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THE ADRENERGIC MECHANISM IN THE NICTITATING MEMBRANE

BY

J. H. BURN, M. J. RAND AND R. WIEN

From the Biological Research Laboratories, May & Baker, Dagenham

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The contractions of the nictitating membrane in response to postganglionic nerve stimulation have been studied in experiments in which cats' heads were perfused. When eserine was added to the perfusion fluid there was a rapid increase in the size of the contractions. In the presence of eserine the contractions produced by injecting acetylcholine into the perfusion fluid were greatly increased. When atropine was injected into the perfusion fluid the contractions caused by postganglionic nerve stimulation returned to the size before eserine was added. In experiments with cats anaesthetized with chloralose, atropine or hyoscine was given first and the effect of eserine on the response to submaximal postganglionic nerve stimulation was determined. Eserine slowly increased the responses to stimulation without increasing the contraction produced by injecting noradrenaline. In other experiments in which maximal stimuli were used, the relation of stimulus frequency to height of contraction was determined. The optimal frequency was low, being 5 to 10 shocks/sec. In the presence of hyoscine, eserine or neostigmine increased the response to stimulation; this increase was greater at lower frequencies, and lessened as the frequency rose to the optimal value.

Previous observations with the nictitating membrane have not shown very clearly that contractions due to postganglionic nerve stimulation were increased in the presence of eserine. Bacq & Fredericq (1935) first observed an increase (using preganglionic nerve stimulation), though it was small. Burn & Rand (1960) carried out experiments with cats previously treated with reserpine, and again the increase in contraction after the injection of eserine was slight. If many cholinergic nerve fibres are present in the nerve supply to the nictitating membrane, there should be no difficulty in demonstrating a considerable effect of eserine.

Observations have therefore been made with the perfused cat's head as described by Burn & Trendelenburg (1954). Experiments have also been performed with anaesthetized cats in which atropine or hyoscine was given first, and later eserine or neostigmine, to see if eserine potentiated any nicotine-like action which acetylcholine, released by postganglionic nerve stimulation, might have in liberating noradrenaline.

METHODS

In perfusion experiments, the fluid was a 3:1 mixture of Locke solution (NaCl, 9 g; KCl, 0.42 g; $CaCl_2$, 0.24 g; $NaHCO_3$, 0.5 g; dextrose, 1 g; in 1 l. of water) and 6% dextran solution, well-oxygenated beforehand in a reservoir at 37° C. The cat was anaesthetized with chloralose (80 mg/kg, intravenously). The contractions of the right nictitating membrane

were recorded by attaching the membrane to an isotonic lever fitted with a frontal writing point. The magnification was about 7 times. Both external jugular veins and both common carotid arteries were dissected clear from other tissues and two ligatures were passed around each of these vessels. The next steps were taken rapidly. A clip was put on one jugular vein and a cannula was inserted below the clip leading away from the head. The other vein was treated similarly. Arterial cannulae pointing towards the head were then placed in the two carotid arteries. A Dale-Schuster pump was used to pump the perfusion fluid to a Y-piece, the ends of which were connected to the two carotid cannulae by polyethylene tubing. Polyethylene tubes were joined to each venous cannula and led the effluent fluid to a collecting jar. A 4-way cannula was placed in the path from the pump to the Y-piece, immediately before the Y-piece, so that a thermometer could be inserted and perfusion pressure recorded. The time from the interruption of the natural circulation to the beginning of the perfusion was 10 to 15 min, and observations were almost always completed in 20 min from the start of the perfusion.

Shielded electrodes were placed on the postganglionic nerves just beyond the superior cervical ganglion. The nerve was stimulated with rectangular pulses, from a constant voltage stimulator at a frequency of 10 to 20 shocks/sec and duration of 1 to 2 msec. The voltage of the pulses was below that required to give a maximal contraction. Trains of pulses were applied for 5 to 10 sec every 2 min.

