Br. J. Pharmac. Chemother. (1968), **33**, 145–153.

THE ACTION OF SOME CHOLINERGIC AGONISTS AND ANTICHOLINESTERASE AGENTS ON THE DORSAL MUSCLE OF THE LEECH

BY

W. FLACKE AND T. S. YEOH*

From the Department of Pharmacology, Harvard Medical School, Boston, Massachusetts

(*Received December* 4, 1967)

The leech muscle is one of the oldest preparations for the bioassay of acetylcholine and has served in this capacity on many venerable occasions. The last systematic pharmacological study was carried out by Minz (1932). Minz found the contraction of the eserinized leech muscle practically specific for acetylcholine. Several other agents either were not potentiated by physostigmine or were simply inactive save in very high concentrations.

No information is available concerning the effects on the leech muscle of a number of agents which are known to, or assumed to, interact with acetylcholine receptors and which have become available only within the past 20 years. We consider it of sufficient interest and importance to try and fill in these gaps. The results of these investigations indicate an even closer pharmacological similarity between the leech muscle and the end-plate of vertebrate skeletal muscle than previously recognized. Succinylcholine and decamethonium were found to be agonists in the leech, but could clearly be differentiated from acetylcholine, carbachol, and nicotine.

In addition, the ability of some anticholinesterase agents to potentiate the effect of acetylcholine and related agonists has been studied. These studies, too, take on an added significance in view of the close pharmacological similarity between vertebrate endplate and leech muscle.

METHODS

The dorsal muscle of the leech (*Hirudo medicinalis*) was dissected free of all tissue adhering to its inner surface as described by MacIntosh & Perry (1950). Two strips of muscle were prepared by dividing along the midline. The paired muscles were mounted vertically in identical Lucite organ baths of 10 ml. capacity and suspended in a bicarbonate Ringer solution. The lower end was secured to a hook at the bottom of the bath and the upper end attached by a length of inelastic thread to a strain gauge tension transducer (Grass model FT 03) mounted on an adjustable rack. On isolating and mounting, the muscles tend to go into spasm. This could be overcome by perfusing the organ bath with the Ringer solution at a rate of approximately 3 ml./min. An interval of at least an hour was allowed before testing with drugs was started. During this period, as the muscle gradually relaxed, the strain gauge transducer was raised so that the final resting tension was 1–2 g.

* Research Fellow, sponsored by the China Medical Board of New York, Inc. Present address: Department of Pharmacology, University of Singapore, Singapore.

The composition of the solution was (in mM) NaCl 112, KCl 5.64, $CaCl_2$ 2.16, $MgCl_2$ 2.05 and $NaHCO_3$ 27. This solution was aerated with a mixture of 97% oxygen and 3% carbon dioxide resulting in a pH of 6.9–7.1 at room temperature (20°–25° C). Anticholinesterase agents were allowed to equilibrate with the tissue for 30 min. Final concentrations of drugs to be used for testing were made up in the same solution used to bathe the muscle. Response of the muscle was measured isometrically and was recorded by a Grass direct-writing oscillograph. Agonistic drugs were allowed a contact time of either 90 or 120 sec after which the organ bath was drained and refilled three times. The duration of the contact time was determined by the speed of response caused by the agonist and was such as to allow the maximum or near maximum response to be achieved. During the interval between tests, the bath fluid was changed at intervals of about 5 min. The tension developed during the contact time was measured as the difference between the final recorded and the initial resting tension.

For determining a concentration-effect curve, a three-fold geometric increase in concentration of the agonist was used until the maximum response was reached.

Drugs

The drugs used in this study were: acetylcholine chloride (Merck); carbaminoylcholine chloride (Merck); nicotine hydrogen tartrate (BDH); succinylcholine chloride (Burroughs Wellcome); decamethonium bromide (Burroughs Wellcome); physostigmine sulphate (Merck); neostigmine methylsulphate (Roche); di-isopropyl-fluorophosphate (Mann).

All drug concentrations are expressed in terms of the molar concentration of the bases. A stock solution of di-isopropyl-fluorophosphate (10^{-2}M) was prepared in anhydrous propylene glycol and diluted with Ringer solution for use as required. The diluted solution was used within 10 min of preparation.

