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METHODS FOR STUDYING THE REFLEX ACTIVITY OF THE FROG'S SPINAL CORD

BY

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This paper outlines a method for testing the actions of drugs on the frog's spinal cord. The preparation described here has proved useful for demonstrating the classical properties of spinal reflexes and is more predictable in its behaviour than the spinal or decerebrate cat. Moreover, the frog preparation is sensitive to many drugs with known actions on the mammalian cord and should be found convenient for testing new compounds. Our primary reason for the development of this method was, in fact, the need for a simple preparation which might help in a search for hypothetical humoral transmitters in spinal reflex arcs (Burgen and MacIntosh, in press; Brock, Coombs, and Eccles, 1952). This interest defined the requirements of our preparation and we consequently sought a system which would provide:

(*a*) isolation of the spinal cord from the systemic circulation in such a manner that spinal reflexes remained excitable for several hours,

(*b*) a means of recording continuously both reflex excitation and reflex inhibition, and

(*c*) a slow flow of Ringer's solution over the active cord.

The method we have found most successful is unusual in that the vascular system is perfused with gaseous oxygen.

METHODS

Principal Method

Perfusion of the Vertebral Canal (Fig. 1).—The Ringer solution used contained 6.5 g. NaCl, 0.14 g. KCl, 0.12 g. $CaCl_2$, 2.0 g. dextrose, and 30 ml. of 0.15 M-sodium phosphate buffer (*p*H 7.4) per litre. This fluid flowed from a reservoir bottle (A), some 30 cm. above the frog, fitted with a Marriotte tube to ensure a constant pressure-head. The outlet from the reservoir supplied the frog through two parallel alternative channels; each channel contained a drop-counter (B, C). The common outlet from the drop-counters was through a T-tap (E), and the flow was adjusted by means of a screw-clamp (G) on the single plastic tube of 1.0 mm. internal diameter leading to the vertebral cannula. The latter was made by grinding off the sharp tip of a No. 18 intravenous needle and sawing off the butt.

During control periods the vertebral canal was supplied through drop-counter B (Fig. 1); test solutions were introduced through the heavy rubber tubing beneath drop-counter C, which was connected to an air-trap (D). The counter used for the test solutions was fitted with a tap (F) for rinsing. The double route for perfusion fluid made it easy to change from the control to a test perfusion and back again without interruption or change of flow.

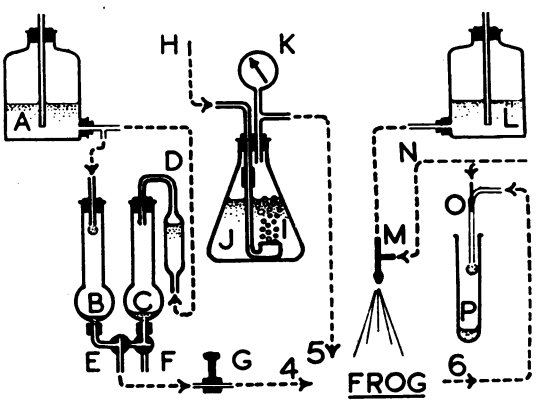

FIG. 1.—A diagram of the apparatus. The numbers 4, 5, and 6 correspond with the same numbers in Fig. 2. A, Reservoir containing Ringer solution fitted with Marriotte tube. B, Drop-counter for control perfusion. C, Drop-counter for test solutions. D, Air trap. E and F, T-taps. G, Screw clamp on plastic tube leading to cannula. H, Gas line from cylinder. I, Bubbler for saturating gas with water vapour. J, Ringer solution in saturating flask. K, Pressure gauge. L, Reservoir containing Ringer solution for spray. M, Atomizer for spraying frog's skin and muscle. N, Compressed air line. O, Hypodermic needle pushed into fine plastic tube. This provides suction. P, Test tube for collecting effluent from the vertebral canal. 4, Ringer fluid supply to vertebral canal. 5, Gas supply to vascular system. 6, Fine plastic tubing for collection of effluent from the vertebral canal.

