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FURTHER OBSERVATIONS ON THE PHYSIOLOGY AND PHARMACOLOGY OF A SYMPATHETIC GANGLION.

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FELDBERG and GADDUM [1934] have shown that, when the superior cervical ganglion of the cat is perfused with warm, oxygenated Locke's solution containing a small proportion of eserine, acetylcholine appears in the venous effluent whenever the cervical sympathetic nerve is effectively stimulated, and only then. They suggested that the liberation of a small quantity of acetylcholine provides the mechanism by which the effect of a preganglionic impulse passes the synapse. The appearance of acetylcholine, on this view, provides the direct stimulus to the output by a ganglion cell of a postganglionic impulse.

It is obviously desirable to bring this suggestion to the test of further experiment. As pointed out in the earlier paper, it requires the assumption that the acetylcholine detected in the venous fluid is liberated actually at the synapses in the ganglion, in immediate proximity to the ganglion cells. The suggestion has been criticized by Eccles [1934], chiefly on the ground of his own failure to observe any potentiation by eserine of the electrical potential changes in the ganglion and the postganglionic fibres following a single volley of preganglionic impulses, or any prolongation by eserine of the persistent excitatory state following such a volley. The evidence which we here present indicates more definitely the site and the conditions of liberation of the acetylcholine, and deals with the effects of eserine and of other substances, when applied to the reactive structures in the ganglion through the artificial perfusion.

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METHODS.

The method for perfusing the ganglion was essentially that used by Feldberg and Gaddum [*loc. cit.*]. The following modification was introduced in the preparation of the ganglion. The central end of the vagus was tied and cut beyond the ganglion near its entrance into the skull. This made it easier to tie the arterial branches accompanying the postganglionic fibres into the skull. In the absence of this precaution, if acetylcholine was injected into the perfusion cannula (see later), some of it leaked into the general circulation and caused a general fall of arterial blood-pressure, as recorded from the femoral artery. If such evidence of leakage still appeared after starting the perfusion, the small arterial branches responsible for it were found and tied.

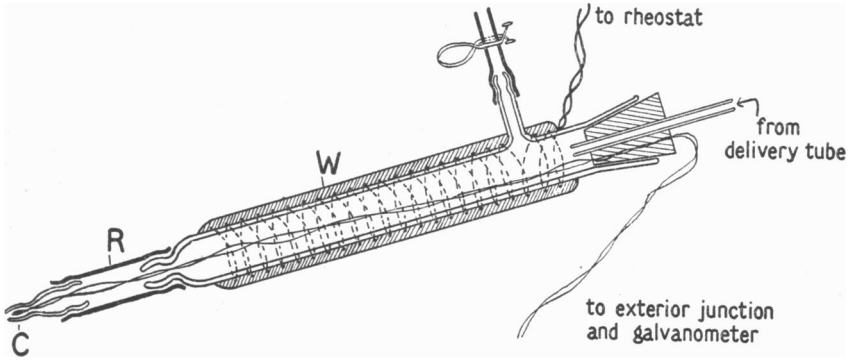

Fig. 1. Cannula (Gaddum) for controlling the temperature of a slowly perfused fluid.

In connection with the technique of some of the injections, it is desirable to describe a little more fully the cannula already used by Feldberg and Gaddum for controlling the temperature of the arterial fluid (see Fig. 1). From the delivery tube the filtered Locke's solution passed through the warming tube (*W*), a thin walled tube of glass 10 cm. in length and 8 mm. in internal diameter. The upper end of the warming tube was widened to receive a small rubber stopper, which gave passage to the glass end of the delivery tube, and to the small insulated wires leading to the thermocouple. A small side tube, furnished with a rubber tube and clip, near the upper end of the warming tube, allowed any bubbles of gas which collected to be expelled. The warming tube was warmed by a current in a coil of resistance wire with insulating sheath, controlled by a rheostat. To the lower end of the warming tube the small glass arterial cannula (*C*) was joined by a short length of rubber tubing (*R*).

In this arterial cannula lay the thermocouple, and the heating of the coil was so controlled by the rheostat as to maintain the temperature of the fluid in the cannula as constant as possible. Injections were made by puncturing the rubber tube (*R*). Since the resistance was on the arterial side of the cannula, practically the whole of a volume of fluid so injected passed immediately back into the warming tube, to be delivered therefrom through the ganglion at the slow rate of perfusion. The injection of a small volume, such as 0·1 c.c., as used in testing the stimulating effects of various substances, caused no important change in the temperature of the fluid entering the ganglion. Such a small injection, it will be understood, was subjected to an unknown dilution by admixture with the perfusion fluid. The dosage could only be expressed, therefore, in terms of the weight injected of the substance under test. It is realized that differences in the rate of perfusion in different experiments, or in the degree, at different stages in the same experiment, of the œdema which the ganglion always showed with prolonged perfusion, would cause rather wide variations in the rate at which a given dose of the drug reached the ganglion cells, and even in the proportion of it which reached them at all. In spite of these factors of variation and uncertainty, the threshold dose of a drug often retained a remarkable constancy through a long period of one experiment. When larger volumes, of the order of 10 c.c., were injected, in order to subject the ganglion for a period of some duration to the action of an approximately known concentration of a drug, such as eserine or nicotine, precautions had to be taken to exclude serious alterations in the temperature of perfusion. The fluid in the warming tube was normally maintained at such a temperature that the thermocouple in the arterial cannula registered a temperature of about 38°–39° C., so that the fluid, which still had to pass slowly through the carotid stump and its small branch to the ganglion, would reach the latter at a temperature slightly lower. It was found that if a large injection, practically all of which would pass immediately back into the warming tube and even beyond it into the delivery tube, was made with fluid at room temperature or prewarmed to over 40° C., the temperature in the arterial cannula fell or rose to such an extent as to complicate the specific effect of the drug under test, and thus to obscure the interpretation of the result. When the possibility of a potentiating effect was under test, it was particularly important to exclude a temporary rise of temperature. It was found by trial that if the fluid was injected at a temperature of 37° or 38° C., only a slight initial fall of temperature occurred, before the heating coil, with but

small adjustment of the rheostat, restored the cannula temperature to, and kept it at, the previous constant level of 38°–39° C.

In the experiments of the earlier series, where the fact of the liberation of acetylcholine by preganglionic stimuli was the main point to be demonstrated, the nerve was laid for stimulation on platinum electrodes. In some of the experiments here described it was necessary to ensure the constancy of successive groups of stimuli, in order to test the potentiation or depression of their effects by application of drugs to the ganglion. For this purpose we used Collison's [1933] modification of the Brown-Garrey fluid electrode, filled with the blood of the animal under experiment. The cervical sympathetic nerve having been drawn into the electrode, the latter was fixed in a clamp, and left without adjustment till the experiment was completed. Groups of equal break shocks at the chosen rate were obtained with Lewis's rotating interruptor, connected to an induction coil fed by a storage cell.

Results.

The site of the release of acetylcholine.

