

RESUME OF LITERATURE

Chemical mediators in autonomic nervous system

The concept of neurohumoral transmission holds that nerve impulses elicit responses in smooth muscle, cardiac muscle, skeletal muscle, exocrine glands and post-synaptic neurones through the liberation of specific chemical substances.

Dubois-Reymond (1877) provided the first reference to the possibility of involvement of chemical substances in transmission, that is the transmission of nerve impulses may be produced either electrically or chemically by exciting substances such as ammonia or lactic acid formed at the surface of nerve endings.

Transmitter at cholinergic nerve ending:

Dixon (1907) reported the similarity between the effect of para-sympathetic nerve stimulation and that of alkaloid muscarine. This led him to suggest that stimulation of vagus nerve releases a muscarine-like substance which acts as a chemical transmitter. Hunt in the same year, announced his study on acetylcholine (ACh) and other choline esters.

Dale (1914) reinvestigated the pharmacological properties of ACh; he made the most interesting observation that ACh reproduced the responses to stimulation of parasympathetic nerves, and he introduced the term "parasympathomimetic" to characterize its effects.

Loewi (1921) established the first proof of chemical mediation of nerve impulses by peripheral release of specific chemical agents. He published a simple but convincing demonstration. He stimulated the vagus nerve of a perfused (donor) frog heart and allowed the perfusion fluid to come in contact with the second (recipient) frog heart which was used as a test object. A substance was liberated from the first heart (donor) which slowed the second heart (recipient). Loewi referred to this substance as "Vagusstoff" (vagus substance, parasympathin). Subsequently Loewi and Navratil (1926) presented evidence for its identification as ACh. Loewi also discovered that an accelerator substance similar to adrenaline was liberated into perfusion fluid when the action of sympathetic fibres in frog's vagus nerve predominated over

that of inhibitory fibres. Rylent (1927) duplicated Loewi's observation by using perfused rabbits' hearts. Feldberg and Krayner (1933) extended the work by using intact cats and dogs. They stimulated the cardiac vagus and were able to detect the liberation of the vagus substance by its effects on leech muscle treated with physostigmine and on the blood pressure of assay cats.

Many other investigations established quite conclusively that a chemical mediator had been instrumental in the transmission of excitation not only to the heart but also to other parasympathetically innervated structures. The experiments of Dale and Feldberg (1934) demonstrated the presence of the vagus-substance in the eserinizied perfusion fluid collected from the stomach of a dog whose vagi had been stimulated. Similar experiments proved the chemical transmission of parasympathetic nerve impulses to the small intestine (Bunting; et al , 1935) and to salivary glands (Babkin et al, 1932).

Transmitter at sympathetic nerve ending

Lewandowsky (1898) and Langley (1901) noted independently the similarity between the effect of injection of extract of

adrenal gland and stimulation of sympathetic nerves. Few years later, Elliot (1904) suggested that the impulses in sympathetic nerves release adrenaline-like substance as chemical transmitter at the sympathetic neuro-effector junction. He also noted that after degeneration of sympathetic nerve, effector organ still responded characteristically to the hormone of adrenal medulla. This suggestion was generally accepted with the reservation that the transmitter might not be adrenaline itself but something closely related to it.

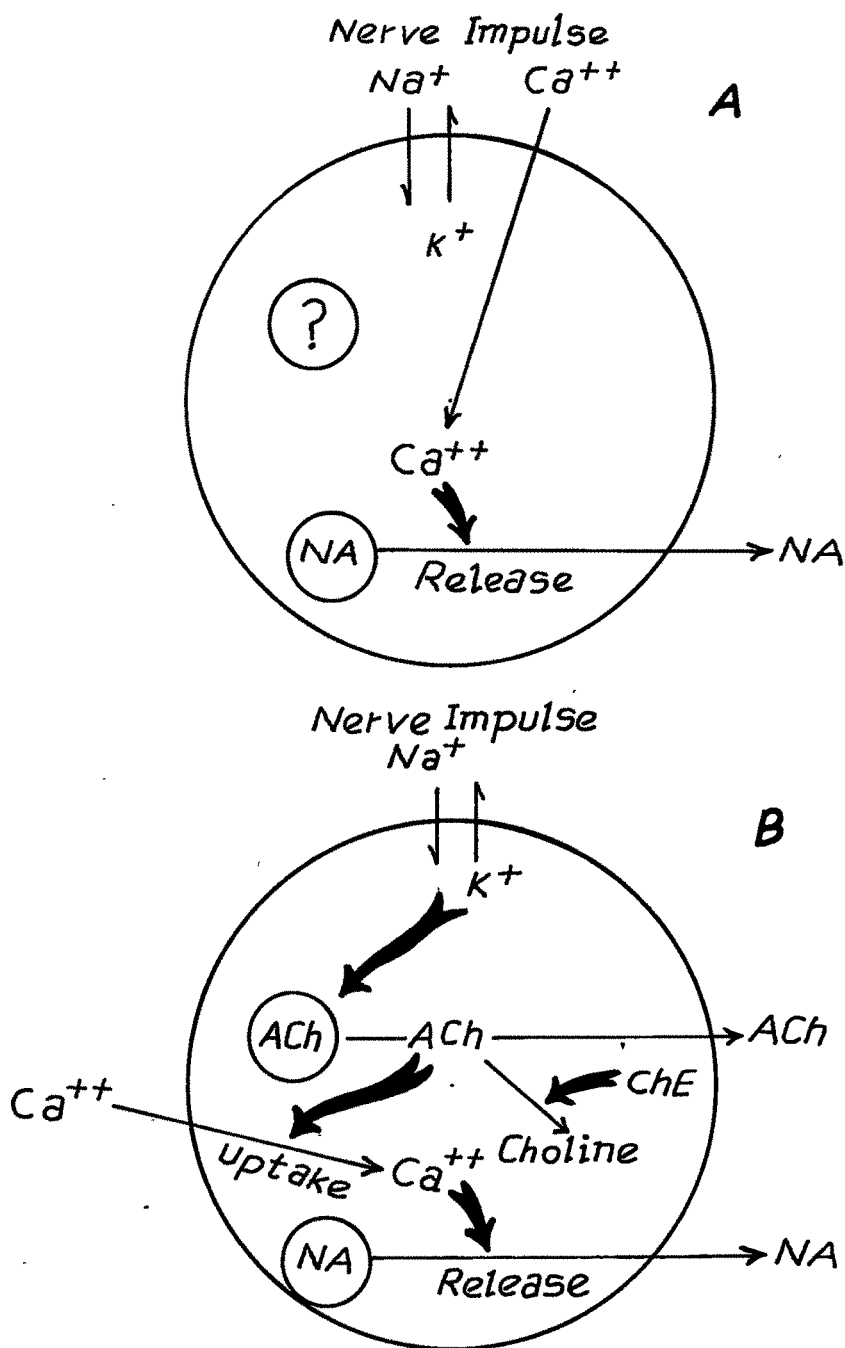
Cannon and Uridil (1921) reported that the stimulation of sympathetic hepatic nerves released an adrenaline-like substance that increased blood pressure and heart rate but failed to dilate the pupil. With subsequent experiments, Cannon and his co-workers called this substance liberated by sympathetic nerve impulse at neuro-effector junctions as "sympathin". Bacq (1931, 1933) found that stimulation of the cervical sympathetic nerves caused the chemical mediator to appear in the aqueous humor of the eye. The mediator was liberated not only under experimental circumstances but also

physiologically in the intact animal; as during struggle and excitement; it was likewise released during shamrage in decorticate animals. Von Euler (1946) and Peart (1949) finally showed that the transmitter at postganglionic sympathetic neruo-effector junction for the most part was noradrenaline (NA). Since then the release of NA due to the stimulation of postganglionic sympathetic nerves is accepted as the "classical theory".

