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# THE ANTICHOLINESTERASE ACTIVITY OF SOME ANTIADRENALINE AGENTS

BY

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The contractions of the isolated guinea-pig vas deferens in response to stimulation of the sympathetic hypogastric nerve were potentiated by low concentrations and inhibited by high concentrations of the antiadrenaline agents tolazoline, yohimbine, ergotamine, phenoxybenzamine and piperoxan. Eserine potentiated the contractions of the vas deferens produced by hypogastric nerve stimulation. The cholinesterase activity of an extract of vas deferens was decreased by the antiadrenaline agents. The potentiation of responses to sympathetic stimulation by antiadrenaline drugs, which also possess anticholinesterase activity, can be explained on the basis of a cholinergic sympathetic mechanism.

The effects of antiadrenaline agents on the responses to stimulation of a sympathetic nerve do not always run parallel to their effects on responses induced by noradrenaline. Thus Varagić (1956b) found that tolazoline potentiated the response of the rabbit uterus to sympathetic nerve stimulation, and Huković (1959) reported that phenoxybenzamine increased the response to sympathetic nerve stimulation in the isolated atria of the rabbit.

We have investigated the effects of tolazoline and other antiadrenaline agents on the responses of the isolated vas deferens to sympathetic nerve stimulation, noradrenaline and acetylcholine, and on the cholinesterase activity of the vas deferens.

## METHODS

*Isolated Innervated Vas Deferens.*—The isolated vas deferens with intact hypogastric nerve was prepared by the method of Huković (personal communication). Guinea-pigs weighing about 500 g. were killed by a blow on the head. The abdomen was opened in the midline and the distal colon retracted to one side. The hypogastric nerves were identified and dissected free. The vasa deferentia were cut from their attachments to the epididymis at one end and the urethra at the other and removed, each with its accompanying nerve. The vas deferens was mounted in a 50 ml. organ bath containing McEwen's (1956) solution gassed with 95% oxygen and 5% carbon dioxide and maintained at 29°. The nerve was passed through a stimulating electrode of the type described by Burn and Rand (1960a) consisting of a 1 mm. tube containing 2 adjacent platinum rings. Stimulation was by 2 msec. pulses at a frequency of 10/sec. applied at supramaximal strength (usually 3.0 mA.) for 10 sec. in every 2 min. The time cycle of stimulation was automatically controlled. Contractions were recorded by an isotonic frontal writing lever.

*Isolated Vas Deferens.*—For experiments in which nerve stimulation was not required the vas deferens was suspended in a 5 ml. bath.

*Cholinesterase Activity.*—The vasa deferentia from a guinea-pig were ground with Tyrode solution (11 ml.) in a mortar. The whole extract was divided into 2 ml. aliquots to serve as source of enzyme in each reaction mixture. The drugs examined for anticholinesterase activity were mixed with the enzyme 10 min. before the addition of substrate and brought to 32°. Acetylcholine as substrate was added to reach a final concentration of 0.5 $\mu$g./ml. in a final volume of reaction mixture of 5 ml. At various time intervals after this, the reaction mixture was agitated and 0.1 ml. samples were withdrawn and assayed for acetylcholine on a strip of guinea-pig ileum suspended in Tyrode solution in a 5 ml. bath. A dose-response curve for acetylcholine was obtained upon the ileum at the beginning and the end of each experiment.

## RESULTS

*Sympathetic Stimulation.*—The contractions of the isolated vas deferens in response to stimulation of the sympathetic hypogastric nerve for 10 sec. at 2 min. intervals maintained a steady amplitude over long periods of time. The vas deferens was contracted by acetylcholine and noradrenaline.

