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RELEASE OF ACETYLCHOLINE AT VOLUNTARY MOTOR NERVE ENDINGS

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In a note published some time ago [Dale and Feldberg, 1934], two of us gave a preliminary description of experiments which indicated that something having the properties of acetylcholine (ACh.) is liberated, when impulses in motor nerve fibres excite contraction of a voluntary, striated muscle. Several earlier observers had recorded observations of this kind, but their significance had not been clear. Geiger and Loewi [1922] estimated the choline present in extracts from frog's voluntary muscle, and observed an apparent large increase (five- to tenfold) after prolonged direct and indirect stimulation. Plattner and Krannich [1932] and Plattner [1932, 1933] found that the substance present in such extracts was rapidly inactivated by fresh blood, like acetylcholine, and that the quantity present had a general correspondence to the wide differences in sensitiveness of different muscles to the stimulating action of acetylcholine. Faradic stimulation of the nerve increased the yield; but Plattner associated the apparent presence of the acetylcholine in the muscle, and its increase on mixed nerve stimulation, with a "parasympathetic" innervation of the blood vessels. In the tongue, excised from a cat treated with eserine, and divided longitudinally into halves, he found that stimulation of the chorda-lingual nerve caused increase of acetylcholine in the extract from one half, while stimulation of the hypoglossal nerve did not significantly increase the yield of the other.

Hess [1923], Brinkman and Ruiter [1924, 1925] and Shimidzu [1926], all perfused the muscles of a frog's hindlimbs and tested the effluent Ringer's solution on isolated preparations sensitive to acetylcholine, with some variations of method. All found evidence of the liberation, when the nerves supplying the muscles were stimulated, of

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something acting like acetylcholine. Brinkman and Ruiter observed that the liberation still occurred, when curare was present in sufficient amount to prevent the motor impulses from causing contractions.

It will be seen that these different observations, though suggestive, are not clear in significance. Something like acetylcholine was apparently liberated, when the mixed nerves to voluntary muscles were stimulated; but the identification of the substance was incomplete, and there was little to indicate what kind of nerve fibres were responsible for its liberation. There is no real evidence, indeed, for a secondary "parasympathetic" nerve supply to the voluntary muscle fibres themselves, responsible for maintenance and variation of tone, such as Frank and his coworkers have assumed [Frank and Katz, 1921; Frank, Nothmann and Hirsch-Kauffmann, 1922]. There is evidence, however, that the sympathetic nerve supply to the blood vessels of the leg muscles contains a cholinergic component in the cat [Hinsey and Cutting, 1933] and in the dog [Bülbring and Burn, 1935]; and the same might be the case in the frog. As regards the vaso-dilator action of sensory axon branches, the mode of its chemical transmission is still in doubt; it is still possible, though in the light of recent evidence no longer probable, that it may also be cholinergic. It is clear, in any case, that the liberation of acetylcholine in a voluntary muscle, as the result of stimulating a mixed nerve containing sensory and sympathetic as well as motor fibres, could not be regarded as having any necessary connection with motor nerve impulses, and the transmission of their excitatory action to the voluntary muscle fibres.

Our object has been to discover whether stimulation of the motor nerve fibres innervating voluntary muscle fibres, to the complete exclusion of the autonomic or sensory fibres running with them in a mixed nerve, causes the liberation of acetylcholine in appreciable quantities; and, if so, to endeavour to obtain evidence as to the site of such liberation.

Such an enquiry formed a natural sequel to the experiments which, during the past few years, have produced evidence of the liberation of acetylcholine when a nerve impulse reaches the ending of a preganglionic fibre of the autonomic system, whether that ending makes contact with a cell of the suprarenal medulla [Feldberg, Minz and Tsudzimura, 1934] or with a nerve cell in a ganglion [Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1934; Barsoum, Gaddum and Khayyal, 1934]. The acetylcholine is, in these cases, liberated in contact with cells which are responsive to that aspect of its activity in which it resembles nicotine [Dale, 1914]. The direct stimulant action of acetylcholine on certain voluntary muscles after degeneration of their motor nerve supply,

also belongs to its "nicotine" action, and not to its "muscarine" action [Dale and Gasser, 1926]. It had further been shown long ago, by Langley and Anderson, that voluntary motor nerve fibres and preganglionic autonomic fibres are functionally interchangeable in crossed regeneration; so that, as evidence for a cholinergic function of preganglionic fibres accumulated, the presumption of a cholinergic function for the motor fibres to voluntary muscle increased [cf. Dale, 1935*a*, *b*). On the other hand, it was clear that the task of demonstrating the liberation of acetylcholine by nerve impulses reaching the endings of motor nerve fibres in a voluntary muscle, would be attended with difficulties of a different kind from those involved in the experiments on a ganglion. For the small substance of the ganglion is closely packed with the synaptic endings of preganglionic fibres; so that if acetylcholine were liberated by impulses arriving at the synapses, it might be expected to appear in relatively high concentration in the slow-dropping venous effluent; and this expectation had been realized in experiment. In a voluntary muscle, on the other hand, the bulk of the tissue, requiring effective perfusion to maintain its functional activity, is relatively enormous in relation to the motor nerve endings. If acetylcholine were liberated by the arrival of impulses at these endings, it could not, therefore, be expected to appear in the perfusion fluid in more than a very low concentration. This expectation has again been realized in our experiments, but we have, nevertheless, been able with regularity to detect the appearance of a substance having the recognizable characters of acetylcholine, when the purely motor nerve supply to a voluntary muscle has been stimulated under the conditions of our experiments.

Most of our experiments have been made on the muscles of cats and dogs. These mammalian muscles are usually regarded as completely insensitive to the action of acetylcholine when their motor nerve supply is intact. Recent evidence, to be discussed later, shows that they are not, in fact, indifferent to acetylcholine in relatively large doses, applied through the circulation or directly; but their response, under such conditions, is by twitches or fibrillation, and they do not exhibit the slow contracture with which many muscles of the frog and other lower vertebrates respond to acetylcholine in low dilutions. It was accordingly of special importance to discover whether acetylcholine was liberated when motor impulses, causing only quick contractions, passed down the motor nerve fibres to such normal, mammalian muscles. A few experiments were also made on frog's muscles, with stimulation of motor fibres separately from the other components of the mixed nerve.

Methods

All our successful experiments on mammalian muscles have been made by perfusing them with Locke's solution at 37° C., pre-oxygenated to saturation, and containing 1 part of eserine in 5×10^5. Perfusion was carried out with the Dale-Schuster pump. In a few experiments the attempt was made to demonstrate the liberation of acetylcholine during stimulation of motor nerve fibres to a muscle with normal circulation, eserine being given to the whole animal with sufficient atropine to prevent excessive circulatory depression. Feldberg [1933*a*], who under such conditions had readily detected acetylcholine in blood of the lingual vein of the dog when the vaso-dilator chorda-lingual nerve was stimulated, had failed to find any when he stimulated the motor hypoglossal nerve. We had a like failure in experiments with natural circulation on the tongue and on the gastrocnemius, stimulating only motor fibres. Franel [1935], in experiments apparently suggested by our preliminary account, also failed in most cases to detect acetylcholine in venous blood from a leg, the muscles of which were thrown into contraction by stimulating the whole limb. The same author, had, indeed, no greater proportion of success when eserinized Locke's solution was used for perfusion. The conditions of these experiments, however, so differed from those of our own that we cannot profitably discuss the cause of their mostly negative results. Our own success in perfusion experiments, with Locke's solution containing eserine, was regular, in contrast to our failure in the few experiments made with normal circulation.

