Biochemical Pharmacology, 1965, Vol. 14, pp. 79–89. Pergamon Press Ltd., Printed in Great Britain.

CHOLINESTERASES IN THE BLADDER OF THE TOAD (*BUFO MARINUS*)

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(*Received* 10 *June* 1964; *accepted* 24 *June* 1964)

Abstract—A study of the nature of the cholinesterases present in the bladder of the toad (*Bufo marinus*) has been undertaken in an attempt to explain why eserine fails to potentiate the responses to either applied acetylcholine or pelvic nerve stimulation.

Comparison with mammalian bladder cholinesterases shows that the enzymes in the toad bladder exhibit pronounced resistance to eserine. However, both are sensitive to inhibition by neostigmine. Organ bath studies show that the responses of the toad bladder to applied acetylcholine and pelvic nerve stimulation are potentiated by neostigmine (5×10^{-7}–5×10^{-6} g/ml). The effects of eserine and neostigmine are discussed and the properties of the toad bladder cholinesterases are examined in terms of evolutionary trends.

INTRODUCTION

RECENT studies in this laboratory have been concerned with comparative aspects of the functional organization of the autonomic nervous system.[1,2,3] Investigations of the innervation of the bladder of the toad (*Bufo marinus*) have revealed that this organ is supplied only with motor nerves, which are probably all cholinergic.[1] However these workers found that the cholinesterase inhibitor physostigmine (eserine) did not potentiate the responses to either applied acetylcholine or to extrinsic nerve stimulation. Two possible explanations of this phenomenon are:

(i) Nervously-released acetylcholine is not hydrolysed by tissue stores of cholinesterase, but instead by cholinesterase circulating in the blood. This explanation is supported by Dumont's[4] report that he was unable to demonstrate cholinesterases in the frog bladder by histochemical techniques.

(ii) The cholinesterase present in the tissue is resistant to inhibition by eserine, as reported for frog brain by Hawkins and Mendel.[5]

In an attempt to clarify this situation, the presence and nature of the cholinesterases in the toad bladder was investigated and compared with those found in the guinea pig bladder, where the response to pelvic nerve stimulation is potentiated by low concentrations of eserine.

MATERIALS AND METHODS

Toads were pithed and their bladders removed using the following procedure. The ventral body wall was opened along the midline and 0·1 ml of heparin (5000 units/ml) was injected into the aortic arch. The pelvic girdle was cut through the midline and the blood vessels to the legs ligated as far as possible from their junction with the dorsal aorta. The intestine was then ligated above its junction with the bladder. The

dorsal aorta was cannulated, the anterior abdominal vein cut, and the bladder was perfused with 8–10 ml of 0·9% saline. The bladder was then separated from its surrounding connective tissue and cut out approximately 0·5 cm above its junction with the rectum. It was opened under saline and washed, blotted on filter paper and weighed. It was then snipped finely with scissors and stored on dry ice until use.

In experiments when the bladder of the guinea pig was used the animal was killed by a blow on the head and the ventral body wall opened. The dorsal aorta was cannulated and the inferior vena cava cut. The hindquarters of the animal was then perfused with 15–20 ml of 0·9% saline. Owing to the fragility and situation of the vessels supplying the legs these were not ligated. However, removal of all visible blood from the bladder vessels was possible without this precaution. Following perfusion the bladder was freed from adipose tissue, cut from the animal and washed, weighed and stored as described above.

Bladder homogenates were prepared in a medium having the composition: 100 pts. 0·9% NaCl, 4 pts. 1·15% KCl, 1 pt. 2·11% KH_2PO_4, 1 pt. 3·82% $MgSO_4$. $7H_2O$, in a Servall Omni-Mix at a speed of about 30,000 rev/min and a temperature of 0°C. The final concentration of tissue was approximately 80 mg/ml.

