ACETYLCHOLINE IN ADRENERGIC TRANSMISSION[1,2]

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The subject discussed in this article, in particular certain aspects of it, has been studied by many workers, and their contributions have been very numerous. For example, it would be possible to quote at least 50 references to those who have demonstrated that acetylcholine (ACh) has actions similar to those of sympathetic stimulation. We have not been able to refer to these workers because of limitation of space. We make this apology for many omissions.

The cholinergic link.—The conception that ACh might play a part in the release of norepinephrine from the postganglionic fibers was prompted by several considerations. It had been shown that ACh and nicotine caused effects like those of sympathetic stimulation in many organs. Hoffmann et al. (47) observed this action in the heart, showing that it was followed by the release of an epinephrine-like substance. These effects of ACh and of nicotine were also seen in many other organs, for example in skin vessels (26) and pilomotor muscles (28, 32). Nicotine was shown to have a similar effect in the nictitating membrane (24), in the colon, and in the ileum (45). The effects were absent when the organs were taken from animals treated with reserpine, and were, therefore, due to the release of norepinephrine.

The second consideration arose from Brücke's observations in 1935 (16) on the pilomotor muscles of the cat's tail; these have an adrenergic innervation. The injection of a small amount of ACh into the skin caused piloerection, but a large dose of ACh caused a transient erection and then abolished the response to sympathetic stimulation. Epinephrine, however, still caused piloerection. Thus, Brücke showed that ACh in large dose blocked the effect of stimulating the sympathetic fibers at their terminations.

On the assumption that the nerve impulse directly releases norepinephrine, there is no explanation of this observation. ACh might block a cholinergic fiber, but ACh in a cat treated with reserpine has little or no pilomotor action (24). Thus, it is the release of norepinephrine which causes the pilomotor action, yet a large dose of ACh has been found to block this release. Therefore, Brücke's observation could only be explained by supposing that a large dose of ACh blocked the release of norepinephrine. This suggested to us that the normal release of norepinephrine might be mediated by ACh.

The third consideration was the structure of the substances which were

[1] The survey of literature pertaining to this review was concluded in June, 1964.
[2] The following abbreviation will be used: ACh (acetylcholine).

discovered to block the postganglionic fiber. The first of these compounds was xylocholine. It is a compound which might be expected to block the action of ACh. Exley (39) demonstrated that xylocholine and the next compound, bretylium, did not interfere with conduction along postganglionic fibers and that their action was not that of a local anesthetic. Burn & Rand (28) showed that these compounds did not interfere with the release of norepinephrine by tyramine. Therefore, it seemed probable that these blocking agents acted in the same way as a large dose of ACh appeared to act, by preventing the release of norepinephrine by the nerve impulse. This again suggested to us that the nerve impulse might first release ACh which then in turn released norepinephrine.

The cholinergic fibers.—In 1931, it was shown that the stimulation of some sympathetic nerves, which normally resulted in the release of an epinephrine-like substance, did, in fact, release ACh as well. Euler & Gaddum found such fibers present in the sympathetic supply to the mucous membrane of the buccal cavity and lips of the dog (38). Bülbring & Burn (17) found them in the supply to the hindlegs of the dog, while Folkow et al. (41) found that ACh was released by stimulation of the accelerator nerves to the heart and by the stimulation of the sympathetic supply to the hindlegs of the cat. These authors concluded that among the adrenergic fibers there was an admixture of cholinergic fibers. It was never contemplated that ACh and the adrenergic transmitter might be released from the same fiber. However, it was not clear what the function of the cholinergic fibers might be.

The hypothesis that the main purpose of the release of ACh (in some fibers at least) was to release, in turn, norepinephrine served to suggest a function for the ACh. It was, however, a working hypothesis which was put forward by Burn & Rand (27) in 1959 and as such was used to indicate lines of investigation which must be followed in order to validate the hypothesis. Thus, cholinergic fibers in postganglionic sympathetic fibers were known only in a few situations and, if the hypothesis was correct, they ought to be present in all sympathetic postganglionic supplies. Cholinergic fibers had never been shown to be present in the splenic nerves, and they had not been shown to supply the vessels of the skin; nor had they been shown to be present in the supply to the intestines. Moreover, the evidence for cholinergic fibers to the nictitating membrane was weak. The ensuing investigations demonstrated that in all these organs stimulation of the sympathetic postganglionic fibers released ACh; therefore, cholinergic fibers were present either as fibers separate from the adrenergic fibers or identical with them. Thus, the introduction of the hypothesis led to the discovery that cholinergic fibers were present in all sympathetic postganglionic supplies.

The splenic nerves.—To see whether ACh was released on stimulation of the splenic nerves, experiments were made in which cats were injected with reserpine. Stimulation caused dilatation of the spleen, and this dilatation was increased following the intravenous injection of physostigmine. The dilatation was abolished by atropine [Burn & Rand (28)].

Other experiments were made by perfusing the spleen of a cat treated with reserpine and allowing the fluid leaving the spleen to superfuse a strip of guinea pig ileum. When neostigmine was added to the perfusing fluid for a period of 30 min, it was found that stimulation of the splenic nerves was followed by a contraction of the guinea pig ileum. This contraction was due to the appearance of ACh in the outflow from the spleen, since the contraction was not seen when the ileum was treated with atropine [Brandon & Rand (13)].

To determine the existence of fibers liberating ACh in the postganglionic supply to the vessels of the rabbit ear, the ear was perfused by the method of Gaddum & Kwiatkowski (43). It was found that when physostigmine was added to the perfusion fluid stimulation caused the appearance of a substance in the venous effluent which made the leech contract (28). The action of this substance was abolished by tubocurarine, and the substance was therefore presumed to be ACh.