RESULTS

Agonists

Acetylcholine and nicotine are well known to cause a contraction of the leech muscle. In these experiments, carbachol, succinylcholine, and decamethonium were found to do the same.

Effective concentrations

Acetylcholine is unique in that it is a weak agonist in the absence of an anticholinesterase. In the presence of physostigmine, however, it is the most potent of all the agonists tested (Table 1). This potentiation of the acetylcholine effect is generally assumed to be the result of inhibition of the leech muscle cholinesterase, which was reported by Kahlson & Uvnäs (1935) to be of very high activity. When instead of physostigmine, either neostigmine or di-isopropyl-fluorophosphate (DFP) was used to block the esterase (see below), however, the potency of acetylcholine was about 1/10 of that in the presence of physostigmine and of the same order of magnitude as the potency of carbachol and nicotine (Table 1). Succinylcholine and decamethonium were again about 1/10 as potent as the group consisting of carbachol, nicotine, and acetylcholine with DFP or neostigmine (Table 1). The concentration response curves for all agents were roughly parallel. Potassium in increasing concentrations up to 120 mM also caused a contraction (Fig. 1).

Size of the maximal response

Figure 1 shows an experiment in which as representative agents carbachol, succinylcholine, and potassium were tested in pieces of muscle from the same leech. The result

shows not only the differences in the concentrations producing effects, but also the differences in the size of the maximally obtainable contraction at any concentration. Column 3 of Table 1 shows that the grouping according to effective concentrations (Table 1, column 2) is repeated when the compounds are grouped according to the size of the maximal response obtainable. Carbachol was chosen as standard of reference for these determinations because it is capable of producing the maximal contraction without requiring the presence of an enzyme inhibitor. The ordinate of Fig. 1 is calibrated in absolute units ; the standard errors are quite small and conversion to percent of maximum was unnecessary. Equally, conversion of absolute tensions developed to tension per cross-sectional area did not improve the statistics because the muscle pieces used were of fairly uniform dimensions.

TABLE 1

COMPARISON OF EFFECTIVE CONCENTRATION (EC50) AND SIZE OF MAXIMALLY PRODUCED EFFECT OF SOME AGONISTS ACTING ON LEECH MUSCLE

The size of the maximal responses of the various agonists are compared with that of carbachol, which is given the value of 1. Figures in parentheses refer to the number of experiments.

Agonist	EC50 ± S.E. (molar concentration)	Ratio of maximally attainable effect ± S.E.
Acetylcholine (ACh)	$3{\cdot}3 \pm 0{\cdot}6 \times 10^{-4}$ (22)	—
ACh + physostigmine 10^{-5}	$2{\cdot}5 \pm 0{\cdot}6 \times 10^{-7}$ (8)	$0{\cdot}991 \pm 0{\cdot}012$
ACh + neostigmine 10^{-4}	$1{\cdot}4 \pm 0{\cdot}2 \times 10^{-6}$ (6)	—
ACh + DFP 3×10^{-4}	$3{\cdot}1 \pm 0{\cdot}7 \times 10^{-6}$ (8)	—
Carbachol	$3{\cdot}7 \pm 0{\cdot}2 \times 10^{-6}$ (18)	1·0
Nicotine	$5{\cdot}9 \pm 0{\cdot}5 \times 10^{-6}$ (12)	$0{\cdot}971 \pm 0{\cdot}016$
Succinylcholine	$5{\cdot}6 \pm 0{\cdot}4 \times 10^{-5}$ (14)	$0{\cdot}708 \pm 0{\cdot}008$
Decamethonium	$4{\cdot}2 \pm 0{\cdot}4 \times 10^{-5}$ (10)	$0{\cdot}668 \pm 0{\cdot}03$
Potassium	$3{\cdot}1 \pm 1{\cdot}4 \times 10^{-2}$ (12)	$0{\cdot}798 \pm 0{\cdot}025$