Perfusion of the Vascular System with Gas (Fig 1).—Gas was supplied from standard commercial cylinders fitted with pressure regulators. It was saturated with water vapour by passing it in small bubbles through water or Ringer solution (I, J) from which it ran through 1.0 mm. bore plastic tubing to a

cannula, made from a No. 20 intravenous needle. A gauge (K) attached to the saturating vessel registered pressure. A mixture of 97% O_2 and 3% CO_2 was used for most of the experiments, but the effects of some other gas mixtures were investigated.

Operative Procedure.—The frogs used were all *Rana pipiens*. After the brain had been destroyed by pithing, the skin of the thigh was slit over the biceps femoris muscle, and the distal two-thirds of the muscle freed from adjacent fascia and the underlying blood vessels and sciatic nerve. A thread was tied around the tendon close to its insertion at the knee. The skin was then slit over the vertebral column and the spinal cord was exposed in the brachial region by dissecting away the tops of the neural arches. Further preparation of the vertebral canal for perfusion was left until after the gas perfusion of the vascular system had been established.

The frog was then turned on to its back and the abdominal cavity was opened. A ligature was tied around the base of each lung and another around the two systemic arches just superior to the origin of the dorsal aorta. These ligatures excluded the lungs, the viscera, and the posterior part of the frog from the general circulation. The heart was then exposed and the cannula attached to the gas supply line was tied into the truncus arteriosus. The cannula was made from a No. 18 intravenous needle. Perfusion with gas was begun, the efferent gas escaping from a slit made in the sinus venosus. The gas was supplied at constant pressure, between 100 and 140 mm. Hg in different experiments.

The test muscle and the peripheral nerves supplying it were isolated from both perfusions. In spite of this isolation, reflex responses remained satisfactory for periods as long as 6–8 hr.

The frog was turned back on to its belly and the cannula for the Ringer perfusion was pushed forwards into the vertebral canal through the gap made by flexing the joint of the urostyle and the vertebral column proper. If this was properly done the cannula remained firmly in place, with no leakage of fluid around its shank. The Ringer perfusion was then started, the fluid flowing out of the canal in the brachial region.

The frog was pinned firmly in position on the cork board (Fig. 2). If the knee joints are pinned so that the two femurs lie in a straight line, distortion of the record due to occasional movements of the frog is reduced. Finally, the exposed section of the spinal cord was crushed with forceps and removed, leaving a cavity from which the cord perfusate was collected by gentle suction (Fig. 1, O, P; Fig. 2, 6).

Throughout the experiment the exposed tissues and the remaining skin of the frog were kept moist by a spray of Ringer solution (Fig. 1) of the same composition as that used for the cord perfusion except that it contained no dextrose. The atomizer (M) was of the ordinary nasal spray type and was supplied with Ringer from a stock bottle (L) and a constant current of air from the compressed air mains (N).

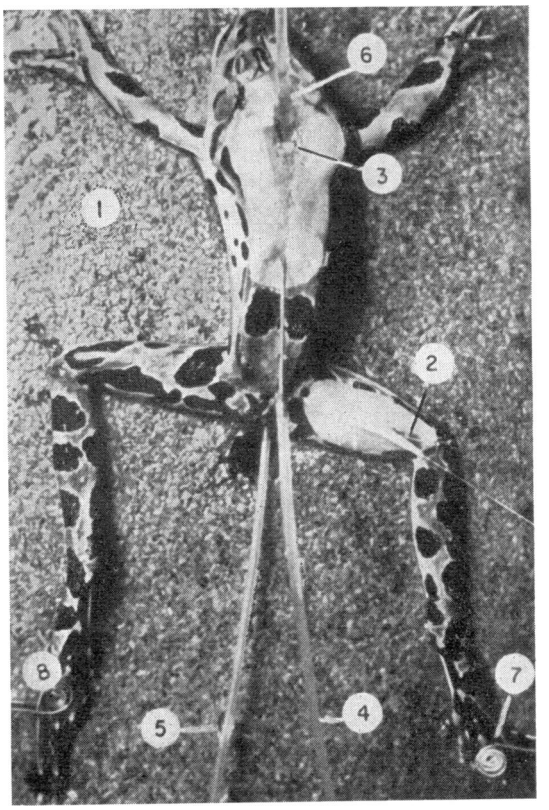

FIG. 2.—A frog prepared for an experiment. 1, Cork mounting board. 2, The biceps femoris muscle. 3, The cut end of the spinal cord. 4, The Ringer solution supply line to the vertebral canal. 5, The gas supply line to the vascular system. 6, The suction tube for collecting the effluent perfusate. 7 and 8, The stimulating electrodes in position on the feet.

