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BIOCHEMICAL AND PHARMACOLOGICAL PROPERTIES OF ACRYLOYLCHOLINE, AN INHIBITOR OF CHOLINE ACETYLTRANSFERASE

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Abstract—Acryloylcholine, an unsaturated choline ester was found to be a strong inhibitor of rat and pigeon brain choline acetyltransferase. Substitution of the hydrogen atoms of the C-2 and C-3 carbon atoms in the acylgroup by methylgroups resulted in less potent inhibitors. Propionylcholine, the saturated choline ester analogue, was 1000-fold less potent. The rat brain enzyme was not protected against the inhibition by acryloylcholine by high concentration of acetyl-CoA or choline and the inhibition was neither affected by variation in the concentration of sodium chloride nor by variation in pH. The inhibition of rat brain choline acetyltransferase was uncompetitive with respect to choline and acetyl-CoA. The inhibition of pigeon choline acetyltransferase was completely reversible whereas the inhibition of rat choline acetyltransferase could only be reversed with difficulty.

Acryloylcholine inhibited acetylcholinesterase reversibly and to the same extent as propionylcholine. Acryloylcholine was a better substrate for buturylcholinesterase than for acetylcholinesterase.

Acryloylcholine caused a neuromuscular block in the rat diaphragm, but this block was probably not due to inhibition of choline acetyltransferase. The ester contracted the esterinized frog rectus abdominus muscle to the same extent as propionylcholine.

ALTHOUGH there exist several groups of inhibitors of acetylcholinesterase (AChE, acetylcholine hydrolase EC 3.1.1.7.), only a few inhibitors are described for choline acetyltransferase (ChAc, acetyl-CoA: choline-O-acetyltransferase EC 2.3.1.6.). Inhibitors for ChAc would be of value in characterizing the enzyme and would be a valuable tool in studying the biosynthesis of ACh. At present only two groups of ChAc inhibitors are known, namely those containing ringstructures separated by a double bond[1–4] and halogenated analogues of acetylcholine, acetyl-CoA and bromoacetonyltrimethylammonium bromide.[5–7]

The present investigation shows that choline esters with an unsaturated bond in the acylgroup are strong inhibitors of ChAc. Some of these esters are present naturally and have been isolated from various snails.[8,9] The kinetics and reversibility of the inhibition of rat ChAc by acryloylcholine, the most potent of these inhibitors, was studied in detail. The hydrolysis of acryloylcholine by cholinesterases and its pharmacological properties were reinvestigated.

MATERIAL AND METHODS

Chemicals

(1-^{14}C)acetyl-CoA (sp. radioactivity 49·8 mCi/m-mole) was obtained from New England Nuclear and diluted with unlabelled acetyl-CoA (96 per cent pure) from

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Schwarz-Mann Res. Lab., to give a final sp. radioactivity of 16·3 mCi/m-mole. (1-^{14}C)-acetylcholine (sp. radioactivity 13·7 mCi/m-mole) was supplied by the Radiochemical Centre, Amersham.

Acryloylcholine, metacryloylcholine, 3:3-dimethylacryloylcholine and crotonylcholine were synthesized as the iodide salts by Dr S. Øksne (unpublished results) in our laboratory. The three former compounds were synthesized by reacting the corresponding acid chloride with dimethylaminoethanol and then methylating the product with methyl iodide. Metacryloylcholine was synthesized by methylating *N*:*N*-dimethylaminoethylmetacrylate with methyl iodide. The esters were purified by fractional crystallization and analysed by NMR (Dr S. Øksne unpublished results) and paperchromatography.[10] The paperchromatograms were developed with butan-1-ol–ethanol–acetic acid–water (8:2:1:3, by vol.) and the spots visualized by iodine vapour.

Preparation of ChAc. ChAc from rat brain and pigeon brain were purified by acidification and ammonium sulphate fractionating as previously described.[11] The rat enzyme was further purified by ion exchange chromatography.[12]

ChAc from *Buccinum undatum* was obtained by homogenizing the head ganglia of the whelk in 25 mM-sodium phosphate buffer, pH 7·4, with a Potter-Elvehjem homogenizer.

The homogenate was centrifuged at 20,000 *g* for 15 min and the clear supernatant used directly.

AChE. Bovine erythrocyte AChE was supplied by Schwarz-Mann Res. Lab., and horse serum cholinesterase by Koch-Light Lab. The other cholinesterases were prepared by solubilizing brain membranes with 0·5% (w/v) Triton X-100.[13] Rat serum was obtained by centrifugation of heparinized rat blood.

