removed from the kymograph, is laid onto the paper and the proper area is marked off and cut out. The paper is cut to allow about 3 cm overlap. In order to apply the cut paper strip to the drum, lay it, glossy side down, on the table and moisten the non-glossy (now upper) surface with a sponge and place the drum (which lies on its side) over its center. Lift the two ends of the strip up and join them on top of the drum. Orient strip and drum in such a way that the top of the drum faces away from you. The ends of the kymograph paper must then overlap so that the end of the left half lies over that of the right half. Apply a fast drying glue or cement to the end strip of the right half and press the end flap of the left half of the kymograph paper over it. The procedure is depicted in Fig. 1.

As the paper dries, it stretches and tautly fits the drum. The seam is arranged in such a way that the writing stylus cannot be caught by the edge of the paper when the drum turns in the normal clockwise direction.

Smoking the drum

When illuminating or natural gas is bubbled through benzene, it picks up enough benzene vapor to give a very sooty flame when ignited. The benzene–gas mixture can be conducted (by way of rubber tubing) to a piece of glass tubing with a narrow orifice (about 2 mm diameter). The arrangement is shown in Fig. 1. When the gas is turned on and the gas–benzene mixture is ignited where it emerges from the glass nozzle, the resulting sooty flame can be directed against the rotating drum; the kymograph paper will not burn

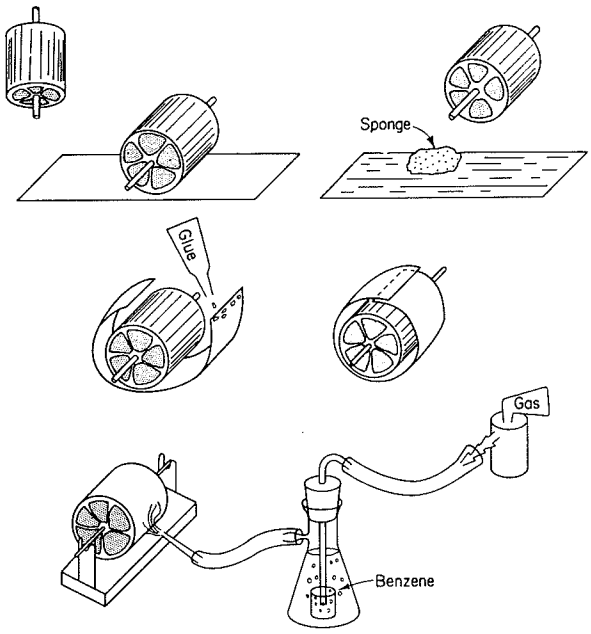


FIG. 1. How to fasten the paper to the drum and how to "smoke" it. If there is not sufficient time to prepare the drum in advance, the paper can be fastened with adhesive tape and the moistening can be omitted. The paper will not be as smooth, however, and the writing tip of the lever might get caught on the tape.

because the metal drum below it conducts the heat away. It is best to place the kymograph drum with its spindle on a stand, as illustrated in Fig. 1, so that it can be rotated horizontally. By moving the flame back and forth the paper can be covered with an even, thin layer of soot. Care must be taken not to touch the paper surface afterwards. The drum is now ready to be mounted on the kymograph.

ADJUSTING THE LEVER

Before a freshly prepared drum is used for recording, the lever and writing stylus must be moved into proper position. It is important that the lever be adjusted so that it moves in the plane of the tangent of that point at which the writing stylus touches the circumference of the drum (see Fig. 2). Both kymograph and the stand holding the lever must be absolutely vertical. Even so the tip of the stylus tends to leave the paper as it moves up or down from the original horizontal position in which it touches the paper. This is due to the fact that the curvature of the drum and that of the arc through which the recording stylus move are at right angles to each other. To avoid the con-

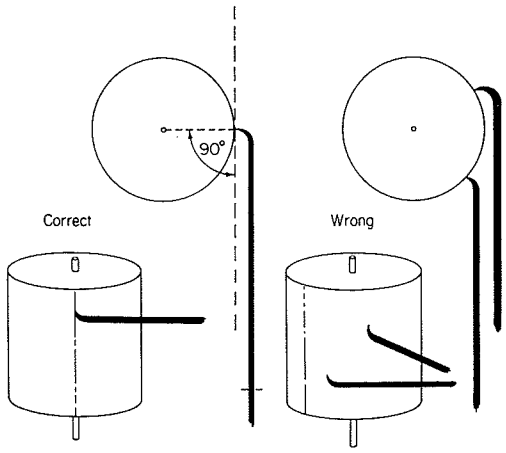
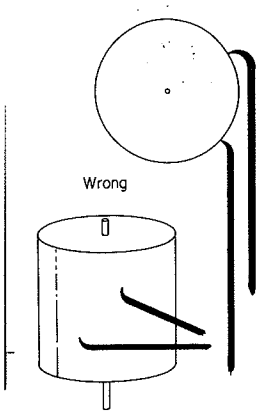


FIG. 2. Diagrams to illustrate the correct placement of the writing lever and some of the common misuses of the kymograph.

sequences of this geometry, levers and styli have been designed that compensate for this and keep the stylus tip on the kymograph drum. Two mechanisms are employed: (1) springs, and (2) double pivoting joints. The springs have the advantage of simplicity but the disadvantage that the pressure with which the tip is pressed against the drum varies: it is greatest with the lever in the horizontal position. The most common arrangement uses spring-like material for the writing stylus itself: paper, celluloid or plastic. Other constructions use a rigid stylus but incorporate a metal spring in the writing arm of the lever which keeps the stylus pressed against the drum.

Levers with a double pivoting arrangement have the great advantage that the stylus writes with constant pressure. Two types are in use. The first employs a Cardanic suspension of the lever. This is modified so that the writing arm of the lever is attached to a freely rotatable spindle that is not vertical but inclined towards the drum so that the writing arm of the lever "falls" against the drum (Fig. 3). The effective weight of the lever arm and the degree of inclination of the spindle determine the pressure of the stylus on the drum. The second type of "constant pressure" lever looks like a conventional type of lever except that the writing tip is mounted on a pivoting hook as shown in Fig. 4. The latter type of lever can easily be made in the laboratory from a bit of soft glass tubing and sealing wax. It is particularly suited for use with delicate heart preparations and organs that exert only small forces on a lever.

Here are instructions to make an isotonic, light lever with a pivoting "constant pressure" stylus. If you do not already have a proper heart lever



placement of the writing lever and some of the

nd styli have been designed that com-
on the kymograph drum. Two mecha-
(2) double pivoting joints. The springs
the disadvantage that the pressure with
rum varies: it is greatest with the lever
common arrangement uses spring-like
paper, celluloid or plastic. Other con-
porate a metal spring in the writing arm
essed against the drum.
ngement have the great advantage that
sure. Two types are in use. The first
he lever. This is modified so that the
to a freely rotatable spindle that is not
m so that the writing arm of the lever
e effective weight of the lever arm and
e determine the pressure of the stylus on
ant pressure" lever looks like a conven-
iting tip is mounted on a pivoting hook
ever can easily be made in the laboratory
sealing wax. It is particularly suited for
and organs that exert only small forces

n isotonic, light lever with a pivoting
o not already have a proper heart lever

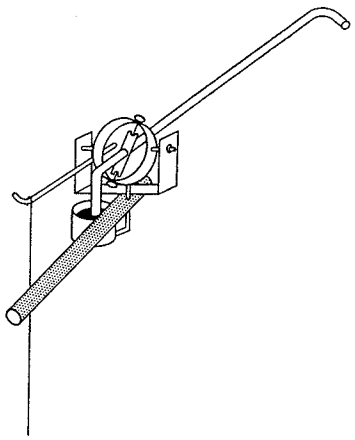


FIG. 3. Diagram of an ink writing gimbal lever; the lever is doubly suspended so that it falls against the drum. In this ink writing version the lever consists of steel capillary tubing; one end dips into an ink well. Note that the load arm is attached to the suspension ring and is not a continuation of the writing arm of the lever.

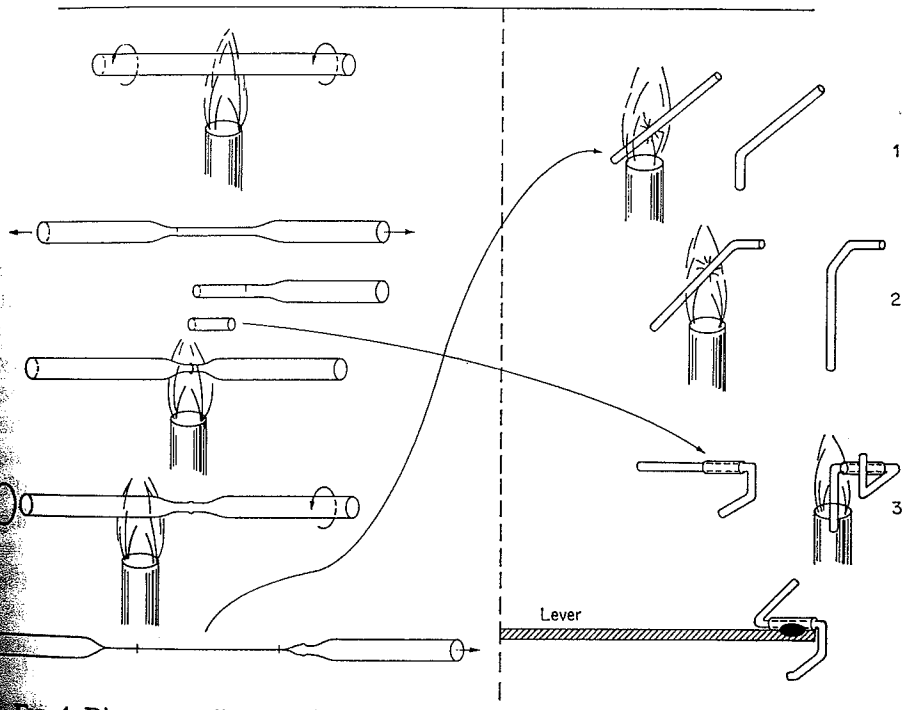


FIG. 4. Diagrams to illustrate the manufacture of a constant pressure stylus. The procedure is explained in detail in the text.

K

available, you can make one from a stick of wood, 0·5 cm in diameter, and a drinking straw (natural, paper or plastic): simply stick a pin through the straw about 5 cm from one end and insert it into the rounded end of the wooden stick. If you have proper kymograph accessories available, select a light heart lever.

Prepare a pivoting writing tip as follows. Heat the middle region of a piece of soft glass tubing (5–8 mm diameter) over the Bunsen burner while continually rotating it with your fingers. Wait until it is soft and pliable, then remove it from the flame and stretch until the thinnest region is no more than about 2 mm in diameter. Nick the center with a file and break the tubing at that point. Nick one of the capillary points again about 15 mm from the tip and break this off. Hold this short piece of capillary with forceps and touch it at both ends to the lower part of the flame. Withdraw it from the flame the instant the flame changes color at the point of contact. The edges of the capillary are now firepolished and smooth.

Next, pick up the two separated parts of the drawn out glass tubing and fuse them over the flame. Now apply the flame to a region to one side of the point of fusion and as soon as the flame turns yellow, pull and move the glass out of the flame all in one motion. This will produce a fine capillary. Break off an 8 cm portion of this.

Now reduce the flame. Hold the thin capillary with one hand at one end and let it touch the flame at a point about 15 mm from the other end. If the flame is touched properly, that is, at its periphery, the heating will be localized and the glass will soften at that point only. Be careful to hold the capillary horizontal. The tip will now drop downward at a right angle. As the tip begins to droop, remove the glass from the flame.

Now hold the capillary at the bent tip in such a way that all parts of the tubing are horizontal. Touch it to the flame as before, but at a point about 15 mm inward from the bend. This will accomplish a second bend of 90°.

Slip the long arm of the bent capillary through the piece of 2 mm capillary prepared before. Now hold, with two fingers, both pieces of tubing so that the bend rests against the shoulder of the short capillary. Turn them so that the terminal, bent portion of the inner, thin capillary points upward. Touch the thin capillary to the flame just where it emerges from the other end of the wider, short capillary. Remove from the flame immediately when the bending begins but allow this to complete a right angle.

All that is left to do now is to touch the very tip of the hook part of the bent inner capillary to the flame to melt the rough edges into a tiny bead.

The writing tip is now ready to be mounted onto the lever. Simply attach it with a drop of molten sealing wax.

When properly balanced (see below) levers of this type do not require more than a pull of 20 mg from the moving organ.

FIXING THE RECORDS

The soot-blackened kymograph paper with the inscribed traces of recorded lever-movements must be fixed in order to make the records permanent. For this purpose the paper must be carefully removed from the kymograph drum and be submerged in a fixative. The procedure is shown in Fig. 5.

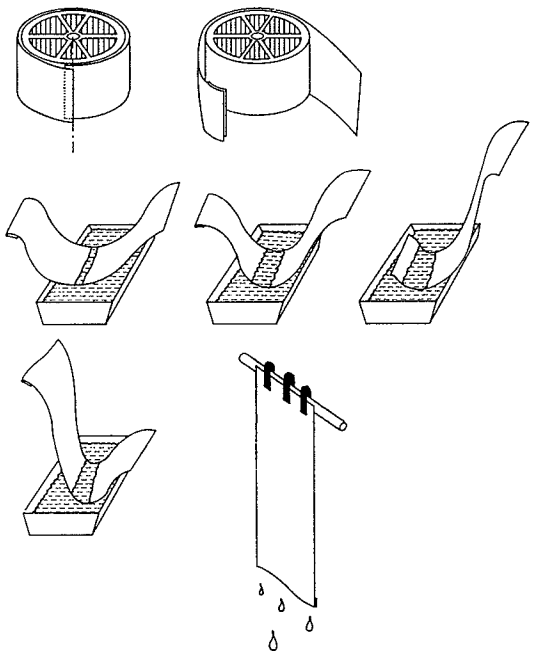


FIG. 5. Diagrams to illustrate the procedure involved in fixing a kymograph record. For details see text.

Do not remove the paper while the drum is still on the kymograph. Remove the drum first from its stand and spindle and set it on a paper-covered table top. Locate the seam where the paper strip had been glued together. With a razor blade make a cut just above the edge of the underlying end of the paper strip. Avoid cutting into the metal surface of the drum. Be sure to place a finger over the first part of the cut so that the paper strip does not suddenly snap off the drum and fall, sooty side down onto the table. Remove the paper strip from the drum, being careful to touch it only near the cut edges (the former seam).

Now, with the sooty side facing upwards, dip the strip into the fixing solution. This is done as follows. First lower the middle portion until it just

touches the surface. As the fixative penetrates through the soot from below, immerse further, allowing the liquid to pass over the surface of the middle portion. Now gradually pull the paper strip through the solution, keeping it submerged—first one way towards the left, then the other way towards the right. Pull it through the fixative once again and hang it up to dry on that end which had last been in the fixative.

PREPARATION OF THE FIXATIVE

The most widely used fixative consists of a solution of shellack in alcohol. Dissolve 1 unit weight (e.g. 500 g) of shellack in 2 vol (e.g. 1 liter) of alcohol (96% denatured) and add to each 100 ml of solution 1 ml of castor oil. The latter addition makes the dried film of lacquer more flexible and prevents cracking when the papers are rolled up for storage.

A convenient and clean method of transferring the fixing solution from the storage container to the fixing trough and back is shown in Fig. 6.

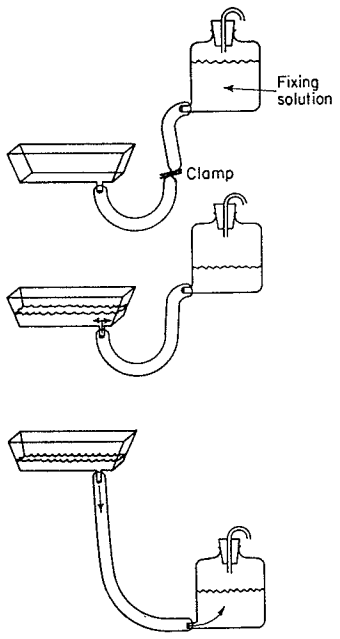
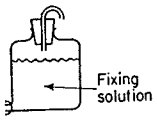


FIG. 6. Method of transfer of fixing solution. The fixing trough has at its bottom an inlet-outlet tube. This is connected by way of rubber tubing with the aspirator bottle that contains the fixative. By raising or lowering the bottle, the trough can be filled or drained. Note the air inlet through the stopper.

