Br. J. Pharmac. (1976), **56**, 93–99

THE EFFECT OF EDROPHONIUM ON ERYTHROCYTE ACETYLCHOLINESTERASE AND NEUROMUSCULAR FUNCTION IN THE RAT

H.E. BARBER, T.N. CALVEY, K.T. MUIR & K. TAYLOR
Department of Pharmacology and Therapeutics, University of Liverpool, Liverpool L69 3BX

1 The relation between the concentration of edrophonium in plasma, inhibition of red cell acetylcholinesterase, and neuromuscular transmission was studied in the rat.

2 In both *in vivo* and *in vitro* conditions, red cell acetylcholinesterase activity was predictably related to the concentration of the quaternary amine.

3 After low doses of edrophonium (1.0 μmol/kg) there was a significant correlation between the monophasic potentiation of twitch tension and the plasma concentration of the drug. In contrast, with higher doses of edrophonium (4.0 or 10.0 μmol/kg) a biphasic potentiation of twitch tension was observed; this was only correlated with the plasma concentration of the drug during the secondary decline in neuromuscular facilitation. Subsequent recovery of normal neuromuscular transmission invariably occurred at a constant plasma concentration of edrophonium.

Introduction

It has been recognized for many years that edrophonium chloride has a rapid and transient effect on neuromuscular transmission (Randall, 1950). Nevertheless, the mechanism of action of this phenolic quaternary amine on the motor end-plate has not been precisely defined. Edrophonium reversibly inhibits acetylcholinesterase (acetylcholine acetylhydrolase; EC 3.1.1.7) and has a direct action on the motor end-plate (Randall, 1950; Wescoe & Riker, 1951), although these effects may play only a subsidiary role in the facilitation of neuromuscular transmission. For instance, it has been suggested that the drug acts by inducing antidromic discharges in presynaptic terminals that are disseminated by local axon reflexes (Blaber, 1972). In these conditions, the pharmacological effects of edrophonium may not be predictably related to its plasma concentration or to acetylcholinesterase inhibition. Previous experiments have shown that the unchanged drug is rapidly removed from the circulation (Back & Calvey, 1972b); the relation between its plasma concentration and neuromuscular transmission remains a matter of conjecture.

This paper is concerned with two main aspects of this problem. In the first place, we have studied the relation between the concentration of edrophonium in plasma and the inhibition of acetylcholinesterase activity in rat erythrocytes. These experiments have been carried out in both *in vivo* and *in vitro* conditions. Secondly, the relation between the clearance of edrophonium from plasma, the facilitation of neuromuscular transmission, and the subsequent recovery of normal twitch tension has been investigated. A preliminary account of this work has been presented to the British Pharmacological Society (Barber, Calvey, Muir & Taylor, 1975).

Methods

In vitro *studies*

Male Wistar rats (250–300 g) were anaesthetized with ethyl carbamate (14% w/v in distilled water; 1.4 g/kg, i.p.) and a carotid artery was cannulated with polyethylene tubing. Heparin sodium (approximately 200 u in 0.2 ml) was injected into the arterial cannula and the animals were then exsanguinated.

In most experiments, aqueous solutions of edrophonium chloride (1 μmol/l, 2 μmol/l, 5 μmol/l, 10 μmol/l, 20 μmol/l, 50 μmol/l, 100 μmol/l, 200 μmol/l, 500 μmol/l, 1 mmol/l; 10 μl) were added to rat blood (1.0 ml). A capillary tube was filled with a sample of blood (about 50 μl), sealed at one end, and centrifuged (12,000 ***g***; 2 minutes). The concentration of edrophonium in plasma was calculated from the haematocrit value. It was assumed that edrophonium was solely distributed in plasma, and that the quaternary amine was not bound to (or did not penetrate) the red cell membrane. The validity of this

assumption was confirmed by concurrent studies with [^{14}C]-edrophonium (ethyl[1-^{14}C]-dimethyl (3-hydroxyphenyl)-ammonium chloride, specific radioactivity = 13.7 mCi/mmol; The Radiochemical Centre, Amersham, Bucks). In these experiments, [^{14}C]-edrophonium chloride (100 nCi in 10 µl) was added to rat blood (1.0 ml) and the concentration of the drug in plasma was determined by liquid scintillation spectrometry. Similar results were obtained in both types of experiments.