Further observations were made by Holton & Rand (49) on the changes of circulation produced in the ear of the anesthetized rabbit by postganglionic stimulation. They used a photocell on which light fell after passing through the ear. The effect of stimulation was diphasic, a constriction being produced followed by dilatation. The dilatation was shown to be increased by physostigmine and usually abolished by atropine. The diphasic response persisted after section and degeneration of the preganglionic fibers to the superior cervical ganglion. When guanethidine was injected, the initial constrictor response was abolished, but the second dilator response remained. These results supported the view that stimulation of the postganglionic fibers to the rabbit ear liberated both norepinephrine and ACh.

The nictitating membrane.—The nictitating membrane has been the subject of much controversy. Observations were made by Burn & Rand (28) of the effect of stimulating the postganglionic fibers to the membrane in reserpinized cats under chloralose. It was observed that stimulation produced a small contraction of the membrane, about 20 to 40 percent of that seen in normal animals, which was abolished by atropine (1.5 mg per kg).

It was important to investigate the action of physostigmine and to demonstrate an increase produced by it. The difficulty appeared to be in ensuring that sufficient physostigmine reached the nerve endings in the membrane. Burn, Rand & Wien (29), therefore, perfused the head of the cat with Locke's solution by means of a pump. This ensured a circulation through the nictitating membranes, which was unaffected by the addition of physostigmine to the perfusion fluid. When the perfusion fluid contained physostigmine (10^{-6} g per ml), the response to stimulation was rapidly increased by 40 to 80 percent. The increase was abolished by the injection of 1 mg atropine into the perfusing fluid.

In other experiments in which cats were anesthetized with chloralose, physostigmine was shown to produce an increased response to stimulation when atropine (1 mg per kg) or hyoscine (0.2 mg per kg) had been injected

first. These substances prevented physostigmine from lowering the blood pressure and the heart rate. These results established that cholinergic fibers were present.

Gardiner, Hellmann & Thompson (44) found that physostigmine did not increase the response to postganglionic stimulation, but they did not perfuse the head and did not give atropine or hyoscine first. They administered physostigmine by putting it in the conjunctival sac, so that the amount in the blood stream was not known. Mirkin & Cervoni (58) studied the response of the nictitating membrane to postganglionic stimulation in normal cats and in cats treated with reserpine. They found that atropine diminished the response in normal cats by only 21 percent, but it diminished the response in cats treated with reserpine by 69 percent. Yet they drew the surprising conclusion that the action of atropine was the same in both cases! They thought there was no evidence that ACh played a part. They used a semi-isometric method of recording the contractions which may be unsuited to the nictitating membrane, which always contracts isotonically.

A different conclusion was reached by Nystrom (60), who recorded changes in electrical potentials in the cat nictitating membrane when the postganglionic sympathetic fibers were stimulated. He observed a double response consisting of an initial wave which was increased by physostigmine and abolished by atropine and a second slow potential which was diminished by phenoxybenzamine. He considered that the first wave was due to the release of ACh and the second to the release of norepinephrine. Thus, he thought there was a dual innervation of the membrane, one cholinergic and the other adrenergic.

In our view, the positive evidence in favour of the presence of cholinergic fibers to the nictitating membrane outweighs the negative evidence.

Sympathetic supply to the intestine.—Observations have been made by Gillespie & Mackenna (45) on the effect of stimulating the sympathetic fibers to the isolated colon and to the isolated ileum of the rabbit. In preparations taken from rabbits which had been treated with reserpine, the authors demonstrated the presence of cholinergic fibers accompanying the adrenergic fibers and thought that they were parasympathetic in origin. It is, of course, impossible to refute this view of the origin of the cholinergic fibers in an organ which has both a parasympathetic and a sympathetic innervation; but the observations are also consistent with the presence of cholinergic fibers in the sympathetic supply.

Evidence from electron microscopy.—The foregoing evidence showed that all sympathetic postganglionic fibers liberate ACh on stimulation; there are differences of opinion about the supply to the nictitating membrane. Thus, in all sympathetic fibers, including those to the uterus of the dog and the vas deferens of the guinea pig, there is some evidence of the presence of cholinergic fibers which is, in several cases, conclusive. Are these fibers few, or do they represent a large proportion of the fibers? The splenic fibers provide

information on this. In the cat spleen, Brandon & Rand (13) found that the amount of norepinephrine was 1.13 μg per g, while the amount of ACh was 0.472 μg per g. After section of the splenic nerves and time for degeneration, the amount of norepinephrine in the spleen fell to 0.205 μg per g and the amount of ACh to 0.105 μg per g. Thus, after nerve degeneration, the norepinephrine fell to 18 percent of the normal amount and the ACh fell to 22 percent. Therefore, it may be concluded that the 82 percent of norepinephrine and the 78 percent of ACh which disappeared on denervation were in the splenic nerve terminations, and that the amount of ACh normally present in the nerve terminations was as much as 40 percent of the amount of norepinephrine. If the cholinergic fibers are separate from the adrenergic fibers, they should be numerous and it would be expected that the electron microscope would reveal nerve endings of two kinds, namely: (*a*) endings containing vesicles in which ACh is held; and in addition, (*b*) other endings containing only granular vesicles of the kind in which norepinephrine is held.