Inhibition and assay of ChAc

The enzyme reaction was usually carried out at 37° for 1 min. The reaction rate was linear for about 4 min with the enzyme concentration used. The standard procedure adopted was to add 5 μl of the substrate mixture, usually containing the inhibitors, to 2 μl of enzyme solution. The standard incubation mixture contained (final conc): 8 mM-choline bromide, 0·28 mM-(1-^{14}C)acetyl-CoA, 200 mM-NaCl, 25 mM-sodium phosphate buffer, 4 mM-EDTA, 0·6 mM-physiostigmine and the inhibitor (Fig. 1). The final pH was 7·4. The enzyme reaction was terminated by the dilution

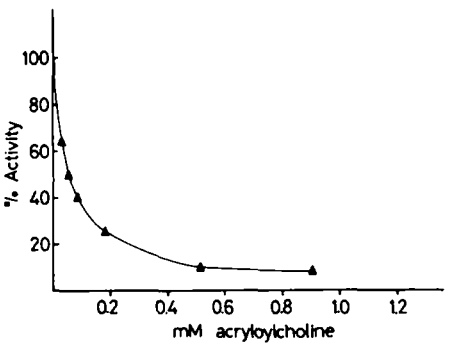


FIG. 1. The inhibition of rat brain choline acetyltransferase by varying concentrations of acryloylcholine. The inhibitor was added to the substrate mixture and the enzyme assayed at 37° for 1 min.

of the incubation mixture (7 μl) with 5 ml 10 mM-sodium phosphate buffer, pH 7·4, and the labelled ACh isolated by extraction with tetraphenylboron (Kalignost) in ethylbutylketone.[14]

Preincubation of ChAc with substrates. The protection of the enzyme by substrates was investigated by preincubating ChAc with either varying conc. of choline bromide (0·5 50 mM) or acetyl-CoA (0·05–0·5 mM) in the presence of 50 mM or 280 mM-sodium chloride for 3 min at 37°. Two μl of this mixture was added to 5 μl of the incubation mixture containing the other constituents in normal concentration and in addition 0·087 mM acryloylcholine (Table 2).

Inhibition by a mixture of acryloylcholine and CoA (Table 3)

Acryloylcholine (1 mM) was preincubated with varying concentration of CoA (13 1·6 mM) for 1 min at 37°. Samples of this mixture were added to the standard incubation mixture and the enzyme assayed as before.

Reversal of inhibition of ChAc

Gelfiltration. Concentrated rat and pigeon brain enzyme was preincubated with 0·5 mM-acryloylcholine and 0·1 mM-physostigmine (to prevent hydrolysis of the inhibitor) for 10 min at 0°. The inhibitors were removed by gel-filtration on Sephadex G-50 fine, equilibrated with 25 mM-sodium phosphate buffer, pH 7·4. The column used was 9 cm high and the diameter was 0·4 cm. Fractions of 0·14 ml (six drops) were collected. The column was standardized with uninhibited ChAc, (1-^{14}C)acetylcholine and acryloylcholine. (1-^{14}C)Acetylcholine was determined directly by liquid scintillation counting in a Triton- toluene mixture. Acryloylcholine was determined by its inhibition of ChAc.

Washing a "membranebound" ChAc preparation. A crude mitochondrial fraction (Fraction P_2) was prepared and hypoosmotically treated with 2 mM-sodium phosphate buffer pH 6·3.[15] The suspension was also treated with ether to release any occluded enzyme activity. Treatment with Triton X-100 would dissolve part of the membrane and decrease the efficiency of the membrane binding. Five ml of the suspension was incubated with varying concentrations of acryloylcholine for 15 min at 0°. Two μl of the suspension was added to the standard substrate mixture and the proportion of inhibited ChAc was determined. The remaining solution was diluted to 12·5 ml with aqua dest. and centrifuged for 30 min at 100,000 *g*. Under these conditions the enzyme remains membrane-bound and is recovered in the pellet. The pellet was resuspended in 5 ml of sodium phosphate buffer and the enzyme activity determined as before.

AChE assay

The substrate specificities of the different cholinesterases at 37° were determined with manometric technique.[13] The inhibition of AChE at 25° by choline esters in the presence of 0·2 mM acetylthiocholine was determined spectrophotometrically.[17]

Pharmacological experiments

The phrenic nerve diaphragm preparation was used.[18] The AChE in the preparation was inhibited by incubating with 30 μg/ml DFP for 10 min at 37°. The preparation was washed and left for 20 min before the addition of acryloylcholine.

Bioassay on frog rectus. The frog rectus was prepared in a bath containing 9 ml of eserinized frog Ringer solution.