Even on the reasonable assumption that the release of acetylcholine, in response to stimulation of the cervical sympathetic nerve, occurs in the superior cervical (sympathetic) ganglion, its appearance might, theoretically, be connected with the passage of impulses over any of the structures in the ganglion which they traverse—the preganglionic fibres before they reach the synapses, the bodies of the ganglion cells, or the postganglionic fibres on their way out from the ganglion.

(*a*) *The presynaptic nerve fibres.* It is impossible to restrict the perfusion to the superior cervical ganglion, the closely adherent ganglion of the vagus trunk being, of necessity, perfused with it. This necessity, however, provides the opportunity for testing one possibility. If the acetylcholine were released from the presynaptic nerve fibres in the ganglion, *i.e.* by the passage of impulses through nerve fibres in continuity, impulses in the fibres passing through the sensory vagus ganglion, without synaptic interruption, ought to be similarly effective. This possibility was easily put to the test with the usual perfusion scheme, by stimulating alternately the central end of the cut cervical vagus and the cervical sympathetic, and collecting and testing in parallel the venous fluids corresponding to the periods of stimulation of each nerve. The eserinized leech preparation was used as the test object, and the perfusion

fluid contained eserine in a dilution of 1 in 5×10^5. Fig. 2 reproduces the record obtained. The cervical sympathetic nerve was stimulated for two periods, and an equal period of central vagus stimulation, with identical shocks, was interposed between the two. *A* and *C* in Fig. 2 show the effects produced by tenfold dilutions of the fluids collected during the first and second periods of cervical sympathetic stimulation; at *B* in the same figure the fluid obtained during the intervening period of vagus stimulation, diluted only $2\frac{1}{2}$ times, was applied, without any perceptible effect. It is safe to conclude that, if acetylcholine is released in any amount by the passage of impulses over nerve fibres in continuity,

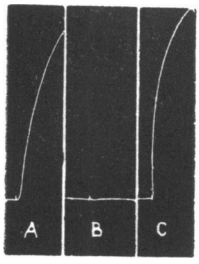

Fig. 2.

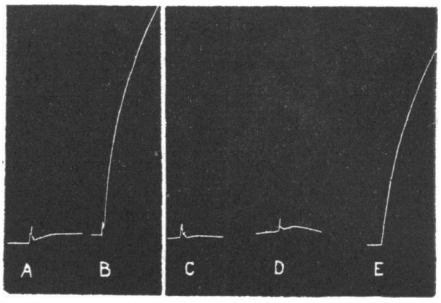

Fig. 3.

Fig. 2. Leech muscle. Effects of ganglion effluents during stimulation of cervical sympathetic (*A* and *C*) and vagus (*B*).

Fig. 3. Leech muscle. Effects of ganglion effluents; *A* and *C* without stimulation, *B* and *E* during preganglionic, and *D* during postganglionic stimulation.

as in the vagus ganglion, the amount is far too small to contribute significantly to the output detected when the cervical sympathetic nerve is stimulated. It seems reasonable, by analogy, to exclude from further consideration the presynaptic fibres in the sympathetic ganglion.

(*b*) *Postganglionic fibres and ganglion cells.* These structures could be tested together, as potential sources of the acetylcholine, by employing a "backfiring" method of stimulation, analogous to that used by Sherrington and his co-workers for sending impulses back through motor fibres into the motor-horn cells of the spinal cord. As in the experiment with the vagus, equal periods of identical stimulation were applied alternately to the preganglionic fibres of the cervical sympathetic nerve, and to the postganglionic bundle beyond the ganglion. Each stimulation period consisted of 3 min. stimulation, 1 min. pause and a second 3 min. stimulation, the venous fluid being collected for the whole

7 min. and for a further 1 min. after the end of the stimulation. Eserine, 1 in 5×10^5, was again added to the perfusion fluid. Records of contraction of the nictitating membrane gave, in this case, an additional check on the effective stimulation of the postganglionic bundle.

Fig. 3 shows the effects of the different venous fluids on the leech preparation. At *A* the control fluid, collected before stimulation, was applied in a dilution of 1 to 1·4; at *B* the fluid from a first period of preganglionic stimulation, diluted 1 to 9; at *C* a second control fluid, 1 to 1·4; at *D* the fluid from a period of postganglionic stimulation, 1 to 1·4; and at *E* the fluid from a second period of preganglionic stimulation, 1 to 9.

It will be clear that the fluid, collected during the stimulation causing passage of impulses through the postganglionic fibres into the bodies of the ganglion cells, applied at *D*, exhibits no perceptible acetylcholine action even in a dilution of 1 to 1·4. It seems proper to conclude, therefore, that the passage of impulses through either of these structural elements plays no significant part in the release of the acetylcholine, which appears when the preganglionic fibres are stimulated.

The record of the effects of stimulation on the contractile activity of the nictitating membrane led incidentally to observations of some probable interest. In this, as in many other experiments, stimulation of the cervical sympathetic nerve with rapidly repeated induction shocks produced a characteristic complex. At the outset of stimulation the nictitating membrane was withdrawn rapidly to the maximal extent, but partial relaxation from this maximum rapidly set in during continued stimulation, often to be followed in turn by slow recovery to an intermediate level of contraction. We have not yet examined the possibility of a connection of this sequence with the artificial perfusion of the ganglion, or with the presence of eserine in the perfusion fluid. It was not seen when the postganglionic fibres were stimulated; in response to such stimulation the membrane was rapidly withdrawn to the maximal extent, and the contraction remained maximal until stimulation ceased. There can be no doubt, therefore, that the stimulation applied to the postganglionic fibres was maximal. It may further be concluded that the partial relaxation, seen during continued stimulation of the cervical sympathetic, was due to a partial failure of transmission of the effect across the synapses to the ganglion cells. Of greater interest was the fact that a further period of stimulation, applied to the preganglionic fibres about a minute after the maximal postganglionic stimulation had ended, produced no retraction of the nictitating membrane. That the post-

ganglionic fibres and the plain muscle were still fully responsive was shown subsequently, by stimulating the postganglionic fibres, maximal retraction being again produced. We can only conclude that, during this second period of preganglionic stimulation, following the period of "backfiring" impulses, the excitation failed to pass the synapses because the ganglion cells had been rendered insensitive. Whether this condition was wholly due to the impulses fired back into them in the foregoing period, or whether the effect of these was reinforced by the prolonged perfusion with eserine, is not clear. The point of immediate importance for our purpose is that, although the ganglion cells were sending out no effective impulses in response to the preganglionic stimulation, acetylcholine was liberated in as large an amount (cf. Fig. 3, *E*) as when they were fully active.

The observation makes it additionally clear that neither the output of impulses from the ganglion cells, nor the passage of impulses from them along the postganglionic fibres, is responsible for the output of the acetylcholine detected in the venous fluid. We shall later find evidence in the same direction in dealing with the paralytic effects of larger doses of eserine and of nicotine.