But the results have been different. Results obtained with the electron microscope have been in agreement with the hypothesis that there is only one kind of fiber. In the termination of each sympathetic postganglionic fiber, two kinds of vesicles have been found. One kind contains a dense granule, and the other kind is an empty or homogenous vesicle. This was the finding in the splenic nerve of the rat (35). Similar observations have been made in the vas deferens of the rat. After treatment of the rat with reserpine, the granular vesicles disappeared. Autoradiography, combined with electron microscopy, demonstrated that the granular vesicles stored exogenous [^{3}H] norepinephrine. The vesicles without granules were found to be closely similar to the synaptic vesicles of central nervous system synapses and motor endplates (69). Similar observations were made in the adrenergic nerve endings of the pineal gland of the rat, where 60 to 70 percent of the vesicles were without granules and were homogenous, while 30 to 40 percent contained granules. The proportion of granulated vesicles diminished between 2 to 48 hr after a single injection of reserpine (61). It is important to note that similar observations have been made in the nictitating membrane of the cat (2), and an investigation has also been made in the anterior hypothalamus of the rat which had "two well-defined populations of vesicles, one of clear vesicles of 510 Å and another of granular vesicles with a diameter of 1300 Å." There was "practically no superposition between the two maxima, which are widely apart. This is in contrast to the interpretation that both types represent stages in the development of a single entity" (61a). Thus, in each situation where vesicles containing granules are found, it being agreed that the granules contain norepinephrine, there are also vesicles which contain no granules and which are similar to the vesicles in central synapses or in the terminations of the motor nerves. It is not yet known whether these vesicles contain ACh, but recent observations by Richardson (63) on the iris show that the sphincter muscle has nerve endings with agranular vesicles which

must certainly contain ACh, while the dilator muscle has endings which contain both agranular and granular vesicles.

Histochemical observations.—Histochemical investigations have given more puzzling results. Observations have been made by Koelle (53), by Holmstedt & Sjöqvist (48), and Fredericsson & Sjöqvist (42) on the amount of acetylcholinesterase in sympathetic ganglia. While some ganglia show the presence of the enzyme quite clearly, others show an intermediate amount, and the majority show none. Holmstedt & Sjöqvist have been able to identify the cells in the stellate ganglion of the cat which supply the sweat glands; but apart from these cells, they find no others which stain by the thiocholine method. Thus, they find no evidence of cholinergic fibers in the nervi accelerantes which go to the heart. Folkow et al. (41) showed quite conclusively that stimulation of these fibers in the cat and the dog caused liberation of ACh when the heart was perfused. In support of this result was the finding of Day & Rand (34), that when the postganglionic fibers to the isolated atria of the cat were stimulated, the acceleration produced by the stimulation was slowly abolished when guanethidine was added to the bath, and then stimulation caused inhibition. Boura & Green (10) made similar observations in the cat under chloralose, using bretylium. These observations show that fibers arising from cells in the stellate ganglion of the cat, which do not stain for acetylcholinesterase, liberate ACh when stimulated.

Koelle (53) has been impressed by the difference between the rabbit and the monkey on the one hand, the sympathetic ganglia of which stain for acetylcholinesterase, and the cat on the other hand, the sympathetic ganglia of which do not stain. He is therefore inclined to the view that in the cat the impulses passing along postganglionic fibers release norepinephrine directly. However, Brandon & Rand (13) produced conclusive evidence that stimulation of the splenic nerves releases ACh, and the evidence already given that stimulation of the postganglionic fibers to the nictitating membrane of the cat releases ACh is certainly strong.

Further evidence that staining by the thiocholine method cannot be used to prove the absence of cholinesterase is given by Gardiner, Hellmann & Thompson (44) who were unable to detect, by this method, the presence of cholinesterase in the nictitating membrane of the cat. Burn & Philpot (25) used the manometric method and examined 20 cats. They used ACh, acetyl-β-methylcholine, and butyrylcholine as substrates. They showed that the amount of cholinesterase in the two membranes was similar in any one cat, but varied considerably in different cats. They demonstrated a fall in the amount of acetylcholinesterase, but not of butyrylcholinesterase, in the nictitating membrane after the degeneration of the postganglionic fibers. The extensive and consistent results by the manometric method showed that the thiocholine method cannot be used to prove the absence of cholinesterase and the failure to stain the sympathetic ganglia of the cat is no indication

that the postganglionic fibers do not include a cholingergic link in the transmission of the impulse.

Release of norepinephrine by ACh and other drugs.—As mentioned above, there is evidence that ACh and nicotine, when applied to isolated cardiac tissue in the presence of atropine, cause release of epineprhine-like substances. Lee & Shideman (55) observed that nicotine increased the force of contraction of the papillary muscle of the cat heart. This increase was not seen when the sympathetic fibers in the heart had degenerated and when the catecholamines had disappeared. They made interesting observations on the still-uninnervated 4-day-old embryonic chick heart. They found that both nicotine and ACh caused an increase in the amplitude, the increases being 25 and 18 percent, respectively. They demonstrated that these embryonic chick hearts contained catecholamines, the amount being 1.2 μg per 100 mg nitrogen. From these results, it seems that catecholamines can be released by nicotine from sites in the heart other than those in postganglionic terminations. Reserpine diminished the catecholamine content of these noninnervated hearts (54).

Action in vessels.—In the vessels of the perfused rabbit ear, the action of ACh in the presence of atropine and the action of nicotine are regularly constrictor. These vessels are skin vessels and their behaviour differs from that of the main vessels of the dog hindleg. When nicotine is injected into the iliac artery, sometimes vasoconstriction is seen; but this is not the rule, and nicotine usually causes vasodilatation.