Burn and Rand (1959) proposed a modification of this theory. According to them ACh is released due to stimulation of postganglionic sympathetic nerves which in turn releases NA from its store in the nerve endings. Hence there are two accounts of the mechanisms of NA release, the cholinergic link hypothesis and the classical theory.

The cholinergic link hypothesis and the "classical theory" are very similar. Both state that the release of NA follows the inflow of calcium ions. The difference between them lies in the events between the action potential and the calcium inflow. In the cholinergic link hypothesis these events consist of the release and action of ACh (Fig. 1).

Fig. 1



EVENTS LEADING TO RELEASE OF NORADRENALINE FROM ADRENERGIC TERMINALS: A, ACCORDING TO "CLASSICAL" THEORY; & B, ACCORDING TO CHOLINERGIC LINK THEORY
 O, STORE OF NEUROHUMOR; NA, NORADRENALINE; ACh, ACETYLCHOLINE; ChE, ACETYLCHOLINESTERASE.
 ↑, FACILITATORY PROCESS; ↗, DIRECTION OF CHANGE.
 (AFTER C. B. FERRY, 1966).

Development of cholinergic link hypothesis

The hypothesis of cholinergic link in adrenergic neuro-effector transmission was developed in order to explain several discrepancies in the classical theory. Burn (1928) during perfusion experiments on the vessels of dog hindleg observed that the stimulation of postganglionic fibres of the lumbar sympathetic chain caused vasodilatation instead of vasoconstriction. This phenomenon was attributed to the anoxia from which the leg suffered during the preparation resulting in damage to the vasoconstrictor fibres without causing damage to the vasodilator fibres. This concept required one to suppose that store was rapidly depleted in the absence of oxygen and therefore, stimulation of postganglionic sympathetic lumbar chain caused vasodilatation due to the release of ACh. Bulbring and Burn (1935) from their experiments on stimulation of postganglionic lumbar sympathetic chain of cat and dog hindleg perfusion preparations, suggested that among the adrenergic fibres there was an admixture of cholinergic fibres. Brucke (1935) observed with pilomotor muscles of cat's tail that injection of small amount (5 μ g) of ACh into the skin caused piloerection, but a large dose of ACh (0.2 mg) caused a

transient erection and then abolished the response to sympathetic stimulation. Coon and Rothman (1940) confirmed this finding, and showed that nicotine had a similar action. These workers began by studying the effect of ACh in causing gooseflesh in human forearm and then observed that small amounts of ACh and nicotine caused erection of tufts of hairs on the cat's tail after intradermal injection. They observed that this action was unaffected by atropine. They also showed that the action of nicotine and ACh was absent after degeneration of the sympathetic nerves. Hoffmann et al. (1945) observed the effect of ACh and nicotine on isolated hearts of cat and rabbit in the presence of atropine. ACh caused an increase in the rate and force of contraction of heart which suggested the release of an adrenaline-like substance. Folkow et al. (1948) found that ACh was released by stimulation of the accelerator nerves of the heart. Later, they concluded that among adrenergic fibres there was an admixture of cholinergic fibres. It was never contemplated that ACh and the adrenergic transmitter might be released from the same fibre. However, it was not clear what the function of cholinergic fibres might be. Kottegoda (1953 a) described the

action of nicotine and of ACh in the presence of atropine as causing acceleration and increased contraction of isolated rabbit atria. In the same year Kottegoda (1953 b) described the action of nicotine and ACh in the presence of atropine causing vasoconstriction in the perfused vessels of rabbit ear, and also found that this vasoconstriction was reversed to vasodilatation when tolazoline was added to perfusion fluid. Since tolazoline reverses the constrictor action of NA and adrenaline in the rabbit ear vessels, he concluded that nicotine and ACh caused vasoconstriction by liberating an adrenaline-like substance. Thompson (1958) found that nicotine caused contraction of the isolated nictitating membrane of the cat. The stimulant action of nicotine on the smooth muscle of the cat's isolated nictitating membrane on subsequent analysis was shown to be an indirect one involving the release of NA from within the muscle. It was concluded that in this preparation, nicotine affects the release of NA by exciting the large number of fine postganglionic nerve fibres which course among the smooth muscle fibres before

innervating them. The possibility that the effect was due to stimulation of local ganglion cells was excluded by complete absence of these cells on histological examination. Murray and Thompson (1957), Burn and Rand (1958) and Burn et al. (1959) measured the effect of ACh and nicotine in relation to that of adrenaline on the pilomotor response and vessels of rabbit ear, before and after treatment with reserpine. They found that the action of ACh and nicotine were greatly reduced after treatment with reserpine.

These observations seemed to be consistent with the working hypothesis introduced by Burn and Rand (1959) that sympathetic postganglionic fibres which convey the impulses to the terminals, were cholinergic, and that ACh liberated by them, liberates in turn NA from the nearby store.

Hukovic (1959) observed that stimulation of sympathetic fibres to isolated rabbit atria caused acceleration which was converted to inhibition in atria obtained from animals treated with reserpine. In the presence of physostigmine

the inhibition was greater and it was absent in the presence of atropine. Hence the inhibition indicated the presence of cholinergic fibres in sympathetic supply. Burn and Rand (1960) made similar observation on the spleen of cats treated with reserpine. Stimulation of splenic nerve in the reserpine treated cat produced dilatation of the spleen. The dilatation was increased in the presence of physostigmine and was abolished by atropine. This observation was further strengthened by the work of Brandon and Rand (1961) who perfused the spleen of the cat treated with reserpine and allowed the fluid leaving the spleen to superfuse the isolated guinea pig ileum. When neostigmine was added to the perfusion fluid, it was found that stimulation of the sympathetic fibres was followed by contraction of the guinea pig ileum. Since this effect was abolished by atropine, it was evident that ACh had been liberated by the stimulation of splenic nerves. Leaders and Dayrit (1965) found that hemicholinium (HC-3) did not prevent the contractile response of dog spleen to sympathetic nerve stimulation. However, HC-3 did reduce the amount of

ACh released. This led them to suggest separate cholinergic and adrenergic fibres rather than a cholinergic junction in the adrenergic fibres.

