Biochemical Pharmacology, Vol. 20, pp. 669–682. Pergamon Press, 1971. Printed in Great Britain

CHOLINE ESTER HYDROLASES IN DIAPHRAGM MUSCLE

W. K. BERRY and J. P. RUTLAND

Chemical Defence Establishment, Porton Down, Wilts., England

(*Received* 15 *June* 1970; *accepted* 12 *August* 1970)

Abstract—By the use of centrifugal fractionation, specific substrates and selective inhibitors evidence was obtained that the diaphragm muscle of guinea pig, man and rat contain both soluble and particulate acetylcholinesterase (EC 3.1.1.7) and a soluble cholinesterase (EC 3.1.1.8). This is believed to be the first report of naturally-occurring soluble mammalian acetylcholinesterases. Human and rat diaphragms also contain a physostigmine-resistant enzyme which hydrolyses butyrylcholine, and is probably a B-esterase. It was noted incidentally that a modified Koelle histochemical method, using thiocholine esters, has a specific requirement for iodide ion which has not previously been reported.

THE RESPONSE of animals to drug treatment of organophosphorus anticholinesterase poisoning varies with animal species. Guinea pigs and rabbits respond well, but rats and mice poorly, whether atropine and P2S (2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate)[1] or atropine and physostigmine[2] are used. As part of a programme to try to assess the causes of these species differences, a study of the choline ester hydrolases of diaphragm muscle was undertaken.

The only previous work of this nature was reported by Ord and Thompson[3] and by Davison,[4] who examined the hydrolysis of specific substrates by unfractionated homogenates of rat diaphragm. They agreed that AChE* was present. Ord and Thompson[3] found that the homogenates did not hydrolyse benzoylcholine, a substrate then believed to be hydrolysed by ChE's but not by AChE's.[5] They therefore concluded that ChE was not present. However Davison[4] found that the homogenates hydrolysed BuCh, recommended by Cohen, Kalsbeek and Warringa[6] as a better specific substrate for ChE and therefore concluded that this enzyme was present. Several other papers, notably one by Koelle,[7] have persuaded us that benzoylcholine is hydrolysed by enzymes other than ChE, and that the use of BuCh is preferable.

Despite this, most other workers studying the relationship between neuromuscular function of the rat phrenic-diaphragm preparation and muscle "cholinesterase" activity have acted on the assumption that only AChE was present[8–14] and used ACh as substrate.

* Abbreviations used: ACh, acetylcholine; AChE, acetylcholinesterase, EC 3.1.1.7; ATCh, acetyl thiocholine; BuCh, butyrylcholine; BTCh, butyryl thiocholine; ChE, cholinesterase, EC 3.1.1.8; DPDA, *N,N'*-diisopropyl phosphorodiamidic anhydride; MCh, acetyl-β-methylcholine; 62.c.47, 1,5-di(trimethylaminophenyl)pentan-3-one diiodide; ethopropazine, 10-(2-diethylaminopropyl)-phenothiazine hydrochloride.

We have defined the hydrolases according to the following criteria:

(1) AChE is inhibited by low concentrations of physostigmine[15] and 62.c.47.[16] It hydrolyses ACh and MCh but not BuCh.[5,17] Hydrolysis of ACh is inhibited by excess substrate.[18]

(2) ChE is inhibited by low concentrations of physostigmine[15] and ethopropazine.[16] It hydrolyses BuCh about 2·5 times as fast as ACh[19,20] but MCh not at all.[5]

(3) Hydrolysis of choline esters which is not inhibited by low concentrations of physostigmine has been mentioned in the literature but the enzymes have not been defined. In the Appendix one of us suggests that BuCh may be hydrolysed by a B-esterase. These are characterized by a high rate of hydrolysis of phenolic esters, very low rates of hydrolysis of acetates, sensitivity to inhibition by organophosphorus compounds, and resistance to inhibition by physostigmine.[21,22]

We have not used phenolic esters in studying the diaphragm because they are hydrolysed by authentic choline ester hydrolases, as well as B-esterases.

Ethopropazine and 62.c.47 are selective inhibitors of ChE and AChE respectively.[16] There is no published evidence whether sensitivity and selectivity differ according to the animal species from which the enzymes are derived. We have therefore used a range of concentrations with each preparation. Theoretical curves (Fig. 1) show that if selectivity between two enzymes acting on the same substrate is high there is a marked inflexion in the curve relating percent activity to the logarithm of the concentration of inhibitor. If selectivity is not high the inflexion may not be noticeable,

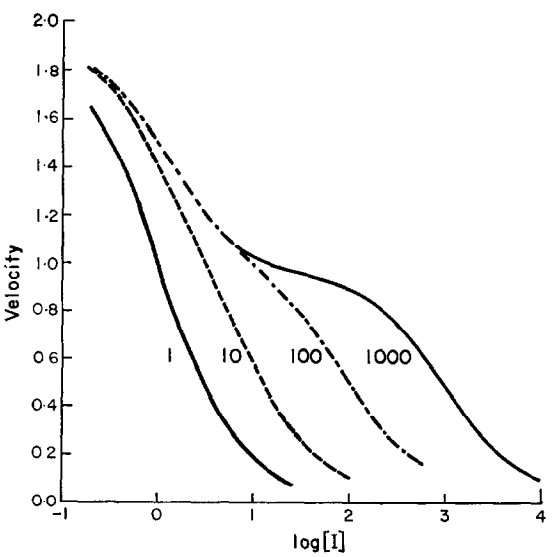

FIG. 1. Theoretical curves showing relationship between velocity and inhibitor concentration in a system containing equal parts of two enzymes of different sensitivities to the inhibitor. In all cases there is an enzyme for which $K_I = 1$