It may be that the relatively low concentration of acetylcholine released by motor stimulation cannot be protected from the blood esterase by eserine in doses insufficient, by themselves, to cause general twitchings of the voluntary muscles. It is possible, on the other hand, that the artificial conditions of saline perfusion in some other way facilitate the escape of the acetylcholine from the site of its origin into the blood vessels. Whatever the reason, artificial perfusion with an eserine-containing saline fluid has been necessary for success. This has caused a limitation of the period of an experiment in which success was possible. Perfusion must be continued long enough to wash the blood thoroughly out of the vessels; on the other hand, it if is continued too long, the muscle becomes more quickly insensitive to motor nerve impulses. In our few experiments on the frog, the skinned hindlimbs were perfused with Ringer's solution containing eserine at the room temperature. Mammals, after preliminary anæsthesia with ether, were given a stable anæsthesia

with chloralose, administered intravenously, before the dissection was begun.

Cat's tongue. All branches of both common carotid arteries and all tributaries of both external jugular veins were tied, excepting only the lingual arteries and veins respectively. The transverse vein connecting the jugulars at the hyoid level was left open, small tributaries to it being tied, so that the effluent from both lingual veins could later be collected from one jugular, the other being then tied. Both hypoglossal nerves were tied as near as possible to their exits from the skull, cut and dissected free from the accompanying lingual arteries up to the point of their entry into the tongue muscles. As soon as these dissections had been completed, an injection of "Chlorazol Fast Pink" was given intravenously, to render the blood incoagulable, and thus facilitate its complete removal by perfusion. Cannulæ, united by a Y-junction to the tube leading from the perfusion pump, were then tied into the peripheral ends of both common carotid arteries, the perfusion was begun, and the fluid collected from one jugular vein, as above described. It is, of course, impossible to isolate the vascular system of the tongue from anastomatic communications. While the heart is still beating the perfusion fluid does not become free from blood, unless the pump is so adjusted as to give a comparatively high perfusion pressure and rapid perfusion rate. Our procedure was to cut down the throw of the pump until the venous outflow was about 1–3 c.c. per minute, and then to kill the animal by incising the heart. The last traces of blood then quickly disappeared from the venous fluid. Only an uncertain part of the perfusion fluid pumped into the lingual arteries was thus recovered from the outflow through the lingual veins, a substantial proportion passing by arterial and venous anastomoses and flowing from the open heart cavities into the chest. This unavoidable and unmeasured loss prevented any calculation of the total quantity of acetylcholine liberated in the tongue muscles during stimulation, and restricted observation to a comparison of the acetylcholine contents of the fluid from the lingual veins, when the tongue was at rest and when it was stimulated through its motor nerve. In most of the experiments on the cat's tongue the hypoglossal nerves had been freed from their sympathetic component by aseptic removal of both superior cervical ganglia, under ether anæsthesia, a few weeks before the experiment was made.

Gastrocnemius. In experiments on the gastrocnemius muscle of the cat and dog, the stimulation was in most cases through ventral spinal roots. In the animal under chloralose the sympathetic chain, on the side

chosen for experiment, was first removed, through an abdominal incision, from the fourth lumbar to the first sacral ganglion. The muscles covering the lumbar vertebræ were then dissected away, and the neural arches removed from as many vertebræ as necessary, for later exposure of the required roots, with careful hæmostasis at each stage. The popliteal artery and vein were then prepared, all branches except those to and from the gastrocnemius being tied. The Achilles tendon was isolated and a strong ligature passed under it, round the whole of the other tissues of the leg above the ankle. The saphena veins were separately tied. Mass ligatures were also tied round the thigh muscles above the knee, leaving the main vessels and the sciatic nerve free. The crural nerve was cut at the groin. A stout steel rod was pushed through a hole drilled in the lower end of the femur, enabling that bone to be fixed by a clamp, so that muscular contractions would not drag on the popliteal vessels and interfere with the perfusion. The roots to be stimulated were now exposed by opening the *dura mater*. The dorsal roots were separated, and, unless required for control stimulation, were completely removed, so as to leave good lengths of ventral roots for stimulation, without danger of stimulating dorsal roots by escape of current. The ventral roots of the side not under experiment were similarly removed. The last lumbar and first two sacral roots on the experimental side were then tied with fine ligatures close to the cord. These roots were separately tested with short faradic stimuli, those being kept which caused contractions of the gastrocnemius. These were tied together for stimulation, or, in some cases, left attached to a segment of the cord, which could be raised with them for stimulation. The cannulæ were then tied into the popliteal vessels and the perfusion begun. If the exclusion of collateral circulation had not been sufficient to render the venous effluent free from blood, this was effected by lowering the blood-pressure by bleeding. It was not considered desirable, in this case, to deprive the whole nervous pathway, from the roots to the gastrocnemius, completely of its blood supply by killing the animal. In a few experiments, however, in which the whole sciatic nerve was stimulated, or in which the gastrocnemius muscle was stimulated directly, the preparation was isolated completely after the perfusion had been started, by killing the animal and cutting through the femur.

Quadriceps extensor femoris. To exclude by degeneration the sympathetic nerve endings on the blood vessels, which could not easily be effected with the gastrocnemius, a few experiments were made on the quadriceps extensor mass of the dog's thigh. By aseptic operation under ether, the sympathetic chain, from the third lumbar to the first or second

sacral ganglion on the experimental side, together with the first sacral ganglion of the other side, was removed some weeks beforehand. At the experiment the quadriceps was isolated by dividing all the other muscles of the thigh, the ilio-psoas and the glutæal muscles between ligatures. The leg was amputated at the knee joint. The perfusion cannulæ were tied into the femoral artery and vein, all branches except those to the quadriceps being tied. Further to minimize collateral circulation, a ligature was tied round the quadriceps mass above the entry of the vessels and nerves supplying it. Stimulation was through the appropriate lumbar ventral roots, or, in some cases, through the crural trunk.

Frog's legs. Large specimens of Hungarian *R. esculenta* were used. The frog was killed by decapitation and skinned. The abdominal sympathetic chains of both sides were removed. Perfusion was through the abdominal aorta and the anterior abdominal vein, the renal portal veins being tied. The roots of the lumbo-sacral plexus were exposed in the spinal canal, dorsal and ventral roots being tied separately and prepared for stimulation. The perfusion fluid was oxygenated Ringer's solution, containing 1 part of eserine in $3–5 \times 10^5$. A perfusion pressure of 25–35 c.c. of solution was found to give a convenient rate of outflow of about 1 c.c. per minute.

Stimulation. For stimulation the nerves or roots were laid across a suitable pair of chlorided silver electrodes. Direct stimulation of muscles was made through electrodes in the forms of chlorided silver pins, one impaling the muscle near its tendon of insertion, and the other, of several pins, penetrating the muscle near its origin. The stimuli used were maximal break shocks from a secondary coil, Lewis's rotating interruptor being used so as to give from 5 to 15 shocks per second. This method, causing a rhythmic series of twitches, was chosen in preference to tetanization, so as to avoid impediment to the perfusion during the activity of the muscle.