Taking together these results of stimulating the vagus and the postganglionic sympathetic fibres, we may exclude the presynaptic fibres, the ganglion cells and the postganglionic fibres, as sources of the acetylcholine which comes from the ganglion when the cervical sympathetic nerve is stimulated. There remain only the synapses as the site of its liberation.

Liberation of acetylcholine with natural circulation.

It seemed possible that the appearance of acetylcholine in the venous effluent, concurrently with stimulation, might be an abnormal phenomenon, due to the conditions of artificial perfusion. Its significance would remain, even if this were so; but it was of interest to enquire whether, under conditions of perfectly normal circulation, stimulation would cause acetylcholine to appear in the venous blood from the ganglion. For this purpose the preparation was made as for perfusion, so far as concerned the ligature of all branches of the carotid artery and the internal jugular vein, except those supplying and draining the ganglion. The natural relations of the ganglion, however, were undisturbed; it was kept carefully covered and warm, with its normal circulation. The blood was rendered incoagulable with "Chlorazol Fast Pink," and a cannula inserted into the internal jugular vein, from which

the slowly dropping venous blood from the ganglion was collected. Half an hour before the first sample was taken 5 mg. of eserine sulphate were injected intravenously, with 1 mg. of atropine sulphate to prevent excessive retardation of the heart beat.

Fig. 4 gives a record from the testing of the successive blood samples on the eserinized leech preparation. At *A* the first "control" sample was tested in a dilution of 1 to 9; at *B* a sample collected during maximal stimulation of the cervical sympathetic, again at 1 to 9 dilution; at *C* a second control sample, after a further injection of 1 mg. of eserine, in this case at 1 to 5 dilution; at *D* a sample collected during a second stimulation, diluted 1 to 10, and at *E* the same sample diluted 1 to 20;

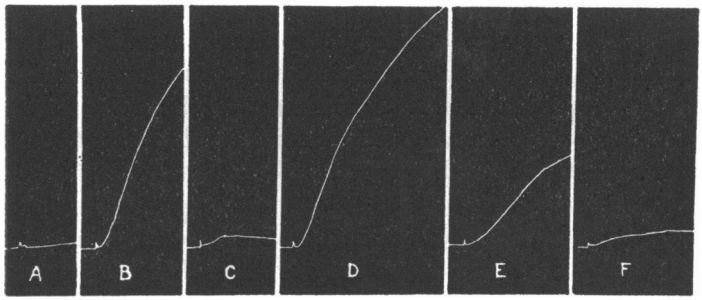

Fig. 4. Leech muscle. Blood from ganglion with natural circulation; *A*, *C* and *F*, without stimulation, *B*, *D* and *E* during preganglionic stimulation.

at *F* a further control sample, collected after an interval of a few minutes from the end of the stimulation period, and diluted only 1 to 2.

The record needs little exposition. When mammalian blood is tested on the leech, even when diluted, slight non-specific effects are occasionally seen, and can be discounted. Such effects as those shown in Fig. 4 at *C* and *F*, with blood diluted 5 and 2 times respectively, have no significance, especially when compared with the effects of a "stimulation" sample, diluted 10 and 20 times, at *D* and *E*. A comparison of *E* with the effects, on the same leech preparation, of standard dilutions of acetylcholine, showed it to be equivalent to A.C. 1 in 5×10^8; so that the original blood, before the twentyfold dilution, would have contained A.C. 1 in $2{\cdot}5 \times 10^7$, or 40γ per litre. This is a figure well within the range of those obtained with the saline effluent of an artificial perfusion. The latter, therefore, represents with reasonable accuracy the rate of escape of acetylcholine, during stimulation, into the blood vessels of the ganglion

with normal circulation. It need hardly be added that, under such normal conditions, in the absence of poisonous doses of eserine, it would be destroyed even before it reached the blood.

The quantity of acetylcholine released by a single preganglionic volley.

Feldberg and Gaddum [1934] gave some data as to the amount of acetylcholine entering the venous outflow from the perfused ganglion in unit time, during a period of maximal stimulation of the cervical sympathetic nerve. They were using, however, the automatic interruptor, and their figures furnished no precise information as to the amount corresponding to each shock. They only showed that this amount would be too small to be detected by any methods available. The information could be obtained, however, by using shocks at a rather slower and accurately known rhythm, and collecting the fluid from the ganglion during a known number of maximal shocks, and a sufficient subsequent period to ensure the collection of any acetylcholine entering the vessels during the stimulation. From the quantity corresponding to a known number of shocks, that corresponding to a single maximal preganglionic volley could be calculated, on the assumption that the amount per shock would be independent of the number administered. The latter point could be put to the test of experiment. The ganglion was perfused as usual with warm oxygenated Locke's solution containing 1 part of eserine in 5×10^5. The fluid electrode was used. Two periods of stimulation were given, of 30 shocks in 17 sec., and 120 shocks in 68 sec. respectively. In each case the venous fluid was collected for the period of the stimulation and for 1 min. after its cessation. A control sample taken after this period of collection was found, in each case, to be inactive. The rate of venous outflow remained constant at about 1 c.c. in 4 min.

The two stimulation samples, after dilution to convenient volumes, were tested on the eserinized leech preparation in comparison with known dilutions of acetylcholine. The first sample, corresponding to 30 maximal shocks, showed in a dilution volume of 2 c.c. an acetylcholine dilution of 1 in 10^9. This indicates the output of $0{\cdot}002\gamma$ by 30 shocks, or $0{\cdot}000066\gamma$ per shock. The second sample, corresponding to 120 shocks, diluted to 4 c.c., showed an acetylcholine dilution of 1 in 5×10^8. This indicates an output of $0{\cdot}008\gamma$ by 120 shocks, or again $0{\cdot}000066\gamma$ per shock. A portion of this 120-shock sample was given a further twofold dilution, so that its strength now corresponded to a dilution of the original volume to 8 c.c.; its action was now indistinguishable from that of the first, 30-shock sample, made up to a volume of 2 c.c. In another similar experiment

two different samples, each corresponding to 60 shocks, showed identical activities corresponding to 0·0001γ per shock. In this instance the ganglion from the unperfused side, when dissected out cleanly, weighed 12 mg. From data given by Billingsley and Ranson [1918] we may assume that the superior cervical ganglion in the cat contains some 120,000 nerve cells. On the basis of 100,000 cells, the amount of acetylcholine liberated by a single impulse at a single synapse would be of the order of 10^{-15} g., *i.e.* about 3 million molecules. Since, however, we have no evidence as to what fraction of the total amount thus liberated escapes into the circulation and is collected in the venous fluid, the figure obtained has no accurate significance.

Actions of acetylcholine and eserine.