Other experiments were made with cats, anaesthetized with chloralose, and with intact circulations. In three of these experiments the preganglionic nerve fibres were divided on one side in an aseptic operation 5 to 7 days before the final experiment. Shielded electrodes were applied just beyond the superior cervical ganglion. In the first group of experiments submaximal stimuli were used at a frequency of 10 shocks/sec and a pulse duration of 0.5 msec. Stimulation was applied for 10 sec at 2 min intervals. In the second group of experiments, maximal stimuli of 0.5 msec pulse duration were used at various frequencies, the total number of shocks being kept constant at 100 pulses. In both groups of experiments atropine sulphate (1 mg/kg) or hyoscine hydrobromide (0.1 mg/kg) was initially injected intravenously and then, 20 min later, eserine sulphate or neostigmine methylsulphate (0.5 mg/kg) was injected. Tests were made to ensure that the direct action of acetylcholine on the nictitating membrane was excluded; in these, 0.1 or 0.2 mg of acetylcholine chloride was injected. Amounts of drugs referred to in Results are in terms of the salts mentioned above.

RESULTS

Perfusion experiments

In the first 10 min of the perfusion, the contractions resulting from submaximal postganglionic nerve stimulation decreased in size, rapidly at first and then more slowly. When they were approximately constant, the perfusion fluid was changed to one containing eserine sulphate (10^{-6} g/ml.); the change was effected without stopping the perfusion or altering the pump. The contractions in response to submaximal postganglionic nerve stimulation began to increase in height almost at once. Fig. 1 shows results from three experiments. In the top record, A shows the contractions before eserine was added. Eserine was then added, and 8 min later the contractions were 80% greater (B). Atropine sulphate (1 mg) was then injected into the perfusion fluid, and the contraction was at once reduced to the original height (C).

In a second experiment, illustrated in the middle record, A shows the contractions in response to the injection of 25 μg of acetylcholine into the perfusion fluid and to nerve stimulation. The perfusion fluid was changed to one containing eserine (B), and the contraction due to stimulation increased by 40% while that to acetylcholine

