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THE RELEASE OF ACETYLCHOLINE FROM PERFUSED SYMPATHETIC GANGLIA AND SKELETAL MUSCLES

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The theory that acetylcholine (ACh) is the transmitter at ganglionic and neuromuscular junctions was originally based on evidence derived from perfusion experiments. When a sympathetic ganglion was perfused, ACh was found in the effluent only when the preganglionic nerve fibres had been stimulated (Feldberg & Gaddum, 1934; Feldberg & Vartiainen, 1934); and when a voluntary muscle (deprived of its vasodilator innervation) was perfused, ACh was found in the effluent only when the motor nerve fibres had been stimulated (Dale, Feldberg & Vogt, 1936). In these investigations the perfusion fluid was ordinary Locke's solution, to which eserine had been added in order to prevent the enzymic hydrolysis of ACh. The structures under study were thus exposed to an abnormal environment and they showed sign of abnormal function: for example, the perfused tissues rapidly became oedematous, transmission failed prematurely in ganglia subjected to repetitive preganglionic stimulation, and voluntary muscles progressively lost their contractile power. It has even been suggested (Fleisch, Sibul & Kaelin, 1936; Lorente de Nó, 1938; Nachmansohn, 1946) that the release of ACh in such perfusion experiments is unphysiological, and may be regarded as evidence that the perfused tissues have suffered damage.

Attempts have indeed been made to demonstrate the liberation of ACh, both in ganglia and in muscle, when the circulatory conditions were more nearly normal. Thus Feldberg & Vartiainen (1934) reported one experiment in which they isolated the superior cervical ganglion of a cat as for perfusion, but allowed it to retain its natural blood supply. They gave the cat a large dose of eserine, and could then observe that when the preganglionic trunk was stimulated, ACh appeared in the blood leaving the ganglion in about the same concentration as they had found in their earlier experiments, in which the ganglion was perfused with Locke's solution. The flow of blood through the

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ganglion must, however, have been much slower than the flow of Locke's solution, because of the higher viscosity and the lowering of the arterial pressure by eserine; and the *quantity* of ACh collected must therefore have been much below that obtained during perfusion. Lorente de Nó (1938), who carried out similar experiments, found that ACh was released during stimulation only when there was evidence of tissue damage, or reason to suspect it; when he took elaborate precautions to keep the ganglion in good condition, no ACh entered the blood. Finally, MacIntosh (1938) was unable to detect more than a trace of ACh in the blood leaving a stimulated ganglion, although when he used blood diluted tenfold with Locke's solution, he found ACh to be released in the same quantity as when he perfused the ganglion with Locke's solution alone.

Similarly, Dale *et al.* (1936), in their experiments on voluntary muscle, were only able to detect the release of ACh by motor-nerve stimulation when they tested the effluent from a muscle perfused with Locke's solution; when they examined the venous blood from a muscle whose arterial supply was intact, their results were always negative. Feldberg (1933) had previously reported that ACh appeared in the venous blood of the tongue when he stimulated the vasodilator fibres running in the lingual nerve, but not when he stimulated the hypoglossal nerve; and Fleisch *et al.* (1936) had found the ACh content of venous blood from the hindlimbs to be unaffected by stimulation of the motor nerves. In all these negative experiments eserine, often in very large dosage, had been given to the animal.

In these experiments, then, the release of ACh in substantial quantity by preganglionic or motor nerve stimulation occurred regularly when the tissue was perfused with Locke's solution, irregularly or not at all when it retained its natural blood supply. Several possible explanations for this discrepancy have been suggested by the authors cited above. It might be that in the whole animal the amount of eserine, needed to inhibit effectively the cholinesterase of the blood and tissues, is greater than would be expected from the minute amounts which suffice when an artificial perfusion fluid is used. Or there might be, during a saline perfusion, some failure of oxygenation or some increase of permeability above the normal, which would facilitate the escape of ACh from the synaptic regions. Thus under physiological conditions ACh might be removed by some eserine-resistant mechanism linked to oxidative metabolism, for instance by resynthesis to a 'precursor' (MacIntosh, 1938; Eccles, 1944). A further possibility that occurred to us was that ACh might only be released under conditions of abnormal alkalinity. The perfusion fluid generally used for demonstrating the release by the above-named authors and others (Barsoum, Gaddum & Khayyal, 1934; Brown & Feldberg, 1936*a*, *b*; Lorente de Nó, 1938; Kahlson & MacIntosh, 1939; Harvey & MacIntosh, 1940; Paton & Zaimis, 1951; Perry, 1953) was Locke's original (1901) solution

containing $NaHCO_3$ as its only buffer, and so having a pH of about 8·5. The oxygenated diluted blood used by MacIntosh (1938) had about the same pH.

The experiments described below were intended to test the significance of these various departures from normality for permitting the release of ACh to be detected. They were done several years ago, and a preliminary note was published at that time (Emmelin & MacIntosh, 1948).

METHODS

Cats anaesthetized with chloralose were used in all the experiments. The superior cervical ganglion was perfused by the method of Kibjakow (1933), essentially as described by Feldberg & Gaddum (1934). The main postganglionic bundle was left intact, and the contractions of the nictitating membrane of the same side were recorded so as to provide an index of the effectiveness of preganglionic stimulation. The stimuli used were supramaximal induction shocks timed by a thyratron circuit and delivered through shielded silver electrodes: the frequency varied in different experiments between 16 and 26 per sec. Oscillographic records, for which we are indebted to Dr J. A. B. Gray, showed that the shocks were too brief to fire the nerve fibres repetitively. The perfusion fluid was delivered from a constant-pressure reservoir or from a pump: it seemed not to matter whether the pressure was steady or pulsatile. The pressure was usually between 80 and 120 mm Hg at first, and was raised if the flow slackened greatly; for defibrinated blood the pressure was about twice as high, but even so the rate of flow was much slower than with Locke's solution or plasma. The fluid was warmed either by exposing the whole perfusion system to radiant heat, or by means of an electrically heated wire spiral surrounding the arterial cannula, or by passing the tubing which led to the cannula up the cat's oesophagus from abdomen to neck. The temperature of the fluid was measured with the aid of a thermocouple junction placed within the tip of the arterial cannula; it was usually $38 \pm 1°$ C.