Preliminary substrate specificity studies revealed the presence in the toad bladder of considerable quantities of aliesterases. It seemed possible that these might be responsible for some hydrolysis of choline esters. In some experiments, therefore, the aliesterases were partially removed and the results compared to those obtained using whole homogenate. Subcellular studies have revealed that while the highest percentage of ChEs is localized in the microsomal fraction, a high proportion of aliesterases is present in the nuclear and mitochondrial fractions.[6,7]

Approximately 1 gm tissue was homogenized in 5 ml 3 M sucrose in the presence of 10^{-5}M EDTA, using a glass-to-glass homogenizer. The suspension was centrifuged for 10 min at 1000 *g* and 2°C in a Servall refrigerated centrifuge with an SS-34 head, and the resultant supernatant was recentrifuged at 10,000 *g* and 2°C for 20 min. In both cases the precipitates were washed with a further 5 ml sucrose and respun at the appropriate speeds, the same sample of sucrose being used to wash both precipitates. Following centrifugation at the higher speed both samples of supernatant were combined and used as a source of microsomal ChE.

Cholinesterase (ChE) activity was measured manometrically using a Warburg apparatus. The temperatures used were the approximate body temperatures for each species: 37°C for guinea pig and 25°C for toad. All determinations were carried out in an atmosphere of 95% N_2 and 5% CO_2. The tissue homogenate and inhibitors were placed in the main body of the flask, and the substrate in the side-arm. The final volume of fluid in each flask was 3·0 ml, the volumes of both homogenate and substrate being 0·4 ml. The buffered medium used had a pH of 7·4, the final $NaHCO_3$ concentration being 0·028 M and other salts being present in the proportions stated above. The system was equilibrated for 30 min before reading was commenced. Th first reading was taken immediately before tipping and thereafter every 10 min for 40 min. Enzymic activity is expressed as the QCO_2 (μl CO_2/g tissue/hr), adjustment being made for non-enzymatic hydrolysis.

Substrates used were: acetylcholine perchlorate, B.D.H. (ACh); butyryl choline iodide, Biochem. Research (BuCh); benzoylcholine chloride, B.D.H. (BzCh); acetyl-β-methyl-choline chloride, N.B.C. (MeCh); succinylcholine chloride, I.C.N. (SuCh);

and tributyrin, B.D.H. (Tb). Supplies of Propionylcholine were unavailable during the course of these experiments.

Inhibitors used were eserine sulphate, I.C.N.; neostigmine bromide, Roche; 1:5-*bis* (4-allyldimethyl-ammonium-phenyl) pentane-3-one diiodide(BW 284C51), Wellcome; tetramono-*iso*propyl pyrophosphortetramide (iso-OMPA), Light. All inhibitors were in contact with the enzyme for 40 min prior to addition of the substrate.

Organ bath experiments were performed using the toad bladder - pelvic nerve preparation. Dissection of this preparation has been described in detail in a previous paper.[1] The isolated bladder with its nerve supply was set in a 50 ml capacity organ bath at room temperature (21–25°C), in a medium of McKenzie's solution (pH 7·25) having the composition: NaCl 6·72, KCl 0·24, $NaHCO_3$ 1·68, $NaH_2PO_4.2H_2O$ 0·49, $MgSO_4.7H_2O$ 0·35, glucose 3·0, $CaCl_2$ 0·14 g/ml.[8] The bladder was mounted on a Perspex clamp-type phrenic nerve electrode and isotonic contractions were recorded on a smoked drum via a thread from the apex of the lobe and a frontal point writing lever. The pelvic nerves were stimulated using a Grass S5 square-wave stimulator delivering pulses with a duration of 1 msec and a strength of 80V. The frequency of stimulation was maximal (25 pulses/sec) or a sub-maximal (5–20 pulses/sec dependent on the reactivity of the preparation) for periods of 10 sec. An interval of 8 min was left between successive stimulations.

Drugs used were: acetylcholine chloride (Roche), eserine sulphate (I.C.N.), and neostigmine methylsulphate (Roche). All the drugs were made up in distilled water and injected in doses of 0·1 ml. The response to acetylcholine was measured during a 2 min contact period, with an interval of 10 min between doses.