Rate of perfusion. As explained above, the unknown and irregular escape of some of the venous fluid by collateral channels made impossible a strict adjustment of the rate of flow to the size of the muscle perfused. A rough adjustment, however, had to be made; and it has already been indicated that venous fluid was collected from a cat's tongue at about 1–3 c.c., and from the hind legs of a large frog at about 1 c.c.per minute. For a cat's gastrocnemius the rate of collection was 3–8 c.c., and for the quadriceps femoris of a large dog, sometimes as high as 25 c.c. per minute. An estimate of total output rate being impracticable, our main concern was to ensure that an increased liberation of acetylcholine was not masked,

on the one hand, by accelerated perfusion, or simulated, on the other, by retarded perfusion during the period of stimulation. Failing adjustment, the rate of perfusion was, in fact, always accelerated during a period of the rhythmic stimulation employed, especially during its latter part. This acceleration was approximately corrected by an assistant, who watched the rate of dropping from the vein cannula, and reduced the throw of the pump as required, so as to ensure that the rate was not unduly accelerated during stimulation, but not, in any case, to retard it below the control rate.

Tests for acetylcholine. Tests were made as usual on the preparation of leech muscle sensitized by eserine. The preparations used usually responded well to acetylcholine in a dilution of 1 in 5×10^8. The tonicity of the mammalian Locke's solution had to be adjusted to that used for the leech by adding distilled water, and was then further diluted, if necessary, in accordance with its activity. The samples as collected were placed on ice and tested with as little delay as possible. In a number of experiments tests were also made on the blood-pressure of cats under chloralose, the sensitiveness of which to small doses of acetylcholine was, when necessary, increased by injecting eserine, and by restricting the circulation volume by removal of the abdominal viscera. For such tests the samples as collected were made acid to congo red by adding a drop of HCl solution, and then kept cold till the perfusion experiment was finished. The stability of the active substance could be tested by either method, and the test on the cat further enabled its sensitiveness to atropine to be demonstrated.

Results

(1) *Stimulation of purely motor fibres to voluntary muscles*

(*a*) *Tongue of the cat.*

The perfusion being started, the rate adjusted, and a bloodless effluent obtained, control samples were tested on the leech. The earlier samples, after the rather prolonged dissection and manipulation, usually showed detectable amounts of a substance acting like acetylcholine, as Hess [1923] observed in his perfusions of frog muscle. The activity might be such as to correspond to an ACh. content of 1 in 4×10^8 of the undiluted fluid. This activity diminished rapidly, however, with successive samples, and, after perfusion for about half an hour, was no longer perceptible by the very delicate test. The hypoglossal nerves were then stimulated rhythmically, the tongue muscles responding with rhythmic contractions. The perfused muscles show rapid fatigue to the effects of

such stimuli, the initially vigorous contractions rapidly becoming weaker, until, at the end of a stimulation period of 2–3 min. they have practically ceased. The stimulation was not continued when the muscles showed signs of this failure. The fluid collected during the stimulation showed a pronounced activity, in comparison with the inactive control. The contrast is illustrated in Fig. 1 A and C. All the fluids in this experiment were tested in 50 p.c. dilutions. The control fluid, at A, has no significant action. At C the fluid collected during a first period of stimulation at 5 shocks per sec. was applied and produced an effect closely similar to that of a control solution of ACh. 1 in 10^8, applied at B; so that the undiluted effluent during stimulation would correspond in activity to ACh. 1 in 5×10^7. The rate of outflow being 3 c.c. per min., the amount of ACh.

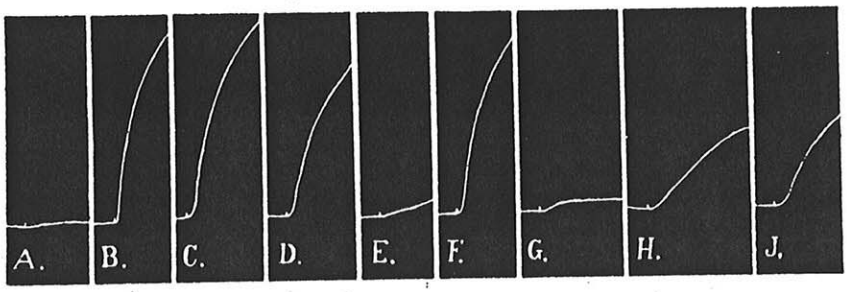

Fig. 1. Leech preparation; eserine 1 in 3×10^5 (same in Figs. 3, 4 and 5). A, C, D, E, F, G, H, venous fluids from cat's tongue, 50 p.c. dilutions (details in text). B and J, control ACh. dilutions; B, 1 in 10^8, J, 1 in 4×10^8.

actually collected in 1 min., apart from what escaped by anastomotic channels, was $0{\cdot}06\gamma$. The active substance was still present in fluid collected after the stimulation period, but in rapidly diminishing concentration in successive samples. Fig. 1 D shows the effect of fluid collected in the 2 min. after the end of stimulation, and E that of fluid collected 20 min. later. A second period of stimulation was then given, again causing vigorous contractions of the tongue at its commencement but with a more rapid onset of fatigue. The fluid collected during this second period, applied at F, was still highly active; and again the stimulating substance had almost disappeared from a further sample, applied at G, which was collected 20 min. after the end of the stimulation. Further periods of stimulation, after the second, were usually progressively ineffective, as regards both the contractile activity evoked in the tongue muscle and the concentration of stimulant substance in the venous fluid. Fig. 1 H shows the effect of the venous fluid collected during a third

and less effective period of stimulation. The 50 p.c. dilution at this stage is rather less active than ACh. 1 in 4×10^8, applied as a control at J.

In the later period of the experiment, the tongue gradually becomes œdematous with the prolonged perfusion; and this not only lowers the activity of the fluid collected during stimulation, but causes the activity to disappear more slowly from successive subsequent samples. Eventually, after four, five or more periods of stimulation, the tongue muscles fail to respond further, and the "stimulation" fluid is then not perceptibly more active than the control.

Identity of substance released. The action on the leech muscle, though closely similar to that of acetylcholine, would not by itself identify it. In several cases, when the concentration in the stimulation effluent was sufficiently high, it was directly tested on the blood-pressure of the cat under chloralose, and the activity again matched against known doses of acetylcholine. In every case the match so obtained was identical with that obtained in the comparison made on the leech muscle. In every case, also, the effect on the blood-pressure was completely annulled by a small dose of atropine. In one experiment a larger volume of the effluent was collected, during several periods of stimulation, stabilized by acidification, and evaporated to complete dryness under reduced pressure. The residue was extracted with dry alcohol, and this was evaporated again to dryness, its residue being finally taken up in saline. This solution was then accurately matched against acetylcholine on the eserinized leech preparation, and a solution of acetylcholine corresponding with it in activity, as so determined, was prepared, the actual concentration required being 1 in 2×10^7. These solutions (*S* and *AC*) were then compared against one another on the blood-pressure of a cat under chloralose, the record of the comparison being shown in Fig. 2. It will be seen that in doses of 1 and 0·5 c.c. the two solutions gave indistinguishable effects. The cat was then given 0·15 mg. of eserine per kg. intravenously, and after 15 min. the comparison of the two solutions was repeated (Fig. 2*b*). It will be seen that 0·5 c.c. of the concentrated effluent again accurately matches 0·5 c.c. of the ACh. solution, the effect of both being intensified to the same extent, in comparison with their earlier effects. Finally, after 1 mg. of atropine, the effects of both solutions were annulled (Fig. 2*c*).