The muscle chosen for perfusion was tibialis anticus. Its arterial and nerve supplies were isolated as described by Brown (1938); the sciatic nerve was laid on shielded electrodes; a bulldog clip was placed on the tibial artery below its branches to the muscle, and a cannula pointing centrally was inserted below the clip. The muscle near its origin was then divided by blunt dissection into four or five bundles, each of which was secured by a ligature and then freed from its tibial attachment. The vein draining tibialis anticus was thus exposed and was freed for cannulation. The muscle was then attached to the limb only by its nerve and blood vessels. The clip on the artery was now removed and reapplied above the branches supplying the muscle, and perfusion begun through the cannula previously inserted lower down. Lastly the vein was cannulated, the muscle was covered with skin flaps, and collection of the venous fluid was begun. The perfusion temperature was $38 \pm 1°$ C, and either a pulsating or a steady pressure was used. We found it convenient to heparinize the animal when the dissection was completed: the perfusion could then be stopped at will, and the natural circulation restored, by moving the clip on the artery to its initial position. This procedure quickly improved the contractile power of the muscle when it had declined as the result of prolonged stimulation in the absence of the normal blood supply.

The venous effluent from ganglion or muscle was collected in ice-cooled vessels, and its activity was matched as soon as possible against that of known dilutions of ACh chloride on the cat's blood pressure, as described by MacIntosh & Perry (1950). Some samples of plasma and blood used for perfusion had a little pressor or depressor activity, but this was never great enough to interfere seriously with the assay.

The identification as ACh of the depressor substance released on stimulation of preganglionic and motor nerves has been made, on sufficient grounds, by earlier workers. We satisfied ourselves in each experiment that the depressor activity attributed to ACh was lost after atropine, but did not think it necessary to make further tests of its identity as a matter of routine. The following observations made in certain experiments confirmed the belief that it was ACh: (1) assay against

ACh on the eserinized leech preparation gave values in agreement with those found in the blood-pressure test; (2) brief exposure to 0·1 N-NAOH at 100° C destroyed the activity; (3) no activity could be detected unless eserine or another anticholinesterase drug had been added in adequate proportion to the perfusion fluid.

Choline in the ganglionic effluent was estimated as ACh after acetylation. Like earlier workers (Roman, 1930) we observed that prolonged heating of choline-containing residues, after desiccation, led to large evaporation losses. Thereafter, 1 ml. portions of effluent were dried in ampoules in a water-bath at 70–80° C; a stream of air was blown into each ampoule to speed the drying, which was stopped as soon as no visible moisture was left in the residue. 1 ml. of acetylcholine was then added, and the ampoule was sealed and placed for 15 min in a boiling water-bath. It was then cooled and opened, acetyl chloride was removed by blowing a stream of dry air into the ampoule, and water was added to restore the initial volume. When small quantities of choline chloride in Locke's solution were treated in this way, ACh was found in theoretical yield (1·30 g ACh chloride per g of choline chloride).

RESULTS

Experiments on the superior cervical ganglion

Perfusion with saline solution of different pH. Two perfusion fluids were used, Locke's solution of the original formula (1901), as employed in most of the earlier studies on the perfused ganglion ('bicarbonate-Locke'), and a solution of the same tonicity containing phosphate buffer at pH 7·4 instead of bicarbonate ('phosphate-Locke'). Each solution contained per l.: NaCl 9 g, KCl 0·42 g, $CaCl_2$ 0·24 g, glucose 1 g. In addition, the bicarbonate-Locke contained 0·5 g/l. $NaHCO_3$, and its pH when oxygenated varied in different experiments between 8·47 and 8·58 (glass electrode at 16° C). The phosphate-Locke was 0·006 M in respect to sodium phosphate and its pH was between 7·39 and 7·46. Each solution contained eserine sulphate 5×10^{-6} g/ml. Phosphate rather than CO_2-bicarbonate buffering was used for the less alkaline fluid, because a rather complicated set-up would have been needed to maintain the correct CO_2 tension of the warmed perfusion fluid.

A standard procedure was adopted. The perfusion having been established, and the last trace of blood washed from the system, samples of the effluent were collected over consecutive 5 min periods. Preganglionic stimulation was applied for the first 3 min of some periods, the remaining 2 min being allowed for the washing out by the perfusion stream of ACh released during the stimulation. Each such period was preceded and followed by a control period, during which no stimulation was applied. The rate of perfusion always fell off during the experiment, but generally remained satisfactory long enough for two or three periods of stimulation.

In three experiments both ganglia were perfused, one with bicarbonate-Locke and one with phosphate-Locke; in the others only one ganglion and one solution were used. The choice of perfusion fluid made no apparent difference to the appearance of the perfused tissue or to the effectiveness of preganglionic stimulation. With either fluid, ACh was always found in measurable concen-

tration in samples of perfusate collected during periods of stimulation; but it appeared only in traces, or not at all, in the control samples. Table 1 summarizes the results. The ACh output is expressed as the weight in pg ($g \times 10^{-12}$) of ACh chloride liberated per single maximal stimulus applied to the preganglionic trunk. In a few experiments, the control samples of the venous fluid contained a little ACh-like activity, and a small correction was made for this when the output per stimulus was calculated.

It will be seen that the ACh output was generally smaller in the later periods of stimulation; we do not know how far this diminution reflected physiological changes in the ganglion or how far it was due to deterioration of the preparation through such causes as the increasing oedema and leakage of perfusion fluid, the blocking of minute vessels, the leaking out of essential metabolites, or to slow replenishment in the presence of eserine of the stores of 'available ACh' (Perry, 1953). The main fact, however, was that the output of ACh on stimulation was of the same order, whether the perfused solution was the over-alkaline fluid of the earlier experiments or was at a physiological pH. The average output, for the first period of stimulation in each experiment, was 28·5 pg/volley with bicarbonate-Locke, and 26 pg/volley with phosphate-Locke. These values agree well with those obtained by previous workers, as may be seen from Table 2, which summarizes all the published data which permit the calculation of the ACh output per volley from the unfatigued ganglion: the data of Lorente de Nó (1938), whose results were very variable, have been omitted. Significantly higher values than the present ones, up to 100 pg/volley, were obtained by Feldberg & Vartiainen (1934), by Perry (1953) and by ourselves in experiments in which a much smaller number of stimuli were applied to the preganglionic nerve; but this discrepancy need cause no concern, since the rate of ACh discharge is known to fall off with prolonged stimulation (Brown & Feldberg, 1936*b*; Kahlson & MacIntosh, 1939; Perry, 1953). The lower values for the output observed in our experiments are thus significant only with respect to the parameters of stimulation we have employed; but they can justifiably be compared with the data obtained in other experiments, in which the perfusion fluids were different but the conditions of stimulation were the same.