RESULTS

(i) *Manometric studies*

Substrate specificity. A comparison was made of the rates of hydrolysis of ACh, BuCh, BzCh, MeCh, SuCh, and Tb (each at $6{\cdot}5 \times 10^{-3}$M) by toad bladder homogenate (Fig. 1a).

The most rapidly hydrolysed substrate was the aliphatic ester tributyrin. Addition of 10^{-5}M eserine inhibited Tb hydrolisys by 26 per cent. Acetylcholine was the most rapidly hydrolysed choline ester. The hydrolysis rates of BuCh and MeCh were 17 per cent and 10 per cent that of ACh respectively. Succinylcholine and BzCh were hydrolysed only very slowly.

The proportions of true ChE and pseudo ChE present. In order to determine the relative proportions of true and pseudo ChEs present, the hydrolysis rate of ACh was measured in the presence of BW 284C51 (5×10^{-6}M) and *iso*-OMPA (4×10^{-6}M), these being selective inhibitors for true and pseudo ChE respectively.[9,10] The result indicated that about 90 per cent of total ACh hydrolysis was due to true ChE. Use of both inhibitors together produced 98 per cent inhibition of hydrolysis. Eserine (10^{-5}M), however, only produced 80 per cent inhibition.

In the guinea pig bladder the proportion of pseudo ChE present was considerably higher, only 60 per cent inhibition of ACh hydrolysis being produced by BW 284C51. In this case, eserine (10^{-5}M) produced 100 per cent inhibition of activity.

Concentration of substrate/activity. The above results indicated that there was a high proportion of true ChE in the toad bladder. To confirm this, the hydrolysis rates of ACh, MeCh and BuCh at various concentrations were compared (Fig. 1b). The

figures obtained again indicated the presence of true ChE.[11,12] Maximal hydrolysis of ACh was seen at a concentration of 5×10^{-3}M, inhibition occurring at higher concentrations.

Using 4×10^{-6}M *iso*-OMPA to inactivate the pseudo ChE present, the relative hydrolysis rates of ACh ($6{\cdot}5 \times 10^{-3}$M) and MeCh ($2{\cdot}5 \times 10^{-2}$M) were determined. The QACh/MeCh ratio so estimated had a value of 8·3. Partial removal of aliesterases from the homogenate by use of the microsomal fraction as an enzyme source reduced this ratio to 6·5.

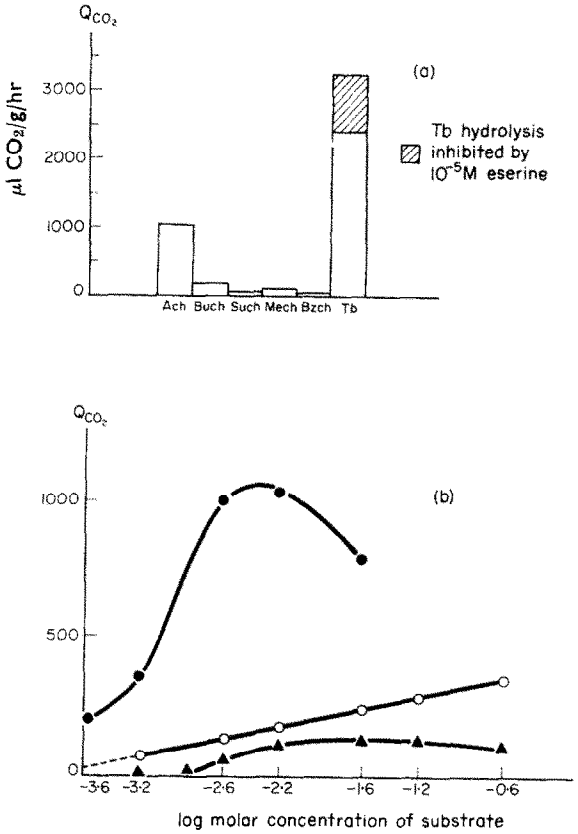

FIG. 1. Toad bladder ChE. (a) Substrate specificity. All substrates used in final concentration of $6{\cdot}5 \times 10^{-3}$M. (b) Substrate activity curves. ● ACh, ▲ MeCh, ○ BuCh.
Abbreviations: acetylcholine (ACh), butyrylcholine (BuCh), succinylcholine (SuCh, acetyl-β-methylcholine (MeCh), benzoylcholine (BzCh), tributyrin (Tb).