(1) second enzyme, $K_I = 1$. } Using the present preparations any inflexions could not be
(10) second enzyme, $K_I = 10$. } detected.
(100) second enzyme, $K_I = 100$. }
(1000) second enzyme, $K_I = 1000$. Inflexion might just be detectable.

yet the slope of the curve is less than if only one enzyme is present. We have therefore presented the results in the form[23]

$$\log (v_o/v_i - 1) = n \log [I] + C$$

where v_o is the normal velocity, v_i is the velocity in presence of inhibitor, and $[I]$ the concentration of inhibitor. If only one enzyme is present, n denotes the number of inhibitor molecules bound to each active centre. A value of n substantially smaller than that found in a control one-enzyme system may indicate the presence of more than one enzyme.

EXPERIMENTAL

Diaphragms

Female guinea pigs (280–400 g) or female rats (180–240 g) were killed by a blow on the head and the diaphragms removed. Human specimens were from persons of either sex who had died other than from infectious disease or from disorder affecting muscle. They were about 10 cm square from unspecified areas of the organ and were usually of muscle about 5 mm thick between sheets of connective tissue up to 1 mm thick, and were received frozen solid. All specimens were kept moist by immersion in 0·15 M KCl while blood, clots, fat and connective tissue were removed as completely as possible. They were blotted on hard filter paper, weighed, minced in a top-drive macerator with some KCl solution and finally ground in an all-glass Potter–Elvehjem homogenizer in the proportions of 1 g muscle plus 3 cm^3 of 0·15 M KCl.

For a few experiments the homogenate was used at this stage, but for others it was fractionated by centrifugation. The "long method" consisted of centifuging in the No. 40 head of a Spinco Model L centrifuge at 500 *g* for 30 min, obtaining a sediment which was diluted to the original volume with 0·15 M KCl and centrifuged again. The combined supernatant and wash liquor was centrifuged at 34,000 *g* for 30 min. This gave a clear supernatant and a sediment which was also washed once. Finally the combined supernatants and washings were centrifuged at 100,000 *g* for 60 min, yielding a tiny pellet which was collected without washing, and a supernatant fraction.

As the result of the preliminary investigation of these fractions a "short method" was devised. The homogenate was centrifuged at 500 *g* for 30 min. The sediment was washed twice and the washings discarded. The supernatant was centrifuged at 100,000 *g* for 60 min and the pellet discarded. The short method yielded an undiluted supernatant fraction, and the material discarded formed only a small proportion of the total esterase present.

At least four animals and sometimes as many as 20 were required to provide enough material for a single experiment. The results therefore reduce the effects of individual variation. Each human specimen provided enough material for several experiments but was not kept longer than a week.

Other enzyme preparations

Purified soluble AChE from bovine red cells and purified soluble ChE from horse serum were provided by Dr. J. J. Gordon. A particulate AChE from rat brain was prepared by homogenizing 1:10 in 0·15 M KCl, centrifuging at 34,000 *g* for 30 min, and washing twice by centrifugation in 0·15 M KCl. The velocity ratio ACh–BuCh

was about 20:1, showing that the proportion of ACh hydrolysis catalysed by ChE was negligible: the preparation could be regarded as a particulate AChE.

Substrates and inhibitors

ACh and BuCh chlorides, ATCh and BTCh iodides, were obtained from B. D. H. (Poole, Dorset), MCh chloride from Koch-Light (Colnbrook, Bucks.), physostigmine sulphate from Boots (Nottingham), ethopropazine from May & Baker (Dagenham, Essex) and 62.c.47 from the Wellcome Research Laboratories. DPDA was provided by Mr. M. Rumens of C. D. E. The compounds were assumed to be adequately pure, and were used as received.

Measurement of esterase activity

Esterase activity was measured by automatic continuous titration at 38°. The reaction mixture contained 3 cm^3 of diaphragm preparation derived from 750 mg of muscle, plus 5 cm^3 of 0·15 M KCl. This was adjusted to pH 7·42 by addition of 0·2 M NaOH and the volume made up to 9·5 cm^3 with 0·15 M KCl, including, as appropriate, 0·5 cm^3 of inhibitor in KCl solution. The reaction was started by adding 0·5 cm^3 of substrate solution and the pH kept constant by addition of NaOH solution, usually 0·02 M, but this could be varied as desired.

Histochemical examination

Choline ester hydrolases were located in intact diaphragms of guinea pig or rat, or in the various particulate fractions isolated by the long method, using Waser and Lüthi's[24] copper-thiocholine method.