Pilomotor muscles.—In pilomotor muscles of the cat, it has been usual to test the action of drugs by intracutaneous injection, and the effects obtained have already been described. Recently, Hellmann (46a) failed to observe the stimulant action of small amounts of ACh. He made a preparation of the skin by removing the whole skin of the tail as a cylinder which was inside out. He placed the skin in a bath and stimulated it by putting one electrode inside the skin and one outside. He recorded the longitudinal contraction of the cylinder of skin. He observed that the contraction caused by stimulation was blocked by bretylium and that it was diminished by adding nicotine or ACh to the bath. However, Brücke (16a) has made a similar preparation, but after taking off the skin he turned it right side out and then observed pilomotor effects. If ACh together with eserine was placed inside the skin, pilomotor stimulation was observed.

Relation of ACh to sympathetic action.—The action of ACh in the presence of atropine, in releasing norepinephrine from sympathetic fibers in different organs, furnished one of the reasons for the view that there was a cholinergic link in the adrenergic fiber. Ferry (40), however, pointed out that whereas hexamethonium blocked the action of ACh and of nicotine, it did not block the response to sympathetic stimulation; he therefore concluded that the action of ACh did not point to it playing such a role in sympathetic trans-

mission. In reply to this, it can be said that bretylium, which blocks the sympathetic fiber, also blocks the action of ACh in releasing norepinephrine. This has been shown in the isolated atria (50), in the perfused vessels of the rabbit ear (50), in the spleen (10, 13), and in the rabbit ileum (22). In all these organs, bretylium blocked the sympathetic impulse and also the action of ACh. It seemed likely that hexamethonium might prevent ACh from entering the sympathetic fiber and, that unless ACh entered the fiber, it could not act. The blocking agent, bretylium, is structurally similar to ACh in having a charged group like trimethylammonium; if hexamethonium, a bis-quarternary compound, prevented ACh from entering the fiber, it should also prevent bretylium from entering. That this occurs has been demonstrated; for, in the presence of hexamethonium, bretylium has no blocking action (22).

Ferry also observed that ACh, when injected into the splenic artery, caused antidromic impulses to pass along the C fibers of the splenic nerve. He concluded that ACh stimulated presynaptic terminals, and through these terminals caused liberation of norepinephrine. That ACh causes antidromic activity is known in other situations. Thus, Randić & Straughan (62) have found that ACh released from motor nerve endings exerts antidromic activity in the phrenic nerve. When the splenic nerves are stimulated, ACh is released (13), and it is therefore possible that this ACh can exert antidromic activity. In Ferry's paper (40), antidromic activity was recorded in one experiment on direct stimulation of the splenic nerves. Thus, the production of antidromic impulses, when ACh is injected into the splenic artery, may have no more and no less significance than antidromic activity set up by stimulation of motor nerves. It does not modify the evidence that ACh is the transmitter at motor nerve terminations. If ACh is injected into the adrenal artery, it might cause antidromic impulses to pass along the C fibers of splanchnic nerves; but ACh is none the less the transmitter at splanchnic terminations.

Sympathetic blocking agents.—ACh blocks the sympathetic postganglionic fibers to pilomotor muscles. Burn & Rand (28) observed that ACh, when added to the fluid perfusing the vessels of the rabbit ear, blocked the constrictor effect of sympathetic stimulation, in a concentration of 2.5 μg per ml, though not that of norepinephrine.

The blocking agents which have been used clinically are bretylium and guanethidine, and their action provides a clue to the mechanism of norepinephrine release just as valuable as that provided by tubocurarine in explaining transmission at the neuromuscular junction. As already pointed out, bretylium is related structurally to ACh; it contains one quarternary nitrogen in the group ethyldimethylammonium, which is similar to the trimethylammonium group in ACh. Guanethidine is at first sight different in structure, but it too has a group, the guanidine group, which carries a charge just as does trimethylammonium, and guanethidine is highly ionized in solution.

It is, however, in their properties that guanethidine and bretylium re-

semble ACh and nicotine most closely. ACh stimulates the release of norepinephrine in smaller doses, but blocks sympathetic stimulation in high doses. Bretylium blocks sympathetic stimulation in low doses, but stimulates the release of norepinephrine in high doses. Thus, the difference between ACh on the one hand and bretylium and guanethidine on the other is quantitative rather than qualitative.

This is reinforced by the evidence that bretylium and guanethidine act at other sites where ACh is recognized to be the transmitter. Thus, both substances block the phrenic nerve diaphragm preparation as tubocurarine does [Dixit, Gulati & Gokhale (36)]. Both substances block the sympathetic ganglion as was shown by Boura & Green (10) for bretylium and by Maxwell et al. (57) for guanethidine.

To test the view that bretylium and guanethidine were acting in the postganglionic fiber as substances which blocked ACh, Burn & Froede (19) chose a substance known only to block the neuromuscular junction in skeletal muscle. This was phenyltrimethylammonium. They found, on examination, that this substance also blocked the sympathetic postganglionic fiber and, like bretylium, it effected a block of stimulation at high frequency first and at low frequency last. Such a block is characteristic of the blocking action of hexamethonium at the sympathetic ganglion and of the blocking action of tubocurarine at the neuromuscular junction. Thus, both the actual structure of bretylium and the other properties of bretylium and guanethidine suggest that their action at the sympathetic postganglionic termination is to block ACh. It is of course of some significance that no one has put forward any explanation whatever of how substances like bretylium, guanethidine, and phenyltrimethylammonium can effect the blockade of a sympathetic impulse if it directly releases norepinephrine.