In vivo *studies*

Male Wistar rats (250–350 g) were anaesthetized with ethyl carbamate (1.4 g/kg i.p.) and a polyethylene cannula was placed in a jugular vein. Respiration was assisted (when necessary) by means of an endotracheal tube and a Palmer miniature respiration pump (C.F. Palmer Ltd., Sandwich, Kent). The contraction of the tibialis anterior muscle in response to supramaximal stimulation of the sciatic nerve (0.33 Hz, 0.5 ms) was measured from both hind limbs by means of a strain gauge. Arterial blood pressure was recorded from a common carotid artery using a polyethylene cannula filled with heparin sodium (approximately 100 u/ml) which was attached to a pressure transducer.

The contralateral common carotid artery was also cannulated and heparin sodium (200 u in 0.2 ml) was injected intravenously at the beginning of each experiment.

[^{14}C]-edrophonium chloride (1.0, 4.0, or 10.0 µmol/kg in 0.9% w/v NaCl solution) was injected into the jugular vein within 5 seconds. Samples of arterial blood (approximately 250 µl) were removed at 1, 2, 3, 5, 10, 15, 30, 45, 60, 90, 120 and 180 minutes. Small amounts of heparin sodium (1000 u/ml) were occasionally used to prevent coagulation in the carotid arterial cannula between the collection of consecutive blood samples. Plasma was obtained from part of each sample by centrifugation, and the remainder of the sample was used for the assay of acetylcholinesterase activity.

Measurement of [^{14}C]-edrophonium in plasma

Samples of plasma (usually 50 µl) were directly added to scintillation fluid (9 ml) of the following composition: butyl-PBD (6.7 mg), toluene (5.0 ml), Triton-X-100 (3.0 ml) and distilled water (1.0 ml). A mixture of isoamyl alcohol and toluene (1:5, v/v; 1.0 ml) was then added to each scintillation vial. Radioactivity in plasma was counted at an efficiency of approximately 60% in a Unilux Nuclear Chicago liquid scintillation spectrometer. Counting efficiency was determined by the channels ratio method; similar efficiencies (±1%) were obtained when [1-^{14}C]-*n*-hexadecane (The Radiochemical Centre) was used as an internal standard.

Unchanged [^{14}C]-edrophonium was separated from its glucuronide metabolite in plasma by descending paper chromatography (Back & Calvey, 1972a). Radioactivity on paper chromatograms was identified in a Tracerlab 4π radiochromatogram scanner. The concentration of [^{14}C]-edrophonium was then determined from the total radioactivity in plasma and the results of paper chromatography.

Measurement of acetylcholinesterase activity in blood cells

Acetylcholinesterase activity in red blood cells was measured by a modification of the method of Smith (1974). Samples of [1-^{14}C]-acetyl-β-methylcholine iodide (specific radioactivity = 9.92 mCi/mmol; NEN Chemicals GmbH; 25.4 nmol in 50 µl sodium phosphate buffer, (40 mmol/l; pH = 7.4)) were placed in glass vials and freeze dried. All measurements of acetylcholinesterase activity were carried out in a cold room at 4°C.

Immediately prior to the assay, blood specimens were thoroughly stirred in a vortex mixer and pre-cooled in an ice-cold water bath. Blood (50 µl) was added to freeze-dried [^{14}C]-acetyl-β-methylcholine; after 1 min, the reaction was stopped by the addition of HCl (100 µl; 200 mmol/litre). [1-^{14}C]-acetic acid was extracted from the mixture as described by Smith (1974), and radioactivity was counted by liquid scintillation spectrometry.