Note on the use of ethopropazine

Contrary to our earlier experiences using bicarbonate buffer in the Warburg apparatus, the present experiments showed that ethopropazine was unstable in the presence of muscle preparations. It was therefore added immediately before substrate, and the velocity recorded for about 10 min, during which interval linear traces were obtained.

RESULTS AND CONCLUSIONS

Studies on enzymes of known purity

The key to the interpretation of the inhibitor studies on diaphragm preparations is the behaviour of preparations which contain effectively only one type of choline ester hydrolase. Since there are no published data on the relationship between concentration of the inhibitors used and the degree of inhibition, a preliminary study was necessary.

Figures 2 and 3 show lines of unit slope fitted to the double-log plots of data describing inhibition of the soluble AChE and ChE by 62.c.47 and ethopropazine. These plots show that the inhibitions involve a 1:1 equilibrium between enzyme active centre and inhibitor.

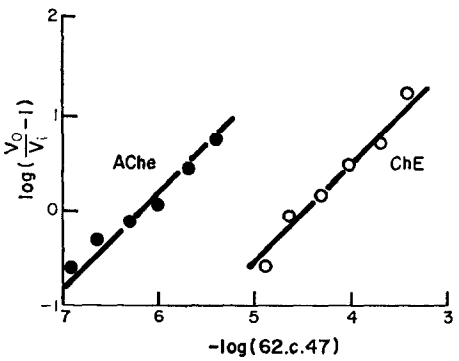

FIG. 2. Inhibition of hydrolysis of 5·5 mM ACh by 62.c.47. Lines are of unit slope. AChE is soluble purified bovine red cell enzyme, ChE is soluble purified horse serum enzyme.

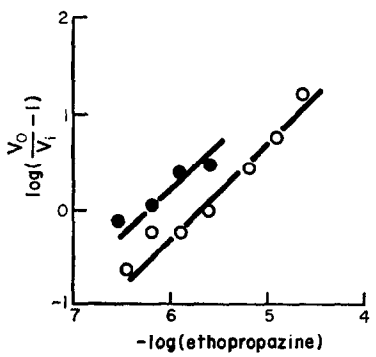

FIG. 3. Inhibition of purified horse serum ChE by ethopropazine. Solid circles, 5·5 mM ACh. Open circles, 10 mM BuCh. Lines of unit slope.

Figure 4 shows that the data on the inhibition of the brain AChE by 62.c.47 are fitted by a double-log plot with a slope of 0·5, which may be interpreted as showing that each inhibitor molecule binds two active centres. This is theoretically possible. Each quaternary end of 62.c.47 could bind to a separate anionic site in each of two active centres.

Thus the kinetics of inhibition of AChE by 62.c.47 depend on the physical state of the enzyme. In solution, the concentration of the bovine AChE is very low, and the probability of a ternary collision to form an E_2I complex remote. Histochemical evidence (see Ref. 25) shows that in the brain the AChE is packed into a very small proportion of the tissue. It is reasonable to suppose that this packing favours the formation of ternary complexes.

Preliminary investigation of unfractionated homogenates

The earlier experiments of Ord and Thompson[3] and Davison[4] have been confirmed and extended (Table 1). All diaphragms hydrolyse MCh, thus showing the presence of AChE. Benzoylcholine is hydrolysed at low rates, if at all, whereas BuCh

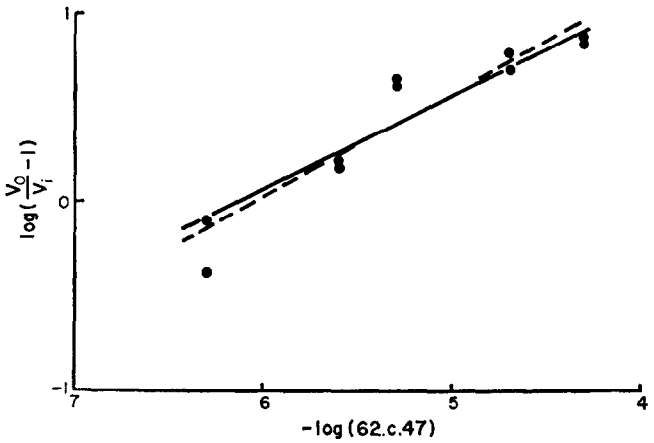

FIG. 4. Inhibition of rat brain particulate AChE by 62.c.47. Substrate, 5·5 mM ACh. Solid line, slope of 0·5; broken line, calculated line of best fit.

is hydrolysed much more rapidly. We agree with Davison[4] in accepting this as evidence that ChE is also present. The proportion increases in the order man, rat, guinea pig.

The hydrolysis of ACh or BuCh by guinea pig diaphragms was inhibited more than 90 per cent by incubation for 30 min with 10 μM-physostigmine before adding substrate, showing that hydrolyses are catalysed by "cholinesterases".[15] Although the hydrolysis of ACh by rat diaphragm was also inhibited more than 90 per cent by by 10 μM-physostigmine, that of BuCh was inhibited only 68 per cent, indicating the presence of a small proportion of an enzyme which hydrolyses BuCh but not ACh, and which is resistant to physostigmine.

