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SECTION V

Adrenergic Transmission

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A. INTRODUCTORY REMARKS

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CALCIUM AND RELEASE OF NOREPINEPHRINE

A study of the part played by calcium in the release of catecholamines from the adrenal medulla which was made by Douglas and Rubin (26, 27) led us to make observations on the release of NE from sympathetic postganglionic fibers (13). We used the isolated rabbit ileum, stimulating the periarterial nerves in the mesentery. Stimulation caused inhibition of the pendular movements. The inhibition produced by a given number of maximal shocks was diminished when the Ca^{++} concentration was reduced, and was increased when the Ca^{++} concentration was raised. If stimulation was maintained for a period of 15 min, the addition of Ca^{++} to the bath during the stimulation increased the inhibition. In the presence of the normal Ca^{++} concentration of about 2 mM, the inhibition was reduced when Mg^{++} was added to the bath.

In their experiments, Douglas and Rubin used acetylcholine (ACh) as a stimulus for the adrenal medulla, and we also used ACh as a stimulus for the release of NE from the sympathetic endings in the heart (15). We used the isolated atria of the rabbit and observed the effect of ACh in the presence of hyoscine 10^{-6} g per ml. We observed that when ACh was added in a concentration of 5×10^{-5} g per ml there was little effect on the rate when the Ca^{++} concentration was 2.2 mM, but there was an increase of 19% in the rate when the concentration was 6.6 mM, and an increase of 46% when the concentration was 13.2 mM. These were the mean figures of 5 experiments. Thus the release of NE by ACh from sympathetic postganglionic fibers in the atria was dependent on the Ca^{++} concentration, as was the release of catecholamines by ACh from the adrenal medulla.

Previously Ferry (30) reached the conclusion that ACh caused a release of NE from sympathetic postganglionic endings by stimulating the fibers; he observed that when ACh was injected into the splenic artery, antidromic impulses passed along the splenic nerves, and he supposed that orthodromic impulses would also pass to the spleen. However Hertting and Widhalm (34) have found that in the perfused spleen, the release of NE by stimulation of the splenic nerves can be blocked by concentrations of bretylium from 2.5×10^{-6} to 10^{-5} g per ml, but that ACh is still effective in releasing NE. The action of ACh is blocked only when the concentration of bretylium is raised to 5×10^{-5} g per ml. These observations show that the release of NE by ACh is not mediated by the splenic nerves, just as

the release of catecholamines from the adrenal medulla by ACh is not mediated by the splanchnic nerves.

Douglas and Rubin showed that when the perfused adrenal gland was kept without Ca^{++} for 20 min, the readmission of Ca^{++} then caused a release of catecholamines. They supposed that in the 20-min period during which Ca^{++} was absent, the chromaffin cell membrane lost some of the bound Ca^{++} and became more permeable. When Ca^{++} was restored, it then entered the cell at once and caused the release of catecholamines. We made experiments on the ileum in which we replaced the ordinary Locke's solution by one which was Ca^{++}-free. When this was done for 2 min only, the pendular rhythm became slow and declined but was fully restored at once when Ca^{++} was replaced. When Ca^{++} was removed for 20 min, the restoration of Ca^{++} at the end of that time did not restore the rhythm at once. Often there was a period of 1 min before any pendular movement was seen, and it then began as a very small rhythm which increased slowly and took 3.5 to 4 min to reach its previous amplitude. We suspected that this delayed resumption of the rhythm was due to the release of NE by Ca^{++} which entered the fiber through a membrane made more permeable by the period of Ca^{++} deficiency. We were able to prove that this was so by taking strips of ileum from rabbits treated with reserpine. In these strips NE was absent, and we found that restoration of Ca^{++} after a period of 20 min without Ca^{++} led to prompt revival of the rhythm. Thus we had evidence that as in the chromaffin cell of the adrenal medulla so in the postganglionic fiber, after a period of Ca^{++}-deficiency, Ca^{++} when replaced could enter and set free NE. Thus we observed a close similarity between the events in the adrenal medulla and those in the postganglionic fiber.

THE CHOLINERGIC LINK

These observations suggest that the hypothesis of Burn and Rand (16) put forward in 1959 can be restated as follows. The sympathetic impulse first releases ACh; this affects the permeability of the membrane of the postganglionic fiber making it more permeable to Ca^{++}, and Ca^{++} then enters the fiber. Within the fiber it causes release of NE from the sites where it is bound.