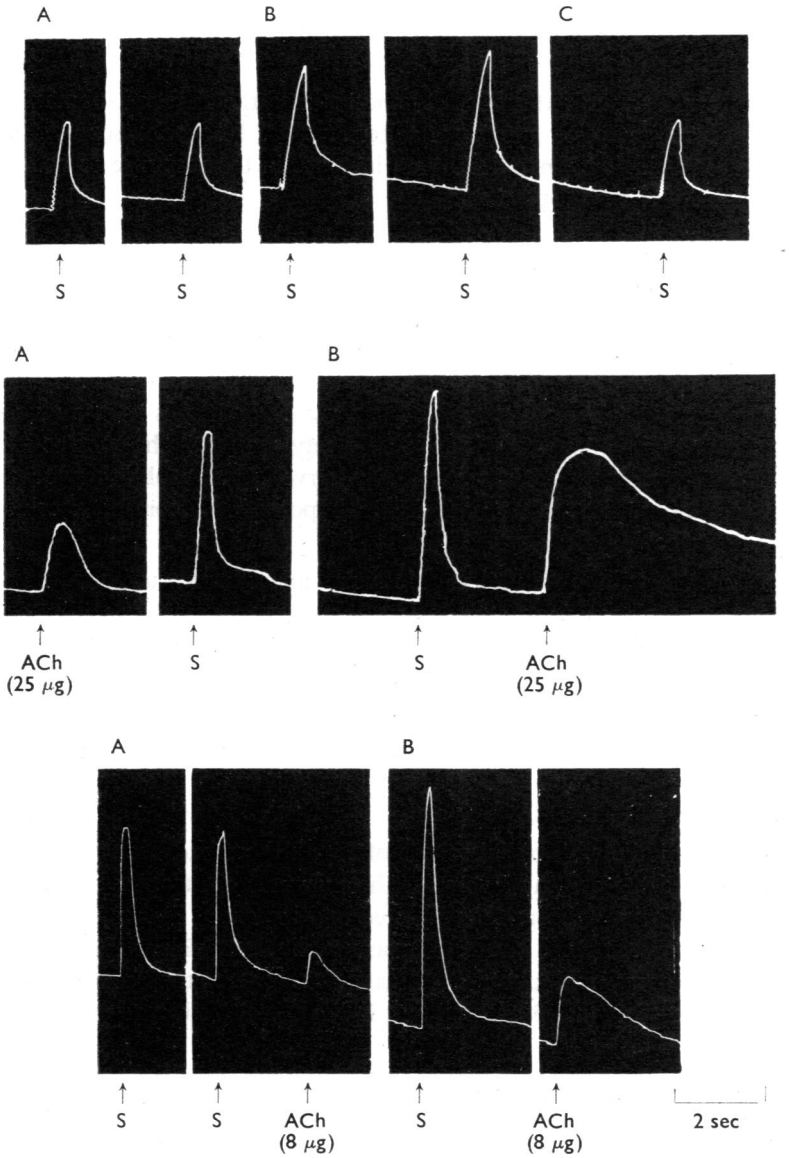

Fig. 1. Contractions of the nictitating membrane in response to submaximal postganglionic nerve stimulation at 10 shocks/sec (S) for 10 sec every 2 min during perfusion of the cat's head with dextran-Locke solution. Top record: A, initial responses; B, responses during perfusion with eserine (10^{-6} g/ml.); C, response after the injection of atropine (1 mg) into the perfusing fluid. Middle record: A, initial contractions in response to acetylcholine (25 μg) injected into the perfusion fluid and in response to postganglionic nerve stimulation (S); B, contractions during perfusion with eserine (10^{-6} g/ml.). Bottom record: A, initial contractions in response to stimulations (S) and to acetylcholine (8 μg); B, contractions in response to nerve stimulation and to acetylcholine (8 μg) during perfusion with eserine (10^{-6} g/ml.).

increased in height and about five-fold in duration. The lowest record, taken from a third experiment, shows that eserine increased the response to nerve stimulation by 60% and that to acetylcholine about five-fold.

These observations showed that eserine potentiated the effect of submaximal postganglionic nerve stimulation applied at a frequency of 10 shocks/sec by 40 to 80%, the potentiation being accompanied by an increase in the response to acetylcholine, and being abolished by atropine.

Anaesthetized cats

The action of eserine after atropine with submaximal nerve stimulation. According to the hypothesis of Burn & Rand (1959), the acetylcholine released by cholinergic fibres in sympathetic nerves may act directly, but its principal function is to liberate noradrenaline. One way of testing the hypothesis was to give atropine and so exclude the direct muscarinic action of acetylcholine. The contraction caused by stimulating a postganglionic sympathetic nerve trunk could then be due only to the release of noradrenaline and, if the hypothesis is correct, eserine should potentiate the effect.

In experiments with cats under chloralose anaesthesia, uniform submaximal contractions were obtained on stimulation of postganglionic nerve fibres and then either atropine (1 mg/kg) or hyoscine (0.1 mg/kg) was injected intravenously.

Fig. 2 shows the results in three experiments. In the top record, taken from an experiment with a cat in which the preganglionic fibres to the membrane had been divided 6 days previously, the responses to submaximal stimulation (A) were reduced after the injection of atropine (B). Eserine was injected, and the responses then increased almost 2.5 times (C).

For both the middle and lower records of Fig. 2 there had been no preganglionic nerve section, and the contractions after the injection of atropine are those shown in B. Eserine (0.5 mg/kg) was then injected and increased the response to stimulation (C). This increase came on slowly, was usually first evident after 15 or 20 min, and continued progressively during the next hour. In the top record of Fig. 2 (C), the maximum increase was 130%, and that in the other two records was 38 and 40% respectively.

Effect of eserine on the response to noradrenaline. The increased contractions after eserine (Fig. 2) might have been the result of potentiating the response to noradrenaline. Burn, Philpot & Trendelenburg (1954) showed that eserine increased the effect of adrenaline on the nictitating membrane of the spinal cat. However, they also showed that this increase disappeared after atropine was injected, and thus it was very unlikely that the effect of eserine on nerve stimulation in the presence of atropine was to be explained in this way. In order to eliminate this possibility several experiments were performed in which contractions of the nictitating membrane were obtained by injecting adrenaline or noradrenaline; the effect of giving atropine first and eserine afterwards was then determined. We usually observed a decline of about 10% in contraction height after atropine (1 mg/kg), and a gradual return to the original height some time after eserine (0.5 mg/kg).