The action of pempidine.—Recently, a paper appeared by Gokhale, Gulati & Joshi (46) in which they said that the pressor action of physostigmine in the rat was abolished by bretylium, but not completely abolished by hexamethonium. However, they found that mecamylamine caused complete block. Now mecamylamine is a ganglion-blocking agent like hexamethonium; however, they differ in that mecamylamine is a secondary amine, while hexamethonium is a bis-quarternary compound. Thus, mecamylamine enters cells with relative ease, while hexamethonium enters with great difficulty. The results described suggested that mecamylamine could act like bretylium, and that on examination mecamylamine might prove to be a sympathetic blocking agent as well as a ganglion blocking agent. Burn & Gibbons (22) showed that this was so and that pempidine, which is a tertiary amine similar in action to mecamylamine, as a ganglion blocking agent, was a substance also capable of blocking the sympathetic postganglionic fiber. Burn & Ng (24a) have further found that the action of pempidine in blocking the sympathetic postganglionic fiber is exerted on stimulation of the highest frequency first and then on stimulation of lower frequencies in due progression. Thus,

the action of pempidine is not that of a local anesthetic. Here, therefore, is a well known ganglion-blocking agent, which acts by blocking ACh, causing block of the sympathetic postganglionic fibers. Blackman & Ray (6) have shown that pempidine blocks the neuromuscular junction in skeletal muscle.

Thus, the blocking agents afford as much support for the presence of a cholinergic step in transmission in the sympathetic fiber as the action of tubocurarine does at the neuromuscular junction, and as the action of hexamethonium does at the sympathetic ganglion. Thompson (66) considered that there was no evidence of a cholinergic link in the nerve supplying the isolated nictitating membrane; Bevan & Su (5) thought the same about their preparation of the isolated pulmonary artery. In both cases, nerve stimulation was blocked by bretylium, and this itself was strong evidence for the cholinergic link. Hemicholinium and physostigmine appear to have little or no action in these preparations, perhaps because they lack the higher penetrating power of bretylium. They cannot reach the site of action easily when added to the bathing fluid.

Action of anticholinesterases.—According to the hypothesis that ACh acts as an intermediate in the release of norepinephrine, substances which have an anticholinesterase action should increase the amount of norepinephrine released. The first observations showing this were made in the perfused vessels of the rabbit ear, when it was demonstrated that the vasoconstriction caused by stimulation of the postganglionic fibers was increased by 40 percent when physostigmine (5×10^{-6}) was added to the perfusion fluid. A similar result was obtained when the stimulation was applied to the mixed nerve at the base of the ear. An increase in the vasoconstriction was recorded when physostigmine (2×10^{-6}) was present in the Locke's solution for 15 min. However, the number of experiments made was small [Burn & Rand (28)].

Observations on the nictitating membrane.—In the nictitating membrane, both norepinephrine and ACh cause contraction; and to discover whether postganglionic stimulation released norepinephrine through the action of ACh, it was necessary to exclude the direct action of ACh on the membrane. This was done by injecting atropine (1 mg per kg) or hyoscine (0.2 mg per kg). Hyoscine has been shown by Thompson (66) to be more potent and more specific. Burn, Rand & Wien (29) observed that in these circumstances, the effect of postganglionic stimulation was increased when physostigmine (0.5 mg per kg) was injected intravenously. The response to norepinephrine did not change.

In experiments in which supramaximal stimuli were used, it was shown that the increase caused either by physostigmine or by neostigmine (0.5 mg per kg) was greatest for stimulation of low frequency, and diminished as the frequency rose. The observations were consistent with the explanation that when a low frequency of 0.5 per sec was used, there was normally time for cholinesterase to destroy ACh between each pulse, and therefore the concentration of ACh could not rise very much; however, at a high frequency of

20 per sec, there was much less time for this destruction. Thus, a rise in frequency was equivalent in its effect to an inhibition of cholinesterase. Therefore, when physostigmine or neostigmine was added, it exerted the greatest effect at low frequency and the effect was progressively less as the frequency rose. The results thus afforded direct evidence that postganglionic stimulation first released ACh, and that this in turn released norepinephrine. Further work on the nictitating membrane has recently been published by Bowman, Callingham & Cuthbert (11). They used pentobarbital, as well as chloralose as anesthetic and, in preparing the postganglionic fibers for stimulation, crushed the ganglion or removed it completely. They cut the external ocular muscles and removed the eyeball. They recorded isometric contractions of the nictitating membrane. The nictitating membrane is a highly sensitive organ which can be used for quantitative observations (23), but it requires the presence of the eyeball for proper function, and it normally contracts isotonically. Stimulation was applied to the postganglionic fibers at rates of 1 per min, and responses to single shocks were also recorded. In a first series of 17 experiments, they observed that physostigmine increased the duration of the contraction in eight instances. In a second series, in which the preparation had been set up for 2.5 hr, so that the authors spoke of the preparation as fatigued, but where the conditions may rather have become stabilized, they observed that in 10 out of 15 experiments, physostigmine caused an increase in the tension developed on stimulation.

The most consistent effect of physostigmine was an increase in the duration of the contraction which was observed in 31 out of 45 experiments, and was the same whether the stimulation was pre- or post-ganglionic, whether the preparation was chronically decentralized or not, and whether atropine was injected previously or not. Such an increased duration is consistent with the idea that prolongation of the period before ACh was destroyed would lead to a release of norepinephrine for a longer period. The results of this investigation substantiate the existence of the cholinergic link.

The dog hindleg.—Bernard & De Schaepdryver (4) have made experiments in which they have stimulated the sympathetic trunk running to the dog's hindleg, measuring the flow through the femoral artery. The dogs were given morphine and were anesthetized with pentobarbital; atropine was injected (1 mg per kg). They used stimulation of low frequency from 0.1 to 3.0 per sec and determined the reduction in the rate of flow. They found that in all experiments, of which they performed 14, the injection of physostigmine, or neostigmine, or sarin (50 μg per kg) caused increased vasoconstriction, the increase being greatest at the lowest frequency. While the authors agreed that their results were compatible with the hypothesis of a cholinergic link, they considered that they might be explained in part by the stimulation of preganglionic fibers, and that eserine, neostigmine, and sarin might act in part at ganglionic sites. Further, they considered that the vasoconstriction might be due to norepinephrine coming from chromaffin cells in

the skin, and that fibers innervating such cells would be preganglionic, so that the transmission of impulses to the cells would be facilitated by anticholinesterases.