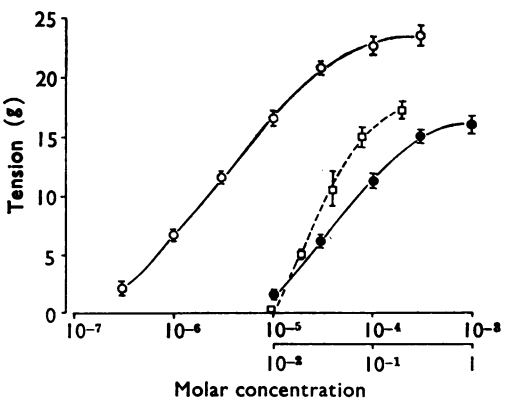

Fig. 1. Response of the leech muscle to carbachol, succinylcholine and potassium. The log concentration-effect curves represent the mean responses and standard errors obtained with four pieces of muscle prepared from the same leech. The response to carbachol is indicated as open circles, to succinylcholine as solid circles, to potassium as open squares. The upper scale on the abscissa is for carbachol and succinylcholine, while the lower scale is for potassium.

It can be seen in Fig. 1 that exposure to 120 mM potassium did not produce the same maximum of tension as did carbachol. The possibility that this result might be caused by diffusion delay and asynchrony of contraction was tested by reducing the cross-sectional area and thus the diffusion distances by dividing a muscle along the middle following the first exposure to potassium. Tension development per cross-sectional area and rate of tension rise in response to potassium were the same both times. Also, the

contraction induced by potassium was maintained for 10 min with only about 10% decline in tension. Thus the contractile response to potassium is well maintained, and a slight asynchrony of activation should have little influence upon the total tension output. When carbachol was added after the response to 120 mM potassium had reached a plateau there was an additional increase in tension of about 25%. This shows directly that the contractile response to potassium was not the maximal response of the muscle system, and that carbachol was able to cause an additional tension output in the depolarized muscle.

The agonists tested can thus be separated into two distinct groups according to effective concentrations and the size of the maximal response obtainable. Acetylcholine (in the presence of DFP), carbachol and nicotine were of about the same potency and induced the same maximum of effect. Succinylcholine and decamethonium were about 1/10 as potent and capable of inducing only about 70% of the tension caused by the agents in the first group. Potassium, too, was unable to induce the same magnitude of tension as the agents of the first group.

Anticholinestrase agents

Physostigmine, neostigmine and DFP did not have any effect by themselves when applied to a completely relaxed muscle preparation in concentrations up to 3×10^{-4}M. As stated above, however, the response to acetylcholine was increased in the presence of these agents. The concentration-response curves to acetylcholine were shifted in parallel to the left. For quantification, the EC50s of acetylcholine were determined in the absence and in the presence of the inhibitors. The term " potentiation factor " is used to denote the ratio of the EC50 of acetylcholine in the absence to their EC50 in the presence of different concentrations of the inhibitors.

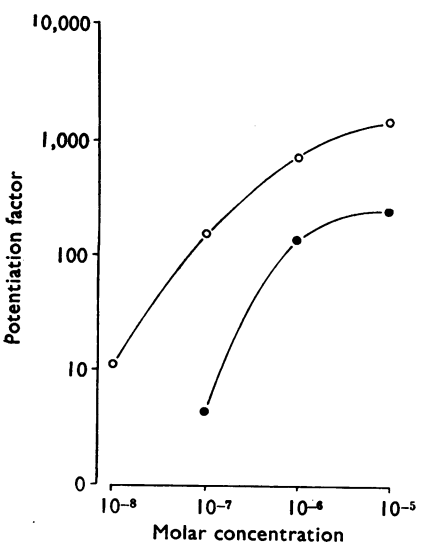

Fig. 2. Potentiation of the contractile response of the leech muscle to ACh. The " potentiation factor " refers to the ACh ratio—that is, EC50 in absence/ EC50 in presence of either physostigmine or neostigmine. The potentiation by phyostigmine (○) is the mean of eight experiments, and that by neostigmine (●) is the mean of six experiments. The standard errors are too insignificant to be shown on a log scale and are given in Table 2A.

In Fig. 2 the potentiation factor resulting from physostigmine and that from neostigmine in increasing concentrations is shown. The curves approach a maximum asymptotically at a concentration of 10^{-5}M. Physostigmine was more potent and its effect reached a greater maximum. The maximum potentiating effect of physostigmine was about six times greater than that of neostigmine (Table 2A).

