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DEMONSTRATION OF SEPARATE ADRENERGIC AND CHOLINERGIC FIBRES TO THE VESSELS OF THE REAR QUARTERS OF THE RAT BY HEMICHOLINIUM AND A PROPOSED ROLE IN PERIPHERAL VASCULAR REGULATION *

BY

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Since the proposal by Burn & Rand (1959) of a cholinergic component in the release of noradrenaline, two conflicting primary mechanisms have been considered for the release of noradrenaline from adrenergic nerve terminals. These may be called the " classical " and the " cholinergic link " hypotheses. Since the introduction of the " cholinergic link " hypothesis, it has been the subject of numerous reviews (Volle, 1963; Zaimis, 1964; Burn & Rand, 1965; Ferry, 1966). Additional discussion would not be purposeful at this time.

It has been accepted for some time that acetylcholine may be released from " adrenergic " nerves to some vascular beds in reserpinized animals (Bülbring & Burn, 1935; Burn & Rand, 1960). The question remaining to be answered, therefore, concerns the source of this acetylcholine. Does it originate in separate cholinergic fibres carried in predominantly adrenergic nerves or in an intermediate cholinergic step within adrenergic fibres ?

Previous experiments in these laboratories using dogs suggested strongly the existence of separate cholinergic and adrenergic components in the sympathetic innervation of the vascular bed of the hind limb (Leaders, 1965) and the spleen (Leaders & Dayrit, 1965). In these studies it was demonstrated that hemicholinium (HC-3) (Long & Schueler, 1954), a compound known to inhibit neurohumoral transmission in cholinergic nerves, could selectively inhibit cholinergic transmission without interfering with adrenergic function. These findings were consistent with the classical concept of nerve innervation but did not explain the inconsistencies in this hypothesis. These data were also consistent with an alternate hypothesis for adrenergic transmission proposed recently by Leaders (1963). This proposal, the " cholinergic-adrenergic interaction " hypothesis, attempts to explain the inconsistencies in both the classical and the " cholinergic-link " concepts.

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Roberts & Stadter (1960) and Leaders & Long (1962) reported data indicating that parasympathetic nerves could in some instances influence sympathetic nerve activity. In 1963 Leaders demonstrated the ability of adrenergic nerve activity to influence cholinergic nerve activity and *vice versa* in preparations of cat atria. This reciprocal involvement of adrenergic and cholinergic portions of the autonomic nervous system was described as a " local cholinergic-adrenergic interaction." The hypothesis suggested that, through mechanisms as yet unknown, both acetylcholine and noradrenaline might be released by stimulation of either branch of the autonomic nervous system. It was further suggested that it is probably the ratio of the neurohumoral agents from the cholinergic and adrenergic fibres which determines the physiological or pharmacological response observed. Pharmacological evidence for such an interaction has been found more recently in preparations of the dog spleen (Leaders & Dayrit, 1965), the dog hind limb (Leaders, 1965), the dog salivary gland (Leaders & Pan, in press), and the dog eye (Leaders & Fray, in press).

The present experiments were designed to distinguish among the various hypotheses for the mechanism of adrenergic transmitter release. The nerves to the vascular beds of the rear quarters of the rat were chosen for this study.

METHODS

Eighty-six Sprague-Dawley rats, weighing at least 200 g, were anaesthetized with pentobarbitone sodium (30 mg/kg) intraperitoneally. An endotracheal tube was placed in all animals. A small animal respirator (Harvard Apparatus, Model 607-A) was used to provide positive pressure ventilation in those animals which received gallamine.

Surgical procedures

A medial abdominal incision was made, the muscle and peritoneum were retracted and the intestines pushed to one side and covered with a sponge moistened with saline. The descending aorta was dissected free and very carefully separated from the vena cava just anterior to the bifurcation of the aorta into the common iliac arteries.

Perfusion system

The perfusion system used was that described previously by Dorr & Brody (1965). Briefly, blood for perfusion was obtained *via* a cannula inserted anteriorally into the aorta. A Sigmamotor pump, Model T-8 (Sigmamotor, Inc.) was used to propel blood through the system. This pump will deliver a fixed volume of fluid virtually independently of perfusion pressures over the range of pressures evaluated in these experiments. Blood was delivered from the perfusion system into the distal portion of the ligated aorta (Dorr & Brody, 1965). The perfusion system used had a capacity of 3.1 ml. and was filled with saline solution containing heparin (5 mg/kg) before the beginning of the experiment.