Intravenous injection. The rats were injected into the femoral vein.

RESULTS

Acryloylcholine inhibited ChAc almost instantaneously at 37°. Thus there were no differences in the proportion of ChAc activity inhibited when the enzyme was preincubated with the inhibitor for 30 sec or 10 min. The inhibition was therefore studied by adding the inhibitor together with the substrate mixture to the enzyme and incubating this mixture for 1 min at 37°. The percentage decrease in enzyme activity was taken as the per cent inhibited ChAc. The proportion of ChAc inhibited was unchanged by preincubating the enzyme with varying concentration of choline, acetyl-CoA and NaCl (Table 1). These compounds were studied at concentrations which

TABLE 1. THE EFFECT OF ACETYL-CoA, CHOLINE AND SODIUM CHLORIDE ON THE INHIBITION OF CHOLINE ACETYLTRANSFERASE (ChAc) BY ACRYLOYLCHOLINE (AcrCh)

Acetyl-CoA (mM)	Choline (mM)	AcrCh (mM)	NaCl (mM)	Activity of ChAc (%)
0·2	8	0	280	100
0·2	8	0·087	280	45
0·2	8	0	50	50
0·2	8	0·087	50	20
0·2	0·6	0	280	57
0·2	0·6	0·087	280	24
0·04	8	0	280	48
0·04	8	0·087	280	25

For experimental details see "Methods" section.

markedly influenced ChAc activities. Thus these compounds did not seem to protect the enzyme against the inhibitor, neither did they change the enzyme conformation to make it more or less susceptible to the inhibitor.

Acryloylcholine was a very efficient inhibitor of rat ChAc at concentrations below 0·1 mM (Fig. 1). Substitution of hydrogen-atoms at carbon-atoms C-2 or C-3 with methyl-groups decreased the inhibition both for rat and pigeon ChAc (Table 2). The

TABLE 2. THE INHIBITION OF CHOLINE ACETYLTRANSFERASE (ChAc) AND ACETYLCHOLINESTERASE (AChE) BY DIFFERENT CHOLINE ESTERS

Choline ester	Acyl group	ChAc Rat (mM)	ChAc Pigeon (mM)	AChE Rat (mM)
Acryloylcholine	$CH_2{=}CHCO$	0·05	0·025	0·34
Crotonylcholine	$CH_3CH{=}CHCO$	0·9	1·1	0·32
Metacryloylcholine	$CH_2{=}C(CH_3)CO$	5·0	6·2	0·33
3:3-Dimethylacryloylcholine	$(CH_3)_2C{=}CHCO$	>5*	>5*	0·61
Acetylcholine	CH_3CO	240	—	0·75
Propionylcholine	CH_3CH_2CO	250	—	0·53
Buturylcholine	$CH_3CH_2CH_2CO$	160	—	0·20

The results are expressed as the inhibitor conc. necessary to give 50% inhibition of the enzyme under standard incubation conditions.

* No inhibition found at this concentration.

inhibition of ChAc with choline esters with saturated bonds in the acylgroup was 1000-fold less efficient. Rat brain AChE did not show any such degree of specificity and its inhibition was not markedly increased by introduction of double bond into the acyl group. It is interesting that acryloylcholine, which is present in *Buccinum undatum*,[8] inhibited the brain ChAc of this mollusc (I_{50} = 0·14 mM).

Acryloylcholine in a concentration of 0·09 mM, inhibited ChAc 60–64% in phosphate buffer pH 6·5, 7·0 and 7·5. The inhibition was therefore independent of pH in this range.

The inhibition by acryloylcholine was not affected by CoA, another inhibitor of ChAc.[19] The inhibition by the two compounds together was almost additive (Table 3).

TABLE 3. INHIBITION OF CHOLINE ACETYLTRANSFERASE (ChAc) BY CoA AND ACRYLOYLCHOLINE (AcrCh)

AcrCh (mM)	CoA (mM)	ChAc inhibition (%)
0·087	0	63
0	0·23	14
0	0·60	39
0	2·90	72
0·087	0·23	72
0·087	0·60	83
0·087	2·90	94

The inhibition was performed as described in "Methods". Preincubation time for acryloylcholine and CoA was 1 min at 37°.

The kinetics of the inhbition of ChAc by acryloylcholine were studied by varying the concentration of acetyl-CoA, choline and acryloylcholine. The results are expressed in double reciprocal plots (Fig. 2). Both plots gave a series of straight and parallel lines, thus indicating a change in both the V_{max} and the K_m of the enzyme. The inhibition of ChAc therefore seemed to be uncompetitive with both substrates.