TABLE 1. HYDROLYSIS OF CHOLINE ESTERS BY DIAPHRAGM HOMOGENATES

Substrate and concentration (mM)	Relative rate of hydrolysis by diaphragm of		
	Guinea pig	Man	Rat
Acetylthiocholine, 6	156	134	153
Benzoylcholine, 30	13	20	0
Butyrylcholine, 10	88	20	41
Butyrylthiocholine, 10	86	16	45
Acetyl-β-methylcholine, 30	46	40	46

Rates of hydrolysis of 5·5 mM ACh = 100.

The data for animals were obtained from the pooled diaphragms of four to five animals. Those for man are the means of two concordant experiments on specimens from men aged 67 and 73.

100 per cent is about 1 μmole/g/min for animals and about 2 μmoles/g/min for man.

Examination of fractions separated by the long method

A less complete substrate specificity study was carried out on the fractions obtained by the long method, ACh being used to measure total esterase activity and BuCh as a marker for ChE. Table 2 shows that most of the esterase activity was in the combined particulate fractions, about 75 per cent in the animal tissues and about 90 per cent in human diaphragm.

To obtain a rough estimate of the proportion of ACh hydrolysis catalysed by ChE, the BuCh figures may be divided by 2·5 (see above), ignoring the small fraction of physostigmine-resistant enzymes. For example the figure of 0·183 (BuCh) for the total specific activity of the rat particulate fractions becomes 0·073 (ACh). The total specific activity of ACh hydrolysis is 0·695. Hence the 0·073 attributable to ChE represents nearly 11 per cent of the total, and the remaining 89 per cent is catalysed by AChE. Similarly 93 per cent of the hydrolysis of ACh by the combined particulate fractions of the guinea pig, or 96 per cent of human, is catalysed by AChE.

Similar calculations on the data for the supernatant fractions show that the proportions of ACh hydrolysis catalysed by ChE are, guinea pig, 57 per cent; man, 41 per cent; rat, 31 per cent.

TABLE 2. DISTRIBUTION OF CHOLINE ESTER HYDROLASE ACTIVITY IN CENTRIFUGALLY SEPARATED FRACTIONS OF DIAPHRAGM HOMOGENATE

	Activity, substrate and species					
	Guinea pig		Man		Rat	
Fraction	ACh	BuCh	ACh	BuCh	ACh	BuCh
500P	0·567	0·122	1·507	0·134*	0·524	0·127
34,000P	0·072	0·000	0·288	0·025*	0·104	0·040
100,000P	0·051	0·000	0·150	0·013*	0·067	0·016
100,000S	0·207	0·296	0·268	0·273	0·219	0·171

Animal data from different pools of tissue from those reported in Table 1. Human data from a third individual. Fractions marked* from four other individuals did not hydrolyse BuCh at all.

P, particulate; S, supernatant; number, relative g. Enzyme activities in μmoles/min/g of original diaphragm. ACh, 5·5 mM; BuCh, 10 mM.

Table 2 also shows that the proportion of particulate esterase activity (ACh) deposited between 500 g and 100,000 g did not exceed 25 per cent. The use of the short method saved time, yielded an undiluted supernatant fraction, and we considered that it did not sacrifice information essential for the present work.

Histochemical examination

Treatment of intact guinea pig or rat diaphragms with a histochemical reagent containing ATCh gave the familiar pattern of staining of end-plates, as illustrated by Waser and Lüthi[24] for mouse diaphragm. Examination of the three particulate fractions prepared by the long method showed that less than 5 per cent of the particles carried a black stain which could be interpreted as a fragment of end-plate. Thus the

homogenizing process had not disturbed the highly concentrated distribution of AChE shown in intact tissue. It could be anticipated that inhibition of ACh hydrolysis by 62.c.47 might involve formation of an E_2I complex, as with the brain preparation (above).

Treatment of the intact diaphragms with a reagent containing BTCh failed to reveal anything, even when the time of incubation was increased from 30 to 90–120 min, roughly proportional to the relative velocities given in Table 1. Guinea pig or rat diaphragms were divided in two laterally. It has been shown that the "cholinesterase" activities of lateral halves are equal.[8,10,12] One half was treated with histochemical reagents, including thorough final washing to remove ammonium sulphide. They were then homogenized. Hydrolysis of ACh by preparations which had been treated with ATCh averaged about 90 per cent the rate of untreated halves, but hydrolysis of BuCh by preparations treated with BTCh was about 50 per cent of the controls. Thus selective irreversible loss of ChE activity caused by treatment with histochemical reagent containing BTCh contributed to the failure to localize the enzyme histochemically.

We have observed incidentally that a reagent prepared with ATCh chloride or methanesulphonate failed to stain, but that when the same diaphragms were re-treated with a reagent containing ATCh iodide they stained normally. Normal staining was also given by a reagent containing the chloride plus (empirically) an equimolar proportion of KI. Sodium bromide did not promote staining. There thus seems to be an absolute requirement for iodide ion. We have not been able to find any earlier reference to this fact; and we are not in a position to investigate further.