Systemic pressure was recorded *via* a side arm in the perfusion system on the proximal side of the pump, and perfusion pressure (usually 120 mm Hg) from the distal side of the pump on a Beckman, Type RB, direct writing oscillograph (Offner Division, Beckman Electronics, Inc.) *via* Statham P23A pressure transducers (Statham Electronics, Inc.).

Sympathetic nerve stimulation

The sympathetic trunk was very carefully dissected free and silver electrodes placed around the chain in the thoracic region. Electrical stimulation was provided by a Grass stimulator Model S4G (Grass Instruments, Inc.) at a pulse frequency of 35 counts/sec, a delay of 0.01 msec and a duration

of 6 msec. Voltage was usually 6 V. This was supramaximal for sympathetic chain stimulation but not for sciatic nerve stimulation. Vascular responses were rapid in onset and lasted 15 to 20 sec.

Sciatic nerve stimulation

These experiments were performed using the following modifications. After cannulation, the iliac artery to the left leg was ligated to restrict blood flow to one hind leg only. The sciatic nerve to this leg was then dissected free and silver electrodes were placed around the nerve as was done previously on the sympathetic trunk.

Drugs were introduced intra-arterially *via* a short piece of rubber tubing near the distal end of the perfusion system (Dorr & Brody, 1965). Agonists included noradrenaline (0.1 μg/kg) (Levophed bitartrate), nicotine bitartrate (100 μg/kg) and acetylcholine chloride (2 μg/kg). Following each drug, perfusion pressure and systemic blood pressure were allowed to stabilize before administration of the next drug or electrical stimulation. Hemicholinium bromide (Aldrich Chemical Co.) (1 mg/kg) was given intra-arterially and the nerves were stimulated for 30 sec intervals every 2 or 3 min for a 30 min period. Antagonists were also administered intra-arterially. These included hexamethonium chloride (Hexameton, Burroughs Wellcome) (1 mg/kg), gallamine triethiodide (Flaxedil) (2 mg/kg), atropine sulphate (2.5 mg/kg) and guanethidine sulphate (Ismelin, Ciba) (1 mg/kg). Reserpine (2 mg/kg) (Serpasil, Ciba) was injected intraperitoneally before surgery and was followed 1 hr later by guanethidine (1 mg/kg) in those experiments where it was used.

Evaluation of data

Perfusion pressures are reported as mm Hg increase or decrease from control readings that were measured immediately before nerve stimulation or drug administration. "Systolic" pressure values were used. The Student *t* test was used throughout the study to evaluate significance of difference between values. A *P* value of less than 0.05 was regarded as significant.

RESULTS

Sympathetic nerve stimulation

In these preparations, electrical stimulation of the sympathetic chain consistently produced an increase in perfusion pressure as did administration of noradrenaline (0.1 μg/kg). Nicotine (100 μg/kg) and acetylcholine (2 μg/kg) both resulted in a decrease in perfusion pressure. These data are illustrated in Fig. 1. Hemicholinium bromide (1 mg/kg) followed by sympathetic nerve stimulation for 30 sec intervals every 2 to 3 min for 1 hr did not significantly alter the perfusion pressure response to nerve stimulation, acetylcholine or noradrenaline. The decrease in perfusion pressure induced by nicotine became much greater after hemicholinium and the responses to administered noradrenaline became extremely variable.

Additional experiments were performed in which the same time course was followed but in which hemicholinium was not administered. Following stimulation of sympathetic nerves as described above, responses to nerve stimulation and to administration of nicotine, acetylcholine and noradrenaline were not significantly altered. The magnitude of the responses to noradrenaline administration varied markedly after the passage of time as in the preceding experiments.

Sympathetic nerve stimulation; reserpine and guanethidine treated

Nerve stimulation and noradrenaline administration increased perfusion pressure in preparations treated with reserpine (2 mg/kg) before the start of the experiment.