Eserine sensitivity. Using ACh as substrate, eserine inhibition curves for toad and guinea pig bladder ChEs were estimated, and the pI50 values compared (Fig. 2a). The results showed that toad bladder ChEs are 100 times more resistant to eserine than guinea pig bladder ChEs. Use of the microsomal fraction of the homogenate as an enzyme source produced no alteration in eserine sensitivity.

Neostigmine sensitivity. Using ACh as a substrate, neostigmine inhibition curves for the toad and guinea pig bladder ChEs were estimated (Fig. 2b). In this case, comparison of the pI50 values showed only an eightfold sensitivity difference between the ChEs from the two sources.

Organ bath experiments

Neostigmine. In concentrations of $5 \times 10^{-7} - 5 \times 10^{-6}$ g/ml, neostigmine produced potentiation of the responses to applied ACh and to pelvic-nerve stimulation, both at maximal and sub-maximal stimulation frequencies (Fig. 3). The latency period of contraction was also reduced by 25–33 per cent dependent on the original period, a longer latency being more markedly reduced. The initial potentiation produced by 5×10^{-6} g/ml neostigmine was increased after washing out the bath. This increase was not seen with 5×10^{-7} g/ml neostigmine. No alteration in tone or spontaneous activity was observed at either dosage. After washing out the neostigmine, the responses to nerve stimulation and applied ACh returned to normal after about six hours.

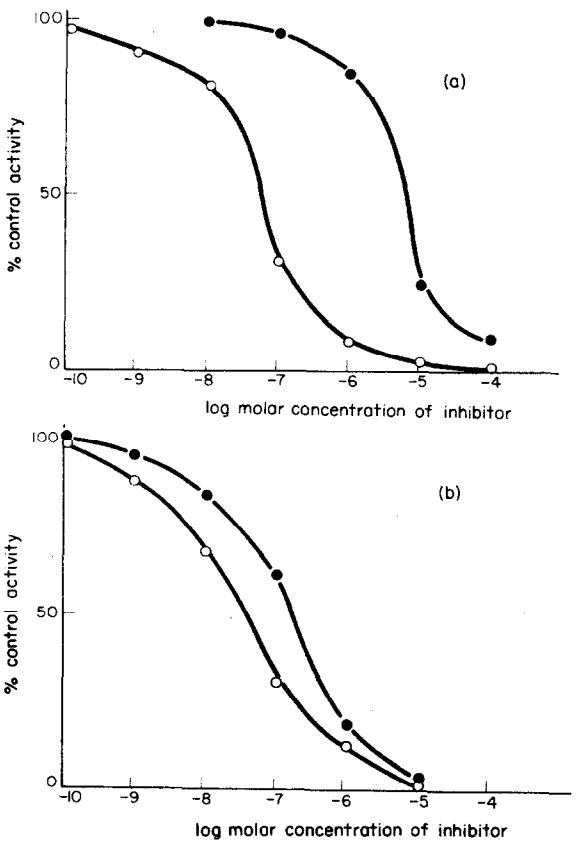

FIG. 2. Sensitivity of toad and guinea pig ChEs to inhibition. Substrate ACh in final concentration of $6{\cdot}5 \times 10^{-3}$M. (a) Eserine, (b) Neostigmine. ● toad ○ guinea-pig