Criteria applicable to evidence for cholinergic link

A number of criteria that must be fulfilled in order to establish a substance as a transmitter at a synapse were drawn up some years ago. The criteria that ought to be fulfilled to substantiate the cholinergic link hypothesis are similar, but differ in detail. If the release and action of ACh are intracellular events as Burn and Froede's experiments (1963) with blocking agents suggest, there would be no necessity to show its release into the perfusion fluid. However, it would be necessary to show that drugs affecting the cholinergic link gain access to it. In this section the following points are discussed: (i) presence of ACh in adrenergic nerve terminals, (ii) presence of choline acetylase in adrenergic nerve terminals, (iii) release of NA by ACh, (iv) block of adrenergic and cholinergic transmission by pharmacological agents and (v) facilitation by anticholinesterases of adrenergic transmission.

Presence of ACh in adrenergic nerve terminals. In order to establish the presence of a cholinergic link it is of paramount importance to demonstrate clearly that adrenergic nerve terminals contain ACh. The experiments that have been done have resulted in the demonstration of the presence of ACh in sympathetically innervated organs and its disappearance together with the NA, after degenerative section of the anatomically sympathetic nerves (Arnin^{by Grant}, et al. 1953; Brandon and Rand, 1961). The experiments on organs such as spleen and rabbit ear treated with drugs that deplete the stores of NA have shown that ACh was released when the anatomically sympathetic nerves were stimulated (Brandon and Rand, 1961; Burn and Rand, 1960; Holton and Rand, 1962). These experiments show that the anatomically sympathetic nerves contain cholinergic fibres. However, the composition of these nerves is uncertain; they do not consist of adrenergic fibres only; for, Kuntz and Jacobs (1955) found that sympathetic ganglion cells appeared along the length of anatomically postganglionic sympathetic nerves. Many organs are innervated by parasympathetic fibres in the anatomically

sympathetic nerves. It may be possible that some of the total ACh content of the organs is derived from preganglionic sympathetic fibres or parasympathetic fibres. It is so important to demonstrate unequivocally that the adrenergic fibres contain ACh that in the interpretation of experiments on the total ACh content of organs, it must be assumed that aberrant cholinergic fibres are present, and their contribution to the ACh content must be estimated.

Presence of choline acetylase in adrenergic nerve terminals.

The demonstration that nerve fibres contain choline acetylase is considered by Hebb (1961) to be important evidence favouring their cholinergic nature. Thus, in addition to showing that adrenergic fibres contain ACh, it must also be shown that they can synthesize this substance.

Choline acetylase cannot yet be detected histochemically, therefore, its presence must be deduced by measuring the ability of the adrenergic fibres to synthesize ACh. It is important to show that choline acetylase is present at the

adrenergic nerve terminals, the site of proposed cholinergic link. This means that the total organ ACh synthesizing ability must be measured and the location of the enzyme system in adrenergic neurons deduced by the exclusion of the presence of cholinergic neurons.

There is little information now available about the presence of choline acetylase in adrenergic nerves. Comline (1947) found that preparations of horse spleen synthesized ACh, as did the liver, heart, and placenta; the locus of this activity is unknown. Nordenfelt (1965) found that parasympathetic denervation caused a fall in the choline acetylase content of salivary glands to about 10% of normal; degeneration of the sympathetic as well as parasympathetic nerves caused no additional fall in the content of choline acetylase. Thus it seems that the sympathetic nerves to salivary glands contain no choline acetylase.

It has been suggested that choline acetylase migrates peripherally from the cell body to the nerve terminals. Therefore, the detection of choline acetylase in the cell

bodies would be important. The ACh synthesizing activity of the superior cervical ganglion of cat was investigated by Feldberg (1943) and by Banister and Scrase (1950). They found that the synthetic ability disappeared after degenerative section of the preganglionic fibres. Hebb and Waites (1956) measured the choline acetylase content by an improved technique with similar results. It appears that ganglion cells do not contain choline acetylase..

Release of NA by ACh. The cholinergic link hypothesis states that NA is released from adrenergic fibres as a consequence of the action of ACh. Provided that the extracellularly applied ACh can reach the cholinergic link, it ought to be shown that injected ACh releases NA by acting in a physiological way.

The sympathomimetic effect of ACh and nicotine have been described in rabbit heart (Folkow et al. 1948; Hukovic, 1959); rabbit ileum (Burn and Gibbons, 1964); rabbit colon (Gillespie and MacKenna, 1960); rabbit ear vessels (Kottagoda, 1953; Burn et al. 1959); pilomotor muscles of cat tail (Coon and Rothman, 1940; Burn et al. 1959, 1960); nictitating

membrane of cat (Burn et al. 1959; Thompson, 1958) and spleen (Daly and Scott, 1961; Brandon and Rand, 1961). In these sympathetically innervated organs, ACh and nicotine produced effects similar to the excitation of the adrenergic nerves. The effects depended on the functional integrity of adrenergic neuro-effector transmission, for they were prevented by degeneration of sympathetic supply, by reserpine, by bretylium, by adrenergic blocking agents and also by hexamethonium and other ganglion blocking agents. They were not affected by atropine. In some organs it has been shown that the injection of ACh or nicotine caused the appearance of catecholamines in the perfusate.

There is thus a great deal of evidence that ACh, nicotine and other ganglion stimulants cause the release of NA. In some cases release of catecholamines from chromaffin tissue might be implicated (Muscholl and Vogt, 1964), but the experiments showing loss of the sympathomimetic action of these drugs after degeneration of the sympathetic supply suggest an action on the adrenergic neurons. The sympathomimetic action of ACh suggests the participation of this

substance in adrenergic neuro-effector transmission. However, it has been known for some time that nicotine and ACh can excite mammalian sensory nerve fibres near their terminations. Sensory nerves to skin and to mesentery (Brown and Gray, 1948; Douglas and Gray, 1953), carotid sinus baroreceptor fibres (Diamond, 1955) and C fibres from cutaneous touch receptors (Douglas and Ritchie, 1960) were excited by ACh or nicotine. This excitant action could be prevented by hexamethonium or by curare. Armett and Ritchie (1960, 1961, 1963 a, b), have shown that ACh, nicotine and carbachol depolarized desheathed rabbit vagal C fibres trunks. The response could be prevented by physostigmine, high doses of ACh, hexamethonium, curare, large doses of atropine, local anaesthetics, and substances with tertiary and quaternary nitrogen. ACh acted by increasing the permeability of the membrane to sodium ions and divalent cations such as calcium. The possibility that sympathomimetic effect of ACh was due to the excitation of the adrenergic fibres was investigated and it was found that

the closearterial injection of ACh into the cat's spleen excited the intrasplenic portions of the C fibres of normal and decentralized splenic nerves. The centripetal discharge of nerve impulses was unaffected by atropine or hydergine, but was prevented by hexamethonium. Cabrera and Torrance (1964) have shown that ACh excited antidromic impulses in the sympathetic C fibres to the cat's skin. They stimulated preganglionically and recorded a centrifugal volley in the postganglionic C fibres and those sensory fibres of the saphenous nerve ascending with sympathetic nerve fibres. The closearterial injection of ACh into the skin provoked a centripetal burst of impulses that collided with the centrifugal volley and completely extinguished it. The centrifugal volley was abolished by hexamethonium, and this suggests that it was carried mainly in postganglionic fibres. ACh must have excited the adrenergic fibres to the skin in addition to the sensory fibres at the point near their terminations. Viveros (1964) stimulated preganglionic fibres in order to demonstrate activation of the postganglionic fibres. He found that the injection of ACh into the left atrial appendage

excited antidromic impulses in the majority of the post-ganglionic C fibres. Hence there is good evidence that the rapid closearterial injection of ACh into sympathetically innervated organs can excite impulses in the post-ganglionic fibres.