Perfusion with heparinized plasma. As a nearer approach to physiological conditions, the ganglion was perfused in four experiments with cat's plasma made incoagulable with purified heparin. The plasma was oxygenated and eserine sulphate (10^{-5} g/ml.) was added. Otherwise the procedure was the same as in the experiments with Locke's solution. Heparinized plasma proved to be a very satisfactory perfusion fluid: there was no oedema and almost no leakage, and the flow was better maintained than with the saline fluids. Preganglionic impulses were normally transmitted; and ACh was released on preganglionic stimulation, but did not appear at other times. The average

output per volley, 22 pg, did not differ significantly from the average with the two forms of Locke's solution (Table 1).

TABLE 1. Release of ACh on preganglionic stimulation from ganglia perfused with different fluids. The values for ACh in each experiment refer to successive 3-min periods of stimulation at 20–25/sec. The mean values for each perfusion fluid are based on the output in the first stimulation period of each series.

Expt. no.	Ganglion	Perfusion fluid	ACh output per maximal volley (pg)	
1	Right	Bicarbonate–Locke (eserine sulphate 5×10^{-6})	34, 21	
2	Left	Bicarbonate–Locke (eserine sulphate 5×10^{-6})	26, 27, 18	Mean 28·5
3	Right	Bicarbonate–Locke (eserine sulphate 5×10^{-6})	19, 10	
4	Left	Bicarbonate–Locke (eserine sulphate 5×10^{-6})	35, 18, 9	
1	Left	Phosphate–Locke (eserine sulphate 5×10^{-6})	22, 24, 26	Mean 26
2	Right	Phosphate–Locke (eserine sulphate 5×10^{-6})	33, 30	
3	Left	Phosphate–Locke (eserine sulphate 5×10^{-6})	30, 25	
5	Right	Phosphate–Locke (eserine sulphate 5×10^{-6})	30, 30, 22	
6	Right	Phosphate–Locke (eserine sulphate 5×10^{-6})	14	
7	Right	Plasma (eserine sulphate 10^{-5})	26, 26, 13	
8	Right	Plasma (eserine sulphate 10^{-5})	10, 8	
9	Right	Plasma (eserine sulphate 10^{-5})	12, 10	
10	Right	Plasma (eserine sulphate 10^{-5})	40, 22, 18	
11	Right	Plasma (eserine sulphate 10^{-4})	30, 24	
12	Right	Plasma (DFP 5×10^{-5})	13, 15	Mean 20·5
13	Right	Plasma (DFP 5×10^{-5})	16, 16	
14	Right	Plasma (TEPP 2×10^{-4})	22, 23	
15	Right	Plasma (prostigmine methyl-sulphate $1{\cdot}7 \times 10^{-5}$)	16, 14	
16	Right	Blood (eserine sulphate 10^{-4})	18	Mean 21
17	Right	Blood (eserine sulphate 10^{-4})	17	
18	Right	Blood (eserine sulphate 10^{-4})	21, 12	
19	Right	Blood (eserine sulphate 10^{-4})	29, 27, 23	
20	Right	Blood (eserine sulphate 10^{-4})	19, 6	

TABLE 2. Release of ACh on preganglionic stimulation from ganglia perfused with eserinized Locke's solution

Authors	No. of expts.	No. of stimuli applied	Frequency of stimulation	ACh output per stimulus (pg)
Feldberg & Vartiainen (1934)	2	30, 120; 60, 60	1·8/sec	66, 66; 100, 100
Brown & Feldberg (1936*b*)	3	5100; 2700; 5000	10; 15; 17/sec	18; 26; 18
MacIntosh (1938)	26*	900 to 5400	10/sec	24 (mean)
Kahlson & MacIntosh (1939)	1	6000	10/sec	27
Paton & Zaimis (1951)	1	1800	10/sec	28, 26
Perry (1953)	—	600–240,000	5–100/sec	<2–100, depending on parameters of stimulation
Emmelin & MacIntosh (present paper)	10	3250 to 4500	18–25/sec	27 (mean)

* Perfusion with 10% blood in eserinized Locke's solution.

It was of interest to know how far the ACh output depended on the concentration of eserine in the perfusion fluid. In one experiment, therefore, the concentration was raised tenfold to $10^{-4} \times$ g/ml.: the ACh output was 30 pg/volley. The concentration of eserine can thus be varied within wide limits without any important effect on the ACh output, provided of course that

enough of the drug is present to inactivate the esterases of the plasma and the ganglion.

The opportunity was also taken of using anticholinesterase drugs other than eserine. The plasma used for perfusion contained di*iso*propyl fluorophosphonate (DFP) 5×10^{-5} g/ml. in two experiments, tetra-ethyl pyrophosphate (TEPP) 2×10^{-4} g/ml. in one experiment, and prostigmine methylsulphate $1{\cdot}7 \times 10^{-5}$ g/ml. in one experiment. The output of ACh in these experiments was a little, but not significantly, below the average found in the experiments with eserine (Table 1).

Perfusion with blood. We then decided to repeat the earlier experiments on ganglia with their circulation intact, in the hope of finding out why ACh usually failed to appear in the venous blood under these conditions. The dissection and ligation of vessels was carried out as for a perfusion, but the common carotid artery was left intact so as to maintain the normal arterial supply of the ganglion. When the dissection was finished, the cat was heparinized and given 1 mg/kg of eserine sulphate by vein; in one instance a further mg was injected through the stump of the lingual artery so that a good deal of it must have reached the ganglion. Collection of the slowly dropping blood from the venous cannula was then begun without delay, and the samples were assayed immediately. In no instance, however, was any ACh detected, even with maximal preganglionic stimulation for 10 min. A probable reason for this failure was soon discovered when it was observed that ACh added to the venous blood was almost entirely destroyed within a few minutes. We next tried to determine how much eserine must be given by vein to a cat in order that ACh entering its blood might be preserved long enough for a reliable assay. We found, however, that even the largest doses that could be given, without lowering the blood pressure to any point when hardly any blood flowed through the ganglion (a preliminary small dose of atropine was given in order to lessen the circulatory effects of the eserine), were still inadequate. Thus, blood was taken from a cat 10 min after the injection of 25 mg/kg of eserine sulphate: when ACh was added to this blood, the final concentration being 10^{-8} and the temperature 35° C, 50% was destroyed within 30 min. We concluded that it was impracticable to determine the true output of ACh from a ganglion with its natural circulation. The ACh liberated from such a ganglion would remain for some minutes at least in contact with warm, incompletely eserinized blood within the ganglionic vessels and the venous cannula: and it seemed likely that the cholinesterase of the ganglion itself might be even more active than that of the blood.