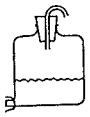
trates through the soot from below, pass over the surface of the middle trip through the solution, keeping it eft, then the other way towards the ain and hang it up to dry on that end

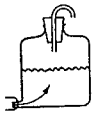
THE FIXATIVE

of a solution of shellack in alcohol. llack in 2 vol (e.g. 1 liter) of alcohol ml of solution 1 ml of castor oil. of lacquer more flexible and prevents or storage.
nsferring the fixing solution from the d back is shown in Fig. 6.



Clamp





on. The fixing trough has at its bottom an rubber tubing with the aspirator bottle that e bottle, the trough can be filled or drained

2. The Ink Writing Technique

There can be no question about the advantages of ink writing kymography. No smoking apparatus is required. The drum can be prepared quickly for recording and the records can be stored without requiring fixation.

The drawbacks of ink writing are that the friction of an ink writing stylus is greater than that of a soot writing one. When levers are used that feed the ink writing stylus by capillary action, clogging occurs not infrequently. When simple ink boats are used as attachment to the writing tip, the changing weight, as the ink is used up and evaporates, changes the load on the lever and alters the recording conditions.

The best kinds of levers are those which use as the writing arm a stainless steel capillary tube that is fed from a stationary ink reservoir, and which have the writing arm cardanically suspended (gimbal lever, as shown in Fig. 3). Although the manufacturers recommend a non-glossy writing surface, glossy paper must be used when minimal friction is required.

Ink writing levers must be thoroughly cleaned at the end of each experiment. After ink container and capillary have been cleaned with distilled water, they should be stored in alcohol. If there is any suspicion that the ink to be used contains dust, it must be filtered before use. Only slow drying, recording ink should be used. Ordinary ink, as used in fountain pens, is unsuitable.

General Hints on the Use of the Kymograph Apparatus

1. Prepare a hand rest to facilitate writing on the kymograph drum. A horizontally held short metal rod held by a clamp on a suitable, independent table stand is very convenient. The height of the rod should be set so that the hand resting on it can comfortably reach a place just to the left or below the recording writing tip of the lever.

2. Select a lever that is suitable for the particular preparation. The contractions of strong muscles should not be recorded with a light heart lever! Some preparations require a counterweight to keep the muscle extended and to facilitate relaxation after induced contractions. The light heart levers to be used for delicate hearts (lamellibranchs, crayfish) and other "weak" organs should be carefully balanced before the organs are connected. Balancing is best done with pieces of plasticene or suitably sticky wax.

3. Although the connecting thread must be securely attached to the organ from which one wishes to record, the other end of the thread should not be tied on to the lever unless one is dealing with a tough and mechanically very resistant organ. It is better to attach the thread by simply pressing it into a small lump of wax affixed to the load arm. Should the lever, in the course of an exciting experiment, be jolted or accidentally hit, this will not tear the organ but merely release the thread.

4. Set the drum at the lowest possible position on its spindle so that it can later on be raised when the first round of recording has been completed.

5. Before beginning the recording make sure that the drum is securely fastened and is not in danger of slipping.

6. Adjust the writing lever so that its tip touches the paper near the upper margin of the drum just to the right of the seam where the paper had been glued together. Make sure that the lever is in a horizontal position when the preparation is attached and in its normal, resting position. When recording from a beating heart, the lever should be horizontal when the heart is in diastole. Only if this precaution is rigidly and consistently observed, can the records later be interpreted properly. Remember that the recording is curvilinear, that is the y-axis is represented by a semicircle the radius of which is given by the length of the writing arm of the recording lever.

As has been stated before, it is essential that the plane of the up-and-down movements of the writing arm lies parallel to the tangent through the drum radius that goes through the writing tip (see Fig. 2).

When adjustments have to be made, be sure to leave the thread slack that connects the load arm of the lever to the preparation. Otherwise the inevitably jerky movements might tear and injure it.

7. On the upper left margin of the paper covered drum (just to the right of the seam) write the date, your name, the kind of animal and preparation used and other information concerning the nature of the forthcoming experiment. Make it a rule to follow this procedure whenever you start with a "new drum". Nothing is more embarassing later, than to be confronted with a record that has no identifying marks on it: the recorded heart beats may have been those of a crayfish or of a clam.

8. Equally important: calibrate the "speed" of the drum before you start recording. Although special timers are available that place time marks on the kymograph paper as this revolves, these are not really necessary if one takes the precaution of marking off suitable time intervals, such as 1 min, 10 sec, or even 2 sec in the case of fast turning drums.

B. The Clam-heart Technique of Bioassay for Acetylcholine

This is the most specific, convenient and rapid bioassay technique. The ventricle of a clam is isolated and suspended for mechanical recording in a suitable organ-bath of known volume. Known and unknown amounts of acetylcholine (ACh) are added to the bath and their effects on the heart are compared. Equal effects indicate identical amounts of ACh present in the bath. A simple calculation permits determination of the amount of ACh originally present in the extracted tissue.

PREPARATION OF THE HEART (FIG. 7)

Best results are usually obtained with species of the genus *Protothaca* or *Tapes*, but other genera, such as *Mya*, *Mercenaria* (=*Venus*), *Cyprina* and *Ostrea*, can be used also. The following genera of lamellibranchs are unsuitable: *Pecten*, *Mytilus*, *Modiolus*, *Anodonta*, *Crassostrea*, *Serripes*, *Tresus*, *Solen*, *Dinocardium*, *Clinocardium*, *Saxidomus* and *Chlamys*. These statements rely on observations by the author and on the reports by Welsh and Taub (1948), Welsh (1954), Hughes (1955), and Greenberg (1964).

Animals obtained live at fish markets are perfectly suitable. They should be kept in cool (10–15°C) sea water that is aerated. They will live for several weeks without food. In an aquarium with non-circulating sea water, not more than two animals should be kept per liter of sea water.

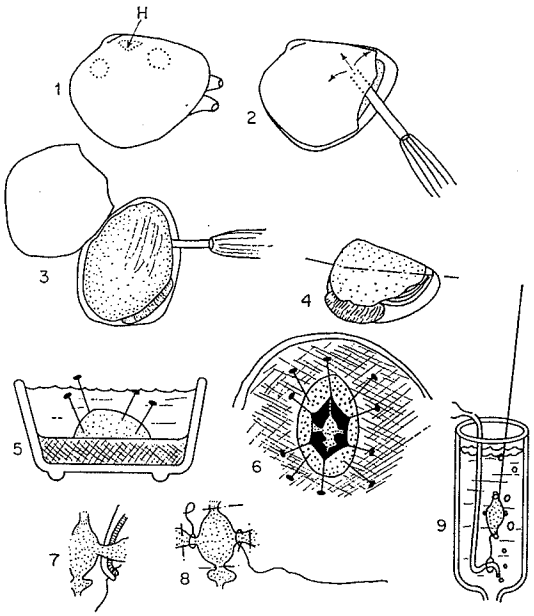


FIG. 7. Sketches illustrating the preparation of a clam ventricle for use in the bioassay of acetylcholine. (1) Outline of the animal with indication of the siphons, the attachments of the adductor muscles and of the heart (*H*). (2) and (3) A screwdriver is used to free the organism from its attachments to the shell. (4) The dashed line indicates where to cut off the dorsal portion of the animal which is to be mounted (5) in a dissecting dish. (6) A top view of the dissecting dish; a longitudinal incision has been made and the dorsal body wall and pericardium have been retracted laterally, exposing the ventricle and the lateral attachments of the auricles. (7) and (8) Details of the heart showing the tips of forceps used in tying the ligatures (7) and the position of the cuts that will free the ventricle; the bulbous structure below the ventricle is the accessory heart. The muscle chamber is shown in (9); the ventricle is anchored on the tip of a long hypodermic needle through which air is bubbled into the bathing saline solution.

For the dissection the following instruments are needed: one screwdriver; one pair of large, one pair of fine scissors; two pairs of small, curved forceps. A dissecting microscope of low power (5 or 10×) is helpful. One also needs fine thread and about ten pins.

The dissection should be carried out with the preparation under sea water. A glass bowl, about 5 cm deep and of about 15 cm diameter with a bottom of black "dissecting wax" (at least 1 cm thick), is most convenient for this.

1. Find a suitable animal and gently tap the edge of the shell with the handle of the screwdriver until a small fragment breaks loose. Now insert the blade of the screwdriver and scrape along the inner surface of one halfshell until the closer muscles are detached. Now remove the remaining portion of the broken halfshell taking care not to injure that part of the animal that lies close to the hinge. With the aid of the blade of the screwdriver free the animal from the other half shell.

2. With the larger pair of scissors cut through the animal in such a way that the entire ventral portion of the animal with its foot can be removed.

Now transfer the dorsal part of the animal to the dissecting dish and pin it down, dorsal side up.

3. With the fine scissors make a superficial sagittal cut along the dorsal body wall. This exposes the pericardial cavity. Retract the body wall laterally.

4. Slip the tips of a pair of curved forceps underneath the junction between one of the auricles and the ventricle. Open its jaws slightly and grasp the tip of a short length of thread. Pull the thread through and, using the other pair of curved forceps, tie a ligature around the proximal portion of the auricle, leaving the junction itself intact.

5. Prepare another piece of thread by tying a small loop at one end. Use it to tie a ligature around the other auricle thereby attaching the loop. Cut through the auricles distal to the ligatures and sever the arteries and the gut anterior and posterior to the ventricle. The ventricle is now free and can be removed (with the proper lengths of thread attached) to the muscle bath.

Mounting the Heart and Recording Apparatus (Fig. 8)

MOUNTING THE HEART

For the muscle chamber and heart holder the following are needed: the barrel of a 2 ml hypodermic syringe, the barrel of a 1 ml tuberculin-type syringe, a hypodermic needle (No. 17–No. 20) at least 6 cm long, a 10 cm length of thin rubber tubing to be slipped over the nozzle of the 2 ml syringe and a spring clip or pinch cock to be clamped onto the tubing. Plastic syringes are preferable because their flanged ends can be easily cut off. In the case of the tuberculin syringe this facilitates slipping over it the rubber tubing that

ıments are needed: one screwdriver;
ɜ; two pairs of small, curved forceps.
5 or 10×) is helpful. One also needs

vith the preparation under sea water.
out 15 cm diameter with a bottom of
ick), is most convenient for this.
p the edge of the shell with the handle
.t breaks loose. Now insert the blade
nner surface of one halfshell until the
e the remaining portion of the broken
art of the animal that lies close to the
screwdriver free the animal from the

hrough the animal in such a way that
with its foot can be removed.
ıimal to the dissecting dish and pin it

perficial sagittal cut along the dorsal
:avity. Retract the body wall laterally.
:eps underneath the junction between
pen its jaws slightly and grasp the tip
ead through and, using the other pair
l the proximal portion of the auricle,

tying a small loop at one end. Use it
icle thereby attaching the loop. Cut
res and sever the arteries and the gut
The ventricle is now free and can be
ead attached) to the muscle bath.

:CORDING APPARATUS (FIG. 8)

THE HEART

holder the following are needed: the
the barrel of a 1 ml tuberculin-type
-No. 20) at least 6 cm long, a 10 cm
ıed over the nozzle of the 2 ml syringe
ımped onto the tubing. Plastic syringes
ds can be easily cut off. In the case of
lipping over it the rubber tubing that

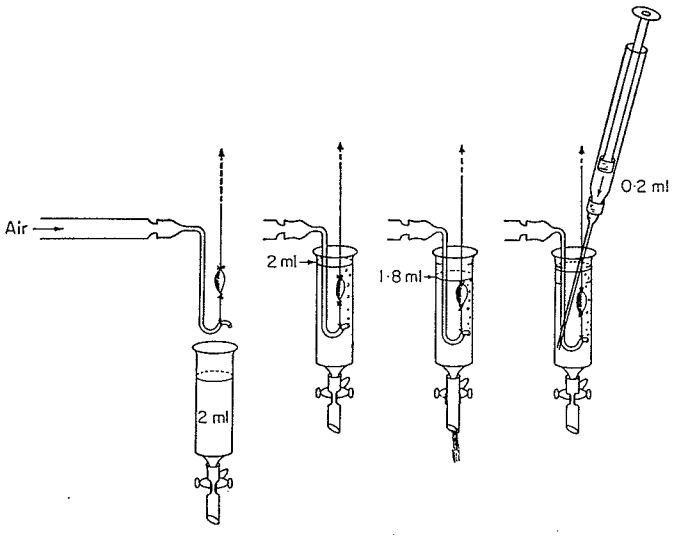


FIG. 8. Muscle chamber and heart holder, as explained in the text, are shown on the left. Also shown is the method of adjusting the level of fluid in the chamber preparatory to the application of ACh and of test samples. The diagram on the right shows how the test samples and ACh are applied.

supplies compressed air, in the case of the 2 ml syringe barrel this reduces the extra volume and length above the calibrated portion.

The 2 ml barrel is held upright by a suitable clamp mounted on a rack and pinion device. The 1 ml barrel with the hypodermic needle attached is held horizontal by another clamp. The hypodermic needle is bent downwards, its tip is bent S-shaped, so that it forms a hook.

The loop attached to the heart preparation is slipped over the hook while the muscle chamber (the 2 ml barrel) is kept lowered below the tip of the needle. The thread attached to the other auricle is attached to the writing lever (see below).

Now the muscle chamber must be filled with sea water and raised to totally submerge the preparation. The air flow through the tuberculin syringe and needle-hook should be adjusted to produce a stream of small air bubbles.

THE RECORDING APPARATUS

The heart preparations are rather delicate. Only if the recording device offers minimal frictional resistance can adequate records be obtained. Heart levers writing on smoked kymograph paper are to be preferred over ink writing ones. In any case, only glossy kymograph paper should be used. The levers must be of the "constant friction" type (see p. 224 and Figs. 3 and 4).

PROCEDURE

1. PREPARATION OF PRIMARY STANDARDS

Select twenty clean test tubes of at least 20 ml capacity, Set them into a rack. With the aid of a 10 ml pipette add to each of eight tubes 9 ml of buffered artificial sea water of pH 6·5 (see Appendix). Cover all test tubes. From a fresh stock solution of acetylcholine chloride (see Appendix) transfer 1 ml into the first test tube, using a 1 ml pipette (blow out type). Gently blow air through the pipette to mix the contents of the test tube. Draw the solution up into the pipette, and blow it out. Repeat five times. Now blow more air through the pipette, holding the tip of the pipette against the bottom of the test tube. The air should displace enough of the solution so that this rises to the top of the test tube and rinses off any drops of an incompletely mixed (thus more concentrated) ACh solution. Using a gummed label or a wax pencil, mark the test tube "ACh 10^{-4}".

Now draw exactly 1 ml of the freshly mixed solution of ACh 10^{-4} into the pipette, withdraw the pipette from the test tube and wipe it with a fresh piece of tissue paper. Immerse the tip of the pipette into the next tube and blow out and repeat the mixing procedure. Be sure to keep the covers over the other test tubes! Remember that 1 drop of an ACh solution of 10^{-4} that accidentally splashes over into another tube will establish there an ACh concentration of about 5×10^{-7}, a concentration that is more than a hundred times stronger than the minimum concentration required to completely stop the test heart!

Continue the stepwise dilution until you have the following standard concentrations: 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, 10^{-10}, 10^{-11}. Be sure to label all test tubes after the mixing is completed.

2. ESTABLISHING THE RANGE OF SENSITIVITY

It is now necessary to find the minimal concentration of ACh that will cause complete cessation of the heart beat. For this as well as the subsequent bioassay it is necessary to know the precise concentration of ACh in the bathing chamber of the test heart. The following procedure should be followed. Fill the bath with exactly 2 ml of sea water and then submerge the heart and aeration needle by raising the bath to the necessary level (see Fig. 8). Adjust the air to the desired flow rate (this should provide a continuous stream of fine bubbles but not so strong as to cause spraying). Now mark the level of the fluid. Remove 0·2 ml with a tuberculin syringe and mark this level also. For exceptionally large hearts you may have to use a larger bath chamber and use 5 ml of sea water from which you withdraw 0·5. In either case the upper mark indicates 10 vol, the lower mark 9 vol.

When the test heart has established a regular heart rate, add (see below)

1 vol of ACh 10^{-11} to 9 vol of bathing solution, using a tuberculin syringe and squirting the ACh solution into the bath with the needle submerged. Do not aim at the heart. The air bubbles help in the mixing process and within a few seconds the heart will be bathed by a solution of ACh 10^{-12}. It is likely that no effect is noticeable, but there are exceptionally sensitive clam ventricles which will show a diminution of amplitude even at this low a concentration of ACh.