When we attempted to block the response to sympathetic stimulation with the antiadrenaline

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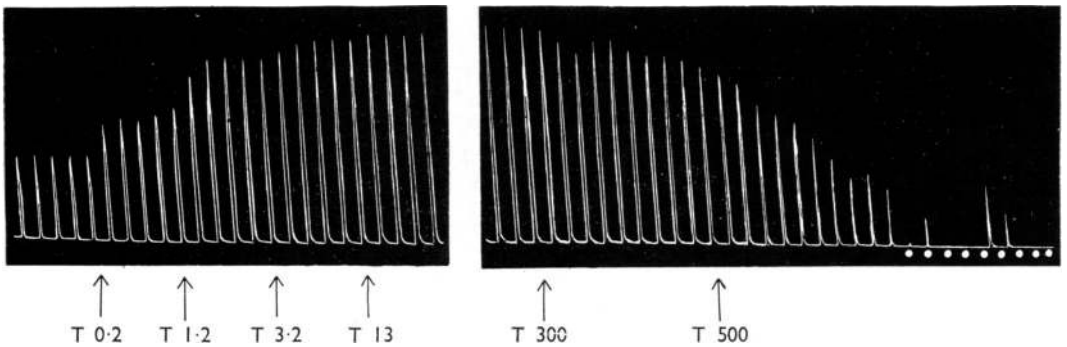


FIG. 1.—Contractions of the isolated guinea-pig vas deferens in response to hypogastric nerve stimulation at 2 min. intervals (white dots indicate stimulation not followed by contractions). At T, tolazoline was added to the bath to produce the concentrations indicated in μg./ml.

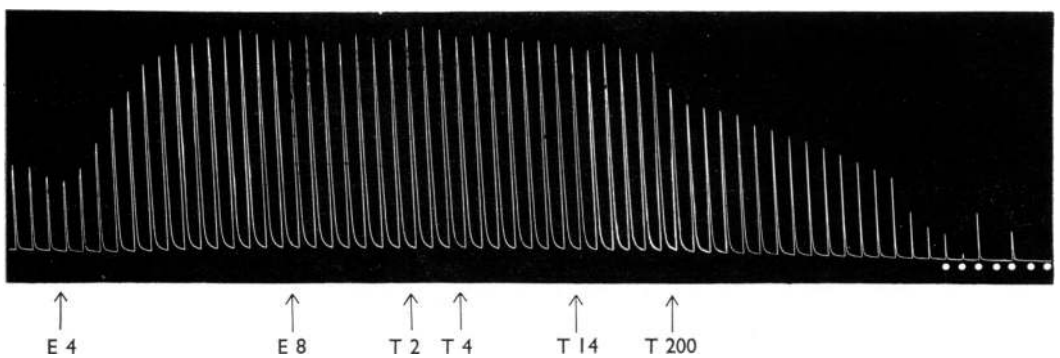


FIG. 2.—As Fig. 1. Eserine was added to the bath at E, and tolazoline at T, to produce the concentrations indicated in μg./ml.

agent tolazoline, it was observed that low doses of tolazoline produced a potentiation of the response (Fig. 1). The concentration of tolazoline required to inhibit the response to nerve stimulation was several hundred times higher than the dose which produced a clear potentiation. Thus in Fig. 1 a potentiation of the response can be seen with 0.2 μg./ml., whereas 500 μg./ml. was required to block.

In other experiments we had found that the response of the vas deferens to sympathetic nerve stimulation was potentiated by eserine. This observation led us to consider that the potentiation of the responses to sympathetic nerve stimulation that we had observed with low doses of tolazoline may be explained if it too possessed an anti-cholinesterase action. In the experiment shown in Fig. 2 the responses of the vas deferens to nerve stimulation were potentiated by eserine in a concentration of 4 μg./ml. An additional 4 μg./ml. when added to the bath had no further