TABLE 2

POTENTIATION OF THE ACh RESPONSE BY ANTICHOLINESTERASE AGENTS ACTING ON THE LEECH MUSCLE

The potentiation factor refers to the ratio of the EC50 of ACh in the absence to the EC50 in the presence of an anticholinesterase agent.

	Number of experiments	Anticholinesterase(s) Molar concentration	ACh EC50 ± S.E. Molar concentration	Potentiation factor ± S.E.
A				
	8	Physostigmine 10^{-8}	$3{\cdot}3\pm0{\cdot}9\times10^{-5}$	11± 2
	8	Physostigmine 10^{-7}	$2{\cdot}3\pm0{\cdot}8\times10^{-6}$	159± 21
	8	Physostigmine 10^{-6}	$5{\cdot}0\pm1{\cdot}4\times10^{-7}$	702± 15
	8	Physostigmine 10^{-5}	$2{\cdot}5\pm0{\cdot}6\times10^{-7}$	1,444± 33
	6	Neostigmine 10^{-7}	$8{\cdot}1\pm1{\cdot}4\times10^{-5}$	4± 0·5
	6	Neostigmine 10^{-6}	$2{\cdot}0\pm0{\cdot}3\times10^{-6}$	138± 6
	6	Neostigmine 10^{-5}	$1{\cdot}3\pm0{\cdot}2\times10^{-6}$	246± 27
B				
	6	DFP 3×10^{-4}	$2{\cdot}9\pm0{\cdot}6\times10^{-6}$	144± 12
	6	DFP 3×10^{-4} Physostigmine 10^{-6}	$1{\cdot}1\pm0{\cdot}3\times10^{-6}$	461± 53
	6	DFP 3×10^{-4} Physostigmine 10^{-5}	$2{\cdot}9\pm0{\cdot}8\times10^{-7}$	1,551± 72
	6	Neostigmine 10^{-4}	$1{\cdot}4\pm0{\cdot}2\times10^{-6}$	220± 29
	6	Neostigmine 10^{-4} Physostigmine 10^{-6}	$7{\cdot}0\pm1{\cdot}8\times10^{-7}$	562±108
	6	Neostigmine 10^{-4} Physostigmine 10^{-5}	$1{\cdot}8\pm0{\cdot}3\times10^{-7}$	2,040±374

DFP was used as prototype of the so-called irreversible organophosphate inhibitors. Preliminary experiments with DFP showed that a concentration of 10^{-4}M for a period of 30 min caused maximal potentiation. In order to ensure complete cholinesterase inhibition, however, a concentration of 3×10^{-4} was used. Physostigmine (10^{-6} and 10^{-5}M), given after 30 min of exposure to DFP 3×10^{-4}M, caused an additional potentiation of the response to ACh. Neostigmine, when tested in place of physostigmine, did not cause an additional potentiation. With neostigmine substituted for DFP and followed by physostigmine, however, an additional potentiation resulted. The findings are illustrated in Fig. 3 and summarized in Table 2B. They suggest that physostigmine has a potentiating effect above that of maximal concentrations of DFP or neostigmine.

The time course of the potentiation by the various enzyme inhibitors is shown in Fig. 4. Within 1 min of the exposure to physostigmine, the potentiating effect was already greater than the maximal effect of DFP. On the other hand, at this time there was no noticeable potentiation from DFP. These results agree in principle with those reported concerning the time course of enzyme inhibition by the different inhibitors (Nachmansohn, Rothenberg & Feld, 1948). After 30 min, a maximal inhibitory effect had been reached with all agents. The additional potentiation observed when physostigmine was added after exposure to DFP or neostigmine (see above) was already present 2 min after addition of the alkaloid.

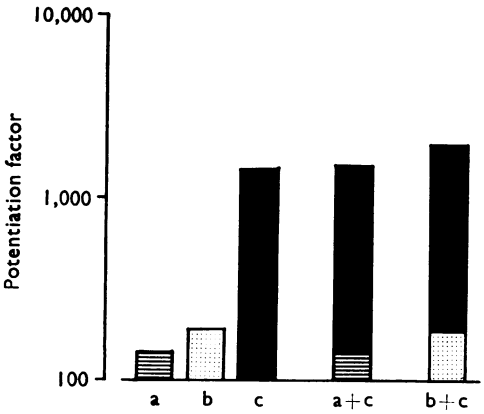

Fig. 3. Ability of some anticholinesterase agents to potentiate the ACh response in the leech muscle. Columns a, b and c indicate the potentiation in the presence of DFP (3×10^{-4}M), neostigmine (10^{-4}M) and physostigmine (10^{-5}M) respectively. The last two columns indicate the combined effect of DFP with physostigmine (a+c) and of neostigmine with physostigmine (b+c).