After 1 min, drain the bath and from a wash bottle rapidly fill the bath. Drain again, fill, drain once more and then carefully fill to the 10 vol mark. Wait for at least 1 min. Now drop the level of bathing fluid to the 9 vol mark. Add 1 vol of ACh 10^{-10}. After mixing this will give a concentration of 10^{-11}. After 1 min drain the bath, fill with sea water, drain again, fill, drain once more and then fill to the 10 vol mark. Wait for at least 1 min before the next addition of ACh.

Proceed with the addition of higher and higher concentrations until you find the one that causes complete cardiac arrest.

3. PREPARATION OF THE TEST STANDARDS

The bioassay using the clam ventricle is very accurate and it is necessary to have standard solutions that differ in their ACh concentration by not more than 20%. Such standards can now be prepared economically since you know the sensitivity of the test ventricle. What is needed now is a series of dilutions covering the range between the minimal concentration that causes cardiac arrest (as determined in procedure 2) and the next lower standard concentration. For instance, if ACh 10^{-9} (final dilution) stops the heart, then the test standards need to cover the range between ACh 10^{-8} and 10^{-9} (the final concentrations reaching the heart will thus be 10^{-9}–10^{-10}).

The following test standards will be needed designating the maximum standard concentration as determined by procedure 2 as 10^{-n}:

$1{\cdot}0 \times 10^{-(n+1)}$	$1{\cdot}6 \times 10^{-(n+1)}$	$2{\cdot}9 \times 10^{-(n+1)}$	$5{\cdot}0 \times 10^{-(n+1)}$	$8{\cdot}5 \times 10^{-n(+1)}$
$1{\cdot}2 \times 10^{-(n+1)}$	$2{\cdot}0 \times 10^{-(n+1)}$	$3{\cdot}5 \times 10^{-(n+1)}$	$6{\cdot}0 \times 10^{-(n+1)}$	$1{\cdot}0 \times 10^{-(n)}$
$1{\cdot}4 \times 10^{-(n+1)}$	$2{\cdot}4 \times 10^{-(n+1)}$	$4{\cdot}2 \times 10^{-(n+1)}$	$7{\cdot}2 \times 10^{-(n+1)}$	

These test standards are easily prepared as follows. First take a clean 100 ml or 125 ml Earlenmeyer flask, add 99 ml of buffered (pH 6·5) artificial sea water (see Appendix) and 1 ml of the ACh standard $10^{-(n-2)}$. For example, if the minimum concentration causing stopping of the heart beat was ACh 10^{-9}, add 1 ml of ACh 10^{-7} to the 99 ml of artificial sea water. This gives a concentration of 10^{-n} (or 10^{-9} in the example quoted).

Now transfer with suitably accurate pipettes the following quantities into the remaining twenty test tubes: 1·2, 1·4, 1·6, 2·0, 2·4, 2·9, 3·5, 4·2, 5·0, 6·0 7·2

and 8·5 ml. To each of these tubes add enough buffered (pH 6·5) artificial sea water, to give a total volume of 10 ml. Thus you add 8·8, 8·6, 8·4, 8·0, 7·6, 7·1, 6·5, 5·8, 4·0, 2·8 and 1·5 ml. Carefully label all tubes.

4. ESTABLISHING THE RANGE OF EFFECTS

Before the beginning of the bioassay proper, it is advisable to establish the effects of the different standard concentrations of ACh. If afterwards extracts of unknown ACh content are applied, it is very easy to tell, at least approximately, what the range of the contained ACh concentration is, as long as the effect is not supramaximal. This saves much time in matching the effect with that of known ACh standards.

Apply, in subsequent trials, the entire series of test standards, from $1{\cdot}0 \times 10^{-(n+1)}$ through $1{\cdot}0 \times 10^{-n}$. Follow the following schedule, which, in fact, is the same that will be employed in the bioassay proper. Allow the heart to beat undisturbed for 1 min. If the beat is steady and regular, stop the kymograph (turning off the motor or disengaging the clutch, whichever is more convenient). Lower the bath level to the 9 vol mark. Add 1 vol of test standard concentration $1{\cdot}0 \times 10^{-(n+1)}$. Wait for exactly 30 sec. Turn on the kymograph and record for exactly 1 min. Turn off the kymograph, drain the bath, fill with sea water, drain, fill with sea water, drain once more and fill with sea water to the 10 vol mark. Wait another 30 sec, or until 30 sec after the drum was stopped, then turn on the kymograph and record the heart beat for 1 min. During this minute flush the syringe you used to apply the ACh standard and then fill it with 1 vol of the next higher test standard concentration ($=1{\cdot}2 \times 10^{-(n+1)}$). Proceed as described before until all test standards have been applied.

Note. It is imperative that no air bubbles are trapped in the syringe when the standard (and later on when the unknown extract dilutions) are applied. As this air is expelled into the bath it pushes out the fluid contained in the needle and introduces a sizeable error: the needle volume must not be added to the bath. Make sure that you expel all air from the syringe when you adjust the contained solution to the 1 vol mark (in most cases this will be 0·2 ml).

5. THE BIOASSAY PROPER (FIG. 9)

If the extracts to be assayed have been made up to have a concentration of 1 g tissue (wet weight) in 10 ml of extract ($=1 : 10$) or (10^{-1}), prepare dilutions of 10^{-2}, 10^{-3} and 10^{-5}. If the extract concentration is not 1 : 10 but another ratio, make it first up to 10^{-1} or 10^{-2} before diluting further by factors of 10, 100, etc. Apply extracts in the same manner as you applied the

REY

enough buffered (pH 6·5) artificial
ml. Thus you add 8·8, 8·6, 8·4, 8·0,
fully label all tubes.

ANGE OF EFFECTS

roper, it is advisable to establish the
itions of ACh. If afterwards extracts
is very easy to tell, at least approxi-
ACh concentration is, as long as the
uch time in matching the effect with

ire series of test standards, from
ow the following schedule, which, in
the bioassay proper. Allow the heart
eat is steady and regular, stop the
lisengaging the clutch, whichever is
to the 9 vol mark. Add 1 vol of test
Wait for exactly 30 sec. Turn on the
.. Turn off the kymograph, drain the
sea water, drain once more and fill
another 30 sec, or until 30 sec after
ie kymograph and record the heart
h the syringe you used to apply the
ol of the next higher test standard
ed as described before until all test

bles are trapped in the syringe when
known extract dilutions) are applied.
ushes out the fluid contained in the
he needle volume must not be added
air from the syringe when you adjust
(in most cases this will be 0·2 ml).

ROPER (FIG. 9)

a made up to have a concentration of
xtract (=1 : 10) or (10^{-1}), prepare
xtract concentration is not 1 : 10 but
or 10^{-2} before diluting further by
the same manner as you applied the

standard ACh concentrations. Always add 1 vol to 9 vol of bathing solution. Keep the same time schedule as described in section 4 of this outline.

It is very likely that one of the concentrations of a given extract will give an intermediate depression of the heart rate. Looking over the established range of effects of various ACh concentrations (test standards) you can readily select an ACh test standard that will give a slightly smaller effect. If you find this to be the case, apply the extract again (same concentration as before), and next apply a concentration of ACh that gives a stronger effect.

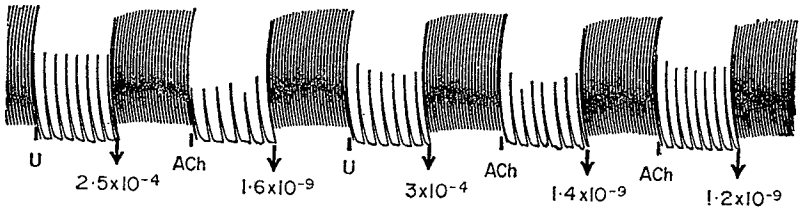


FIG. 9. Example of an actual bioassay record obtained with a ventricle of *Protothaca staminea*. U, Extract of unknown ACh content; ACh, known acetylcholine. The figures refer to the final concentrations (g/ml) in the bath. ACh $1{\cdot}6 \times 10^{-9}$ has a stronger action than U 3×10^{-4}. ACh $1{\cdot}4 \times 10^{-9}$ is only slightly more active than U 3×10^{-9} but ACh $1{\cdot}2 \times 10^{-9}$ is definitely less active. Two interpretations are permissible: (1) U 3×10^{-4} = ACh $1{\cdot}4 \times 10^{-9}$; (2) U 3×10^{-4} = ACh $1{\cdot}3 \times 10^{-9}$ (the average of $1{\cdot}2 \times 10^{-9}$ and $1{\cdot}4 \times 10^{-9}$). The results can thus be stated as: $4{\cdot}6 \times 10^{-6}$ g or 4·6 (±12·5%) μg/g, and as $4{\cdot}33 \times 10^{-6}$ g or 4·33 μg (±7·15%)/g respectively. By comparison, U $2{\cdot}5 \times 10^{-4}$ is slightly less active than ACh $1{\cdot}2 \times 10^{-9}$ and more active than ACh $1{\cdot}0 \times 10^{-9}$ (not shown); thus its activity is intermediate between the two ACh concentrations and the result of $4{\cdot}4 \times 10^{-6}$ g or 4·4 μg (±10%)/g. (From Florey, 1967).

The aim in the bioassay is to "bracket in" the extract, so that the record of its effect is preceded by that of a lower, and followed by that of a higher ACh concentration. This procedure defines the lower and upper limit of the possible ACh concentration present in the extract. The more closely matched, the better will be the agreement in the actual ACh concentrations between unknown and test standards.

It is important to know that the differences in the effects of various ACh concentrations become larger the closer the concentrations come to that which causes complete cessation of the heart beat. Where great accuracy is required it is advisable, therefore, to adjust the extract concentrations by suitable dilution (4×10^{-3}, or $6{\cdot}5 \times 10^{-2}$ etc.) so that their effects nearly match those of near maximal concentrations of the ACh test standards.

The sensitivity of a test heart may vary with time. For this reason it is not possible to rely on the range of effects established under procedure 4. It is necessary to alternate between application of test standards and of extract throughout the entire bioassay procedure.

6. CALCULATIONS

From the results of the bioassay it is easy to calculate the ACh content of the original tissue. This is done in the following manner.

Decide which test standard gives the same effect as the optimal extract concentration. If you find that a given extract concentration has effects intermediate between two test standards that differ by 20%, you can assign to it a standard concentration 10% lower than the higher of the two test standard concentrations. For instance, if the effect of an extract $2{\cdot}5 \times 10^{-4}$ is smaller than that of ACh 6×10^{-9} but larger than that of ACh 5×10^{-9}, you can equate the extract concentration of $2{\cdot}5 \times 10^{-4}$ with an ACh concentration of $5{\cdot}5 \times 10^{-9}$ (accuracy $\pm 10\%$). *Note.* The accuracy of the assay should always be given. It is roughly equal to the percent difference between the effectiveness of the extract and the nearest higher and the nearest lower ACh standard, or $\pm 0{\cdot}5$ times the percent difference between the nearest higher and lower ACh standards tested.

When you have established the ACh concentration that corresponds in effectiveness to that of the particular extract concentration, multiply the ACh concentration with the reciprocal of the extract concentration. For example, in the case given above the extract concentration of $2{\cdot}5 \times 10^{-4}$ has the same effect as ACh $5{\cdot}5 \times 10^{-9}$ ($\pm 10\%$). The ACh concentration of the extracted tissue therefore is

$$5{\cdot}5 \times 10^{-9} \times \frac{1}{2{\cdot}5 \times 10^{-4}} \quad \text{or} \quad \frac{5{\cdot}5 \times 10^{-9}}{2{\cdot}5 \times 10^{-4}} \quad \text{or} \quad \left(\frac{5{\cdot}5}{2{\cdot}5}\right) \times 10^{-9(-4)}$$

$$= 2{\cdot}2 \times 10^{-5} (\pm 10\%) \text{ g ACh per g of tissue.}$$

It is customary to express the ACh content in terms of μg of acetylcholine chloride per g wet (or dry) weight of extracted tissue. In the example given the ACh content of the extracted tissue would thus be $22{\cdot}0$ ($\pm 10\%$) μg/g, since 1 μg is equal to $1{\cdot}0 \times 10^{-6}$ g.

7. DETERMINATION OF SPECIFICITY OF THE ASSAY

Several compounds can accomplish inhibition of the clam ventricle and it is possible that the effect of a tissue extract is due to a component other than ACh. This is particularly true if the extract had to be applied in relatively high concentration or if the assay indicated only a low ACh content of the tissue. So far, no compound has been found that is more effective than ACh in inhibiting the clam ventricle. Of the ones that have been tested, carbamyl choline is the most active. On the heart of *Mercenaria* (=*Venus*) *mercenaria* it has 1/80 of the activity of ACh (Welsh and Taub, 1948). Table I lists the

TABLE I

Relative molar quantities of various cholinesters that cause inhibition equivalent to that produced by a molar quantity of acetylcholine equal to 1

	Mercenaria (Welsh and Taub, 1948)	*Protothaca*
Acetylcholine	1	1
Carbamylcholine	80	18
Propionylcholine	105	37
Benzoylcholine	15 000	292
Butyrylcholine	625	4 800
Acetyl-β-methylcholine	1 100	541
Choline	14 000	58 000

relative effectiveness of various compounds as tested on the hearts of *Mercenaria* and of *Protothaca*. From these data it is quite evident that if any of the compounds shown were present in an extract, and if the inhibitory effect were entirely due to this compound, its amount in the extracted tissue would have to be many times larger than that of the amount of ACh indicated by the assay. Thus, if the assay indicated 1 μg of ACh per g and the activity were due to propionylcholine, about 100 μg of this compound must have been present in 1 g of extracted tissue, and if the compound were acetyl-β-methylcholine the assay would indicate 1 000 μg/g.

From such considerations one may conclude that the assay becomes the more specific for ACh the higher the amount of (apparent) ACh found in the assay. It is very unlikely that an effect corresponding to 100 μg of ACh per g of extracted tissue is actually due to propionylcholine, much less to acetyl-β-methylcholine or choline; if this were so the tissue would have contained no less than 10 mg of propionylcholine, 100 mg of acetyl-β-methylcholine or 1·5 g of choline!

To test for specificity in the case of lower yields of ACh, the following procedures are recommended.

(a) *Biological procedures*

To test whether the active agent is a cholinester, add a small, measured amount of lyzed erythrocytes of human or horse blood to a quantity of extract. The erythrocytes of both species contain acetycholinesterase of high specificity. Blood should be drawn into a heparinized syringe; oxalate or citrate should be avoided because they may affect the calcium activity of the extract. One ml of blood is sufficient for 44 ml of extract. Centrifuge the blood at 1 000 *g* for 10 min, siphon off the plasma and save it. Add 9 vol of distilled water to the 1 vol of red blood cells. Stir until a homogeneous

mixture is obtained. Add 1 part of this to 9 parts of an aliquot of extract. Shake and allow to stand at room temperature for 30 min, then place the mixture into a boiling water bath for 2 min in order to inactivate the enzyme. The subsequent bioassay should indicate that all ACh has been destroyed by the procedure. Remaining activity is due to compounds other than ACh and is most likely not due to a cholinester. The erythrocyte cholinesterase will not appreciably interfere with butyrylcholine and benzoylcholine, however. These compounds can be destroyed by serum-cholinesterase. To the extract, previously treated with erythrocyte-cholinesterase, add 1 part of serum and incubate at room temperature for 30 min, then place the tube into boiling water for 2 min. If the subsequent bioassay indicates that the inhibitory activity has disappeared it is likely that the agent responsible for the previously obtained inhibition was either buytryl or benzoyl choline. Further tests would have to be performed to identify the esters.

(b) *Pharmacological procedures*

Clam hearts have weak cholinesterase activity. They can be sensitized to ACh and closely related cholinesters by the application of anticholinesterase compounds. The sensitivity towards ACh usually increases two- to fivefold. At the end of the bioassay, add eserine (=physostigmine salicylate) to the washing and bathing solution to give a final concentration of 10^{-5} g/ml. No eserine needs to be added to the test standards or the extract samples. Allow the eserine to take effect by bathing the heart for 30 min in eserine-sea water (10^{-5}), then repeat the bioassay. If the inhibitory effects of the extract(s) is due to ACh, the results of the assay should be the same as those obtained before the application of eserine. If a significantly different value is obtained, it is to be assumed that part or all of the activity are due to a cholinester other than ACh.