Stimulation.—The electrical stimuli applied to the animal's feet were sinusoidal at 60 c.p.s. and variable between 0–20 volts. Two channels of stimulation were available and the strengths of the stimuli applied to each foot of the frog could be varied independently. The timing of the stimulation periods was controlled by standard, commercial, adjustable commutators driven by synchro-motors connected to the A.C. mains (R. W. Cramer & Co.). These were arranged so that :

1. Every n sec. the biceps femoris muscle was reflexly stimulated by a train of stimuli (S_e) of duration d_e applied to the ipsilateral foot. n was variable between 10–120 sec., but was usually 30 sec.; d_e was variable between 0–10 sec., but was usually 1 sec.

2. Every $2n$ sec. a train of stimuli (S_i) of duration d_i could be given to the other foot in order to produce reflex inhibition of the response to S_e. d_i was variable but was set so as to be $2d_e$, and the timing was arranged so that S_i began before S_e and finished after S_e was over.

The bipolar stimulating electrodes were flat coils of silver wire, and these were applied above and below a foot (Figs. 2, 7, and 8).

(In the experiments reported here, stimulation has been regularly applied at two sites only, the foot ipsilateral to the dissected muscle, and the contralateral foot; the responses are flexor responses presumably involving a polysynaptic reflex path. The method should, however, be adaptable to the investigation of monosynaptic reflexes or to other reflexes, evoked by stimuli restricted to the skin or to chosen nerve trunks.)

Recording of the Muscular Contractions.—The tendon of the muscle was severed close to the knee, and the thread already attached to it was passed under a pulley to a lever writing on a slowly moving smoked drum; the muscle lay in its natural position during the experiment. The recorded contractions were isotonic, the muscle working against a weight of 2 g. The electrodes were put in position on the two hind feet and stimulation was carried out at regular intervals, as described above. Since alternate stimuli to the foot on the same side as the dissected muscle were accompanied by stimulation of the contralateral foot, the records show a regular succession of uninhibited and inhibited contractions. Fig. 2 shows the frog prepared for the experiment, and Fig. 3 a control record.

In this record the responses are stable for a period of over half an hour and very regular. It is always possible to produce such a regular record in a good preparation, provided that the cord has been transected behind the brachial enlargement, and that the stimulus strength is such as to produce a maximal or nearly maximal response on ipsilateral stimulation. When the stimulus is well below maximal, the responses often become less regular, as the preparation is easily affected by small changes in the area of contact of the electrodes, or in the afferent input from the skin and viscera supplied by the remaining section of cord posterior to the brachial region. Submaximal stimuli have nevertheless been preferred, since with these it was easier to detect the augmentation of the reflex response which occurred with some procedures. In Fig 4, the submaximal responses in the control period, and again after recovery, were stable; in Figs. 5 and 6 the responses, especially the inhibited responses, show a greater irregularity, but the average level was sufficiently constant for any important change due to altered conditions to be unmistakable. In general, a control period was regarded as satisfactory if the uninhibited responses did not show random variations from the mean of more than $\pm 15\%$ and showed no steady trend.

Other Methods Investigated

Before the adoption of the technique described above, several other methods of maintaining a preparation that would show good reflex activity for some hours were tried. Two of these procedures were successful. Although not suitable for investigating the possible liberation of transmitting substances in the cord, they could be used to test the effects of drugs on spinal reflexes. In neither of the two methods was the vascular system perfused. Circulation was arrested by excision of the heart, and the cord supplied with oxygen and Ringer solution through the vertebral canal perfusion. The first method described below, in which the vascular system was perfused with oxygenated Ringer solution, was a failure.