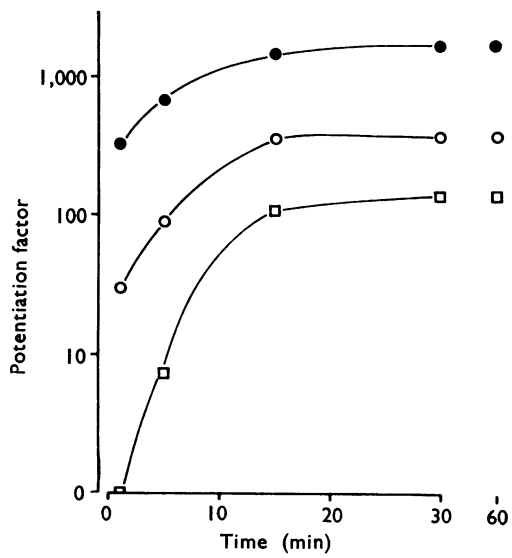

Fig. 4. Time course of the effect of anticholinesterase agents to potentiate the action of acetylcholine on the leech muscle. The curves are from two experiments with each anticholinesterase. The concentrations of physostigmine (●), neostigmine (○) and DFP (□) were 10^{-5}, 10^{-4} and 3×10^{-4}M, respectively.

Neostigmine also had an effect on the leech muscle which seemed unrelated to its anticholinesterase action. At a concentration of 10^{-4}M, neostigmine caused irregular contractile activity of different character than those seen with other agonists. These contractions did not follow immediately after addition of the agent. They were not sustained and were followed by spontaneous relaxations. They varied in magnitude from 2 to 10 g tension and occurred at intervals varying from 1 to 3 min. The contractions ceased immediately when the drug was washed out and were unaffected by curare. The only other substance we have observed to have such an effect on the leech muscle was veratridine, although in much lower concentration (10^{-7}M).

Other drugs found to act as agonists on the leech muscle, namely, carbachol, nicotine, succinylcholine and decamethonium, were tested for potentiation by DFP and by subsequent treatment with physostigmine. None of them were potentiated by DFP treatment. Only carbachol was potentiated by a factor varying from two to three by physostigmine.

DISCUSSION

It has been known for some time (MacIntosh & Perry, 1950) that, pharmacologically, the leech muscle resembles the vertebrate motor endplate. It was known to be sensitive to acetylcholine and nicotine, and the effect of these agents is not affected by atropine, but by curare. The present experiments have extended this similarity. Succinylcholine and decamethonium, which are known to depolarize the mammalian endplate region (del Castillo & Katz, 1957) also induced a contraction of the leech muscle. There was, however, a clear distinction between acetylcholine, carbachol and nicotine on the one hand, and succinylcholine and decamethonium on the other. This distinction was apparent in the effective concentrations of the two groups of agonists as well as in the size of the maximal response obtainable.

Potassium differs from other "agonists" in causing a depolarization, not by combining with specific receptors and thereby altering membrane conductances, but by virtue of eliminating the electrochemical gradient. It is interesting that the contraction induced by potassium was lower than that caused by acetylcholine and related agents and about equal to that caused by succinylcholine and decamethonium. It has been shown that acetylcholine is capable of causing a contraction (and changes in membrane permeabilities) in the presence of high potassium solutions (del Castillo & Katz, 1955; Evans, Schild & Thesleff, 1958). It seems possible that the difference between acetylcholine, carbachol and nicotine on the one hand, and succinylcholine, decamethonium and potassium on the other, is causally related to the ability of the autonomic agonists to activate the contractile protein in a depolarized cell. Thus these agents might act by virtue of the depolarization and by a second effect not related to the electrical membrane functions (Evans *et al.*, 1958). The "depolarizing agents" and potassium possess only the first type of activity, whether achieved by a change in membrane permeability or by elimination of the electrochemical gradient. Acetylcholine has been shown to cause an increase in conductance in the endplate even in high potassium solutions (del Castillo & Katz, 1955).

