

## THE USE OF THE SOUTH AFRICAN FROG (*XENOPUS LAEVIS*) IN THE STUDY OF SPINAL REFLEX PHYSIOLOGY

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### INTRODUCTION

Frogs have been valuable as test animals in neurophysiological and related investigations for many centuries. Basic facts discovered by means of frog preparations include the existence of animal electricity (Galvani, 1791), the anatomical basis of a reflex arc (Hall, 1850), the speed of nerve impulse conduction (Helmholtz, 1850), the inhibition exercised by brain centres on the cord (Sechenov, 1863) and the existence of membrane potentials (Bernstein, 1902). According to Lloyd (1943), the history of contemporary neurophysiology began with the use of the cathode ray oscillograph by Gasser & Erlanger (1922) and the discovery of the compound nature of sciatic nerve action potentials by Erlanger, Bishop & Gasser (1926). In these studies frogs were also employed.

The study of reflexes, however, necessitates some means of assessing the degree of excitability of motoneurons which constitute the final link of the reflex arc. The most satisfactory method of measuring motoneurone excitability is monosynaptic testing, a stimulus of known intensity being applied to those dorsal root fibres which have direct synaptic connections with the studied motoneurons. Because in a monosynaptic reflex arc only one synapse is interposed between the stimulated fibres and the motoneurons, the number of excited motoneurons will always be the same if the intensity of the stimuli applied to the dorsal root is kept constant (Lloyd, 1943). Only under such circumstances can the response of the motoneurons be considered as a criterion of their excitability.

The presence of a single synapse in the simplest nervous pathway in the spinal cord of the cat was demonstrated by Renshaw (1940). The result of this observation was that the majority of studies on spinal reflex activity were thereafter performed on mammals, especially cats. The existence of monosynaptic connections between afferent dorsal root fibres and motoneurons in the spinal cord of the frog was only recently demonstrated (Holemans, Meij & Meyer 1966), and the monosynaptic method of testing motoneurone excitability is thus possible in this species as well as in mammals. Since it is so much easier to conduct experiments of this type on frogs than on cats, this paper is devoted to the description of techniques in which the South African frog is used for the study of spinal physiology.

### EXPERIMENTAL

#### *Preparation of the test animals*

The frogs are kept in a refrigerated incubator at 12° C for periods of three days or more prior to the experimentation. This procedure has been adopted since it was observed that the preparations remained viable for longer periods and reacted more strongly in winter than in summer.

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The frogs are made spinal by transection of the cord below the fourth ventricle. A quarter inch incision, at the height of and parallel to the posterior edge of the occipital bone is made in the skin with a small pair of pointed scissors, which is then used to cut the occipito-vertebral ligament joining the medial part of the occipital bone with the processus spinalis of the first vertebra. This incision must not be allowed to extend laterally, or damage will be done to the vertebral artery which begins its posterior downward course in this area. A small, blunt dentist's spatula is now inserted between the posterior edge of the skull and the first vertebral arch, and the spinal cord is severed from the cranial contents. The completeness of the separation can be checked by turning the frog on its back. If this results in righting reactions, there must be connections between the brain and the cord which are still intact, and the incompleteness of the section must be remedied.

The spinal preparation is placed in position on a double-layered brass rectangle of  $7 \times 5$  in. through which water can be circulated (Plate 1), and which fits into a perspex tray of  $8 \times 6$  in. (Plate 2). Two screws furnished with nuts are attached to one end of the tray, and the other end is provided with a rack and pinion support for a small, strong hook. This hook is inserted behind the posterior edge of the skull to hold it in a fixed position. The hind limbs are held in position by hooks which grip the gastrocnemius tendon and these hooks are fastened with string to the screws by tightening the nuts. Adjustment of the rack and pinion permits the tension exerted on the vertebral column to be adjusted so as to obtain the desired degree of immobilization (Plate 3). To prevent the preparation from drying out the tray is filled with water to a level just below the dorsal surface of the frog. The environmental temperature of the frog should be kept low during the experiments, and for this purpose fluid of the desired temperature is circulated through the double brass rectangle.