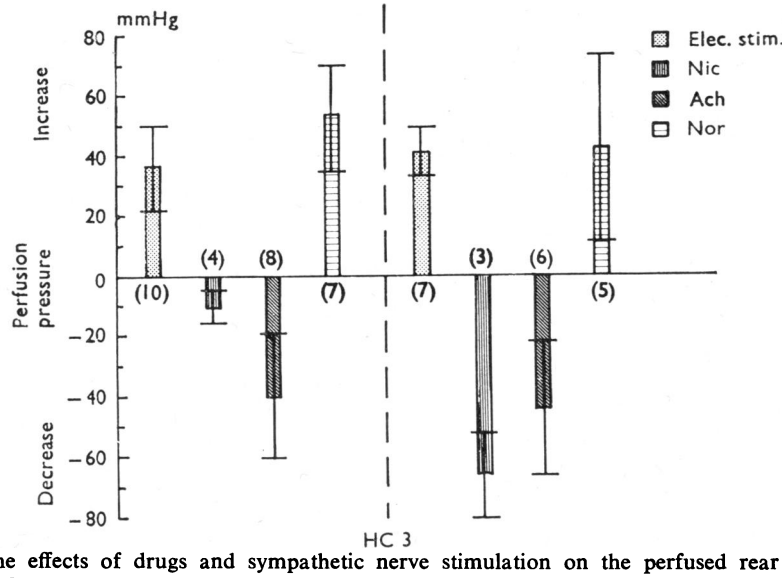

Fig. 1. The effects of drugs and sympathetic nerve stimulation on the perfused rear quarters of rats, HC-3, hemicholinium, 1 mg/kg followed by intermittent nerve stimulation ; Nic, nicotine, 100 μg/kg ; Ach, acetylcholine, 2 μg/kg ; Nor, noradrenaline, 0.1 μg/kg. Numbers in parentheses indicate the number of observations used to derive mean values. Limits are 95% confidence intervals for the means.

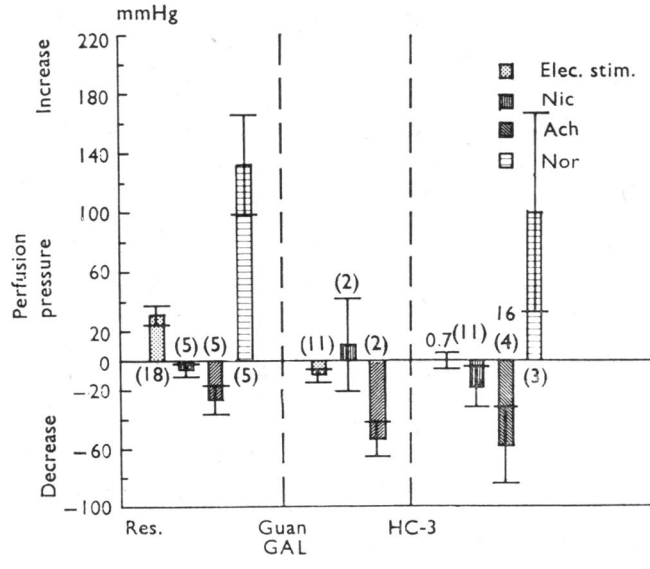

Fig. 2. The effects of drugs and sympathetic nerve stimulation on the responses of the perfused rear quarters of rats previously treated with reserpine and guanethidine. Res, reserpine, 2 mg/kg ; Guan, guanethidine, 1 mg/kg ; GAL, gallamine, 2 mg/kg ; HC-3, hemicholinium, 1 mg/kg, followed by intermittent nerve stimulation ; Nic, nicotine, 100 μg/kg ; Ach, acetylcholine, 2 μg/kg ; Nor, noradrenaline 0.1 μg/kg. Numbers in parentheses indicate the number of observations used to derive mean values. Limits are 95% confidence intervals for the means.