EXISTENCE OF CHOLINERGIC FIBERS

As long ago as 1931 it had been shown that sympathetic postganglionic fibers in several places liberated not only NE but also ACh. Thus fibers from the superior cervical ganglion to the buccal mucous membrane of the cat and the dog were shown to liberate ACh by Euler and Gaddum (29). Fibers to the vessels of the muscles in the hindleg of the dog and the cat (10), fibers to the uterus of the dog (46) and fibers to the heart of the dog and the cat (31) were also shown to liberate ACh as well as NE. More recently the splenic nerves have been shown to liberate ACh as well as NE (7, 17, 38) and also the postganglionic fibers to the skin vessels of the rabbit ear (17, 36) the postganglionic fibers to the ileum and to the colon (33) and the fibers to the pilomotor muscles of the cat tail (49). Thus there is no known exception to the rule that all sympathetic postganglionic fibers liberate ACh as well as NE.

TABLE 1

Amounts of ACh and of NE in cat spleen

	Normal Spleens	Denervated Spleens	D/N%
$\mu g/g$			
NE	1.13	0.205	18
ACh	0.472	0.105	22

AMOUNT OF ACh RELEASED

Brandon and Rand (7) have measured the amount of NE and ACh present in the normal cat spleen. They have also measured it in the cat spleen after section and degeneration of the splenic nerves. Their results are shown in table 1.

The results indicate that denervation affected the ACh and the NE to the same extent, reducing the ACh to 22% and the NE to 18%. Thus it can be concluded that the ACh as well as the NE was mostly present in the splenic nerves. Thus the results indicate that the amount of ACh in the splenic nerves was more than 40% of the NE in the splenic nerves; the ACh is not present in an insignificant amount, but in an amount of a similar order of magnitude. It is therefore important to discover what the function of this large amount of ACh is. So far no hypothesis applying to all organs has been put forward other than that of Burn and Rand.

SUBSTANCES WHICH BLOCK THE RELEASE OF NE

Both ACh and nicotine block the release of NE from the postganglionic fiber. Block by ACh was first demonstrated by Brücke (8) in the pilomotor muscles of the cat's tail. Block by ACh has also been demonstrated in the perfused vessels of the rabbit ear (17). Block by nicotine has been demonstrated in the pilomotor muscles of the cat's tail (17, 23) and in the rabbit ileum (2). If the sympathetic impulse directly releases NE, there is no explanation for block by ACh and by nicotine. The blocking action of these two substances at once suggests that ACh plays a part in the release of NE.

ACTION OF HEMICHOLINIUM

The hemicholinium called by Schueler HC-3 (45) prevents the synthesis of ACh by interfering with the transport of choline to the intraneuronal site where the synthesis takes place (39). The action of HC-3 is overcome by choline. HC-3 has been shown by Brandon and Rand (7) to abolish the contraction of the spleen caused by stimulating the splenic nerves. The abolition was complete only after 4 hr and 23 min; the contraction was restored by choline. Other results obtained by Rand and his colleagues are shown in table 2.

These results show that HC-3 was found to block the postganglionic fibers to the spleen, the heart, the intestine and the blood vessels. This is a wide selection which indicates that the block is a general phenomenon.

Several workers, however, have failed to confirm the action of HC-3 in post-

TABLE 2

Block of response to sympathetic stimulation by HC-3 (7, 22, 43)

Organ	Concentration	Time	Choline
	μg/ml	*hr*	
Cat spleen	50	4.5	Restored
Cat atria	500	4.5	Restored
Guinea-pig colon	50	5.5	Restored
Rabbit ear-vessels	50	3.0	Restored

ganglionic sympathetic fibers. The main reason for this failure is they have not allowed sufficient time for the HC-3 to act. Thus HC-3 was found to have no effect on the isolated preparation of the nictitating membrane, but the longest time during which HC-3 acted was more than 1 hr (47). HC-3 was found to have no effect on the isolated preparation of the pulmonary artery when sympathetic stimulation was applied. The longest time of application was 2 hr (4). Leaders and Dayrit (38) found that HC-3 had no effect in the dog spleen, but the longest time of application was "more than 30 min". The observations of Rand and his colleagues show that times up to 5½ hr are often required to abolish the response to sympathetic stimulation, but that even at the end of that time the response can be restored by choline. When an effect of HC-3 is observed, it cannot be claimed as a specific effect, unless it is reversed by choline. Leaders and Dayrit (38) found that the liberation of ACh from the perfused dog spleen by stimulation of the splenic nerves slowly diminished in the presence of HC-3 but they failed to show that the liberation was restored by choline. They cannot, therefore, refute the criticism that the diminution of the release of ACh was probably explained by the slow death of their preparation.