1. *Double Perfusion using a Ringer Solution in both the Intravertebral and Vascular Perfusion Systems.*—Some attempts were made to supply the cord with oxygenated Ringer flowing through the anterior part of the vascular system, using at the same time a slow intravertebral perfusion with Ringer as a route for the introduction of drugs. No successful preparation was obtained in this way: with low vascular perfusion pressures responses rapidly declined; with high perfusion pressures all tissues became oedematous. The use of gum in the Ringer prevented the oedema, but slowed the perfusion rate so much that even at high pressures oxygenation became inadequate. Attempts to improve this method were abandoned when the gaseous perfusion was found to be effective.

2. *Intravertebral Perfusion with Oxygenated Ringer Solution.*—The vertebral canal was perfused with

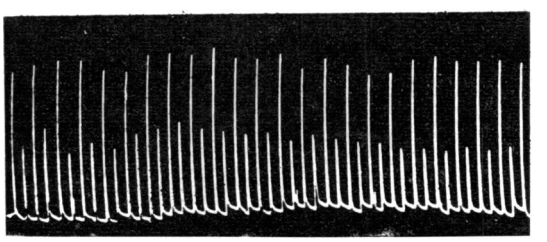

FIG. 3.—Reflex contractions of biceps femoris muscle. A control record. The long strokes are the reflex responses of the muscle to stimulation of the ipsilateral foot, the short strokes the inhibited responses which result when the two feet are stimulated together. Interval between stimuli, 30 sec.

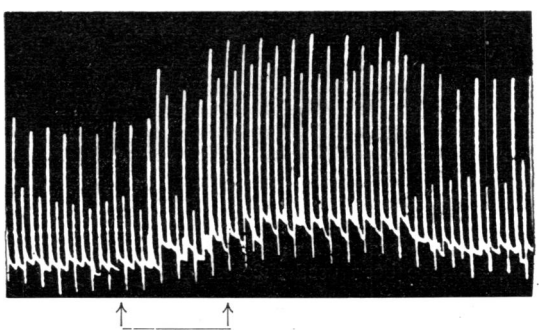

FIG. 4.—Reflex contractions of biceps femoris muscle. The effect of six minutes of anoxia. N_2 was substituted for O_2 during the period between the arrows. Interval between stimuli, 30 sec.

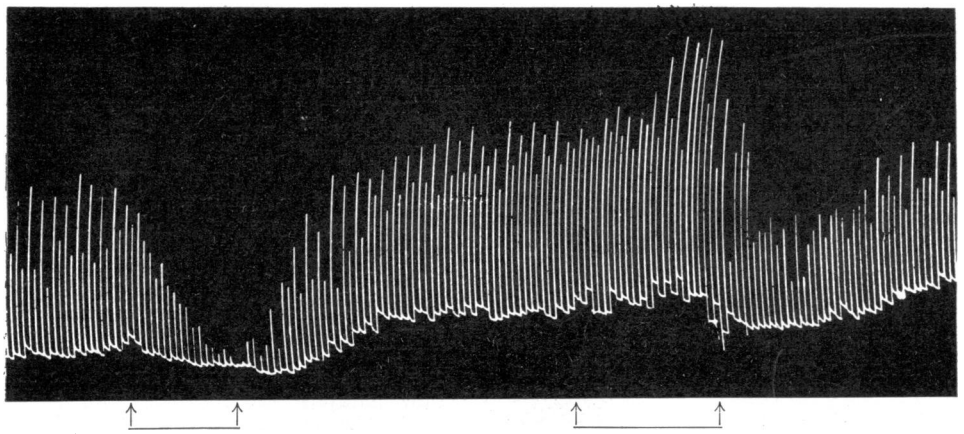

FIG. 5.—Reflex contractions of biceps femoris muscle. The effects of N_2O and anoxia. N_2O was substituted for O_2 for a 10 min. period between the first pair of arrows, N_2 for O_2 during an 11 min. period between the second pair of arrows. The complete recovery from anoxia is not shown; it was a slower process than the recovery from N_2O, which was complete in 7.5 min. Interval between stimuli, 30 sec.