Nicotine produced a very small decrease in perfusion pressure while acetylcholine decreased perfusion pressure more dramatically. When the preparations were treated with guanethidine (1 mg/kg) 1 hr later, stimulation of the sympathetic nerves resulted in a small decrease in perfusion pressure. Gallamine (1 mg/kg) was administered to simulate preparations in which the sciatic nerve was stimulated. The perfusion pressure decrease following acetylcholine administration became significantly greater. Hemicholinium was then administered followed by sympathetic nerve stimulation as described previously. This treatment abolished the vasodilator response to sympathetic nerve stimulation. The responses to acetylcholine, nicotine or noradrenaline did not change significantly from those recorded following their previous administration. Mean values for these experiments are summarized in Fig. 2.

In other experiments using animals treated with reserpine and guanethidine, hemicholinium was administered but the nerves were not stimulated during a 30 min period following hemicholinium administration. The decrease in perfusion pressure induced by nerve stimulation was not altered by this treatment. The response to administration of acetylcholine was enhanced after hemicholinium administration as it was in the experiments in which the nerves were stimulated.

Sciatic nerve stimulation

Gallamine (1 mg/kg) was administered before stimulation of the sciatic nerve to inhibit muscle contraction. Electrical stimulation of this nerve resulted in a biphasic response in perfusion pressure, a rise followed by a fall. The fall was abolished by administration of atropine (2.5 mg/kg). Nicotine and acetylcholine again produced a decrease in perfusion pressures. These responses are illustrated in Fig. 3. Hexamethonium (1

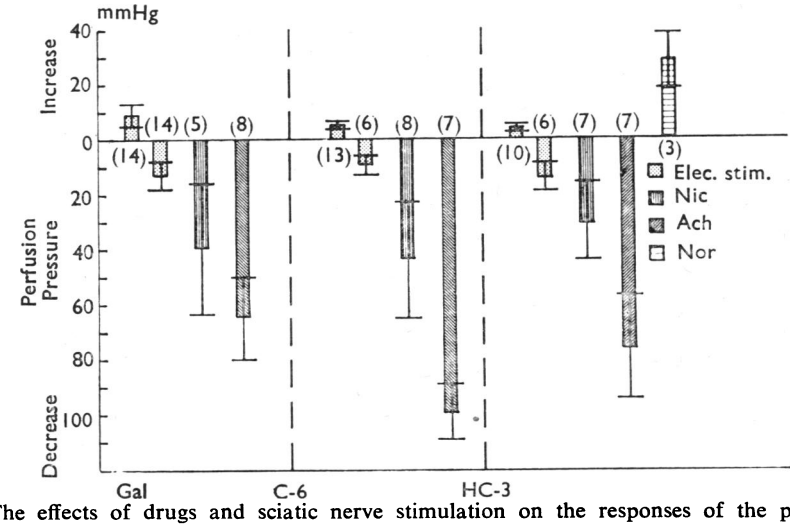

Fig. 3. The effects of drugs and sciatic nerve stimulation on the responses of the perfused rear quarters of rats. Gal, gallamine, 2 mg/kg; C-6, hexamethonium, 1 mg/kg; HC-3, hemicholinium, 1 mg/kg followed by intermittent nerve stimulation; Nic, nicotine 100 μg/kg; Ach, acetylcholine, 2 μg/kg; Nor, noradrenaline, 0.1 μg/kg. Numbers in parentheses indicate the number of observations used to determine mean values. Limits are 95% confidence intervals for the means.

mg/kg) slightly diminished both phases of the response to nerve stimulation and significantly enhanced the depressor response of acetylcholine. However, addition of hemicholinium followed by repeated sciatic nerve stimulation for 30 sec intervals every 2 to 3 min over a period of 1 hr failed to block either phase of the response to nerve stimulation. The perfusion pressure responses to the drugs administered were not significantly altered.

