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EXPERIMENTS WITH PERFUSED FROG'S SPINAL CORD

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The study of the effects of drugs on spinal reflexes and the identification of substances liberated during nervous activity are of interest in connexion with the hypothesis of the chemical transmission of nervous impulses. The object of the present work was to study the effects of drugs on spinal reflexes in the frog, and to try to detect the liberation of active substances during activity.

The method described by Bunzl, Burgen, Burns, Pedley, and Terroux (1954) has been found suitable, with some modifications, for this investigation. In this method gaseous oxygen is perfused through the blood vessels in order to supply oxygen to the spinal cord, and Ringer solution containing glucose is allowed to run through the vertebral canal. Drugs which are added to the Ringer solution then act directly on the spinal cord without interference from other tissues, and substances liberated at synapses in the cord may appear in the fluid perfusing the vertebral canal without being exposed to possible destruction by blood.

METHODS

Dissection.—*Rana temporaria* was used. The spinal cord was divided in the brachial region and the portion of the central nervous system above this was destroyed. The body cavity was opened and ligatures were tied around the base of each lung and around the confluence of the two systemic arches to form the aorta. General circulation was thus reduced to the branches coming from the systemic arches, some of which run to the spinal cord. Immediately after, a cannula made from an injection needle and connected to the oxygen supply was tied into the truncus arteriosus and gaseous perfusion was started. The gas was allowed to escape from a slit made in the sinus venosus. A constant pressure of 120 mm. Hg always gave satisfactory results. Bunzl *et al.* found that ligation of the systemic arches above the abdominal aorta excluded the posterior parts of the frog from the general circulation. With the frogs used here it was found that when this was done oxygen passed into the femoral arteries so that the muscle, the contraction of which was recorded, was supplied with oxygen. This did not appear, however, to be essential, since satisfactory results were also obtained when this was prevented by ligation of the femoral artery. After gaseous perfusion had been started the frog was laid prone and the *biceps femoris* was freed from its fascia, blood vessels, and sciatic nerve. The tendon was tied close to its insertion at the knee and cut between the thread and the insertion. When dissection was completed the thread, passing under a pulley, was connected to a writing lever, to give a tension of 2 g. Contractions were isotonic. A cannula made from an injection needle was then pushed into the vertebral canal through the gap made by flexing the urostyle-vertebral-column joint. The insertion into the vertebral canal of the cannula, if not properly done, can cause lesions of the spinal cord or nerves, after which intense spontaneous activity follows. The whole body of the frog was kept moist by a very fine spray of Ringer fluid with no dextrose.

Perfusion of the Vertebral Canal.—The technique used was different from that of Bunzl *et al.* Fig. 1 shows our apparatus : fluid from the reservoir *A* runs into the tube *B* and the rate of flow is controlled with a screw clip so as to maintain a slow overflow through b_1. The tube *B* is marked at b_3 corresponding to 1 ml. below the level of the overflow, so that it is possible to calculate the velocity of flow by stopping the flow from the reservoir and timing the fall of the level of the fluid. The tube b_2 can be opened by a clamp, so that the tube *B* can be emptied rapidly. The tube *c* makes it possible to collect fluid from the tube *B*. The tube *B* is connected with the cannula *D* which has a diameter of about 1 mm. and lies in the lower part of the vertebral canal. Flow in *D* and consequently in the vertebral canal is controlled by a screw clip with a very fine thread. With this apparatus changes of pressure are negligible and the rate of flow is sufficiently constant. The composition of the Ringer is that indicated by Bunzl *et al.*: NaCl 6.5 g., $CaCl_2$ 0.12 g., KCl 0.14 g., dextrose 2 g., and 30 ml. of 0.15 M-sodium phosphate buffer (*p*H 7.4) in a litre.

Stimulation.—With the apparatus described by Bunzl *et al.* two difficulties were encountered : (a) reflex activity could be obtained only for a comparatively short time ; (b) the electric stimuli were apt to escape and cause contractions of the muscles which were not due to reflexes. Bunzl *et al.* used two stimulators delivering alternating current (frequency

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60 c/sec., voltage 0–20). The apparatus used here consisted of two completely independent neon stimulators in each of which the current was derived from dry batteries. The frequency and strength of the stimuli were controlled by suitable resistances and a neon lamp. The connexion between the stimulators and each electrode was through a condenser (0.1 μF). The positive electrode consisted of a piece of platinum foil (1 cm. × 1 cm.) placed under the leg of the frog. The negative electrode, consisting of a spiral of silver wire on which chloride had been deposited, was placed on the foot. With this arrangement the flow of current was concentrated on the foot. In order to avoid loss of current to earth through the body of the frog, the table on which the frog was placed was supported on plexiglass. The right hind foot was stimulated at regular intervals of 30–60 sec. by volleys of stimuli lasting 1 sec. under the control of an electric clock (Palmer). The frequency was 10–15/sec. and the voltage was adjusted to give a suitable response. This stimulus caused a flexion reflex of the right leg; the contractions of the right *biceps femoris* were recorded. In some experiments, in order to study spinal inhibition, alternate stimuli were combined with stimuli on the left hind foot. This stimulation, contralateral to the side of recording, started 1 sec. before the homolateral stimulation and stopped at the same time, so that its duration was 2 sec. The normal effect of this contralateral stimulus was to diminish the effect of the homolateral stimulus, so that in the records responses to homolateral stimulation (uninhibited) alternate with smaller (inhibited) responses due to the stimulation of both feet. When stimuli were submaximal, reflex responses were regular for 6–8 hr., during which the size of the response diminished slowly. Fig. 2 shows constant responses during 4 hr. in an experiment where no drugs were used. When attempts were made to detect active substances in the perfusate both hind feet were stimulated together.

