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ACETYLCHOLINESTERASE ACTIVITY IN INTACT AND HOMOGENIZED SKELETAL MUSCLE OF THE FROG

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WITH AN APPENDIX BY P. C. MOLENAAR

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SUMMARY

1. Enzymatic hydrolysis of acetylcholine (ACh) was determined in intact frog sartorius muscles or their homogenates. The V_{max} was 29 nmol min^{-1} in intact muscles and 46 nmol min^{-1} per muscle in homogenates, and the K_m was 6 and 0·2 mM, respectively. The muscle was divided into small segments, which were homogenized; the junctional cholinesterase (ChE) accounted for 60 % of total enzyme activity.

2. At low substrate concentrations the rate of hydrolysis was up to 30 times higher in homogenates than in intact muscles. This difference was greatly reduced at very high substrate concentrations. It appears that most of the ChE in intact muscle is 'occluded' to external ACh, mainly because the ChE at the edges of the synaptic cleft prevents the ACh from reaching the enzyme situated further inwards, which consequently does not contribute to its hydrolysis; homogenization makes all synaptic ChE accessible to added ACh.

3. Incubation of sartorius muscles with collagenase caused an 80 % decrease in ChE activity (determined in homogenates) of end-plate-containing parts which became similar to that in end-plate-free parts on which collagenase had little effect.

4. Histochemistry showed that the tendon–muscle junction contained folds which were stained intensively for ChE.

5. Diethyldimethylpyrophosphonate, neostigmine, eserine, and di-isopropyl-fluorophosphonate inhibited ChE activity in this order of potency. The I_{50} values (i.e. the concentrations of the drugs which caused a 50 % inhibition) were about 5 times higher in intact than in homogenized tissue.

6. Neostigmine, 0·15 and 0·4 μM, increased the time constant of miniature end-plate currents 1·3- and 1·8-fold, and slowed down ChE activity of muscle homogenates by 1·4 and 2·1 times, respectively, without significantly affecting ACh hydrolysis by intact muscles. This indicates that synaptic ChE is not present in large excess.

7. It is concluded that ChE activity measured in homogenates presents a better

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picture of *in situ* ChE activity than that measured in whole muscles especially for evaluating the effect of ChE inhibitors. A mathematical model for ChE-hindered diffusion of ACh is presented in an Appendix.

INTRODUCTION

Application of acetylcholine (ACh) at the neuromuscular junction produces membrane noise due to the random opening and closing of ionic channels (Katz & Miledi, 1970, 1971; Anderson & Stevens, 1973). Spectral analysis of the noise reveals that the mean life of the unitary event (the opening of a single channel) is similar to that of the miniature end-plate current (m.e.p.c.) caused by the release of a multimolecular amount of ACh from the motor nerve terminals (Katz & Miledi, 1972, 1973*a*; Anderson & Stevens, 1973). This suggests that the time course of the m.e.p.c. is largely determined by the closing rate of the channels. Apparently the action of synaptic cholinesterase (ChE) is so rapid that an ACh molecule has little chance to activate more than one receptor. On the other hand, when ChE is completely inactivated, the effect of a released quantum of ACh lingers on until it is terminated by diffusion of the transmitter from the synaptic cleft (Katz & Miledi, 1973*b*, see also Kordaš, 1977).

In most studies on the relationship between ChE activity and end-plate currents (e.p.c.s), attention has been focussed on the effects of *complete* inactivation of the enzyme, and it is not clear how far the shape of the m.e.p.c. is influenced by the rate of enzymatic activity. This can be investigated by studying the effects of different degrees of ChE inhibition on the shape of the m.e.p.c. However, the problem arises of how to determine the enzymatic activity. There are several reports showing that intact muscles hydrolyse ACh at a much slower rate than their homogenates (Feng & Ting, 1938; Marnay & Nachmansohn, 1938; Mittag, Ehrenpreis & Hehir, 1971; Lund Karlsen & Fonnum, 1977). Marnay & Nachmansohn (1938) suggested that in intact frog muscle the relative slowness of the enzymatic hydrolysis of external ACh is due to its slow diffusion into the muscle, and Hobbiger (1976) suggested that with assays on intact muscle the ChE activity in the synapses is greatly underestimated by hindered diffusion of external ACh into the synaptic cleft. On the other hand, in mammalian muscle the difference in ChE activity between intact and homogenized preparations has been attributed mainly to the liberation of intracellular esterase by the homogenization. Some workers have therefore suggested that the data obtained with intact preparations are more relevant to the physiological function of the enzyme at the synapses than data obtained with homogenates (Mittag *et al.* 1971; Lund Karlsen & Fonnum, 1977).

In the present experiments we examined the effects of several ChE inhibitors on ACh hydrolysis by intact frog sartorius muscles or muscle segments, and in homogenates prepared from them. We also studied the effects of partial ChE inhibition on the shape of the m.e.p.c. and found, among other things, that the rate of hydrolysis of ACh in homogenates was a much more sensitive index for the effects of ChE inhibitors than the hydrolysis by intact muscle or muscle segments. A small degree of enzyme inhibition, as determined in the homogenate, was accompanied by a significant increase of the amplitude and the decay time of the m.e.p.c. Part of this work has been presented in preliminary form (Miledi, Molenaar & Polak, 1981).

METHODS

The experiments were made on sartorius muscles of male and female frogs (*Rana temporaria*, unless otherwise stated). In a few frogs, the sartorius of one leg was denervated under ether anaesthesia by cutting the sciatic nerve in the pelvis (Miledi, 1960). The muscles were often divided into an end-plate-free pelvic segment (non-e.p.), 4–5 mm long, and a larger segment containing all the end-plates (e.p., cf. Miledi, Molenaar & Polak, 1977). Muscles and muscle segments were incubated in Ringer solution of the following composition (mM): NaCl, 115·6; KCl, 2·0; $CaCl_2$, 1·8; sodium phosphate, 2·0 (pH 7·0). Homogenization of the tissue was done in Ringer solution, in a glass-in-glass homogenizer, after cutting the tissue into fine fragments with scissors.

Electrophysiology. M.e.p.c.s were recorded from end-plates, identified under a compound microscope (Katz & Miledi, 1965), using two micro-electrode voltage clamps, and analysed with a computer (cf. Miledi & Parker, 1980).

Acetylcholinesterase. The method was in principle the same as described by Potter (1967). Intact muscles or muscle segments, were gently shaken in 1 ml Ringer solution with [^{3}H]ACh at different concentrations. To assay esterase activity in homogenates, the tissue was ground in Ringer solution (final concentration between 0·2 and 0·5% (w/v)) and incubated in plastic scintillation vials. Incubations were at 20 °C and lasted usually 15 min. In the homogenates the reaction was stopped by the addition of 0·4 ml 10 M-acetic acid, which converted the [^{3}H]acetate produced during the incubation into the free acid. For the non-homogenized tissue the 1 ml medium was transferred to a scintillation vial and the acetic acid was then added. The [^{3}H]acetic acid was extracted into a 5 ml (homogenate) or a 10 ml (intact tissue) toluene-based scintillator layer containing 20% (w/v) *n*-amylalcohol. There was no need to remove the water layer, containing the unsplit [^{3}H]ACh from the scintillation vial, because ACh is insoluble in the organic phase and, consequently, few counts from the [^{3}H]ACh were observed. In fact most counts in the blanks originated from a trace of [^{3}H]acetate in the substrate preparation. To keep the blanks low, the [^{3}H]ACh was purified every week by precipitation with potassium periodide (Polak & Molenaar, 1974). Values for 100% hydrolysis were obtained in the presence of 1 μg acetylcholinesterase (AChE) from electric eel. The recovery of the [^{3}H]acetate was about 50%.

In a few experiments the hydrolysis of ACh, methacholine and butyrylcholine (1 mM each) was determined by the colorimetric method of Hestrin (1949). In these experiments small pieces of muscle were incubated for 60 min at 20 °C in a volume of 0·15 ml Ringer solution.

Materials. [^{3}H]ACh (250 mCi/mmol) was from Amersham, eserine salicylate from Fluka, neostigmine bromide from Merck, electric eel AChE from Boehringer, Mannheim, and collagenase type I from Sigma. Di-isopropylfluorophosphonate (DFP) and diethyldimethylpyrophosphonate (DEPP) were kindly provided by Dr H. P. Benshop, Chemical Lab./RVO-TNO, Rijswijk, The Netherlands.

RESULTS

Fig. 1 shows the hydrolysis of [^{3}H]ACh at a low, non-saturating, concentration (1 μM) by two intact muscles. The amount of [^{3}H]acetate collected in the medium during the first 15 min period was almost as high as that in the following periods during which it remained stable. Subsequently, when [^{3}H]ACh was replaced by Ringer solution, there was release of some residual [^{3}H]acetate, but 70% of this was already collected in 15 min. This indicated that the diffusion of [^{3}H]ACh into, and that of [^{3}H]acetate out of the muscle are fairly rapid.

The activities of ChE in muscles from the same animal were usually similar. On the other hand large variations were observed among muscles from different animals, but the variations were not correlated with muscle weight (Fig. 2).