The question now arises, are all the sympathomimetic effects of ACh and nicotine due to the generation of impulses in the adrenergic C fibres ? It seems this may not be so, since the application of nicotine to the nictitating membrane in vitro causes a sympathomimetic effect, without any centripetal activity in the sympathetic post-ganglionic nerves (J.W.Thompson, personal communication to Ferry; 1966). Apparently there are two mechanisms involved in the sympathomimetic effect of ACh and nicotine, one involving impulse generation and the other without impulse generation. It is possible, however, that these two mechanisms may be basically identical. An important feature of the generation of impulses after the closearterial injection of ACh was the rapidity of the injection. A small amount of ACh

injected rapidly gave a greater discharge than a much larger dose injected slowly (Ferry, 1963). With a rapid injection of ACh into the circulation, the concentration of ACh at the nerve terminals might be rising rapidly. And if ACh acts by depolarizing the membrane, then the rapid depolarization might generate an impulse. In experiments on the sympathomimetic effects of ACh in vitro, with the addition of ACh to the bath fluid, its concentration near the nerve terminals would rise slowly and the nerve might accommodate to the slowly rising depolarization and impulse might not be generated. Thus it is possible that the release of NA by ACh and nicotine is due primarily to an action on the membrane of nerve terminals, thereby increasing the permeability to ions, leading to release of the adrenergic transmitter. Douglas and Rubin (1961, 1963) have shown that ACh, nicotine, tetramethylammonium and dimethyl phenylpiperazinium (DMPP) evoked the release of catecholamines from the chromaffin cells of adrenal medulla by promoting the uptake of calcium. Since in calcium free perfusion fluid ACh and nicotine fail to evoke the release of

catecholamines, Burn and Gibbons (1964) made similar studies on the postganglionic sympathetic nerve terminal. They stimulated periarterial nerve to rabbit ileum and observed that inhibitory responses were proportional to the calcium concentration present in the organ bath. Similarly they also observed that the sympathomimetic effects of nicotine was proportional to the calcium concentration in the bath. Nicotine or ACh appear to increase the entry of calcium ions in the nerve ending, leading to the release of NA from the granules.

Blocking action of ACh and nicotine. If ACh is necessary for the release of NA from postganglionic sympathetic nerve endings, then it presumably acts on receptors in doing so. Hence it should be possible to block these receptors by an excess of ACh. Earlier Brucke (1935), showed that small doses of ACh produced piloerection in the cat's tail, but a large dose of ACh caused a transient erection and then abolished the response to sympathetic stimulation. Coon and Rothman (1940) confirmed these results. Burn and Rand (1960) found

that 0.2 mg of nicotine or of ACh given intradermally at the base of the tuft of hair on cat's tail, blocked the effect of sympathetic stimulation. However, Hellmann (1963) found that ACh in greater than 0.4 μ g depressed the contractile response to transmural stimulation, but after a preparation had been desensitized to nicotine postganglionic nerve stimulation was still effective. on cat tail piloerector muscles excited by transmural stimulation. Thompson (1958) recorded similar phenomena on isolated cat nictitating membrane. The simplest explanation for these results is that nicotine blocked those receptors mediating the sympathomimetic effect. However, persistence of adrenergic neuro-effector transmission suggests that these receptors are not part of the physiological mechanism for the release of the sympathetic transmitter. Burn and Rand (1960) showed in the perfused rabbit ear, that vasoconstrictor response to nerve stimulation was reduced after perfusion with ACh 2.5 μ g to 200 μ g in saline; this made them to suggest that this blocking action of nicotine and ACh is exerted at the cholinergic link, presumably in a way analogous to their

action in blocking autonomic ganglia. Recently Malik and Ling (1969 a) and Malik (1970) by using superior mesenteric artery of the rat studied the effect of ACh on vasoconstrictor responses due to the stimulation of sympathetic nerves. When ACh was allowed to act for very short time (15 sec), the vasoconstrictor responses to sympathetic nerve stimulation were steadily increased; but when ACh was allowed to act for 20 min the responses to sympathetic nerve stimulation were greatly reduced. This reduction disappeared as soon as ACh was removed.

The blocking action of ACh resembles the blocking action of guanethidine, since the block produced by both substances is diminished by dexamphetamine and also by raising the calcium concentration of the perfusion fluid. The block caused by ACh is reduced by atropine or hyoscine while that produced by guanethidine is not. A similar block is also produced by DMPP (Malik and Ling, 1969 b). The block due to DMPP is not affected by atropine, although it is removed by increasing the concentration of calcium in perfusion fluid or by adding dexamphetamine.

Malik and McGiff (1971) studied the effect of choline (500 $\mu\text{g/ml}$) on postganglionic nerve stimulated responses in perfused rat mesenteric arteries. It was found that when choline was infused for 15 sec, it enhanced the responses to sympathetic nerve stimulation whereas the infusion of the same concentration of choline for 20 min greatly reduced the response to nerve stimulation. However, choline infused for either shorter or longer period did not alter the response to injected NA. In the light of Burn and Rand hypothesis, they explained their finding by suggesting that the effect of infusion of choline for short period presumably either summates with ACh, which is released from adrenergic nerve fibres, or increases the synthesis or release ACh from these fibres as has been shown for choline in preganglionic fibres. This in turn may increase the release of NA from adrenergic nerve terminals and result in greater constrictor response. The infusion of choline for longer period presumably causes accumulation of ACh or interferes with the action of ACh at receptors of the adrenergic nerve endings, resulting

in reduction or abolition of nerve stimulated responses. The block induced by choline was abolished by hyoscine, possibly due to an action on muscarinic receptors on the nerve ending. Since adrenergic nerve terminal like superior cervical ganglion contains nicotinic and muscarinic receptors (Malik and Ling, 1969 b; Lindmar et al. 1968; Haeusler et al. 1968; Malik and Ling 1969 a), the muscarinic and nicotinic receptors may be stimulated or inhibited depending upon the concentration and the time course of action of cholinomimetic agents (Malik and Ling, 1969 a).

Recently Von Euler (1970) studied the effect of ACh on transmurally stimulated guinea pig vas deferens. ACh (10 ng/ml) enhanced the transmural nerve stimulated responses. The effect was rapidly blocked by atropine (100 ng/ml) which did not affect the muscle tone and only caused slight decrease in the stimulation response. It was suggested that "if ACh demonstrated in adrenergic nerves is a true component of the axons, this ACh may have important actions on the neuromuscular transmission process".