TABLE I

CONCENTRATIONS OF ANTIADRENALINE AGENTS TO POTENTIATE AND TO INHIBIT RESPONSES OF VAS DEFERENS TO HYPOGASTRIC NERVE STIMULATION

| | Concentration of Drug Required to Produce | | Maximum Potentiation % |
|---|---|---|---|
| | Potentiation (μg./ml.) | Block (μg./ml.) | |
| Tolazoline .. .. | 0·1–100 | 500 | 280 |
| Phenoxybenzamine | 1·0–30 | 130 | 140 |
| Yohimbine .. | 0·2–1·0 | 30 | 200 |
| Ergotamine .. | 3·0–30 | 70 | 150 |
| Piperoxan .. .. | 0·2–20 | 100 | 150 |

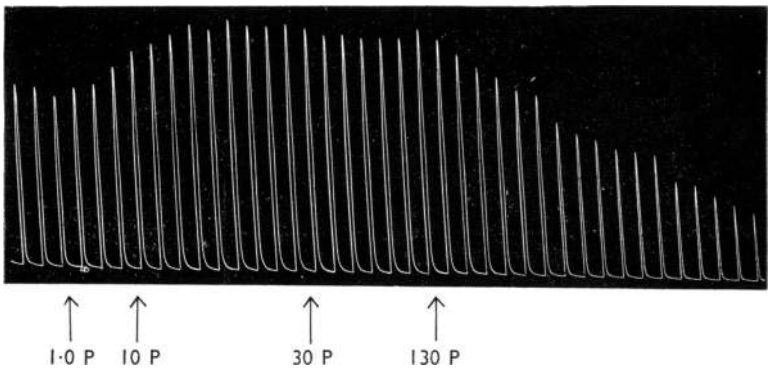


FIG. 3.—As Fig. 1. Phenoxybenzamine was added to the bath at P to produce the concentrations indicated in μg./ml.

effect, thus showing that the potentiation due to inhibition of cholinesterase was complete. Gradually increasing concentrations of tolazoline were next added to the bath without causing any further potentiation of the responses.

The effect of low doses of other antiadrenaline agents was tested on the responses of the vas deferens to hypogastric nerve stimulation. The results obtained with phenoxybenzamine are shown in Fig. 3. The responses were potentiated by a low dose of phenoxybenzamine (1.0 μg./ml.), a higher dose (30 μg./ml.) had no further effect, and finally at a still higher dose (130 μg./ml.) the responses gradually decreased until there was complete block. Similar results were obtained for yohimbine, piperoxan and ergotamine. The findings are summarized in Table I.

*Noradrenaline and Acetylcholine.*—The contractions of the isolated vas deferens in response to acetylcholine added to the bath were potentiated by tolazoline, but the responses to noradrenaline showed no sign of potentiation. Thus in Fig. 4, 30 μg./ml. of tolazoline inhibited the response to noradrenaline but potentiated the response to