As expected, homogenates of sartorius muscles hydrolysed [^{3}H]ACh at a higher rate than intact preparations (Fig. 2). At ACh concentrations below 0·1 mM (cf. Fig. 5), this difference varied between 15 and 30 times in muscles from the same

animal, which is somewhat more than observed for the diaphragm and iris (Mittag *et al.* 1971; Lund Karlsen & Fonnum, 1977). It could be thought that the difference between the rates of hydrolysis is due to ChE on the muscle fibres preventing the added ACh from penetrating deep into the muscle, so that ChE in the interior would not contribute to the hydrolysis. However, muscle thickness was not an important factor because the difference was the same in 150 mg muscles (33-fold) as in 50 mg muscles (31-fold, see Fig. 2). Of course this does not exclude the possibility of a diffusion barrier around each individual muscle fibre.

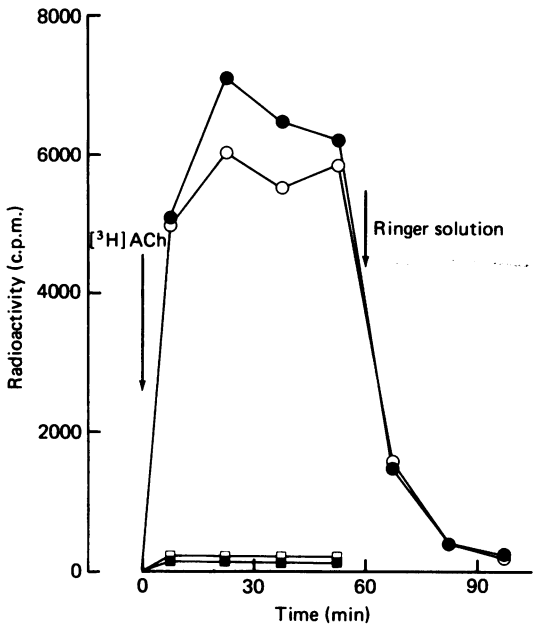

Fig. 1. Hydrolysis of [³H]ACh by intact frog sartorius. Muscles were incubated at 20 °C in Ringer solution containing 1 μM-[³H]ACh (80000 c.p.m.) during four successive periods of 15 min, and then in normal Ringer solution. The formation of [³H]acetate was determined in incubations of two muscles from the same frog (○, ●) and in two incubations in the absence of muscle (□, ■), indicating the incubation blanks, for which the results were not corrected.

Distribution of ChE in the muscle

The distribution of ChE along the length of the muscle fibres is illustrated in Fig. 3. In these experiments small segments of muscle were homogenized and assayed for ChE activity. There were two peaks of activity at positions, where end-plates are mainly located (Feng & Ting, 1938). From the ChE activity at extrajunctional sites it can be calculated, by extrapolation for the whole length of the muscle fibres, that non-junctional ChE accounted for about 40% of the total activity of the muscle. Apparently, about 60% of total enzyme is located at the neuromuscular junctions. Fig. 3 further suggests that the ChE peaks were somewhat reduced by denervation whereas the extrajunctional activity was unaffected.

The distribution of ChE was also investigated in non-homogenized segments of

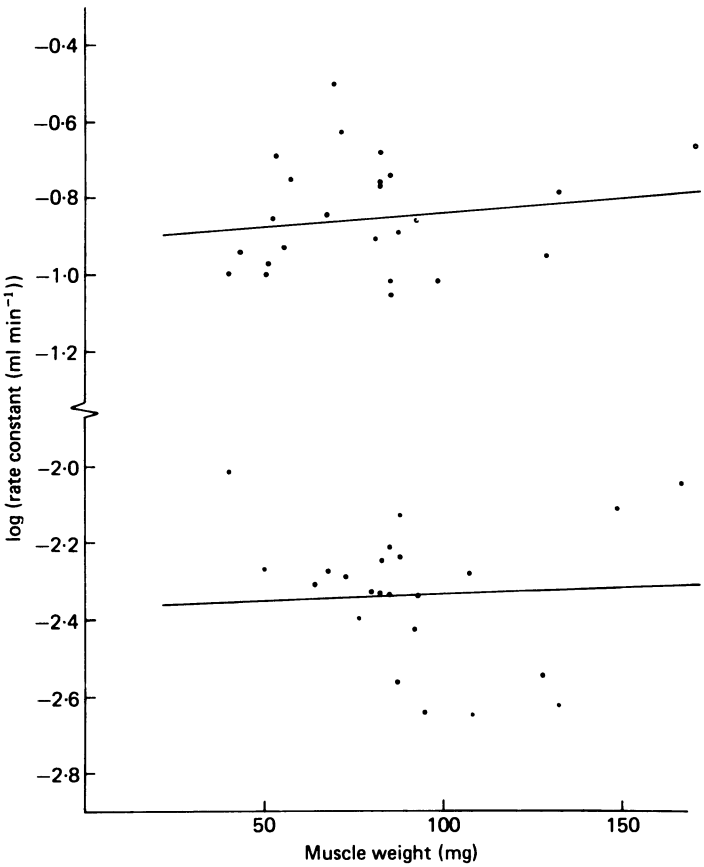

Fig. 2. Hydrolysis of [^{3}H]ACh at 20 °C by ChE in intact (lower line) and homogenized (upper line) sartorius muscles as a function of the wet weight of the muscles. The ordinate presents the $\log_{10}$ of the rate constant, in ml min^{-1} per muscle, which is equal to the slope in a *V vs. S* plot for the enzyme at non-saturating substrate concentrations (cf. Fig. 5).

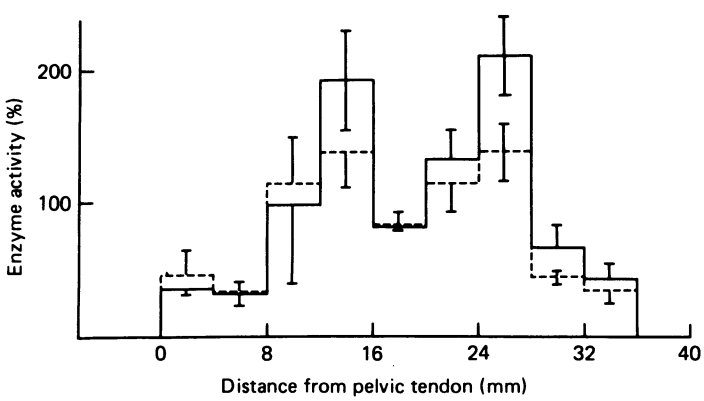

Fig. 3. Hydrolysis of 1 μM-[^{3}H] ACh in normal (continuous line) and denervated (15–20 days, interrupted line) sartorius muscles from *Rana esculenta*. Muscles were divided into nine pieces, which were subsequently homogenized. The values (enzyme activities per gram) are presented as percentages of the activity per gram whole muscle. Means ± S.E. of the means of three experiments.

muscle (Fig. 4). As compared with homogenates, the peaks of ACh hydrolyzing activity were very much reduced at the end-plates, confirming the observations by Marnay & Nachmansohn (1938). It is as if the junctional ChE is in some way occluded in intact end-plates.

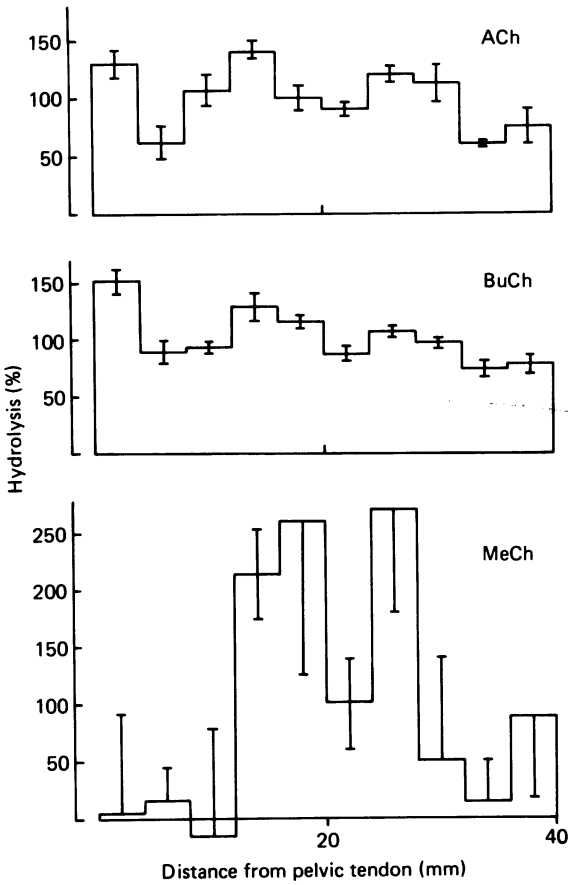

Fig. 4. The hydrolysis of ACh in non-homogenized muscle, after division into ten segments. The hydrolysis of 1 mM-ACh, methacholine (MeCh) and butyrylcholine (BuCh) was determined by a colorimetric method (Hestrin, 1949). The values are plotted in the same way as in Fig. 3. Means ± S.E. of the means of three experiments.