Several drugs are known to block the action of ACh on the clam ventricle. Of these mytolon chloride (=2 : 5 bis (3'-diethylaminopropylamino)-benzoquinon bisbenzyl chloride; in some published papers referred to as benzoquinonium chloride) is the most potent and requires a final concentration of not more than 10^{-6} g/ml to block the action of ACh in concentrations up to 10^{-8} g/ml. The other effective agents are methantheline or banthin (=β-diethylaminoethyl-9-xanthene carboxylate methobromide) phenyltriethylammonium iodide and 3-hydroxyphenyltriethylammonium iodide. These should be applied in a concentration about 500 times stronger (=5×10^{-3} g/ml) for a blocking action similar to that of mytolon 10^{-6} g/ml. (Quantitative data have been reported in papers by Luduena and Brown, 1952; Welsh and Taub, 1953.)

It is always advisable to make use of one or the other of these blocking agents at the end of the bioassay period to make certain that the activity

to 9 parts of an aliquot of extract.
perature for 30 min, then place the
in in order to inactivate the enzyme.
that all ACh has been destroyed by
to compounds other than ACh and
ıe erythrocyte cholinesterase will not
ine and benzoylcholine, however.
ɜerum-cholinesterase. To the extract,
inesterase, add 1 part of serum and
in, then place the tube into boiling
ɔassay indicates that the inhibitory
t the agent responsible for the pre-
buytryl or benzoyl choline. Further
ntify the esters.

e activity. They can be sensitized to
the application of anticholinesterase
Ch usually increases two- to fivefold.
e (=physostigmine salicylate) to the
final concentration of 10^{-5} g/ml. No
ndards or the extract samples. Allow
heart for 30 min in eserine-sea water
e inhibitory effects of the extract(s)
ɜhould be the same as those obtained
gnificantly different value is obtained,
the activity are due to a cholinester

action of ACh on the clam ventricle.
bis (3′-diethylaminopropylamino)-
ɔme published papers referred to as
potent and requires a final concentra-
k the action of ACh in concentrations
agents are methantheline or banthin
boxylate methobromide) phenyltrie-
henyltriethylammonium iodide. These
ıbout 500 times stronger (=5 × 10
at of mytolon 10^{-6} g/ml. (Quantitative
ɹuduena and Brown, 1952; Welsh and

of one or the other of these blocking
ɨiod to make certain that the activity

seen in the assays is indeed due to ACh or at least due to a related cholinester. If the minimal extract concentration that caused complete inhibition of the heart beat before the application of the blocking agent still causes inhibition, this must be due to agents other than ACh.

(c) *Combined chromatography–bioassay*

The easiest method of identification of ACh in the tissue extracts is by means of paper chromatography. This can be carried out on Whatman No. 1 paper or filter paper of equivalent quality. The solvent should be neutral in order to minimize hydrolysis of the cholinesters. Because of the great sensitivity of the clam ventricle to ACh it is possible to carry out the chromatography with very small amounts of tissue extract. Even if the tissue contains not more than 1 μg of ACh per g, no more extract than what corresponds to 10 mg of tissue needs to be applied to the paper. If the ventricle used for the bioassay responds well to ACh 10^{-9}, it is sufficient to extract the cut out strips of the developed chromatogram with 5 ml of buffered sea water. Provided all the ACh is found within one such strip, the final concentration in the eluate would be no less than 2×10^{-9}, and even if the ACh should be distributed over three consecutive strips, enough would be present in the eluates to be detectable in the bioassay.

The technique of paper chromatography is explained on p. 347; therefore no details are given here. The most suitable solvents are water saturated butanol, and a mixture of 5 vol of propanol, 2 vol of benzyl alcohol and 2 vol of water. With these neutral solvents no acid hydrolysis of ACh takes place and the formation of secondary esters is avoided.

If an extract of 100 mg tissue per ml contains acetylcholine-like activity equivalent to that of 0·1 μg of ACh per ml (the original tissue thus contained 1 μg/g), not more than 0·01 ml of the extract need be spotted onto the paper. A spot of authentic acetylcholine chloride, at least 0·01 ml of a solution of 10^{-6}, should be placed near the extract spot. Obviously, the experiment is easier if larger amounts of ACh can be applied.

After development, the papers are air dried (this should be done under the fume hood). The paper strip is then divided into ten equal sections (covering the region from the point of application of the spots to the solvent front) and the strips are shredded into 10 ml beakers. Five milliliters of buffered artificial sea water are then added to each beaker and 2 h allowed for extraction. The eluates thus obtained can then be tested on a clam ventricle. With saturated butanol, ACh migrates to an R_F of 0·1, with propanol-benzyl alcohol-water (5 : 2 : 2) it can be found at R_F 0·45–0·5.

The procedure is sketched in Fig. 10. For a more conclusive proof of the identity of the eluted compound with ACh it is advisable to apply two extract spots and to elute only the paper sections above one of them for bioassay.

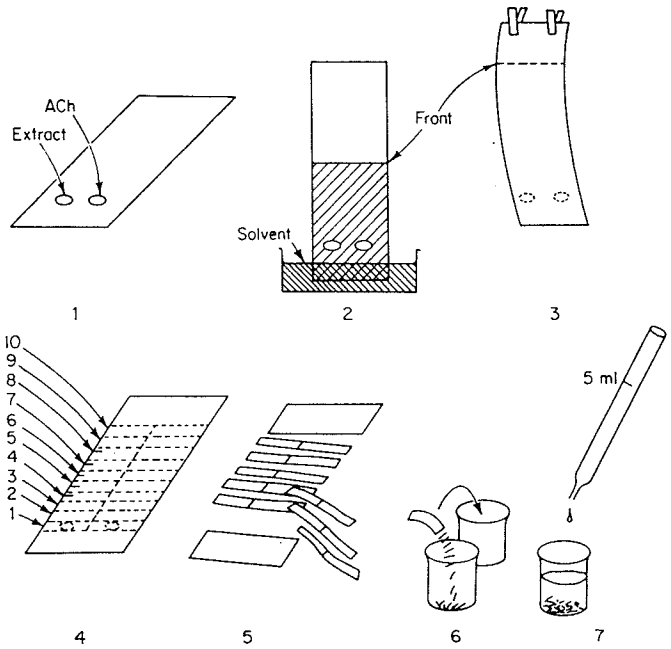


FIG. 10. Sketches of the procedure involved in the identification of tissue acetylcholine by combined paper chromatography and bioassay. (1) The extract and ACh are spotted onto the paper. (2) The chromatogram is developed (the air tight enclosure is not shown). (3) It is removed from the chromatography chamber and dried. (4) The region between origin and "front" is then divided into ten sections, and (5) these are cut out. (6) The left and right portions of each section are then shredded and placed into 10 ml beakers. (7) To each beaker 5 ml of buffered sea-water are added for the elution of ACh. The eluates are tested on the clam ventricle.

When the area to which the activity has migrated has been ascertained in the assay, elute the corresponding area above the other spot with distilled water, allow most of it to evaporate and apply the concentrated eluate to another solvent for development with another solvent. The method has been successfully used to identify ACh in extracts of *Octopus* nerve tissue (Loe and Florey, 1966).

APPENDIX

1. COMPOSITION OF ARTIFICIAL SEA WATER, BUFFERED TO pH 6·5

NaCl453, $MgCl_2$ 52, KCl9 $CaCl_2$ 11 mM, make up to 1 liter. To 1 liter of this add 56 ml of 0·2 N Tris-acid maleate and 44 ml of 0·2 N NaOH.

It is best to prepare this from the following stock solutions: 5 M NaCl (292·2 g of NaCl in 1 liter); 1 M $MgCl_2$ (203·3 g of $MgCl_2.6H_2O$ in 1 liter); 1 M KCl (74·5 g of KCl in 1 liter); 1 M $CaCl_2$ (111 g of $CaCl_2$, or 147 g of

$CaCl_2.2\,H_2O$ in 1 liter); 0·2 M Tris-acid maleate (19·6 g of maleic anhydride and 24·2 g of Tris in 1 liter); 0·2 N NaOH (8·0 g of NaOH in 1 liter).

Proceed as follows. Into a 1 000 ml volumetric flask pipette 90·6 ml of 5 M NaCl, 52 ml of 1 M $MgCl_2$, and 9 ml of 1 M KCl. Add distilled water to a level of about 100 ml below the 1 000 ml mark. Close the flask and shake. Now add 11 ml of 1 M $CaCl_2$, close the flask and shake. When the solution has come to rest, add distilled water up to the 1 000 ml mark. Pour the solution into a polyethylene (or Pyrex) bottle of 1 250–2 000 ml capacity.

Into a 100 ml measuring cylinder pour 56 ml of Tris-acid maleate and fill to the 100 ml level with 0·2 N NaOH. Pour the contents of the cylinder into the bottle that contains the salt solution, close the bottle and shake. Label the bottle indicating the molar composition of the artificial medium, the date and the name of the person who prepared the solution.

2. ACETYLCHOLINE STOCK SOLUTION

It is best to obtain preweighed ACh. Ampules containing 100 mg of acetylcholine chloride can be obtained from Merck Inc. With a glass file, break the ampule and drop it (including the top) into a polyethylene bottle of 100–125 ml capacity. Add 100 ml distilled water (should be slightly acid, pH 5), close the bottle and shake. Carefully label the bottle stating the name of the compound (acetylcholine chloride), its concentration (10^{-3} g/ml of distilled water), the date and the name of the person who prepared the solution. This stock solution should be kept in the refrigerator; it will be stable for several weeks.

BIBLIOGRAPHY

Florey, E. (1967). The clam-heat bioassay for acetylcholine. *Comp. Biochem. Physiol.* **20**, 365–377.

Greenberg, M. J. (1964). A compendium of responses of bivalve hearts to acetylcholine. *Comp. Biochem. Physiol.* **14**, 513–539.

Hughes, B. (1955). The isolated heart of *Mya arenaria* as a sensitive preparation for the assay of acetylcholine. *Br. J. Pharmac.* **10**, 36–38.

Loe, P. R. and Florey, E. (1966). The distribution of acetylcholine and cholinesterase in the nervous system and in innervated organs of *Octopus doflein. Comp. Biochem. Physiol.*, **17**, 509–522.

Luduena, F. P. and Brown, T. G. (1952). Mytolon and related compounds as antagonists of acetylcholine on the heart of *Venus mercenaria. J. Pharmac. exp. Ther.* **105**, 232–239.

Welsh, J. H. (1954). Marine invertebrate preparations useful in the bioassay of acetylcholine and 5-hydroxytryptamine. *Nature, Lond.* **173**, 955–956.

Welsh, J. H. and Taub, R. (1948). The action of choline and related compounds on the heart of *Venus mercenaria. Biol. Bull. mar. biol. Lab., Woods Hole* **95**, 346–353.

Welsh, J. H., and Taub, R. (1953). The action of acetylcholine antagonists on the heart of *Venus mercenaria. Br. J. Pharmac. Chemother.* **8**, 327–333.

C. Preparation of Nerve Extracts for Bioassay of Bound and Free Acetylcholine

One frog; dissecting pan; strong pair of scissors; pair of small forceps; balance, accurate to 1 mg; weighing paper; 10 ml frog-Ringer solution; 5 ml pipette, graduated to 0·1 ml; three test tubes; 500 ml beaker; test tube stand; glass homogenizer, 10 ml capacity; Bunsen burner; wire mesh screen.

Introduction

The acetylcholine (ACh) of nerve tissue occurs in two major fractions: (a) free acetylcholine and (b) bound acetylcholine. The free ACh cannot readily be determined; it is destroyed by the choline esterase as soon as the nerve cells are broken down during homogenizing, i.e. during the procedure that permits separation of free and bound ACh. Bound ACh, on the other hand, can be readily determined and so can the total amount (= free + bound) of ACh.

When nerve tissue is homogenized in an isotonic saline, its free ACh is free to diffuse out of the broken cells, but as it does so it comes in contact with membrane-bound cholinesterase and is destroyed. What remains is the ACh that is bound to certain cell structures, presumably enclosed by membranes (= vesicles). This bound ACh can be released by agents that break down cell structures or that denature proteins. Thus, bound ACh can be released by boiling, treatment with trichloroacetic acid or by "hypo-osmotic shock", i.e. by exposure of the homogenate to a hypo-osmotic medium.

If nerve tissue is dropped directly into boiling distilled water, the cholinesterase is inactivated almost instantly. At the same time the membrane structures of the nerve cells are sufficiently altered to permit outward diffusion of all ACh, that which was formerly bound, and that which was free already. Thus, boiling releases the *total ACh* into the extracting medium.

If isotonic homogenates are boiled, their bound ACh is released and remains in the extract because cholinesterase is destroyed by the boiling.

Method

Decapitate and pith a frog, expose the two sciatic nerves, free them, and remove them from the animal. Weigh each nerve and note the weights. Add about 200 ml of water to the beaker and bring the water to a boil. Multiply the weight of the nerve (expressed in grams) by 100 and add this amount of water to a test tube. For example, if the nerve weighed 33 mg (=0·033 g) add 3·3 ml (=100 × 0·033) of water to the test tube. Place the tube into the boiling water and drop in the nerve, using a glass needle. Remove the test

for Bioassay of Bound and Free

ir of scissors; pair of small forceps;
paper; 10 ml frog-Ringer solution;
e test tubes; 500 ml beaker; test tube
ty; Bunsen burner; wire mesh screen.

JCTION

issue occurs in two major fractions:
acetylcholine. The free ACh cannot
by the choline esterase as soon as the
mogenizing, i.e. during the procedure
und ACh. Bound ACh, on the other
so can the total amount (= free +

in an isotonic saline, its free ACh is
but as it does so it comes in contact
ınd is destroyed. What remains is the
ctures, presumably enclosed by mem-
can be released by agents that break
proteins. Thus, bound ACh can be
hloroacetic acid or by "hypo-osmotic
enate to a hypo-osmotic medium.
ıto boiling distilled water, the cholin-
ly. At the same time the membrane
ıtly altered to permit outward diffusion
ound, and that which was free already,
to the extracting medium.
ıeir bound ACh is released and remains
destroyed by the boiling.

'HOD

the two sciatic nerves, free them, and
each nerve and note the weights. Add
nd bring the water to a boil. Multiply
grams) by 100 and add this amount of
the nerve weighed 33 mg (=0·033 g)
o the test tube. Place the tube into the
using a glass needle. Remove the test

tube 1 min later and allow it to cool. Label the test tube immediately ("SN b ad 1 : 100"—meaning sciatic nerve, boiled, aqua distillata, 1 : 100).

Fill a beaker with crushed ice, pre-cool the glass homogenizer (pestle inserted), add Ringer solution in an amount equal to 100 × the weight of the other sciatic nerve. (Example: if this nerve weighs 28 mg or 0·028 g, add 2·8 ml.) With a suitable glass needle, insert the nerve and then homogenize it, keeping the homogenizer ice cold.

Allow the homogenate to stand for 1 h, then transfer an aliquot of perhaps 1·5 ml to a test tube and label this "SN h ub R 1 : 100" (meaning sciatic nerve, homogenized, unboiled, Ringer solution 1 : 100). Place the homogenizer with the remaining homogenate into the boiling water and heat for 2 min to liberate the bound ACh. Allow the tube to cool and label it "SN h b R 1 : 100" (meaning sciatic nerve, homogenized, boiled, Ringer solution 1 : 100).

Assay

Determine the ACh content of each extract on the clam ventricle, as outlined in Exercise 14B. Expect the total ACh in the original nerve tissue to amount to about 5–10 μg of ACh per g wet weight (= 5–10 × 10^{-6} g), thus a final dilution in the heart muscle chamber of 10^{-3} should have an effect equal to that of a final ACh concentration of 5×10^{-9}–1×10^{-8}.

Remember *SN b ad* gives you the *total ACh*, *SN h b* the *bound ACh*. By subtracting the value obtained for bound ACh from that of the total ACh you obtain the free ACh. Test SN h ub to convince yourself that the bound ACh is physiologically inactive.

Bibliography

Birks, R. I. and MacIntosh, C. F. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Can. J. Biochem.* **39**, 787–827.

Hebb, C. O. and Krnjevic, K. (1962). The physiological significance of acetylcholine. *In* "Neurochemistry", 2nd ed. (K. A. C. Elliott, I. H. Page and J. H. Quastel, eds.), pp. 452–521. Thomas, Springfield.

MacIntosh, F. C. (1963). Synthesis and storage of acetylcholine in nervous tissue. *Can. J. Biochem.* **41**, 2552–2571.