Collection of the Perfusion Fluid.—Fluid was collected by a fine tube lying loosely in the upper end of the vertebral canal, so as not to interfere with the rate

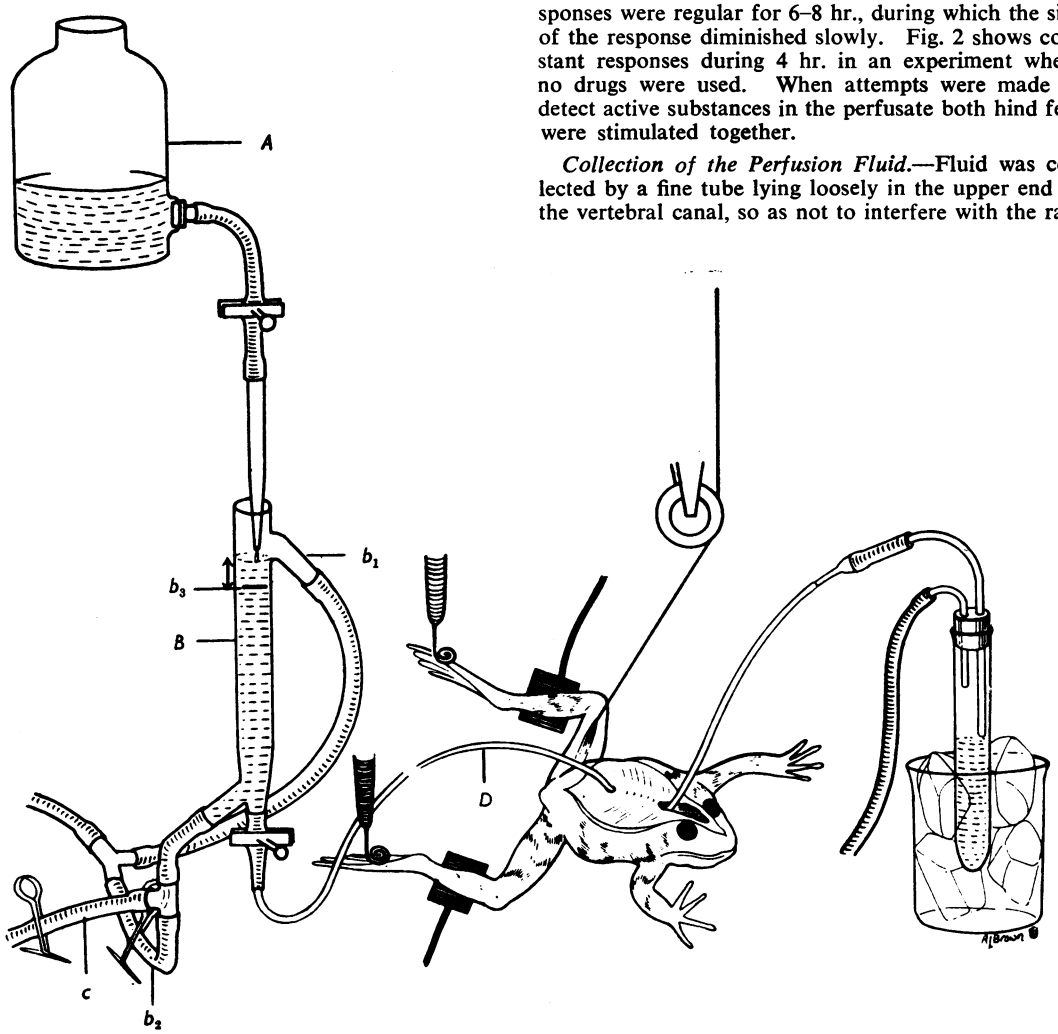

FIG. 1.—A diagram of the apparatus (see text).

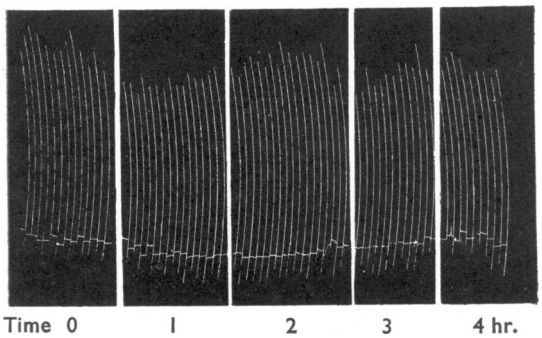

FIG. 2.—Flexor reflex. Interval between stimuli 1 min. Interval between sections of the record 1 hr. Flow 6 ml./hr. Regular responses for 4 hr.

of flow. The tube was connected to a small measuring cylinder to which a negative pressure was applied. This cylinder was immersed in a mixture of ice and water and, as soon as collection was complete, it was frozen in order to preserve active substances for testing later. When drugs were to be tested the velocity of flow was 6–20 ml./hr. When perfusates were to be tested for released substances the flow was about 1–2 ml./hr., but at the beginning of an experiment the perfusion was allowed to proceed for 30–60 min. at a higher velocity in order to wash out tissue debris and remove active substances liberated during dissection. When the perfusate was to be tested for acetylcholine, the perfusion fluid generally contained eserine (8×10^{-6}). Fluid was collected for 1 hr. without stimulation (control period). Stimulation was then applied for 1 hr. and more fluid collected. Sometimes these periods of control and stimulation were repeated.