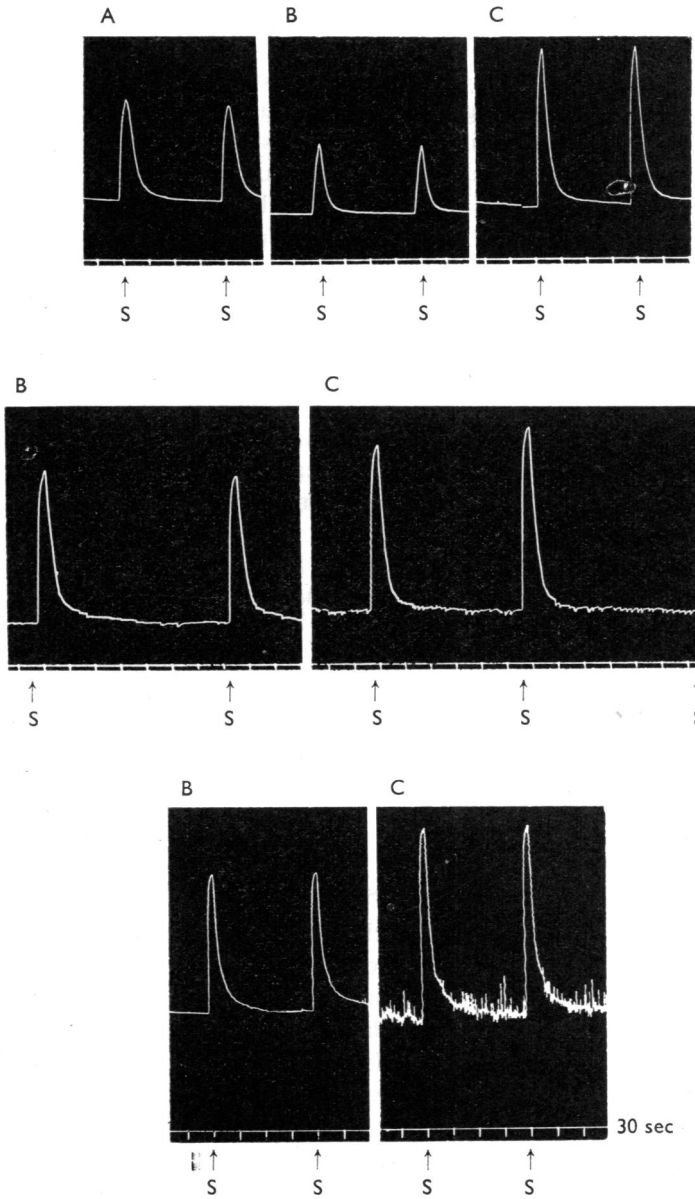

Fig. 2. Contractions of the nictitating membrane in cats anaesthetized with chloralose. Top record: A, initial responses to submaximal postganglionic nerve stimulation (S) in a preparation in which the preganglionic fibres had been cut 6 days previously; B, responses after injection of atropine (1 mg/kg); C, responses after injection of eserine (0.5 mg/kg). Middle and lower records: B, responses after atropine, and C after eserine (0.5 mg/kg), in preparations which had not been denervated.

In other experiments we compared initially the effects of noradrenaline and of submaximal postganglionic nerve stimulation. Then we gave hyoscine, and later eserine. We found an increase in the response to submaximal stimulation after eserine, but no increase in the effect of noradrenaline. In Fig. 3, eserine increased the effect of submaximal postganglionic nerve stimulation by 66% (D), but did not increase the effect of noradrenaline (E) which was less than at first. In another experiment eserine increased the effect of postganglionic nerve stimulation by 110%, but did not increase the effect of noradrenaline. Thus the increase in the response to submaximal stimulation caused by eserine in the presence of atropine or hyoscine was not due to the potentiation of the response to noradrenaline, and was therefore probably due to an increase in the amount of noradrenaline liberated.

Maximal stimulation. Observations were also made using maximal stimuli, and the effect of the same number of shocks was determined at different frequencies. The lowest frequency which gave a maximal contraction was unexpectedly low. As shown in Table 1, this frequency was 5 shocks/sec in experiment 2, and in experiment 3 also there was not much increase in the contractions at higher frequencies. When hyoscine was injected (0.1 mg/kg) the contractions in response to the higher frequencies were unchanged, but those with lower frequencies were reduced. A similar observation has been made for contractions of the vas deferens in response to hypogastric nerve stimulation (Burn & Weetman, 1963).

When eserine or neostigmine (0.5 mg/kg) was injected, the contractions increased in height, the increase being greatest for the lowest stimulus frequency and becoming smaller as the frequency rose (Figs. 4 and 5). This change was easily seen when the increase in contraction at each frequency was expressed as a percentage of the contraction before eserine or neostigmine was added. In experiment 1 (Table 1), the increase at a frequency of 1 shock/sec was 61% ; the increase became less as the frequency rose until at 20 shocks/sec it was only 2%.