Fig. 4 further shows that with butyrylcholine as a substrate (test for pseudocholinesterase), there were two small peaks at the end-plates, which suggests that the junctional ChE of the frog has the ability to hydrolyse some butyrylcholine. Methacholine (test for acetylcholinesterase) was hydrolysed probably only at the junctions, but a low extrajunctional activity may have escaped detection as methacholine was hydrolysed at a much lower rate than the other two substrates, causing considerable errors in the measurements.

It is important to know if all junctional ChE is located extracellularly. For this purpose muscles were treated with collagenase, which solubilizes most, if not all,

extracellular ChE (Hall & Kelly, 1971; Betz & Sakmann, 1973; Sketelj & Brzin, 1977). After collagenase muscles lost about 80 % of total ChE (see Table 1). About 60 % was recovered in the medium. Thus, some activity was destroyed, possibly due to proteolysis of the enzyme caused by proteases in the preparation of collagenase used. Table 1 also shows that the end-plate region of the muscle lost 80 % of the activity and its activity became equal to that of the non-e.p. part. Apparently, the activity associated with the end-plate region (the peaks in Fig. 3) is mainly due to extracellular enzyme.

TABLE 1. Solubilization of extracellular ChE by collagenase

	Control (nmol min^{-1} g^{-1}) (*a*)	Collagenase (nmol min^{-1} g^{-1}) (*b*)	$\frac{b}{a}$	P_2*
Total in muscle	2·3±0·25 (7)	0·5±0·12 (6)	0.22	0.001
Total released into medium	0·07±0·03 (4)	1·2±0·18 (6)	17	0·001
In e.p. segments	2·6±0·42 (5)	0·5±0·07 (5)	0·19	0·003
In non-e.p. segments	0·7±0·15 (5)	0·3±0·11 (5)	0·43	0·09

The muscles were incubated at 20 °C for 90 min in Ringer solution containing 3 mg collagenase ml^{-1}. Subsequently, the collagenase Ringer solution was removed, replaced by Ringer solution and the muscles were kept overnight at 5 °C. The muscles were divided into end-plate-containing (e.p.) and end-plate-free (non-e.p.) segments. The whole muscles or muscle segments were homogenized and ChE activity was assayed at 20 °C in the presence of 1 μM-[^{3}H]ACh.

Values are means±S.E. of the means with the number of muscles in parentheses.

* Student's *t* test.

Plate 1 shows electron micrographs of the muscle–tendon region. It can be seen that there was intensive staining of ChE in folds of the muscle–tendon junction, suggesting that some occlusion of enzymatic activity could also occur at this site. This extra activity was not evident in the biochemical measurements of ChE in Fig. 3, possibly because it was too low to be detectable when pieces measuring several millimetres in length were homogenized.

Enzyme kinetics

The activity of ChE over a wide range of ACh concentrations is shown in Fig. 5. As expected, the activity of the homogenates was linearly dependent on the substrate at low concentrations and levelled off in the range between 0·1 and 1 mM-ACh. The activity in intact muscles showed a similar trend, but levelled off at higher concentrations of ACh. Again, at low substrate concentrations there was a large difference (20 times) between activities of homogenized and intact tissue. At higher concentrations this difference gradually decreased.

The question arose whether the effect of homogenization on ChE activity was due to an effect on the V_{max} or on the K_m of the enzyme: occlusion of enzyme in a compartment, inaccessible to exogenous ACh, would affect the V_{max}, whereas diffusion barriers would affect the apparent K_m by causing a drop in ACh concentration. As shown in Fig. 6, homogenization had only a small effect on V_{max} which increased from 29 to 46 nmol min^{-1}. Thus, 63 % of the V_{max} activity of homogenized muscle could be accounted for by V_{max} of intact tissue, which is lower than the proportion

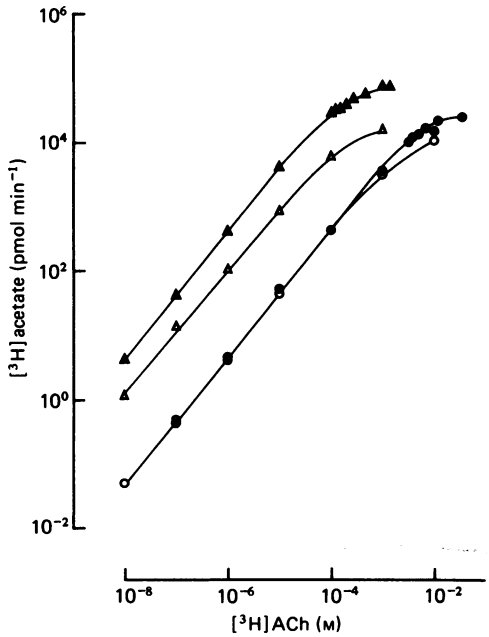

Fig. 5. Hydrolysis of [^{3}H]ACh at different concentrations by homogenates (△, ▲) and intact muscles (○, ●). The open symbols refer to *R. temporaria* and the filled circles to *R. esculenta*. The activities were determined in triplicate; all ACh concentrations were tested on each muscle (either intact or homogenized). Means of two experiments.

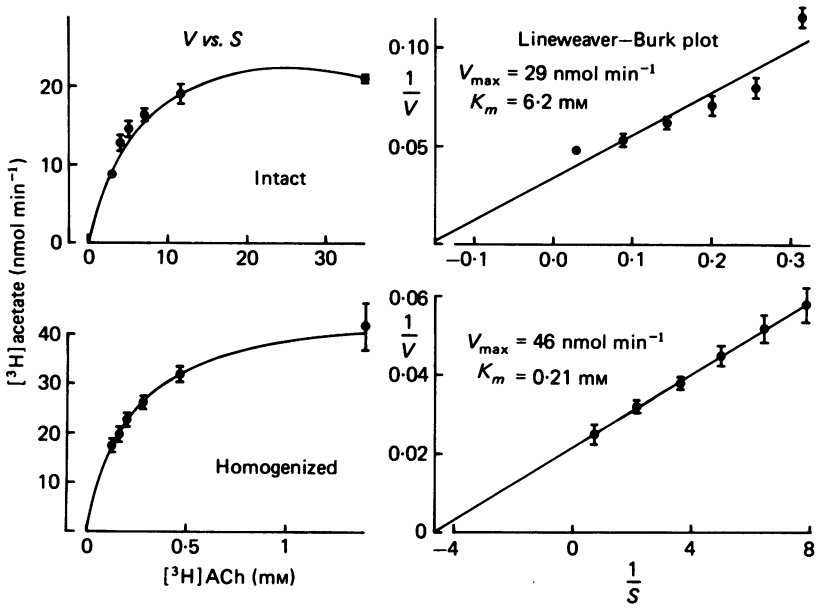

Fig. 6. Estimation of V_{max} and K_m in intact and homogenized sartorius. Note the deviation from Michaelis–Menten kinetics in intact muscles. Means ± S.E. of the means of four experiments.

of the ChE that is extracellularly located (80% in the collagenase experiments). Possibly this discrepancy is due to anomalous Michaelis–Menten kinetics at higher substrate concentrations, which may cause an underestimation of V_{max} in intact tissue (see below). Fig 6 also shows that homogenization had a marked effect on K_m, which was 6 mM for the intact tissue and 0·2 mM in the homogenates. This 30-fold difference in K_m seems to be largely responsible for the difference in activities observed in the linear part of the velocity *vs.* substrate (*V vs. S*) curve, where the slope is equal to V_{max}/K_m.

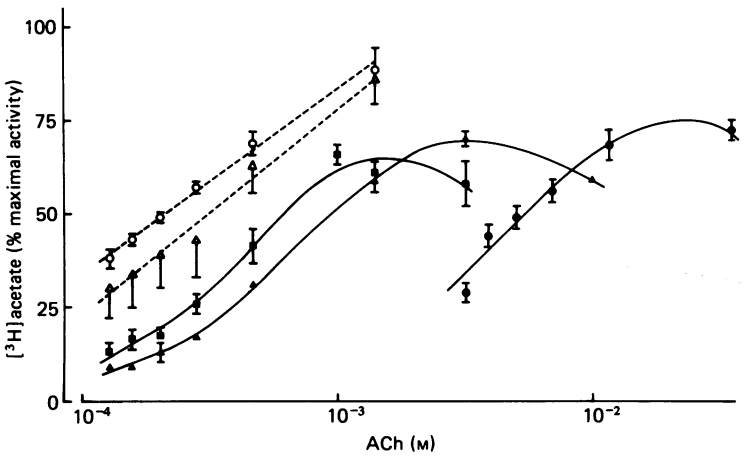

Fig. 7. Hydrolysis of [^{3}H]ACh as a function of substrate concentration in e.p. and non-e.p. segments. The rate of hydrolysis was estimated at 20 °C in non-homogenized segments (●——●, e.p.; ▲——▲, non-e.p.; ■——■, pieces of fibres that were teased free from non-e.p. segments). Thereafter the ACh was washed away, the tissue was homogenized and the hydrolysis rate was measured again at 20 °C (○——○, e.p.; △——△, non-e.p.). The values are percentages of individual V_{max} values, determined for each experiment from Lineweaver–Burk plots (cf. Fig. 6). In four experiments five to ten single fibres were teased free from a non-e.p. segment, attached to a tiny piece of tendon, which served as a handle to pin the specimen to the Sylgard in a Petri dish. The V_{max} values were (in pmol cm^{-1} min^{-1} per fibre): (a) (homogenized) whole muscle 27·6 ± 1·4 (4), non-e.p. 9·4 ± 0·64 (2); (b) (non-homogenized) whole muscle 19·0 ± 5·8 (4), non-e.p. 4·7 ± 0·44 (2), non-e.p. 'free' fibres 7·8 ± 0·5 (4). It was assumed that the sartorius contained 600 fibres. Means ± S.E. of means of 2–4 experiments.