Drugs.—Doses of drugs are given as the weights of the salts, except for 5-hydroxytryptamine, the dose of which is given as the weight of the base. Concentrations refer to the final concentrations in the perfusion fluid. After addition of the drug to the fluid the *p*H was adjusted, if necessary, to 7.4. Drugs were applied until effects were observed and then washed out again. The following drugs were used : acetylcholine hydrochloride (Roche Products), benzoquinonium ("mytolon," Sterling Winthrop), hexamethonium hydrobromide (May and Baker), 5-hydroxytryptamine creatinine sulphate (Abbott Laboratories), lysergic acid diethylamide (Sandoz Products), reserpine (Ciba Laboratories), substance P_4 (Amin, Crawford and Gaddum, 1954), adrenaline hydrochloride, atropine sulphate, chlorpromazine, carbachol hydrochloride, eserine sulphate, histamine hydrochloride, morphine sulphate, nicotine tartrate, noradrenaline hydrochloride, (+)-tubocurarine chloride.

Spinal Cord Extracts.—The frog was frozen, the spinal cord then dissected out, freed of the roots of the nerves, weighed and crushed in a test tube with a known volume of Ringer solution. The supernatant fluid was used for tests. When tests for acetylcholine were to be made, eserine (8×10^{-6}) was run through the vertebral canal before dissection and used for the extraction.

Test Objects

Leech.—Dorsal muscles from leeches were suspended in 2 ml. Ringer solution. The tension was about 5 g. Acetylcholine was used as a control drug and was diluted shortly before use, from a stock solution kept at *p*H 4 in ice. Solutions were tested every 10 min. and allowed to act for 3 min.

Heart of Spisula solida.—The technique used was that described by Gaddum and Paasonen (1955). The heart was suspended in 1–2 ml. of a solution of the following composition : NaCl, 23 ; Na_2SO_4, 4 ; KCl, 0.65 ; $CaCl_2$, 1.1 ; $MgCl_2.6H_2O$, 10 ; $NaHCO_3$, 0.2 g./l. Drugs were allowed to act for 30 sec. and HT was used as a standard. Benzoquinonium (10^{-5}) was sometimes present in the solution as an antagonist to acetylcholine.

Guinea-pig's Intestine was suspended in 2 ml. Tyrode solution at 37°. Drugs were allowed to act for 30 sec. Carbachol and histamine were used as control drugs.

Rat's Uterus was prepared by subcutaneous injection of stilboestrol (10 μg./100 g.) into the rat on the day before the experiment. The uterus was suspended in 2 ml. of Jalon's solution at 30°. Drugs were added in small volumes and allowed to act for 30 sec. ; consistent results were obtained in several experiments with each drug.

RESULTS

Effects of Drugs on the Spinal Reflexes

Preliminary experiments showed that the reflexes were reversibly depressed by ether, chlorbutol and anoxia.

Ether was dissolved in the perfusion liquid or vaporized in the immediate neighbourhood of the

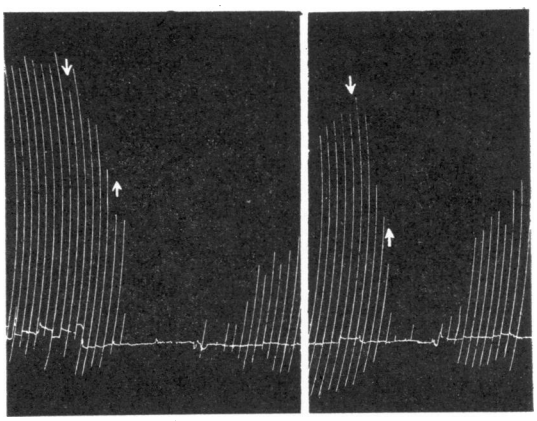

FIG. 3.—Flexor reflex. Interval between stimuli 1 min. Flow 12 ml./hr. Between arrows chlorbutol 10^{-4} for 5 min. (twice in the same experiment). Depression of flexor reflex followed by recovery.

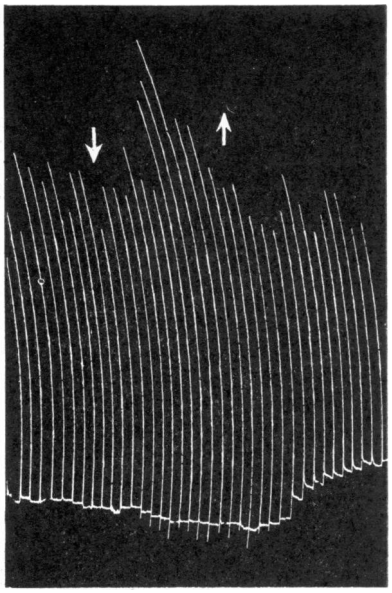

FIG. 4.—Flexor reflex. Interval between stimuli 1 min. Flow 6 ml./hr. Between arrows acetylcholine 3×10^{-4}. Temporary increase of response.

frog. In the latter case the circulation was intact but the brain destroyed. The effect of ether appeared quickly: after a brief period of excitation in which reflex responses were bigger and accompanied by spontaneous contractions, the ether caused progressive reduction of the responses, both inhibited and uninhibited. The degree of depression and the duration of the effect depended on the duration of exposure. At the end of the period of administration the reflexes recovered slowly, but the rate of recovery could be increased by the administration of oxygen. The recovery was complete for both inhibited and uninhibited responses.