The action of anticholinesterases. If for the release of NA from sympathetic postganglionic fibres the release and action of ACh is involved, possible mechanisms for the disposal of ACh would be destruction by acetylcholinesterase or diffusion away from its site of action. Should this be so, the amount of NA would be expected to be greater in the presence of anticholinesterase. The greatest increase would be seen at the lowest frequency of stimulation, and the increase would diminish as the frequency rose. For, if the nerve impulses release ACh, then when a fixed number of pulses is given, and when the interval between pulses is longer, there is more time for cholinesterase to destroy ACh between pulses and this would prevent the concentration of ACh from rising. The hypothesis assumes that as the concentration of ACh rises, more NA will be released. When the frequency of stimulation is higher, there will be less time for cholinesterase to act between each pulse; therefore, the concentration of ACh will rise and more NA will be released and hence the effect of anticholinesterase will diminish at higher frequencies, since the effect of cholinesterase diminishes with the shortening of the time in which it can act.

Physostigmine, neostigmine and other anticholinesterases in the presence of atropine or hyoscine enhance responses to sympathetic nerve stimulation in a variety of organs: rabbit ear vessels (Burn and Rand, 1960 b); cat nictitating membrane (Burn et al. 1963); dog femoral artery (Bernard and DeSchaepdryver, 1964); taenia of guinea pig (Ng, 1966); rabbit heart (Hukovic, 1966); dog retractor penis (Armitage and Burn, 1967); dog renal vessels (McGiff et al. 1967). Recently Malik and Ling (1969 a) and Malik (1970) showed increase by neostigmine of vasoconstrictor response to post-ganglionic nerve stimulation in perfused superior mesenteric artery of rat. However, the results on cat nictitating membrane and cat spleen are controversial.

Another hypothesis involving the inhibition of acetylcholinesterase is based on the suggestion of Cuthbert (1963) and Carlyle (1964) that ACh may be involved in the transmission of activity from one cell to another. Anticholinesterases might facilitate this and thus affect the contraction of the whole muscle. There are several possible other explanations. Physostigmine might act like neostigmine and by increasing the

supernormal period of the nerve terminals (Hubbard and Schmidt, 1961), might permit re-excitation of them by a muscle action potential (Brown and Matthews, 1960) or by the transmitter that had been released (Eccles, 1964; Randic and Straughan, 1964), and thus a single stimulus to the nerve might result in repetitive synaptic activity. Another possibility is that the anticholinesterases, by increasing negative after potential (Hubbard and Schmidt, 1961) in the nerve terminal might increase the amount of transmitter released.

These hypotheses fall into two groups: one based on the inhibition of acetylcholinesterase and the other excluding an action on this enzyme. In order to choose between the two groups, it is most important to verify the presence of acetylcholinesterase near the adrenergic nerve terminals.

Acetylcholinesterase and adrenergic synapses. Early studies on the acetylcholinesterase activity in the sympathetic ganglia revealed marked differences in the intensity of individual ganglion cells, which ranged from strong to weak, (Koelle, 1963). Koelle (1955) reported strongly positive acetylcholinesterase

containing ganglion cells in the sympathetic ganglia. This observation was confirmed by Sjoqvist (1963) in cat's sympathetic ganglia. In the ganglia of many other species, the majority of cells, necessarily including many adrenergic ones, exhibit a moderate or intense acetylcholinesterase activity (Koelle, 1955). The problem became quite interesting when Burn and Rand (1959; 1965) presented the cholinergic link hypothesis. Since subsequent studies showed that catecholamine fluorescence of individual cells of sympathetic ganglia varies (Franko and Harkonen, 1963; Hamberger et al. 1963), it was thought of interest to correlate the acetylcholinesterase activity and amine content in individual ganglion cells.

In several sympathetic ganglia of the cat Hamberger et al. (1963; 1964) observed that the majority of cells exhibited a weak acetylcholinesterase reaction but an intense catecholamine fluorescence, while minority showed a weak fluorescence, but an intense acetylcholinesterase activity. The results were indicated the presence of distinct adrenergic ~~and~~ and cholinergic neurons in the ganglia. However, Franko

and Harkonen (1965) found that weak, moderate or strong NA fluorescence of individual ganglion cells was indiscriminately associated with weak, moderate or strong acetylcholinesterase activity. The high amine content and the intense acetylcholinesterase activity in some of the cells were taken to suggest that these cells are at the same time both adrenergic and cholinergic, which fits well with the Burn and Rand hypothesis (1959). However, the cytoplasmic cholinesterase of these cells may be taken to reflect their cholinceptive rather than cholinergic nature (Shute and Lewis, 1966). Harkonen (1964) reported the presence of nonspecific cholinesterase in the superior cervical ganglion of the rat. However, the nonspecific cholinesterase activity was inversely correlated with the intensity of the catecholamine fluorescence.

Peripheral sympathetic fibres. Eranko et al. (1964) observed that there were many fine fibres in the nerve net of the dilator muscle in the rat iris which exhibited both catecholamine fluorescence and acetylcholinesterase activity, while other fibres were found to contain either catecholamine or

acetylcholinesterase activity, but not both. It was concluded that the fibres containing both catecholamine and acetylcholinesterase are originated from similar cells of superior cervical ganglion. However, catecholamine and acetylcholinesterase may be in closely concomitant fibres which cannot be resolved by light microscopy.

Jacobowitz and Koelle (1965) observed similar fibres containing both catecholamine and acetylcholinesterase in the vas deferens of the guinea pig, as well as in the uterus and tube of the cat. Such fibres could not be found in the vas deferens or in the nictitating membrane of the cat, and their presence remained uncertain in the same organ of the rabbit.

Csillik and Koelle (1965) observed that removal of the superior cervical ganglion of the rat caused a rapid disappearance of all fluorescent fibres from the iris and also a slower degeneration and disappearance of some 15 to 20% of acetylcholinesterase positive fibres. These fibres were assumed to originate from those cell bodies in the

superior cervical ganglion which possess a moderate or high acetylcholinesterase activity. In a similar study, Ehinger and Falck (1965) failed to observe any appreciable reduction in the number of acetylcholinesterase positive fibres after excision of the cervical sympathetic chain to the iris of the rat. On the other hand, a considerable reduction in the number of acetylcholinesterase containing fibres was observed after removal of ciliary ganglion, although no overt reduction of adrenergic nerves was detected.

From the above observations, it seems that (a) some sympathetic fibres contain catecholamine but no acetylcholinesterase; (b) other fibres contain acetylcholinesterase but no catecholamine; (c) fibres which appear single in the light microscope, may in fact enclose several axons and contain both acetylcholinesterase and catecholamine; (d) liberation of catecholamine through a cholinergic link is possible through either of the structural mechanisms given in (c).