In the experiments of Fig. 7 ACh hydrolysis by e.p. and non-e.p. segments and by their homogenates was studied as a function of the ACh concentration. For better comparison of the widely different values under various conditions the rates of hydrolysis are plotted as percentages of the V_{max} values and a log scale was used for the ACh concentrations.

The enzyme kinetics observed in homogenates from e.p. and non-e.p. segments followed Michaelis–Menten kinetics at ACh concentrations below 5 mM but showed some substrate inhibition at higher concentrations. Substrate inhibition has been described as a property of AChE (Augustinsson, 1948). The K_m for homogenates of e.p. and non-e.p. segments was 0·2 and 0·3 mM, respectively and the curves were similar.

Although the enzyme kinetics observed with muscle segments deviated from Michaelis–Menten kinetics, the apparent K_m values were determined. In comparison with the hydrolysis curves in homogenates, there was a shift to the right, the K_m being 6 mM with e.p. segments and 1 mM with non-e.p. segments. In order to analyse the effects of possible diffusion barriers in interstitial space, ACh hydrolysis by single fibre segments, teased free from non-e.p. muscle segments were also studied. In these single fibre pieces a peculiar deviation from Michaelis–Menten kinetics was noted: the hydrolysis increased linearly with the ACh concentration and turned sharply into a plateau at about 1 mM. However, when the results of four experiments were pooled, a much smoother curve was obtained. In the single fibre segments the curve was shifted somewhat to the left (K_m = 0·4 mM) compared to non-e.p. muscle segments and lay closer to the curves obtained with non-e.p. and e.p. homogenates. This might indicate that close packing of muscle fibres slightly impedes the diffusion of ACh. However, the finding that the difference in K_m between homogenized and non-homogenized tissue was so much greater in e.p. than in non-e.p. segments strongly suggests that it is the synaptic cleft which forms the main diffusion barrier to exogenous ACh. It seems likely that this barrier is destroyed completely by homogenization because we found no increase in the hydrolytic activity of homogenates when Triton X-100 was added.

Cholinesterase inhibition

The effect of various concentrations of different ChE inhibitors on the hydrolysis of ACh by intact muscles is shown in Fig. 8. There was a rapid stabilization of the different levels of inhibition when the competitive inhibitors neostigmine and eserine were applied, indicating that their diffusion, like that of ACh (cf. Fig. 1), was equilibrated within 15 min. Organophosphorus inhibitors react chemically with the active site of ChE, and therefore the time course of inhibition depends not only on diffusion, but also on the reaction rate of the inhibitor with the enzyme. The degree of inhibition increased only slowly with time with DFP in concentrations below 1 mM. With 1 μM-DEPP and 1 mM-DFP, on the other hand, the activity was soon practically eliminated. The potency of the inhibitors decreased in the order DEPP, neostigmine, eserine, DFP (see Fig. 9*B*). The relatively weak action of eserine compared to neostigmine in frog muscle is well known (see for instance: Kordaš, Brzin & Majcen, 1975) and the lower potency of DFP on the frog muscle enzyme, compared to its effectiveness on mammalian ChE, has also been reported (Miquel, 1946; Cohen & Posthumus, 1955). On the other hand, frog pseudocholinesterase seems to be very sensitive to DFP (Jacob & Pécot-Dechavassine, 1958).

All inhibitors tested were about 5 times more potent in inhibiting ACh hydrolysis in homogenates than in intact muscle (Fig. 9). This difference does not seem to be due to slow penetration of the inhibitor into the intact muscles because, at least with neostigmine and eserine, maximum inhibition was reached after 15 min. Although it cannot be ruled out that the enzyme *in situ* is less sensitive to inhibitors, the following explanation of the smaller effect of ChE inhibitors on intact muscles seems to be more likely. As mentioned above, we believe that the slow hydrolysis of ACh by intact muscle is mainly due to 'occlusion' of the greater part of the junctional ChE. This means that external ACh is hydrolysed by ChE in the outer parts of the

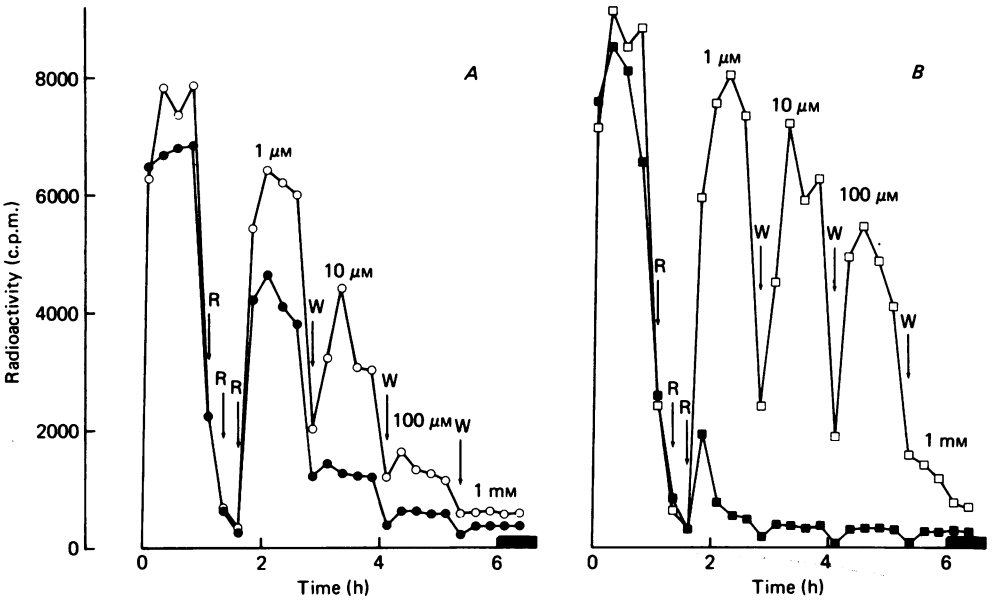

Fig. 8. Hydrolysis of 1 μM-[^{3}H]ACh by intact sartorius in the presence of various ChE inhibitors. The muscles were incubated at 20 °C during successive 15 min periods in Ringer solution which contained in *A*: neostigmine (●——●) or eserine (○——○), and in *B*: DFP (□——□) or DEPP (■——■). Before an increased concentration of an inhibitor was applied the muscles were rinsed with Ringer solution (arrows marked with R) or washed with Ringer solution containing the inhibitor at the same concentration as used in the previous periods (arrows marked with W), in order to avoid contamination of the samples with [^{3}H]acetate left over from previous periods (cf. Fig. 1). The ordinate gives the amount of [^{3}H]acetate formed in 15 min periods. Means of two experiments.

synaptic cleft and does not gain access to more centrally located enzyme. However, when ChE activity is decreased by an inhibitor, ACh is allowed to penetrate deeper into the synaptic gap, and to reach a greater fraction of that part of the enzyme which is still active, the net result being that the effect of an inhibitor on ChE activity is counteracted by better penetration of ACh. Thus in intact muscle the percentage inhibition of the rate of *ACh hydrolysis* would always be less than the percentage inhibition of *enzyme activity*, whereas no such difference is to be expected in a homogenate. However, as more and more enzyme is inhibited the difference between the inhibition of hydrolysis and of enzyme activity should decrease, and consequently, also the difference between the hydrolysis by intact muscle and homogenate. Table 2 shows that this was indeed the case. The hydrolysis of 1 μM-ACh in intact preparations, in the absence of inhibitors, was about 0·07 times that in the homogenate (a 15-fold difference). The difference decreased progressively with increasing degrees of inhibition in the homogenates, and at 98–99% inhibition, i.e. when the residual activity was just measurable above the spontaneous hydrolysis of ACh, the activity in intact muscles approached a value of 0·6 to 0·7 times the value in homogenized tissue, that is a ratio similar to that between the activities of the external and the total enzyme in the tissue (cf. Table 1).

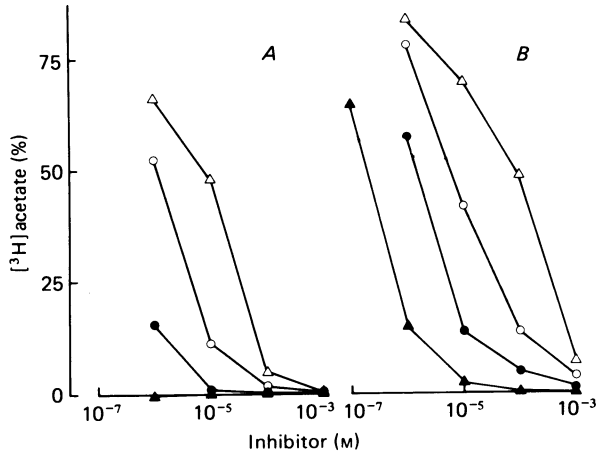

Fig. 9. Log concentration–inhibition curves for various ChE inhibitors. Intact muscles (*B*) were assayed as indicated in Fig. 8. Homogenized tissue (*A*) was pre-incubated for 30 min in the presence of the inhibitor before the [^{3}H]ACh was added. All incubations were at 20 °C. The activity was expressed as a percentage of full activity. The inhibitors were eserine (○——○), neostigmine (●——●), DFP (△——△), and DEPP (▲——▲). Means of two or three experiments.