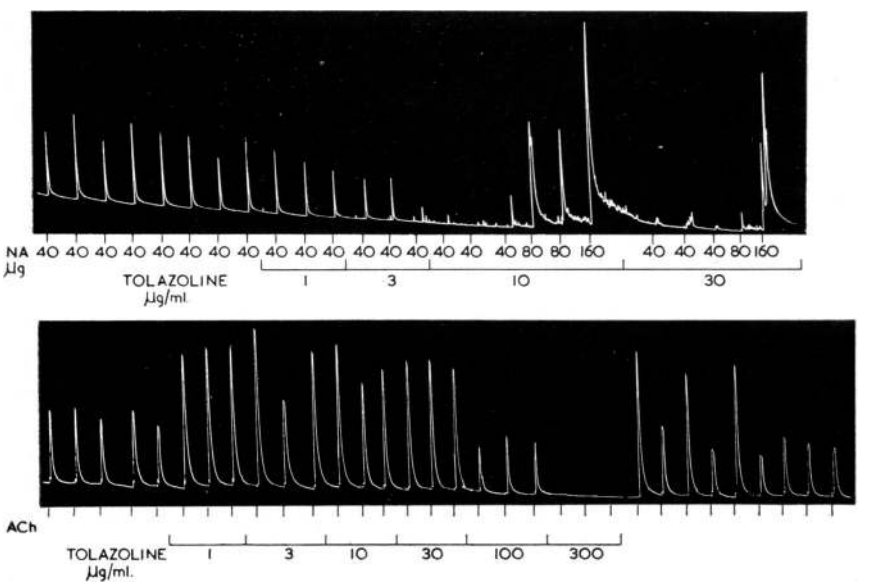


FIG. 4.—Effect of tolazoline on responses of the isolated guinea-pig vas deferens to noradrenaline and acetylcholine. In the upper tracing, the responses to 40 μg. of noradrenaline were abolished by 10 μg./ml. of tolazoline, although higher doses of noradrenaline (80 μg. and 160 μg.) were still effective. In the lower tracing the responses to acetylcholine (5 μg.) were potentiated by 1 to 30 μg./ml. of tolazoline, and inhibited by 300 μg./ml.

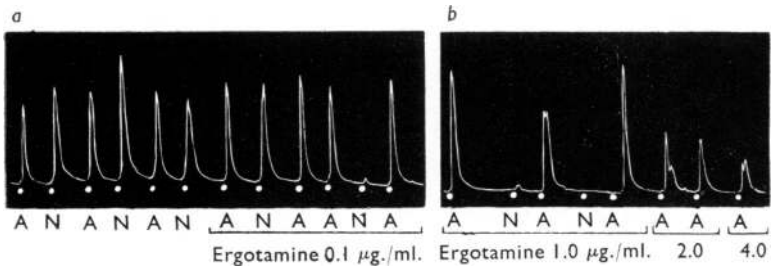


FIG. 5.—Effect of ergotamine on responses of the isolated guinea-pig vas deferens to acetylcholine (A, 0.2 μg./ml.) and noradrenaline (N, 2 μg./ml.).

acetylcholine. The block of noradrenaline by tolazoline was readily overcome by larger doses of noradrenaline.

Ergotamine in concentrations of 0.1 to 1.0 μg./ml. potentiated the responses to acetylcholine as shown in Fig. 5. In higher concentrations (2.0 to 4.0 μg./ml.), ergotamine exhibited an atropine-like action in diminishing the responses to acetylcholine. Similar results were obtained with phenoxybenzamine, piperoxan and yohimbine; low concentrations potentiated the contractions produced by acetylcholine, while higher concentrations reduced them.

We observed no potentiation of the contractions of the vas deferens in response to noradrenaline with ergotamine (Fig. 5), phenoxybenzamine, piperoxan or yohimbine.

*Atropine and Eserine.*—It is apparent that the antiadrenaline agents which we have investigated possess an atropine-like action in that they depress the response of the vas deferens to acetylcholine.

Our findings with tolazoline had led us to suppose that the potentiation of the responses of the vas deferens to hypogastric nerve stimulation could be attributed to its anticholinesterase activity. Since the antiadrenaline agents potentiated the responses to hypogastric nerve stimulation, but in addition possessed an atropine-like action, we investigated the effect of atropine on the potentiation of the responses to hypogastric nerve stimulation produced by eserine. In Fig. 6 the responses to hypogastric nerve stimulation were reduced in the presence of 0.1 μg./ml. of atropine, which was a sufficient dose to block completely the response to acetylcholine. Eserine (4 μg./ml.) when added to the bath still potentiated the responses to sympathetic nerve stimulation.

Fig. 7 illustrates an experiment in which dibenzyline produced a potentiation of the responses of the vas deferens to nerve stimulation although the dose of phenoxybenzamine used was sufficient to abolish the responses to both acetylcholine and noradrenaline.

*Anticholinesterase Activity.*—There were grounds for considering that the action of low doses of the antiadrenaline agents in potentiating the responses of the vas deferens to hypogastric nerve stimulation was due to their anticholinesterase activity. We selected a biological assay method for determining residual acetylcholine in the enzyme reaction mixture since it was the only way in which we could test enzyme activity on low concentrations of substrate. Thus, the acetylcholine content of the reaction mixture was $2.3 \times 10^{-6}$ M; for the manometric determination of anticholinesterase activity concentrations of $1 \times 10^{-2}$ M have generally been employed.