Electron microscopic study. De Robertis and Pellegrino

de Iraldi (1961) did electron microscopic study on the splenic

nerve of the rat and found that in the termination of each sympathetic fibre, two kinds of vesicles were present. One type contains dense granules and the other contains empty vesicles. Similar observation was made on the vas deferens of rat with autoradiography and electron microscopy. It was observed that after treatment of rat with reserpine, the granular vesicles disappeared. It was also demonstrated that granular vesicles could store exogenously added ^3H -nor-adrenaline. The vesicles without granules were found to be closely similar to the synaptic vesicles of central nervous system, synapses and motor end plates (Wolfe et al. 1962). Similar observation was made by Pellegrino de Iraldi and De Robertis (1963) in the adrenergic nerve endings of the pineal gland of rat, where 60 to 70 percent of the vesicles were without granules, while 30 to 40 percent contained granules. The proportion of granuloid vesicles diminished between 2 and 48 hr after a single injection of reserpine. Barer and Thompson (1963) made similar observations on the cat nictitating membrane, and an investigation has also been made on the anterior hypothalamus of rat which had "two well defined populations of the vesicles, one of the clear vesicles with a diameter of 501 \AA^0 and another of granular vesicles with diameter of 1300 \AA^0 ". There was no

superimposition between the two maxima which are widely apart. This is in contrast to the interpretation of Pellegrino de Iraldi et al. (1963) that both types represent stages in the development of a single entity. Richardson (1964) observed that the sphincter muscle of the iris has nerve endings with granular vesicles, which must contain ACh, while dilator muscle has endings which contain both agranular and granular vesicles. However, Tranzer and Thoenen (1967) showed by electron microscopic studies that two terminations were located in the same Schwann sheath, one with agranular vesicle and other with dense-cored vesicle lying side by side. If this is the general rule, it may be that ACh is coming out of one of the fibres to release NA from the other.

Esterhuizen et al. (1968) confirmed the presence of acetylcholinesterase positive axons in the nictitating membrane of cat in their electron microscopic studies. The number of axons was very small, however, less than 0.5% of total axon profiles in the muscle being acetylcholinesterase positive. They also showed that none of these esterase positive axons possessed a close synaptic relationship with the smooth muscle

cells, an observation in accord with that of Gardiner et al. (1962) who suggested that these fibres may end on small blood vessels.

However, it must be added that since cholinergic and adrenergic axons have been observed occasionally in the same nerve bundle in the nictitating membrane muscle, it is possible that in these situations ACh released from the cholinergic axons, is able to influence NA from the neighbouring adrenergic axons.

Recently Franko et al. (1970) did histochemical studies on rat pineal body under light and electron microscopy. The postganglionic sympathetic fibres from superior cervical ganglion which innervate the rat pineal body were found to be invested by acetylcholinesterase. In contrast, Graham et al. (1968) have shown in the cat, that the nerve bundles innervating pancreatic arterioles consist exclusively of adrenergic or cholinergic axons. In addition, they observed some nerve bundles which contained a mixture of discrete adrenergic and cholinergic axons juxtaposed, without any Schwann cell cytoplasm intervening. Cholinesterase staining

elements have also been demonstrated in the artery of the rabbit ear at the medial-adventitial border (Waterson et al. 1970), a region containing a network of noradrenergic nerve terminals (de La Lande and Waterson 1967). The staining associated with cholinesterase enzymes together with NA disappeared after the removal of the superior cervical ganglion. After pretreatment with reserpine NA disappeared but the staining due to the enzymes remained unaffected. Thus clear evidence was provided of a cholinergic element in close relation to an adrenergic element, both being sympathetic in origin.

The action of hemicholinium (HC-3). HC-3 was introduced by Long and Schueler (1954). MacIntosh et al. (1956) showed that HC-3 interrupted the synthesis of ACh by stopping the transport of choline to the intraneuronal site where ACh was synthesized. The interruption was competitive, and the action of HC-3 could be overcome by an excess of choline. Thus in perfused superior cervical ganglion of the cat stimulated through the preganglionic fibres in the presence of HC-3, the transmission of impulses gradually diminished,

the release of ACh declined to zero, and the store of ACh in the ganglion was reduced to very low value.

If ACh is released by sympathetic stimulation and played a part in release of NA, then sympathetic nerve mediated responses should be reduced in the presence of HC-3.

Chang and Rand (1960) studied the effect of HC-3 in series of isolated preparations. In isolated rabbit colon, the inhibitory response to the sympathetic nerve stimulation was blocked by HC-3. The preparation recovered after resting for one hour. Similar results were obtained in guinea pig vas deferens, isolated atria of cat, perfused vessels of rabbit ear and piloerector responses in cat tail. In all these preparations HC-3 blocked the sympathetic nerve stimulated response. The blocking action of HC-3 was reversed by choline in all these preparations. Brandon and Rand (1961) showed that after HC-3, the isolated perfused cat spleen failed to contract when the splenic nerves were stimulated. However, Leaders and Dayrit (1965) found that HC-3 did not

prevent the contractile response of dog spleen to nerve stimulation, but it did reduce the amount of ACh released. In the light of this observation, they tried to strengthen their concept that there are two separate fibres, adrenergic and cholinergic in splenic nerve rather than cholinergic link in adrenergic nerve fibres (Leaders, 1963).

The inhibition of sympathetic-nerve-stimulated responses by HC-3 has also been observed in the guinea pig colon (Rand and Ridehalgh, 1965) and in dog nictitating membrane (Arya and Gulati, 1967). Various workers (Leaders and Dayrit, 1965; Bevan and Su, 1964; Gardiner and Thompson, 1961) have failed to observe an action of HC-3 in other tissues; but this has been because they studied the action for too short a time, whereas Chang and Rand (1961) found it necessary to continue observations for 5.5 hr in the guinea pig colon (Burn , 1968). Vincenzi and West (1965) excised the sinoatrial node of cats or rabbits and applied generalized shocks to the whole tissue. Stimulation caused inhibition followed by acceleration. After exposing the tissue to HC-3 they found that the inhibition disappeared but the acceleration remained,

and concluded that HC-3 did not prevent the response to sympathetic impulses. Blinks (1966) has shown that the sympathetic effect of field stimulation is not blocked by even high concentrations of bretylium, and pointed out that the mechanism of NA release by field stimulation may be quite different from that by normal action potential. Appel and Vincenzi (1970) studied the effect of HC-3 and bretylium in isolated sinoatrial node of the rabbit and found that stimulation of intranodal autonomic nerve fibres resulted in biphasic chronotropic response. The chronotropic response: (negative chronotropic response followed by positive chronotropic response) was due to release of ACh and NA from the intranodal nerve fibres. In the presence of HC-3, the negative chronotropic (cholinergic) response was abolished, while the positive chronotropic (adrenergic) response was unaltered. In the presence of bretylium, the positive chronotropic response was abolished, while the negative chronotropic response was unaltered.


The action of botulinum toxin. Botulinum toxin is known to prevent the release of ACh from all cholinergic nerve endings.

In the mammalian neuromuscular junction, it reduced the amplitude of the end plate potential and the frequency of the miniature potentials, and sometimes abolished them completely. With low dose of botulinum toxin, the effect could be overcome by stimulation of the nerve at high frequency or by increasing the calcium concentration in the bath fluid, procedures that normally increase the frequency of miniature potentials. These findings suggested that botulinum toxin acted at the nerve terminals (Brooks, 1956; Thesleff, 1960). Ambache (1951) studied the effect of botulinum toxin on adrenergic neuro-effector transmission. He found that after the injection of 10 μ g of botulinum toxin type A (LD_{50} : 0.1 mg/kg mouse) into the anterior chamber of the eyes of cats, there was no pupillary constriction, when ciliary nerves were stimulated, but dilatation was seen after the stimulation of the cervical sympathetic nerves. A pupillary dilator response to sympathetic stimulation was still present in cats treated with 200-300 times the threshold dose of toxin (2.5 μ g) for abolishing the pupillary constrictor response to parasympathetic nerve stimulation. The

dilator responses were smaller in the intoxicated eye than control, as treatment with botulinum toxin caused pupillary dilatation. After the injection of 50-500 μ g of botulinum toxin into the nictitating membrane of one side, the tension developed on postganglionic stimulation was 50% of that of the control side. This dose is 50-100 times than that needed to paralyze tibialis anticus, a much larger structure. Hence, even after massive doses of botulinum toxin, part, if not all, of the adrenergic innervation of the iris and nictitating membrane of the cat was still functional. The adrenergic fibres were resistant to botulinum toxin.