Eserine. As previously reported by Burnstock *et al*[1], eserine at 10^{-6} g/ml had no effect on the preparation. At 10^{-5} g/ml depression of the nerve-mediated response was observed (Fig. 4). In two preparations the response to ACh was potentiated, in two others it was unaltered. At 10^{-4} g/ml eserine depressed the responses to both ACh and nerve stimulation. Washing restored these responses towards normal and

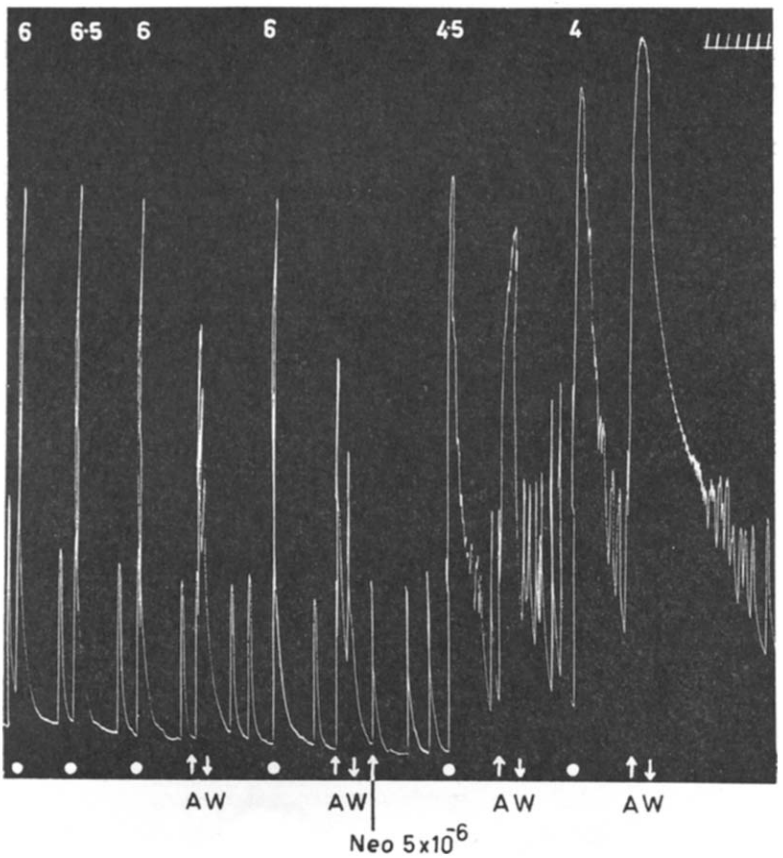

FIG. 3. Effect of neostigmine 5×10^{-6} g/ml on the responses of the isolated toad bladder to acetylcholine 5×10^{-8} g/ml (A) and nerve stimulation (25 pulses/sec, 1 msec, 80V) (white dot). The bath was washed out at W. Figures above each nerve-mediated response represent latency period of contraction. Time marker, 1 min intervals. Temperature 21°C.

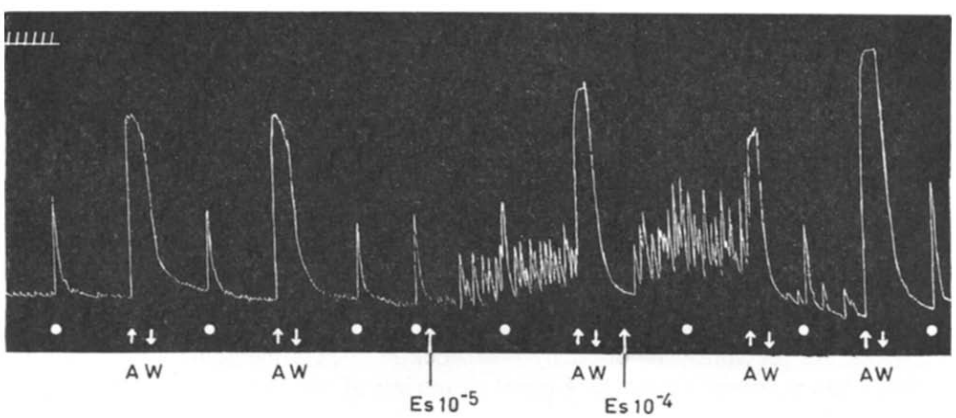

FIG. 4. Effect of eserine 10^{-5} and 10^{-4} g/ml on the responses of the isolated toad bladder to ACh 5×10^{-8} g/ml (A) and nerve stimulation (10 pulses/sec, 1 msec, 80V) (white dot). The bath was washed out at W. Time marker, 1 min intervals. Temperature 25°C.