Results

In vitro *studies*

There was a sigmoid relationship between the concentration of edrophonium in plasma and the activity of red cell acetylcholinesterase (Figure 1). When the concentration of the quaternary amine in rat plasma was increased from 0.1 µmol/l to 2.0 µmol/l, acetylcholinesterase activity decreased from 80% to 10% of control values. Between these limits, there was a linear relationship between the logarithm of edrophonium concentration and acetylcholinesterase activity (Figure 1). When the concentration of the drug was 10.0 µmol/l or greater, no enzyme activity was present in rat erythrocytes.

In vivo *studies*

After intravenous injection of [^{14}C]-edrophonium the plasma concentration of the unchanged drug rapidly decreased within 10–15 min (Table 1; Figure 2). The rapid decline in the concentration of the drug was seen

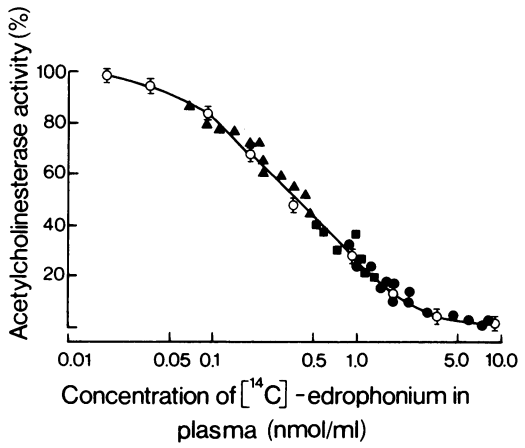

Figure 1 Effect of edrophonium on red cell acetylcholinesterase activity. Each open circle and vertical bar represents the mean ± s.d. of at least 3 observations in *in vitro* conditions. Closed symbols correspond to results obtained in *in vivo* conditions at three different dose levels: (●) 10.0 μmol/kg; (■) 4.0 μmol/kg; (▲) 1.0 μmol/kg.

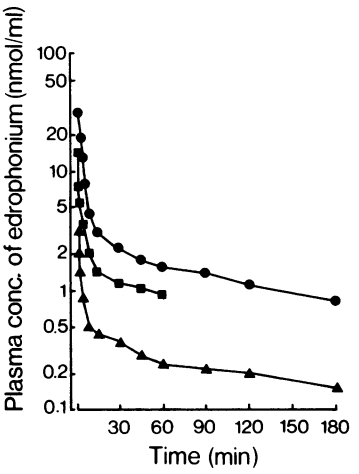

Figure 2 The concentration of unchanged [^{14}C]-edrophonium in plasma after intravenous administration of three different doses of the drug: (●) 10.0 μmol/kg; (■) 4.0 μmol/kg; (▲) 1.0 μmol/kg. Each graph represents the results of a typical experiment.

at all three dose levels. After 10 min, the plasma concentration of unchanged drug decreased more slowly and a semi-logarithmic plot relating edrophonium concentration to time (Figure 2) could be invariably resolved into at least two exponential components by the method of residuals (Riggs, 1963).

Measured concentrations of [^{14}C]-edrophonium in plasma were related to red cell acetylcholinesterase activity (Figure 1). Three different doses of the drug were used in order to compare *in vitro* and *in vivo* enzyme inhibition. After intravenous injection of edrophonium (10 μmol/kg) acetylcholinesterase activity gradually increased to 31% of control values (Figure 1). Corresponding enhancement of enzyme activity was associated with the lower doses of edrophonium. There was a statistically significant correlation between acetylcholinesterase inhibition in *in vivo* and *in vitro* conditions ($r=0.99$, $d.f.=89$; $P<0.001$; Figure 1).