Chlorbutol in a concentration of 10^{-4} in the perfusion fluid caused depression of the flexor reflex. As shown by Fig. 3, the effect appeared almost immediately and reflexes were quickly abolished. At the end of the period of administration reflexes quickly recovered.

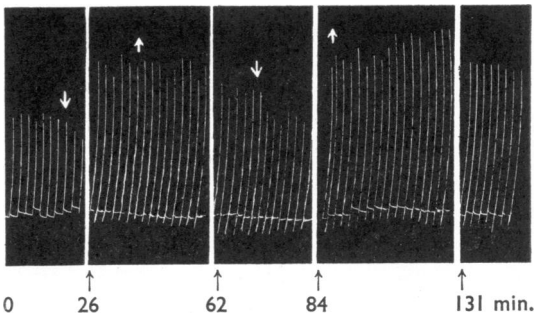

FIG. 5.—Flexor reflex. Interval between stimuli 1 min. Flow 15 ml./hr. Between arrows nicotine tartrate 10^{-4} from 7 to 32 min. and from 67 to 85 min. Delayed increase of the response.

Anoxia due to stoppage of the flow of oxygen caused depression and finally disappearance of both reflexes, inhibited and uninhibited. After periods of 7 min. of anoxia, recovery was complete in 1 hr. It appeared that reflexes could recover even after 15–20 min. of anoxia.

Acetylcholine in a concentration of 10^{-6}–10^{-4} in the perfusion fluid had usually no effect on the reflexes, even when it was accompanied by eserine (5×10^{-5}). High concentrations of ACh ($>10^{-4}$) caused an increase in the reflex response. The effect appeared quickly and was generally of very short duration; in Fig. 4 the effect of ACh is vanishing before the administration is stopped. Sometimes still higher doses appeared to have an inhibitory action.

Nicotine in a concentration of 10^{-4} increased the reflexes, as shown by Fig. 5. At the end of administration recovery was complete in 1 hr. In Fig. 5 a subsequent dose caused a larger response; with higher concentrations there was depression of the flexor reflex.

Mephenesin at a concentration of 2×10^{-5} caused progressive depression of the reflexes or complete suppression. When the drug was removed recovery was complete. After a higher concentration (10^{-4}) recovery was not possible.

Chlorpromazine in a concentration of 10^{-4} depressed the reflexes; when the drug was

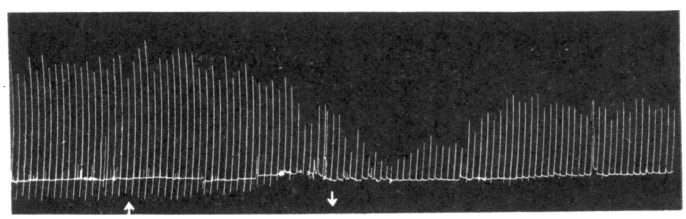

FIG. 6.—Flexor reflex. Interval between stimuli 1 min. Flow 10 ml./hr. Between arrows chlorpromazine 10^{-4}. Depression followed by recovery.

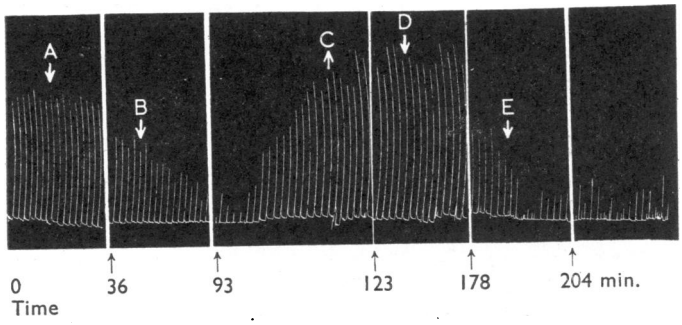

FIG. 7.—Flexor reflex. Interval between stimuli 1 min. 7.5 ml./hr. A–B, reserpine 10^{-7}; depression. B–C, reserpine 10^{-7}+LSD 10^{-6}; recovery after about 1 hr. C–D, no drugs. D–E, reserpine 10^{-7}; depression. E–F, reserpine 10^{-7}+LSD 10^{-6}; no recovery.

removed recovery occurred (Fig. 6). Both the effect and recovery appeared fairly quickly.

Reserpine in a concentration of 10^{-7} depressed or completely abolished the reflexes. There was always a latent period of 30–90 min. The depression of the reflexes was irreversible even after many hours.

Lysergic acid diethylamide in a concentration of 10^{-6} added to the reserpine was found to arrest the gradual development of depression due to reserpine, and sometimes there was recovery of the response (Fig. 7). In other cases, however, it was impossible to demonstrate any such effect.

5-Hydroxytryptamine in a concentration of 5×10^{-6} did not alter the depressant action of reserpine even when administered for a very long time, as in Fig. 8. Reflexes which had been depressed with reserpine were restored by strychnine (Fig. 8).

Morphine in a concentration of 5×10^{-5} quickly caused slight depression of the direct effect of the homolateral stimulation, and definite depression of the inhibitory effect of stimulation of the contralateral foot. At the end of the period of administration reflexes recovered completely (Fig. 9). The depressant effect was obtained more than once in the same preparation, but the spinal cord appeared to become insensitive to the morphine after several doses.