Mann, P. J. G., Tennenbaum, M. and Quastel, J. H. (1939). Acetylcholine metabolism in the central nervous system. *Biochem. J.*, **33**, 822–835.

Quastel, J. H.(1962). Acetylcholine distribution and synthesis in the central nervous system. *In* "Neurochemistry", 2nd ed. (K. A. C. Elliott, I. H. Page and J. H. Quastel, eds.), pp. 431–451. Thomas, Springfield.

Schallek, W. (1945). Action of potassium on bound acetylcholine in lobster nerve cord. *J. cell. comp. Physiol.* **26**, 15–24.

D. The Function of the Cardioregulator Nerves in the Crayfish

As in all decapod crustaceans, the heart muscle of the crayfish contracts in response to nerve impulses sent out by nerve cells located in the *cardiac ganglion*; the heart beat is *neurogenic*. By complex interactions, involving synaptic transmission and electrotonus, the ganglion cells of the heart ganglion coordinate their activity in such a way that they produce at regular intervals bursts of nerve impulses. The cardiac ganglion is situated within the heart muscle. In the European crayfish, *Potamobius astacus* (formerly *Astacus fluviatilis*), there are sixteen neurons in the cardiac ganglion (Alexandrowicz, 1929). This, among decapod crustaceans, is the largest number of heart ganglion cells known; other species have smaller numbers. Our knowledge of the function of the heart ganglion cells stems largely from the studies on the nerve cells in the cardiac ganglion of palinurid lobsters (reviewed by Maynard, 1960, 1961; Hagiwara, 1960). Of the nine ganglion cells, five function as *pacemakers*, that is they are spontaneously and rhythmically active and dictate their rhythm to the other four cells which in turn act as motoneurons whose axons innervate the heart muscle cells. These "follower" cells also influence the behavior of the pacemaker cells by collateral connections. It is very likely that the heart ganglion of crayfish is organized in a similar manner, but no details are known.

The central nervous system exerts control over the activity of the heart ganglion by means of the cardioregulator nerves; in this way it controls, indirectly, the strength and frequency of heart beats.

In crayfish there are, bilaterally, two regulator nerves: one causes inhibition, the other acceleration. They will be referred to as *inhibitory nerves* and *accelerator nerves* respectively. The normal innervation pattern seems to be that of one axon in each nerve, so that the heart ganglion is reached by a total of two inhibitory and two accelerator axons (Florey, 1960). Recently, however, it has come to light that there are individuals within an otherwise "normal" population of crayfish (*Pacifastacus leniusculus*) which have in their "accelerator" nerves not one but three axons: two accelerator and one inhibitory axon. It is thus very possible that variants to the "normal" patterns will be found. In this sense the following laboratory exercise may well lead to a valuable contribution to the research literature.

The dissection is delightfully simple and the preparation can be set up within a few minutes. The experiments provide a most convincing demonstration of excitatory as well as inhibitory effects of neurons and, at the same time, illustrate the role of cardioregulator nerves in the case of a neurogenic heart beat.

The experiments involve electrical stimulation of the exposed cardioregulator nerves, perfusion of the heart with various solutions, and isotonic

REY

lator Nerves in the Crayfish

art muscle of the crayfish contracts
ɔy nerve cells located in the *cardiac*
By complex interactions, involving
the ganglion cells of the heart gang-
way that they produce at regular
ɪrdiac ganglion is situated within the
'otamobius astacus (formerly *Astacus*
he cardiac ganglion (Alexandrowicz,
ns, is the largest number of heart
'e smaller numbers. Our knowledge
lls stems largely from the studies on
of palinurid lobsters (reviewed by
). Of the nine ganglion cells, five
ɪre spontaneously and rhythmically
ɔther four cells which in turn act as
heart muscle cells. These "follower"
pacemaker cells by collateral con-
ganglion of crayfish is organized in
wn.
ɔntrol over the activity of the heart
tor nerves; in this way it controls,
heart beats.
regulator nerves: one causes inhibi-
e referred to as *inhibitory nerves* and
mal innervation pattern seems to be
t the heart ganglion is reached by a
ator axons (Florey, 1960). Recently,
are individuals within an otherwise
ifastacus leniusculus) which have in
hree axons: two accelerator and one
hat variants to the "normal" pattern
laboratory exercise may well lead to
literature.
and the preparation can be set up
ɔrovide a most convincing demonstra
effects of neurons and, at the same
ator nerves in the case of a neuro

stimulation of the exposed cardia
with various solutions, and isotonic

recording of the heart beat which is used as an indicator of the activity of the heart ganglion. The following interpretation is useful. The frequency of heart beats indicates the frequency of electrical activity (burst of nerve impulses) of the heart ganglion. The amplitude of each heart beat indicates the number of nerve impulses (motoneurons of the heart ganglion) per unit time. The duration of the individual contractions indicates the duration of the "burst" or the repetitive spike discharge that reaches the muscle over the motor axons coming from the heart ganglion.

Equipment and Supplies

One kymograph (writing speed about 2 mm/sec), one light isotonic heart lever of the constant friction type (see p. 224); one, but preferably two electronic stimulators capable of delivering 0·5 msec pulses of up to 10 V and frequencies up to 50 per sec. One, but preferably two, double electrodes with platinum tips; one, but preferably two, simple micromanipulators for electrode positioning. *Note*: no shielding cage is required and the electrode cables need not be shielded. One small piece of "tacky" wax or plasticene; one piece of thin sewing thread (1 ft length) to which a pin is attached whose tip is bent into a hook; one old pair of strong scissors; one pair of small, curved forceps; one dissecting needle; one medicine dropper without rubber bulb; three ring stands; one separatory funnel of 500 ml capacity and equipped with a one hole rubber stopper through which a glass tube is inserted so that it reaches almost down to the stopcock of the funnel when the stopper is tightened; one ring clamp to hold the separatory funnel; one clamp to hold the medicine dropper; one clamp to hold the crayfish; one 10 in. length of rubber tubing to be attached to the stems of the funnel; one screw clamp to be placed over the rubber tubing to adjust the flow of solution; one glass adaptor to connect the rubber tubing with polyethylene tubing of 1–2 mm inner diameter; one 1 ft length of polyethylene tubing; one dish to collect waste solution that drips from the preparation.

Saline medium: crayfish solution, buffered to pH 6·5 (see Appendix, p. 254).

The Set-Up

The arrangement of the experimental set-up is illustrated in Fig. 11. Prepare the kymograph drum as described on p. 222. Mount heart lever and crayfish clamp on rings stand (1). Mount medicine dropper on rings stand (2). Mount the ring clamp near the top of ring stand (3). Insert the separatory funnel with tubing attached.

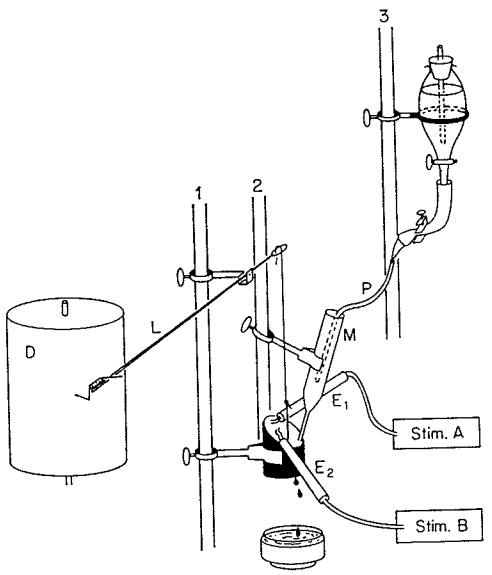


FIG. 11. Arrangement for stimulation of cardio-accelerator nerves, perfusion of the heart and for mechanically recording the heart contractions. The light writing lever (*L*) is mounted on ring stand 1. The preparation is mounted on the same stand. To facilitate the placement of the perfusion cannula (*M*) this is held on a second ring stand (2). A ring clamp mounted on ring stand 3 holds the separatory funnel that serves as constant pressure perfusion reservoir. The perfusion fluid is delivered to the cannula via polyethylene tubing (*P*). E_1 and E_2 are the two electrode holders; the electrode positioners, or micromanipulators, are not shown. The two stimulators (Stim. A and Stim. B) are represented by rectangles. The perfused fluid is allowed to drip into a collecting vessel. *D* represents the kymograph drum. For further detail see Fig. 14.

When properly adjusted, the crayfish clamp occupies the lowest level, the clamp holding the medicine dropper the next. The clamp holding the heart lever should be above that and the ring clamp holding the separatory funnel should occupy the highest position.

Close the stopcock of the separatory funnel, remove the stopper and fill funnel with crayfish solution.

Put the rubber stopper with its glass tube in place and tighten it. Close the screw clamp, then open the stopcock. Now gradually open the screw clamp until a flow rate of about 1 drop every 2 sec is established. Be sure to expel all the air from the tubing. This can be accomplished by squeezing the tubing above the screw clamp and forcing the air up into the separatory funnel. Close the stopcocks.

Balance the heart lever by placing a small lump of wax or plasticene on the load arm.

The Preparation

The dissection and the position of the cardioregulator nerves are illustrated in Figs 12 and 13.

With the old scissors cut off all the legs from a healthy, lively crayfish. Cut along the cervical groove as shown in Fig. 12, but avoid cutting through any of the internal organs. Pull the rostral end of the exoskeleton forward and downward, enough to expose the stomach retractor muscles. Cut through them and pull further: the entire stomach and gut together with the liver and most of the gonads will come out and can be discarded. Be sure to hold the animal upside down, so that any escaping digestive juices can flow out of

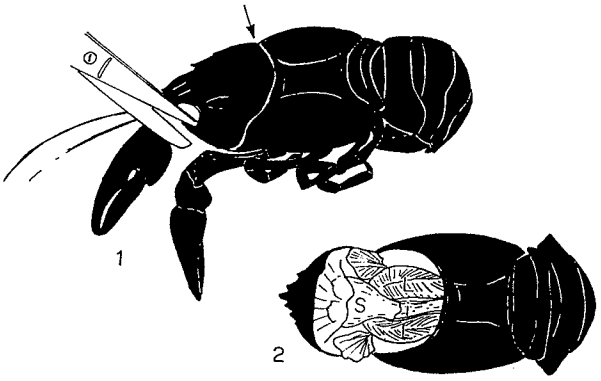


FIG. 12. (1) A crayfish being prepared for evisceration. The scissors cut along the cervical groove indicated by the arrow. It is advisable to cut off the legs before commencing with this operation. (2) When the cut has been made the "head" of the animal can be pulled forward and downward; with it the viscera (liver, *L*; stomach, *S*; intestine, *i*) are pulled out.

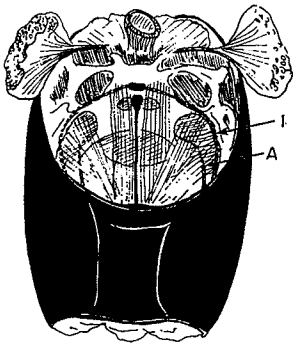
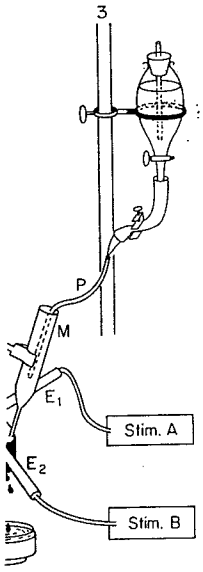


FIG. 13. Dorsal view of the preparation after evisceration and removal of the abdomen. The cardioregulator nerves are indicated by heavy, dashed lines. *I*, Inhibitory nerve; *A*, accelerator nerve.



accelerator nerves, perfusion of the heart
ons. The light writing lever (*L*) is mounted
e same stand. To facilitate the placement
nd ring stand (2). A ring clamp mounted
t serves as constant pressure perfusion
cannula via polyethylene tubing (*P*). E_1
le positioners, or micromanipulators, are
n. B) are represented by rectangles. The
essel. *D* represents the kymograph drum.

amp occupies the lowest level, the
next. The clamp holding the heart
amp holding the separatory funnel

unnel, remove the stopper and fill

e in place and tighten it. Close the
w gradually open the screw clamp
sec is established. Be sure to expel
complished by squeezing the tubing
air up into the separatory funnel

ill lump of wax or plasticene on the

the body cavity and do not come in contact with nerves or heart. Remove the front part of the animal and immediately rinse the body cavity with fresh crayfish solution.

Now flex the "tail" of the animal ventrad until the intersegmental fold between cephalothorax and first abdominal segment is exposed (dorsally). Insert one blade of the scissors and cut through the extensor muscles and on around the entire joint and through all the flexor muscles. This severs the abdomen which should be discarded, unless other experiments are planned which make use of the ventral nerve cord or of the stretch receptors: if in the process of evisceration care is taken to cut the intestine at the boundary between mid- and hindgut before the stomach and appendant organs are removed, the hindgut will remain in position within the abdomen and can then be prepared for a separate experiment.

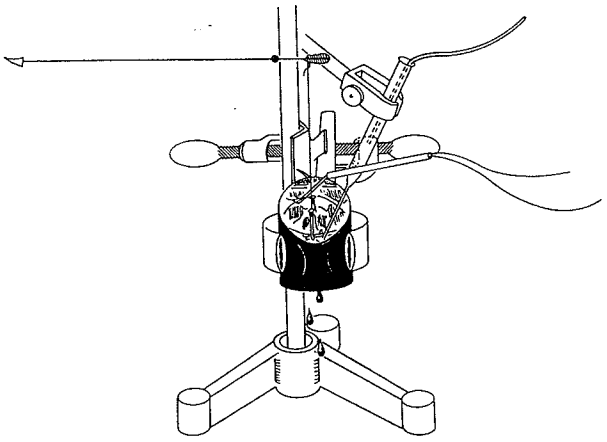


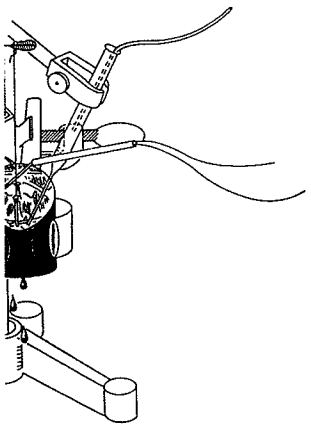
FIG. 14. The relative positions of perfusing cannula, heart hook and electrodes. Diagram of a preparation set up for perfusion, stimulation and recording, showing cannula, heart hook and stimulating electrodes in place. The electrodes stimulate the left inhibitory nerve.

The eviscerated thorax of the animal should be placed in a beaker with aerated crayfish solution as soon as possible. This allows the heart to fill with saline and to expel blood which otherwise would form a clot that prevents proper contact of the perfusion fluids with the heart ganglion.

After 5 min of immersion remove the thorax from the beaker and with fine, curved forceps pick up any remaining portion of the gonads and remove these. Hold a dissecting needle into the flame of a Bunsen burner and, when red hot, push it down the nerve canal to destroy the thoracic ganglia. If this precaution is not observed, the spontaneous activity of the cardioregulator fibers will obscure the results. The thorax can now be set up as shown in Fig. 14.

contact with nerves or heart. Remove
liately rinse the body cavity with fresh

ventrad until the intersegmental fold
minal segment is exposed (dorsally).
through the extensor muscles and on
ll the flexor muscles. This severs the
unless other experiments are planned
cord or of the stretch receptors; if
aken to cut the intestine at the boun-
re the stomach and appendant organs
position within the abdomen and can
nent.



cannula, heart hook and electrodes. Diagram
ation and recording, showing cannula, heart
electrodes stimulate the left inhibitory nerve.

al should be placed in a beaker with
ssible. This allows the heart to fill with
rwise would form a clot that prevents
with the heart ganglion.
e thorax from the beaker and with the
ning portion of the gonads and remove
e flame of a Bunsen burner and, when
to destroy the thoracic ganglia. If the
aneous activity of the cardioregulator
orax can now be set up as shown in

Mount the preparation upright in the crayfish clamp, the dorsal side facing you. Immediately lower the medicine dropper so that its tip pierces the pericardium fronto-laterally. Open the stopcock of the funnel and insert the polyethylene tubing in the dropper. This establishes the perfusion of the heart. Now hook the pin into the anterior portion of the heart muscle (you may have to rip the anterior wall of the pericardium to do this) and connect the thread to the lever, pressing it into the wax or plasticene. Carry out this operation while you hold the lever in such a position that the load arm points downward. Now carefully release the lever. If it does not straighten out and does not pull the thread upwards, move the wax or plasticene closer to the fulcrum until the writing arm is just a bit heavier than the pull on the load arm. The effective length ratio of load to writing arm should be at least 1 : 10 and may be as large as 1 : 20. It is imperative that the point of attachment of the thread to the lever is exactly above the heart. When the heart is in diastole the writing arm should be horizontal (see p. 223). Excursions of the writing tip should be at least 1 cm during normal heart activity. Amplitudes of recorded heart beats may easily reach 5 cm, even with crayfish of not more than 10 cm body length.