In considering the action of HC-3 it must be remembered that it is a large bis-quaternary molecule which can be expected to enter the sympathetic postganglionic fiber only very slowly. HC-3 has, however, another action like that of hexamethonium since it prevents the entry of bretylium into the postganglionic fiber (14). Hence the abolition of the effect on the vas deferens of stimulating the hypogastric nerve, an aboliton obtained in 10 min by a concentration of HC-3 equal to 10^{-4} can be assumed to be a ganglion-blocking action (2). Thus the evidence provided by Rand's observations of the action of HC-3 is most strongly in favour of the cholinergic link.

ACTION OF BOTULINUM TOXIN

Botulinum toxin has been shown by Burgen *et al.* (11) to stop the release of ACh from the endings of motor nerves in skeletal muscle. The work of Ambache (1) demonstrated that parasympathetic nerves were blocked in the same way, and now Rand and Whaler (44) have demonstrated that sympathetic postganglionic fibers are blocked. They have shown this for the fibers to the pilomotor muscles of the cat's tail and for the fibers to the rabbit ileum. In this preparation, set up in an isolated organ bath, they showed that botulinum toxin, present

during 4½ hr, blocked all response, whereas in a comparable piece of ileum, there was no failure whatever during the same period of time. The demonstration was successful in 17 out of 19 experiments. It is of course at once explained if ACh plays a part in the release of NE.

MORPHINE

In 1957 Trendelenburg (48) showed that when morphine in amounts varying from 0.05 to 2.5 mg was injected into cats anaesthetized with chloralose, the response of the nictitating membrane to postganglionic stimulation was reduced or abolished. Since the response to NE itself was not reduced, he suggested that morphine diminished the amount of NE liberated. Paton (42) showed that morphine reduced the amount of ACh liberated from cholinergic nerve endings. These results are then also consistent with the hypothesis that ACh plays a part in the liberation of NE.

THE SYMPATHETIC BLOCKING DRUGS

The mode of action of bretylium and guanethidine has been discussed previously (12, 20) and will be considered here only briefly. Both Rand and I have independently observed that *d*-tubocurarine will block the sympathetic postganglionic ending in the rabbit ileum. The concentration of *d*-tubocurarine required is not very high, but the time is variable. Thus I have observed that a concentration of 7×10^{-5} g per ml has blocked stimulation at different frequencies in 90 min. The converse of this is that bretylium and guanethidine in a concentration of 3×10^{-4} g per ml will block the motor nerve endings in the diaphragm. This was shown by Dixit *et al.* (25) and we have confirmed it, and found that bretylium is only 5 times weaker than decamethonium and that guanethidine is only 8 times weaker (20). A further finding is that mecamylamine will block the sympathetic postganglionic fiber. Thus there is no sharp distinction between bretylium and guanethidine on the one hand, and *d*-tubocurarine and mecamylamine on the other. These substances and others all fall into one class. They are substances which block the action of ACh.

THE ACTION OF ANTICHOLINESTERASES

If ACh is first released by the sympathetic impulse, and then takes a part in the release of NE, it should be possible to demonstrate that an anticholinesterase will increase the amount of NE which is liberated. By increasing the concentration of ACh an anticholinesterase should have two effects. There will be an increased direct effect of ACh; this can be excluded by the injection of atropine or preferably of hyoscine which is more powerful and more specific (47). When the muscarinic action of ACh is excluded in this way, an increased concentration of ACh should show itself by increasing the amount of NE which is liberated. This effect should be greatest when the frequency of stimulation is low, and should diminish as the frequency of stimulation rises. It is noteworthy that Garry and Gillespie (32), who compared the optimal frequency of the parasympathetic nerves to the isolated colon with that of the sympathetic nerves, found two

things. They found that the optimal frequency of sympathetic nerves was much higher than that of parasympathetic nerves. If we assume that the purpose of a high frequency is to build up a high concentration of transmitter, then this finding is the reverse of what would be expected, since NE is much more stable than ACh. The second observation was a much greater delay in the response to sympathetic stimulation than in the response to parasympathetic stimulation. Both these findings are explained if the liberation of the sympathetic transmitter is a double process in the first part of which ACh is concerned.