Feldberg and Gaddum [*loc. cit.*] referred briefly to the stimulant effects on the ganglion cells, as demonstrated by retraction of the nictitating membrane, of injecting small doses of acetylcholine into the perfusion fluid on its way to the ganglion. They mentioned also the potentiating effect on this action of a small proportion of eserine in the perfusion fluid. We have examined these actions in more detail, using a more uniform method of injection. For injections of acetylcholine, or of the other substances of which the action is described later, we have prepared dilutions from a stock solution, such that the required dose was, in each case, contained in 0·1 c.c. of Locke's solution; this volume being rapidly injected by a needle pushed through the rubber junction just above the arterial cannula. In order to demonstrate the changes in reaction to acetylcholine produced by eserine in different concentration, we have perfused the ganglion with plain Locke's solution, determined the threshold dose of acetylcholine under those conditions, and then temporarily changed the perfusion fluid for one containing the required concentration of eserine, by injecting 10 c.c. of the latter through the same rubber junction, with the precautions to avoid temperature changes described under "Methods." The same technique has enabled us to compare the effects of preganglionic stimulation, with and without eserine.

Acetylcholine without eserine.

The threshold dose of acetylcholine under these conditions has varied in different experiments from 1 to 3γ. In this lower range the effect increases quickly with the dose. If the threshold dose is determined, as that which causes a retraction of the nictitating membrane just perceptibly recorded by our lever system, doubling the dose produces a

strong retraction. As the dose is further increased the curve recording the contraction becomes higher and rises more rapidly till the maximum is obtained, but relaxation in every case begins as soon as the full contraction is reached. No increase of dose beyond that maximum, whether by increasing the strength or volume of the injection, causes any prolongation of the contraction; on the contrary, when the dose is increased beyong the maximum, the effect becomes smaller, relaxation setting in at an earlier stage of the contraction.

It seemed clear that the stimulant effect was here becoming complicated by the secondary, paralytic action on the ganglion cells, which was to be expected from Dale's [1914] description of the nicotine-like action of acetylcholine and other choline esters on ganglia. This paralytic effect could be more clearly demonstrated by comparing the responses to equal periods of electrical stimulation of the cervical sympathetic, before and after a dose of acetylcholine. Even with a dose of acetylcholine just producing the maximal response obtainable from the ganglion, a depressant effect on the subsequent response to sympathetic stimulation was evident. It was noted, further, that the paralytic effect was still progressive for some time after the stimulating action of the acetylcholine had subsided. The response to a period of electrical stimulation, given just after the end of the stimulation by acetylcholine, showed already some depression in comparison with that to an earlier control period; but sometimes the depression, as shown by the responses to further test stimulations, increased for a further period of a few minutes. A precisely similar after effect is shown by a stimulant injection of nicotine and is illustrated in Fig. 9. If a large dose of acetylcholine, 20–100γ, is injected, after a brief stimulating effect, the ganglion cells are rendered, for the time, completely unresponsive to preganglionic impulses or to further injections of acetylcholine, until by continued perfusion with clean solution the excess of acetylcholine is washed away, and the responsiveness of the ganglion cells is restored.

Effects of eserine.

(*a*) *Sensitizing action.* During a temporary perfusion of Locke's solution containing eserine in concentrations from 1 in 10^6 to 1 in 5×10^5, the activity of acetylcholine, injected as above, was increased from 8 to 20 times, the threshold dose being lowered to 0·05–0·2γ. Fig. 5 reproduces a record from an experiment in which such eserine perfusion caused a tenfold increase in the activity of acetylcholine on the ganglion cells. We have also tested the effects of such low concentrations of eserine on

the stimulant actions on the ganglion, to be described later, of other substances, such as choline, potassium ions, nicotine and hordenine methiodide. In all cases a slight enhancement of effect was detected; in the case of nicotine the activity appeared, in a few experiments, to be approximately doubled; in the other cases the enhancement was not more than 50 p.c. These actions are further mentioned in a later section. We must conclude, accordingly, that eserine in low dilutions causes a slight, non-specific sensitization of the ganglion cells to a variety of chemical stimuli. The sensitization of their response to injections of acetylcholine, however, is of a higher order, and it is reasonable to regard it as largely due to inhibition of cholinesterase by which, in the absence

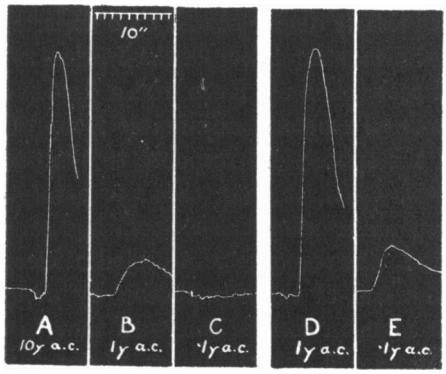

Fig. 5. Retraction of cat's nictitating membrane. Effects of acetylcholine on ganglion. Between *C* and *D*, eserine 1 in 5×10^5 added to perfusion.

of eserine, a large part of it would be destroyed on its way from the blood vessels to the ganglion cells; just as Feldberg and Gaddum found that acetylcholine liberated in the ganglion by nerve impulses only appeared in the venous outflow if eserine was present. As would be expected, on the view that the potentiating effect is chiefly due to this protective action, eserine increases the paralytic, as well as the primary stimulant action of acetylcholine; doses which in the absence of eserine produce only a moderate stimulation become, with a small concentration of eserine, supramaximal and paralytic in action.

The question arose whether eserine would similarly sensitize the ganglion cells to the effects of preganglionic impulses. As suggested already in the paper by Feldberg and Gaddum, it was difficult to predict what the effect might be. Assuming that the arrival of an impulse at a synapse releases a small charge of acetylcholine, which acts

as the direct stimulus to discharge of a corresponding impulse by the ganglion cells, we have no reason to suppose that persistence of the acetylcholine would cause a discharge of further impulses from the same cell; such evidence as we have, indeed, concerning its secondary, paralytic effect, is against such a supposition. If it were released in such relation to the ganglion cell as to be exposed to partial destruction by cholinesterase while reaching the site of its action, eserine might conceivably, by its effect on the esterase, enable an effective charge of acetylcholine to reach a certain number of additional cells. What is known, however, as to the time required for the effect of a stimulus to pass the synapse (see Brown, 1934; Eccles, 1934) seems inadequate to allow for diffusion over such a distance that significant destruction could take place, and protection by eserine be locally effective. Further, though it may seem plausible to suggest that the acetylcholine liberated at a synapse by an impulse, having produced its stimulating effect on the ganglion cell, is immediately removed by esterase, there is no direct evidence to support such a conception. It has been proved that eserine protects acetylcholine which escapes from the synapses, and enables it to appear in the venous outflow from the ganglion, and that it protects acetylcholine injected into the arterial flow during its diffusion to the ganglion cells; but the method of its destruction or removal at the synapses themselves may be entirely different, and uninfluenced by eserine. We were unable to agree with Eccles, therefore, when he used his failure to observe potentiation of the effect of a single preganglionic volley by eserine as an argument against the function of acetylcholine as the transmitter. We have found, moreover that, under appropriate conditions, a clear potentiation by eserine of the effects of preganglionic impulses can be demonstrated.