### *Laminectomy*

In order to stimulate afferent fibres and record action potentials from efferent fibres, access must be gained to the spinal cord and the dorsal and ventral roots. *Xenopus laevis* has ten spinal segments but only eight separate vertebrae, the last few being united in the urostyle. The first spinal nerve emerges just above the first vertebra, the ninth between the eighth vertebra and the urostyle and the tenth through a foramen in the urostyle. The dorsal and ventral roots of VIII, IX and X, being well developed, are employed in the majority of experiments. They are exposed when the laminae of vertebrae VI, VII and VIII are removed. Small toothed forceps and scissors are used to remove the appropriate portion of the skin together with the underlying dorsal muscles so as to expose the vertebral laminae [Plate 4 (1)]. Remnants of muscular tissue adhering to the vertebrae are scraped off by means of a dentist's spatula. Laminectomy is performed under a Zeiss operation microscope. The vertebral arches consist of hard bone which is difficult to cut. They can be removed with good quality scissors, but much practice is required before this can be carried out successfully, since the slightest mechanical injury to the nervous tissue inevitably impairs or even abolishes reflex activity. The most suitable instrument for laminectomy is a high speed drill (air rotor). Number 612 burrs are used at maximal speed (60 lb/sq in. pressure, 300,000 rpm), and no pressure need be exerted when cutting through the laminae. The burrs are cooled when necessary by pressing a button which releases a jet of saline.

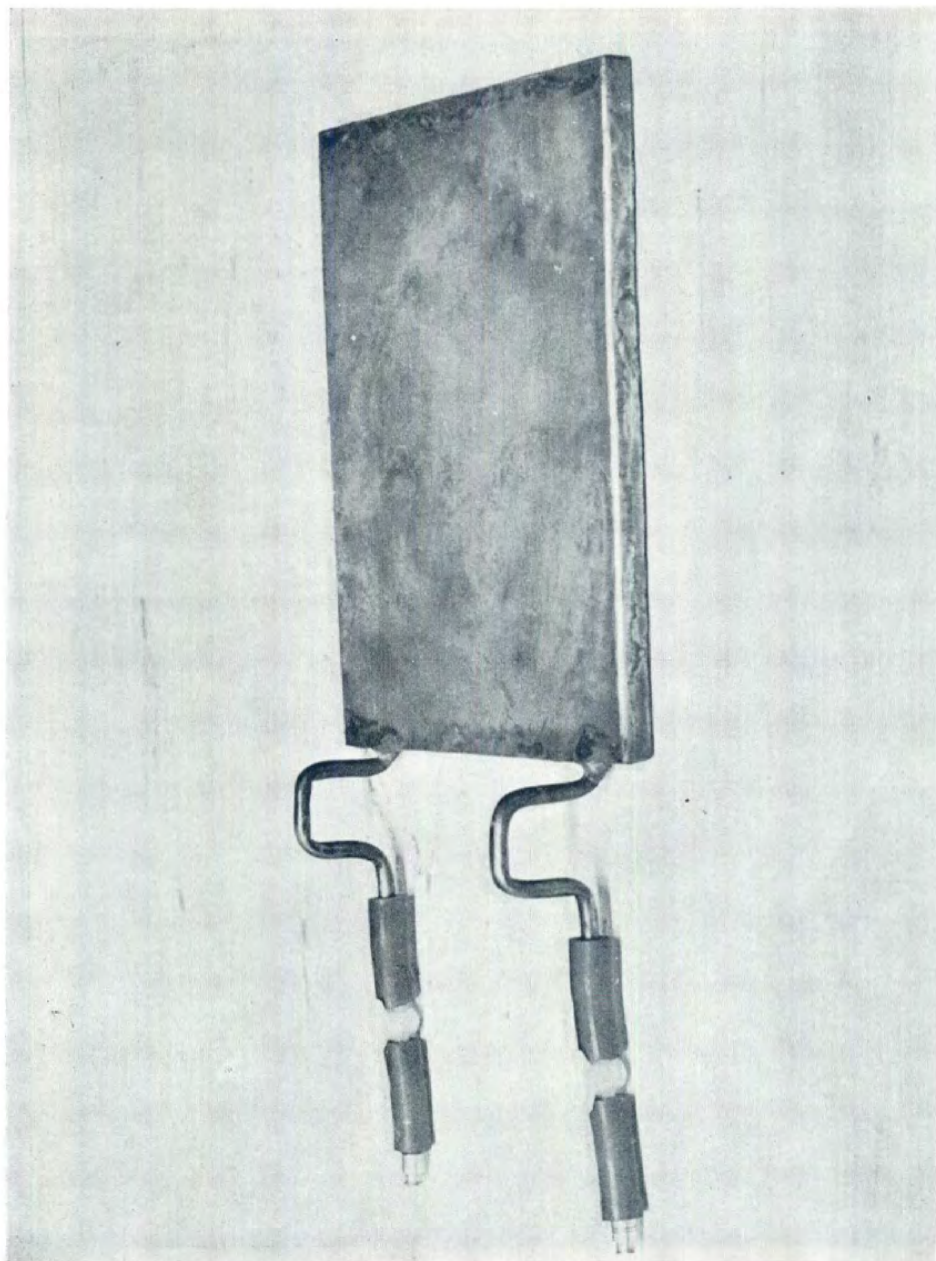


PLATE 1.—An example of a double layered brass rectangle. Liquid at the desired temperature can be circulated between the two layers through tubes connected to the rectangle