We therefore had recourse once more to perfusion, this time with blood containing eserine sulphate in a concentration, 10^{-4} g/ml., sufficient for practically complete inactivation of the esterase of both cells and plasma. Before it was perfused the blood was passed through a coarse sintered glass

filter and gassed with 5% CO_2 in O_2: otherwise the arrangements were as for plasma. Heparinized blood was found to be unsuitable: its cells settled so rapidly within the cannulae and major vessels that the flow soon stopped, although the perfusion pressure was kept high. With defibrinated blood, the cells of which settle more slowly, perfusion was more satisfactory; but even with defibrinated blood the flow was so slow (0·02–0·05 ml/min with arterial pressure of 150–200 mm Hg) that at least 5 min had to be allowed after each stimulation for the collection of all the ACh released. The blood was collected in ice-cooled vessels, containing 0·5–1 ml. of 0·167 M-NaH_2PO_4 for slowing of non-enzymic hydrolysis.

In control experiments it was found that when ACh is added to fully eserinized blood some of it enters the corpuscles; and when the blood is injected into a cat for assay of its ACh content, the ACh in the corpuscles does not contribute to the depressor effect. The ACh content of the red cells when equilibrium had been reached was, volume for volume, about half that of the plasma. A small upward correction was therefore applied to the apparent ACh content of the venous samples from the ganglion, the haematocrit being taken as 35%, and allowance being made for dilution by the phosphate solution in the collection vessels.

In every experiment in which the ganglion was perfused with fully eserinized blood, preganglionic stimulation liberated ACh in the now familiar quantity (Table 1). Transmission of impulses with prolonged stimulation was not well maintained under these conditions of perfusion. The failure of transmission had a time-course similar to that described by Brown & Feldberg (1936*b*) and by Rosenblueth & Simeone (1938), and attributed by these authors to paralysis of ganglion cells by accumulated ACh. The combination of a slow perfusion rate and high concentration of eserine doubtless favoured such an accumulation in our experiments.

Demonstration in vivo *of acetylcholine release from the ganglion.* About half as much acetylcholine is released by stimulation of the preganglionic sympathetic trunk as is released from the perfused submaxillary gland of the cat by stimulation of similar frequency and duration applied to the chorda tympani (Emmelin & Muren, 1950). In the case of the submaxillary gland, however, it has long been known that the released acetylcholine may reach the general circulation in such quantity as to cause a sharp fall of the arterial pressure, provided that a small dose of eserine has been given to the animal beforehand: the discovery of this phenomenon by Babkin, Gibbs & Wolff (1932) gave the first direct evidence for cholinergic transmission in this tissue. One might therefore hope to be able, under favourable conditions, to demonstrate the release of ACh from a ganglion, without recourse to any artifice of perfusion; and this point has been tested in the following way.

The superior cervical ganglia were isolated as for perfusion, but the main

arterial and venous trunks were not interrupted. During the operation all the visible postganglionic fibres were severed. The two cervical sympathetic trunks and the chorda tympani of one side were placed on electrodes. The cat was eviscerated, artificial respiration was begun, and the blood pressure in the femoral artery was recorded. Stimulation of neither the two sympathetics nor the chorda tympani caused any fall of blood pressure. Eserine sulphate (0·2–1 mg/kg) was now given, followed by curare ('Intocostrin', 0·3–0·6 mg/kg) to prevent the muscular twitching caused by eserine; finally, pyranisamine maleate ('Neoantergan', 0·25–1 mg) was given to reduce the depressor effect of histamine liberated by the curare (Alam, Anrep, Barsoum, Talaat & Wieninger, 1939). Stimulation of the chorda tympani now regularly caused a fall in blood pressure after a latent period of 10–15 sec, as described by Babkin *et al.* (1932).

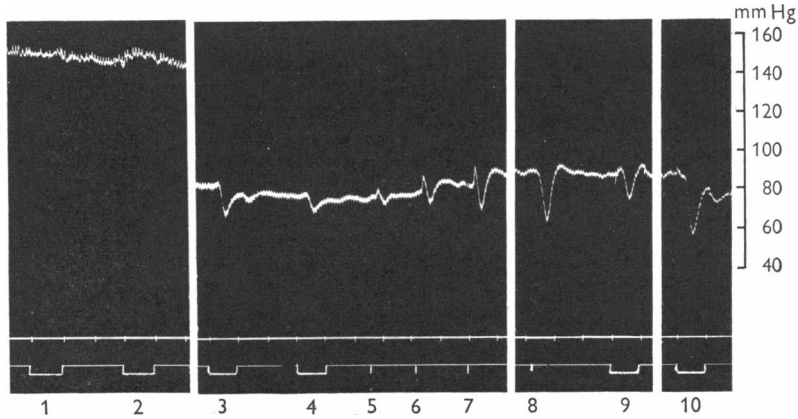

Fig. 1. Blood pressure of cat, 1·7 kg; anaesthetized with chloralose. Stimulation for 1 min 1, of the chorda tympani, and 2, of both cervical sympathetics. Between the two sections of the tracing, injection of 0·4 mg/kg of curare, 0·25 mg of Neoantergan and 0·6 mg of eserine sulphate. 3, Stimulation of both sympathetics; 4, of the left sympathetic. 5, Intravenous injection of 0·001 μg ACh; 6, 0·002 μg ACh; 7, 0·004 μg ACh; 8, 0·010 μg ACh; 9, stimulation of both sympathetics; 10, stimulation of the chorda tympani.