Strychnine in a concentration of 5×10^{-5} first increased the reflex response to homolateral stimulation and abolished the inhibitory effect of contralateral stimulation. The response to homolateral stimulation then became smaller than the response to bilateral stimulation. When the response was recorded on a fast drum it was seen that contralateral stimulation actually caused contraction of the muscle in the fraction of time (1 sec.) by which contralateral stimulation preceded homolateral stimulation. The contralateral stimulation before strychnine caused a small relaxation of the muscle or no effect at all, except a diminution of the response to homolateral stimulation. In Fig. 10 it will be seen that the response to bilateral stimulation which had originally been less than that to homolateral stimulation became greater after strychnine. The effect of strychnine is slow to appear (generally 30 min.) and to develop to the

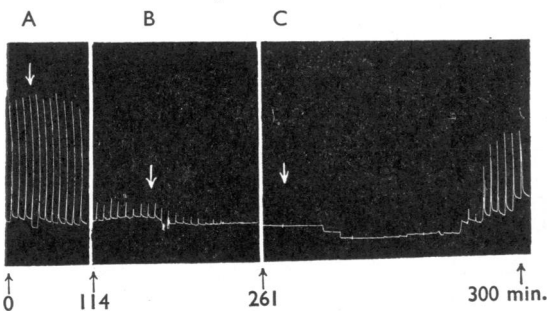

FIG. 8.—Flexor reflex. Interval between stimuli 1 min. Flow 6 ml./hr. A–B, reserpine 10^{-6}; depression. B–C, HT 10^{-5}; no recovery. C-onwards, strychnine 10^{-4}; recovery after 26 min.

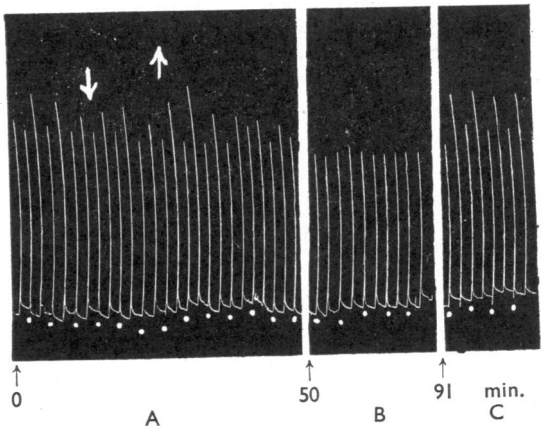

FIG. 9.—Flexor reflex. Interval between stimuli 1 min. At white dots simultaneous stimulation of both feet inhibits response. A—Between arrows morphine 5×10^{-5}; B—depression of both reflexes, especially of the inhibitory one; C—recovery.

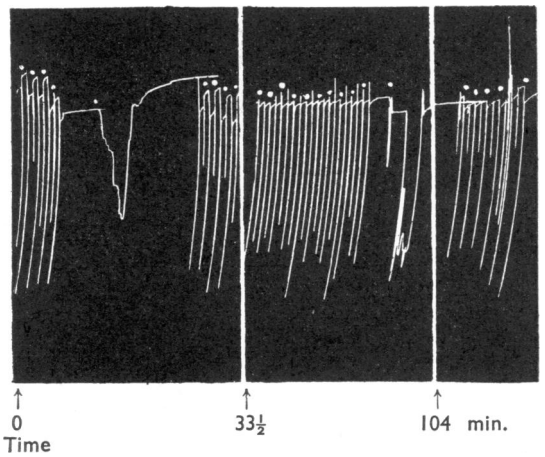

FIG. 10.—Flexor reflex. Interval between stimuli 30 sec. Flow 6 ml./hr. At white dots simultaneous stimulation of both feet before strychnine inhibits response. After strychnine this stimulation increases the response. At 4.5 min. and 104 min. the drum was run quickly for a short time.

stage of complete ineffectiveness of inhibitory stimuli.

No effect on flexor reflexes, uninhibited or inhibited, was demonstrated for *adrenaline, noradrenaline, carbachol, atropine, eserine, hexamethonium, tubocurarine, histamine, 5-hydroxytryptamine, substance P,* or *benzoquinonium,* in concentrations of these substances up to 10^{-4}.

Active Substances in the Perfusates

Perfusion fluids collected from the vertebral canal have been shown to produce effects on leech muscle, *Spisula* heart, guinea-pig ileum and rat uterus. The activity was always comparatively high in fluids collected during the first hour after dissection, whether the reflexes were stimulated or not; it usually fell to a minimum in 3–4 hr. and then remained stable if no stimuli were applied. Crude extracts of the spinal cord had similar effects.

Leech Muscle.—In most experiments eserine (8×10^{-6}) was present both in the perfusion fluid while it was in contact with the spinal cord, and in the fluid bathing the leech muscle. Such perfusion fluids usually caused a contraction of the muscle (Fig. 11). This was compared with that due to acetylcholine, and the apparent concentration of this substance is called the ACh-equivalent (Chang and Gaddum, 1933). Such effects were not observed when the leech had not been treated with eserine, but the presence of eserine in the perfusion fluid in contact with the spinal cord did not seem to be essential, since perfusates collected soon after dissection and containing no eserine sometimes caused contractions of the eserinized leech. However, no effects were seen with perfusates collected in later periods of the experiments in the absence of eserine in the perfusion fluid.

TABLE I

ACETYLCHOLINE-EQUIVALENT IN PERFUSATES COLLECTED IN DIFFERENT EXPERIMENTS

BS, before stimulation; DS, during stimulation; AS, after stimulation; DSA, during spontaneous activity.