The origin of the cardioregulator nerves has not yet been described with certainty. Wiersma and Novitski (1942) felt it likely that they are the second and third superior nerves of the subesophageal ganglion, as originally described by Keim (1915) in his monograph of the crayfish nervous system.

The inhibitory and accelerator nerves emerge through openings in the ventral, inner skeleton. The inhibitory nerve of either side runs along the transverse chitinous ridge at the level of the first thoracic ganglion (see Fig. 13). The accelerator nerves run parallel to the inhibitors but emerge one segment posteriorly. They are covered by the flexor muscles shown in Fig. 13 for the first few millimeters but then emerge to run over the extensor muscles (Fig. 13) exposed to the body cavity. For stimulation it is not necessary to expose the accelerator nerves; it is sufficient simply to press the tips of the stimulating electrodes gently against the mid-region of the flexor muscles.

If two pairs of stimulators and electrodes are available, set both stimulators to give repetitive stimulation at 50/sec at 10 V (pulse duration 0·5 msec). Carefully guide the tips of the first pair of electrodes over one inhibitory nerve. As soon as the heart stops beating, turn off the stimulation. Now guide the second pair of electrodes so that the tips come to rest just over the accelerator nerve of one side. As soon as you notice acceleration, turn off the stimulation. Turn on the "inhibitory" stimulation and gradually reduce the voltage until the heart suddenly starts beating again. Now increase the voltage again to about 1·2 times the threshold voltage. Repeat this procedure with the accelerator stimulation.

If only one stimulator and electrode pair is available, you may begin the

experiment by placing the electrode tips over the inhibitory nerve first, and later on change it to the accelerator nerve, or vice versa.

The following is a schedule of exercises that should be performed in order to gain familiarity with the preparation.

1. *Serial stimulation of the inhibitory nerve.* Set stimulator to 1/sec and stimulate for a period of 5 sec. Wait 30 sec during which time you change the setting to 5/sec. Stimulate again for 5 sec. Wait 30 sec during which time you change the setting to 10/sec. Again stimulate for 5 sec. Repeat this performance until you have recorded responses to stimulation with 1, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50/sec. When you have found a frequency that gives complete inhibition, lower the frequency in steps of 1/sec (keeping the same time schedule) until you have found the threshold for complete inhibition.

2. *Serial stimulation of the accelerator nerve.* Proceed as described under exercise 1, but stimulate with 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 45 and 50/sec.

3. In order to discover whether there is more than one cardioregulator fiber in each of the nerves stimulated, select a frequency of stimulation that gives an intermediate response and stimulate repetitively while continuously varying the voltage. Note any sudden, stepwise, change in the frequency or amplitude of the heart beat. The number of steps indicates the minimum number of cardioregulator fibers present. It is quite likely that no such steps will be found.

The following exercises require two stimulators and two pairs of electrodes.

4. *Summation.* Place one pair of electrodes over one, the other pair of electrodes over the other inhibitory nerve. Stimulate one nerve with the threshold frequency ascertained under exercise 1, then stimulate the other inhibitory nerve with the same frequency to make sure that the threshold is the same. Differences are more likely due to unequal calibration of the stimulators than to inequality of effectiveness of the two inhibitory nerves. This can, of course, be checked by leaving the electrodes in place and exchanging the stimulators.

Now stimulate the left inhibitory nerve with half the threshold frequency and continue stimulating while, with a delay of a few seconds, the other stimulator, previously set at half threshold frequency, is turned on. The two effects should sum to give threshold inhibition.

Now vary the ratios of stimulation and determine the range over which the frequencies are additive. Be sure to allow 1 min periods of rest between periods of stimulation. Repeat this type of exercise but place the electrodes over the two accelerator nerves.

5. *Adaption.* Stimulate the left inhibitory nerve with threshold frequency and continue stimulation until heart beats resume and reach a steady rate. Now, at one instant turn off the "left" stimulator and turn on the "right" one.

(previously set to threshold frequency). If the heart continues to beat, you may conclude that the adaptation to stimulation of the left inhibitory nerve is a post-synaptic phenomenon and not a pre-synaptic one (such as decreasing responsiveness of the inhibitory nerve to stimulation, or decreasing output of transmitter substance from the terminals of the left inhibitory axon(s)).

Place electrodes over the two accelerator nerves. Stimulate the left accelerator at a low frequency, such as 5/sec. Then, while this stimulation continues, add stimulation of the right accelerator nerve, also at 5/sec. Does the result resemble that obtained with stimulation of either accelerator nerve with 10 pulses per sec? Vary the frequencies.

6. *Interaction of accelerator and inhibitory nerve stimulation.* Place one pair of electrodes over one inhibitory nerve, the other over one accelerator nerve. For convenience of expression, we designate the stimulator that activates the inhibitory nerve as the I-stimulator, the other as the A-stimulator.

For the first series of experiments, turn the A-stimulator on, 5 sec later turn the I-stimulator on, 5 sec later turn the I-stimulator off and 5 sec later turn off the A-stimulator. Repeat after 1 min intervals. Set I-stimulator at a subthreshold, threshold and a suprathreshold frequency and vary A-stimulation between 1 and 50/sec in 5/sec steps.

For the second series of experiments turn the I-stimulator on, 5 sec later turn on the A-stimulator, 5 sec later turn off the A-stimulator and 5 sec after that turn off the I-stimulator. Use the same settings as in the first series.

Evaluation of the Records

(a) Plot frequency of stimulation of the inhibitory nerve against heart rate observed during period of maximal effectiveness of the inhibitory nerve at that frequency of stimulation.

(b) Plot frequency of stimulation of inhibitory nerve against percent change in heart rate.

(c) Plot frequency of stimulation of the accelerator nerve against heart rate observed during period of maximal effectiveness of the accelerator nerve at that frequency of stimulation.

(d) Plot frequency of stimulation of the accelerator nerve against percent change in heart rate.

(e) Plot duration of after-effect of "inhibitory stimulation" against frequency of stimulation.

(f) Plot duration of after-effect of "accelerator stimulation" against frequency of stimulation.

(g) Discuss the results evident from plots a–d in relation to the findings obtained in exercises 5 and 6.

(h) Try to interpret the mechanisms underlying the after-effects as described in (e) and (f).

Appendix

Composition of crayfish solution of 10 × stength, to be used as stock solution: NaCl, 120 g; KCl, 4·0 g; $MgCl_2.6\,H_2O$, 5·0 g; $CaCl_2$ (anhydrous) 15·0 g; H_2O up to 1 000 ml.

Method of preparation: (a) Weigh out NaCl, KCl and $MgCl_2.6\,H_2O$ and place the powders into a 1 000 ml volumetric flask. Add 700 ml of H_2O and shake until the salts have dissolved. Weigh out the $CaCl_2$ and dissolve this in 100 ml of H_2O in a beaker. Pour the $CaCl_2$ solution into the volumetric flask, mix well and then add H_2O to the 1 000 ml mark. The solution is stable.

(b) Prepare stock solutions of the following: 5 M NaCl (292·2 g/liter); 1 M KCl (74·5 g/liter); 1 M $MgCl_2$ (203·3 g of $MgCl_2.6\,H_2O$/liter) and 1 M $CaCl_2$ (111 g $CaCl_2$ or 147 g $CaCl_2.2\,H_2O$/liter). Using clean measuring cylinders and pipettes transfer to a 1 000 ml volumetric flask 41.0 ml of NaCl stock solution, 5.4 ml of KCl stock solution, and 2.6 ml of $MgCl_2$ stock solution. Add 700 ml of H_2O and mix. Now add 13.5 ml of the $CaCl_2$ stock solution, mix and fill with H_2O to the 1 000 ml mark.

To prepare crayfish solution for use in the experiments, add 900 ml of H_2O to 100 ml 10 × stock solution. Buffered crayfish solution of pH 6·5 is prepared by adding to this 100 ml of 0·2 M Tris-acid maleate buffer, the latter is made up as follows: dissolve 19·6 g of maleic anhydride and 24·2 g of Tris in 1 liter of H_2O. Take 56 ml of this and add 44 ml of a solution of 8·0 g of NaOH/liter. This gives 100 ml of Tris-acid maleate buffer of pH 6·5 of 0·2 M strength.

References

Alexandrowicz, J. S. (1929) Recherches sur l'innervation du coeur de l'écrevisse. *Folia morph.* **1**, 37–67. (In Polish with French summary.)

Alexandrowicz, J. S. (1932). The innervation of the heart of the Crusteacea. I. Decapoda. *Q. Jl microsc. Sci.* **75**, 182–249.

Florey, E. (1960). Studies on the nervous regulation of the heart beat in decapod Crustacea. *J. gen. Physiol.* **43**, 1061–1081.

Hagiwara, S. (1961). Nervous activities of the heart in Crustacea. *Ergebn. Biol.* **24**, 287–311.

Keim, W. (1915). Das Nervensystem von *Astacus fluviatilis* (*Potamobius astacus* L.). Ein Beitrag zur Morphologie der Kekapoden. *Z. wiss. Zool.* **113**, 485–314.

Maynard, D. (1961). Cardiac inhibition in decapod Crustacea. *In* "Nervous Inhibition" (E. Florey, ed.), pp. 144–178. Pergamon Press, Oxford.

Maynard, D. M. (1960) Circulation and heart function. *In* "The Physiology of Crustacea" (T. H. Waterman, ed.), Vol. 1, pp. 161–226. Academic Press, New York and London.

Wiersma, C. A. G. and Novitski, E. (1942). The mechanism of the nervous regulation of the crayfish heart. *J. exp. Biol.* **19**, 255–265.

E. Pharmacology of the Crayfish Heart

The crayfish heart preparation, as described in the preceding exercise, offers an unusual opportunity for neuro-pharmacological experiments. Since the pioneering studies of MacLean and Beznak (1933) many publications have dealt with the excitatory actions of acetylcholine (ACh) on the crustacean heart. There have been many different interpretations of the significance of this, the majority of workers have assumed that ACh acts as the transmitter substance of the cardio-accelerator nerves (Welsh, 1939, 1942; Smith, 1947; Davenport, 1941, 1942; Wiersma and Novitsky, 1942) and of the motoneurons of the heart ganglion (Davenport). More recent evidence indicates, however, that this is not so and that the heart ganglion cells are cholinoceptive even though the cardioregulator nerves are non-cholinergic (Florey, 1963). In the following exercises the student can convince himself of this.

The heart of decapod crustaceans also responds to certain catecholamines (adrenaline, noradrenaline) and indol-alkylamines (5-hydroxytryptamine, 6-hydroxytryptamine) with pronounced excitation. This is an action on ganglion cells (Florey, 1963). While there is no evidence that catecholamines are present in any crustacean tissue, 5-hydroxytryptamine (Maynard and Welsh, 1959), 5,6-hydroxytryptamine (Carlisle and Knowles, 1959) and 6-hydroxytryptamine (Kerkut and Price, 1964) have been detected in crustacean pericardial organs. These neurosecretory structures are presumed to release these compounds into the venous blood that returns to the heart.

γ-Aminobutyric acid mimics the action of the cardio-inhibitory neurons of the crayfish and may, in fact, be the inhibitory transmitter substance responsible for the inhibition of the activity of the heart ganglion (Florey, 1957).

The following exercise involves (1) application of compounds that mimic transmitter substances and neurohormones (for a distinction between these two terms, see Florey, 1962), (2) comparison of their effects with the actions of the cardioregulator nerves, (3) application of drugs that interfere with the transmitter-like compounds, and (4) experiments to discover whether the latter drugs also interfere with the action of the cardioregulator neurons that, presumably, release transmitter substances.

Equipment and Supplies

The same equipment is used as in exercise 14 D, p. 247., except that one stimulator is sufficient and that four, instead of one, separatory funnels complete with ring clamps, rubber and polyethylene tubing and screw clamps are required. In addition there is need for one test tube stand, twenty test tubes, seven 1 ml and one 10 ml pipettes. The saline medium is the same

solution as that recommended in the preceding exercise. In addition, the following drugs and stock solutions are needed: acetylcholine chloride (acetylcholine bromide is also suitable), γ-aminobutyric acid, eserine (physostigmine salicylate), atropine, picrotoxin, adrenaline (best used in the form of the bitartrate) and 5-hydroxytryptamine (as the creatinine sulfate). Per set-up not more than 1 ml of a solution of 1 mg of each drug in 1 ml of distilled water is required. Of all the drugs listed here, atropine is the most labile; the solutions of others can be stored in the refrigerator for several weeks, provided the distilled water was pure and of a pH of about 5 (due to CO_2). Freshly distilled water should be slightly acidic (pH 5), but distilled water that has been stored in soft glass bottles, may have become slightly alkaline. It is therefore advisable to test the reaction of the distilled water to be used with the aid of a drop of universal indicator, or with indicator paper (the sample containing the indicator, must, of course, be discarded).

Stock solutions are best kept in plastic bottles (50 ml or 100 ml size) with screw caps. Cork stoppers have a habit of turning up in the laboratory's cork supply and once used in contact with a drug solution they can easily confer unwanted pharmacological potencies to other solutions. Do not use spatulas when weighing out drugs: the compounds used here are extremely potent and a small speck of the powder unwittingly transferred to another drug bottle or other container of laboratory chemicals can endanger the future work of an entire laboratory. It is best to gently rotate the opened and inclined bottle that contains the drug until a small quantity of a few milligrams of the drug powder falls onto the preweighed weighing paper. Use an analytical balance and weigh to 0·1 mg. It is preferable to obtain ACh preweighed in ampules (100 mg) since this compound is quite hygroscopic for the preparation of Ach-stock solution see p. 243.

When samples of the different drugs have been weighed, transfer them to the plastic bottle, tapping the weighing paper gently until the last visible granule of the powder has been dislodged. Discard the weighing paper. With an accurate 10 ml pipette, add as many milliliters of distilled water as there were milligrams of the drug weighed out. Do not allow the tip of the pipette to touch the mouth of the plastic bottle because it is quite possible that a bit of drug powder may have got caught there. A contaminated pipette tip should not come in contact with another plastic bottle when the next stock solutions are made up. Now stopper the bottle and shake the contents. Picrotoxin is not very soluble in water and the suspension should be placed in a water bath of at least 70°C. Solution will take place in about 1 h. All stock solutions should be carefully labelled as follows: name of compound, manufacturer, concentration (in this case 10^{-3} g/ml), solvent (in this case distilled water), date, and name of person who prepared the solution.

As a precaution, wash your hands before you continue with the experiment.

preceding exercise. In addition, the
are needed: acetylcholine chloride
γ-aminobutyric acid, eserine (physo-
ı, adrenaline (best used in the form of
: (as the creatinine sulfate). Per set-up
; of each drug in 1 ml of distilled water
ɜre, atropine is the most labile; the
ɜfrigerator for several weeks, provided
ɔH of about 5 (due to CO_2). Freshly
ɔ (pH 5), but distilled water that has
have become slightly alkaline. It is
of the distilled water to be used with
', or with indicator paper (the sample
ɜe, be discarded).
tic bottles (50 ml or 100 ml size) with
ɔit of turning up in the laboratory's
with a drug solution they can easily
ɜncies to other solutions. Do not use
: compounds used here are extremely
er unwittingly transferred to another
ɔoratory chemicals can endanger the
t is best to gently rotate the opened
drug until a small quantity of a few
ɔnto the preweighed weighing paper.
to 0·1 mg. It is preferable to obtain
since this compound is quite hygro-
k solution see p. 243.
; have been weighed, transfer them to
ng paper gently until the last visible
ɡed. Discard the weighing paper. With
ɣ milliliters of distilled water as there
ut. Do not allow the tip of the pipette
e because it is quite possible that a bit
ht there. A contaminated pipette tip
her plastic bottle when the next stock
r the bottle and shake the contents
r and the suspension should be placed
ɪtion will take place in about 1 h. All
ɒelled as follows: name of compound,
case 10^{-3} g/ml), solvent (in this case
son who prepared the solution.
ɜfore you continue with the experiment

Set up and Preparation

Set-up and preparation are the same as in the preceding exercise, except that three separatory funnels with their accessories should be mounted on one of the ring stands in addition to the one used before.