The effect of sympathetic stimulation was variable. In three out of nine experiments there was a steep fall of blood pressure (Fig. 1), which began 15–20 sec after the start of stimulation; in four experiments the fall was a very small one; and in two experiments no depressor response was obtained, although the sensitivity of the animal to injected ACh was high. The response was not seen in the absence of eserine, and when elicited could be suppressed by atropine. The depressor effect of stimulating the two sympathetics was in no experiment as big as that of chorda stimulation. A rough estimate of the amount of ACh that reached the general circulation, with chorda or sympathetic stimulation, could be made on the basis of the corresponding depressor effects. In the experiment of fig. 1, for example, the small dose of eserine used apparently permitted about 20% of the ACh released by chorda stimulation to escape into

the general circulation, but only about 10% of the ACh set free in the ganglia was able to reach the distant vessels. The relative difficulty of demonstrating ACh release from the ganglion, as compared with the gland, was probably due to the slow flow of blood in the veins of the neck after ligation of their extra-ganglionic branches; the liberated ACh would be mostly destroyed in this sluggish venous stream: whereas the circulation through the submaxillary gland is very rapid during stimulation of the chorda tympani.

These observations provide fairly good evidence that significant amounts of ACh can escape from a stimulated ganglion into the blood stream when the natural circulation is preserved. The possibility, however, cannot be completely excluded that a few cholinergic postganglionic fibres survived the operation designed to interrupt them, and that ACh was released from their endings during stimulation of the preganglionic trunk.

Liberation of choline by the uneserinized ganglion. In all the experiments described above, the ganglion was subjected to the action of an anticholinesterase drug. When no such drug is added to the perfusion fluid, no ACh can be detected in the effluent collected during preganglionic stimulation: it should be expected, however, that a corresponding quantity of choline would be discharged under these conditions. Brown & Feldberg (1936*b*), indeed, have already found that when eserine-free Locke's solution is used for the perfusion choline appears in the samples collected during stimulation; when eserine was present they found no choline, but only ACh.

We thought it worth while to repeat the experiments of Brown & Feldberg and to see, further, whether the quantity of choline released during synaptic activity in the absence of eserine agreed well with the quantity of ACh released when eserine was present. Phosphate–Locke was used as perfusion fluid: it contained no detectable choline. During each experiment, the ganglion was perfused alternately with eserine-free and eserine-containing fluid, and the effluent was tested for ACh both before and after acetylation. The inhibition of the ganglion's cholinesterase by eserine, as judged by the appearance or non-appearance of ACh in the venous fluid, developed quickly and was readily reversible. Our first experiments, however, were unsuccessful because all the samples tested, both stimulation and control, contained so much choline that the expected increment due to stimulation could not be detected with certainty: this happened whether or not there was eserine in the Locke's solution. It was found necessary to perfuse the ganglion for at least half an hour to allow this continuous discharge of choline to slacken to a manageable level. When this was done, subsequent control samples contained no more than 0·05 μg of choline (measured as ACh chloride) per 5 min period, and stimulation always produced a clear rise in choline content.

In one such experiment, after 45 min perfusion with eserine-free Locke's solution, the effluent collected during a 5 min control period contained 49 mμg

of choline estimated as ACh chloride. During the next 5 min period the preganglionic trunk was stimulated maximally for 3 min at 25/sec and the output of choline rose to 77 mμg. In the following control period the output fell to 58 mμg. The increment in choline output produced by the stimulation was thus about 23 mμg: none of these samples contained any detectable ACh. The perfusion fluid was now changed for eserinized Locke's solution; and effluent was collected during three successive 5 min periods, during the second of which the preganglionic trunk was stimulated in the same way as before. Assayed immediately on the cat's blood pressure, the samples contained <6, 22 and <6 mμg of ACh; assayed after acetylation, the corresponding figures were 45, 66 and 42 mμg. The increment in choline output due to stimulation was thus about 22 mμg, and was exactly accounted for by the ACh present. Lastly, the ganglion was perfused once more with the eserine-free solution for three 5 min periods, with stimulation during the second period. The successive values for total choline were 45, 67 and 35 mμg, the increment due to stimulation being once more 22 mμg; again, no free ACh was detected. In this experiment, therefore, the total amount of choline released by a given burst of preganglionic stimulation was practically the same, whether the perfusion fluid contained eserine or not; in the presence of eserine the choline appeared entirely in the form of ACh, while in the absence of eserine it appeared entirely in the form of choline. In two other experiments the findings were similar, except that the output of choline (or ACh) ascribable to stimulation fell off steadily as the experiment progressed. These, of course, are the findings to be expected on the assumption that eserine does not affect the liberation of ACh at the preganglionic terminals, but only preserves ACh from destruction after it has been liberated.

Calculation of the amount of ACh liberated by each preganglionic volley in these experiments gave values well below those in Tables 1 and 2. This was true whether ACh was determined by direct assay of the eserinized effluent or in the eserine-free samples after acetylation. In the three experiments the mean output per volley was respectively 5, 9 and 5·5 pg (perfusion without eserine), or 5, 7·3 and 3·1 pg (perfusion with eserine). It is tempting to suppose that these ganglia were so depleted of choline by prolonged perfusion that they were incapable of synthesizing ACh at the normal rate, especially as Brown & Feldberg (1936*b*) have shown that the addition of choline to the perfusion fluid sometimes increases the output of ACh from ganglia subjected to continuous stimulation; but other explanations are, no doubt, equally probable. In one experiment, in which because the assay cat was a particularly good one the increase in choline output due to stimulation could be determined early in the perfusion, a value of 30 ± 10 pg per volley was found, in good agreement with the usual figures for ACh at a comparable stage of perfusion in the experiments with eserine.

'Late firing' of ganglion cells. A curious phenomenon was observed in most of the experiments in which the perfused ganglion was subjected to repetitive stimulation in the presence of an anticholinesterase drug. The nictitating membrane relaxed, at the end of the period of stimulation, almost as rapidly and completely as if no such drug had been added to the perfusion fluid; but it soon began to contract again, and the record rose slowly and smoothly to a maximum that was maintained for many minutes, and was sometimes not far below that seen with preganglionic excitation. Occasionally this after-contraction began within a few seconds after the end of stimulation, before the membrane had fully relaxed. The phenomenon was seen during perfusion with either Locke's solution, plasma or blood, and in the presence of eserine, prostigmine, DFP or TEPP. Its late onset and long duration distinguished it from the after-discharge seen in the eserinized ganglion by Eccles (1935, 1944) and by Rosenblueth & Simeone (1938). It was certainly due to discharge of ganglion cells, for it could be temporarily inhibited by cooling of the post-ganglionic trunk or by the injection of a small dose of procaine hydrochloride into the perfusion stream. It was not, however, due to the persistence of free ACh in the ganglion, since it was not modified by the addition of tubocurarine or hexamethonium to the perfusion fluid. Moreover, no trace of ACh could be detected in the effluent obtained during even the most intense discharge of this type. We have not attempted any further analysis of its origin.