Table 1 The concentration of unchanged [^{14}C]-edrophonium in plasma after intravenous administration of three different dose levels (1.0 μmol/kg, 4.0 μmol/kg and 10.0 μmol/kg)

	Dose of [^{14}C]-edrophonium		
	1.0 μmol/kg	*4.0 μmol/kg*	*10.0 μmol/kg*
Time	*Plasma concentration of edrophonium*		
(min)	*nmol/ml*	*nmol/ml*	*nmol/ml*
1	2.97 ± 0.09 (4)	10.90 ± 1.39 (3)	29.67 ± 1.86 (3)
2	2.03 ± 0.09 (4)	6.35 ± 0.64 (4)	17.77 ± 2.41 (3)
3	1.43 ± 0.08 (4)	4.78 ± 0.40 (4)	12.70 ± 0.89 (3)
5	0.85 ± 0.04 (4)	3.33 ± 0.16 (4)	8.20 ± 1.37 (3)
10	0.44 ± 0.01 (4)	2.06 ± 0.30 (4)	4.93 ± 0.44 (3)
15	0.33 ± 0.04 (4)	1.41 ± 0.04 (4)	3.60 ± 0.25 (3)
30	0.26 ± 0.04 (4)	0.95 ± 0.09 (4)	2.30 ± 0.00 (3)
45	0.25 ± 0.02 (4)	0.75 ± 0.10 (4)	1.84 ± 0.03 (3)
60	0.19 ± 0.02 (4)	0.65 ± 0.10 (4)	1.63 ± 0.03 (3)
90	0.15 ± 0.05 (4)		1.43 ± 0.07 (3)
120	0.12 ± 0.03 (4)		1.15 ± 0.04 (3)
180	0.09 ± 0.02 (4)		0.96 ± 0.02 (3)

Data are means ± s.e. mean, with the number of experiments in parentheses.

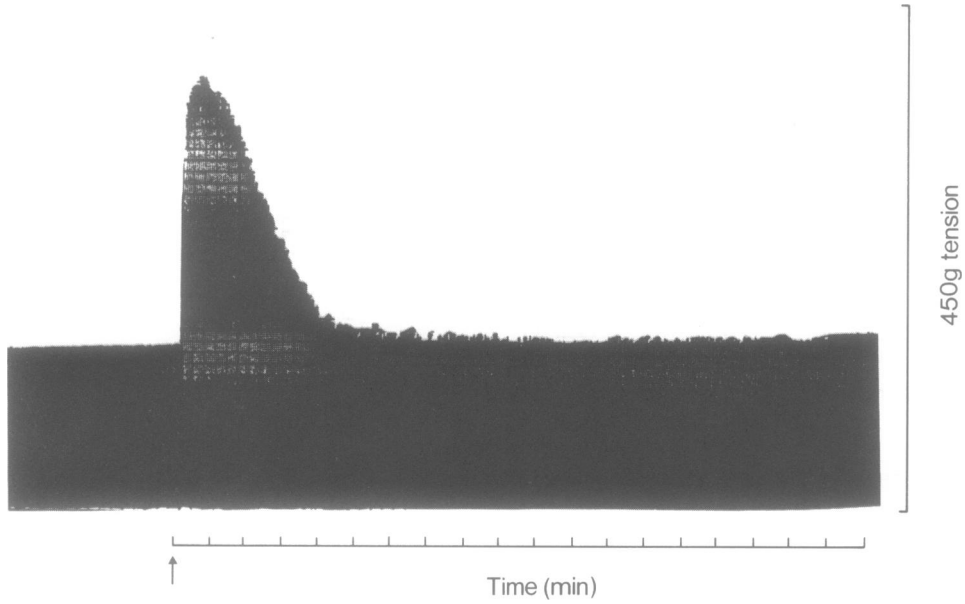

Figure 3 Effect of edrophonium (1.0 μmol/kg) on contraction of tibialis anterior muscle in response to supramaximal stimulation of the sciatic nerve (0.33 Hz, 0.5 ms). Figure retouched.