Rand and Whaler (1965) showed that 17-29 hr after the injection of botulinum toxin type D into the skin of the cat's tail at the base of the tuft of hair, piloerection due to sympathetic nerve stimulation was prevented. They also showed that in the presence of botulinum toxin, there was no inhibitory response to sympathetic nerve stimulation in rabbit ileum, however, the added NA continued to give relaxation. The isolated guinea pig vas deferens failed to respond

to hypogastric nerve stimulation after botulinum toxin. However, Vincenzi (1967) failed to observe such an effect in isolated sinoatrial node of the rabbit. Moreover, the interpretation of Rand and Whaler's finding on the guinea pig vas deferens is complicated by the presence of ganglion cells peripheral to hypogastric nerve and it is very likely that failure of transmission could have occurred at the ganglion. Whaler (personal communication to Ferry, 1966) found that botulinum toxin caused failure of contractile response of vas deferens to stimulation of hypogastric nerve 1-2 cm from the muscle; the contraction could be elicited by stimulating hypogastric plexus, nearer to the muscle or by transmural stimulation. At a time when transmural stimulation of the nerve fibres caused no response, a change in stimulus parameters to stimulate the smooth muscle still evoked contractions. There are suggestions that the innervation of vas deferens contains cholinergic fibres (Della Bella Benalli and Gandini, 1964). In the experiments of Ambache (1951), Ambache and Lessin (1955) and Rand and Whaler (1965) nicotine, in botulinum toxin treated rabbit and mouse ileum, caused



relaxation of the smooth muscle. It may be possible that nicotine acted by depolarizing the nerve cell membrane, increasing its permeability to sodium and calcium ions. Thus nicotine might have some of the effects of a nerve impulse. In botulinum-intoxicated preparation, nicotine was more effective than nerve impulses in causing NA release. The reason for this is unknown. Perhaps nicotine, by causing longer lasting permeability changes than the nerve impulse, might be more effective in releasing NA from the botulinum-intoxicated nerves.

Alternatively, there may be a cholinergic process in the release of NA, and botulinum toxin may act by preventing the release of ACh after the arrival of the nerve impulse at the nerve endings, and thus no NA can be released. Nicotine would, perhaps, short-circuit the botulinum toxin block and release NA by an action on the intracellular receptors for ACh (Ferry, 1966).

The evidence offered by Rand and Whaler for the cholinergic link in adrenergic transmission in cat piloerector

muscles, guinea pig vas deferens and in rabbit ileum is strong, since botulinum toxin is considered to have a specific action.

Other drugs affecting adrenergic transmission

The sympathetic blocking agents choline 2:6 xylyl ether (xylocholine) synthesized by Hey and Willey (1954) was shown to inhibit the sympathetic postganglionic stimulation, but did not impair response to exogenous NA. Exley (1957) suggested that the action of xylocholine on adrenergic nerves might be analogous to that of botulinum toxin on cholinergic nerves. The chemical structure of xylocholine suggested that it might be acting by interfering with cholinergic transmission. Hukovic (1960) found that xylocholine blocked the action of ACh (in the presence of atropine) in causing acceleration of isolated rabbit atria, and vasoconstriction of the vessels of the perfused rabbit ear. Burn and Rand (1960) observed in the nictitating membrane of the cat that xylocholine did not interfere with the release of NA by tyramine. But it interfered with the release of NA by sympathetic nerve stimulation and by ACh.

The second group of blocking agents, bretylium and guanethidine were described by Boura and Green (1959) and Maxwell et al. (1960) respectively. These blocking agents, which have been used clinically as anti-hypertensives, provide a clue to the mechanism of NA release. Bretylium and guanethidine were shown to block sympathomimetic actions of ACh and nicotine in various isolated adrenergically innervated preparations. Hukovic (1960) observed that bretylium blocked ACh-induced acceleration in isolated atria of rabbit in the presence of atropine. Similarly ACh-induced vasoconstriction in rabbit ear artery was also blocked by bretylium. Burn and Rand (1960) found that bretylium blocked the response of the nictitating membrane to postganglionic nerve stimulation; however, tyramine-induced contraction was enhanced. This observation led them to conclude that bretylium interferes with the release of NA from the store at sympathetic nerve endings induced by sympathetic nerve stimulation and by ACh. Guanethidine was also reported to block sympathomimetic effects of ACh in a similar fashion. Both bretylium and guanethidine also block the phrenic nerve diaphragm preparation (Dixit et al. 1961). Hence it is suggested that

bretylium and guanethidine act at sites where acetylcholine is recognised as the chemical transmitter. It has also been suggested that bretylium contains one quaternary nitrogen in the group ethyldimethylammonium which is similar to the trimethylammonium group of ACh. Guanethidine, at first sight appears to have different structure bearing no similarity with ACh molecule; but the guanidine group, which carries a positive charge is similar to trimethylammonium and moreover guanethidine is highly ionized in solution.

Development of adrenergic nerve fibre

The majority of the sympathetic postganglionic fibres differ from other motor fibres in the body in that they exert their effect by releasing NA. Motor fibres to skeletal muscles, both pre- and postganglionic parasympathetic nerves and pre-ganglionic sympathetic fibres including the splanchnic nerves and postganglionic fibres to the sweat glands all release ACh. Thus the postganglionic sympathetic fibres stand alone in releasing NA.

This exception may be specially characteristic of mammals, since Young (1936) found that the sympathetic innervation of the intestine in two teleost fishes *Lophius piscatorius* and *Uranoscopus scaber* was motor. Adrenaline caused the intestine to relax and, therefore, the nerves did not release an adrenaline like substance. Burnstock (1958) studied the sympathetic innervation to intestine of another teleost fish, trout and found that the splanchnic nerve to the intestine was also motor, and that this effect was abolished by atropine. He, therefore, said that the splanchnic nerve was cholinergic.

Burn (1960 b) studied the effect of stimulation of the periarterial nerves which run to the intestine of the chicken, making a preparation like that described by Finkleman (1930) for the rabbit. In the rabbit, the response to stimulation was inhibition. The response in the chicken was mainly motor like that in the trout, and the motor effect was abolished by atropine. However, when the muscle had been in the bath for sometime, he observed a small initial inhibition before the motor response. This muscle was also relaxed by NA so that

it appeared likely that the main motor response was due to ACh.