DISCUSSION

Thompson (1958) showed that acetylcholine has two actions on the isolated nictitating membrane. He observed that low concentrations of acetylcholine caused a contraction which was greatly potentiated by eserine. This contraction was abolished by atropine. However, when he raised the acetylcholine concentration from 0.2 μg/ml. to 10 μg/ml., he obtained a contraction in the presence of atropine which was approximately halved by hexamethonium. Thus Thompson (1958) showed that acetylcholine had both a muscarine-like and a nicotine-like action on the nictitating membrane. Later it was shown that nicotine contracted the isolated membrane, though not after degeneration of the nerves nor if it was taken from a cat treated with reserpine (Burn, Leach, Rand & Thompson, 1959).

Thus by its nicotine-like behaviour acetylcholine may be presumed to act by the release of noradrenaline. Trendelenburg (1962) concluded from experiments that it was " most unlikely " that acetylcholine contracted the nictitating membrane by the release of noradrenaline. However, his results showed that the relation between the dose of acetylcholine and the contraction of the membrane was different for the normal membrane compared with the membrane from a cat treated with reserpine.

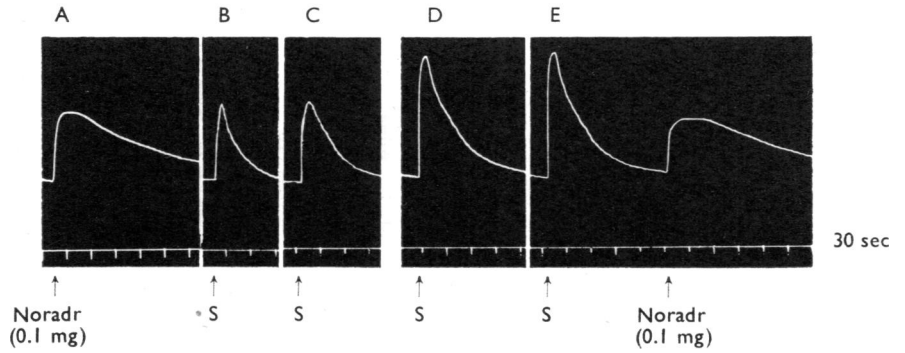

Fig. 3. Cat, chloralose anaesthesia. Preganglionic nerve fibres had been cut 6 days previously. A, contraction in response to 0.1 mg of noradrenaline. B and C, contractions in response to submaximal postganglionic nerve stimulation at 10 shocks/sec for 10 sec, 0.5 msec, 3 V. Hyoscine (0.2 mg/kg) was given before B, and eserine (0.5 mg/kg) before D. Finally, 0.1 mg of noradrenaline was given during E.

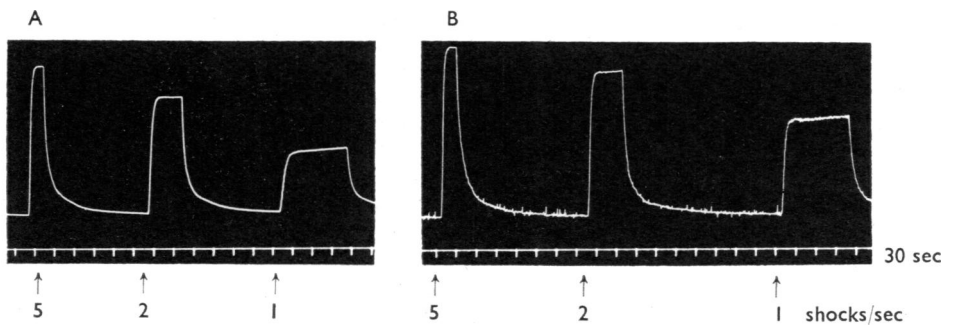

Fig. 4. Cat, chloralose anaesthesia. Observations with maximal stimuli, giving groups of 100 shocks at the frequencies shown, after hyoscine (0.1 mg/kg). A, Before eserine; B, after injection of eserine (0.5 mg/kg). The increase in contraction caused by eserine was greatest for a frequency of 1 shock/sec, and least for a frequency of 5 shocks/sec.