Tolazoline was the most effective of the drugs tested in inhibiting the cholinesterase of guinea-pig vas deferens. Fig. 8 shows that inhibition was detectable with 1 μg./ml., appreciable with 10 μg./ml., and complete with 500 μg./ml. Ergotamine (100 μg./ml.) produced a 35% inhibition

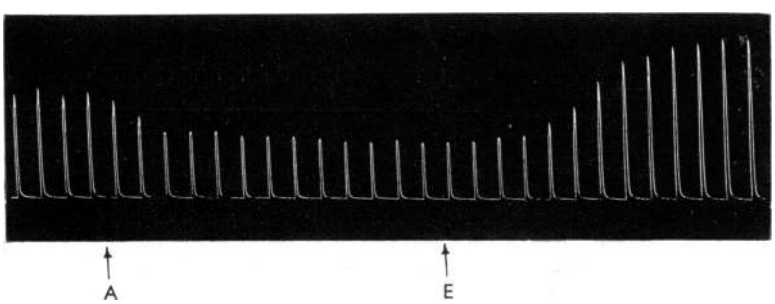


FIG. 6.—As Fig. 1. At A, atropine was added to the bath to produce a concentration of 0.1 μg./ml. At E, eserine was added to produce a concentration of 4 μg./ml.

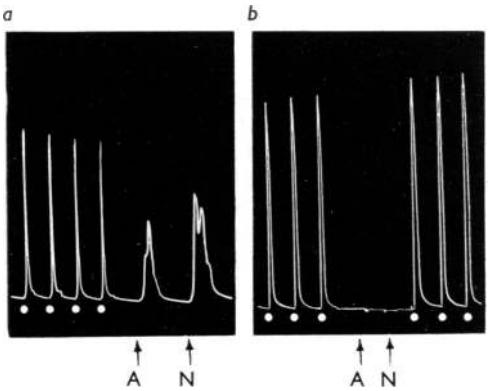


FIG. 7.—Isolated guinea-pig vas deferens. At white dots the hypogastric nerve was stimulated. At A, 10 μg./ml. of acetylcholine, and at N, 10 μg./ml. of noradrenaline was added. Between tracings (*a*) and (*b*) phenoxybenzamine was added to produce a bath concentration of 3 μg./ml.

of cholinesterase activity measured over the first 10 min. of the reaction.

Piperoxan and yohimbine were approximately equiactive; both produced a 30% inhibition of enzyme activity in concentration of 100 μg./ml. Phenoxybenzamine was the least active; 50 μg./ml. producing a 10% inhibition, and 500 μg./ml. a 25% inhibition during the first 10 min. of enzyme action.

## DISCUSSION

Jang (1941) attributed the increase in response to sympathetic stimulation produced by ergotamine, piperoxan, and yohimbine to their action in enhancing the response to adrenaline. He found that small doses of these three drugs increased the vasoconstriction in the rabbit ear produced by adrenaline. Other reports occur in the literature of potentiating action towards adrenaline and noradrenaline of antiadrenaline agents when they are used in low doses. Thus Holzbauer and Vogt (1955) reported that dibenamine, phenoxybenzamine, and dihydroergotamine made the rat uterus more sensitive to the inhibitory action of adrenaline, although tolazoline, yohimbine, piperoxan, and phentolamine had no such effect. In order for Jang's suggestion to hold good it would be necessary to show that the antiadrenaline drugs which potentiated the response of the vas deferens to sympathetic stimulation were effective in potentiating the responses to noradrenaline. We have not observed any increase in response to noradrenaline in the presence of antiadrenaline agents.