Date	Perfusate	ng./ml.
Aug. 26	DSA	22
„ 27	BS	13
	DS	33
„ 30	DSA	11
Sept. 1	BS	8
	DS	11
	AS	7
„ 2	BS	2
	DS I	4
	AS	<1
	DS II	6
„ 3	BS	2
	DS I	4
	DS II	7
„ 5	BS	—
	DS I	—
	AS I	2
	DS II	4
	AS II	<1
	DS III	1

The results are shown in Table I. In the periods of reflex activity due to stimulation of the feet the ACh-equivalent of the perfused fluid increased; and during the next hour it generally fell to values below those observed before stimulation. In some

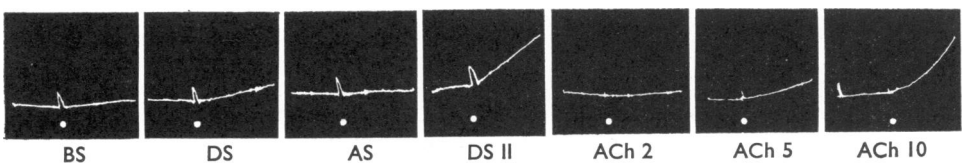

FIG. 11.—Leech muscle (Sept. 3). Eserine 8×10^{-6}. Undiluted perfusate applied to the leech. BS, before stimulation; DS, during stimulation; AS, after stimulation; DS II, during second period of stimulation. Acetylcholine (ACh) doses in nanograms in 1.5 ml.

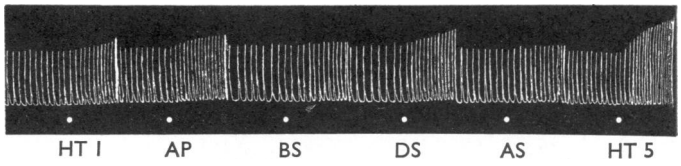

FIG. 12.—*Spisula* heart in 2 ml. bath (Sept. 5). 0.2 ml. of perfusates added to the bath. AP, after pithing; BS, before stimulation; DS, during stimulation; AS, after stimulation. HT doses in nanograms in bath.

experiments the ACh-equivalent during reflex activity was higher than that during the first hour after dissection. Generally it was lower than this, but when sufficient time was allowed after dissection the ACh-equivalent of the fluid collected during stimulation was always greater than those of control fluids, collected just before and after the stimulation. In two experiments a second period of stimulation caused a second rise of the ACh-equivalents (Sept. 2 and Sept. 5). In several experiments intense spontaneous activity of the frog was found to be associated with high ACh-equivalents (Aug. 26 and Aug. 30).

Spisula solida *Heart.*—Perfusates caused an increase of the amplitude and frequency of the beat (Fig. 12). Perfusates collected during the first hour after dissection, or during spontaneous activity of the frog, were more active than those collected later, even if the reflexes were stimulated. On the other hand, when sufficient time was allowed for the activity to fall to low values, stimulation appeared to increase the activity. The effects were compared with those of 5-hydroxytryptamine, and the HT-equivalents are shown in Table II.

In one experiment the HT-equivalent of a perfusate was estimated as 15 ng./ml. on the heart of *Spisula* and 15 ng./ml. on the rat uterus.

Since reserpine is known to liberate 5-hydroxytryptamine (Pletscher, Shore, and Brodie, 1956), experiments were made with this substance. Control samples were first collected during stimulation in the absence of reserpine and compared with samples collected later for 1 hr. during stimulation while reserpine (10^{-6} to 10^{-7}) was present in the perfusion fluid. Contrary to expectation the perfusates which contained reserpine had less effect on the heart than the control samples (Fig. 13). These concentrations of reserpine did not alter the response of the heart to known amounts of 5-hydroxytryptamine, so that the results suggest that in these conditions reserpine actually inhibited the release of 5-hydroxytryptamine-like substance from the spinal cord.

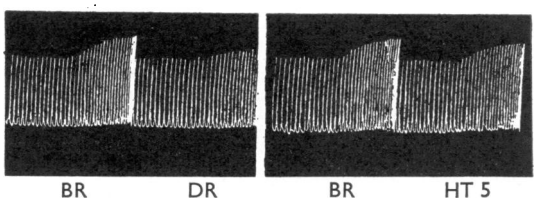

FIG. 13.—*Spisula* heart in 2 ml. bath. 0.2 ml. of the perfusates added to the bath. All samples collected during stimulation. BR, before reserpine; DR, during reserpine 5×10^{-6}. HT doses in nanograms in the bath.

TABLE II

5-HYDROXYTRYPTAMINE-EQUIVALENT IN PERFUSATES COLLECTED IN DIFFERENT EXPERIMENTS

BS, before stimulation; DS, during stimulation; AS, after stimulation; DSA, during spontaneous activity; ^{0}, in this second hour the spontaneous activity was less intense; ^{00}, in this second hour the intensity of stimulation was greater. In some assays benzoquinonium (10^{-7}) was present in the bath.