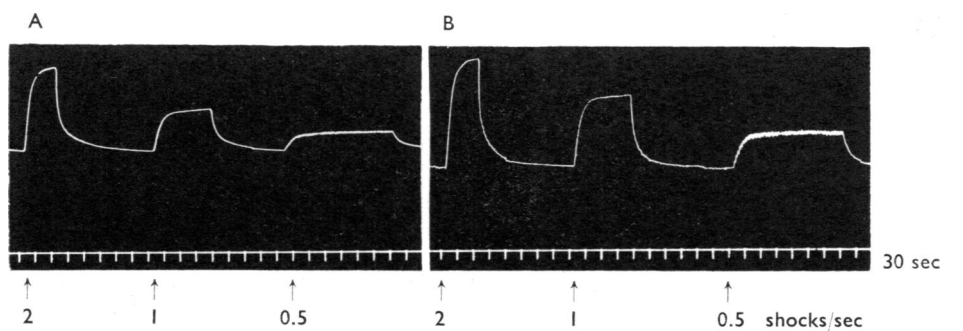

Fig. 5. As Fig. 4, but A, observations before neostigmine; B, observations after the injection of neostigmine (0.5 mg/kg).

TABLE 1

CONTRACTIONS OF NICTITATING MEMBRANE IN RESPONSE TO MAXIMAL STIMULATION OF THE POSTGANGLIONIC NERVE TRUNK AT DIFFERENT STIMULUS FREQUENCIES AFTER THE INJECTION OF HYOSCINE

Contractions (in mm) were measured before and after the injection of eserine (experiments 1 and 2) or neostigmine (experiments 3 and 4). 100 shocks were given at each stimulation

Stimulus frequency (shocks/sec)	Experiment 1			Experiment 2			Experiment 3			Experiment 4		
	Control	After eserine	Increase (%)	Control	After eserine	Increase (%)	Control	After neostigmine	Increase (%)	Control	After neostigmine	Increase (%)
0·5	—	—	—	12	18	50	3·5	8	120	—	—	—
1	18	29	61	26	31	19	9·5	16·5	73	—	—	—
2	35	43	23	38	42	10	19·5	25·5	30	11	18	63
5	45	51	13	48	48	0	25	27	8	28·5	34	19
10	47	51	8	49	49	0	26·5	28	5	35	38	9
20	51	52	2	49	49	0	28	29	3	—	—	—

For example, the contraction produced by the highest dose of acetylcholine was twice as great in the normal membrane as in the reserpine-treated membrane. Further, Trendelenburg (1962) observed that, in the presence of phenoxybenzamine, the dose-response curve for acetylcholine in the normal membrane was shifted towards the curve for the reserpine-treated membrane. Thus his evidence, in fact, supports the view that, in high concentrations, part of the effect of acetylcholine on the nictitating membrane is due to the release of noradrenaline.

In this paper we have further evidence for the presence of cholinergic fibres in the nerve supply to the membrane. Burn & Rand (1958) showed that stimulation of postganglionic fibres contracted the membrane of a reserpine-treated cat, when tyramine (4 mg) failed to cause a contraction. Since there is now proof that tyramine releases noradrenaline (Schümann & Weigmann, 1960; Lockett & Eakins, 1960; Lindmar & Muscholl, 1961) the failure of tyramine to cause a contraction indicated the absence of noradrenaline. Therefore the contraction caused by nerve stimulation was probably due to the release of acetylcholine. Later Burn & Rand (1960) showed that this contraction was abolished by atropine in the reserpine-treated cat. By using the perfused head of the cat we have now demonstrated that the contraction caused by submaximal stimulation is considerably potentiated by eserine, and that the increase in the contraction disappears when atropine is injected. Thus it appears that cholinergic fibres leave the superior cervical ganglion for the nictitating membrane of the cat, just as they leave it for the mucous membrane of the dog's lip (Euler & Gaddum, 1931) and for the blood vessels of the rabbit ear (Burn & Rand, 1960; Holton & Rand, 1962). Ambache (1951) found that botulinum toxin injected into the nictitating membrane reduced the contraction in response to postganglionic nerve stimulation; he suggested that paralysis of cholinergic fibres to the membrane might explain his finding.

Our results support the view put forward by Burn & Rand (1959) that an impulse passing along a postganglionic sympathetic nerve fibre might release acetylcholine, which in its turn might liberate noradrenaline. Thus it has been shown that, when the direct muscarinic action of acetylcholine was prevented by atropine or by hyoscine, eserine or neostigmine increased the contraction caused by postganglionic nerve stimulation. Since, in the presence of atropine or hyoscine, eserine did not increase the contraction produced by an injection of noradrenaline, the increased contraction in response to nerve stimulation after eserine must have been due to the liberation of more noradrenaline. The effects of eserine and of neostigmine therefore imply that the noradrenaline is released by acetylcholine.