Experiments with tibialis anticus

Our results with the blood-perfused ganglion led us to expect that ACh would also be liberated in detectable amounts by blood-perfused muscle, provided that the cholinesterase of the blood and the muscle were fully inhibited under the conditions of perfusion. We have made a few experiments on tibialis anticus, which confirmed this expectation. The results are summarized in Table 3. In our first experiments (nos. 1 and 2 of Table 3), the perfusion fluid was phosphate-Locke or plasma, the proportion of eserine being the same as for the ganglion. The experiments confirmed those of Dale *et al.* (1936) on other skeletal muscles: ACh was absent from control samples of the venous effluent, but appeared when the motor nerve was stimulated. The output of ACh per maximal nerve volley was about 20% of that usually found in the ganglion experiments; and owing to the much greater bulk of tissue perfused the venous fluid contained ACh in much higher dilution than in ganglion perfusions. In the later experiments, in which defibrinated blood containing 10^{-4} eserine sulphate was perfused, the extreme dilution of ACh in the effluent sometimes made the assay difficult. The sensitivity of the test cat to ACh was usually such that 2 or 3 ml. of blood was needed to give a good depressor response. After a few such injections the cat became overeserinized, the blood pressure fell, troublesome twitching developed, and the threshold

for ACh was raised. A reasonably good assay was, however, generally possible if (*a*) the perfusion was kept fairly slow, so that the liberated ACh was not diluted in too large a volume of blood, and (*b*) the samples to be treated were all injected within 10 or 15 min, before the condition of the assay cat had too far deteriorated. In one or two experiments the venous blood from the muscle contained traces of a histamine-like substance, whose concentration was not related to nerve stimulation: a small dose of pyranisamine maleate ('Neoantergan') given to the test cat prevented this material from interfering with the assay.

TABLE 3. Release of ACh from tibialis anticus muscle on motor nerve stimulation at 20–25/sec, for 3 min except where noted

Expt. no.	Perfusion fluid	ACh output per maximal volley
1	Phosphate–Locke (eserine sulphate 5×10^{-6})	4·5
2	Plasma (eserine sulphate 10^{-5})	5·5
3	Blood (eserine sulphate 10^{-4})	3·5
4	Blood (eserine sulphate 10^{-4})	6·5
5	Blood (eserine sulphate 10^{-4})	1·1
6*	Blood (eserine sulphate 10^{-4})	4·5
7*	Blood (eserine sulphate 10^{-4})	4·5, 3·4†

* Sympathectomized muscle, fully curarized.

† Stimulation for 8 min.

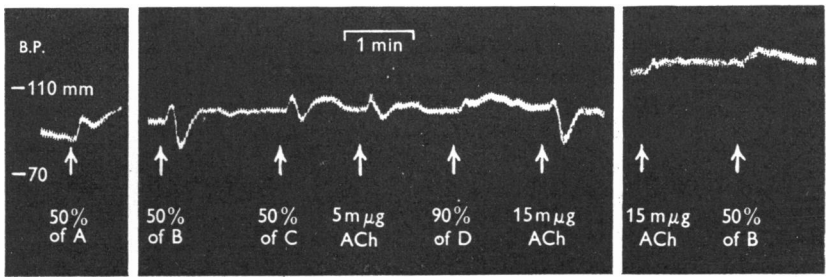

Fig. 2. Perfusion of a cat's tibialis muscle (sympathectomized) with defibrinated blood containing eserine sulphate 10^{-4} and tubocurarine chloride 7×10^{-5}. Effect of successive samples of the perfusate on the blood pressure of a second cat. Each blood sample was collected during a 10 min period. The sciatic nerve was stimulated with condenser discharges at 25/sec during the first 8 min while sample B was being collected; there was no stimulation during the other periods. Atropine sulphate (0·5 mg/kg) was injected into the assay cat before the last part of the record was made.

In each of five perfusion experiments with blood, ACh appeared regularly when the motor nerve was stimulated, but never in the absence of stimulation. As in the experiments on the ganglion, the quantity of ACh released was about the same as when Locke's solution or plasma was used for the perfusion. With the slow perfusion rates required, some ACh was often found in the first control blood sample following a stimulation (Fig. 2), and this was taken into account in reckoning the output per volley.

In contrast to the results obtained with Locke's solution (Dale *et al.* 1936) the muscle retained its ability to contract forcibly throughout the period of blood perfusion, but a prolonged tetanus was not well maintained, probably on account of the high concentration of eserine used. The appearance of ACh in the perfusate was not a consequence of the muscular contraction itself, since it took place as usual when the blood used for perfusion contained tubocurarine chloride in concentrations (7×10^{-5} g/ml.) high enough to produce neuromuscular block even with eserine present. Nor was the ACh liberated mainly by cholinergic sympathetic fibres that reach the muscle via the sciatic nerve, for the usual result was obtained with muscles which had been sympathectomized by the aseptic removal, a week earlier, of both abdominal chains from the third lumbar to the second sacral ganglion. The assay record shown in Fig. 2 is from an experiment on a sympathectomized and curarized muscle. The possibility that ACh was released by the antidromic firing of sensory fibres was not excluded in our experiments: it seems an unlikely one, since Dale *et al.* (1936) failed to detect such a release from perfused muscle when they stimulated the appropriate dorsal roots, in experiments in which ACh regularly appeared on stimulation of the corresponding ventral roots. The identification as ACh of the depressor substance released by nerve stimulation was confirmed by its failure to lower the blood pressure of the atropinized cat and by its failure to appear in the absence of eserine.