If the effluent was made alkaline by adding one-tenth of its volume of *N*/10 NaOH, allowed to stand for 20 min. at the room temperature and then reneutralized with HCl, its actions on the leech and the blood-pressure were found to have disappeared. Since the perfusion fluid contained eserine, it was not possible to test directly the sensitiveness to esterase

of the active substance after collection. Indirectly, however, its liability to esterase was demonstrated by an experiment in which the tongue was perfused with Locke's solution containing no eserine. Under these conditions the muscles responded as usual to hypoglossal stimulation, but no trace of stimulant action on the previously eserinized leech muscle was acquired by the effluent. The stimulation was repeated several times, with a similarly negative result, until the tongue muscle ceased to respond. The perfusion fluid remained throughout free from any trace of a substance acting like acetylcholine. Eserine was then added to the perfusion fluid in the usual concentration of 1 in 5×10^5. In the subsequent period of stimulation there was an obvious renewed response of the tongue muscles to the nerve impulses, and the venous fluid now acquired the

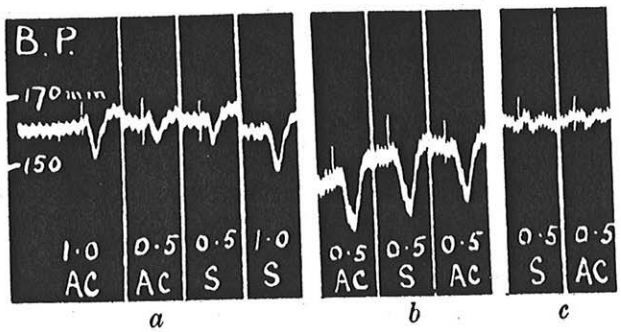

Fig. 2. Carotid blood-pressure of cat under chloralose. Injections (i.v.) of S = concentrated venous effluent from tongue (see text), and AC = acetylcholine in dilution matching S in the leech test. *a*, before eserine; *b*, after eserine; *c*, after atropine.

usual stimulant action on the leech muscle. The revival of muscular response is of interest, but it would not be proper to emphasize its significance on the basis of one observation. For our present purpose the point of importance is the appearance of the stimulant substance in the venous effluent, whereas without eserine it had been consistently absent, even when the muscle contracted well. The substance is normally destroyed on the way from the site of its liberation to the perfusion fluid, but is protected from this destruction by eserine. We are dealing, then, with a substance stable in acid and rapidly destroyed in dilute alkali; protected by eserine from destruction in the tissues; equivalent to the same doses of acetylcholine when tested on the eserinized leech muscle and on the cat's blood-pressure, before and after eserine; and having its action on the cat's blood-pressure annulled by atropine in parallel with

that of acetylcholine. There can be no real ground for doubting that it is acetylcholine itself.

Nerve fibres concerned. The tongue muscles and the hypoglossal nerve were chosen, in the first instance, because of the readiness with which this motor nerve could be freed, by degeneration, from sympathetic fibres joining it from the superior cervical ganglion. In most of the experiments above described, both ganglia had been aseptically removed some weeks before the experiment. The hypoglossal nerve has the further advantage that the sensory supply to the tongue runs separately in the lingual nerve, and is not stimulated. Langworthy [1924], has shown, however, that the cat's hypoglossal nerve often contains a vestigial sensory ganglion, in the form of a few cells of sensory type embedded among its fibres. There is no evidence that these are connected with sensory fibres from the tongue; the available evidence, indeed, connects them rather with fibres from the infrahyoid muscle,[1] not included in our perfusion. There was little likelihood, in any case, that this trivial and inconstant sensory component of the hypoglossal nerve would be responsible for the regular liberation of acetylcholine from the tongue, in the significant amounts which we obtained on stimulating the nerve. The exclusion of all sensory elements, however, was even more complete in the later experiments on leg muscles. Another possibility which gave us some concern was that of a mechanical stimulation, by the muscular contractions, of the fibres and endings of the cholinergic chorda tympani, in connection with the blood vessels of the tongue and the glands in its mucous membrane. In several experiments, accordingly, the chorda tympani was also cut by aseptic operation, at a point before its junction with the lingual nerve, and allowed to degenerate completely before the experiment, without affecting the result; stimulation of the hypoglossal, freed from sympathetic fibres, still caused liberation of acetylcholine from the parasympathetically denervated tongue. In another experiment the whole chorda-lingual nerve was cut on one side by previous operation and allowed to degenerate. The margin of the insensitive half of the tongue had been indented by biting before the experiment was performed. The two halves of the tongue were perfused separately on this occasion, and there was no significant difference in their yields of acetylcholine in response to stimulation of their respective hypoglossal nerves.

We shall see later that the mechanical stimulation of chorda-lingual fibres, together with other possibilities, can be more easily excluded by the use of curarine. At the present stage we had established a very strong

[1] Personal communication from Dr D. H. Barron.

presumption that the observed liberation of acetylcholine was due to the impulses passing along motor nerve fibres to excite voluntary muscle fibres. For the further testing of this presumption we turned to other muscles.

(*b*) *Gastrocnemius.*

Cat. The experiments on the cat's gastrocnemius were less successful than those on the tongue. After the rather long preliminary preparation, and the further period required to obtain a bloodless perfusate, we found, in several cases, that the muscle failed to respond to maximal stimulation of the ventral roots, or responded very weakly. In such cases we failed to observe any liberation of acetylcholine during the stimulation. The sciatic nerve was then isolated in the thigh, cut and stimulated peripherally, and the resulting contractions of the gastrocnemius were accompanied by the appearance of acetylcholine in the venous fluid. This latter form of stimulation, however, involved the sympathetic and sensory fibres running in the nerve, and the result was not beyond criticism for our purpose. We may confine attention, therefore, to two experiments in which the roots remained satisfactorily sensitive, the muscle responding when they were stimulated. As in the tongue perfusion, the first samples of perfusion fluid, after it had become free from blood, still contained perceptible traces of acetylcholine. These, however, rapidly disappeared with continued perfusion. When blank controls had been obtained, the roots were stimulated for a period, and with the contractions of the gastrocnemius acetylcholine appeared in the venous fluid, rapidly disappearing again with continued perfusion after the stimulation was stopped. The concentration reached during the stimulation was never high, being about 1 in 4×10^8; on the other hand, the response of this muscle to the root stimulation was never vigorous, even in the most successful experiments.

Dog. The sensitiveness of the dog's roots, as shown by the response of the muscle to their stimulation, survived the conditions of the experiment much better. The first samples of clear venous fluid showed a weak activity on the leech muscle, but practically inactive controls were soon obtained with continued perfusion. Before the motor roots were stimulated, in some experiments, a further control sample was collected during a period of stimulation of the corresponding sensory roots. Fig. 3 shows the record from such an experiment. In all cases the venous fluid was applied in 75 p.c. dilution. A shows the negative effect of a blank control, B that of the fluid collected during stimulation of the sensory roots, and

C the response of the leech muscle to ACh. 1 in 10^9, given for calibration. At D a further resting control sample was applied, and at E the venous fluid collected during a period of stimulation of the motor roots, causing contractions of the gastrocnemius. At F a sample collected 2 min. after the end of stimulation was tested, and at G a sample collected after a further 20 min. perfusion, by which time the effluent was again practically inactive. The sequence could be repeated with a second period of stimulation, but with later periods the response of the muscle, and the concurrent output of acetylcholine, declined together. As with the tongue, if perfusion was continued till the muscle had become visibly œdematous, the appearance in the effluent, of the acetylcholine liberated during stimulation, was delayed and prolonged. Results like the above could be obtained regularly in experiments on the dog's gastrocnemius, stimulated

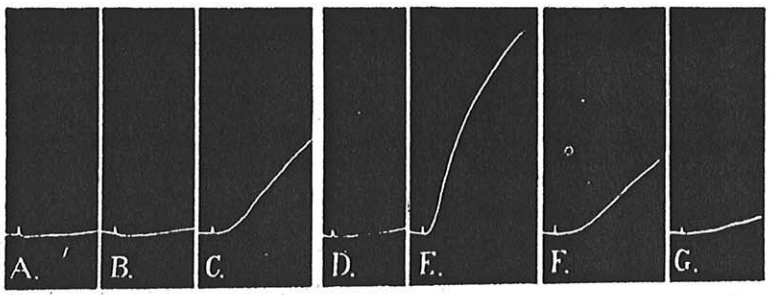

Fig. 3. A, B, D, E, F, G, venous fluids from dog's gastrocnemius, in 75 p.c. dilution (see text). C, ACh. 1 in 10^9.

through the motor roots. The concentration of acetylcholine in the stimulation effluent did not rise above about 1 in 2×10^8, and was accordingly lower than that observed in successful experiments on the tongue. On the assumption that the acetylcholine is liberated by the arrival of impulses at the motor nerve endings, for which evidence will be given later, it may be suggested that the leg muscles, with their relatively long fibres and consequently large mass perfused in relation to the number of nerve endings, might be expected to yield a lower concentration in the venous fluid than the comparatively short-fibred muscles of the tongue. The crude adjustment of the perfusion rate to the size of the muscle, however, forbids any attempts at calculation.