ChE inhibitors and m.e.p.c.s

Fig. 10 shows that 10 μM-DEPP, which inactivates the ChE completely, increased the time constant of the decay of the m.e.p.c. from about 1 ms to 5·5 ms, and the amplitude of the m.e.p.c. by about 40 %. The amplitude of the m.e.p.c. in the presence of DEPP was not underestimated, since at a much higher concentration, 100 μM, DEPP still has no curarizing action (Miledi, Molenaar & Polak, 1980), in contrast to neostigmine which at concentrations above 1 μM depresses slightly the m.e.p.c. amplitude. As shown in the middle part of Fig. 10, 0·15 and 0·4 μM-neostigmine increased the time constant of the decay of the m.e.p.c. 1·3- and 1·8-fold, respectively. In these concentrations it slowed the hydrolysis of ACh 1·4- and 2·1-fold in muscle homogenates, whereas it had hardly any effect on ACh hydrolysis by intact muscle.

The effect of different concentrations of neostigmine on the hydrolysis of ACh, the time constant of decay and the amplitude of the m.e.p.c.s are compared in Fig. 11. At 0·15 μM-neostigmine the hydrolysis time of ACh, the time constant and amplitude of the m.e.p.c. were altered by about the same factor. At higher concentrations the curves diverged, among other things because the diffusion of ACh from the synaptic cleft becomes relatively more important for the shape of the m.e.p.c. (Katz & Miledi, 1973*b*). The amplitude reached a plateau at low degrees of inhibition, while the time constant of decay was still increasing.

If the sensitivity of non-junctional ChE to neostigmine is different from that of junctional ChE, the degree of inhibition measured in homogenates of whole muscle, as used in Figs. 10 and 11, would not be representative for the inhibition of ChE at the neuromuscular junction. However, it was found that the junctional and extrajunctional enzymes were inhibited to the same extent by neostigmine in sub-micromolar concentrations.

TABLE 2. Hydrolysis of [^{3}H]ACh by intact and homogenized sartorius muscles in the presence of ChE inhibitors

Inhibitor (μM)	Neostigmine I (*a*)	Neostigmine H (*b*)	Neostigmine Ratio (*a*/*b*)	Physostigmine I (*c*)	Physostigmine H (*d*)	Physostigmine Ratio (*c*/*d*)	DFP I (*e*)	DFP H (*f*)	DFP Ratio (*e*/*f*)
				I_{50} (μM)					
Variable	1·2±0·3 (3)	0·26±0·03 (5)	4·6	7·4±3 (2)	1·7	4·4	55±29 (2)	7·3	7·5
				ChE activity (pmol min^{-1})					
0	5·3±0·6 (2)	78±19 (2)	0·068	6·2±1·6 (2)	97	0·064	7·2±1·9 (2)	97	0·074
1	3·1±0·44 (2)	14±5 (2)	0·22	5·0±1·7 (2)	59	0·085	6·0±2·0 (2)	73	0·082
10	0·74±0·065 (2)	1·1±0·4 (2)	0·65	2·7±1·1 (2)	13	0·21	5·2±1·9 (2)	53	0·098
100	—	—	—	1·0±0·5 (2)	1·8	0·56	3·8±1·9 (2)	5·6	0·68
1000	—	—	—	—	—	—	0·6±0·3 (2)	0·7	0·86

Intact (I) and homogenized (H) muscles were pre-treated for 30 min with each inhibitor and subsequently incubated in the presence of 1 μM-[^{3}H]ACh. The concentration of the drug which caused a 50% inhibition (I_{50}) was derived for each experiment from an activity *vs.* log [I]plot. The Table also shows the ChE activity in pmol min^{-1} and the ratio between the activities in intact and homogenized tissue. Determining these ratios at higher degrees of inhibition than here presented, gave unreliable results due to the fact that the corresponding ChE activities were close to the radiochemical blanks. Mean±S.E. of the mean with number of experiments in parentheses. The values in each experiment were determined in triplicate.

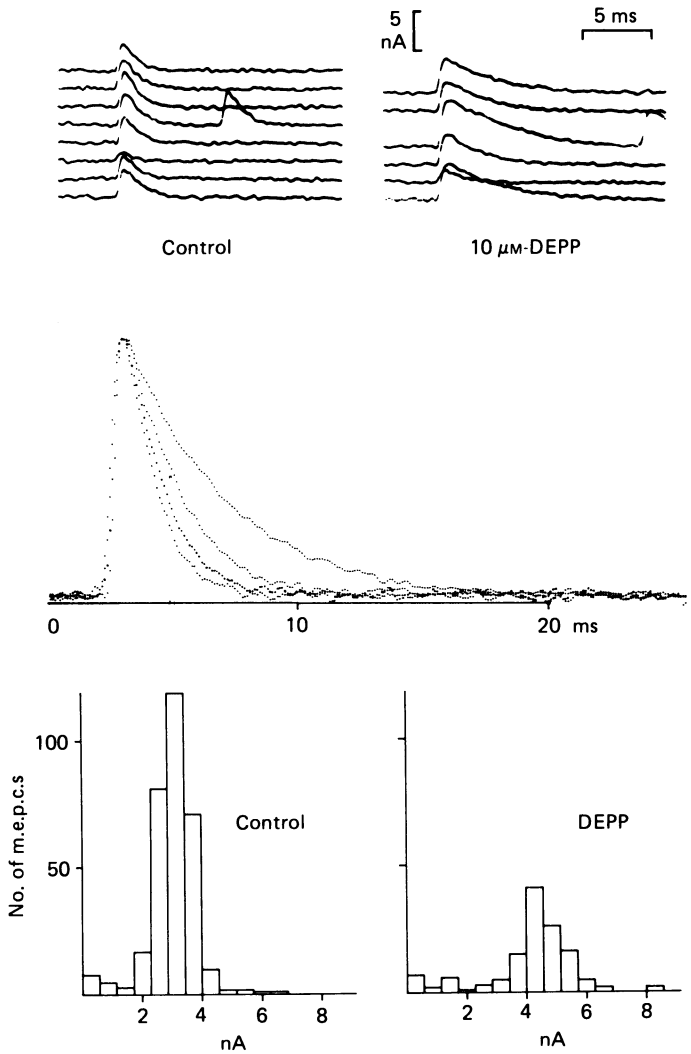

Fig. 10. Effect of ChE inhibitors on time course and amplitude of m.e.p.c.s. Upper Figure: m.e.p.c.s before (left) and after (right) complete inhibition with DEPP. Middle Figure: computer-averaged m.e.p.c.s, normalized to amplitude, with increasing decay time, from a muscle bathed in Ringer solution, in Ringer solution containing 0·15 μM-, 0·40 μM-neostigmine, and 10 μM-DEPP. Lower Figure: histograms of m.e.p.c.s under control conditions and in 10 μM-DEPP. Note a small shift towards higher amplitudes. Temperature 21–22 °C.

DISCUSSION

In the present experiments the high rate of ACh hydrolysis in homogenates, with respect to intact muscle, can only for a small part be due to the liberation of intracellular ChE, because this fraction accounted for less than 40% of the total enzyme content of the muscle. Instead, results of various types of experiments all indicate that diffusion of ACh into the synaptic cleft is hindered by ChE action, and

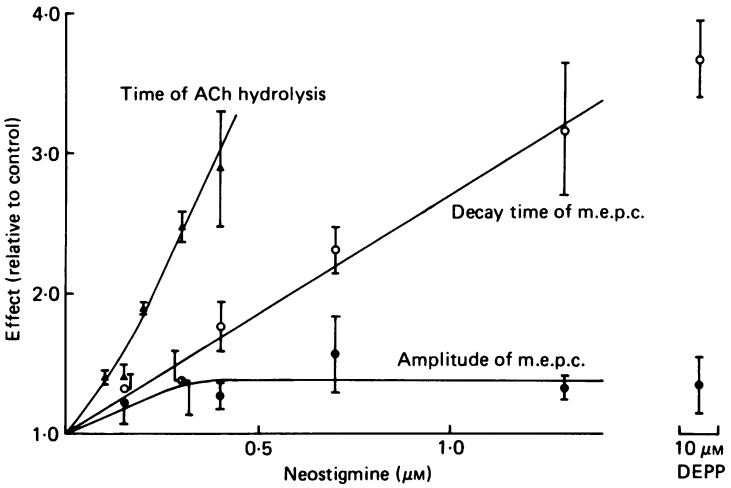

Fig. 11. The effect of neostigmine on ACh hydrolysis by homogenates of sartorius muscles and on m.e.p.c.s. The values are plotted as multiples of control values in the absence of neostigmine. The hydrolysis of ACh is plotted in an inverse fashion, that is the time needed to hydrolyse a unit amount of ACh. For comparison the effect of 10 μM-DEPP on decay time and amplitude of the m.e.p.c. is given. This concentration of DEPP caused more than 99·5% inhibition, without affecting ACh receptors. Means ± S.E. of the mean.

that poor penetration of ACh is responsible for the low hydrolytic activity that is observed in intact muscle (cf. Hobbiger, 1976). In particular the results obtained with ChE inhibitors were revealing in that the difference between intact and homogenized muscle was very much reduced, when the ChE was nearly completely inhibited. That the inner regions of the cleft are poorly supplied with exogenous ACh is also evident from the well-known fact that the depolarizing effect of bath applied ACh is strongly potentiated by ChE inhibitors. For instance, Katz & Miledi (1977) showed that the sensitivity of the frog sartorius to bath-applied ACh (measured as the 'H-effect') increased more than 10-fold upon ChE inhibition, i.e. a factor of similar magnitude as found in the present experiments between ACh hydrolysis in intact and homogenized muscle.