The most effective conditions for this demonstration were found in recording the retractions of the nictitating membrane caused by equal periods of submaximal shocks, applied to the cervical sympathetic nerve with a rhythm of about 2 per sec. Fig. 6 shows the effects produced by successive groups of 18 shocks in 10 sec., applied at intervals of 3 min. The successive small responses were quite uniform until a control injection was made of Locke's solution prewarmed to 37° C. This caused a temporary fall in the cannula temperature of 2° C., and the following responses show a consequent small depression. Thereafter, with re-establishment of the original temperature, the sequence of responses regained the original regular dimensions. Two of them are recorded at *A* and *B* in Fig. 6. After *B* 10 c.c. of Locke's solution containing 1 part

of eserine in 10^6 and prewarmed to 37° C. were similarly injected, and again caused a temporary fall of 2° C. in the cannula temperature. In spite of this the ensuing responses, *C* and *D*, are obviously much larger than those obtained in the absence of eserine. We have obtained quite similar effects in several experiments of this kind, and definite though less pronounced potentiation with periods of stimulation at 10 to 30 shocks per sec., and with stronger, though still submaximal, shocks.

We are not able to offer with confidence a complete explanation of this effect. The fact that eserine, apart from its protective action on acetylcholine, causes some sensitization of the ganglion cells to chemical stimuli in general, must certainly be considered as a possible factor. We suspect, however, that the protective action may also have some

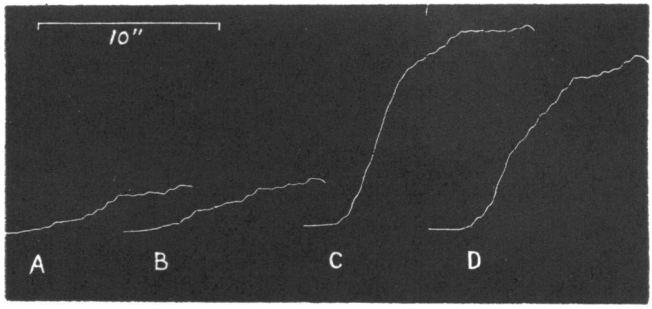

Fig. 6. Nictitating membrane. Responses to equal groups of submaximal shocks to cervica sympathetic; *A* and *B* before, *C* and *D* during perfusion of eserine 1 in 10^6.

influence in this phenomenon. Acetylcholine, liberated by preganglionic impulses, can diffuse into the blood vessels in a concentration sufficient, on occasion, to act as a stimulus on reinjection. It is at least not improbable, therefore, that when eserine is present in the ganglion, acetylcholine may reach by diffusion, and in a stimulating concentration, other ganglion cells than those in direct relation to which it is liberated by the submaximal shocks. Impulses from these secondarily stimulated cells might be expected to undergo a temporal summation with those from the cells primarily stimulated, increasing the total response of the nictitating membrane. Such a possibility could obviously be better tested with an electrical record of the postganglionic impulses, and it is hoped that this may later be obtained.

(*b*) *Paralysing action.* Even with weak concentrations of eserine, such as 1 in 5×10^5, prolonged application by perfusion can lead to a secondary depression of the response to preganglionic stimulation. This

depressant action had been observed in some of the earlier experiments by Feldberg and Gaddum in which stronger solutions of eserine were used, and was the only effect of eserine detected by Eccles [1934], who observed it when larger doses of eserine were given. The effect is most clearly seen by a temporary perfusion with a higher concentration, such as 1 in $1–2 \times 10^4$. If 10 c.c. of such a solution, prewarmed, were injected as described above into the warming tube, the response to preganglionic stimulation rapidly declined, and, after a few minutes' perfusion, was completely annulled. Simultaneously, the response to injections of acetylcholine was abolished, and likewise that to injections of the other stimulant substances, nicotine, choline, arecoline and potassium ions, of which the actions are described later. Fig. 7 illustrates the fact that the ganglion cells, under the influence of such a concentration of eserine,

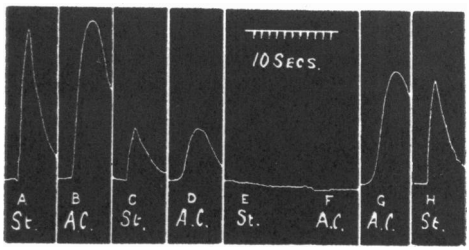

Fig. 7. Nictitating membrane. Responses to acetylcholine and preganglionic stimulation, before (*A*, *B*), during (*C*, *D*, *E*, *F*), and after (*G*, *H*) perfusion with eserine 1 in 10^4.

lose their sensitiveness to preganglionic impulses and to injections of acetylcholine in a closely parallel manner, and, as the excess of eserine is washed away, show similarly parallel rates of recovery to these two methods of stimulation. In the cases of arecoline and hordenine methiodide, the extinction of their effects by perfusion with relatively strong eserine solutions showed a less complete parallel with that on preganglionic stimulation, the effect of the latter being completely suppressed rather earlier than that of the drugs.

This secondary, paralytic effect of eserine probably explains the rather wide variations in sensitiveness of different ganglia to acetylcholine, observed by Feldberg and Gaddum, who, in perfusions with eserine in a dilution of 1 in 5×10^5 over relatively long periods, must certainly have made some of their tests when the response of the ganglion cells was depressed. Since the effect of all drugs stimulating the cells is depressed, as well as that of preganglionic impulses, the paralytic effect

would appear to be on the ganglion cells, and not on any other part of the synapse. It is, in fact, closely similar to the secondary, paralytic effect of nicotine on the ganglion cells, the action of eserine, in these larger doses, differing from that of nicotine only in showing no trace of primary, stimulant action.

The simultaneous paralysis of the effects of preganglionic impulses and of acetylcholine artificially applied, was obviously in full accord with the view that the latter acts as transmitter at the synapse. If the paralytic action of eserine, as the facts suggested, was limited to the ganglion cells, it would be expected that preganglionic impulses would still release acetylcholine at the synapses, transmission being blocked by the failure of the ganglion cells to respond to it. An experiment proved that this was, indeed, the case. A ganglion was perfused, in the first instance, with eserine 1 in 10^6, and a period of maximal preganglionic stimulation caused full retraction of the nictitating membrane and the usual output of acetylcholine, as demonstrated by testing the venous fluid in tenfold dilution on the sensitized leech preparation. The perfusion fluid was now changed for one containing eserine 1 in 10^4. This, as always, caused some lessening of the rate of perfusion, presumably by causing constriction of the vessels of the ganglion. A sample was collected without stimulation, in order to test the possibility that strong eserine, by itself, might cause acetylcholine to appear in the fluid even from the resting ganglion. This sample, however, in tenfold dilution, had no effect on the leech muscle. This absence of any output of acetylcholine in response to strong eserine by itself, was shown in another experiment by tests on the arterial pressure of a cat, on which the effluent could be tested without dilution. It was entirely without effect in a dose of 2 c.c., though the cat gave a clear response to 2 c.c. of A.C. 1 in 5×10^8. A second similar period of stimulation of the cervical sympathetic then caused no movement of the nictitating membrane, the ganglion being under full eserine paralysis. The venous fluid, however, collected during this peripherally ineffective stimulation, was found to contain a somewhat greater concentration (about 1 in 10^7) of acetylcholine than that collected during the first, effective stimulation. The retardation of the perfusion by the eserine makes it impossible to attribute any significance to this greater strength of the venous fluid; but we can, at least, conclude that eserine in paralytic concentration does not depress the liberation of acetylcholine by the arrival of preganglionic impulses at the synapses. The effect of the strong eserine is to render the ganglion cells unresponsive to these impulses, as to acetylcholine artificially applied. The fact that

the paralysis was limited to the perfused ganglion was demonstrated by subsequent stimulation of the postganglionic bundle, which caused full retraction of the nictitating membrane.