DISCUSSION

For demonstrating the release of ACh at ganglionic and neuromuscular synapses at least one departure from physiological conditions is necessary: the cholinesterase of the tissue under study and of the blood flowing through it must be put out of action. We have been concerned to discover whether any further abnormality is required. In the earlier experiments carried out in this laboratory, uniform success was achieved only when artificial perfusion media were used. The two fluids employed, bicarbonate–Locke (Feldberg & Gaddum, 1934; Feldberg & Vartiainen, 1934; Brown & Feldberg, 1936*b*; Kahlson & MacIntosh, 1939; Harvey & MacIntosh, 1940) and bicarbonate–Locke containing 10% of blood (MacIntosh, 1938) were unphysiologically alkaline, and were deficient in the normal solutes of plasma and in oxygen-carrying power. In the experiments described above we have used several different perfusion fluids: each of these was abnormal in some respect, but there was no abnormality common to all of them. The experiments with phosphate–Locke showed that the discharge of ACh can take place at a physiological pH; the experiments with defibrinated blood show that neither anoxia, nor dilution of the constituents of serum, is necessary for the discharge; and the experiments with plasma exclude the remote possibility that the discharge might be inhibited in the presence of fibrinogen or prothrombin. Nor is it essential that the perfusion

fluid should contain eserine, for in the ganglion at least the chemically unrelated compounds DFP and TEPP, as well as the related base prostigmine, are also effective. There is, furthermore, no substantial or even significant variation in the quantity of ACh discharged at the preganglionic endings, despite the considerable variations in their fluid environment imposed by the different conditions of perfusion. Finally, the observation that when no esterase inhibitor is present the perfused ganglion on stimulation liberates choline, in an amount corresponding to the ACh it releases when its cholinesterase is inhibited, provides evidence that ACh is also released when that enzyme is normally active, though the release cannot then be demonstrated directly. The only reasonable interpretation of these findings is, we think, that the release of ACh on stimulation is a physiological event, and that the quantity of ACh released under physiological conditions, by a known number of impulses in a particular group of nerve fibres, is a characteristic quantity that can be measured with tolerable accuracy.

In the superior cervical ganglion, under the conditions of our experiments (3250 to 4500 maximal preganglionic volleys in 3 min.) about 25 pg of ACh was liberated per volley. In tibialis anticus, stimulated in the same way, about 5 pg was liberated per motor nerve volley. It may be assumed, however, that the earlier volleys of each series released more ACh than the later ones, for it is well known (Feldberg & Vartiainen, 1934; Brown & Feldberg, 1936*b*; Kahlson & MacIntosh, 1939; Perry, 1953) that with prolonged stimulation the ACh output falls approximately exponentially to reach a steady low level: in fact, we can confirm the observations of Feldberg & Vartiainen and of Perry that in the ganglion the initial output of ACh per volley is about 100 pg. In the later part of each stimulation period the output per volley no doubt fell below the values we have given above. Perry indeed has found, in experiments in which the preganglionic trunk was subjected to prolonged stimulation at varying frequencies, that the ACh output eventually becomes constant at about 4 pg/min, whatever the frequency of stimulation: the output per volley under these conditions must then have varied inversely with the rate of stimulation. It is not therefore the absolute value of the ACh output per shock that we wish to emphasize, for this will depend on the frequency and duration of the stimulation; rather it is the fact that with a given kind of stimulation, the output of ACh per shock remains the same in spite of substantial changes in the composition of the fluid perfusing the ganglion.

In earlier investigations (Feldberg & Vartiainen, 1934; Lorente de Nó, 1938; MacIntosh, 1938) in which attempts were made to detect liberated ACh in the venous blood leaving a ganglion whose arterial supply was intact, only a trace of ACh or none at all was found, in spite of the massive doses of eserine which had been administered. Our present experiments on blood-perfused ganglia make it clear that these negative results were due to incomplete inhibition of

cholinesterase. A nearly maximal inhibition of the enzyme by eserine cannot be achieved in the whole animal, unless it is protected by appropriate blocking agents against the effects of accumulated ACh. When the cholinesterase of the blood and the ganglion is fully inhibited by contact with eserine sulphate at a concentration of 10^{-4} g/ml., the ACh output corresponds to that found for the saline-perfused ganglion. It is accordingly unnecessary to suppose, as MacIntosh (1938) and Eccles (1944) have done, that in the ganglion with natural circulation released ACh is for the most part removed by resynthesis to an inactive precursor rather than by enzymic hydrolysis. Perry (1953), on the basis of perfusion experiments in which the preganglionic stimulation was more prolonged than in our own, has suggested that in the absence of eserine the choline derived from liberated ACh (though not the ACh itself) may be recaptured by the preganglionic endings and used for the synthesis of readily releasable ACh. Our data provide no test of this idea, but we agree with him in finding that for stimulation periods lasting a few minutes only, the output of choline from the uneserinized ganglion corresponds to the output of ACh from the eserinized ganglion.

It may be worth while at this point to compare the amount of ACh liberated by stimulation with the amount required to excite the ganglion cells, since it has been suggested (Nachmansohn, 1946) that the former is very much smaller than the latter, and so can have no significance as evidence for synaptic transmission by ACh. In our tests the amount of ACh liberated by a single preganglionic volley was about $2{\cdot}5 \times 10^{-11}$ g on the average. Assuming this to be equally distributed through the volume of the ganglion (approx. 12 μl.), the concentration of the ACh would be about 2×10^{-9} g/ml. A discharge of impulses in the postganglionic fibres, equal to or greater than that following a preganglionic volley, is regularly produced by injecting 0·1 μg of ACh, dissolved in 0·2 ml of eserinized Locke's solution, into the perfusion stream above the ganglion: the concentration of ACh in the ganglion would be at most 5×10^{-7}. The ratio of these two concentrations is 1:250. But it cannot be concluded that the amount of ACh liberated by a volley is only 1/250 of the amount required to excite the ganglion cells: for it has still to be taken into account that if the liberated ACh comes solely from the preganglionic endings, as there is good reason to believe, then at the moment of its liberation it is not distributed throughout the whole volume of the ganglion, but is confined to the synaptic regions. If we may suppose that the total space in which free ACh is dissolved at the moment of its action is less than 1/250 of the volume of the ganglion, we shall then have completely resolved the apparent discrepancy between the liberated and the effective quantity of the transmitter.