Identification. The substance from the gastrocnemius, like that from the tongue, behaved like acetylcholine in its instability to alkali, and its action on the cat's arterial pressure, abolished by atropine. It was further

shown, in one experiment, that an effluent, active on the leech muscle previously sensitized by eserine, had no immediate action on a control strip which had not been so treated.

(*c*) *Quadriceps extensor femoris* (*dog*).

As mentioned earlier, a few experiments were performed on this muscle mass because it could be freed from sympathetic nerve endings, by degeneration following removal of the abdominal sympathetic chain at a preliminary operation. The results obtained by stimulating the appropriate ventral nerve roots were closely similar to those obtained with the gastrocnemius, acetylcholine in 1 in 4×10^8 appearing in the venous effluent, during contractions of the muscle evoked by such stimulation. The mechanical stimulation of cholinergic sympathetic fibres and endings could, therefore, be excluded as a possible source. In one experiment the dorsal roots were also stimulated, with negative result.

(*d*) *Hind leg muscles of the frog.*

The object of these experiments was to identify the nerve fibres, responsible for the output of something like acetylcholine from the perfused frog's muscles, which Hess [1923] and others had observed with stimulation of the mixed nerve supply. The results were very similar to those we had already obtained with mammalian muscles. At the beginning of the perfusion, perceptible amounts of acetylcholine appeared in the venous fluid from the unstimulated muscles, and these disappeared as the perfusion was continued. Stimulation of the ventral roots, the sympathetic chain being extirpated, then regularly caused the appearance of acetylcholine in the venous fluid. The concentrations were low, 1 in 2×10^8 being the maximum obtained. The activity of the fluid, as in the mammalian experiments, slowly disappeared with continued perfusion, and reappeared on renewed stimulation. In two experiments the sympathetic chain was preserved and directly stimulated, causing vaso-constriction, as shown by retarded perfusion; but no acetylcholine appeared. In several experiments the dorsal roots were stimulated with completely negative results. In one experiment only, a perceptible

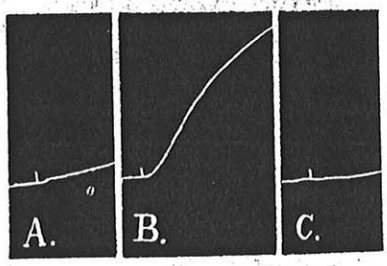

Fig. 4. Venous fluids from skinned hind-limbs of frog. A, before; B, during stimulation of motor roots; C, same fluid as B, after treatment with alkali.

quantity of something acting on the leech like acetylcholine appeared in the venous fluid at an interval of 10 min. after a period of sensory root stimulation. This result cannot properly be regarded as related to the stimulation, and it is only recorded as a presumably accidental, and obviously doubtful exception to otherwise completely negative results. Fig. 4 shows tests, on the eserine treated leech preparation, of a control fluid at A, fluid collected during effective motor root stimulation at B, and a portion of the same fluid after standing with alkali and reneutralization, at C. These experiments show that the substance detected by previous observers, as liberated during the stimulation of the mixed nerve supply, is produced by stimulation of the motor fibres to voluntary muscle, and has the instability to alkali of acetylcholine.

(2) *Direct stimulation of muscles*

We have seen that, when stimulation of the motor fibres failed to produce contractions of the muscle, acetylcholine was no longer liberated. It was possible, therefore, that it might come from the muscle fibres themselves, as a by-product of the contractile process. It was further possible that fluid collecting in inadequately perfused areas, or in the tissue between the muscle fibres, might acquire acetylcholine from some source, and that contractions might mechanically press some of it into the perfusion stream. To test these possibilities we first studied the effects of producing contractions of the muscle by direct stimulation. A normally innervated muscle cannot be effectively stimulated without stimulating, at the same time, the branches and endings of motor nerve fibres in its substance. We accordingly made comparative experiments on normal muscles, and on corresponding muscles denervated by degeneration. The muscles chosen were again the gastrocnemius of the cat and the dog and the quadriceps extensor femoris of the dog, the sciatic or crural nerve on one side having been divided aseptically under ether 10 days previously.

The results with direct stimulation of the normally innervated muscles were not different from those produced by stimulating a similar muscle through its motor nerve supply. The first samples of venous effluent contained some acetylcholine, which disappeared with further perfusion. Stimulation of the muscle then caused acetylcholine to appear, as with motor nerve stimulation, and it disappeared as usual with further perfusion. With the denervated muscle the results were entirely different. In the first place, even the earliest samples of venous fluid, after it had become free from blood, showed no significant activity

on the most sensitive leech preparation. Further, though the muscle contracted powerfully in response to direct stimulation, no trace of activity was shown by the fluid collected during or after the stimulation period. A comparison between the results obtained with a normal cat's gastrocnemius and the denervated gastrocnemius of the other leg, similarly perfused and tested in succession, is illustrated in Fig. 5. The venous fluids were tested in 75 p.c. dilutions. At A the control fluid from the denervated muscle was tested, and at B the fluid collected while the muscle was contracting vigorously in response to direct stimulation. In neither case is any trace of activity to be detected. (The small and brief rises of the writing point are accidental mechanical effects of emptying

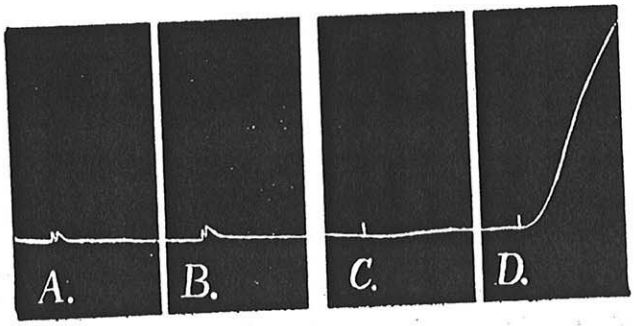

Fig. 5. Venous fluids from gastrocnemius muscles of cat, in 75 p.c. dilution. A and B from denervated muscle, A resting, B during stimulation; C and D from normal muscle, C resting, D during stimulation.

the testing bath and refilling with the test fluid.) At C the control fluid from the normal muscle was tested, and at D that collected during direct stimulation of that muscle.