Examination of the fractions separated by the short method

(1) *Guinea pig, supernatant fraction.* Figure 5 shows that inhibition of the hydrolysis of 5·5 mM ACh by ethopropazine was practically constant over a considerable range of concentrations, suggestive of an inflexion on a more extensive curve. The lowest concentration used would still be high enough to cause total inhibition of ChE, but the highest attainable by reason of limited solubility was just beginning to inhibit AChE. The constant inhibition amounts to 40 per cent, which is in reasonable agreement with the value of 57 per cent deduced above from the relative velocities of hydro-

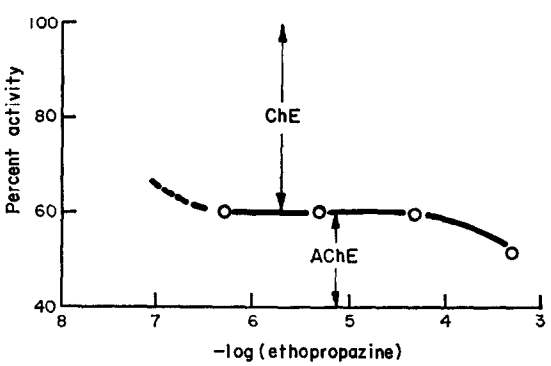

FIG. 5. Guinea pig diaphragm supernatant fraction, inhibition of the hydrolysis of 5·5 mM ACh by ethopropazine.

lysis of ACh and BuCh, when it is realised that two different preparations were involved.

The data show that the diaphragm ChE is considerably more sensitive to inhibition by ethopropazine than is the purified horse serum ChE.

Figure 6 shows that the double-log plot of data relating concentration of 62.c.47 to inhibition of hydrolysis of ACh has a slope of about 0·35. It is extremely unlikely that this signifies the formation of an E_3I complex. We interpret this as confirmation of the deduction made above that there are two enzymes present, viz. AChE and ChE, which, in this tissue, do not differ extremely in their sensitivities to the inhibitor.

The hydrolysis of 10 mM-BuCh was inhibited by 62.c.47 in a range of concentrations higher than those found for horse serum (Fig. 7). The two points on the right of Fig. 7 are consistent with a double-log plot of unit slope.

(2) *Human, supernatant fraction.* The hydrolysis of ACh was inhibited by excess substrate, giving a bell-shaped curve (Fig. 8) typical of AChE's. Hydrolysis of 5·5 mM ACh was totally inhibited by 62.c.47 over the range 500–0·5 μM, and 15 per cent by 0·05 μM. This indicates an AChE about 100 times more sensitive to inhibition by 62.c.47 than the bovine red cell AChE. Further, althought the range of concentrations

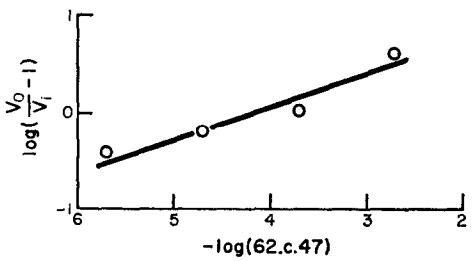

FIG. 6. Guinea pig diaphragm supernatant fraction, inhibition of hydrolysis of 5·5 mM ACh by 62.c.47. Slope is about 0·35.

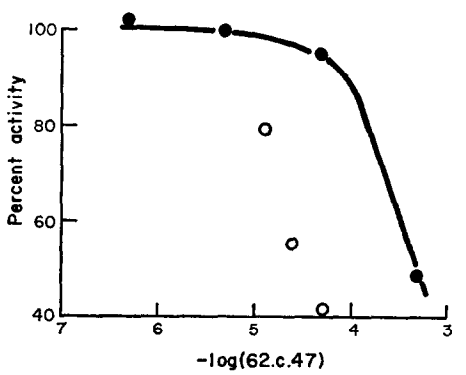

FIG. 7. Guinea pig diaphragm supernatant fraction, inhibition of hydrolysis of 10 mM BuCh by 62.c.47 (solid circles). Open circles show data obtained with horse serum ChE and 5·5 mM ACh. The use of 10 mM BuCh would probably shift the serum data to the right, if only because of the higher substrate concentration.

covered that found to give appreciable inhibition of horse serum ChE there were no inflexions indicative of a ChE. This in turn implies that if a ChE is present it must be much less sensitive than the serum enzyme.

MCh was hydrolysed at a rate about 60 per cent that of ACh, showing the presence of a high proportion of AChE.

As shown in Table 2 the relative rate of hydrolysis of BuCh was fairly high, suggesting the presence of esterases other than AChE. This hydrolysis was inhibited 57 per cent by 50 μM ethopropazine and 73 per cent by 10 μM physostigmine. The latter figure indicates the presence of a small proportion of physostigmine-resistant enzyme.