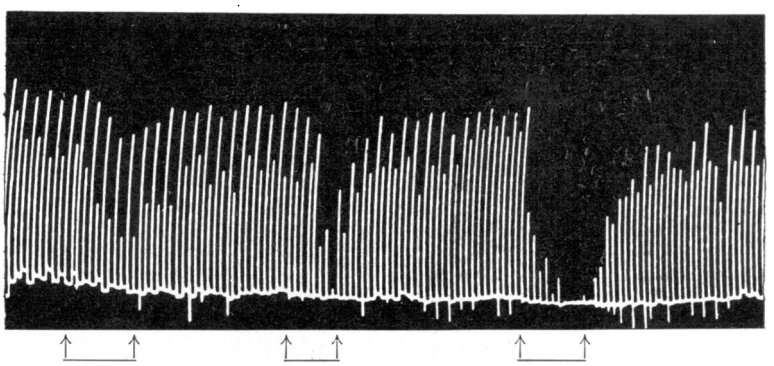

FIG. 6.—Reflex contractions of biceps femoris muscle. The effect of ether. Ether was introduced in solution in the Ringer fluid perfusing the vertebral canal: between the first pair of arrows, ether 10^{-3} for 5 min.; between the second pair of arrows, ether 2×10^{-3} for 2.5 min.; between the third pair of arrows, ether 2×10^{-3} for 4 min. Interval between stimuli, 30 sec. Flow, 0.5 ml./min.

Ringer solution oxygenated by bubbling a mixture of 97% O_2 and 3% CO_2 through the reservoir bottle. This single perfusion will maintain good reflex activity for several hours provided that the flow along the vertebral canal exceeds 3 ml. per min. This method is simple, but the rapid flow not only uses up large volumes of Ringer solution, but would result in such a dilution of any liberated transmitter that the chances of demonstrating its presence would be vanishingly small. If the flow rate is decreased, the reflex responses invariably decline. This decline must be due to insufficient oxygen supply, since a slow flow of Ringer does not depress the responses when oxygen is supplied through the vascular system. In the latter case flow rates as low as 0.05–0.1 ml. per min. have been used successfully.

3. *Intravertebral Perfusion with Ringer and Gas Mixtures.*—In this variant of the single perfusion technique, a hypodermic needle was inserted into the Ringer supply line so that its tip projected into the lumen of the vertebral canal cannula; through it was introduced the O_2 and CO_2 gas mixture. It was possible to adjust the flow of gas so that the jet of bubbles did not jar the cord, which remained reflexly active for a period of hours even with very slow rates of fluid perfusion. This method is more economical of Ringer's fluid than the previous one, and has been used successfully in experiments for testing the effects of drugs and anoxia on the cord. It has the disadvantage that small changes in the rate of flow of gas alter the Ringer perfusion rate, so that it is more difficult to keep experimental conditions constant than with the double perfusion system described earlier.

RESULTS

Preliminary tests of the sensitivity of the preparation to anoxia and a number of drugs have demonstrated its usefulness. The concentrations of drugs given below are not necessarily the lowest concen-

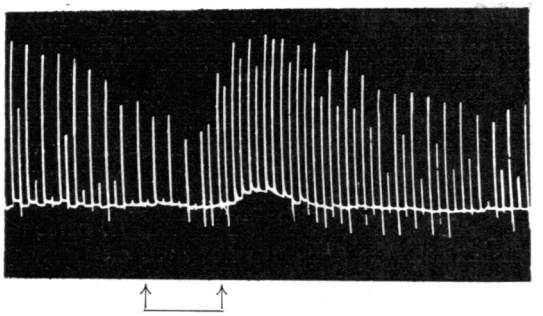

FIG. 7.—Reflex contractions of biceps femoris muscle. The effect of nicotine tartrate. Ringer solution containing nicotine tartrate in a concentration of 2×10^{-5} flowed through the vertebral canal for 6 min. in the period between the arrows. Interval between stimuli, 30 sec. Flow, 0.75 ml./min.