An experiment on a dog's quadriceps, deprived of its sympathetic supply by degeneration but otherwise normally innervated, showed that it gave the usual yield of acetylcholine to direct stimulation. Another experiment on a sympathetically denervated quadriceps gave a result of special interest. This muscle had initially responded normally by contractions, with output of acetylcholine, to motor nerve stimulation. With continued perfusion successive periods of such stimulation had been progressively less effective, till finally the muscle no longer contracted or yielded acetylcholine, with renewed stimulation of the nerve. Direct stimulation being now applied, the muscle contracted vigorously, but no trace of acetylcholine appeared in the venous fluid. The mechanism concerned with transmission of the excitatory process from the nerve to the

muscle fibres being exhausted, acetylcholine was no longer liberated, though the muscle contracted well. The muscle, in this respect, behaved now like one which had been denervated.

(3) *Curarine*

Some of the results described in the foregoing section might still find a conceivable explanation in the passage of acetylcholine from some source into stagnant fluid in the muscle, and the mechanical expression of some of this into the circulation by the contractions. It would be necessary then to assume, indeed, that this source disappeared with degeneration of the nerves and their endings, and that it could be exhausted with repeated stimulation of the nerve to the perfused muscle. It was important, however, to find conditions under which the effects of motor nerve stimulation could be tested, in the complete absence of muscular response. The use of curarine provided the required condition, and gave a decisive result.

Experiments were made on the perfused cat's tongue, stimulated through the hypoglossal nerves, deprived by degeneration of their sympathetic fibres. A little difficulty was experienced in adjusting the dose of curarine, in the necessary presence of eserine in the perfusion fluid, since eserine is well known to be an antagonist of the paralytic action of curare. With our usual concentration of eserine (1 in 5×10^5) curarine even in tenfold concentration (1 in 5×10^4) did not always produce an immediate complete paralysis to the onset of a series of maximal break shocks, applied to the hypoglossal nerves. The experimental stimulation, however, for collection of the venous sample was not carried out until all trace of such initial response had disappeared, the tongue remaining completely passive during the whole period of stimulation. A venous sample was collected, as usual, during a control period, and another during the ineffective stimulation. The presence of curarine made it difficult to test the fluids accurately on the leech muscle, the response of which to acetylcholine is slowly paralysed by curarine. Their activity on the blood-pressure of a cat under chloralose, however, could be readily determined with some accuracy, in comparison with those of known concentrations of acetylcholine. The Locke's solution, containing eserine and curarine in the indicated concentrations, had by itself no perceptible effect on the blood-pressure. Fig. 6 shows a comparison of the depressor effects of the venous effluents collected in such an experiment, with those of two dilutions of acetylcholine. The volume injected was in each case 2 c.c. At A the control venous fluid, collected just before stimulation,

was injected; at B the fluid collected during hypoglossal stimulation; at C and D acetylcholine in dilutions of 1 in 2×10^8 and 1 in 10^8 respectively. It will be seen that the stimulation effluent is slightly less active than ACh. 1 in 10^8 and much more active than ACh. 1 in 2×10^8. That is to say, its activity in terms of acetylcholine is not different from that of a venous effluent obtained during hypoglossal stimulation from the uncurarized and actively contracting tongue, in an average, successful experiment. Between D and E 1 mg. of atropine was given, at E acetylcholine 1 in 10^8 and at F the stimulation effluent.

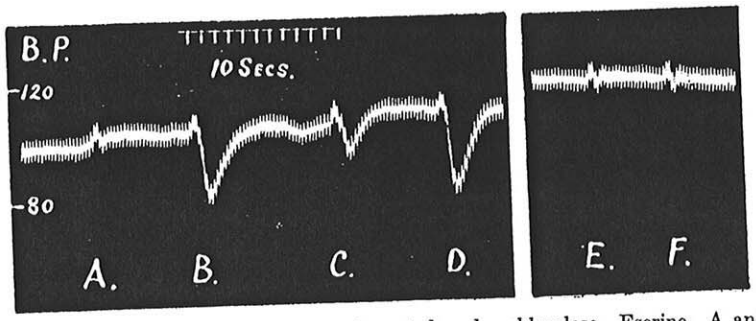

Fig. 6. Carotid blood-pressure of cat, eviscerated under chloralose. Eserine. A and B, 2 c.c. (i.v.) of venous fluids from cat's tongue under curarine. A before, B during motor nerve stimulation. C and D, 2 c.c. of ACh. dilutions; C, 1 in 2×10^8, D, 1 in 10^8. E and F, same as at B and D, after 1·75 mg. atropine.

The leech muscle is not rendered immediately insensitive to acetylcholine by curarine, so that, although no comparison can be obtained between different fluids containing curarine applied in succession to the same preparation, a qualitative contrast was easily demonstrated by applying them to two symmetrical strips of leech, previously sensitized by eserine. A preliminary test with acetylcholine showed that one strip was slightly more sensitive than the other. The control venous fluid from the tongue before stimulation was then tested on the more sensitive strip, and produced no perceptible effect, while the fluid collected during the period of hypoglossal stimulation caused prompt contraction of the other, less sensitive strip. A further application of the same fluid, however, was ineffective, the muscle being now affected by the curarine.

Brinkman and Ruiter [1924] had already shown that curare did not prevent the liberation from the frog's muscles, during nerve stimulation, of the substance stimulating the plain muscle of the cloaca. They stimulated the mixed nerves, however, and it seemed desirable to repeat the observation with stimulation of voluntary motor fibres only, through

the ventral roots, and with a different physiological test for acetylcholine. The fluids were therefore compared on symmetrical leech strips, as above described, the hindlimbs of the frog being perfused with Ringer's solution containing both eserine and curarine. As with the tongue, the fluid collected before stimulation was inactive on one strip, while that collected during motor root stimulation, without contraction of the leg muscles, caused the usual contraction of the other leech preparation.

The experiments on the curarized tongue gave opportunity for a passing observation on another point. In the experiments with eserine but without curarine, we had always observed a conspicuous acceleration of the venous outflow when the hypoglossal nerves, freed from sympathetic fibres, were rhythmically stimulated. We had suspected that vasodilatation due to acetylcholine had some part in this effect; but we could not exclude mechanical action of the rhythmically contracting muscles, or the action of products of the contractile metabolism. In the experiments with curarine in addition to eserine contractions were abolished, but the outflow was still accelerated, though only to the extent of 10 p.c., when the hypoglossal nerve was stimulated. When Locke's solution containing curarine, without eserine, was used for perfusion, no vasodilatation was caused by hypoglossal stimulation, just as we had earlier found that, if eserine was not present, no acetylcholine appeared in the venous effluent. We may safely attribute this residual vaso-dilator effect, therefore, to acetylcholine leaking from the motor nerve endings and, if eserine is present, reaching the blood vessels, where it causes arterial dilatation and diffuses into the fluid passing through the capillaries. It should be pointed out that, in the absence of heavy doses of eserine, this action could play no part in the vaso-dilatation which accompanies the contraction of a muscle with normal circulation.

These experiments with curarine show quite definitely that the appearance of acetylcholine in the perfusion fluid is not directly or indirectly connected with contraction of the muscle fibres. Feldberg and Vartiainen, in their experiments on the vagus and sympathetic ganglia, failed to find evidence for the liberation of acetylcholine by impulses passing along uninjured nerve fibres in continuity. We have had the privilege of reading in advance a forthcoming paper by Gaddum and Khayyal, who have observed the liberation of a small quantity of a substance acting on the leech like ACh., when faradic stimulation is applied to the end of a longer stretch of vagosympathetic (preganglionic) nerve. Without anticipating the details of this publication, we may say that, if the intramuscular portions of motor nerves liberated acetylcholine

at the maximum rate observed by Gaddum and Khayyal, it would not make a significant contribution to the amount which we have obtained from the tongue during motor nerve stimulation. The only supposition which accords with our facts is that a motor nerve impulse, on reaching the nerve ending, there liberates a small charge of acetylcholine, in close proximity to the motor end plate or other structure immediately subjacent to the nerve ending; and that this liberation is not affected by curarine, in a dose sufficient to prevent the response of the muscle fibre to excitation by the nerve impulse.