Anticholinesterase agents

The question whether or not anticholinesterase agents possess pharmacological activity unrelated to enzyme inhibition has been examined by many investigators, often in a more comprehensive fashion than in this study (for review, see Werner & Kuperman, 1963). It is possible to summarize these studies as showing that anticholinesterase agents may indeed have agonistic as well as antagonistic cholinergic activity, although this cannot be demonstrated in all species and in all tissues.

Relevant information on the leech comes from Kahlson & Uvnäs (1935, 1938). These workers reported that the enzymatic activity of the leech is much higher than that of frog muscles (rectus and gastrocnemius). They also found that physostigmine in a concentration of 10^{-5} (w/v) caused a 1,500-fold potentiation of ACh, a value close to that found by us. They concluded from a comparison of *in vitro* inhibitory activity of several agents with the potentiation of the effect of ACh in the muscle, that enzyme inhibition alone could not be considered responsible for the observed ACh potentiation.

This conclusion differs from that of Bhattacharya & Feldberg (1958) who attributed the greater potency of eserine in potentiating the contractile response to acetylcholine

of the leech muscle to the greater potency of this alkaloid in inhibiting acetylcholinesterase prepared from leech. Unfortunately, the authors did not report whether the maximum attainable by the two inhibitors differed. The difference in potency in potentiating the contractile response to acetylcholine is confirmed in the present findings. This does not, however, suffice to explain the greater ceiling of potentiation obtainable with eserine after neostigmine and DFP had been administered in supramaximal concentrations (Fig. 2; Table 2) and for a longer period of time than required to produce a maximal effect (Fig. 4).

In our observations, there was no direct evidence of either agonistic or antagonistic activity of the enzyme inhibitors used, such as has been observed in other tissues. None of the agents caused a contraction by itself when applied to a completely relaxed preparation. In the other direction, there was no reason to assume an antagonistic effect because the curve relating the potentiation of ACh to inhibitor concentration did not show a declining limb even at concentrations higher than those necessary to cause a maximal potentiation. An antagonistic activity present in the same concentration range and becoming maximal at the same concentration as the enzyme inhibitory effect could not have been detected by this criterion. The only evidence which points towards an action not related to enzyme inhibition is the additional sensitization effected by physostigmine in comparison with DFP and neostigmine. This effect certainly cannot be caused by a depressant activity of DFP and neostigmine missing in physostigmine, because physostigmine exerted its greater potentiation even in the presence of DFP or neostigmine. All in all, it seems most reasonable to ascribe this phenomenon to an action of physostigmine on the interaction of ACh with the muscle receptor not directly related to enzyme inhibition. The potentiating effect of the anticholinesterase agents was quite specific. None of the other agonists tested, except carbachol, was potentiated by the inhibitors. Carbachol was potentiated by physostigmine only. The effect of carbachol may be explained if one assumes that carbachol may act, in addition to its direct action, by releasing acetylcholine from tissue stores as reported by Volle & Koelle (1961). The carbachol potentiation may thus be caused by potentiation of released acetylcholine, but it also may be caused by potentiation of carbachol by physostigmine directly, not related to esterase inhibition.

In comparison with other tissues responding to the nicotinic actions of acetylcholine, the leech muscle seemed to demonstrate the effect of enzyme inhibition by anticholinesterase agents relatively uncomplicated by direct agonistic or antagonistic actions of these agents. For example, Hobbiger (1950), found in the frog rectus that the concentrations of neostigmine, which potentiated the response to acetylcholine, were very close to concentrations exhibiting agonistic and antagonistic activity in muscles treated with tetrapyrophosphate. Kahlson & Uvnäs (1938) pointed out that the sensitivity to acetylcholine of the frog rectus in the absence of enzyme inhibitors is greater than that of the leech muscle, while the potentiation of the acetylcholine response by anticholinestrase agents is much greater in the leech.