Preliminary Procedure

Fill one of the separatory funnels with crayfish solution, to each of the other three add 99 ml of crayfish solution. Make sure the stopcock is closed before the saline is added! With the aid of separate 1 ml pipettes add to funnel no. 2 1 ml of eserine 10^{-3}, to funnel no. 3 1 ml of atropine 10^{-3} and to funnel no. 4 1 ml of picrotoxin 10^{-3}. Mix well. Return the stock solutions to the refrigerator.

Now close all the screw clamps on the rubber tubing connected with each funnel, then open the stopcocks. Gradually open the screw clamp of the first funnel until a flow rate of about 2 ml/min is established when the tip of the thin polyethylene tubing is at the level it will occupy when inserted into the positioned intrapericardial cannula (medicine dropper). Repeat the procedure with the other funnels.

Set up serial dilutions of acetylcholine (ACh), γ-aminobutyric acid (GABA), 5-hydroxytryptamine (5-HT), and adrenaline (Adr) as follows. In a test tube rack set up three rows of five, three, five and six test tubes respectively. Add to each 9 ml of crayfish solution. Cover them all with a sheet of paper. Now expose the first tube of the first row and add, with a 1 ml pipette 1 ml of ACh stock solution. Use the same pipette to mix the dilution; insert it all the way into the test tube and gently blow air into the solution so that this rises up in the test tube and rinses both the outside of the pipette and the entire inside of the test tube. Draw some of the solution up into the pipette and release again, repeat five times, then withdraw 1 ml of the mixture, wipe the outside of the pipette with a fresh piece of tissue paper, and transfer to the next test tube. Repeat there the mixing procedure. Always keep all the other test tubes covered. Continue until all the tubes in the first row have been mixed. Carefully label each tube: you now have a series of dilutions ranging from 10^{-4} to 10^{-8} g/ml. Using a new 1 ml pipette, prepare similar dilution series for GABA, 5-HT and Adr. Return the stock solutions to the refrigerator and briefly rinse the pipettes before placing them into the wash-receptacle.

Now dissect a crayfish and mount the heart preparation. Establish perfusion with crayfish solution and record the heart beat. Make all necessary adjustments to obtain good records on the kymograph. Move the stimulating electrodes over the inhibitory nerve of the left or the right side and determine the threshold for complete inhibition, using the experience gained in the

preceding exercise. Then change the electrode position so you can stimulate an accelerator nerve. Record the effects of stimulation at 5, 10, 15, 20, 25 and 30 pulses per sec.

Experiments

1. Use a 1 ml tuberculin syringe with a fine needle (no. 23 or thinner) and inject a "slug" of 1 ml of ACh 10^{-8} into the rubber tubing of funnel no. 1 (crayfish solution) below the screw clamp. Observe the effect, if any, and record it. Using the same syringe, inject a similar "slug" of ACh 10^{-7} and so forth until strong excitation is obtained. Now open the stopcock of funnel no. 2 (eserine), close stopcock of funnel no. 1 and exchange the polyethylene tubing in the cannula (medicine dropper) that delivers the perfusion fluid to the heart. Allow the eserine solution to flow for 5 min, then repeat the sequence of injections (into the tubing of no. 2 funnel) of increasing concentrations of ACh. (*Note*: rinse the syringe before going from 10^{-4} or 10^{-5} to 10^{-8}!).

When a normal, or near normal heart rate is restored, stimulate the accelerator nerve at the same frequencies that were tried before. Change electrode position and stimulate the inhibitory nerve at the frequency that before gave threshold inhibition and try to find the new threshold frequency, should this have changed.

2. Open stopcock of funnel no. 3 (atropine) and close that of no. 2. Exchange the polyethylene tubing. Allow 5 min perfusion with the atropine solution then inject again increasing concentrations of ACh in 1 ml slugs.

When the heart rate has again become steady, stimulate the inhibitory nerve and find out whether there has been a change in the "inhibitory threshold". Then change electrode positions again and stimulate the accelerator nerve at 5, 10, 15, and so forth.

You should now be in a position to evaluate the action of ACh when you can answer the following questions. (a) Is the action of ACh similar to that of the accelerator nerve? (b) Does eserine enhance both the action of ACh and of the accelerator neurons? (c) Does atropine block both the action of applied ACh and that of the transmitter released by the accelerator nerve?

3. Open stopcock of funnel no. 1 (crayfish solution), close stopcock of funnel no. 3 and exchange the polyethylene tubing in the cannula. After an interval of 10 min inject 1 ml of 5-HT 10^{-9}. Note the effect, if any, then inject 1 ml of 5-HT 10^{-8} and so forth, until strong excitation (acceleration and an increase in amplitude) is obtained.

4. Inject 1 ml of Adr 10^{-8}, observe the effect, if any, and follow this with injections of increasing adrenaline concentrations, until strong excitation is obtained.

You can now determine the relative sensitivity of the heart to the two drugs. Remember, however, that the concentrations are given in g/ml and not as molar concentrations. Evaluate the effects in terms of the interpretation suggested in the introduction to the preceding exercise (14 D, p. 247) and discuss whether the drugs act predominantly on the muscle, or on the ganglion cells.

5. Inject 1 ml of GABA 10^{-7}, note the effect, if any, then inject higher concentrations until complete inhibition is obtained.

6. Place the electrodes over the inhibitory nerve and determine once more the inhibitory threshold. Open stopcock of funnel no. 4 (picrotoxin) and close stopcock of funnel no. 1. Exchange the polyethylene tubing in the cannula. At 1 min intervals stimulate the inhibitory nerve at threshold frequency. As inhibition diminishes, increase the frequency. When substantial blocking of the inhibitory action is obtained, inject 1 ml slugs of increasing concentrations of GABA and determine the new threshold concentration for complete inhibition.

7. Shift the electrode position to the accelerator nerve and stimulate serially. Inject a concentration of ACh and/or 5-HT that was previously found to be effective.

You can now evaluate the results of the last experiments (5–7) by answering the following questions. (a) Does GABA mimic the action of the inhibitory nerves? (b) Does pictotoxin block both the action of GABA and that of the inhibitory nerves? (c) Is the action of picrotoxin specific, or does it also affect excitatory actions? (d) Are the actions of GABA and of picrotoxin restricted to synapses between cardioregulator neurons and ganglion cells, or do they also affect the neuromuscular synapses?

References

Bain, W. A. (1929). The action of adrenaline and of certain drugs upon the isolated crustacean heart. *Q. Jl exp. Physiol.* **19**, 297–308.

Carlisle, D. B. and Knowles, F. G. (1959). "Endocrine Control in Crustaceans." Cambridge University Press, Cambridge.

Davenport, D. (1941). The effects of acetylcholine, atropine and nicotine on the isolated heart of the commercial crab, *Cancer magister*, Dana. *Physiol. Zool.* **14**, 178–185.

Davenport, D. (1942) Further studies on the pharmacology of the heart of *Cancer magister*, Dana. *Biol. Bull. mar. Biol. Lab., Woods Hole* **82**, 255–260.

Florey, E. (1957). Further evidence for the transmitter-function of Factor-I. *Naturwissenschaften* **44**, 424–425.

Florey, E. (1962). Neurohormones. *Physiologist, Lond.* **5**, 285–293.

Florey, E. (1963). Acetylcholine in invertebrate nervous systems. *Can. J. Biochem.* **41**, 2619–2626.

Kerkut, G. A. and M. A. Price (1964). Chromatographic separation of cardiac accelerators (6-HT and a mucopeptide) from *Carcinus* heart. *Comp. Biochem. Physiol.* **11**, 45–52.

MacLean M. N. and Beznak, A. B. L. (1933). The effects of sympatheticomimetic *Arb. ung. biol. Forsch. Inst.* **6**, 258–272.
Maynard, D. M. and Welsh J. H. (1959). Neurohormones of the pericardial organs of brachyuran crustaceans. *J. Physiol., Lond.* **149**, 215–227.
Smith, R. I. (1947). The action of electrical stimulation and of certain drugs on cardiac nerves of the crab, *Cancer irroratus. Biol. Bull. mar. Biol. Lab., Woods Hole* **93**, 72–88.
Welsh, J. H. (1939). Chemical mediation in crustaceans. I. The occurrence of acetylcholine in nervous tissue and its action upon the decapod heart. *J. exp. Biol.* **16**, 198–237.
Welsh, J. H. (1942). Chemical mediation in crustaceans. IV. The action of acetylcholine on isolated hearts of *Homarus* and *Carcinides*. *J. cell. comp. Physiol.* **19**, 271–279.
Wiersma, C. A. G. and Novitski, E. (1942). The mechanism of the nervous regulation of the crayfish heart. *J. exp. Biol.* **19**, 255–265.

F. Spontaneous Activity of the Crayfish Hindgut and its Control by Drugs

The hindgut of the crayfish extends as a straight tube through the dorsomedial hemocoel of the abdomen. When removed from the animal and placed into a muscle chamber (see below) it exhibits frequent spontaneous contractions. There are two muscle layers: an inner layer of longitudinal muscle bands and an outer circular muscle layer. Situated between them is a nerve plexus. There is also an external superficial plexus composed of the terminals of efferent nerve fibers which reach the hindgut through the intestinal nerve from the sixth abdominal ganglion. Associated with the longitudinal muscle bands are numerous bipolar nerve cells. Further nerve cells extend long processes towards the inner muscosal surface of the gut; they are most likely sensory cells. The histology of the crayfish hindgut has been described in detail by Alexandrowicz (1909) and by Janisch (1924).

The muscle fibers undergo coordinated contractions which give rise to peristalsis. Although it is difficult to follow the behavior of the ring muscles that of the longitudinal muscles can be recorded easily and is the subject of the following exercise. The contractions of the longitudinal muscles are enhanced by acetylcholine (ACh); the effect is potentiated by anticholinesterases and blocked by atropine (Florey, 1954). Thus the hindgut exhibits the typical response pattern of a cholinoceptive organ. The spontaneous as well as the ACh-induced contractions are inhibited by γ-aminobutyric acid (GABA) and several related amino acids (Tsuchiya, 1960; Florey, 1961). This inhibition can be blocked by picrotoxin. The hindgut thus shows much of the pharmacological behavior of crustacean skeletal muscle. Glutamic acid and some related compounds cause contraction as well as inhibition (Florey, 1961; Jones, 1962), the latter response can be prevented by the application of picrotoxin.

Materials

For the dissection and preparation of the hindgut the following dissecting instruments are recommended. One pair of coarse scissors with one blunt and one pointed blade, two pairs of small forceps with curved tips, one glass plate (microscope slide), fine sewing thread, and one glass beaker (50–100 ml). In addition, each set-up should be provided with a test tube rack, 20 test tubes, six 1 ml pipettes graduated to 0·1 ml, one 10 ml pipette graduated to 1 ml, two 100 ml beakers, one bowl for waste solutions, a bottle with 1 liter of saline, and six 1 ml syringes with No. 22 needles. The basic saline medium should have the following composition: NaCl 200 mM; KCl 5·4 mM; $MgCl_2$ 2·6 mM; and $CaCl_2$ 13·5 mM per liter. To 9 volumes of this add 1 volume of 0·1 M phosphate buffer. The buffer must be prepared from the sodium salts. It is convenient to prepare 0·1 M stock solutions of Na_2HPO_3 and NaH_2PO_3 and to combine equal volumes of the two just before addition to the saline, the pH will be close to 6·5. The stock solutions are stable, particularly when kept under refrigeration, but the mixture is not. Per experimental set-up about 1 liter of buffered saline should be adequate for the exercise outlined here.

The set-up requires a muscle chamber and a recording device. The chamber described for the clam heart bioassay (p. 233) is perfectly adequate for the purposes of this exercise, otherwise a chamber can be made as shown in Fig. 15. It consists of a short length (about 6 cm) of glass tubing of about 1cm inner diameter, closed at one end by a one-hole rubber stopper. A short piece of glass tubing of suitable diameter is inserted into the hole without protruding through the upper surface of the stopper. It serves as an outflow. A 3 cm piece of thin rubber tubing slipped over the lower end of this tubing permits attachment of a pinchcock. A hypodermic needle (No. 23) pushed through the stopper is used for aeration and mixing.

It is best to use a light, well-balanced heart lever for recording on a kymograph (see Section A). The kymograph should provide a drum speed of about 5 cm per min. Laboratories equipped with mechanoelectric transducers and chart recorders can use these to advantage, provided the transducers offer sufficient sensitivity for tensions in the 100 mg range.

Freshwater crayfish of the genera *Astacus*, *Pacifastacus*, *Cambarus*, *Procambarus* and *Orconectes* have been used successfully; other genera are probably equally suitable. The animals should have a minimal body length of 10 cm.

For each set-up 1 ml quantities of the following drug stock solutions (10^{-3} g/ml) should be provided: acetylcholine chloride (bromide, iodide), atropine, eserine (physostigmine salicylate), sodium glutamate, GABA and picrotoxin. Of these only the atropine solution needs to be made fresh, the

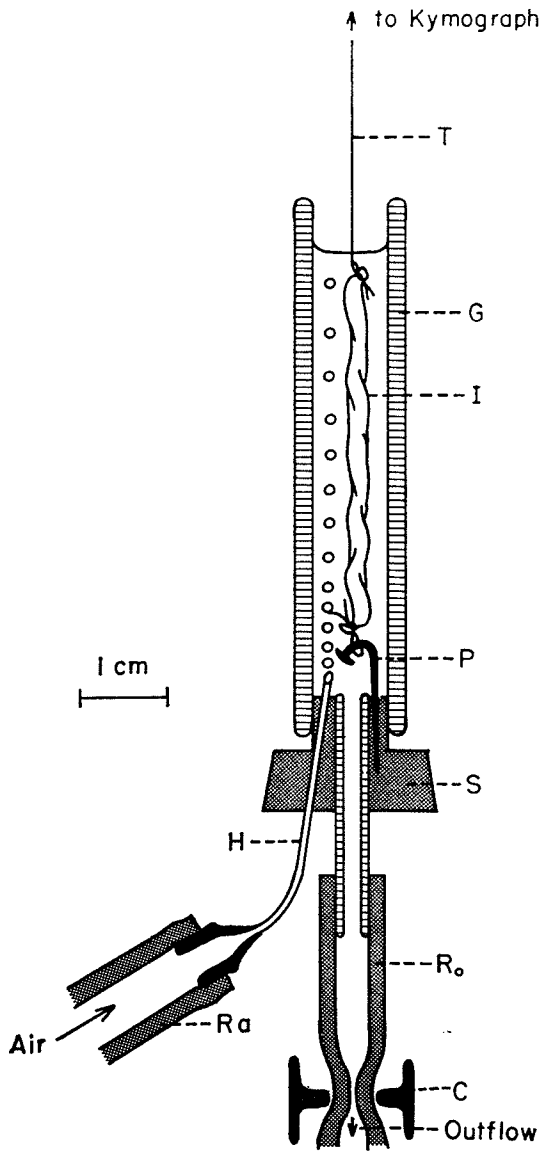
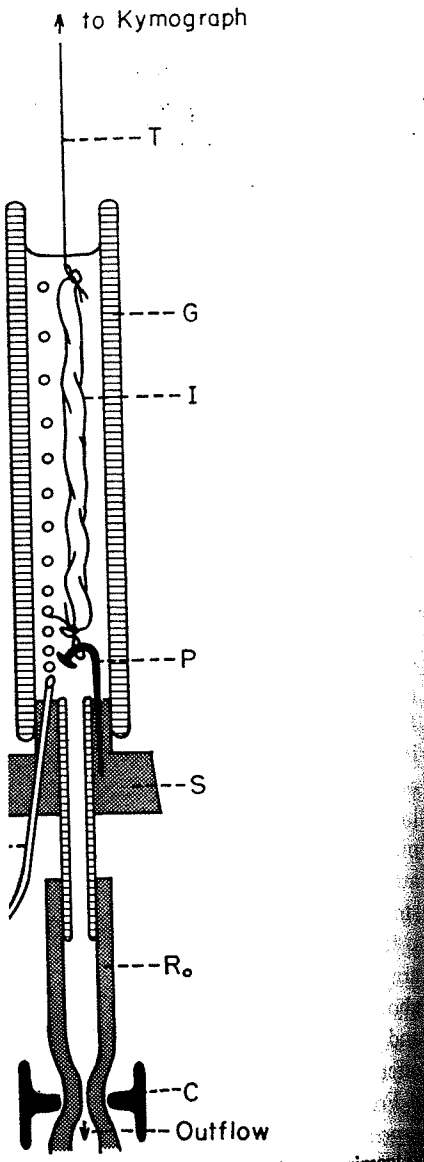


FIG. 15. Diagram to explain the construction of a muscle chamber for the experimen
the crayfish hindgut. The chamber is made of glass tubing (*G*) and closed off at the bot
by a suitably trimmed rubber stopper (*S*). The latter is penetrated by a thinner glass
that serves as an outflow; an attached piece of rubber tubing (R_o) and a pinch clamp
serve as outflow valve. Aeration is accomplished by way of a No. 22 hyperdermic n
(*H*) connected to the air supply with rubber tubing (R_a). A loop of thread attached
rectal end of the hindgut is slipped over a bent pin (*P*) inserted in the stopper (*S*).
anterior end of the hindgut (*I*) is connected to the writing lever by means of the thread



...n of a muscle chamber for the experiment... glass tubing (*G*) and closed off at the bott... ...e latter is penetrated by a thinner glass ... of rubber tubing (R_o) and a pinch clamp ... ...shed by way of a No. 22 hyperdermic nee... tubing (R_a). A loop of thread attached to ... bent pin (*P*) inserted in the stopper (*S*) ... ...) the writing lever by means of the thread ...

others are stable for several weeks when kept under refrigeration. Stock solutions are best prepared by weighing out a certain number of milligrams of the particular drug and then adding an equal number of milliliters of distilled water. Picrotoxin will go into solution only when heated. Place the bottle with the suspension into a waterbath of 70–90°C for an hour. It is important that all bottles containing stock solutions be appropriately labelled, indicating name of drug, concentration, solvent, date and name of person responsible for the preparation of the solution.