in some cases reversed them to potentiation (Fig. 4). On addition to the bath, eserine produced a rise in tone and an increase in spontaneous activity of the bladder. In contrast, neostigmine had no effects of its own, although it caused rapid spontaneous activity during the slow recovery following contractions induced by nerve stimulation or applied ACh

DISCUSSION

High levels of ChEs have been shown to be present in the toad bladder. Most of the activity is due to true ChE, only a small proportion being due to pseudo ChE. This is in contrast to the situation in the guinea pig bladder, where the proportion of pseudo ChE was much higher. Histochemical studies of the toad and frog spinal cords[13] and the frog brain[14] revealed that structures which in mammals contain substantial levels of pseudo ChE contained only true ChE.

Sippel[15,16] reported that pseudo ChE was absent in the hearts of amphibians, and appeared in progressively higher levels in the hearts of reptiles, birds and mammals. The proportions of the two ChEs in the bladders of toad and guinea pig reinforce the theory put forward by Chacko and Cerf[13] that phylogenetic increase in the proportion of pseudo ChE is a widespread phenomenon in both neural and non-neural tissues.

Hydrolysis of the aliphatic ester Tb was only 26 per cent inhibited by 10^{-5}M eserine, as compared to the 80 per cent reduction in ACh hydrolysis seen at the same eserine concentration. This indicates that as well as ChEs, the toad bladder contains high levels of aliesterases.[17]

That these enzymes play a small part in ACh hydrolysis was shown by the fact that their partial removal from the homogenate resulted in a lowering of the QACh/MeCh ratio. However, this procedure did not affect the pI50 value obtained with eserine. The eserine resistance of the tissue could therefore not be explained by the presence of aliesterases.

The properties of the true ChE from the toad bladder differ from those of classical mammalian true ChEs. Comparison of the hydrolysis rates of ACh and MeCh at their respective optimum concentrations gave a QACh/MeCh ratio of 6·5–8·3. Mammalian true ChEs usually yield a ratio of about 3.[18] Variations in this ratio have also been reported by Hawkins and Mendel[5] who found the true ChEs from frog brain and planarian to yield QACh/MeCh values of 4·4 and 16·5 respectively.

Resistance to inhibition by eserine of ChEs from amphibian sources has previously been widely reported. Loewi and Navratil[19] observed that although the negative inotropic effect of ACh on the frog heart was prolonged by eserine it was not consistently increased. Lissak[20] found no significant sensitization of the frog heart to ACh with eserine. Hawkins and Mendel[5] found *in vitro* eserine resistance in the frog brain as well as in the planarian. The toad rectum shows similar properties to the toad bladder: pharmacological evidence indicates cholinergic innervation. but eserine (10^{-7} – 10^{-5} g/ml) does not potentiate the response either to applied ACh or to nerve stimulation.[3] Finally, Porter and de la Lande[21] and Xavier *et al.*[22] have reported that eserine is many times less effective than neostigmine in sensitizing the toad rectus abdominus muscle to ACh.

Several theories as to the basis of eserine resistance have been proposed. Using guinea pig tissues, Hobbiger[23] found that the degree of ChE inhibition by TEPP

in vitro was increased following an increase in the volume of tissue extract used as an enzyme source. He concluded that inhibition could be decreased by non-specific binding of the inhibitor to tissue constituents other than ChE, and suggested this as an explanation for Hawkins and Mendel's results. These workers, on the other hand, postulated that the eserine resistance was a function of the enzyme. The ChE in planarian was even more resistant to inhibition by Nu863 than by eserine, compared to mammalian brain ChE. However both enzymes showed equal sensitivity to neostigmine. It was postulated from this result that decreasing the number of constituents on the nitrogen atom, from neostigmine through eserine to Nu863 lowered the efficacy of the inhibitor.