Time from the End of Dissection (hr.)	Date	Perfusate	HT-equivalent (ng./ml.)	
			Benzoquinonium Absent	Benzoquinonium Present
0	Aug. 26	Washing	—	—
0·5		DSA	59	—
1·5		DS	47	—
2·5		AS	4	—
0	,, 29	Washing	—	—
0·5		DSA	47	—
1·5		DSA^{0}	37	—
2·5		DS I	10	—
3·5		DS II^{00}	23	—
0	Sept. 3	Washing	—	—
0·5		BS	>25	>25
1·5		DS I	>25	9
2·5		AS	11	4
3 5		DS II	12	16
0	,, 5	Washing	—	—
1		BS	12	9
2		DS I	7	6
3		AS I	4	5
4		DS II	20	13
5		AS II	6	4
6		DS III	—	—
0	,, 7	Washing	—	—
1		BS	6	—
2		DS	17	—
3		AS	11	9
4		DS II	14	12
0	,, 8	Washing	—	—
1		BS I	20	—
2		BS II	4	—
3		DS	9	—
4		AS	16	—

Guinea-pig intestine usually contracted when 0.2 ml. of a perfusate was added to the bath (Fig. 14). Fluids collected during stimulation generally had more effect than fluids collected before or after stimulation (controls), but sometimes responses

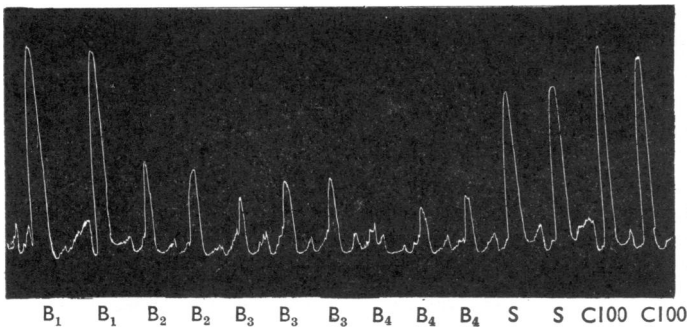

FIG. 14.—Guinea-pig ileum in 2 ml. bath. 0.2 ml. of the perfusates added to the bath. B_1–B_4, successive samples before stimulation (30 min. each); S, during stimulation sample (30 min.). C, carbachol doses in nanograms in bath.

were irregular and obscured by the delayed effects of previous applications of the perfusates, and no clear evidence could be obtained.

The response was not altered by mepyramine (10^{-6}), but atropine had a variable effect upon it. In some experiments it was unaffected by a concentration of 10^{-7}, and in others it was almost completely inhibited by a concentration of 10^{-9}. The concentration causing complete inhibition was generally 5–10 times that which would just cause no inhibition. Concentrations of atropine just below those which inhibited the response to perfusates caused a partial inhibition of the response to equivalent doses of 5-hydroxytryptamine (25–50 ng./ml.), but did not alter the response to equivalent doses of substance P (0.25–0.5 U./ml.). Slightly higher concentrations diminished the responses both to the perfusates and to a preparation of substance P.

Rat uterus usually contracted when 0.1 to 0.2 ml. of a perfusate was added to the bath. The response was not altered by mepyramine (10^{-6}) or atropine (10^{-7}). Few experiments were made and no evidence was obtained of any difference between perfusates collected during stimulation and control perfusates.

DISCUSSION

Evidence for chemical transmission in the central nervous system can be obtained by investigating (a) the effects of drugs on the C.N.S. and (b) the liberation of substances during activity of the C.N.S.

The Effects of Drugs on the C.N.S.

The value of experiments on the effects of drugs by the method described here is limited by our ignorance of the factors which determine whether the drugs present in the perfusion fluid can pass through the meninges and reach their site of action. The technique used for stimulating the reflexes and recording the responses is, of course, crude compared with some of the methods used by electrophysiologists. Moreover, by this technique it is only possible to study the polysynaptic flexor reflex; it is not directly applicable to monosynaptic reflexes. In the light of these considerations and of the fact that only frogs were used, negative and uncertain results can have only a limited significance, particularly when the drugs used are known to act on the central nervous system. For example, adrenaline has been found to increase flexor reflexes (Bülbring and Burn, 1941) and choline esters increase the height and duration of action potentials in the spinal roots (Malcolm and Wurzel, 1955).

According to Häusler and Sterz (1952) histamine stimulates reflexes in the perfused spinal cord of a frog. This result has not been confirmed by the present experiments.

The absence of results with noradrenaline, atropine, hexamethonium, 5-hydroxytryptamine, substance P and benzoquinonium does not mean that these drugs have no actions on reflexes.

The action of eserine on the spinal cord is important because it provides indirect evidence for or against the presence of cholinergic nerves. This action has been demonstrated by many workers (for literature before 1950 concerning acetylcholine and the nervous system, see Feldberg's reviews, 1945, 1950). According to Wikler (1945) eserine only increases monosynaptic reflexes and has no action on polysynaptic reflexes. When eserine is injected intravenously it increases the sensitivity of the cells of Renshaw to acetylcholine injected intra-arterially (Eccles, Eccles, and Fatt, 1955). In the results recorded here eserine (4×10^{-6}) had no apparent effect on the reflex responses. This negative result has little meaning: we cannot be sure that the eserine reached the nervous tissue in sufficient concentration; even strychnine did not appear to reach the tissues in less than 20–30 min.

A direct action of acetylcholine on the spinal cord has been recorded in many ways. It has

usually been injected intra-arterially (Feldberg, 1945, 1950; Feldberg, Gray, and Perry, 1952; Marrazzi, 1953; Eccles *et al.*, 1955), but has also been applied locally to the surface of the cord (Feldberg, 1945, 1950) or by micro-injection (Kennard, 1951). In the present experiments it has not always been possible to show an effect, and in any case the doses were rather high—10^{-4} when the rate of flow was 6 ml./hr. in the absence of eserine. However, this concentration is not very different from that used by Eccles *et al.* (1955) to stimulate the cells of Renshaw. The main effect, when present, was an increase of the reflex response. In some cases still higher doses appeared to have an inhibitory action.