The potentiation of the responses to sympathetic stimulation produced by low concentrations of antiadrenaline agents can be understood in the light of their anticholinesterase action. There are two ways in which an inhibition of the

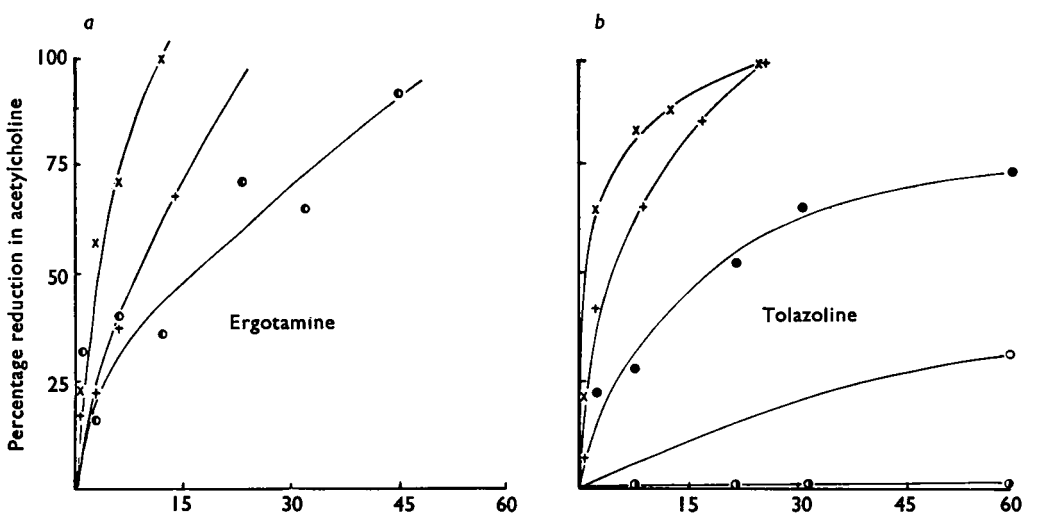


FIG. 8.—The rate of the destruction of acetylcholine by an extract of guinea-pig vas deferens in the presence of various concentrations of ergotamine (*a*) and tolazoline (*b*). X=Control. +=1 μg./ml. ●=10 μg./ml. O=50 μg./ml. ◐=100 μg./ml. ◑=500 μg./ml. Ordinate: % reduction in acetylcholine concentration. Abscissa: time in min.

destruction of acetylcholine from cholinergic sympathetic fibres could increase the responses of the vas deferens to hypogastric nerve stimulation: by increasing the direct action of acetylcholine on effector cells, or its intermediary action in liberating noradrenaline. The mechanism by which acetylcholine and cholinergic sympathetic fibres might liberate noradrenaline has been discussed by Burn and Rand (1959, 1960b). The direct action of acetylcholine on the vas deferens was blocked by atropine, but inhibition of cholinesterase by eserine still led to potentiation of the responses to sympathetic stimulation (Fig. 6) which favours the idea that it is the action of acetylcholine as an intermediary in noradrenaline release that is being potentiated. Thus ergotamine, phenoxybenzamine, piperoxan, and yohimbine, which had atropine-like activity, nevertheless potentiated the responses to sympathetic nerve stimulation.

The potentiation of the responses to sympathetic stimulation by anticholinesterase drugs has been observed in the nictitating membrane (Bacq and Fredericq, 1935a), in the rabbit ear (Burn and Rand, 1960b), in some experiments in the rabbit uterus (Varagić, 1956b) and by us in the guinea-pig vas deferens.

Potentiation of responses to sympathetic stimulation by low concentrations of anti-adrenaline drugs has been observed in isolated rabbit atria with dibenzyline (Huković, 1959), in the isolated rabbit uterus with tolazoline (Varagić, 1956b), in the cat nictitating membrane with piperoxan (Bacq and Fredericq, 1935b) and with yohimbine and ergotoxine (Jang, 1941), and in the rabbit ear with piperoxan (Jang, 1941).