Actions of nicotine.

The actions of nicotine, in stimulating and subsequently paralysing sympathetic ganglia, have been known since the classical description given by Langley and Dickinson [1889]. A point of some doubt has been the exact location of these actions. Langley [1901], finding that stimulation of sympathetic ganglia by nicotine survived degeneration of the preganglionic fibres, located this action in the cells, and not at the synaptic nerve endings. The fact that its paralytic effect was not produced on spinal ganglia, even in those of the skate, where the cells are bipolar, left open the question as to whether the paralytic action was also produced on autonomic ganglion cells, or was a separate effect on the synapses. The close analogy between these actions of nicotine and those of acetylcholine led us to expect that they would both prove to be actions on the ganglion cells, and our experiments confirm this view. We have already mentioned the fact that paralytic doses of eserine, which leave the synaptic release of acetylcholine unaffected, abolish the stimulant action of nicotine.

Fig. 8 illustrates both actions of nicotine, as applied by injection to the perfused ganglion. *A*, *B*, *C* and *D* show records of retraction of the nictitating membrane produced by 1, 0·5, 0·2 and 0·1γ of nicotine tartrate, applied by the usual method of injection. It will be seen that 0·1γ represents about the threshold dose of nicotine. This dose is of a lower order than the threshold dose of acetylcholine when injected in the absence of eserine, and of about the same order as that of acetylcholine when eserine is present to protect it on the way to the site of action. *E* shows the retraction produced by a short period of 10 sec. of stimulation of the cervical sympathetic nerve with the secondary coil at 21 cm. and 25 stimuli per sec. An injection of 5 c.c. of nicotine 1 in 10^5 was then given, and produced, on first reaching the ganglion, the retraction shown at *F*. It will be seen that, although nicotine at this concentration continued to perfuse through the ganglion for the period represented in the remainder of this record, the retraction curve reached a lower maximum, relaxation setting in earlier than when a single injection of 0·1 c.c. of the same solution was given at *A*. The phenomenon is precisely similar to that already described as produced by supramaximal doses of acetylcholine. With continued perfusion of nicotine of

this strength, strong stimulation of the cervical-sympathetic for 10 sec. at *G*, with the sec. coil at 13 cm. and 25 stimuli per sec., produced no response, the paralysis of the ganglion being complete. Acetylcholine and other substances, such as hordenine methiodide (*v. infra*), which

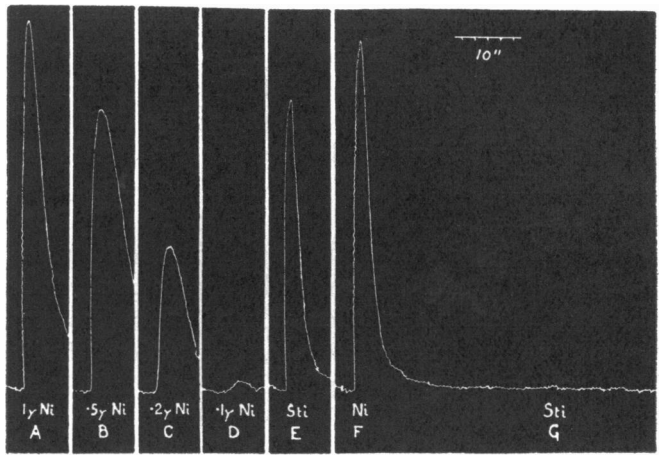

Fig. 8. Nictitating membrane. Stimulating and paralysing effects of nicotine on ganglion.

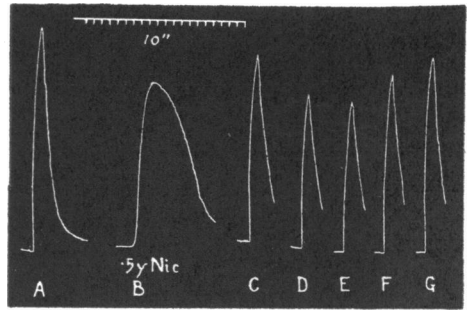

Fig. 9. Nictitating membrane. Sympathetic stimulation before and after 0·5γ of nicotine, injected by perfusion.

normally stimulate the ganglion cells, were similarly devoid of effect during the paralysis by nicotine. Later, a further injection of 10 c.c. of nicotine 1 in 10^5 was given, without any stimulating effect. After washing out the nicotine the sensitiveness of the ganglion reappeared again.

Fig. 9 illustrates a sequence of effects similar to that already described in the case of acetylcholine. It shows the temporary depression of the ganglionic response to preganglionic impulses, caused by injection of a small dose (0·5γ) of nicotine into the perfusion cannula, so that it passes into the ganglion and is removed by the further perfusion. Under such conditions the time relations of the primary stimulant and secondary depressant effects can be studied. At *A*, *C*, *D*, *E*, *F* and *G* equal short periods of stimulation of the cervical sympathetic nerve were given with the fluid electrode. Between *A* and *C* 0·5γ of nicotine, in 0·1 c.c. of Locke's solution, was injected. On reaching the ganglion it produced its stimulant effect, causing the retraction recorded at *B*. When this had just subsided a further period of stimulation of the cervical sympathetic produced, at *C*, a somewhat diminished effect. The subsequent stimulations were given at 3 min. intervals from this, and it will be seen that the maximum of the depressant effect of the nicotine injection is reached at *E*, 6 min. after the stimulant effect of the nicotine had completely subsided, and 8 min. after it was injected.

As in the case of eserine, the question presented itself whether the paralytic effect of nicotine was due only to annulment of the response of the ganglion cells, or affected also the liberation of acetylcholine by preganglionic impulses. The leech-muscle preparation could not, in this case, be used to test the venous fluid for acetylcholine, since it is stimulated by nicotine in the concentration required to paralyse the ganglion. A suitable test for this purpose was provided by a cat under chloralose, the circulation of which was restricted by removal of the abdominal viscera, and to which eserine had been given intravenously. In such a preparation an intravenous injection of as little as 0·002γ of acetylcholine (1 c.c. of 1 in 5×10^8) caused a definite and regular fall of arterial pressure. On the other hand, 1 c.c. of nicotine 1 in 10^5, the concentration used to paralyse the ganglion, produced by itself no effect on the arterial pressure, and the addition of nicotine in this concentration to a maximal dose of acetylcholine did not significantly alter the depressor effect of the latter.