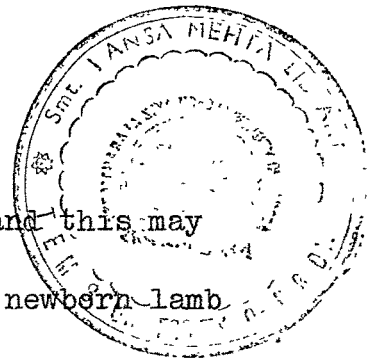
Although in the adult rabbit, the inhibition of the intestine caused by sympathetic nerve stimulation is due to the release of NA there is evidence that ACh is also released. When stimulation is applied at low frequency, the inhibition is increased after atropine is added in the bath. This suggests that at low frequency both ACh and NA are released, and that former opposes the action of the latter.

Burn (1968 b) argued "if it is true that there has been a change in sympathetic function in the course of evolution, and that the motor responses of the intestine seen in the trout and chicken have become changed to an inhibitory action in the rabbit, then, since ontogeny recapitulates phylogeny, some sign should be seen in the newborn rabbit". He, therefore, carried out experiments on rabbits 3 or 4 days old. In one of these, it was found that sympathetic stimulation had a motor effect at all frequencies, while in another there was a motor effect at 3-Hz and 5-Hz, but an inhibitory

effect at 10-Hz and 20-Hz. The motor effect in response to stimulation at 3-Hz was abolished by atropine. Two rabbits 8 days old, were examined from one litter; the response of the ileum to sympathetic stimulation was motor at all frequencies in one, and was inhibitory at all frequencies in the other. A previous study by Day and Rand (1961) showed that in a rabbit 12 days old, sympathetic stimulation at 50-Hz caused inhibition.

Boatman et al. (1965) had made similar observations in the newborn dog. They perfused the hindleg vessels of puppies, 1 day to 8 weeks old, and found that stimulation of the lumbar sympathetic chain caused vasodilatation in 1 day to 2 week old puppies. Since vasodilatation was blocked by atropine they concluded that it was due to the release of ACh. In puppies 4 weeks old, stimulation of the sympathetic fibres caused vasoconstriction. Moreover, vasodilatation was observed at 1 day of age despite the low blood pressure (30 mm Hg). The blood pressure steadily rose as age increased.

These results suggest that in newborn dogs and rabbits, the sympathetic stimulation is cholinergic, but becomes adrenergic in the second or later weeks of life. It is during this period that the uptake of NA by the rat heart begins (Glowinski et al. 1964). The uptake of NA, however, may not be so important as the uptake of its precursors, and this was studied by Burn and Rand (1960 a) in the rat treated with reserpine. In such rat, tyramine when injected intravenously produced a very small rise in the blood pressure, but after the infusion of NA or of its precursors, tyramine caused a much greater rise and the effect of (-)-dopa and also of phenylalanine in increasing the response to tyramine lasted very much longer than the effect of infusing NA itself. This suggests the possibility that a conversion of sympathetic postganglionic fibres occurs in the second and later weeks of life. At birth they are fibres releasing ACh. When the uptake process begins, the fibres may take up phenylalanine from the blood and convert it to NA. The fibres then become adrenergic. The time at which adrenergic fibres are first developed will depend on the



time at which the uptake process begins, and this may depend on the length of gestation. In the newborn lamb after a gestation lasting 149 days, the adrenergic fibres may be already developed.

During ontogenesis, the levels of catecholamines, their synthesis, storage, inactivation, release and uptake and response to adrenergic neurotransmitter is quite low.

It has been established that myocardium of the embryonic chick receives its sympathetic innervation on the 5th day (Romanoff, 1960; Hamilton, 1952). By employing younger and older hearts, it is possible to study the effects of pharmacological agents on innervated and non-innervated hearts. McCarty et al. (1960) have shown that the sensitivity of embryonic heart to diminished catecholamines does not change with sympathetic innervation. It appears, therefore, that the myocardial receptors which respond to sympathomimetic amines are present and fully functional prior to sympathetic innervation.

Ignarro and Shideman (1968 b) performed series of experiments to study the appearance and concentration of catecholamines and their biosynthesis in embryonic and developing chicks. They found that NA and adrenaline were present in embryonic chick heart prior to sympathetic innervation and suggested that the early embryo obtained NA and adrenaline from extraembryonic source, since these biogenic amines could not be synthesized by the embryo at this time. Further, they suggested that endogenous NA and adrenaline appeared in the fertilized egg from the first day, and this endogenous NA was received from maternal source and retained in the yolk, which gradually passed to embryo by diffusion or through blood islands which developed on the 4th day of incubation.

Caston (1962) demonstrated a sequential appearance of dopamine, NA and adrenaline in the whole embryo and isolated neural crests of *Rana pipens*.

During the initial stages of embryogenesis of the chick, similarly there appeared to be a somewhat orderly

sequential appearance of NA and adrenaline and their precursors (Ignarro and Shideman, 1968 b).

Burack and Badger (1964) conducted experiments on developing chick embryo to study dopa decarboxylase, dopamine-beta-oxidase and phenylethanolamine-N-methyltransferase concerned with the biosynthesis of catecholamines and found that the appearance of these enzymes was in orderly sequence; dopa decarboxylase activity was detected on the first 1-2 days of incubation and dopamine-beta-oxidase and N-methyltransferase activities were first detected in 3 - 4 day old and 5 - 6 day old embryos respectively. Similar results were reported by Ignarro and Shideman (1968 b) in developing chick embryo. After injection of labelled tyrosine, dopa and dopamine were detected on 1st and 2nd day respectively. NA and adrenaline were first detected on 3rd day. As there appeared to be somewhat orderly sequential appearance of these catechols, in the whole embryo, in accordance with the scheme of their biosynthesis, the order of appearance of each of the

enzymes responsible for their synthesis was determined. Tyrosine hydroxylase, dopa decarboxylase, dopamine-beta-oxidase and phenylethanolamine-N-methyltransferase activities were first detected on 1st, 2nd, 4th and 6th days of incubation respectively.

Glowinski et al. (1964) showed that the ability of the rat heart to accumulate ^3H -NA rapidly developed between 6th and 15th day after birth. Since uptake and retention of ^3H -NA are thought to occur mainly in postganglionic sympathetic neurons (Axelrod, 1965), the ability of tissues to accumulate ^3H -NA may indicate the density of sympathetic postganglionic innervation in such tissues. Iversen et al. (1967) studied the uptake and metabolism of ^3H -NA and the endogenous content of NA in the heart, spleen, salivary glands and intestine of developing rats. They observed differences in the relative concentrations of endogenous NA in various tissues at birth, the heart having the lowest level and the intestine the highest level. These differences were also evident in the rate at which the

adult level of NA was attained in the various tissues. In heart and spleen, the ability to accumulate ^3H - NA developed parallel to the endogenous NA content whereas the ability of the intestine and salivary glands to accumulate ^3H - NA was already fully developed at birth, although these tissues lacked normal NA content at this time. Mirkin (1969) reported that exogenously administered ^3H - NA is not retained and not bound to microsomal fractions of foetal rat heart until 21st day of gestation. This indicates that foetal heart is fully innervated by 21st gestational day.