Prior to the experiment proper, the following solutions should be prepared in test tubes: ACh (salt) 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} g/ml; atropine 10^{-4} g/ml; eserine 10^{-4} g/ml; sodium glutamate 10^{-4} g/ml; GABA 10^{-4} g/ml, 10^{-5} g/ml; and picrotoxin 10^{-4} g/ml. A fresh 1 ml pipette should be used for each drug. Fill all tubes to be used with 9 ml of saline, then add 1 ml of stock solution of the different drugs to one tube each and label (ACh 10^{-4}; Atr 10^{-4}; Es 10^{-4}; Glu 10^{-4}; GABA 10^{-4}; Pi 10^{-4}). Add 1 ml of the 10^{-4} dilutions of ACh and GABA (using the originally used 1 ml pipettes) and transfer to a tube containing 9 ml of saline. Label ACh 10^{-5} and GABA 10^{-5}. Continue serial dilution of ACh until 10^{-7} is reached.

Dissection and Mounting of the Hindgut

Decerebrate the crayfish by piercing the carapace behind one of the eyes with the pointed blade of the scissors. Cut off all the legs at the coxa, then deflect the abdomen downwards until the membrane between cephalothorax and first abdominal segment is exposed. Cut through the extensor musculature immediately below and cut through the exoskeleton of the abdominal segments on both sides keeping the blades of the scissors parallel to the ventral surface of the abdomen and cutting ventral of the segmental joints. The point of the inserted blade should be aimed along the hemocoelic cavity just dorsal of the abdominal artery that lies just over the intestine. During this dissection the transversal flexor muscles of the abdominal segments will be cut. The dorsal part of the exoskeleton can be removed and saved for the preparation of stretch receptors, if desired. The abdominal artery is now to be pulled off the gut and the latter to be sectioned near its exit from the thoracic cavity and close to the anus. The gut can now be removed from the animal and should be laid on a glass plate. By holding the anterior end and gently stroking it with the closed jaws of a second pair of forceps the contents of the intestine can be forced out the posterior opening. A length of thread should now be tied to the anterior end of the organ and a small loop attached to the posterior end. The prepared hindgut can now be transferred to a beaker containing some saline until everything is ready for mounting it.

It is important to rinse the bathing chamber with fresh saline before use and to adjust the airflow so as to achieve a gentle stream of air bubbles when the chamber is filled with saline. The heart lever should be carefully balanced before the intestine is connected to it. When the recording set-up is properly adjusted the hindgut preparation should be mounted as shown in Fig. 15.

Procedure

1. Adjust balance and magnification factor of writing lever until optimal recording is obtained. Allow at least 15 min for the preparation to come to equilibrium performance. Meanwhile note on the record the following information: name of experimenters, date, sex, size and species of crayfish used, length of hindgut, nature of saline, pH, magnification factor of lever, speed of kymograph (or chart speed).

2. Drain the bathing chamber and use the 10 ml pipette to fill the chamber with a measured volume leaving space for an additional 1/9 of this volume. For a chamber that easily holds 2 ml it is most convenient to add 1·8 ml, chambers holding 3 ml should be filled with 2·7 ml, etc. Carefully mark this level. Ideally the entire preparation should be submerged. For convenience this marked level will be referred to as the 9 vol mark.

3. Now add 1 vol of ACh 10^{-7}, using a 1 ml tuberculin syringe and squirting the solution into the bath (previously adjusted to the 9 vol mark). The tip of the needle should be well submerged in the bathing saline and the emerging stream of ACh solution should be directed against the wall of the chamber rather than against the intestine. Observe the effect during 1 min, then drain the bath, fill with saline, drain and fill again to the 9 vol mark.

4. Repeat with the next higher ACh concentration.

5. Add ACh 10^{-5}, and wash as before.

6. Add 1 vol of eserine 10^{-4}. If there is any residual ACh left from the previous applications there will be gradually increasing contractile activity. When this occurs, wash and reapply eserine.

7. After eserine has been allowed to act for 10 min, lower the fluid level in the bath to the 9 vol mark and add 1 vol of ACh 10^{-7}. Observe the effect for 1 min. Then drain the bath, fill with saline, drain and fill again.

8. When the preparation has resumed its normal "resting" activity, add 1 vol of atropine 10^{-4} and allow this to act for 5 min. Lower the bath level to the 9 vol mark.

9. Add 1 vol of ACh 10^{-5}. After 1 min of observation, wash twice, then add 1 vol of atropine.

10. Add 1 vol of ACh 10^{-4}. Observe action for 1 min, then wash twice. Repeat washings 2 min later.

11. Select a concentration of ACh that previously (before atropine) gave an

intermediate response. Add 1 vol to see whether responsiveness to ACh has returned. After 1 min, wash. If necessary repeat this sequence until response returns.

12. Apply 1 vol of GABA 10^{-5}, observe effect (if any) for 1 min, then lower fluid level to 9 vol mark and add 1 vol of ACh of a concentration previously established as being effective (step 11). Observe the effect (if any) for 1 min, then wash twice.

13. Repeat but use GABA 10^{-4}. Wash at least twice.

14. Apply 1 vol of glutamate 10^{-4}. After 1 min lower fluid level to the 9 vol mark and add 1 vol of ACh of the same concentration that was applied in parts 11–13. Observe effect for 1 min, then wash twice. Repeat this sequence.

15. Apply 1 vol of picrotoxin 10^{-4} and allow this to act for 10 min. Lower the fluid level in the bath to the 9 vol mark and apply 1 vol of GABA 10^{-4}. Observe effect (if any) for 1 min, then lower fluid level to 9 vol mark and add 1 vol of ACh as in the previous sections of this exercise. After 1 min wash twice.

16. Apply 1 vol of picrotoxin 10^{-4} and 1 min later after adjusting the fluid level add 1 vol of glutamate 10^{-4}. One minute later adjust fluid level to the 9 vol mark and add 1 vol of ACh. One minute later wash twice.

Evaluation of Results

The records should be carefully inspected, particularly with regard to the following.

1. Does eserine potentiate the effect of applied ACh?
2. Does eserine enhance the spontaneous contractions (both in frequency and amplitude)?
3. Does atropine depress the response to ACh?
4. Does atropine depress the spontaneous contractions?
5. Does GABA inhibit spontaneous contractions and cause relaxation? Does it cause initial contraction?
6. Does GABA depress the response to ACh?
7. Does glutamate enhance or depress the spontaneous contractions?
8. Does glutamate interfere with the action of ACh?
9. Does picrotoxin prevent the action of GABA?
10. Does picrotoxin interfere with the action of ACh?
11. Does picrotoxin interfere with the excitatory or inhibitory actions of glutamate?

The evaluation of the results should permit at least preliminary answers to the following questions. Is it likely that ACh is involved in the generation of spontaneous contractile activity, perhaps as a transmitter substance released

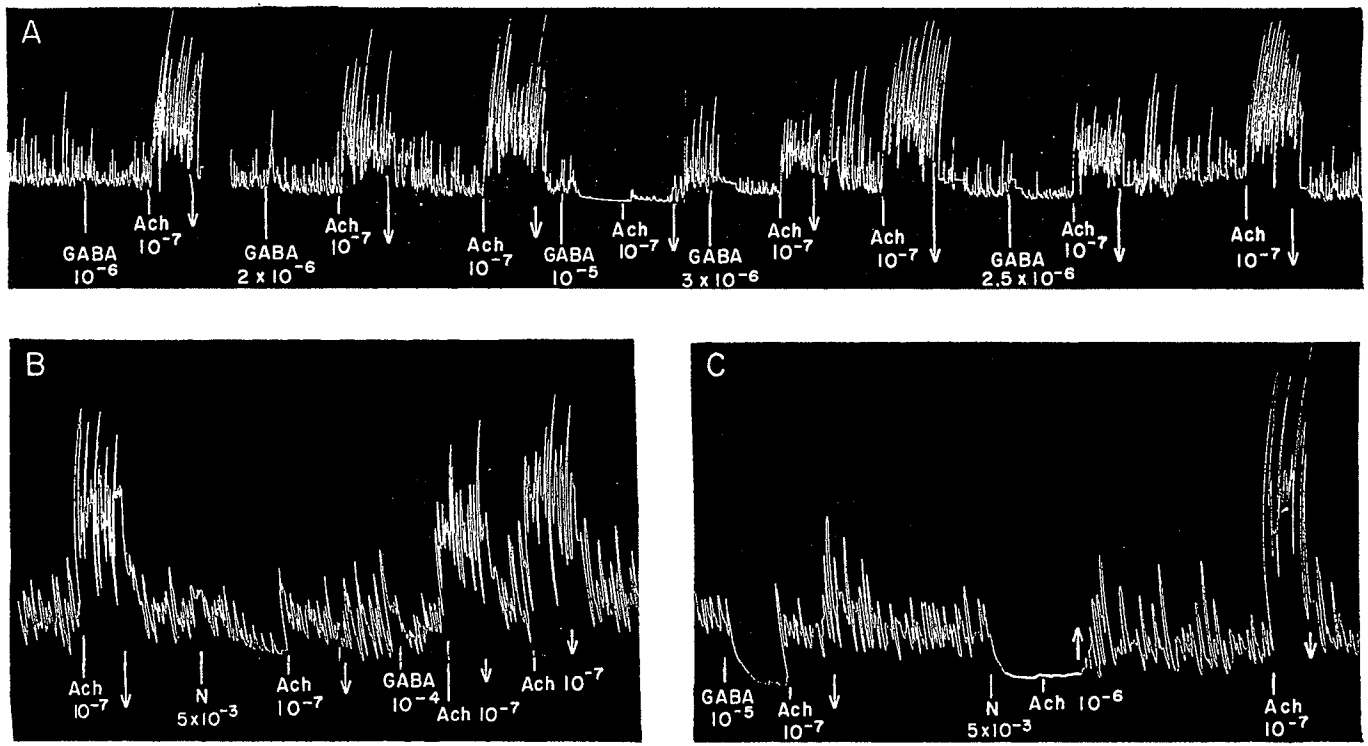
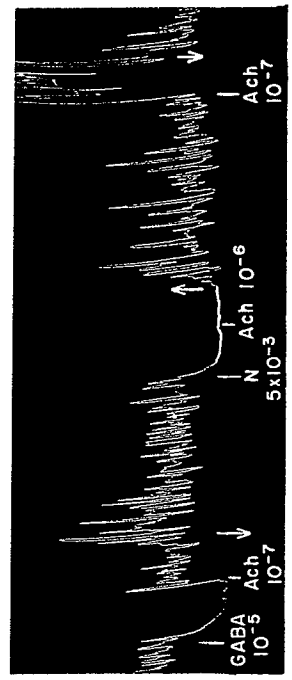


FIG. 16. Sample records obtained from an isolated hindgut of the crayfish, *Pacifastacus leniusculus*, to show the depression of spontaneous contractions and of the effect of applied acetylcholine (ACh) by γ-aminobutyric acid (GABA) and by GABA containing nerve extract, and the blocking of GABA action by picrotoxin. The nerve extract was prepared by boiling a piece of crab peripheral nerve in a tenfold [illegible] A. Interaction of GABA and ACh; note the graded response. B. Onset of the action of picrotoxin which is maintained [illegible] GABA even in high concentration is unable to prevent the action of ACh; the inhibitory action [illegible] the inhibitory actions of GABA and N are restored. The A was obtained from



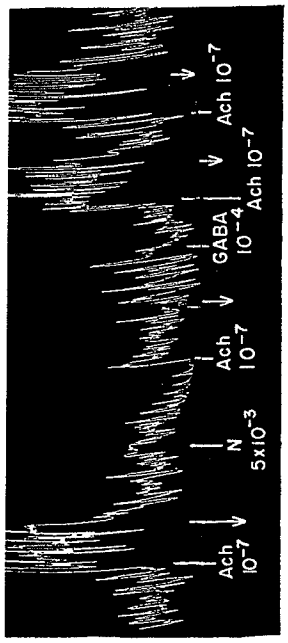


FIG. 16. Sample records obtained from an isolated hindgut of the crayfish, *Pacifastacus leniusculus*, to show the depression of spontaneous contractions and of the effect of applied acetylcholine (ACh) by γ-aminobutyric acid (GABA) and by GABA containing nerve extract, and the blocking of GABA action by picrotoxin. The nerve extract was prepared by boiling a piece of crab peripheral nerve in a tenfold volume of saline. A. Interaction of GABA and ACh; note the graded response. B. Onset of the action of picrotoxin which is maintained at a concentration of 10^{-6} g/ml; note that GABA even in high concentration is unable to prevent the action of ACh; the inhibitory action of a nerve extract (*N*) is partially blocked. C. After 15 min washing the inhibitory actions of GABA and *N* are restored. The A was obtained from a different organ from those of B and C. The arrows indicate washing. In B the washing solution contained picrotoxin. (*From Florey, 1961.*)

by neurons of the intrinsic nervous system of the hindgut? Does the action of picrotoxin indicate that inhibitory elements are involved in the spontaneous coordination of muscular activity of the gut? If GABA and glutamate act on subsynaptic membranes of either nerve or muscle cells do they act on both excitatory and inhibitory synapses?

Discuss the results in relation to the innervation of the hindgut from the sixth abdominal segment and the possibility that ACh, glutamate and GABA might be transmitter substances of regulatory exciting or inhibiting efferent nerve fibers. Consider also that ACh might be a transmitter substance of sensory neurons (Florey and Biederman, 1960).

REFERENCES

Alexandrowicz, J. S. (1909). Zur Kenntnis des sympathischen Nervensystems der Crustaceen. *Jena. Z. Naturw.* **45**, 395–444

Florey, E. (1954). Ueber die Wirkung von Acetylcholin, Adrenalin, Nor-adrenalin, Factor I und anderen Substanzen auf den isolierten des Flusskrebses *Cambarus clarkii* Girard. *Z. vergl. Physiol.* **36**, 1–8.

Florey, E. (1961). A new test preparation for bio-assay of Factor I and gamma-amino-butyric acid. *J. Physiol., Lond.* **156**, 1–7.

Florey, E. and Biederman, M. A. (1960). Studies on the distribution of factor I and acetylcholine in crustacean peripheral nerve. *J. gen. Physiol.* **43**, 509–522.

Janisch, E. (1924). Der Bau des Enddarms von Astacus fluviatilis (*Potamobius astacus* L.). *Z. wiss. Zool.* **121**, 1–63.

Jones, H. C. (1962). The action of L-glutamic acid and of structurally related compounds on the hindgut of the crayfish. *J. Physiol., Lond.* **164**, 295–300.

Tsuchiya, K. (1960). Studies on some inhibitory agents to the contraction of the isolated intestine. I. On the effects of some ω-amino acids on the isolated intestine of crayfish, rabbit and guinea-pig. *Kobe J. med. Sci.* **6**, 35–51.