The complexity of the histological picture is such that this 'effective synaptic space' can hardly be calculated on the basis of direct measurements: especially as it is not known whether ACh is released over the whole surface of the terminal

preganglionic twigs or only close to the junctional interface; nor is it known how far the diffusion of ACh may be limited by the pre- and post-synaptic membranes and by other structures in the vicinity of the synapse. The ratio of 1:250, which has just been calculated, may indeed overestimate the discrepancy between the concentration of free ACh in the whole ganglion after one volley and the threshold concentration for excitation; and if this is true the 'effective synaptic space' may exceed 1/250 of the ganglionic volume. In the first place, it is likely that ACh injected into the perfusion stream, and thence diffusing toward the ganglion cells, will stimulate the latter less effectively than ACh suddenly released in the same concentration at the post-synaptic surface. In the case of muscle, at any rate, the effectiveness of intra-arterially injected ACh in eliciting a contraction is closely dependent on the speed of the injection (Brown, Dale & Feldberg, 1936). In the second place, the ganglion cells will not be maximally responsive to ACh in the presence of eserine, which is known to have some ganglionic blocking action of the competitive type (Feldberg & Vartiainen, 1934; Feldberg & Hebb, 1948; Paton & Perry, 1953). Birks (1954, personal communication) has found that when TEPP is used instead of eserine to inhibit cholinesterase, the cells of the perfused ganglion regularly respond to 5×10^{-8} ACh. In the third place, our value of 25 pg as the quantity of ACh liberated by a single preganglionic volley is probably an underestimate: unless there has been prolonged repetitive firing just beforehand, the quantity, as we have already noted, will be nearer to 100 pg. If we substitute these values in our calculation, we can estimate 'the effective synaptic space' as high as 5–10 % of the ganglionic volume and still allow a sizeable safety factor for transmission.

End-bulbs in this ganglion appear to be sparsely scattered over the surface of the perikaryon and its dendrites, in comparison with their abundance at the surface of motoneurones and other cells of the central nervous system: the largest number counted by Gibson (1940) on a single neurone and its prolongation was 13. Even if one assumes that many end-bulbs are not made visible by the available staining techniques, and that ACh is also released from presynaptic structures, it is hard to suppose that the total bulk of the presynaptic terminations amounts to more than 1–2 % of the volume of the ganglion. The effective synaptic space itself cannot be many times larger than this. On an electrical theory of transmission the net flux of ions would initially be mainly confined to the immediate neighbourhood of the junction; and on a chemical theory the transmitter action would have to be completed within the 2 msec of the ganglion's refractory period, during which time any released transmitter could have diffused only a few micra from its site of release (Ogston, 1955) and would have lost activity through dilution and perhaps through enzymic action. Indeed, it seems probable on the basis of such considerations that ACh, in the quantity which perfusion experiments show to be released by a single volley,

must at the moment of its liberation have represented a considerably supra-liminal stimulus for most of the ganglion cells.

A similar quantitative argument could be put forward to deal with the discrepancy between ACh output and ACh threshold in muscle, which Nachmansohn (1946) has cited as further evidence against the theory of synaptic transmission by ACh. Some calculations of this kind have already been made by Acheson (1948), Eccles (1953), Fatt (1954) and del Castillo & Katz (1955).

Our experiments do, however, support the idea that more than one process may be involved in terminating the transmitter action (cf. Eccles, 1953, Fatt, 1954). Assuming that an effective amount of ACh can be liberated at the nerve ending within the period of synaptic delay, we must suppose this ACh to be somehow removed or made ineffective within the refractory period of the ganglion cell or motor end-plate. The most obvious way in which this could happen is through hydrolysis by cholinesterase; and in mammalian voluntary muscle, drugs which inhibit that enzyme do produce the anticipated repetitive firing of indirectly excited fibres, and other effects which seem to indicate the persistence of a transmitter. In sympathetic ganglia, however, the effects of such drugs are relatively unimportant: there is a summation of the negative 'synaptic' potential produced by a preganglionic tetanus, and a greater tendency to after-discharge following a preganglionic tetanus (Eccles, 1944); and submaximal preganglionic stimuli are more effective (Feldberg & Vartiainen, 1934), probably through spatial rather than temporal extension of the transmitter action. The failure of eserine and similar drugs to prolong more strikingly the effective life of the transmitter seemed to support the suggestion, which has already been referred to, that the evanescent action of the liberated ACh was due to its speedy removal, not by cholinesterase, but by recombination with the material of the axon endings to resynthesize the complex from which it had just been liberated. This idea, as we have noted, no longer seems attractive. It seems clear, however, that in the past too little attention has been given to the possibility that the main removal of ACh at the synapse may be by physical, rather than chemical, means. The most important process involved might be diffusion, but some part might be played by the change of electrical charge at neuronal surfaces in modifying the mobility of the ACh ion, or by the rapid spread of ACh along lipoprotein-water interfaces at these surfaces. As an indication of the rapidity with which diffusion might lower the concentration of ACh within a volume of synaptic dimensions, Ogston (1955) has calculated that if ACh were suddenly liberated at the centre of a spherical volume of radius 1 μ located within an aqueous medium and allowed to dissipate by diffusion alone, the mean concentration of ACh within the sphere would be lowered by 90% in 1·7 msec. Moreover, it may be unnecessary for the whole of the free ACh to be removed within the refractory period, for it is possible that ACh stimulates the ganglion cell or end-plate most effectively

when its concentration is rising steeply, or when the boundary between the depolarized and polarized regions of the cell membrane is fairly sharp. Such conditions for effective stimulation by a chemical mediator would have obvious analogies with those for excitation of nerve by an electric current; but a more elaborate discussion of them would be out of place here, and indeed would hardly be warranted by the facts now available. We raise these considerations only because the assumption has often been made that hydrolysis by cholinesterase, and possibly resynthesis to a precursor, are the only possible methods by which ACh could be inactivated at the synapse.

SUMMARY

1. The amount of acetylcholine liberated from the cat's superior cervical ganglion by a given number of preganglionic volleys has been determined under various conditions of perfusion.

2. It is the same, within fairly close limits, whether the perfusion fluid is Locke's solution at pH 8·5 or pH 7·4, heparinized plasma, or defibrinated blood, so long as a cholinesterase inhibitor is present in adequate concentration. This inhibitor may be eserine, prostigmine, di*iso*-propyl fluorophosphonate, or tetraethyl pyrophosphate.

3. In the absence of any anticholinesterase drug, a corresponding amount of choline, instead of acetylcholine, appears in the perfusion fluid during stimulation.

4. In the cat's tibialis anticus muscle, perfused with Locke's solution, plasma or defibrinated blood, the amount of acetylcholine liberated by motor nerve stimulation is likewise the same for each perfusion fluid.

5. It is concluded that the amounts of ACh released under physiological conditions by nerve impulses arriving at cholinergic terminals can be reliably estimated by measuring the ACh discharged into a perfusion fluid. These amounts are high enough, in both ganglion and muscle, to justify the belief that acetylcholine acts as a synaptic transmitter in these tissues.

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