The electron-microscope observations that ChE is concentrated at the muscle–tendon junctions are in agreement with other findings (Couteaux, 1953). It is possible that hindered diffusion of ACh also occurs at these sites. However, it has been reported that there is little or no potentiation by ChE inhibitors of the electrical effect by ACh applied at the muscle–tendon junction (Katz & Miledi, 1964), and it is still possible that the ChE at these sites is less concentrated than the end-plate ChE.

It is not clear whether the small diffusion barrier to ACh at extrajunctional sites is due to impaired access of ACh to extracellular enzyme or whether there is some diffusion of ACh across the muscle membrane towards intracellular enzyme, for instance to T-tubules, in which products of ChE 'reaction' have been demonstrated (Miledi, 1964). Adding to this uncertainty is the fact that collagenase did not significantly reduce extrajunctional ChE; the ChE remaining after collagenase need not necessarily be intracellular but may simply be resistant to collagenase.

Estimation of in situ activity of synaptic ChE

It would be important to determine by biochemical means the activity of ChE in intact synapses with endogenous ACh as substrate. However, at present the best one can do is to estimate synaptic ChE activity with exogenously applied ACh. As mentioned above, intact muscles pose the problem that exogenous ACh has to diffuse into the deep fibres and into the synaptic clefts, whereby its concentration decreases (see Appendix). In principle this problem could be overcome simply by incubating the muscles with a very high concentration of ACh, say 50 mM, which would saturate the ChE in the cleft (see Fig. 6), but this would raise the problem that the yield of [^{3}H]acetate (because of label dilution) would decrease, and approach the amounts generated by spontaneous hydrolysis. At lower concentrations of ACh the hydrolysis of ACh by intact synapses is underestimated with respect to extrajunctional ChE, but assays with intact muscles could be relevant if at least the ACh hydrolysis were proportional to the amount of synaptic enzyme. Our results obtained with ChE inhibitors show that this is not the case: inhibition of hydrolysis of ACh by intact muscle was much less than the inhibition of the enzyme measured in homogenates (see Appendix), and low doses of neostigmine which hardly affected the hydrolysis by intact muscle, significantly influenced ACh hydrolysis in the homogenate and also the shape of the m.e.p.c.s.

The hydrolysis of ACh by homogenates gives a less distorted picture of synaptic ChE activity, provided that a correction is made for non-junctional (extrajunctional plus intracellular) activity, which was 40 % of total ACh hydrolysis. Thus, the values of V_{max} (Fig. 6) in homogenates, 28 nmol min^{-1}, and in intact muscles, 23 nmol min^{-1}, were similar (both values corrected for non-junctional activity, see Figs. 3, 4 and Table 1). The question then arises whether the K_m in homogenates is representative for the synaptic enzyme *in situ*. Here we have much less direct evidence, but the following suggests that this is the case. First, when the hydrolysis of ACh was nearly completely inhibited by DFP (an irreversible inhibitor that affects V_{max} but not K_m of ChE) the hydrolysis in homogenates came close to that in intact muscles. Since the ChE activity at low substrate concentrations depends on the ratio V_{max}/K_m, and since it is likely that the V_{max}s of the enzyme *in situ* and in the homogenate are decreased to the same extent by the inhibitors the two K_ms must also be similar. Hence, our evidence is in favour of the idea that the kinetic properties of the ChE in the synapse are not altered drastically by homogenization.

Radiolabelled DFP has been used to measure the number of ChE sites in mammalian tissue, for instance by radioautography (Salpeter, 1969). This method has not been applied to frog muscle. However, we may estimate the number of ChE sites in sartorius synapses, assuming a turnover number of $7{\cdot}4 \times 10^5$ min^{-1} per site (obtained under optimum substrate conditions for ChE from electric eel, Wilson & Harrison, 1961). In the sartorius, each homogenized end-plate hydrolyses ACh with a $V_{max} = 28$ pmol min^{-1} (assuming 10^3 end-plates per muscle). Consequently, the number of sites in an end-plate is $28 \times 10^{-12} N / 7{\cdot}4 \times 10^5 = 2{\cdot}3 \times 10^7$. This value exceeds by a factor of roughly five the number of ACh molecules released normally by the nerve action potential (assuming a quantal content of 500 (Katz & Miledi, 1979) and 10^4 molecules per quantum (Kuffler & Yoshikami, 1975; Miledi, Molenaar & Polak,

1982)). The estimated number of ChE sites is similar to the value of $3–4 \times 10^7$ α-bungarotoxin binding sites in frog muscle (Barnard, Chiu, Jedrzejcyck, Porter & Wieckowski, 1973; Matthews-Bellinger & Salpeter, 1978). In the rat diaphragm the number of ChE and ACh receptor sites is also similar (about 5×10^7: Barnard, Rymaszewska & Wieckowski, 1971; Miledi & Potter, 1971; Fambrough & Hartzell, 1972). The concentration of ChE in the frog synapse is somewhat lower than that in the synapse of the rat diaphragm, but it is still quite high. Assuming a synaptic space of $5{\cdot}9 \times 10^{-11}$ cm^3 (disregarding the volume of the basement lamina, Katz & Miledi, 1977) it is $6{\cdot}4 \times 10^{-4}$ mol sites per litre.

ChE activity and m.e.p.c.s

An unexpected finding was that a 35 % inhibition of the ChE by neostigmine caused as much as a 30 % increase of the decay time of m.e.p.c.s. In this connexion it should be pointed out that neostigmine does not increase the lifetime of the membrane channel opened by ACh (Katz & Miledi, 1973*a*). Further, neostigmine in the concentrations used had no curarizing side effects, which otherwise might have facilitated diffusion of ACh out of the synaptic cleft (Katz & Miledi, 1973*b*).

How rapidly is an ACh quantum hydrolysed by the synaptic ChE? Shortly after its release the ACh passes through the basement lamina that contains the ChE. At this stage the local concentration of ACh is probably higher than the K_m of the enzyme, and the active sites may become locally saturated (Matthews-Bellinger & Salpeter, 1978), or inhibited by excess substrate (cf. Augustinsson, 1948). Because of the high affinity of the receptors for ACh, most transmitter molecules subsequently combine with the receptors and the ACh concentration decreases to values, probably far below the K_m of ChE (Wathey, Nass & Lester, 1979), i.e. to a range of concentrations in which the enzyme activity is linearly dependent on substrate concentration. The lifetime of ACh is then dependent on a rate constant ($k = V_{max}/K_m$ in mol min^{-1} (mol l^{-1})$^{-1}$), and also on the kinetic constants of the ACh-receptor interaction. One may calculate the lifetime of ACh, if we disregard, for the sake of simplicity, the interactions with receptors and ignoring morphological factors such as the post-synaptic folds. From the data in Fig. 5, and after correction for extrajunctional activity, a rate constant 0·0848 cm^3 min^{-1} is obtained, and consequently we derive a lifetime (volume cleft/rate constant) $\tau = 42$ μs. (The rate of enzyme action has a lower limit of 10 μs, which is the half-time of formation of the acetyl-enzyme, the step that produces free choline (Wilson & Harrison, 1961).) However, the actual lifetime of ACh in the synapse is much greater than 42 μs. While a part of the quantum when crossing the gap is eliminated at an extremely high rate, the over-all elimination by ChE is slowed down, as soon as the greater part is bound to receptors. The elimination of ACh then becomes dependent on both ChE action and the rates at which ACh combines with and comes off the receptors. The fact that small concentrations of neostigmine increased the decay time of the m.e.p.c.s, implies that a little decrease in ChE activity causes more ACh molecules to act repetitively on ACh receptors, and that the normal activity of synaptic ChE has more influence on the shape of the m.e.p.c. than was anticipated (see Introduction). Perhaps the present approach of measuring ChE, and its inhibition by neostigmine, in homogenates of frog muscle, could be useful in testing the influence of the rate constant of ChE

(k_{ChE}) in reconstruction models of m.e.p.c.s and e.p.c.s (Magleby & Stevens, 1972; Kordaš, 1977; Matthews-Bellinger & Salpeter, 1978; Rosenberry, 1979; Wathey *et al.* 1979; Adams, 1980).