Rat and pigeon ChAc were inhibited 97 and 93 per cent respectively by 5×10^{-4} M-acryloylcholine. Dilution of these enzyme-inhibitor mixtures 11-fold with 25 mM-sodium phosphate buffer, pH 7·4, resulted in a regain of 48 per cent of the activity of pigeon ChAc, but in no increase in the rat enzyme activity. The inhibitor concentration after dilution was expected to inhibit 40 and 50 per cent of native ChAc of pigeon and rat respectively. A 30-fold dilution yielded 58 per cent of the original pigeon ChAc activity, but still no increase of rat enzyme activity. Uninhibited enzymes were diluted simultaneously as controls.

When the inhibitor was removed by gelfiltration, pigeon ChAc was fully reactivated whereas less than 10 per cent of rat ChAc activity was found. The column used was well capable of separating the inhibitor from the enzyme (Fig. 3).

The contamination of the enzyme fractions by acryloylcholine was estimated to be less than 10^{-6} M. When the inhibitor was removed by washing "membrane bound" ChAc (Table 4), the enzyme activity from rat brain was partially recovered. The activity did not increase further after standing for 24 hr at 0°. In conclusion the inhibition of ChAc from pigeon was easily reversible whereas the inhibition of rat ChAc was reversed only with difficulties.

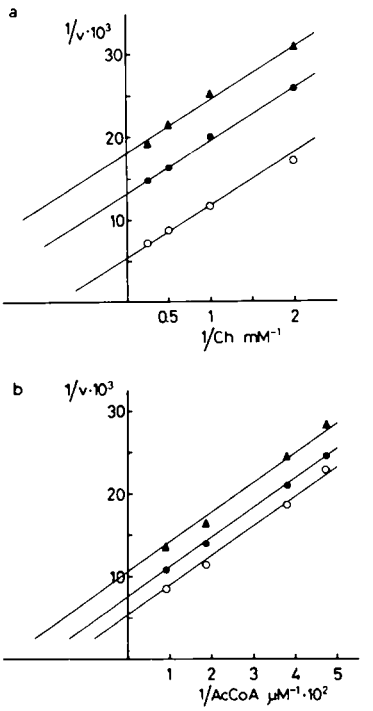


FIG. 2. Double reciprocal plots of the activity of rat brain choline acetyltransferase vs the concentration of choline and acetyl-CoA: (a) The concentration of acetyl-CoA was kept constant at 318 μM. The choline concentration was varied between 0·25–4 mM. (○), 0 mM acryloylcholine; (●), 0·1 mM acryloylcholine; (▲), 0·2 mM acryloylcholine. (b) The concentration of choline was kept constant at 8 mM and the concentration of acetyl-CoA was varied between 20 and 100 μM; (○), 0 mM acryloylcholine; (●), 0·05 mM acryloylcholine; (▲), 0·1 mM acryloylcholine.

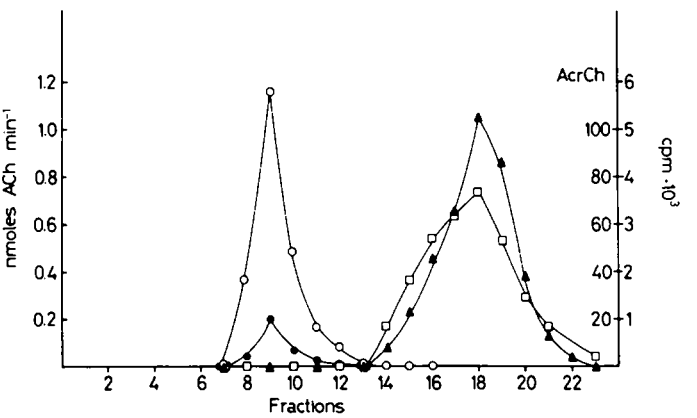


FIG. 3. Gelfiltration of rat brain choline acetyltransferase preincubated with (●) and without (○) acryloylcholine (5×10^{-4} M) on Sephadex G-50 fine. (1-^{14}C) acetylcholine (▲) was added to the inhibited enzyme immediately prior to the gelfiltration to show complete separation of small and large molecules. Acryloylcholine was run in a separate experiment (□). Acryloylcholine is expressed as the per cent inhibition of an untreated enzyme preparation.