The difference of opinion concerning the presence of cholinesterase in the nictitating membrane is of interest in this connexion. Burn & Philpot (1953) published the results of manometric experiments which showed the presence of both specific and non-specific cholinesterase. They compared the right and left membranes of twenty cats, examining them after removing the Harderian gland, and used acetylcholine, acetyl-β-methylcholine and butyrylcholine as substrates; thus they made 120 estimations. They observed considerable variation between different cats, but had good agreement between the right and left membranes of each cat. Thompson (1958) showed that eserine potentiated the effect of acetyl-

choline in the isolated nictitating membrane. In spite of these results, Hellmann & Thompson (1961) failed to find evidence that cholinesterase was present in the nictitating membrane, and concluded that it was absent; they stained for cholinesterase by the thiocholine method. It is becoming increasingly obvious from the results of various workers that the histochemical method cannot prove the absence of cholinesterase, and that precedence must be given to a biochemical method. However, it is evident both from the work of Burn & Philpot (1953) and from that of Hellmann & Thompson (1961) that the amount of cholinesterase present is small.

The optimal stimulus frequency of some postganglionic sympathetic nerve fibres is high. Thus Gillespie & Mackenna (1961), with the isolated rabbit colon, used a stimulus frequency for sympathetic nerves of 50 shocks/sec, while for parasympathetic nerves they used 10 shocks/sec. Since noradrenaline is more stable than acetylcholine, this difference in optimal stimulus frequency is the reverse of what would be expected on the assumption that a sympathetic nerve impulse releases noradrenaline directly. However, if a certain concentration of acetylcholine is necessary to release noradrenaline, then a high stimulus frequency may be necessary to enable the acetylcholine to accumulate. At a high stimulus frequency, the cholinesterase would have less time to act, and the frequency necessary to liberate a large amount of noradrenaline would depend on the amount of cholinesterase present. If there was much cholinesterase, the optimal frequency would be high, but if there was very little cholinesterase the optimal frequency might be quite low. In the nictitating membrane, the optimal frequency is sometimes as low as 5 shocks/sec, and we know that the amount of cholinesterase is small.

Eserine and neostigmine had their greatest effect in increasing the contractions in response to stimulation at the lowest stimulus frequency, and the effect became smaller as the frequency rose. That is to say, the effect of a very low stimulus frequency could be increased either by raising the frequency, which would give cholinesterase less time to act, or by adding eserine which would inhibit the cholinesterase.

On stimulating the hypogastric nerve and recording contractions of the vas deferens, Burn & Weetman (1963) found that, when eserine was added, the response to stimulation was increased when the stimulus frequency was low, but was reduced when the frequency was high. There was probably a block due to excess of acetylcholine. We have not observed the depression in our experiments on the nictitating membrane, though Gardiner, Hellman & Thompson (1962) have done so.

Gardiner & Thompson (1961) found that hemicholinium failed to depress the response of the isolated nictitating membrane to nerve stimulation. Chang & Rand (1960) reported that hemicholinium caused a failure of response to sympathetic nerve stimulation in a number of other isolated organ preparations, but there was a considerable variation in susceptibility between different organs. Furthermore Bowman & Rand (1961) found that hemicholinium more readily caused failure of the responses to motor nerve stimulation of the cat's tibialis anterior than of the soleus muscle, and they suggested that this difference might be related to the fact that the soleus muscle, being concerned with posture, is adapted for prolonged activity, and might contain greater reserves of acetylcholine or a higher capacity

for its synthesis. The nictitating membrane may be resistant to hemicholinium for the same reason, for when a cat is awake the membrane is always contracted.

Nystrom (1962) has recently concluded that there are two separate sets of fibres supplying the nictitating membrane, one cholinergic and one adrenergic. Nystrom (1962) recorded changes in electrical potentials in the nictitating membrane when its postganglionic nerve fibres were stimulated and observed a double response, an initial wave which was increased by eserine 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 acetylcholine and the second to release of noradrenaline. Because the waves were separated in time he thought that there was a dual innervation of the membrane. The observations do not require two sets of nerve fibres ; they are equally well explained by a single set of nerve fibres which release acetylcholine, which acts first directly and later, when its concentration is sufficiently high, releases noradrenaline. Nystrom (1962) did not test the effect of eserine in the presence of atropine.

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