DISCUSSION

The nerves to the vascular beds of the rear quarters of the rat were chosen to study the source of acetylcholine released from sympathetic "adrenergic" nerves to the vasculature. It was of interest to ascertain whether this acetylcholine originated in separate cholinergic fibres carried in the predominantly adrenergic nerves or from an intermediate cholinergic step in adrenergic fibres. Hemicholinium was used as a tool to separate these possibilities. Hemicholinium is known to inhibit neurohumoral transmission in cholinergic nerves (Wilson & Long, 1959; MacIntosh, 1961). Although this drug may have several additional properties such as bretylium-like (Long, 1961) and ganglionic blocking activity (Birks & MacIntosh, 1961), it has been used previously under controlled experimental conditions selectively to inhibit cholinergic transmission without interfering with adrenergic function (Leaders, 1965; Leaders & Dayrit, 1965). Hence, if discrete cholinergic and adrenergic fibres exist, selective inhibition of these separate cholinergic fibres should be possible at a time when the adrenergic vasoconstrictor fibres are still functional. If a cholinergic junction were present in the adrenergic fibres to this vascular bed, hemicholinium should abolish the adrenergic vasoconstrictor response at a time and in a concentration roughly similar to that at which the cholinergic response is abolished following administration of reserpine and guanethidine. Hemicholinium followed by repeated sympathetic nerve stimulation did not inhibit the adrenergic vasoconstrictor response (Fig. 1).

It was therefore necessary to demonstrate that hemicholinium used similarly could inhibit the cholinergic response. Cholinergic vasodilatation was "unmasked" by acute administration of reserpine (2 mg/kg) followed 1 hr later by guanethidine (1 mg/kg). The resulting vasodilator response was abolished by hemicholinium and repeated nerve stimulation but not by hemicholinium alone, when the nerves were not stimulated. Hemicholinium, under the conditions of this study, does abolish the cholinergic component in the innervation of the vasculature of the rear quarters of the rat. Inhibition of this cholinergic component, however, apparently does not interfere with the adrenergic vasoconstrictor function of these nerves. This indicates clearly the distinct nature of the cholinergic and adrenergic mechanisms of these nerves. If, as has been suggested, a cholinergic link exists in some adrenergic fibres in addition to the discrete cholinergic fibres, this link is not a primary source of the cholinergic transmitter which is responsible for vasodilatation following sympathetic nerve stimulation in reserpine and guanethidine treated animals.

Since the acetylcholine found in predominantly sympathetic nerves is apparently not part of a cholinergic link in the adrenergic synaptic mechanism but is present in discrete cholinergic fibres, of what physiological significance can it be?

The " local cholinergic-adrenergic interaction ' 'hypothesis (Leaders, 1963) may provide a partial answer to this question. Such an interaction could occur with equal facility between discrete cholinergic and adrenergic fibres in a single nerve trunk as well as between cholinergic and adrenergic fibres from different nerves. " Cholinergic sympathetic " pathways have been reported previously (Lindgren & Uvnäs, 1953, 1954, 1955). These pathways apparently may fire simultaneously with or independently from the dominant adrenergic vasoconstrictor system. The cholinergic vasodilatation produced by stimulation of sympathetic nerves (in reserpinized animals) has been reported to be of a much shorter duration when compared to the adrenergic vasoconstriction induced by similar stimulation of these nerves (in non-reserpinized animals) (Dorr & Brody, 1965). The release of acetylcholine for a brief period of time from these " cholinergic sympathetic " pathways could bring about one of several vascular responses dependent upon the simultaneous release of catecholamines. If activated alone they could bring about a transient cholinergic vasodilatation. If activated in conjunction with the adrenergic fibres they could bring about a more rapid and intense initial release of local stores of catecholamines *via* a local cholinergic-adrenergic interaction. Conversely, if these cholinergic fibres were inactive during adrenergic nerve activity, catecholamines from nearby sympathetic endings could bring about the release of acetylcholine which would tend to moderate the adrenergic response. Physiologically, a mechanism may exist for the potential fine adjustment of tissue perfusion from one discrete local area to another *via* this type of innervation. Such a mechanism would allow selective modification of peripheral blood flow by the central nervous system to meet moment-to-moment local requirements.