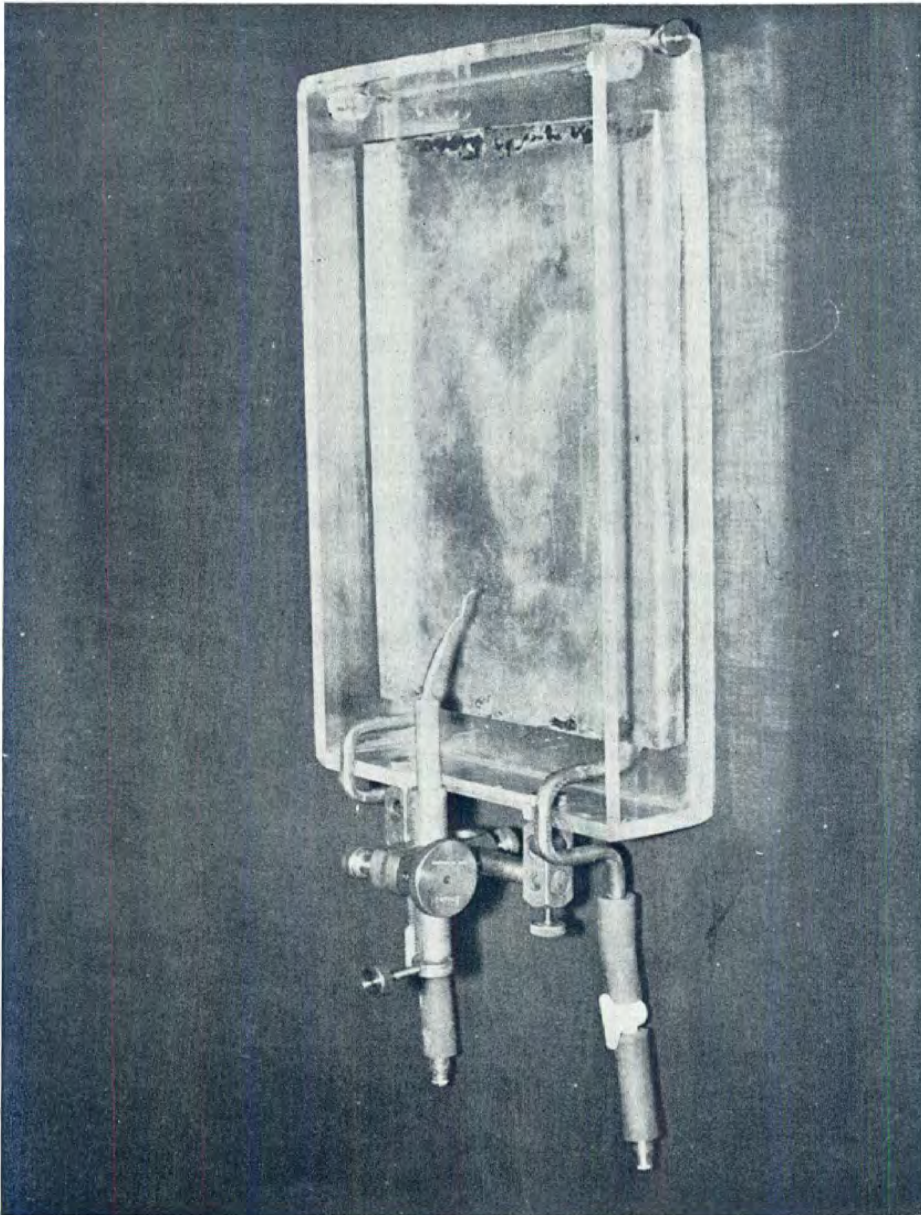


PLATE 2.—The perspex tray into which the brass rectangle fits. The rack and pinion support, carrying the hook, is attached to the one end of the tray, while two screws with nuts are fixed on to the other end