Anticholinesterase activity has been reported previously for ergotamine with horse blood as the source of enzyme (Matthes, 1930), with horse serum (Gautrelet and Scheiner, 1939), with defibrinated cat blood (Brügger, 1938), and with human serum (Thompson, Tickner, and Webster, 1955). Schär-Wüthrich (1943) found that tolazoline (and two other imidazolines) inhibited cholinesterase from human serum and brain. Some actions of tolazoline can be interpreted as due to its inhibition of cholinesterase. For example, tolazoline decreased the rate of isolated rabbit atria and potentiated acetylcholine-induced contraction in the frog rectus and guinea-pig ileum (Gowdey, 1948). The gastro-intestinal stimulating action and the effect on heart rate could be prevented by atropine (Ahlquist, Huggins, and Woodbury, 1947).

Varagić (1956a) found that tolazoline altered the response of the isolated rabbit colon to sympathetic stimulation from a simple inhibition to a biphasic response consisting of an initial contraction followed by relaxation. This observation could not be explained in terms of an antagonism or reversal of the inhibition by tolazoline since Ahlquist *et al.* (1947) showed that tolazoline did not influence the inhibitory action of adrenaline, and Gowdey (1948) found tolazoline reduced but did not reverse the inhibitory action of adrenaline on the rabbit duodenum. Gillespie and Mackenna (1959) found that rabbit colon taken from a reserpine-treated rabbit contracted in response to sympathetic stimulation, and the contraction was abolished by atropine. This can be construed as evidence for cholinergic nerves running in the sympathetic supply to the colon. The action of the acetylcholine liberated from such nerves might be enhanced by an anticholinesterase so that the normal colon would be exposed to the combined effect of acetylcholine and noradrenaline to result in the biphasic response observed by Varagić (1956a). In fact, Varagić observed that eserine only produced a biphasic response in 2 of 11 preparations, but that eserine could enhance the effect of tolazoline. The initial contraction of the colon in response to sympathetic stimulation in the presence of tolazoline was abolished by atropine.

Brown and Gillespie (1957) found that the amount of noradrenaline appearing in the splenic venous blood after stimulating the splenic nerves was increased in the presence of phenoxybenzamine. Huković (1959) suggested that the appearance of a greater amount of transmitter (noradrenaline) might explain both his finding that phenoxybenzamine increased the effect of sympathetic stimulation on the rabbit atria, and Varagić's (1956b) finding that the response of the rabbit uterus to sympathetic stimulation was increased by tolazoline. We believe that an increased amount of transmitter could be released in the presence of an anticholinesterase (such as phenoxybenzamine or tolazoline) if there were a cholinergic process involved in the release of noradrenaline. Such a mechanism has been proposed by Burn and Rand (1959, 1960b) and additional evidence for it has been obtained recently when it was found that hemicholinium produced transmission failure in isolated vas deferens stimulated via its hypogastric sympathetic nerve (Rand and Chang, 1960).

Goodman and Gilman (1956) have pointed out that, " Practically every important class of drug and innumerable compounds of lesser interest have been tested for their effects on cholinesterase activity. The drugs reported to inhibit various cholinesterases outnumber those that are

apparently ineffective. Numerous exorbitant claims have been made that certain agents act principally through cholinesterase inhibition." Nevertheless the use of antiadrenaline agents as tools for investigating mechanisms must be evaluated in the light of our findings that they possess anticholinesterase activity. Thus Burnstock (1958) found that piperoxan increased the tone of longitudinal muscle in the trout stomach. He attributed this to the antagonism of an inhibitory action of adrenaline on the gut and the resultant was an acetylcholine-induced spasm. Our results show that another explanation may be the potentiation of acetylcholine by virtue of the anticholinesterase activity of piperoxan.

Finally, we wish to point out that we have no evidence to suggest that the antagonistic action of antiadrenaline agents is associated with their anticholinesterase activity.

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