Relation between plasma concentration of edrophonium and facilitation of neuromuscular transmission

After intravenous injection of edrophonium (1.0 μmol/kg), neuromuscular transmission was facilitated in a monophasic manner. Tibialis twitch tension rapidly increased, but returned to its control value within 5 min (Figure 3). The maximum plasma concentration of edrophonium (2.97 ± 0.09 nmol/ml; Table 1) was associated with an increase in twitch tension to at least twice control values (Figure 4). Twitch tension returned to its control value after 5 min at a plasma concentration of 1.32–1.80 nmol/ml. There was a statistically significant correlation between the concentration of edrophonium in plasma and facilitation of neuromuscular transmission, as assessed by the percentage increase in control twitch tension ($r = 0.87$, $d.f. = 14$; $P < 0.001$; Figure 4).

Different results were obtained after intravenous administration of higher doses of edrophonium (4.0 μmol/kg or 10.0 μmol/kg). In these conditions, neuromuscular transmission was enhanced biphasically (Figure 5). An immediate, evanescent increase in twitch tension rapidly declined, and was succeeded by a second facilitatory phase that decreased to control levels within 15 min (Figure 5). When the experimental data preceding the peak of the second facilitatory phase were considered, there was no significant correlation between the concentration of edrophonium

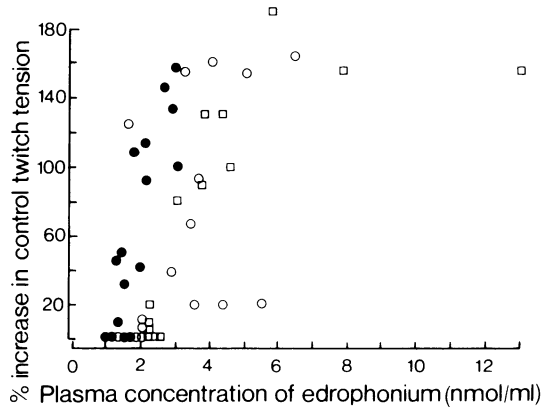

Figure 4 Correlation between the plasma concentration of edrophonium and the percentage increase in twitch tension of the tibialis muscle compared with control values. Dose of edrophonium = 1.0 μmol/kg (●), 4.0 μmol/kg (○) or 10.0 μmol/kg (□). At the higher dose levels (4.0 or 10.0 μmol/kg), results obtained before the peak of the second phase of neuromuscular facilitation were omitted.

in plasma and the percentage change in twitch tension, at either 4.0 μmol/kg ($r = -0.36$, $d.f. = 9$, $P > 0.05$) or 10.0 μmol/kg ($r = -0.12$, $d.f. = 12$, $P > 0.05$). A wide range of plasma concentrations of edrophonium