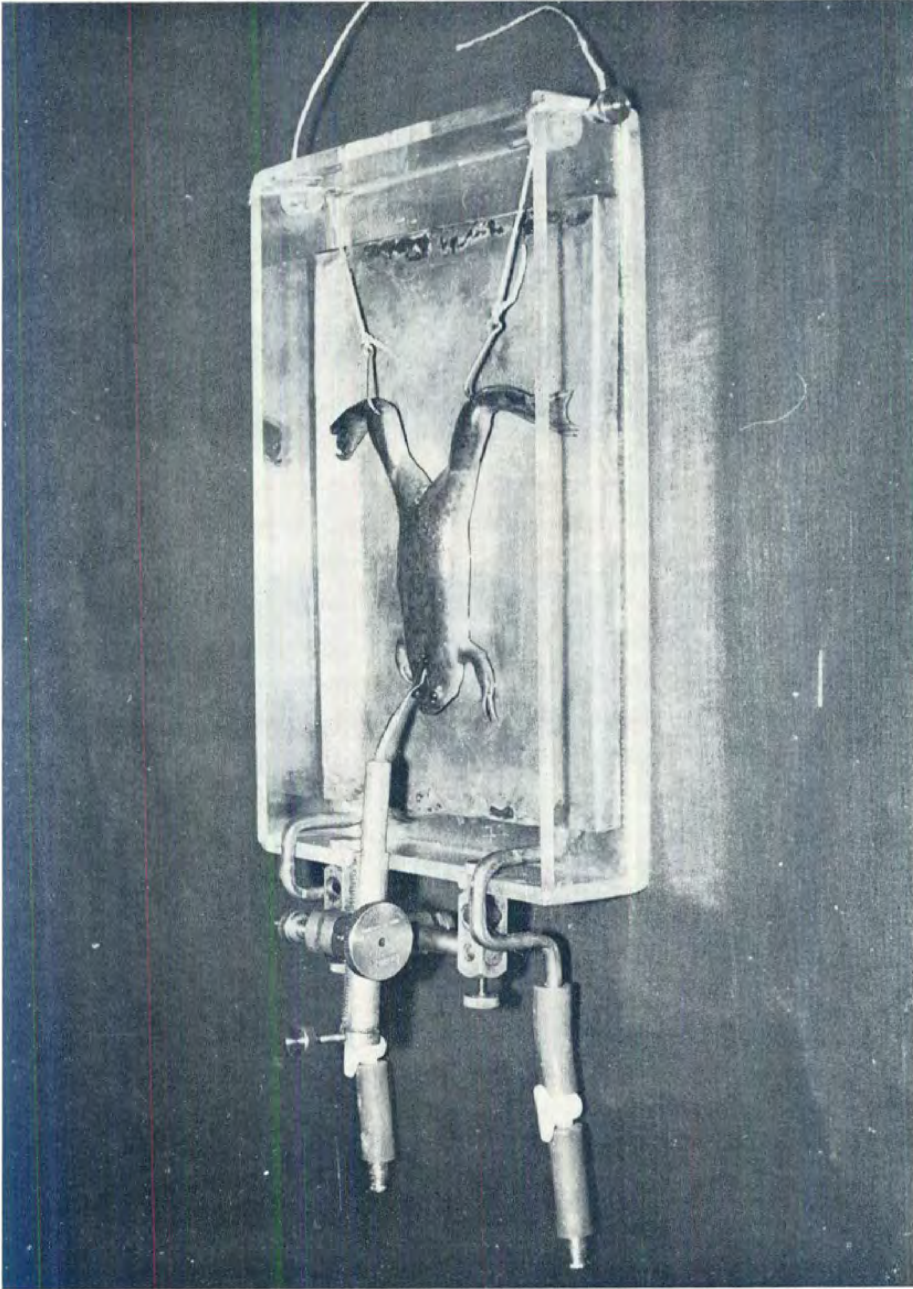


PLATE 3.—The test animal is secured in an immobile position on the brass rectangle in the tray. A hook is inserted behind the posterior edge of the skull. The hind limbs are pierced by hooks which grip each gastrocnemius tendon and these hooks are secured by means of string by the screws and nuts