If ACh is liberated at a low frequency of stimulation, it will be unable to accumulate between the pulses, because there will be time for cholinesterase to destroy it. When, however, it is liberated at a high frequency of stimulation, ACh will accumulate and will then exert a greater effect. It is clear that if anticholinesterases are to increase the release of NE, their effect should be found to be greater at low frequencies than at high frequencies.

The first observations were made in the vas deferens of the guinea-pig in response to stimulation of the hypogastric nerve. Burn and Weetman (21) found that physostigmine, acting in the presence of hyoscine, increased the response to stimulation at a frequency of 5 per sec and decreased it at a frequency of 20 per sec. Thus physostigmine affected the response in the same way as it affected the response of the diaphragm to stimulation of the phrenic nerve (9). Neostigmine acted like physostigmine.

The significance of this result was doubtful because the fibers in the hypogastric nerve which were stimulated were preganglionic, and the effect of the anticholinesterase might have been exerted at the ganglion rather than at the postganglionic terminations. Since such large effects have never been observed at a ganglion, this explanation was unlikely, but it was conceivable.

Results were obtained free from this doubt, however, when the postganglionic fibers to the nictitating membrane of the cat were stimulated, the cats being anaesthetized with chloralose, and injected with hyoscine, so that direct action of ACh on the membrane was excluded. In figure 1 is shown a result in which stimulation was applied at frequencies of 5 per sec, 2 per sec and 1 per sec. The control observations before the injection of hyoscine are shown in the top panel, and the slightly smaller responses after the injection of hyoscine are shown in the middle panel. In the lower panel are shown the much larger responses after the injection of physostigmine 0.5 mg per kg. The response to the frequency of 5 per sec was increased in height by 13 %, the response to 2 per sec was increased by 24 % and the response to 1 per sec was increased by 60 %. Thus the effect of physostigmine was greatest at the lowest frequency. Burn *et al.* (19) obtained other results which were equally clear both with physostigmine and neostigmine. Bowman *et al.* (6) who made more elaborate observations, in which they removed the eyeball and recorded the contractions of the nictitating membrane isometrically, have found that physostigmine caused an increase in the tension developed on stimulation in 10 out of 15 experiments, and that it increased the duration of the contraction in 31 out of 45 experiments.

A second preparation in which observations have been made is the taenia of

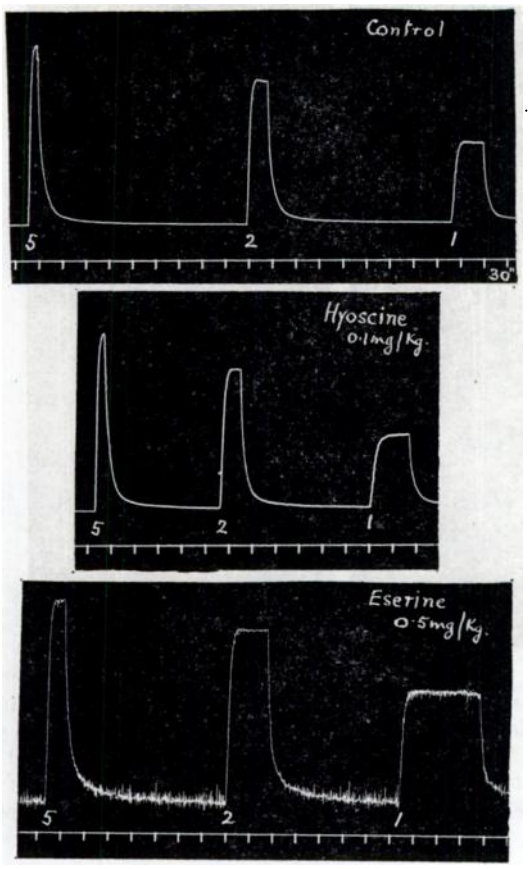

FIG. 1. Record of contractions of nictitating membrane of cat under chloralose. Upper panel shows control observations when postganglionic fibers were stimulated with maximal stimuli at frequencies 5 per sec, 2 per sec and 1 per sec (total of 50 shocks). Middle panel shows the smaller responses after the i.v. injection of hyoscine 0.1 mg per kg. The bottom panel shows the greater responses after the i.v. injection of physostigmine 0.5 mg per kg. (In this panel a total of 100 shocks was given, but it is the height of the contraction and not the duration which is relevant.)