Eserine resistance of true ChE has been reported widely in amphibia but, as far as we know, in no other vertebrate class. The activity of amphibian brain and bladder ChE and planarian ChE towards MeCh is lower than that seen with mammalian ChEs. The toad bladder true ChE also shows virtually no activity towards the non-specific esterase substrate α-naphthyl acetate.[24] These anomalous substrate specificities support the view that the eserine resistance is due to some property of the enzyme.

In the organ bath, eserine depressed the response to nerve stimulation at 10^{-5} – 10^{-4} g/ml, and at the higher dosage also depressed the response to ACh. Washing restored both responses to normal and on occasions converted the depression to potentiation. Some increased potentiation following washing was also seen with 5×10^{-6} g/ml neostigmine. although this effect was absent at 5×10^{-7} g/ml. These results suggest that both inhibitors, as well as inactivating ChE, can exert a direct depressant action on the post-junctional receptors. In the case of neostigmine the anti-ChE action at the dosage used overrides the depressant effect. In the case of eserine, however, the resistance to inhibition of the ChE and the much greater lipoid-solubility of the molecule, which facilitates access to the receptor region, causes the depressant effect to predominate.

Eserine, but not neostigmine, increased the tone and spontaneous activity of the bladder. A similar action of eserine has been reported for the toad rectum.[3] The comparative lipoid-solubilities of the two compounds suggest that these actions of eserine are due to inhibition of intracellular ChEs located within the muscle fibres. Histochemical studies on the toad bladder have revealed ChE localised within the muscle cells.[24] A similar difference in the effects of eserine and neostigmine on the tone of the chick amnion was reported by Cuthbert.[25] It is not clear why eserine at 10^{-5} g/ml caused potentiation of the ACh response in some preparations although the response to nerve stimulation was depressed in all cases. A possible explanation is that the response seen is due partly to an intracellular action of ACh unrelated to the nerve-endings. Easier accessibility of the eserine molecule to the nervous receptors than to these intracellular sites would then mean that the depressant effect on the nerve-mediated response at a lower dosage than that at which it would affect the response to applied ACh. This situation is being further studied in this laboratory. The pharmacological work previously cited indicated probable cholinergic innervation.[1] ACh caused contraction of the bladder, while adrenaline and noradrenaline caused relaxation. Atropine only reduced the response to pelvic nerve stimulation by up to 50 per cent, but atropine resistance is characteristic of

bladder preparations.[2,26] Adrenergic blocking agents reduced or blocked the nerve-mediated response only in doses which also blocked the effect of applied ACh. The fact that neostigmine in the organ bath not only increases the effect of applied ACh but also potentiates the nerve-mediated response and shortens the latency period of contraction, lends additional support to the previous pharmacological evidence for the cholinergic innervation of the toad bladder. Our results support Hawkins and Mendel's warning regarding the interpretation of evidence as to the nature of transmission mechanisms following the failure of eserine to potentiate responses. More widely they emphasize that caution must be used in extrapolation of mammalian pharmacological and biochemical results to the lower vertebrates. At least with ChE, substrates specificity and the response to a number of specific inhibitors remain the safest criteria by which the presence and nature of the enzyme in different animal classes can be compared.

Acknowledgements—Thanks are due to Miss G. Lang for carrying out some of the initial manometric work, and to Dr. L. Austin and Mr. G. Campbell for helpful criticism and discussion. We also thank Dr. Austin for a gift of BW 284C51, and Dr. R. Davis for a gift of neostigmine bromide. This work was supported in part by the National Institute of Neurological Diseases and Blindness (P.H.S. Grant B-2902), National Health and Medical Research Council of Australia and by the Wellcome Trust.

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