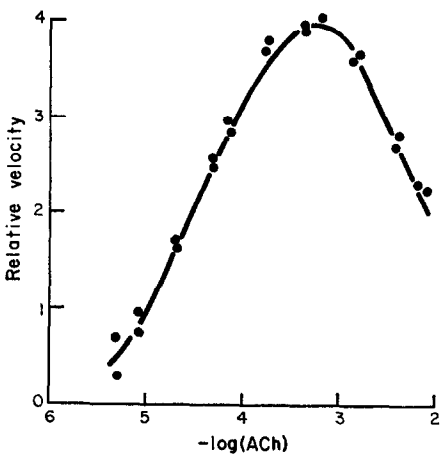

FIG. 8. Hydrolysis of ACh by the supernatant fraction from human diaphragm.

We conclude that there are three choline ester hydrolases present, AChE, ChE and a physostigmine-resistant enzyme.

(3) *Rat, supernatant fraction.* The double-log plot of data on the inhibition of hydrolysis of 5·5 mM ACh by 62.c.47 had a slope of about 0·4 (Fig. 9). As with the guinea pig soluble fraction, this probably represents the presence of two enzymes of moderately different sensitivities. A similar plot of data on inhibition of hydrolysis of BuCh by ethopropazine (Fig. 10) had a slope of about 0·4. Since one of the basic postulates is that BuCh is not measurably hydrolysed by AChE the data indicate a ChE and some other enzyme.

The tentative conclusion is that there are three choline ester hydrolases present, an AChE, a ChE and a third as yet not defined.

(4) *Guinea pig, particulate fraction.* Some deductions were made above about the proportion of ChE in the combined particulate fractions. By the same reasoning the proportion of ACh hydrolysis catalysed by ChE in the 500 g fraction is less than 10 per cent. This proportion is too small to cause noticeable inflexions in curves relating inhibitor concentration to degree of inhibition. When therefore the double-log plot describing inhibition of hydrolysis of 5·5 mM ACh by 62.c.47 is found to have a slope of 0·4, it is reasonable to take this as evidence that the AChE forms an E_2I complex.

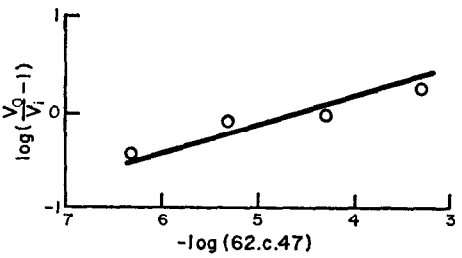

FIG. 9. Rat diaphragm supernatant fraction, inhibition of the hydrolysis of 5·5 mM ACh by 62.c.47. Slope is about 0·4.

The hydrolysis of ACh was inhibited by excess substrate, confirming that the predominant enzyme is AChE.

We consider that the rate of hydrolysis of BuCh is too low to justify studies with inhibitors. Attempts to solubilize the particulate fraction by ultrasonic vibration, with a view to further purification, merely destroyed esterase activity.

(5) *Human, particulate fraction.* Although the specimen reported in Table 2 hydrolysed BuCh, several other preparations failed to hydrolyse it at all. This fact alone is evidence of the absence of ChE from the diaphragms of a high proportion of persons. One preparation which hydrolysed BuCh at 5 per cent the rate of ACh also hydrolysed MCh at a relative rate of 44 per cent. Hydrolysis of ACh by another specimen was inhibited by excess substrate. We conclude that the fraction contains predominantly or exclusively AChE.

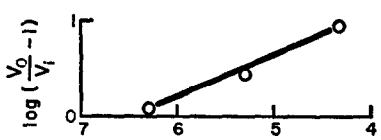

FIG. 10. Rat diaphragm supernatant fraction, inhibition of hydrolysis of 10 mM BuCh by ethopropazine.

(6) *Rat, particulate fraction.* The relative rates of hydrolysis of ACh and BuCh are similar to those of the guinea pig 500 g fraction, and the same conclusion may be reached, that the proportion of ChE is very low. A double-log plot of data on inhibition of hydrolysis of 5·5 mM ACh by 62.c.47 had a slope of about 0·4 and it may again be concluded that the AChE forms an E_2I complex. Hydrolysis of ACh was inhibited by excess substrate.

Although we thought that the rate of hydrolysis of BuCh was too low to justify inhibitor studies, we made an attempt, in view of the widespread use of the rat phrenic-diaphragm preparation. By slowing the chart speed of the pen recorder to 1·25 mm/sec we obtained some rather wavy traces—our instrument is not very steady at low velocities—from which it appeared that the hydrolysis of BuCh was inhibited only 20 per cent by 10 μM-physostigmine. Thus the physostigmine-resistant enzyme indicated from studies on the whole homogenate is present in the particulate fraction.

DISCUSSION

The specific activities of the tissue preparations used in this work have been on the low side. This has frustrated any attempts to study the effects of inhibitors on a wide range of substrates. The conclusions relate therefore to the enzymes that are probably present, rather than to a rigorous description of enzymes that are undoubtedly present.

There is nevertheless adequate evidence that there is a high proportion of AChE present, located mostly in the particulate fractions sedimented up to 100,000 *g*. There appears also to be some soluble AChE, especially in human diaphragm. It has hitherto been accepted that mammalian AChE's are so firmly attached to particulate material that drastic methods are needed to solubilize them, e.g. the use of surface-active agents, proteolytic enzymes, ultrasonic vibration, etc.[26,27] This is believed to be the first evidence for the existence of naturally-occurring soluble mammalian AChE.