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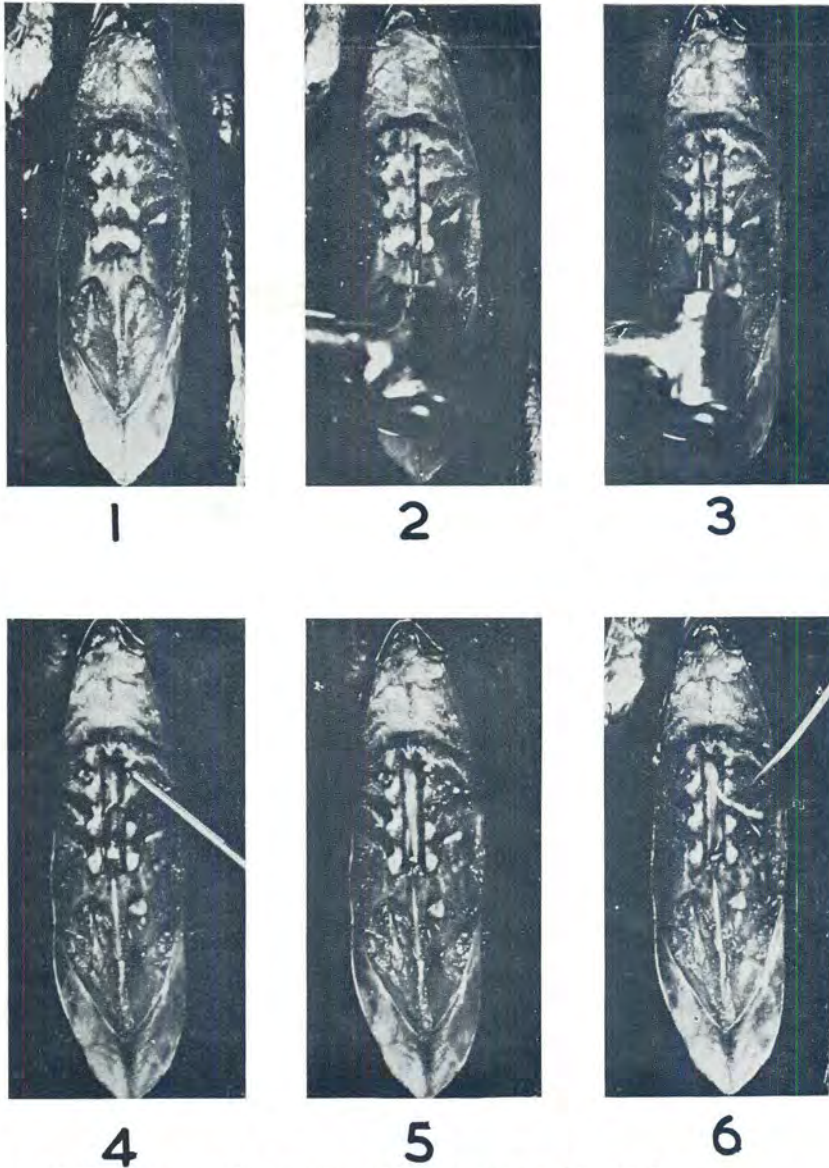


PLATE 4.—Different steps in the procedure of laminectomy are illustrated

- (1) The skin and muscles over the last three vertebrae (VI, VII and VIII) and the urostyle have been removed
- (2) The drill has cut through the laminae along the lateral processes of vertebrae VI, VII and VIII on the right side
- (3) The drill has cut along the lateral processes of the last three separate vertebrae on both sides, as well as the upper edge of vertebra VI
- (4) The arches of all three vertebrae are removed by means of small forceps
- (5) The cauda equina and conus terminalis of the cord are exposed
- (6) After identification, the required roots are each in turn lifted up, severed as distally as possible and carefully shifted laterally

The laminae are cut along the lateral processes of vertebrae VI, VII and VIII [Plate 4 (2) and (3)]. The laminae should not be completely severed, the drilling being discontinued as soon as the shimmering black surface of the meninges becomes visible. The arches of all three vertebrae are then removed by means of small hooked forceps [Plate 4 (4)]. The meninges are carefully torn away, the cauda equina and conus terminalis of the cord being thereby exposed [Plate 4 (5)]. The large medial dorsal vein in relation to the cauda equina is left untouched. If accidentally injured, it may be removed with the meninges. Bleeding from sectioned capillaries is controlled by placing pieces of cotton wool at the upper and lower ends of the wound to suck the blood away.

The different dorsal and ventral roots are now easily identified and the required roots lifted up and severed as far distally as possible with a small pair of scissors [Plate 4 (6)]. The cut roots are carefully moved to the side, keeping them in their correct order so that they can easily be identified later. Traction on these roots should be avoided as it is likely to abolish reflex activity.

#### *Additional surgical proceedings*

Longitudinal incisions are made on either side of the urostyle to expose the lumbo-sacral nerve plexus. The spinal nerves VIII, IX and X are identified and severed proximal to their point of junction to form the lumbo-sacral plexus. The reason for severing the nerves on both sides is twofold: (i) reflex activity is rather widespread in the frog and a stimulus applied to a dorsal root elicits diffuse muscle contractions and muscle action potentials which interfere with the oscilloscope record of nerve potentials, and (ii) spinal nerves may be used either for recording ventral root impulses, provided that the corresponding dorsal root is severed (de-afferented spinal nerve), or for applying stimuli which will be conducted through the dorsal roots, provided that the corresponding ventral roots are cut (de-efferented spinal nerve). Recording in this way from de-afferented peripheral nerves and stimulation of de-efferented peripheral nerves are standard procedures in neurophysiology.

#### *Electrodes used for stimulating and recording*

The fibres to be stimulated are placed on a pair of hooked wire electrodes, and are held in position by the hooks. The fibres from which the recordings are to be made are placed on a similar single electrode. The electrodes should be secured in such a position that neither they nor the severed nerve ends rest on the preparation or have contact with blood or other body fluids (Plate 5). To avoid excessive desiccation of the nervous tissue, it should be covered with liquid paraffin oil.