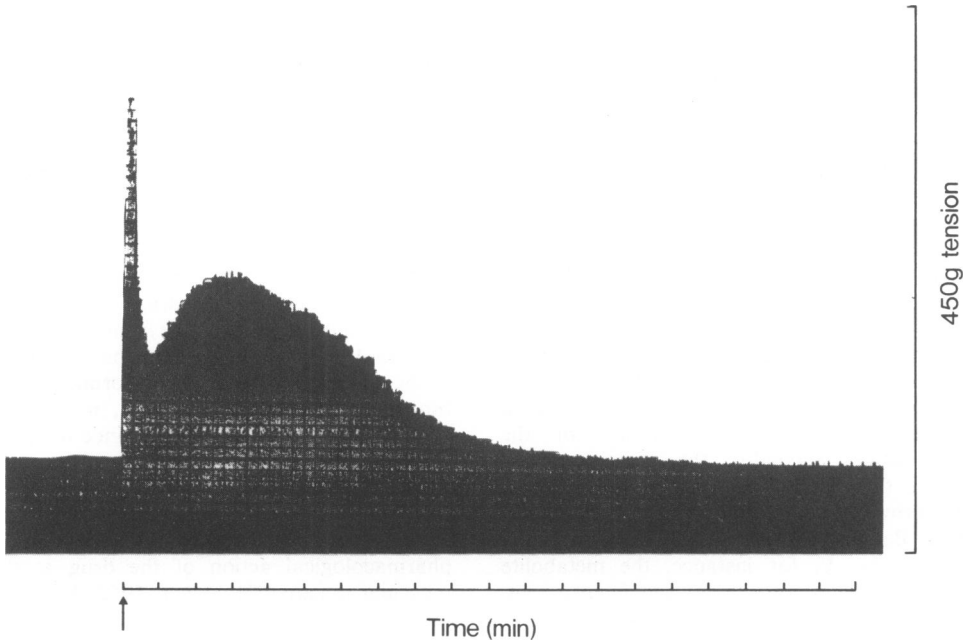

Figure 5 Effect of edrophonium (4.0 μmol/kg) on contraction of tibialis anterior muscle in response to supramaximal stimulation of the sciatic nerve (0.33 Hz, 0.5 ms). Figure retouched.

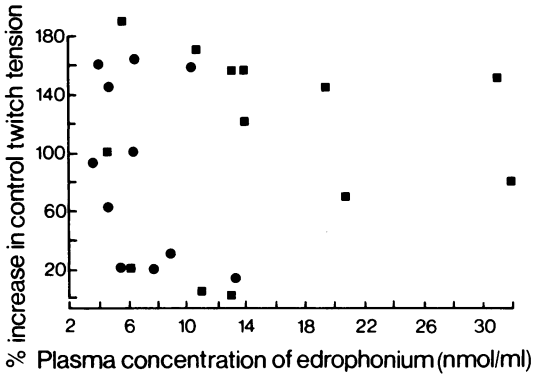

Figure 6 Relation between the plasma concentration of edrophonium and the percentage increase in twitch tension over control values before the peak of the second phase of neuromuscular facilitation. Dose of edrophonium =4.0 μmol/kg (●) or 10.0 μmol/kg (■).

(4.1–31.0 nmol/ml) was associated with enhancement of the tibialis twitch tension to more than twice its control value (Figure 6). In contrast, the decline in tibialis twitch tension from its peak value during the second facilitatory phase (Figure 5) was significantly correlated with the clearance of the drug from plasma at both 4.0 μmol/kg ($r=0.53$, $d.f.=15$; $0.05>P>0.01$) and 10.0 μmol/kg ($r=0.73$, $d.f.=12$; $0.01>P>0.001$) (Figure 4). The plasma concentration at which tibialis twitch tension returned to normal was remarkably constant (1.97 ± 0.12 nmol/ml; mean ± s.e. mean).

Discussion

Since edrophonium chloride combines reversibly with cholinesterase (Wilson, 1955), the activity of the enzyme should be predictably related to the concentration of the quaternary amine. Unfortunately, many of the available methods used to determine cholinesterase activity (for instance, techniques involving dilution or significant concentrations of high affinity substrates) cannot be reliably applied to the assay of reversible enzyme inhibitors. Radiometric determinations of acetylcholinesterase activity (Smith, 1974) are less subject to these limitations, since they depend on the use of relatively small concentrations of lyophilized, low affinity substrate. These methods have been used in the present experiments; although some reversal of enzyme inhibition must occur on addition of the substrate, measurements of acetylcholinesterase activity should be closely related to the concentration of edrophonium in both *in vivo* and *in vitro* conditions.