the caecum of the guinea-pig, to which sympathetic fibers run in the perivascular nerves in the mesentery. Kevin K. F. Ng (41) has shown that this muscle, when suspended in an isolated organ bath, is caused to contract by ACh and to relax by NE. Stimulation of the perivascular nerves in the mesentery causes relaxation at higher frequencies, but a variable much smaller response, sometimes contraction and sometimes relaxation, at low frequencies. However, in the presence of hyoscine, the response to all frequencies was relaxation. This relaxation was unaffected by hexamethonium but was blocked by bretylium.

The experiments were therefore made in the presence of hyoscine, 10^{-7} g per ml. The results in one experiment are shown in figure 2 in which stimulation was applied at frequencies of 10 per sec and 20 per sec. Control observations are

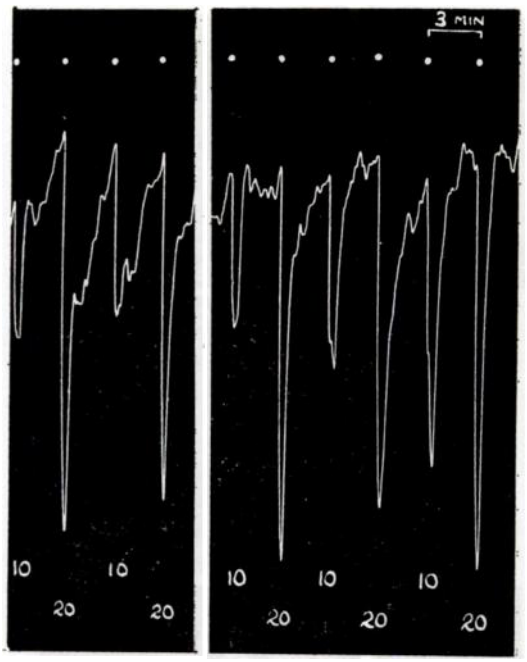

FIG. 2. Inhibitory responses of the taenia of the guinea-pig. The perivascular nerves in the mesentery were stimulated at the dots, stimulation being applied at intervals of 3 min. All observations made in the presence of hyoscine 10^{-7}. Left hand panel shows the responses to maximal shocks at a frequency of 10 per sec and of 20 per sec; 200 shocks were given. The right hand panel shows the responses 12 min after the addition of physostigmine 5×10^{-8} to the bath. Note the increase in the response to stimulation at 10 per sec. (Experiment of Kevin K.F. Ng.)

shown in the left hand panel, and observations after the addition to the bath of physostigmine, 0.05 μg per ml, are shown in the right hand panel. After the addition of physostigmine, the response to a frequency of 10 per sec steadily increased, while that to a frequency of 20 per sec was almost unchanged. Similar observations were made in other experiments when dyflos (diisopropylfluorophosphonate) and mipafox [bis (isopropylamino) fluorophosphine] were used as anticholinesterases; in these stimulation was applied at frequencies of 1 per sec, 2 per sec and 5 per sec, and it was found that the greatest increase was obtained at the lowest frequency. The observations in the innervated taenia therefore support those made in the nictitating membrane.

A start has been made with observations on the retractor penis muscle in the dog. The dog is anaesthetized with chloralose and is then eviscerated to make access to the sacral sympathetic ganglia easier. Electrodes can then be applied directly to the first sacral ganglion. The motor effect of NE on the retractor penis is reduced by simultaneous injection of ACh, and therefore hyoscine is injected to exclude the action of ACh released by sympathetic stimulation. Hyoscine, 0.2 mg per kg, increased the response to stimulation slightly. When physostig-

mine, 0.5 mg per kg was injected, the height of the contraction in response to 10 maximal shocks given at a frequency of 1 per sec was increased by 30%, while the height of the contraction in response to the same number of shocks at 5 per sec was increased only by 3.5%. Thus physostigmine increased the amount of NE liberated by sympathetic stimulation, the increase being much greater at the low frequency of 1 per sec than at the higher frequency of 5 per sec.