APPENDIX

Diffusion of bath-applied ACh into the synaptic cleft and its breakdown during a steady state

By P. C. Molenaar

For the calculations below, we require numerical values of k, the rate constant of ChE in the synaptic space, a, the half-width of the synaptic cleft, and D, the diffusion constant of ACh in the cleft. The time constant of ACh hydrolysis in the cleft as calculated in the Discussion, 42 μs, is equivalent to $k = 2{\cdot}38 \times 10^4$ s^{-1}. The width of the cleft, $2a$, is assumed to be $2{\cdot}4 \times 10^{-4}$ cm (Katz & Miledi, 1977).

The value of D in the cleft is uncertain. Whereas D_{ACh} in a physiological medium is about 10×10^{-6} cm^2 s^{-1} (see for instance Krnjević & Mitchell (1960); a value of $8{\cdot}7 \times 10^{-6}$ cm^2 s^{-1} was measured in a diffusion cell, calibrated with HCl at 20 °C), the value of D in the synaptic cleft might be considerably smaller than that in saline, for instance, because of higher viscosity of the fluid in the synaptic space. Matthews-Bellinger & Salpeter (1978) calculated a value of D of about 1×10^{-6} cm^2 s^{-1} from the spread of ACh over a so-called 'critical area' of post-synaptic membrane and the rise time of the m.e.p.c., but Land, Salpeter & Salpeter (1981) reported that in a kinetic simulation model a higher value of D (4×10^{-6} cm^2 s^{-1}) gave the best fit for the observed behaviour of the m.e.p.c. A similar value was used by Wathey *et al.* (1979), namely $D = 3 \times 10^{-6}$ cm^2 s^{-1}, in a numerical reconstruction of quantal events, but they noted that 'this assumption could well be an over-estimate, because a diffusion constant of 1×10^{-6} cm^2 s^{-1} characterizes several small aromatic amines in 8% gelatine at 25 °C.'

For the present purpose we can make a rough estimate of D from electrophysiological observations made after inhibition of ChE, when the decay of the m.e.p.c. is exclusively dependent on diffusion of transmitter from the cleft. Katz & Miledi (1973*b*) used a low dose of α-bungarotoxin which caused a large, but not complete, inhibition of ACh receptors, in order to minimize binding of ACh to the receptors, which would otherwise slow down the diffusion of ACh from the cleft. Under these circumstances these authors found that externally recorded miniature end-plate potentials (which have a time course similar to that of the m.e.p.c.s) decayed with a time constant ($\tau_{miniature}$) of about 3 ms. Assuming that this time constant is directly related to the clearance of ACh from the cleft (with a time constant τ_{ACh}), we may calculate the amount of ACh (A_t) remaining in the cleft with a width of $2a$:

$$A_t = \frac{8A_{t=0}e^{-(\pi/2a)^2 Dt}}{\pi^2},$$

(an approximation for not too small values of t (Mellor, 1912; Eccles & Jaeger, 1958)). Since we assume that $\tau_{ACh} = \tau_{miniature}$, we get from the logarithmic slope with $\tau = 3$ ms, a value of $D = 2 \times 10^{-6}$ cm^2 s^{-1}. It should be pointed out that D thus

obtained from diffusion out of the cleft is not a true value, dependent on viscosity, because other parameters, such as subsynaptic folds, which could retard diffusion, are likely to play a role. However, the same parameters should be involved during diffusion *into* the cleft and the present value of 'D' will be used for the calculations below.

(*i*) *Concentration profile of ACh in the synaptic cleft*

The diffusion of oxygen into muscle tissue has been treated mathematically by Hill (1929), with the underlying assumption that oxygen is used up by the tissue at a constant rate, independent of its concentration. However, this approach is not valid for diffusion of ACh into the synaptic cleft, as ACh consumption is not constant, but *proportional* to its concentration, and a somewhat more complex mathematical relation seems appropriate.

Let $2a$ be the width of the synaptic cleft, and c_0 the concentration of ACh outside the cleft (about equal to c_{bath}, see Discussion). The increase in ACh concentration at a distance x from the edge of the cleft as a result of diffusion-mediated transport is counteracted by hydrolysis. From Fick's second law we get $\partial c/\partial t = D\partial^2 c/\partial x^2 - kc$, in which c is the concentration of ACh at a distance x. This differential equation can be simplified, since we are mainly interested in the concentration profile after some time, when steady state has been reached, i.e. when $\partial c/\partial t = 0$:

$$d^2c/dx^2 = kc/D. \tag{1}$$

Let us assume that $c = e^{mx}$ is a solution for eqn. (1). Then $m^2e^{mx} = ke^{mx}/D$ and $m = \pm\sqrt{k/D}$. Consequently,

$$c = c_1e^{mx} + c_2e^{-mx}, \tag{2}$$

is also a solution. In order to determine c_1 and c_2 we use the boundary conditions. Where $x = 0$, $c = c_0$, and, consequently,

$$c_0 = c_1 + c_2. \tag{3}$$

In the middle of the cleft, where $x = a$, eqn. (2) becomes zero, after differentiation to x, because there cannot be a net transport of ACh in the middle of the cleft:

$$dc/dx = c_1me^{ma} - c_2me^{-ma} = 0. \tag{4}$$

Rearranging eqn. (4) and using eqn. (3) we obtain $c_1 = c_0e^{-2ma}/(1+e^{-2ma})$ and $c_2 = c_0/(1+e^{-2ma})$. Substituting the values of c_1, c_2 and m in eqn. (2) we get

$$c_x = \frac{c_0(e^{(x-2a)\sqrt{k/D}} + e^{-x\sqrt{k/D}})}{1+e^{-2a\sqrt{k/D}}}. \tag{5}$$

The calculated values for c_x/c_0 are presented in Fig. 12 at different levels of ChE inhibition. With full ChE activity c_x rapidly decreases to zero with increasing x. For 95 % enzyme inhibition there still is a marked decrease in c_x. Even at 99·5 % inhibition the ACh concentration in the middle of the cleft (at $a = 1{\cdot}2 \times 10^{-4}$ cm) is still about 30 % below c_0.

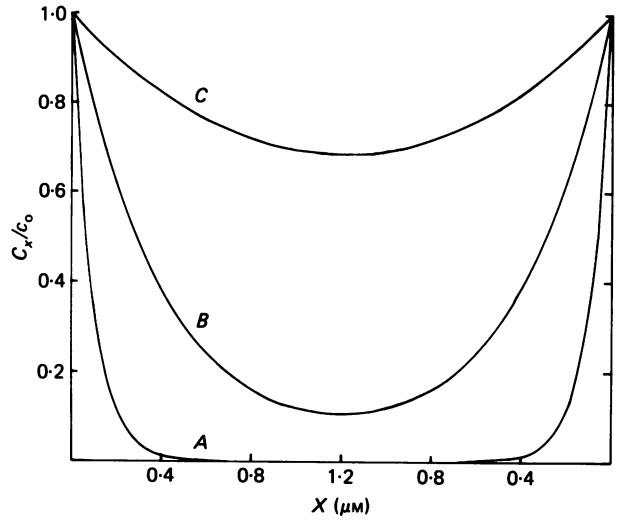

Fig. 12. Concentration profile of ACh in the synaptic cleft as a function of distance from the edge (x), calculated with eqn. (5). The concentrations are plotted relative to the concentration outside the cleft, c_0. The curves refer to full ChE activity (A), 95 % (B) and 99·5 % (C) ChE inhibition.

(*ii*) *Hydrolysis of ACh by the intact end-plate*

The rate of hydrolysis of ACh by an end-plate, i.e. the mean of the rates at different values of x can be expressed as a function of k, a and D. At any x the hydrolysis velocity $v_x = kc_x$ and the mean velocity is $\bar{v} = k\bar{c}$, in which $\bar{c}$ is the mean concentration. Further,

$$\bar{c} = \int_{x=0}^{x=a} \frac{c_x \mathrm{d}x}{a}. \tag{6}$$

By integration between the limits a and 0 we get:

$$\frac{\bar{v}}{v_0} = \frac{\bar{c}}{c_0} = \frac{(1-\mathrm{e}^{-2a\sqrt{k/D}})}{(1+\mathrm{e}^{-2a\sqrt{k/D}})} \times \frac{1}{a\sqrt{k/D}}, \tag{7}$$

in which $v_0 = kc_0$ is the rate of hydrolysis at the edge of the cleft, i.e. the maximal rate in the absence of diffusion barriers. The calculated values for $\bar{v}/v_0$ are presented in Fig. 13 as a function of a. When $a = 1{\cdot}2 \times 10^{-4}$ cm, $\bar{v}$ becomes, roughly, 0·08, 0·35 and 0·8 times as low as v_0, for 0, 95 and 99·5 % inhibition, respectively.

These values suggest that in the reverse situation, i.e. diffusion of endogenous ACh from the cleft, a very high degree of inhibition of ChE is required in experiments in which released ACh is to be collected (but the mathematical treatment of ACh overflow is much more complicated than that of eqns. (5) and (7)).