There is not much evidence about the action of nicotine on the spinal cord, but its effect appears to be excitation followed by depression. Nicotine excites the cells of Renshaw, but its action differs from that of acetylcholine in not being increased by eserine (Eccles *et al.*, 1955). In small doses nicotine accelerates the response to strychnine (Bonnet and Bremer, 1952). The results shown in Fig. 10 confirm the observation of Bunzl *et al.* (1954) that nicotine increases the response in the flexor reflex of the frog.

Tubocurarine is said only to influence monosynaptic reflexes (Bernhard, Taverner, and Widen, 1951). It had no action on the polysynaptic reflex studied here.

The results with strychnine agree with those of Bradley, Easton, and Eccles (1953) and of Bunzl *et al.* (1954). The inhibitory effect of the contralateral stimulation appeared to be converted into an excitatory effect. This confirms the results of Sherrington (1906), but does not disprove the conclusions of Creed and Hertz (1933) regarding the mechanism of this effect.

Wikler (1945) found that morphine increased monosynaptic reflexes and depressed polysynaptic reflexes. In the present experiments morphine depressed two polysynaptic reflexes—the flexor reflex, and the inhibition of this reflex by contralateral stimulation. Inhibitory reflexes in the spinal cord, but not excitatory reflexes, may develop tolerance for morphine (Wikler and Carter, 1953). Sometimes when the same dose of morphine was repeated, the response of both reflexes to the second dose was weaker and briefer.

Mephenesin had a powerful inhibitory action on the reflexes—as was to be expected, since this drug is known to inhibit polysynaptic but not monosynaptic reflexes (Hennemann, Kaplan, and Unna, 1949).

No published information about the action of chlorpromazine on the spinal cord has been found. The effect was purely depressor.

The actions of reserpine have not yet been systematically studied, but its main action is depression of the central nervous system (Bein, 1953; Symposium, 1954). In the present experiments low concentrations progressively depressed or abolished the reflexes after a long latent period (30–45 min. or more). This depression was reversed by strychnine and sometimes appeared to be antagonized by lysergic acid diethylamide.

The Liberation of Substances During Activity of the C.N.S.

The results of the present experiments appear to show that reflex activity in the frog is associated with the release of at least 3 active substances in the perfusion fluid. These 3 substances were detected by tests which are sensitive to, and fairly specific for, acetylcholine, 5-hydroxytryptamine and substance P, but it has not yet been possible to obtain sufficient evidence to identify any of these substances with certainty.

The effects on the leech may be due to acetylcholine. It is *a priori* possible for this substance to diffuse out of the spinal cord, since it has been found in the cerebrospinal fluid (Feldberg, 1945, 1950) and its concentration may be increased in experimental convulsions (McIlwain, 1955). The activity on the leech muscle detected in our perfusates cannot be attributed with certainty to acetylcholine, since this test is not absolutely specific. The fact that no activity is recorded in the complete absence of eserine suggests a choline ester, but further experiments will be necessary before it is known whether it is acetylcholine or not. The amounts liberated in the early stages after dissection were so large that it was not necessary for eserine to be present in the perfusion fluid. In the later stages no activity was detected unless eserine was present both in the perfusion fluid and in the leech bath. The continuous liberation of small quantities during rest need cause no surprise. The increase in the concentration found during stimulated reflex activity suggests that the release of this substance plays a part in this activity.

The heart of *Spisula solida* is a specific reagent for 5-hydroxytryptamine, and the effects of the perfusates may be due to this substance. The agreement in one experiment of the quantitative determination of the activity of a perfusate on *Spisula* heart and rat uterus provides some evidence that the effects were probably due to 5-hydroxytryptamine. They were certainly not due to acetylcholine, which inhibits this heart. It is unlikely

that they were due to adrenaline, since the heart is about 1,000 times less sensitive to adrenaline than it is to 5-hydroxytryptamine. The results suggest that the liberation of 5-hydroxytryptamine may play a part in spinal reflexes.

There is evidence that reserpine liberates 5-hydroxytryptamine from its natural sites in the body: the intestine (Pletscher, Shore and Brodie, 1956; Erspamer, 1956a and b) and the brain (Pletscher, Shore and Brodie, 1956; Paasonen and Vogt, 1955; Correale, 1956), and the theory has been proposed that the central effects of reserpine are due to this liberated 5-hydroxytryptamine (Shore, Silver and Brodie, 1955a and b). The results recorded here showed, contrary to expectation, that reserpine decreased the amount of the substance active on *Spisula* heart present in the perfused fluid.

The substance present in the perfusates which caused contraction of the guinea-pig ileum was not acetylcholine or histamine, because its action was not inhibited by concentrations of atropine and mepyramine which inhibited the responses to these drugs. The results suggest that it is more likely to have been substance P than 5-hydroxytryptamine, but further work will be needed to identify it with certainty and to decide whether its rate of liberation is increased during reflex activity.

Summary

1. The spinal cords of frogs were perfused by a modification of the method in which gaseous oxygen flows through the blood vessels and Ringer solution flows slowly over the cord. The flexor reflex gave regular responses to electric stimulation of the skin of one foot once a minute for 6–8 hr.

2. The reflex was reversibly depressed by anoxia, ether, chlorbutol, mephenesin, chlorpromazine, reserpine or morphine; it was increased by acetylcholine, nicotine or strychnine. Lysergic acid diethylamide appeared to antagonize reserpine.

3. Electric stimulation of the skin of both feet caused the appearance in the perfusates of substances acting like acetylcholine and 5-hydroxytryptamine on eserinized leech and *Spisula* heart, but the evidence on the nature of these substances is still incomplete.

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