The results of this study are entirely consistent with this concept. There was a sigmoid relationship between the logarithm of plasma edrophonium concentration and acetylcholinesterase activity in *in vitro* conditions. At plasma concentrations of 0.1–2.0 nmol/ml, the relation between these parameters was linear. In addition, when [^{14}C]-edrophonium was injected intravenously, and the measured concentration of the drug in plasma was related to acetylcholinesterase activity, a similar relationship was observed. The results of these experiments have two important implications. In the first place, since acetylcholinesterase activity is directly related to the plasma concentration of edrophonium in both *in vitro* and *in vivo* conditions, the relation between the two parameters can be used to measure the plasma concentration of the quaternary amine. Secondly, the present results confirm that the glucuronide metabolite of edrophonium (ethyldimethyl-(3-oxyphenyl) ammonium glucuronide; Back & Calvey, 1972a) has little or no biological activity. If, for instance, the metabolite inhibited acetylcholinesterase to any significant extent, the relationship between the concentration of edrophonium in plasma and enzyme activity would differ in *in vivo* and *in vitro* conditions.

After three different doses of edrophonium the plasma concentration of the unchanged drug decreased in a qualitatively similar manner. An initial rapid fall was followed by a slower declining phase, and a semi-logarithmic plot relating plasma concentration to time could be resolved into more than one exponential component. Similar results were obtained in an earlier study (Back & Calvey, 1972b) in which the clearance of a single dose of edrophonium was studied in the rat. The present experiments were carried out at three different dose levels; in general, the results are consistent with the rapid removal of edrophonium from plasma, irrespective of the dose.

The effects of edrophonium on neuromuscular transmission have been studied by many authors (Randall, 1950; Hobbiger, 1952; Katz & Thesleff, 1957; Blaber & Bowman, 1959; Ferry & Marshall, 1971; Blaber, 1972; Whittaker, 1975). In spite of the use of sophisticated electrophysiological techniques, its precise mechanism of action on the neuromuscular junction has not been unequivocally established. Studies on the isolated tenuissimus muscle of the cat suggest that edrophonium produces antidromic discharges in presynaptic nerve terminals. These antidromic impulses are spread by axon reflexes to other nerve terminals, resulting in repetitive end-plate potentials (Blaber, 1972). Other authors consider that their experimental data can be best explained in terms of the action of the drug on end-plate acetylcholinesterase. Thus in the rat isolated diaphragm the prolongation of the end-plate potential is due to inhibition of the enzyme (Ferry & Marshall, 1971). Similarly, in *in vivo* conditions the effect of edrophonium on miniature end-plate potentials, end-plate potentials, and the quantal release of transmitter suggest that acetylcholinesterase inhibition is probably responsible for the facilitation of neuromuscular transmission (Whittaker, 1975). In the present work, we have been primarily concerned with the relation between the pharmacological action of the drug and its clearance from plasma. After low doses of edrophonium, there was a statistically significant correlation between the potentiation of twitch tension and the plasma level of the drug. A similar relationship was seen during the recovery from facilitation induced by high doses of edrophonium; thus the concentration of the drug in plasma at which normal neuromuscular function returned was constant, irrespective of the dose of the quaternary amine. Since the plasma level was directly related to erythrocyte acetylcholinesterase activity, these results are not apparently inconsistent with the classical view that reversible inhibition of the enzyme is responsible for the pharmacological action of the drug at the neuromuscular junction (Hobbiger, 1952; Katz & Thesleff, 1957; Ferry & Marshall, 1971; Whittaker, 1975). However, it cannot be necessarily assumed that acetylcholinesterase activity in red blood cells and at the neuromuscular junction are closely related. If edrophonium acts on presynaptic nerve terminals (Blaber, 1972), the high concentrations of acetylcholine released locally may rapidly reverse enzyme inhibition at the motor end-plate but not on the red cell membrane. In these conditions, inhibition of erythrocyte cholinesterase may not be directly related to enzyme activity or other presynaptic and postsynaptic events at the neuromuscular junction. Thus, the significance of the correlation between the potentiation of tibialis twitch tension and red cell acetylcholinesterase activity is a matter of conjecture. It is, perhaps, more likely that both enzyme activity and the pharmacological effects of edrophonium at the motor end-plate are independently correlated with the plasma level of the drug.