The nature of the enzymes hydrolysing BuCh is not so well defined. There is probably some ChE plus a small proportion of a physostigmine-resistant enzyme which, as one of us suggests in the Appendix, may be a B-esterase with negligible activity against ACh. We have already calculated, above, that the proportion of the BuCh-hydrolases in the particulate fractions is so small as to have little influence on the hydrolysis of ACh. It may be noted that Klinar and Zupancic[28] claimed to have found ChE at the end-plates of rat diaphragm. Although they used a reagent containing BTCh and a specific inhibitor of AChE, their incubation times were excessively prolonged in comparison with those needed to locate AChE. It is possible that they localized B-esterase which, as we have shown, appears to be present in small proportion in the particulate fraction of the rat diaphragm.

There can be little doubt that the particulate AChE is associated with neuromuscular function. Our data show that it can be estimated with quite small error simply by using ACh as substrate. However the use of ACh with unfractionated homogenate[8–14] involves much larger errors. In the rat diaphragm, for example, only 75 per cent of the hydrolysis of ACh is catalysed by particulate AChE.

Whether the soluble AChE is the "reserve" non-functional AChE whose existence Fleisher *et al.*[10] claimed to have demonstrated in rat diaphragm, remains to be proved. It is clear that their evidence, based partly on the use of unfractionated homogenates, is no longer acceptable.

We have alluded in the Results section to findings that the sensitivities to inhibitors of the diaphragm enzymes differ quite considerably from those of the purified bovine red cell, rat brain or horse serum enzymes. These results reinforce Holmstedt and Sjöqvist's[29] warning that histochemical studies involving selective inhibitors should be accompanied by biochemical controls: and, we would add, on the same material. Studies on model enzymes are useless.

APPENDIX

THE HYDROLYSIS OF BUTYRYLCHOLINE BY B-ESTERASE

J. P. RUTLAND

In an investigation of the hydrolysis of choline esters by male rat red cells Davies and Rutland[30] observed a rapid breakdown of BuCh which was markedly inhibited by DPDA, a selective inhibitor

of ChE.[20,31] The red cells did not hydrolyse benzoylcholine, nor was the hydrolysis of ACh affected by DPDA. They concluded that BuCh was hydrolysed by a ChE which, unlike typical enzymes of this group, did not hydrolyse ACh. Witter[32] reported that the hydrolysis of BuCh was not inhibited by physostigmine. This Note presents evidence that the untypical ChE is not a "cholinesterase" at all, but a B-esterase (EC 3.1.1.1).

Red cells from male rats were washed with 0·154 M NaCl. In some studies they were haemolysed in 25 mM $NaHCO_3$, but in others they were haemolysed in water and centrifuged at 100,000 *g* for 60 min to yield a red supernatant, an intermediate layer containing the stroma, and a residual layer of negligible esterase activity.[27] Esterase activities were determined in the Warburg apparatus, as described by Aldridge.[21] The concentrations of choline esters, in aqueous solutions, were known, but those of the other substrates, present as emulsions, were not.[21] The inhibitors were allowed to to react with enzyme for 20 min at room temperature (18–20°) plus 10 min at 38° before adding substrate. The reaction volumes totalled 3 cm^3.

Table 3 shows that the esterases of the unfractionated haemolysate can be divided into two groups, which are or are not inhibited by 10 μM-physostigmine. Hydrolysis of ACh and MCh was suppressed, showing the presence of an AChE. Hydrolysis of BuCh and other substrates was not affected. The use of DPDA showed that esterases hydrolysing butyrates were inhibited, but those hydrolysing acetates were not.

TABLE 3. RELATIVE RATES OF HYDROLYSIS OF ESTERS BY HAEMOLYSED RAT RED CELLS

Substrate	Haemolysate			Supernatant		
	Relative velocity	Percent inhibition by		Relative velocity	Percent inhibition by	
		Phys*	DPDA†		Phys*	DPDA†
Acetates						
ACh	102	90	20	12	‡	‡
MCh	87	97	15	‡	‡	‡
Glyceryl triacetate	368	0	3	272	—	2
Ethyl acetate	74	2	20	56	2	10
Phenyl acetate	1420	8	22	1280	0	—
p-Nitrophenyl acetate	6900	7	7	7530	0	5
Butyrates						
BuCh	100§	0	93	100‖	1	98
Glyceryl tributyrate	342	2	100	215	—	95
Ethyl butyrate	61	0	100	41	0	100
Phenyl butyrate	660	0	50	405	0	—
p-Nitrophenyl butyrate	900	0	50	840	3	63

* 10 μM-physostigmine.
† 200 μM.
‡ Uninhibited velocity too low for inhibition studies.
§ 100% = 0·353 μmoles/min/cm^3 of packed red cells.
‖ 100% = 0·294 μmoles/min/cm^3 of packed red cells (equivalent).