Discussion

There is an obvious analogy between the release of acetylcholine by impulses arriving at motor nerve endings in voluntary muscle, for which evidence has been here presented, and its release by impulses reaching the endings of preganglionic fibres in ganglionic synapses. In both cases the chief interest of the phenomenon centres in the question of its relation to the transmission of the excitatory process, with very little delay, across the anatomical discontinuity usually regarded as existing between the nerve terminal, on the one hand, and the ganglion cell or muscle fibre on the other. It may be noted that Samojloff [1925] concluded, from its high temperature coefficient (Av. = 2·37), that the conduction of excitation from motor nerve ending to voluntary muscle involved a chemical process of some kind. He even suggested that a stimulant substance might be liberated at the nerve endings, and drew an analogy from Loewi's observations on the nervous control of the heart.

In the case of the sympathetic ganglion, the direct excitatory effect upon the cells exhibited by acetylcholine with artificial application is of the "nicotine" type [Dale, 1914], being annulled by the secondary, depressant effect of nicotine itself, or by curarine in sufficient concentration [Brown and Feldberg, 1936], but resistant to atropine. It has been shown [Feldberg and Gaddum, 1934; Feldberg and Vartiainen, 1934] that acetylcholine may escape into the fluid perfusing a ganglion, during preganglionic stimulation, in a concentration which excites the cells to the output of impulses, when it is artificially injected into the perfused ganglion; and it has been argued that this indicates its release at the synapses in a concentration which cannot be without excitatory effect. In the case of the voluntary muscle the position is less clear, owing to the relatively low concentration of acetylcholine in the perfusion effluent during motor stimulation, the reason for which has been already discussed, and to the more complex nature of the stimulant actions of acetylcholine, when artificially applied to various types of voluntary

muscle. Striated, involuntary muscle fibres can, indeed, be found, such as the outer, striated muscle coat of the intestine in certain fishes [Méhes and Wolsky, 1932], and the sphincter of the pupil in birds, innervated by parasympathetic nerve fibres and responding to the application of acetylcholine with a type of contraction closely simulating the effects of impulses in those nerves; and in such cases the effects of acetylcholine and of parasympathetic impulses differ from those on analogous layers of plain muscle, only in the quickness of the contractile response and in its suppression by curare instead of by atropine.

The reactions to acetylcholine shown by the voluntary striated muscles of different vertebrates, on the other hand, are complex and variable, and it is necessary to consider them in some detail. In the first place, certain normal muscles of the frog, the tortoise and the bird exhibit a prolonged type of contracture, of low tension, in response to nicotine and to various bases which resemble it in action. Riesser and Neuschlosz [1921] first showed that acetylcholine produced this type of effect in relatively low concentrations. It was subsequently shown [Sommerkamp, 1928; Wachholder and Ledebur, 1930] that there are wide differences in the sensitiveness of different muscles of the frog and the tortoise to this action of acetylcholine, corresponding to differences in the prominence of contracture in their natural functions; and, according to Plattner and Krannich [1932], the same muscles show corresponding differences in the amounts of acetylcholine which they yield to artificial extraction. This contracture is relatively resistant to curare, and somewhat sensitive to atropine [Riesser and Neuschlosz, 1921]. Although it seems likely that this type of response has some relation to the normal, functional contractures exhibited by such muscles, it would be difficult to make a case for a participation of acetylcholine in the excitation of voluntary muscle fibres to quick contractions by motor nerve impulses, if this were the only detectable type of reaction shown by skeletal muscle to its artificial application. There is, however, another type of reaction, which the predominant interest given to muscles showing the contracture has been apt to obscure.

Langley [1907], in his experiments with nicotine on the frog's sartorius, showed that it first produced twitches, and a quick type of contraction resembling a short tetanus, prior to the slowly developing contracture. He made the significant observation that, with punctiform application of the alkaloid in low concentrations, the quick reactions could only be elicited from the neighbourhood of the nerve endings, from which the excitation was apparently propagated, whereas the contracture,

while it could be produced in any part of the muscle fibres, usually remained localized to the region of application. Sommerkamp [1928] found that some frog's muscles give only quick contractions when immersed in acetylcholine, while others respond mainly by contracture. In the ilio-fibularis muscle the contracture was limited to a small part of the muscle, separable by dissection; the remainder, freed from it, gave only a quick, evanescent contraction, when immersed in ACh. 1 in 10^5. The normally innervated voluntary muscles of the mammal, with the exception of the small muscles moving the eyeball [Duke-Elder, 1930], are often regarded as insensitive to acetylcholine. They do, indeed show a striking contrast in this respect, under ordinary experimental conditions, to muscles denervated by motor fibre degeneration, which respond to very small injections of acetylcholine with a slow type of contraction. Feldberg and Minz [1931] and Feldberg [1933*b*], however, observed quick contractions and fibrillation of normal mammalian muscles when acetylcholine was injected in moderate doses (0·01–0·2 mg.) into the arteries supplying them. According to the Simonarts [1935*a*] the response of normal mammalian muscles to acetylcholine is very readily depressed by ether. In rabbits and cats in the early stages of anæsthesia by a barbiturate, or in spinal preparations freed from the preliminary ether by prolonged ventilation, he observed quick contractions of the muscles, unaffected by fresh section of the nerve supply, when doses of acetylcholine of the order of 1 mg. were injected intravenously. The reaction was unaffected by atropine, which, indeed, had to be given in advance, to eliminate the effects of such doses of acetylcholine on the heart and the blood vessels. On the other hand, the Simonarts found them to be suppressed by a quaternary ammonium salt having a curare action. Acetylcholine solutions applied to the bared surface of a muscle, or injected by a fine needle into its substance, caused brisk and fugitive contractions of fibres, or of whole bundles, localized to the neighbourhood of the application. In a more recent paper A. Simonart [1935*b*] describes results obtained with the more effective method of arterial injection. As was to be expected, the threshold dose of ACh. for normal cat's muscle was much lower by this method, and the tensions recorded with larger doses were of the order of those obtained with maximal indirect faradization. Curare readily abolished these effects. We hope to deal further with this aspect of our problem in a later paper.

Admittedly there are points still requiring further investigation. The nature of the reaction of denervated mammalian muscle to the quicker but less sensitive reaction of normal mammalian muscle, on the one

hand, and to the persistent contracture of low tension shown by certain frog muscles on the other, does not seem to be adequately defined by the present evidence. In the case of the mammalian muscles, the facts still suggest that the presence of the normal nerve ending in some way hinders the access, to the sensitive point on the muscle fibre, of acetylcholine applied from without; just as we found that, with normal circulation, there appears to be some hindrance to the escape of acetylcholine from the neighbourhood of the motor nerve endings, where it is liberated by nerve impulses. It may be remarked that such restriction would not be unexpected, in a muscle consisting of fibres which can act as independent physiological units. However that may be, the point requiring emphasis for our purpose is the distinction between the quick contractions elicited by acetylcholine, apparently, on Langley's evidence, by excitation of the structures immediately subjacent to the motor nerve endings, and the persistent contractures produced in certain amphibian and other muscles.