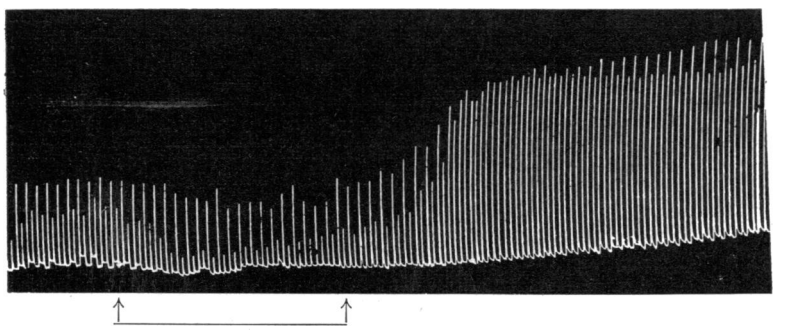

FIG. 8.—Reflex contractions of biceps femoris muscle. The effect of strychnine sulphate. Ringer solution containing strychnine sulphate 10^{-4} was perfused through the vertebral canal for 24 min. during the period between the arrows. Interval between stimuli, 30 sec. Flow, 0.1 ml./min.

trations which could produce an effect, as a wide range of concentrations has not yet been used. Moreover, the effect of any drug upon the cord will depend on the flow of saline and the duration of exposure.

Effects of Changing the Gas Mixture in the Vascular System.—Anoxia was produced by substituting N_2 for O_2, the concentration of CO_2 being kept at 3% in both cases. An increase of the reflex contractions occurred, as it does in mammals during anoxia. Kirstein (1951) has reviewed the pertinent literature and Brooks and Eccles (1947) have shown that the synaptic potentials of mononeurones are increased during anoxia. Our records show, in addition, that stimuli which produced definite inhibition in the control period became ineffective in depressing the reflex response during anoxia. If the period of anoxia was short, these effects were rapidly and completely reversible (Fig. 4). Continued anoxia depresses and finally abolishes both the uninhibited and the inhibited reflex responses.

Nitrous oxide substituted for O_2 had a purely depressant effect, which was rapidly reversible on the readmission of O_2. This immediate depression contrasts with the initial potentiating effect of anoxia. Fig. 5 shows the very different effects of N_2O and N_2 on the same preparation.

Effects of Drugs Introduced into the Ringer Perfusing the Vertebral Canal.—Ether was introduced dissolved in the perfusate of the vertebral canal. Like N_2O, its effect was to depress the reflex contractions, the degree of depression depending on the concentration and the duration of the exposure of the cord to it (Fig. 6).

Nicotine tartrate increased the reflex responses. There was typically a rise in tone with the first dose of nicotine (Fig. 7). Subsequent doses produced less or no rise in tone, and smaller increases in the reflex contraction.

Strychnine sulphate augmented the reflex response, and inhibitory stimuli became ineffective. Under the conditions of these experiments, the effects of strychnine were slow to develop (Fig. 8). It can be seen from the record that inhibition not only failed, but ultimately bilateral stimulation produced a greater reflex contraction than did ipsilateral stimulation alone. The same phenomenon has been reported in the toad by Kato (1934). In mammals a similar increase in flexor contractions occurs after the injection of strychnine (Bernhard, Taverner, and Widen, 1951; Bradley and Eccles, 1953). Anoxia in the frog (Figs. 4 and 5) produces comparable increases in the muscular response, with the same failure of inhibitory stimulation.

DISCUSSION

The use of a frog rather than a mammal simplifies both the operative procedure and the subsequent conduct of the experiment: preliminary anaesthesia is not necessary, the cord is more accessible, and muscles isolated from any circulation remain capable of contraction for hours; in no case has an experiment been terminated because of fatigue of the muscle. An added convenience is that the experiment can be carried out with the animal at room temperature if a sufficient supply of oxygen to the cord can be arranged.

The use of the double perfusion system described here for the frog allows (1) changes in the composition of the gas mixture in the vascular system without interfering in any way with the Ringer

perfusion of the vertebral canal, and (2) changes in the chemical composition of the fluid perfusing the canal without interference with the oxygen supply to the cord. It has the added advantage that very slow flows of Ringer may be used, so that any substances liberated during cord activity will not be too greatly diluted in the perfusate, which is an important consideration if the liberation of synaptic transmitters in the cord is to be demonstrated.