The inhibition observed in these experiments following hemicholinium could have been due to pharmacological blockade at either the cholinergic neuroeffector junction or at ganglia located either in the sympathetic chain or more peripherally. The small size of the sympathetic chain in the rat precluded stimulation of efferent autonomic fibres peripheral to the sympathetic chain. To determine if ganglionic blockade was initiated by hemicholinium, additional experiments were performed in which the sciatic nerve was stimulated. The sciatic nerve carries efferent autonomic nerves to the vascular bed as well as motor fibres to the muscles. These autonomic nerve fibres should be post-ganglionic. Gallamine, which blocks somatic neuroeffector junctions, was administered to eliminate movements during nerve stimulation. Nerve stimulation produced a vasoconstriction followed by vasodilatation of approximately equal magnitude. Since neither response was abolished by hexamethonium, both were presumably due to post-ganglionic stimulation. Hemicholinium followed by repeated nerve stimulation failed to inhibit the vasodilator response to sciatic nerve stimulation, but abolished the cholinergic responses of the " cholinergic sympathetic " endings. Failure to inhibit the cholinergic response in this experiment suggests that the acetylcholine release is not primarily from cholinergic autonomic endings but from " extra-autonomic " sources. A likely source of this " extra-autonomic " acetylcholine would be he somatic motor endings. This neural hormone, liberated by somatic neurone activity, could diffuse into the vasculature with a resultant vasodilatation.

Noradrenaline, acetylcholine and nicotine were administered as test drugs in each experimental situation to provide additional information about the mechanisms involved. In the rat, however, most drug responses varied quite widely from experiment to experiment.

The vascular responses to administration of noradrenaline became increasingly variable during the course of most experiments. The vessels were still responsive, however, to chemical adrenergic stimulation at the termination of each perfusion procedure.

The failure of hemicholinium significantly to alter the vasodilatation produced by acetylcholine in any of the experimental situations tested demonstrated the cholinergic receptors to be functional throughout the experiments. The administration of guanethidine and reserpine in combination and of hexamethonium in " sciatic nerve " preparations resulted in significant increases in the magnitude of the acetylcholine response. These changes may be due to alterations in sympathetic tone caused by the antagonist drugs administered or they could be artifacts resulting from the gradual rise in perfusion pressure baseline that occurred throughout most experimental procedures.

Nicotine has been reported by some workers to act post-ganglionically through a " cholinergic " mechanism (De Burgh Daly & Scott, 1961 ; Leaders, 1965). In the present experiments hemicholinium alone or in conjunction with sciatic or sympathetic chain stimulation was unable to block the vasodilator response to nicotine. Either of two mechanisms might explain the action of nicotine. First, nicotine could mimic acetylcholine by a direct action on the receptor, or, second, it could release endogenous acetylcholine from stores not depleted by hemicholinium and intermittent nerve stimulation. If the second hypothesis were true, the acetylcholine released could come from somatic neuroeffector junction stores.

SUMMARY

1. Hemicholinium was used to differentiate three hypotheses for the mechanism of adrenergic transmitter release, the classical concept, the " cholinergic-link " proposal of Burn & Rand (1959) and the " local cholinergic-adrenergic interaction " hypothesis of Leaders (1963).

2. Hemicholinium in these experiments selectively inhibited the cholinergic component in the innervation of the vasculature of the rear quarters of rats pretreated with reserpine and guanethidine. This inhibition was at a time and in a concentration which did not interfere with the adrenergic vasocostrictor resposes of these nerves.

3. The data suggest the presence of separate cholinergic and adrenergic fibres in the nerves to the vascular beds of the rear quarters of the rat. This is consistent with either the classical concept or the " local interaction " concept of adrenergic transmission.

4. A physiological function in peripheral vascular regulation is proposed for these cholinergic fibres which is consistent with the local cholinergic-adrenergic interaction hypothesis.

5. Hemicholinium, as used in these experiments failed to inhibit cholinergic vasodilatation following sciatic nerve stimulation in gallamine-treated preparations. The possibility that this vasodilatation may be caused by release of acetylcholine from somatic nerves is discussed.

6. Nicotine produced vasodilatation in these experiments which could not be inhibited by hemicholinium. Two mechanisms for the response to nicotine are discussed. One is through a direct action on the receptor, the other through release of acetylcholine stores not depleted by hemicholinium. The involvement of somatic stores of acetylcholine in the second mechanism is proposed.

REFERENCES

BIRKS, R. & MACINTOSH, F. C. (1961). Acetylcholine metabolism of a sympathetic ganglion. *Can. J. Biochem. Physiol.*, **39**, 787–827.