A variable number of electrodes are required for stimulating and recording in the small area of about two square centimetres where the nerves and nerve roots are exposed. With the Stoelting Universal stereotaxic apparatus for rats as modified by us (Plate 6), as many as eight electrodes may be used simultaneously. Perspex wedges 5 cm in length, through which protrude the silver electrodes of 34 gauge wire, are fixed to the electrode carriers. The perspex tray which holds the preparation is placed in the stereotaxic outfit and the electrodes arranged in the desired position. During use silver chloride is precipitated on to the electrodes, this being sufficient to avoid excessive polarization in the stimulating electrodes. The recording electrodes carry only small currents and here polarization is no problem. Stimulation is caused by depolarization of the fibre membrane, viz. by outward flowing currents. When the nerve fibres are stimulated through externally applied electrodes, only the cathode is effective. The anode hyperpolarizes the nerve fibre membrane, and as this could block impulses which originate under the cathode, it should be placed distally and the cathode proximally.

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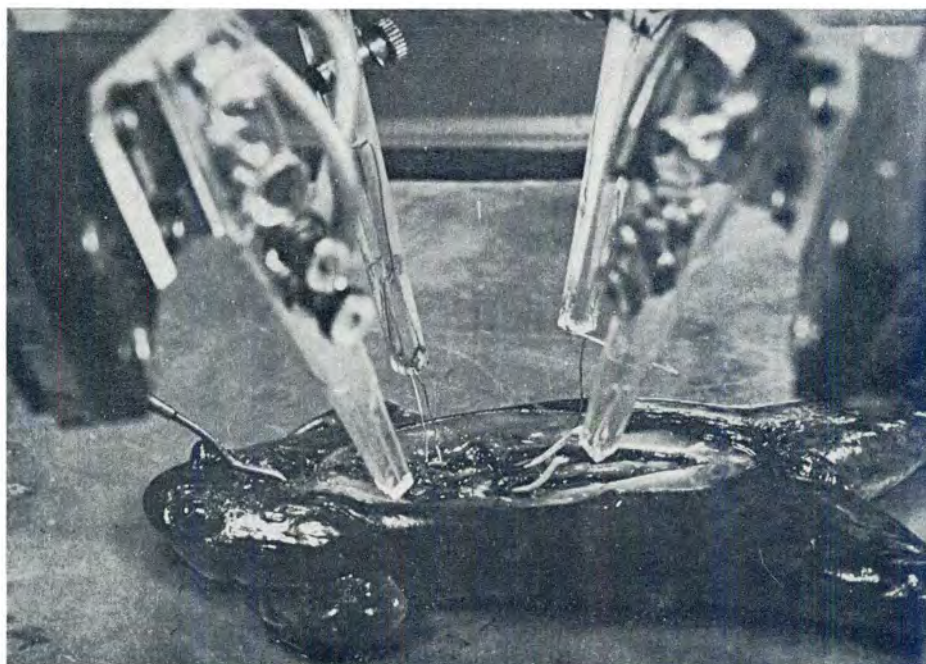


PLATE 5.—The arrangement of nerve roots and peripheral nerves on stimulating and recording electrodes