Lastly I should mention the observations of Bernard and De Schaepdryver (3) who measured the changes in flow through the femoral artery of the dog in response to stimulation of the sympathetic trunk. Having given atropine they stimulated at frequencies from 0.1 to 3 per sec and determined the reduction in the rate of flow. In all 14 experiments the injection of physostigmine or neostigmine or sarin (methyl-isopropyl phosphonofluoridate 50 μg/kg) caused increased vasoconstriction, the increase being greatest at the lowest frequency. However, they thought that their results might be explained by an action of the anticholinesterases on a junction between sympathetic fibers and chromaffin cells in the skin. Of this it is to be said that Coupland (24) has shown that cells in the skin thought to be chromaffin cells are mast cells, and also that Muscholl and Vogt (40) have shown that sympathetic stimulation does not liberate NE from chromaffin cells. Another possibility put forward by Bernard and De Schaepdryver was that they were stimulating preganglionic fibers, and that the anticholinesterases acted at the ganglionic synapse. There is little evidence, however, that anticholinesterases increase transmission through ganglia in the body, as Bowman *et al.* (6) found in their experiments on the nictitating membrane; the response to preganglionic stimulation was not affected by physostigmine differently from the response to postganglionic stimulation. I conclude that the evidence of Bernard and De Schaepdryver is in favour of the cholinergic link.

It seems clear from the evidence on the nictitating membrane, on the taenia of the guinea-pig, on the retractor penis muscle of the dog, and, I would like to add, on the femoral blood flow, that anticholinesterases increase the liberation of NE in a way demanded by the hypothesis.

However some workers have failed to observe effects of physostigmine when stimulating the splenic nerves. For example Hertting and Widhalm (34) failed to do so. But they did not say whether they made their observations in the presence of atropine or hyoscine, and they did not state the frequency of stimulation. It seems clear that the test must be made at a low frequency and that direct effects of ACh must be excluded. Again Bogaert and De Schaepdryver (5) failed to observe an effect of physostigmine, neostigmine or sarin on the increase in rate of the dog's heart produced by postganglionic stimulation. These observations were made in the presence of atropine and at low frequencies. They were made in 21 dogs and are certainly negative results. There seem to me only two criticisms. The dogs received 1 mg per kg of morphine, which may interfere with the release of ACh, though it did not do so in the experiments on femoral artery flow. Experiments of this kind would be more suitably made in the isolated heart, than in the whole animal under anaesthesia. It should not be forgotten that it has been well established that stimulation of the cardiac nerves releases ACh.

THE ACTION OF ACh

Reference should be made to one dissimilarity between the effect of postganglionic stimulation and the effect of ACh and nicotine. Both stimulation and the injection of these substances release NE, but while hexamethonium and some other ganglion-blocking agents do not modify the response to stimulation, they block the effect of ACh and nicotine. For example Heymans and Bennati (35) have shown that tetraethylammonium will prevent ACh from causing a rise in the rate of the dog heart, and they conclude that ACh acts on "intracardiac synaptic sympathetic structures." In experiments on isolated tissues there is evidence that ganglion-blocking agents have another action as well as that of blocking ganglia or synapses. Thus Burn and Gibbons (14), using the isolated rabbit ileum, and stimulating the sympathetic nerves in the mesentery, found that the blocking of the inhibitory response to stimulation by bretylium was not seen when hexamethonium was present in the bath. This was interpreted to mean that hexamethonium prevented the entry of bretylium into the sympathetic fiber, and they thought that it would prevent the action of ACh in the same way. A second observation is that of Huković (37) who showed that the action of ACh in increasing the rate and force of contraction of isolated rabbit atria was blocked when bretylium was added to the bath, but that the block was removed when the bretylium was washed out. This is in contrast to the block of the sympathetic impulse by bretylium which remains when the bretylium is washed out. It would therefore seem more reasonable to believe that the block of ACh and of nicotine by hexamethonium, bretylium and similar substances is nothing more than a block of entry into the sympathetic fiber.

HISTOLOGIC EVIDENCE

Evidence obtained by histologic methods was recently discussed by Burn and Rand (18) and will not be given here. It is interesting, however, to observe that Eränkö and Räisänen (28), using a method which makes both NE and acetylcholinesterase fluorescent, have found that in the superior cervical ganglion of the rat there are cells which give fluorescence for both substances. They have also shown that in the nerve net of the rat iris there are fibers containing both substances.

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