It could be argued that the numerical solution of eqn. (7) gives erroneous values, as not only ChE but also ACh receptors are expected to slow down diffusion of ACh into the cleft, and that the present value of 'D' is taken too high (see above). However, it was found that after blocking the receptors with curare, the hydrolysis of [^{3}H]ACh by intact muscle was not increased (P. C. Molenaar, unpublished obser-

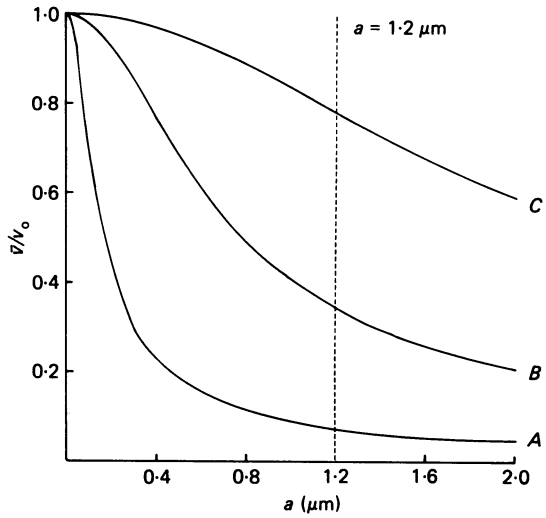

Fig. 13. The hydrolysis of bath-applied ACh by intact end-plate, as calculated with eqn. (7). The hydrolysis is plotted relative to the theoretical maximal hydrolysis in the absence of diffusion barriers, v_0, as a function of a (the half-width of the cleft). The curves refer to full ChE activity (A), 95% (B), and 99·5% (C) ChE inhibition.

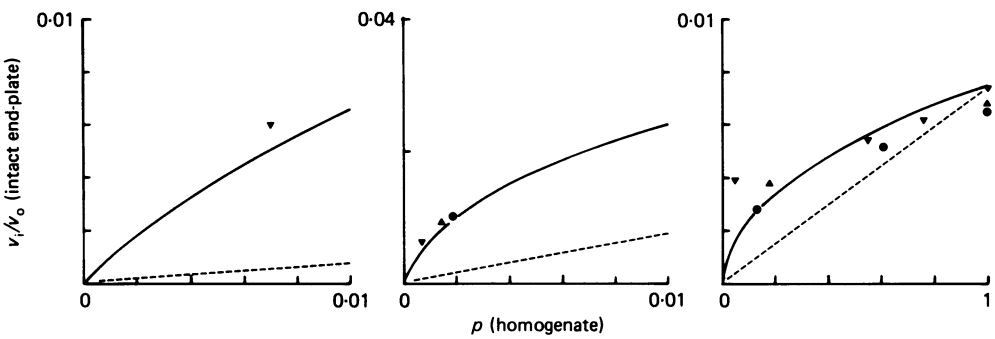

Fig. 14. Relationship between ACh hydrolysis in intact tissue (ordinate) and ACh hydrolysis by homogenized synaptic ChE (abscissa, normalized to 1, for full ChE activity), according to eqn. (8). Three ranges are given, corresponding to 0–1%, 0–10%, and 0–100% of the full, uninhibited, activity of the enzyme. Experimental data obtained from Table 2 have been plotted (DFP, ▼——▼, neostigmine, ▲——▲, eserine, ●——●).

vations) as might have been expected on the basis of facilitated diffusion. The lack of an effect by curare may be explained by the consideration that ACh is not hydrolysed while it stays attached to the receptor and that the chance of hydrolysis during jumps from receptor to receptor is dependent only on the time of free diffusion.

(iii) Inhibition of ACh hydrolysis in the intact end-plate by anti-esterases

Let us calculate the rate of ACh hydrolysis by intact end-plates, v_i, during ChE inhibition as a function of the residual relative ChE activity, p, in order to see whether our experimental data obtained with ChE inhibitors in both intact and homogenized

muscles are in agreement with the theory. Thus, $k = pk_0$, where k_0 denotes the rate constant of ChE in the absence of inhibitor. Subsequently, from eqn. (7) we get:

$$\frac{v_i}{v_o} = \frac{pk_0\bar{c}}{k_0 c_o} = \left[\frac{(1-e^{-2a\sqrt{k_0 p/D}})}{(1+e^{-2a\sqrt{k_0 p/D}})}\right] \times \frac{\sqrt{p}}{a\sqrt{k_0/D}}. \quad (8)$$

At a low degree of inhibition, say $0{\cdot}1 < p < 1$, eqn. (8) is proportional to $\sqrt{p}$, but at high degrees of inhibition the factor in brackets in eqn. (8) causes a substantial deviation from the square-root function. In Fig. 14 the interrupted line indicates the relation between v_i/v_o and p, if the activity of intact muscle were linearly dependent on p, which was tacitly assumed by Mittag *et al.* (1971). It is clear that while the error of taking a straight line instead of eqn. (8) is moderate when $0{\cdot}1 < p < 1$, it is enormous at lower values of p.

The individual points of Table 2 are indicated in Fig. 14. There is satisfactory numerical agreement of the experimental data with the theory over a wide range of values of p.

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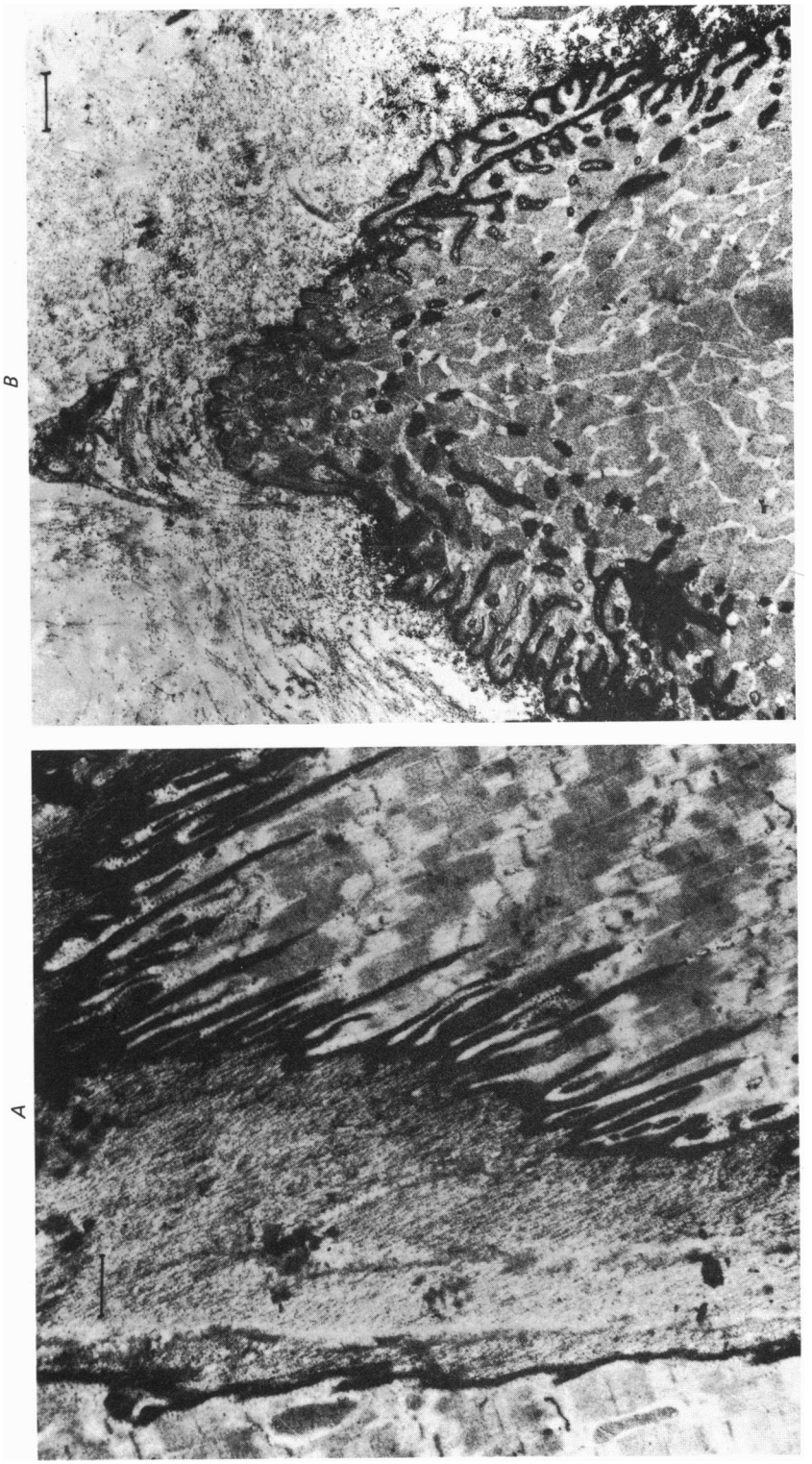
EXPLANATION OF PLATE

Muscle–tendon region of frog sartorius. Muscles were fixed, stained for ChE, and processed for electron microscopy as described earlier (Miledi, 1964). Longitudinal section (*A*) and cross-section (*B*). Note the ChE staining in folds. Calibration bar 1 μm.


B
A