SUMMARY

1. The action of several agonists on the isolated dorsal muscle of the leech (*Hirudo medicinalis*) was studied.

2. In addition to acetylcholine, carbachol, and nicotine, which are known to stimulate the leech muscle, succinylcholine and decamethonium were found to cause a contractile response.

3. The EC50 of succinylcholine and decamethonium was about 5×10^{-5}M. The EC50 of carbachol, nicotine, and of acetylcholine in the presence of di-isoprophyl-fluorophosphate was between 3 and 6×10^{-6}M.

4. The maximal tension developed by succinylcholine and decamethonium was about 70% of that caused by acetylcholine, nicotine and carbachol, and was similar to the magnitude of the contractile response to isotonic potassium chloride.

5. Carbachol, added when the tension response to potassium was maximal, increased tension by about 25%.

6. Physostigmine, neostigmine and di-isopropyl-fluorophosphate did not cause a contraction when applied to a relaxed muscle.

7. The response to acetylcholine was potentiated by di-isopropyl-fluorophosphate and neostigmine by a factor of about 150 and 250 respectively.

8. The response to acetylcholine was potentiated by physostigmine by a factor of about 1,500. Addition of physostigmine after potentiation by di-isopropyl-fluorophosphate and neostigmine had reached its maximum produced additional potentiation.

9. The greater potentiating effect of physostigmine is ascribed to an action not related to the inhibition of the enzyme.

10. The action of anticholinesterase agents is relatively selective in comparison with other tissues such as the rectus muscle of the frog.

This work was supported by grants from the USPHS NB 21760 and HE 09398.

REFERENCES

BHATTACHARYA, B. K. & FELDBERG, W. (1958). Comparison of the effects of eserine and neostigmine on the leech muscle preparation. *Br. J. Pharmac. Chemother.*, **13**, 151–155.

CASTILLO, J. DEL & KATZ, B. (1955). Local activity at a depolarized nerve-muscle junction. *J. Physiol., Lond.*, **128**, 396–411.

CASTILLO, J. DEL & KATZ, B. (1957). A comparison of ACh and stable depolarizing agents. *Proc. R. Soc.*, B, **146**, 362–368.

EVANS, D. J. L., SCHILD, H. O. & THESLEFF, S. (1958). Effects of drugs on depolarized plain muscle. *J. Physiol., Lond.*, **143**, 474–485.

HOBBIGER, F. (1950). The action of carabamic esters and tetraethylpyrophosphate on normal and curarized frog rectus muscle. *Br. J. Pharmac. Chemother.*, **5**, 37–48.

KAHLSON, G. & UVNÄS, B. (1935). Zur Theorie der Sensibilisierung für Azetylcholin, zugleich Bericht über eine erregbarkeitssteigernde Wirkung des Fluorids. *Skand. Arch. Physiol.*, **72**, 215–239.

KAHLSON, G. & UVNÄS, B. (1938). Die Bedeutung der Acetylcholinesterase sowie der spezifischen Rezeptoren für die Acetylcholin-empfindlichkeit kontraktiler Substrate. *Skand. Arch. Physiol.*, **78**, 40–58.

MACINTOSH, F. C. & PERRY, W. L. M. (1950). Biological estimation of acetylcholine. *Meth. med. Res.*, ed. Gerard, R. W., Vol. 3, 78–92. Chicago: Year Book Publishers.

MINZ, B. (1932). Pharmakologische Untersuchungen am Blutegelpräparat, zugleich eine Methode zum biologischen Nachweis von Azetylcholin in Anwesenkeit anderer pharmakologisch wirksamer körpereigener Stoffe. *Arch. exp. Path. Pharmak.*, **168**, 292–304.

NACHMANSOHN, D., ROTHENBERG, A. M. & FELD, E. A. (1948). Studies on cholinesterase. V. Kinetics of the enzyme inhibition. *J. biol. Chem.*, **174**, 247–256.

VOLLE, R. L. & KOELLE, G. B. (1961). The physiological role of ACh in sympathetic ganglia. *J. Pharmac. exp. Ther.*, **133**, 223–240.

WERNER, G. & KUPERMAN, A. S. (1963). *Handbuch der experimentellen Pharmakologie*, ed. Koelle, G. B., vol. 15, 577–678.