While it may be true that some of the fibers they were stimulating were preganglionic, there is very little evidence that anticholinesterases increase transmission through ganglia in the body, as Bowman et al. found in their experiments on the nictitating membrane; preganglionic stimulation was not different from postganglionic stimulation. Zaimis (70) has stated that the effects of anticholinesterases on ganglionic transmission are difficult to demonstrate when contractions of the nictitating membrane are used as an index. This is likely to be true generally, since the superior cervical ganglion is a typical ganglion. The increase in vasoconstriction due to eserine, which was observed by Bernard & De Schaepdryver (4), was large. Thus, at a rate of 0.1 shock per sec, the vasoconstriction was increased immediately after eserine, by 45 percent, and 90 min after eserine, by 90 percent. At the rate of 3 shocks per sec, the corresponding figures were 16 percent and 27 percent.

With regard to the other suggestion of Bernard & de Schaepdryver, that their results might be explained by an action of anticholinesterases on a junction between sympathetic fibers and chromaffin cells in the skin, it can be said that there is no good evidence of the presence of chromaffin cells in the skin [Coupland & Heath (33)] and no evidence that chromaffin cells are innervated by sympathetic fibers (Muscholl & Vogt (59)). Thus, the evidence of Bernard & de Schaepdryver seems on the whole to be in favour of the cholinergic link.

The anti-epinephrine substances.—Recent work has attracted attention to anti-epinephrine substances, as having an anticholinesterase action. Rothlin (64) showed that ergotamine increased vagal action on the heart and the depressor action of ACh. Loewi & Navratil (56) said that ergotamine acted like physostigmine in their experiments on the heart of the toad. Ergotamine prolonged the effect of vagus stimulation and that of ACh; moreover, it inhibited the enzyme in the toad heart which destroyed ACh. More recently, Rand et al. (12) observed that when they recorded the contractions of the isolated guinea pig vas deferens in response to stimulation of the hypogastric nerve, the effect of stimulation was increased when either physostigmine, tolazoline, yohimbine, piperoxan, ergotamine, or phenoxybenzamine was added to the bath. They suggested that these anti-epinephrine substances acted as anticholinesterases in producing this effect, and showed that these anti-epinephrine compounds inhibited the destruction of ACh by the cholinesterase present in the vas deferens. Recently, Hobbiger (see 20) has shown by manometric observations, using guinea pig brain and intestine as sources of cholinesterase, that both tolazoline and phenoxybenzamine have anticholinesterase activity.

These observations are of interest in relation to the work of Brown and his colleagues (7, 8, 9, 14) who studied the amount of norepinephrine liber-

ated in the splenic vein when the splenic nerves were stimulated in the cat. When stimulation was applied at a frequency of 10 per sec, the amount of norepinephrine which appeared in the vein was small; but when it was applied at 30 per sec, the amount was five times as great. However, when an anti-epinephrine substance like phenoxybenzamine or Hydergine (mixed methane sulfonates, of dihydroergocornine, dihydroergocristine, and dihydroergokryptine) was present, stimulation at 10 per sec caused an eight times larger amount of norepinephrine to appear in the vein, though it did not greatly increase the amount liberated by stimulation at 30 per sec. They considered that the norepinephrine released by stimulation at low frequency was either destroyed by the receptors or taken up again by the nerves in the spleen and therefore did not appear in the vein. In the presence of the anti-epinephrine substance, all the norepinephrine liberated by the nerve appeared in the vein, uptake being blocked.

This explanation took no account of the observations that anti-epinephrine substances have been shown not only to diminish the action of epinephrine, but to increase the effect of sympathetic stimulation. Jang (51) showed this for piperoxan in the rabbit ear vessels and for ergotoxine and yohimbine in the nictitating membrane. Varagić (67) showed it for tolazoline in the rabbit uterus, and Kirpekar & Cervoni (52) for phenoxybenzamine in the rabbit colon. The increase in the effect of sympathetic stimulation described by Jang was accompanied, in each case, by a diminution in the response to epinephrine. This diminution made the possibility of explaining the increased response to stimulation as due to block of uptake unlikely.

Another approach to this problem has also been made. Burn & Weetman (30) used the guinea pig vas deferens and showed that the effect of physostigmine and of neostigmine on the response to stimulation of the hypogastric nerve was the same as their effect on the phrenic nerve diaphragm. These anticholinesterases increased the response to stimulation of low frequency and decreased the response to stimulation of high frequency, the decrease being due to a depolarization block produced by excess of ACh. Burn & Gibbons (20) showed that phenoxybenzamine had exactly the same effect as physostigmine and neostigmine in the guinea pig vas deferens. They also used the isolated ileum of the rabbit in which stimulation of the peri-arterial nerves in the mesentery causes inhibition. These nerves are postganglionic sympathetic fibers and the inhibition they cause is not affected by hexamethonium. In this preparation, it was observed that, as in the vas deferens, the presence of either phenoxybenzamine or tolazoline increased the response to sympathetic stimulation of low frequency and decreased the response to sympathetic stimulation of high frequency. Burn & Ng (24a) observed the same double action with Hydergine both in the vas deferens and in the rabbit ileum. An effect on response which differs in direction according to frequency cannot be explained in terms of block of uptake, for an effect depending on block of uptake would be changed in the same direction at all frequencies.