To test the effect of the nicotine the ganglion was first perfused, in the usual manner, with eserine 1 in 5×10^5, and a control period of maximal stimulation applied to the cervical sympathetic, the venous fluid being collected. During this stimulation the ganglion was fully active, as shown by full retraction of the nictitating membrane. The effect of 1 c.c. of this first venous fluid on the arterial blood-pressure of the cat is shown at *A* in Fig. 10. When the venous fluid, as shown by a

test, had again become inactive, perfusion was begun with a fluid containing nicotine 1 in 10^5 in addition to the eserine. A second similar period of cervical sympathetic stimulation was now completely without effect on the nictitating membrane, but 1 c.c. of the venous fluid collected during its application produced the depressor effect shown at *B* in Fig. 10. The records at *A* and *B* could hardly be more similar, if two equal injections of the same fluid had been given. *C* represents the effect of 1 c.c. of the nicotine-containing venous effluent, collected after the stimulation period. It will be seen that the acetylcholine is reduced to a trace. An earlier injection of this effluent, collected before the stimulation was applied, was free from acetylcholine, so that nicotine by itself caused no output of acetylcholine from the ganglion.

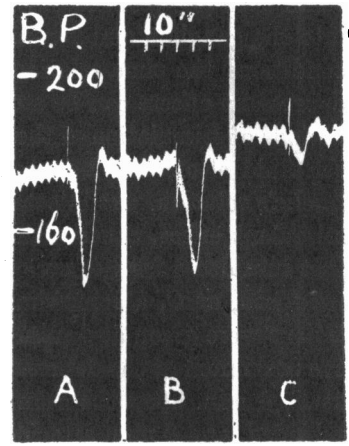

Fig. 10. Arterial blood-pressure, cat. 0·3 mg. eserine i.v. Effects of ganglion effluents during sympathetic stimulation, *A* before and *B* during complete nicotine paralysis.

The paralytic effect of nicotine on the ganglion is, therefore, entirely similar to that of eserine. The response of the ganglion cells to preganglionic impulses is annulled, but the liberation of acetylcholine at the synapses is unimpaired. The ganglion cells are insensitive to the artificial application of substances which normally stimulate them, including acetylcholine. Both the stimulating and paralysing actions of nicotine are exerted directly on the ganglion cells and not on the synapses, and the observations are, again, in complete accord with the conception of acetylcholine as transmitter of the effects of preganglionic impulses.

Actions of some other stimulant alkaloids.

The opportunity was taken to test the effects of choline, arecoline and hordenine methiodide, when injected into the perfusion cannula, and these can be briefly described.

Choline. As was to be expected [cf. Dale, 1914] choline had stimulating and paralysing effects on the ganglion cells similar to those of acetylcholine, but of a lower order. The threshold dose for stimulation varied in different experiments from 25 to 100γ. With an injection of 1 mg. or more, stimulation of the ganglion cells was followed by paralysis of their response to preganglionic impulses, to choline itself, or to other

stimulant substances. The effects of eserine and nicotine on the action of choline have been described above.

Arecoline. Feldberg, Minz and Tsudzimura [1934] showed that arecoline, in addition to its known muscarine-like action, had an action on the suprarenal medulla resembling that of nicotine. It was not surprising therefore to find that it shows on the ganglion cells both phases of a nicotine action, stimulating them in doses from 50γ upwards, and producing the now familiar paralytic action in the larger doses.

Hordenine methiodide is a quarternary salt obtained by complete methylation of tyramine, and is known to have a strong nicotine-like action [Barger and Dale, 1910; Dale, 1914]. On the perfused ganglion the stimulant effect was exhibited by injections of from 2·5γ upwards, and larger doses (100γ) produced the typical paralysis.

Inactivity of certain substances.

The opportunity was taken of testing the effects, when directly applied to the ganglion by perfusion, of substances having such powerful peripheral actions that effects on the ganglion cells, if they existed, might otherwise escape detection. Adrenaline and histamine were thus tested in doses from 0·1 to 100γ, and adenosine in doses up to 1 mg., without any trace of stimulant or paralytic action being detected in any case.

Actions of atropine and strychnine.

Atropine. The peripheral action of atropine, on effector cells innervated by postganglionic fibres of the parasympathetic system, is as well known as that of nicotine on ganglion cells. With smaller doses of the two alkaloids the distinction holds, and enabled Dale [1914] to distinguish the peripheral, muscarine actions of acetylcholine, paralysed by atropine, from its action on ganglion cells, which nicotine abolished. Feldberg, Minz and Tsudzimura [*loc. cit.*], in describing actions on the suprarenal medulla, showed that with larger doses the distinction is not so sharp. Similarly atropine in low concentrations, already sufficient to annul any "muscarine" action, has no effect of any kind on the response of the cells of the superior cervical ganglion to preganglionic impulses or to acetylcholine. If a larger dose, however, such as 100γ, is injected into the perfusion, it causes a complete paralysis of response to preganglionic impulses and to acetylcholine. Responses to both appear again together as the atropine is washed away by further perfusion.

Strychnine. Only two experiments were made. In neither could we detect any trace of a stimulating or sensitizing action of strychnine on the ganglion cells. In larger doses, from 10 to 50γ, it caused, like atropine, a temporary paralysis of response to preganglionic stimuli or to stimulant alkaloids.

Action of potassium ions.

This deserves separate consideration, as providing the only example we have yet encountered of a substance which in higher dosage directly stimulates the output of impulses from the ganglion cells, and in subliminal doses sensitizes them to the effects of preganglionic impulses and

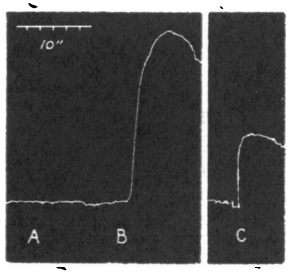

Fig. 11.

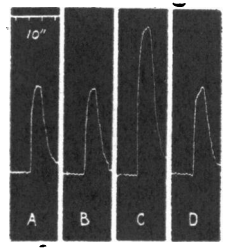

Fig. 12.

Fig. 11. Nictitating membrane. Effects of KCl on perfused ganglion. *A* and *C* 0·1 mg., *B* 0·2 mg.; between *B* and *C* eserine 1 in 5×10^5 added to perfusion.

Fig. 12. Nictitating membrane. Response to equal groups of submaximal preganglionic stimuli. Between *B* and *C* 0·1 mg. KCl injected by perfusion.

of stimulating alkaloids. The minimal dose of KCl which, injected into the perfusion cannula, caused stimulation of the ganglion cells, as shown by contraction of the nictitating membrane, varied in different experiments between 0·2 and 0·4 mg. In the experiment illustrated in Fig. 11, 0·1 mg. of KCl at *A* was without direct stimulating action, but 0·2 mg at *B* caused pronounced retraction. Between *B* and *C* the perfusion fluid was replaced by one containing 1 in 5×10^5 eserine, and a further dose of 0·1 mg. KCl, at *C*, now had a definite effect, though much less than that of 0·2 mg. before eserine. This illustrates the slight sensitizing effect of eserine on the ganglion cells to stimuli in general, described in an earlier section. It will be seen, however, that it does not increase the effective equivalent of a dose of KCl by more than 50 p.c., in contrast to the eightfold to twentyfold increase in the effectiveness of acetylcholine (see p. 113).