BÜLBRING, E. & BURN, J. H. (1935). The sympathetic dilator fibres in the muscles of the cat and dog. *J. Physiol.*, **83**, 483–501.

BURN, J. H. & RAND, M. J. (1959). Sympathetic postganglionic mechanism. *Nature, Lond.*, **184**, 163–165.

BURN, J. H. & RAND, M. J. (1960). Sympathetic postganglionic cholinergic fibres. *Br. J. Pharmac. Chemother.*, **15**, 56–66.

BURN, J. H. & RAND, M. J. (1965). Acetylcholine in adrenergic transmission. *A. Rev. Pharmac.*, **5**, 163–182.

DE BURGH DALY, M. & SCOTT, M. J. (1961). The effects of acetylcholine on the volume and vascular resistance of the dog's spleen. *J. Physiol.*, **156**, 246–259.

DORR, L. D. & BRODY, M. J. (1965). Functional separation of adrenergic and cholinergic fibers to skeletal muscle vessels. *Am. J. Physiol.*, **208**, 417–424.

FERRY, C. B. (1966). Cholinergic link hypothesis in adrenergic neuroeffector transmission. *Physiol. Rev.*, **46**, 420-456.

LEADERS, F. E. (1963). Local cholinergic-adrenergic interaction: Mechanism for the biphasic chronotropic response to nerve stimulation. *J. Pharmac. exp. Ther.*, **142**, 31–38.

LEADERS, F. E. (1965). Separation of adrenergic and cholinergic fibers in sympathetic nerves to the hind limb of the dog by hemicholinium (HC-3). *J. Pharmac. exp. Ther.*, **148**, 238–246.

LEADERS, F. E. & DAYRIT, C. (1965). The cholinergic component in the sympathetic innervation to the spleen. *J. Pharmac. exp. Ther.*, **147**, 145–152.

LEADERS, F. E. & FRAY, R. (1967). Study of the biphasic response of the dog's iris to nerve stimulation. *Archs int. Pharmacodyn. Ther.* In press.

LEADERS, F. E. & LONG, J. P. (1962). Mechanism of the positive chronotropic response to nicotine. *J. Pharmac. exp. Ther.*, **137**, 206–212.

LEADERS, F. E. & PAN, P. J. (1967). Cholinergic-adrenergic interaction in the dog salivary gland prepara tion. *Archs int. Pharmacodyn. Ther.* In press.

LINDGREN, P. & UVNÄS, B. (1953). Activation of sympathetic vasodilator and vasoconstrictor neurons by electric stimulation in the medulla of the dog and cat. *Circulation Res.*, **1**, 479–485.

LINDGREN, P. & UVNÄS, B. (1954). Postulated vasodilator center in the medulla oblongata. *Am. J. Physiol.*, **176**, 68–76.

LINDGREN, P. & UVNÄS, B. (1955). Vasoconstrictor inhibition and vasodilator activation—two functionally separate vasodilator mechanisms in the skeletal muscles. *Acta physiol. scand.*, **33**, 108–119.

LONG, J. P. (1961). Hemicholiniums: structure activity relationships and actions on the peripheral nervous system. *Fedn Proc. Fedn Am. Socs exp. Biol.*, **20**, 583–586.

LONG, J. P. & SCHUELER, F. W. (1954). A new series of cholinesterase inhibitors. *J. Am. pharm. Ass., Sci. Ed.*, **43**, 79–86.

MACINTOSH, F. C. (1961). Effect of HC-3 on acetylcholine turnover. *Fedn Proc. Fedn Am. Socs exp. Biol.*, **20**, 562–568.

ROBERTS, J. & STADTER, R. P. (1960). Effect of reserpine on ventricular escape. *Science, N.Y.*, **132**, 1836–1837.

VOLLE, R. L. (1963). Pharmacology of the autonomic nervous system. *Rev. Pharmac.*, **3**, 129–152.

WILSON, W. & LONG, J. P. (1959). The effect of hemicholinium (HC-3) at various peripheral cholinergic transmitting sites. *Archs int. Pharmacodyn. Ther.*, **120**, 343–352.

ZAIMIS, E. (1964). Pharmacology of the autonomic nervous system. *A. Rev. Pharmac.*, **4**, 365–400.