Hydergine is an anticholinesterase like phenoxybenzamine, for it was shown to increase the response of the toad rectus abdominis muscle to ACh; and since the effects of phenoxybenzamine and of Hydergine on the response to stimulation are characteristic of the effects of anticholinesterases at the neuromuscular junction in skeletal muscle, it is clear that these effects point to the existence of the cholinergic link in the sympathetic postganglionic pathway. Even the observations on the output of norepinephrine in the splenic vein made by Brown & Gillespie (14) are, in part, to be explained in this way; in that paper, while most observations were made at low and moderate frequencies, some were also made at high frequency. The output of norepinephrine was modified by phenoxybenzamine in the same way as was the physiological response to sympathetic stimulation. It was increased by low frequency, changed but little at moderate frequency, and decreased at high frequency. From this, it may be concluded that the observations concerning the effect of phenoxybenzamine on the output of norepinephrine in the vein are, in part, explained as the effect of phenoxybenzamine on the amount of norepinephrine liberated by the splenic nerves.

The question of uptake.—The effects of phenoxybenzamine have recently been interpreted by Blakeley, Brown & Geffen (9) as due simply to block of uptake. The uptake of norepinephrine has recently been measured by Gillespie & Kirpekar (44a) who infused norepinephrine into the spleen at a rate of 0.625 μg per min for a period of 20 min. They found that of the norepinephrine infused, 70 percent was retained in the spleen. In the decentralized spleen, the amount retained was less, being only 55 percent. In the presence of phenoxybenzamine or of cocaine, the amount retained in the spleen was very small, being only 16 to 20 percent. The action of cocaine was exactly the same as that of phenoxybenzamine. Both of these substances block uptake to the same extent.

However, Blakeley, Brown & Ferry (8) found that whereas phenoxybenzamine increased the output in the splenic vein from 200 pg per stimulus to 1710 pg per stimulus, following stimulation at 10 per sec, cocaine did not increase it at all. Kirpekar & Cervoni (52) did exactly similar experiments. They found that cocaine had a slight effect on the amount of norepinephrine in the splenic vein, increasing it from 230 to 370 pg per stimulus, which was observed in 16 out of 19 experiments. But this effect was much less than the effect of phenoxybenzamine which increased it from 230 to 860 pg per stimulus. Therefore, the action of phenoxybenzamine cannot be explained as due to block of uptake alone, since cocaine, which blocks uptake equally, has so much less effect.

In the decentralized spleen, Gillespie & Kirpekar (44a) have found that the uptake of norepinephrine is less. Instead of an uptake of 70 percent, they observed an uptake of 55 percent. Therefore, the effect of blocking uptake in the decentralized spleen should be less than its effect in the normal spleen. But Brown, Davies & Ferry (15) found that the effect of phenoxybenzamine

in the decentralized spleen was to cause a greater rise in the output in the splenic vein from 200 pg per stimulus to 3060 pg per stimulus. This evidence also shows that the effect of phenoxybenzamine is not only block of uptake.

Blakeley, Brown & Geffen (9) used the isolated spleen perfused with blood and stimulated the nerves with successive trains of 200 stimuli at frequencies of 30 per sec at intervals of 10 min. They found that the amount of norepinephrine released with each stimulation remained approximately the same, though falling during the course of 8 stimulations to about 75 percent of the initial value. They supposed that part of the norepinephrine released by the nerve was taken up again by the nerve endings, so that these were kept well loaded with transmitter. The addition of phenoxybenzamine to the blood first increased the output of norepinephrine in the vein considerably; but as further trains of stimuli were applied, the output fell steadily to zero. This fall was apparently thought to be due to the gradual exhaustion of the supply of transmitter in the nerve endings because there was no uptake. But they said that the amount of norepinephrine present in the spleen was not reduced. Therefore, the steady diminution in the amount of liberated norepinephrine must have had a different explantion. We suggest that phenoxybenzamine acted as an anticholinesterase, first raising the amount of norepinephrine released, and then diminishing the amount by a steadily increasing block.

Hydergine, like phenoxybenzamine, is an anticholinesterase; it potentiates the action of ACh on the toad rectus in a concentration of the same order as that of physostigmine. The anti-epinephrine compounds may prove to have more anticholinesterase action at sympathetic endings than physostigmine and neostigmine. Thus, Varagič (67) found that tolazoline regularly increased the response of the rabbit uterus to stimulation of the hypogastric nerve, while physostigmine did so only in 2 out of 14 experiments. Such a difference would explain the observation of Blakeley, Brown & Ferry (8) that, whereas the injection of eserine did not increase the output of norepinephrine in the splenic vein, the subsequent injection of Hydergine did so. What is needed is a quantitative comparison of the anticholinesterase action of physostigmine, neostigmine, and several of the anti-epinephrine compounds, made in several isolated organs.

The action of hemicholinium.—Hemicholinium, and more specifically the compound HC3 (65), has been shown to block the neuromuscular junction and also the sympathetic ganglion. It interferes with the synthesis of ACh. If impulses come down the nerve at a slow rate, so that not much ACh is liberated in a unit of time, HC3 has little or no effect. But if impulses come quickly, then HC3, by interfering with the transport of choline, causes a gradual failure of transmission.