As in the case of the ganglion synapses, the failure of eserine under normal conditions to facilitate or to prolong the excitatory effect of a motor nerve impulse, appears to have been regarded as evidence against the participation of acetylcholine in the transmission of the excitatory process [Kruta, 1935]. In neither case does the argument appear to us well founded. The motor nerve ending is in such immediate contact with the nucleated end plate or other structure, from which the excitatory process in the muscle fibre must start, that there is no room for destruction of acetylcholine during diffusion to its points of action, from which eserine might protect it. On the other hand, when acetylcholine has to reach muscle fibres by diffusion from a solution in which the whole muscle is immersed, the effect of low dilutions in producing contracture should be enhanced by eserine, as Kruta found it to be; just as we have found that eserine is necessary to protect acetylcholine during diffusion from the points of its release into the blood vessels.

The transmission of the effects of nerve impulses by a chemical substance, reaching the effector cells by diffusion, is now an accepted fact in the case of simpler types of contractile cells and tissues, usually displaying an automatic activity which the nerve impulses may modify in either direction. Such transmission by a diffusible stimulant can now be traced in the nervous control of most involuntary muscle, including, as we have seen, some which is striated and relatively quick in contraction. The question which here concerns us is whether in voluntary striated muscle, specialized for the quick contraction of individual fibres in response to nerve impulses, and normally at rest in their absence, this more primitive,

chemical method of transmission has been superseded by an entirely different one, in which the chemico-physical disturbance constituting the nerve impulse passes, by continuous propagation, on to the muscle fibre; or whether, on the other hand, the required specialization has been effected by concentrating the release and the action of the chemical stimulant at the point of immediate contact of the nerve ending with the muscle fibre. An analogous question has already arisen in connection with the response to nerve impulses of more than one plain muscle structure. Henderson and Roepke [1934] find that stimulation of the pelvic nerve causes two kinds of reaction of the plain muscle of the urinary bladder—a quick contraction and a maintained tonus. They find that atropine readily abolishes the maintained tonus, just as it abolishes the response to acetylcholine applied from without, leaving the quick contraction. Similarly Bacq and Monnier [1935] distinguish a quick and a slow component in the response to stimulation of the cervical sympathetic nerve of the plain muscle retracting the nictitating membrane. They find that a synthetic substance "F. 933" depresses the slow component, and with it the response to adrenaline, leaving the quick reaction unaffected, or even apparently enhanced. In both cases the observers suggest that the slower reaction is due to chemical transmission, but that this form of control is supplemented by the presence of certain nerve fibres ending directly in plain muscle cells, the propagated change constituting the nerve impulse being directly continued from these to the plain muscle, without chemical intervention. The number of nerve endings in such sheets of plain muscle is known to be small, in relation to the number of muscle cells. It appears to us that the quick reactions, in both these cases, may equally well be explained by the liberation of the transmitter in high concentration in immediate relation to, possibly within the limiting membranes of, the directly innervated cells; the slow reaction being then evoked by its escape and secondary diffusion on to other cells, in a manner analogous to its artificial application through the blood stream or from the surface. In the case of the vaso-dilator effect of the chorda tympani on the blood vessels of the tongue, which is typically resistant to atropine, there is direct evidence of such escape by diffusion of a chemical transmitter with all the properties of acetylcholine, causing contracture of adjacent voluntary muscle fibres if these have been denervated [Bremer and Rylant, 1924; Dale and Gaddum, 1930], and appearing in the blood or perfusion fluid flowing from the tongue [Feldberg, 1933*a*; Bain, 1933].

If Henderson and Roepke, Bacq and Monnier were right in postulating a supplementary, direct transmission of nerve impulses to

plain muscle, when quick contraction is required, we should expect it to supersede entirely the chemical method of transmission, for nerve impulses causing excitation of the very rapid and individually reacting fibres of skeletal muscle, giving a single twitch, with minimal transmission delay, in response to each impulse. Similarly we should expect such a direct method of transmission at a ganglionic synapse, where each preganglionic impulse can evoke a single postganglionic impulse, again with minimal transmission delay. Such a conception, however, leaves us with no explanation of the release of acetylcholine at the preganglionic and the motor nerve endings. This can hardly be the survival of an archaic form of transmission, no longer having any function. In the ganglion acetylcholine has been shown to be liberated in a concentration which effectively stimulates ganglion cells; while in the muscle we have shown that, when the liberation of acetylcholine fails by exhaustion, the excitation of the muscle no longer occurs. There seem to be two possibilities.

(1) That the propagated disturbance in the nerve fibre is directly transmitted to the effector cell, but that the latter cannot accept it for further propagation unless sensitized by the action of the acetylcholine, which appears with its arrival at the nerve ending. Such an hypothesis might be stated in terms of Lapicque's well-known conception, by supposing that the action of acetylcholine shortens the chronaxie of the nerve cell, or of the motor end plate of the muscle fibre, so that it is momentarily attuned to that of the nerve. H. Fredericq [1924] has observed, indeed, a shortening of the chronaxie of heart muscle by acetylcholine.

(2) That the acetylcholine, in these as in other cases, acts as the direct stimulant of nerve cell or muscle end plate, releasing an essentially new propagated wave of excitation in postganglionic nerve or muscle fibre, which, however, may so resemble that in the preganglionic or motor nerve fibre as to simulate an unbroken propagation. On this view there is no introduction of a new form of transmission, in evolution from the slowest and most primitive to the most rapid and specialized. The required rapidity of transmission is attained by concentrating the release of the chemical transmitter on the actual surface of the responsive structure.

Of the two possibilities, the latter appears to us to be more easily reconciled with the facts yet available concerning transmission at ganglionic synapses. The former would provide an explanation, alternative to that which we have considered earlier, for the apparently low sensitiveness of some normal muscles to stimulation by acetylcholine. The shortness of the delay in transmission appears to cause no greater diffi-

culty for one conception than the other. The action of curare is explicable, in either case, as rendering the receptive element resistant to the action of acetylcholine, whether this be merely to sensitize or directly to stimulate. On the existing evidence we favour the second conception, while admitting that further facts are required for the exclusion or the establishment of either.

As in the case of transmission at the ganglionic synapse [cf. Feldberg and Vartiainen, 1934], either of the above conceptions of the function of acetylcholine, in the transmission of excitation to the voluntary muscle fibre, would require not only its liberation in immediate relation to the excitable structure, but presumably its very rapid disappearance when the excitatory wave in the muscle had been started. The known extreme liability of acetylcholine to the action of an esterase naturally comes to mind in that connection; but there is no direct experimental evidence to justify an assumption that this esterase is, in fact, responsible for removing acetylcholine from the site of its action in this case, and that eserine would, therefore, increase its persistence at that site. Nor can we predict the effect of such persistence on the transmission under particular conditions, if it could be proved to occur. All that our evidence shows is, that acetylcholine which has escaped from the sites of its liberation requires protection by eserine to enable it to diffuse into a fluid perfused through the blood vessels.

Summary

1. Stimulation of the motor nerve fibres to perfused voluntary muscle causes the appearance of acetylcholine in the venous fluid.

2. Direct stimulation of a normal muscle, or of one deprived only of its autonomic nerve supply, has a similar result; but when the muscle is completely denervated no acetylcholine appears in response to effective stimulation.

3. When transmission of excitation from the nerve to the perfused muscle is prevented by curarine, stimulation of the motor nerve fibres causes the usual release of acetylcholine.

4. When conduction from motor nerve fibres to perfused muscle fails from exhaustion, after repeated stimulation, acetylcholine is no longer released by stimulation of either nerve or muscle.

5. The function of acetylcholine in the transmission of excitation from nerve to voluntary muscle is discussed.

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