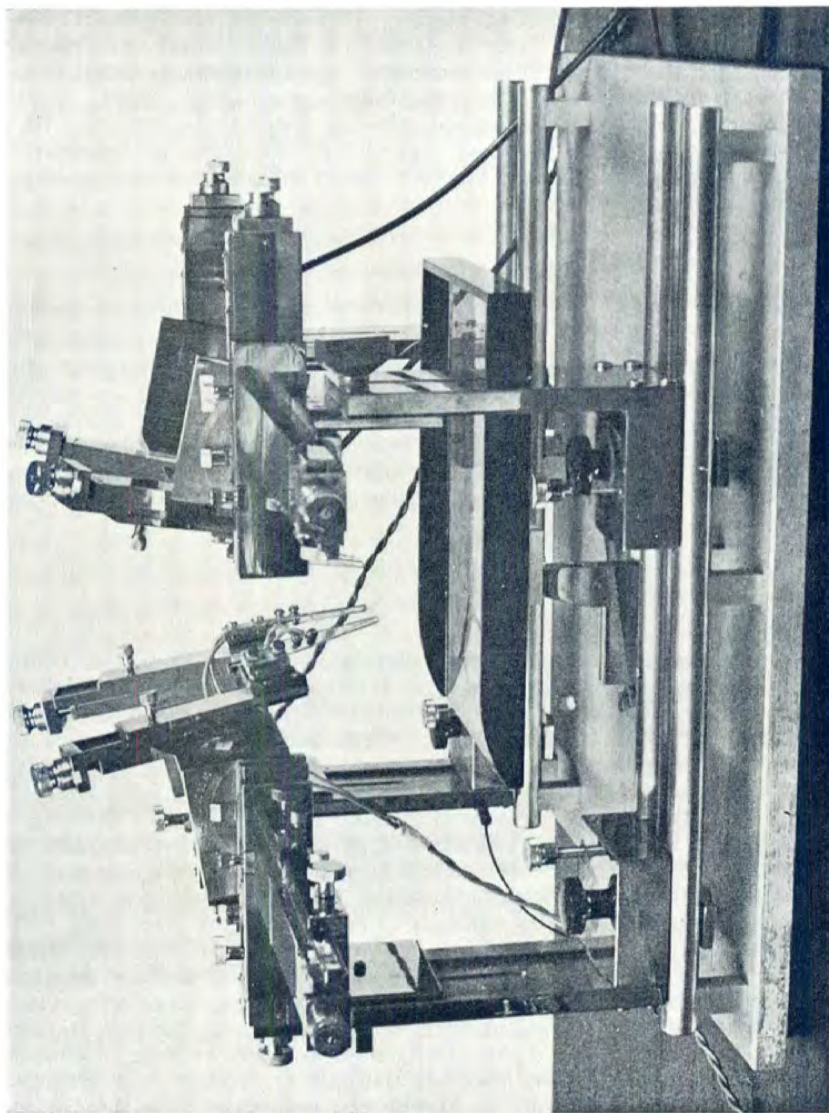


PLATE 6.—A Stoelting Universa stereotaxic apparatus for rats, adapted for frog preparations. Perspex wedges, approximately 5 cm in length, are fixed to the four electrode carriers. Two silver wire electrodes protrude through each of these, thus eight electrodes can be employed simultaneously

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### *Temperature control*

The environmental temperature of the preparation is regulated by circulating fluid of the required temperature through the double brass rectangle in the tray. The temperature of the circulating fluid can be lowered and maintained thermostatically by means of a TK 1 cooling machine (Rheinische Gerätebau GMBH, Buchs sg. Switzerland). The cold finger is placed in a Dewar vessel containing the fluid which is pumped through the brass rectangle. An electrothermometer is very useful to check the temperature of the preparation occasionally. Reflex activity in the South African frog has been studied between temperature extremes of 6° to 29° C and the observed reflex responses were highly dependent on temperature, as the reflex latency, spike duration and intensity varied with changes in temperature (Meij, Holemans & Meyer, 1966).

### *Electrical connections*

To avoid contamination of the oscilloscope recordings with 50 cycles alternating current (AC) a good earth lead is necessary. The double brass rectangle is connected to the frame of the stereotaxic apparatus and to the earth leads of the oscilloscope as well as of the stimulators. To avoid AC interference, coaxial cables are used to connect the stimulating electrodes to the stimulators and the recording electrodes to the oscilloscope. The shielding of these cables must also be carefully earthed.

### *Stimulators*

A commercial electronic square wave stimulator [DISA Multistim (DISA Elektronik A/S Herlev-Denmark)] (Plate 7) is suitable. The stimulator delivers one or two consecutive pulses; the intensity (0–50 Volt) and duration (0·05–1000 msec) of each can be varied independently. When both pulses are applied the interval between them can be varied (0–5000 msec). Either pulse, or both pulses can be applied singly or repeatedly with frequencies varying from once every 10 seconds to several hundred times per second (faradic stimulation). In the frog, it was found convenient to apply reflexogenic stimuli once every five seconds, the influence of fatigue not being noticeable at this frequency. The two pulses of the stimulator are delivered to a single pair of electrodes, but with the aid of an extension box the two pulses can be delivered through different pairs of electrodes. The stimulator is furnished with a radio frequency isolation transformer to reduce artefacts on the oscilloscope records. The instrument is furthermore equipped with a prepulse output which, if connected to the oscilloscope, triggers the sweep of the electron beams. The interval between the prepulse and the first stimulus is chosen arbitrarily. The purpose of this arrangement is firstly to have the electron beams of the oscilloscope to function only when stimuli are applied, and secondly, in order to record a correct base line, the stimuli can be applied after the electron beams have travelled some distance over the screen. The prepulse output of the stimulator can also be used to trigger a second stimulator. In most experiments on the frog more than two independent stimuli are required and an additional stimulator is in these cases necessary. The moment of stimulation can be indicated on the oscilloscope screen through a marking circuit in the stimulator.