Fig. 12 illustrates the sensitizing effect of a subliminal dose of KCl. In this experiment the threshold dose for direct stimulation was 0·4 mg. Equal periods (10 sec.) of submaximal stimulation were applied to the cervical sympathetic nerve, with fluid electrodes at intervals of 3 min. (sec. coil, 19 cm., 18 stimuli per sec.). *A* and *B* show the effects of two control periods. Between *B* and *C* 0·1 mg. of KCl was injected, and produced no direct effect. The ensuing stimulation at *C*, during the passage of the KCl through the ganglion, shows a definite potentiation, but, with the washing out of the extra potassium ions, a further period of stimulation produces a response of the original amplitude, shown at *D*. The stimulating effect of acetylcholine, choline, or other stimulant alkaloids is similarly enhanced by subliminal doses of potassium ions. It is easier, in such a case, to obtain a numerical value from the sensitization, by determining equivalent doses with and without potassium. The increase of sensitiveness is thus found to be of the order of 50 p.c.—a definite but not very large effect.

Discussion.

The observations here recorded have been separately discussed in connection with their detailed presentation. Taken together they seem to us not only to be compatible with the conception of transmission at the ganglionic synapses by the liberation of acetylcholine, but to give it additional support.

The only important arguments yet advanced against this conception are those based by Eccles on his observations on the effects of eserine. Recording the electrical potential changes in the ganglion and the post-ganglionic nerve bundle, in response to single preganglionic volleys, with the natural circulation intact, Eccles administered eserine by the general circulation in different doses, and recorded observations which appeared to him to be incompatible with transmission by acetylcholine. These observations were as follows:

(1) With smaller doses of eserine he observed no potentiating effect on the electrical response to a single preganglionic volley, and with larger doses he observed a depressant effect. We have already discussed these points, and have shown that eserine, applied by perfusion in a weak, accurately known dilution, does, in fact, potentiate the summed effect of a group of submaximal preganglionic volleys, and that its paralysing action on the ganglion cells in higher concentration, when examined in detail, is entirely compatible with the theory of transmission by acetylcholine.

(2) The facilitating action of a first preganglionic volley, on that of a second submaximal volley, following at an interval of 10–100σ, was found by Eccles to be unaltered by a moderate dose of eserine. This negative observation cannot have any significance for the question of transmission by acetylcholine, unless it is assumed that the persistent facilitation, or excitatory state, is due to persistence of the synaptic transmitter. If this assumption could be justified, it might be used by itself, without any reference to eserine, as an argument against acetylcholine as the transmitter; for our own observations suggest that persistence of acetylcholine at the site of its action, after its immediate stimulant effect had been produced, would be more likely to depress than to facilitate the effect of a further stimulus. We have discovered no ground, however, for the assumption. The available evidence suggests that the facilitation is due to the persistence in the ganglion cell of a condition following and caused by its own response to a previous stimulus, whether effective in the output of an impulse or not, rather than to persistence of the transmitter, which acted as the stimulus. It would be difficult otherwise to explain Eccles' own observation that the facilitation increases after 10σ from the first stimulus, to reach a maximum at 15σ. His observation that application of weak nicotine to the ganglion abolishes the facilitation before transmission is affected, taken with our own evidence that nicotine affects the ganglion cell, and not the synaptic mechanism, is again in favour of locating the effect responsible for facilitation in the ganglion cell. We have no direct evidence as to the nature of a change in a ganglion cell which, persisting after its response to one excitation, would lower the threshold of its response to another. It is justifiable, however, to recall in this connection the fact that we have found in potassium ions a stimulant of ganglion cell activity, which in subliminal concentration produces a facilitation of the response of the cells to preganglionic impulses, or to artificially applied acetylcholine. If the response of a ganglion cell to a stimulus involved the mobilization of potassium ions in its substance or on its surface, our evidence suggests that the cell would, in fact, be rendered more excitable to a further stimulus so long as this condition persisted; but we mention such a possibility only to show that the phenomenon of facilitation may find an explanation, without assuming, against the evidence as we read it, that persistence of the synaptic transmitter must be the cause. In relation to such an alternative explanation, the lack of an action by eserine on facilitation would clearly be irrelevant.

SUMMARY.

1. Impulses passing along the vagus fibres through its ganglion, without synaptic interruption, liberate no acetylcholine, and antidromic impulses into the cells of the sympathetic ganglion are similarly ineffective. It is concluded that the liberation of acetylcholine by preganglionic impulses occurs at the synapses.

2. Preganglionic impulses liberate acetylcholine in the ganglion with natural circulation, as in that artificially perfused.

3. The quantity of acetylcholine appearing in the venous effluent from the ganglion, in response to a single maximal preganglionic volley, was from 6×10^{-5} to $1 \times 10^{-4}\gamma$, or of the order of $10^{-9}\gamma$ (10^{-15} g.) per synapse.

4. Acetylcholine, choline, hordenine methiodide, arecoline and nicotine, applied to the ganglion by perfusion, all stimulate the ganglion cells in small doses and paralyse their response to such stimuli, or to preganglionic impulses, when applied in larger doses.

5. Eserine, perfused through the ganglion in weak concentration (*e.g.* 1 in 10^6), strongly sensitizes the ganglion cells to injections of acetylcholine, lowering the threshold dose 8 to 20 times. It has a weak, non-specific sensitizing action, lowering the threshold dose by $\frac{1}{3}$ to $\frac{1}{2}$ for the other substances mentioned under (4), and for potassium ions. Similar low concentrations of eserine cause a definite potentiation of the response to a group of submaximal preganglionic stimuli.

6. Eserine in stronger concentrations (*e.g.* 1 in 10^4) completely abolishes the response of the ganglion cells to nervous impulses or chemical stimulants. The effect resembles the paralytic effect of nicotine. Paralysis by either alkaloid leaves the release of acetylcholine by preganglionic impulses unaffected. The responses of the cells to such impulses and to acetylcholine disappear and reappear together.

7. Adrenaline, histamine and adenosine have no effects on the ganglion. Atropine and strychnine in moderate doses have no specific actions, but paralyse the ganglion cells in larger doses.

8. Potassium ions stimulate the ganglion cells, the threshold dose of KCl, injected by perfusion, being 0·2–0·4 mg. A subliminal dose of KCl raises the excitability of the ganglion cells, to preganglionic stimuli, to acetylcholine and to other chemical stimulants, during its passage through the ganglion.

9. It is suggested that these new items of evidence entirely support the conception of transmission at ganglionic synapses by liberation of acetylcholine.

We desire to make grateful acknowledgment to the Medical Research Council for hospitality, and to Sir Henry Dale for his constant help, by advice and suggestion during the course of the experiments, and in the presentation of their results.

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