In contrast, the rapid increase in twitch tension induced by large doses of edrophonium, and its subsequent decline, were quite independent of the plasma level of the drug or red cell acetylcholinesterase inhibition. In these circumstances, the explanation for the biphasic response to edrophonium is quite obscure, although the initial facilitation may be followed by the onset of 'receptor inactivation' (Hubbard, 1973). In *in vitro* conditions, the amplitude of extracellular end-plate potentials is increased by low concentrations of edrophonium, although opposite effects are produced by high concentrations of the drug (Ferry & Marshall, 1971). The results of the present experiments are not inconsistent with these concepts, although they do not unequivocally exclude other mechanisms of action.

The authors are grateful to Dr G.A. Buckley for helpful discussions, and the technical assistance of Miss S. Newby is gratefully acknowledged. This research was supported by grants from the Peel Medical Research Trust and the Muscular Dystrophy Group of Great Britain. Correspondence to T.N.C.

References

BACK, D.J. & CALVEY, T.N. (1972a). Excretion of ^{14}C-edrophonium and its metabolites in bile: role of the liver cell and the peribiliary vascular plexus. *Br. J. Pharmac.*, **44**, 534–543.

BACK, D.J. & CALVEY, T.N. (1972b). The removal of ^{14}C-edrophonium from the circulation. *Br. J. Pharmac.*, **46**, 355–357.

BARBER, H.E., CALVEY, T.N., MUIR, K.T. & TAYLOR, K. (1975). The relation between the plasma concentration of edrophonium, inhibition of erythrocyte acetylcholinesterase, and the facilitation of neuromuscular function in the rat. *Br. J. Pharmac.*, **54**, 263P.

BLABER, L.C. (1972). The mechanism of the facilitatory action of edrophonium in cat skeletal muscle. *Br. J. Pharmac.*, **46**, 498–507.

BLABER, L.C. & BOWMAN, W.C. (1959). A comparison between the effects of edrophonium and choline in the skeletal muscles of the cat. *Br. J. Pharmac.*, **14**, 456–466.

FERRY, C.B. & MARSHALL, A.R. (1971). Electrophysiological estimation of inhibition of acetylcholinesterase with normal transmitter release. *J. Physiol.*, **218**, 66–67P.

HOBBIGER, F. (1952). The mechanism of anticurare action of certain neostigmine analogues. *Br. J. Pharmac. Chemother.*, **7**, 223–236.

HUBBARD, J.I. (1973). Microphysiology of vertebrate neuromuscular transmission. *Physiol. Rev.*, **53**, 674–723.

KATZ, B. & THESLEFF, S. (1957). The interaction between edrophonium (Tensilon) and acetylcholine at the motor end-plate. *Br. J. Pharmac. Chemother.*, **12**, 260–264.

RANDALL, L.O. (1950). Anticurare action of phenolic quaternary ammonium salts. *J. Pharmac. exp. Ther.*, **100**, 83–93.

RIGGS, D.S. (1963). *The mathematical approach to physiological problems*, pp. 146–161. Baltimore: The Williams and Wilkins Company.

SMITH, M.H. (1974). Determination of blood acetylcholinesterase inhibition by reversible inhibitors *in vivo*. *Biochem. Med.*, **10**, 236–244.

WESCOE, W.C. & RIKER, W.F. (1951). The pharmacology of anticurare agents. *Ann. N.Y. Acad. Sci.*, **54**, 438–455.

WHITTAKER, R. (1975). Neuromuscular actions of edrophonium in the lateral segmental tail muscle of the rat *in vivo*. *J. Pharm. Pharmac.*, **27**, 420–424.

WILSON, I.B. (1955). The interaction of tensilon and neostigmine with acetylcholinesterase. *Arch. int. Pharmacodyn. Thér.*, **104**, 204–213.

(Received July 3, 1975)