A simple preparation in which the muscle responses are recorded as described, but the natural circulation of the frog is left intact except for the damage inevitable in pithing, has proved very useful in teaching for the demonstration of the classical properties of spinal reflexes. Electrodes may be placed in any suitable position, and the facilitory or inhibitory effects of changing the stimulus site, frequency, or strength studied. If in addition a Ringer perfusion of the vertebral canal is used, the effects of drugs or anaesthetics on the reflex activity of the cord can be readily demonstrated. Such a method does not meet the more rigid requirements for research, since with the natural circulation intact the diffusion in drugs into the spinal vessels would result in their distribution to tissues other than the cord.

Many of the previous experiments in which the effects of varied stimuli and drugs were tested on amphibian spinal reflexes have been done on spinal frogs kept alive on their normal circulation. Whether the drugs were injected intravascularly (Bremer and Moldaver, 1934 ; Bonnet and Bremer, 1937) or dripped on to an exposed section of cord (Lefebvre and Minz, 1936), such experiments do not exclude the possibility that part of the observed change in the responses is due to contributing effects from actions of the drug elsewhere than on the cord itself ; and if the perfusate is to be tested for transmitters, blood is an unsuitable vehicle. Torda (1940) in her experiments perfused a spinal toad with Ringer, isolating her test muscle, the gastrocnemius, from communication with the rest of the body except via the sciatic nerve, thus excluding the muscle from the effect of the circulating drug. This method again, because it does not restrict perfusion to the spinal cord, is unsuitable for our purposes.

A method similar in some respects to ours has recently been published by Häusler and Sterz (1952). The test frog (*Rana esculenta*) was pithed and eviscerated. Perfusion of the intradural space with Ringer containing dextrose was effected by putting in a cannula through the pithing hole, and cutting across the posterior end of the cord to allow the fluid to escape. A second Ringer perfusion supplied the forelimbs, and the flexion of one forelimb to stimulation of the contralateral forelimb was recorded. This preparation gave good reflex responses for hours with flows as slow as 1–2 ml./min. over the cord. The Ringer was saturated at a low temperature with oxygen and allowed to warm to room temperature before it was perfused through the frog (Häusler, personal communication). We have not tried oxygenating Ringer in this way, but our preparations did not remain in good condition on such slow flows of Ringer saturated at room temperature with oxygen. Whether the difference in survival time between our frogs and those of Häusler and Sterz is due to differences in saturation of the Ringer fluid with oxygen, to a lower " room temperature " in Graz than in Montreal, or to a species difference, we do not know, as we have not tried to repeat their experiments.

Kato and his co-workers (Kato, 1934) sometimes used a preparation consisting of the spinal cord with its attached nerve roots, the sciatic nerve, and the gastrocnemius muscle. The effects of drugs on the reflex responses were tested by immersing the cord in the appropriate solution. The reflexes of a frog's completely isolated spinal cord have been studied by Eccles (1946) using the electrical response of a ventral root as the sign of reflex activity. The cord, kept in Ringer solution in a chamber with a stream of oxygen passing through it, gave uniform reflex responses on appropriate stimulation of the dorsal root for a period of hours. The effects of drugs on the reflex could be tested by adding them to the Ringer in the chamber. The method requires that the experiments must be carried out at the low temperature of 10° C. (Eccles, personal communication) if the cord is to survive.

There seems no reason why the method we have described should not be adaptable to the conditions of electrical recording. Some modification would obviously be required if access to the spinal cord or its roots was needed, but the maintenance of reflex activity by oxygen-perfusion should prove useful, since the experiments could then be carried out at room temperature.

Summary

1. Methods of keeping the perfused spinal cord of the frog reflexly active for several hours are described.

2. The usefulness of the different methods in demonstrating the classical properties of spinal

reflexes, and in testing the effects of drugs on uninhibited and inhibited reflex responses, is discussed.

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