Centrifugation has effected a fairly clean separation of physostigmine-sensitive and -resistant enzymes. The supernatant contained the resistant esterases. Hydrolysis of ACh and MCh was almost negligible, whereas that of BuCh was about 80 per cent of that of the unfractionated haemolysate. Again the use of DPDA has shown the division into acetate- and butyrate-esterases.

The simplest interpretation of these results is that BuCh is hydrolysed by an esterase which is not inhibited by physostigmine but is inhibited by an organophosphorus compound. The esterase also hydrolyses glyceryl tributyrate and phenyl butyrate more rapidly than BuCh. The hydrolysis of *p*-nitrophenyl butyrate is only partly inhibited by DPDA, so its hydrolysis may be catalysed by two different enzymes. The proportion attributable to the DPDA-sensitive esterase shows a much higher relative velocity than when BuCh was substrate. These properties of the BuCh hydrolase resemble those of

B-esterase, as described by Aldridge[21] and by Mounter and Whittaker.[22] It seems more appropriate to recognise that a choline ester can be hydrolysed by a carboxylesterase (EC 3.1.1.1) than to postulate a special class of physostigmine-resistant cholinesterases.

Acknowledgements—We thank Mrs. E. M. Joy and Mr. M. P. Maidment for technical assistance, and Mr. H. F. Colgrave for histochemical examination of the diaphragm particulate fractions.

REFERENCES

1. D. R. DAVIES, A. L. GREEN and G. L. WILLEY, *Br. J. Pharmac.* **14,** 5 (1959).
2. W. K. BERRY and D. R. DAVIES, *Biochem. Pharmac.* **19,** 927 (1970).
3. M. G. ORD and R. H. S. THOMPSON, *Biochem. J.* **46,** 456 (1950).
4. A. N. DAVISON, *Biochem. J.* **54,** 583 (1953).
5. B. MENDEL, D. B. MUNDELL and H. RUDNEY, *Biochem. J.* **37,** 473 (1943).
6. J. A. COHEN, F. KALSBEEK and M. G. P. J. WARRINGA, *Acta brevia neerl.* **17,** 32 (1949).
7. G. B. KOELLE, *Biochem. J.* **53,** 217 (1953).
8. J. A. B. BARSTAD, *Archs int. Pharmacodyn.* **107,** 21 (1956).
9. J. A. B. BARSTAD, *Archs int. Pharmacodyn* **128,** 143 (1960).
10. J. H. FLEISHER, P. J. KILLOS, J. HANSA and C. S. HARRISON, *J. Pharmac.* **130,** 461 (1960).
11. C. VAN DER MEER and O. L. WOLTHUIS, *Biochem. Pharmac.* **14,** 1299 (1965).
12. W. K. BERRY, D. R. DAVIES and J. P. RUTLAND, *Biochem. Pharmac.* **15,** 1259 (1966).
13. P. F. HEFFRON and F. HOBBIGER, *Proc. 4th Int. Congr. Pharmac. Basel,* p. 135 (1969).
14. B. BOSCOVIC, *Proc. 4th Int. Congr. Pharmac., Basel,* p. 181 (1969).
15. D. RICHTER and P. G. CROFT, *Biochem. J.* **36,** 746 (1942).
16. B. BAYLISS and A. TODRICK, *Biochem. J.* **62,** 62 (1956).
17. L. A. MOUNTER and V. P. WHITTAKER, *Biochem. J.* **47,** 525 (1950).
18. G. A. ALLES and R. C. HAWES, *J. biol. Chem.* **133,** 375 (1940).
19. L. M. STURGE and V. P. WHITTAKER, *Biochem. J.* **47,** 518 (1950).
20. L. AUSTIN and W. K. BERRY, *Biochem. J.* **54,** 695 (1953).
21. W. N. ALDRIDGE, *Biochem. J.* **53,** 110 (1953).
22. L. A. MOUNTER and V. P. WHITTAKER, *Biochem. J.* **54,** 551 (1953).
23. F. H. JOHNSON, H. EYRING and R. W. WILLIAMS, *J. cell. comp. Physiol.* **20,** 247 (1942).
24. P. G. WASER and U. LÜTHI, *Archs int. Pharmacodyn.* **112,** 272 (1957).
25. G. B. KOELLE, in *Cholinesterases and Anticholinesterase Agents* (Ed. G. B. KOELLE) Springer Verlag, Berlin (1963).
26. K-B. AUGUSTINSSON, in *Cholinesterases and Anticholinesterase Agents* (Ed. G. B. KOELLE) Springer Verlag, Berlin (1963).
27. J. J. GORDON and J. P. RUTLAND, *Nature, Lond.* **214,** 850 (1967).
28. B. KLINAR and A. O. ZUPANCIC, *Archs int. Pharmacodyn.* **136,** 47 (1962).
29. B. HOLMSTEDT and F. SJÖQVIST, *Bibl. anat.* **2,** 1 (1961).
30. D. R. DAVIES and J. P. RUTLAND, *Nature, Lond.* **178,** 697 (1956).
31. W. N. ALDRIDGE, *Biochem. J.* **53,** 62 (1953).
32. R. F. WITTER, *Toxic. appl. Pharmac.* **4,** 313 (1962).