The compound HC3 has another action which resembles that of hexamethonium. Thus, when the isolated rabbit ileum is inhibited by stimulation of the periarterial nerves in the mesentery, the inhibition is blocked by

bretylium; the blocking action of bretylium is, however, not seen if either hexamethonium or HC3 is present in the bath in a concentration of 2×10^{-4} g per ml (22). Thus, HC3 not only interferes with ACh synthesis, but also has what appears to be a ganglion-blocking action, exerted only in high concentration. HC3, like hexamethonium, is a bis-quaternary compound; it has been shown to lead to a failure of transmission at the sympathetic postganglionic terminal, also. Chang & Rand (31) demonstrated this failure in the guinea pig vas deferens, using a concentration as low as 20 μg per ml; they showed that it did not occur at low frequencies of stimulation, but only at higher frequencies. The failure was reversed by choline. Bentley (3) obtained an immediate failure of response in this preparation, when he used a concentration of 100 μg per ml, and restored the response by adding norepinephrine to the bath. This concentration of HC3 was almost certainly acting like hexamethonium and was not the specific action of HC3. Chang & Rand showed that HC3 blocked the effect of sympathetic stimulation on the vessels of the rabbit ear, and on the isolated atria of the cat. Brandon & Rand showed a block in the spleen (13).

The compound HC3 does not always block sympathetic postganglionic terminations, just as it does not always block the neuromuscular junction in skeletal muscle. HC3 has a structure which makes its entry into cells difficult, and it is not surprising that in the isolated nictitating membrane (66) and in the isolated pulmonary artery (5) the response to sympathetic stimulation is unaffected. HC3 does not penetrate. However, in both these preparations, bretylium, which has a high power of penetration, blocks the sympathetic impulse. As we have argued above, block by bretylium is itself evidence of the presence of the cholinergic link.

Botulinum toxin.—Botulinum toxin prevents the release of ACh from cholinergic nerves (18). Ambache (1) examined its action on the nictitating membrane and found that it always reduced the response to sympathetic stimulation. In one of his figures, it was reduced by 75 percent, though the average reduction was 51 percent. He made his injections of botulinum toxin into the membrane itself; and by such a route, it would be impossible to ensure that all postganglionic fibers were affected. Recently, Whaler (68) has found that botulinum toxin, when injected into the skin of the cat's tail at the base of a tuft of hair, prevents sympathetic stimulation from causing erection of that tuft. He also found that botulinum toxin, added to the isolated organ bath, blocks both the effect of sympathetic stimulation in causing inhibition of the rabbit ileum and also the effect of hypogastric stimulation in causing contraction of the vas deferens. The evidence in the rabbit ileum showed complete block of sympathetic action in 19 out of 22 experiments. The results in the pilomotor muscles were also clear. Thus the action of botulinum toxin indicates the presence of the cholinergic link.

The cholinergic link and calcium.—On the assumption that the sympathetic

impulse first releases ACh, and this in turn releases norepinephrine, it is necessary to consider how this second step is effected. Recent work by Douglas & Rubin (37) has shown that ACh releases catecholamines from the adrenal medulla through the agency of calcium. When the adrenal gland is perfused with Locke's solution, the injection of ACh into the artery leading to the gland has no effect if calcium is absent; and when calcium is present, the amount of catecholamines released increases with the calcium concentration in the perfusion fluid. No other cation is important, and ACh still releases catecholamines when the perfusion fluid contains only dextrose, sucrose, and calcium. Burn & Gibbons (21) have shown that the situation is similar at the sympathetic postganglionic termination. The inhibition produced in the rabbit ileum by stimulating the peri-arterial nerves in the mesentery is proportional to the concentration of calcium present in the bath. They were also able to show that nicotine produced an inhibition when hyoscine was present, and that the magnitude of the inhibition was increased as the calcium concentration increased. They obtained a little evidence that ACh also caused inhibition which depended on the concentration of calcium.

Thus, it would appear that if the sympathetic impulse releases ACh, the effect of the ACh is to permit the entry of calcium into the terminal part of the nerve ending; the calcium then releases norepinephrine from the granules which are present within vesicles. Thus, ACh may act by altering the permeability of the fiber membrane to calcium. We do not know whether ACh first passes out of the membrane and then acts on the outside, or whether it acts on the inside.

Summary and conclusions.—Perhaps the most convincing part of the evidence in favour of the cholinergic link comes from the action of blocking agents like bretylium and guanethidine. These are considered together with the action of agents which interfere with the synthesis and release of ACh such as HC3 and botulinum toxin, especially the latter. It is surely significant that bretylium and guanethidine both have the property of blocking ACh at the neuromuscular junction and at the sympathetic ganglion. It is surely also significant that a ganglion-blocking agent like pempidine, which is a tertiary amine, but not one like hexamethonium, which is a bis-quaternary compound, can also block the sympathetic postganglionic fiber.

However, it is conceivable to some people that the blocking action of these substances is some general kind of depolarizing action, and therefore does not show the existence of a cholinergic link. But when, side by side with this evidence, there is the evidence that botulinum toxin blocks the sympathetic postganglionic fiber, the situation seems to be altered. Botulinum toxin was shown by Burgen, Dickens & Zatman (18) to prevent the release of ACh, and its action is considered to be characteristic of cholinergic nerves. This combination of evidence seem very difficult to refute.

The evidence from anticholinesterases in vessels and in the nictitating membrane is good, but more such evidence is needed, since it is not confirmed by some and is interpreted differently by others.

The evidence from the electron microscope is fully consistent with the hypothesis, if it does not prove it. But this evidence seems to be against any idea that the sympathetic impulse directly releases norepinephrine.[3]

[3] *Note added in proof:* Kevin K. F. Ng and M. J. Rand have recently used a preparation of the same taenia coli with the attached mesentery in which they stimulated the periarterial nerves. They made observations in the presence of hyoscine, stimulating at different frequencies and observing inhibition. They found that when physostigmine was added, the response to low frequencies (10 per sec) was greatly increased, while that to 20 per sec was increased very little. These observations are the counterpart of those on the nictitating membrane by Burn, Rand & Wien (29).

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