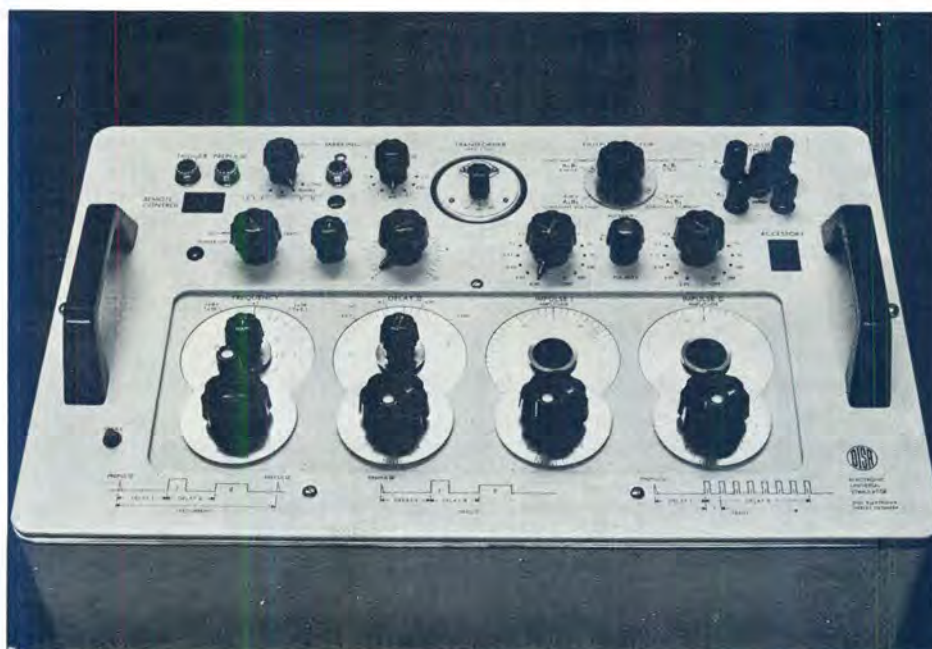


PLATE 7.—An electronic square wave stimulator (DISA Multistim), suitable for applying stimuli of known intensity (0–50 volt) and duration (0.05–1000 msec) to nervous tissue

### *Oscilloscope*

Owing to the speed of neurophysiological events, oscilloscopes are the only practical instruments for recording these phenomena. The Tektronix 502 and especially the 502 A models (Tektronix Inc. Oregon U.S.A.) are suitable for recording nerve potentials. Their input impedance of 1 Megohm is rather low for recording single unit activity, but it can be increased sufficiently by means of special preamplifiers. The input capacitance of 47 pF is adequate for most purposes. The sensitivity is adjustable from 20 volt up to 100 microvolt per cm beam deflection. The amplifiers can be operated either in AC (for fast potentials) or direct current (DC) mode (slow potentials), both with single or differential input. The vertical amplifiers for the two beams are independent of each other, but the horizontal movement is synchronized.

### *Recording of action potentials*

In our experiments, action potentials are recorded by a single electrode, its potential being pitted against the earth. The oscilloscope is operated in AC mode, using one input terminal of the amplifiers only. With this method of recording, the action potentials of a nerve approaches the recording electrode, but does not pass it, as the nerve is severed distally. Under these circumstances, a practically monophasic positive deflection can be expected (Lorente de N6, 1947). This is displayed on the oscilloscope screen as a downward spike, according to the convention of neurophysiologists of recording negative potentials by upward deflections of the electron beam.

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### *Recording of slow potentials*

If the duration of the potential deviations of the recording electrode exceed 10 to 20 msec, the oscilloscope amplifiers are operated in DC mode to avoid distortions of such slow potentials. Slow dorsal and ventral root potentials have a duration of several hundred msecs and can thus only be studied with directly coupled amplifiers. These potentials are recorded by means of two electrodes of which one is placed as close as possible to the cord and the second as distally as the length of the severed root permits. The proximal electrode is connected to the common earth and the distally placed electrode to the grid of the first amplifier valve (Eccles, 1946).



PLATE 8.—A practical arrangement of apparatus for studying the spinal physiology of the frog

- A. TK 1 cooling machine
- B. Stereotaxic apparatus
- C. Oscilloscope with camera
- D. Electrothermometer
- E. Stimulator

The practical combination of stereotaxic apparatus, cooling and/or heating installation, electrothermometer, stimulators and oscilloscope is illustrated in Plate 8. For measuring reflex phenomena, the reflex is elicited with intervals of five sec and measured from four to ten times directly on the graticule of the oscilloscope screen. The tracing of the spot remains visible for several seconds, owing to the coating of the cathode ray tube with medium speed phosphorus. For permanent recordings, an oscilloscope camera is fixed in front of the screen and the shutter is opened before the sweep is triggered and shut immediately afterwards. A high contrast, slow speed (10 ASA) process film (Gevaert Duplo Pan) has been found satisfactory.

SUMMARY

The use of the South African frog (*Xenopus laevis*) in studies on spinal neurophysiology is introduced for the study of monosynaptic reflexes. Motorneurone excitability in this species may be measured by the classical procedure of monosynaptic testing. Operational procedures for exposing the spinal cord and the roots of the spinal nerves are described. Detailed information on stimulating and recording techniques which have been found satisfactory for the South African frog are given.

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