TABLE 4. REACTIVATION OF INHIBITED RAT BRAIN CHOLINE ACETYLTRANSFERASE (ChAc) BY WASHING "MEMBRANE BOUND" ENZYME

Original concentration of AcrCh (M)	% ChAc activity	
	Originally	Washed
10^{-4}	13 ± 3	48 ± 7
4×10^{-5}	43 ± 5	66 ± 8
2×10^{-5}	70 ± 5	81 ± 8

The results are the average of three separate experiments and are expressly mean values ± S.D.

The inhibition of AChE by acryloylcholine was completely reversed by diluting the enzyme-inhibitor solution. The rate of hydrolysis of this ester by AChE was low. Propionylcholinesterases and butyrylcholinesterases were slightly more effective (Table 5). Acryloylcholine was therefore both a reversible inhibitor and a substrate of cholinesterases.

TABLE 5. THE SUBSTRATE SPECIFICITIES OF CHOLINESTERASES FROM DIFFERENT SPECIES

Choline esters	Frog brain	Bovine erythrocytes	Rat brain	Chicken brain	Horse serum	Rat serum
Acetylcholine	100	100	100	100	100	100
Propionylcholine	50 ± 2	74 ± 5	86 ± 2	131 ± 2	171 ± 3	229 ± 6
Acryloylcholine	5 ± 2	10 ± 2	18 ± 2	16 ± 2	37 ± 5	35 ± 3

The results are expressed relative to the rate of hydrolysis of acetylcholine which was taken as 100. All results are presented within 95 per cent confidence limits.

In view of the fact that acryloylcholine was found to be a strong and not readily reversible ChAc inhibitor, we decided to reinvestigate its effect on the rat phrenic nerve diaphragm preparation. Acryloylcholine caused a neuromuscular block in the rat diaphragm after DFP* inhibition. Unlike the inhibition of ChAc the neuromuscular block was completely reversible. The threshold value for this effect was found to be 1 μM and a complete block was obtained with 20 μM-acryloylcholine on stimulating 1/10 sec (Fig. 4). Even after inhibiting the rat diaphragm for 2 hr with 0·2 mM-acryloylcholine, the effect was completely reversed. Samples of the solution in the latter cases were removed and assayed for acryloylcholine on the frog rectus muscle. This confirmed that the ester was not hydrolysed. The blocking effect on rat diaphragm was therefore, due to its reversibility, probably not caused by inhibition of ChAc.

* DFP diisopropyl phosphonofluoridate.

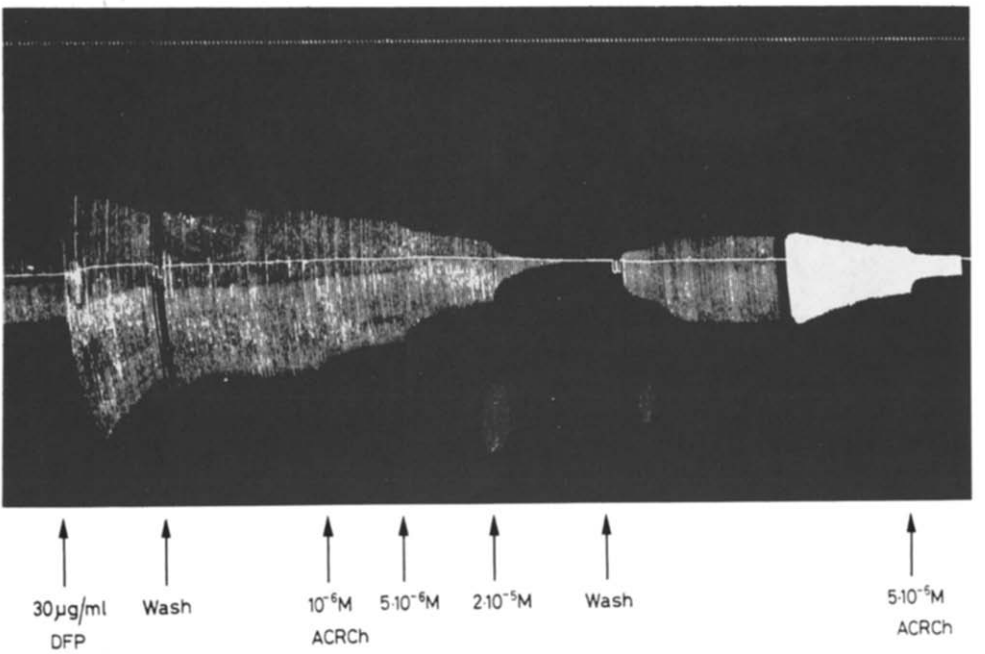


FIG. 4. The effect of acryloylcholine on the rat phrenic diaphragm preparation. The cholinesterase activity was inhibited with 30 μg/ml DFP. The recording shows the decrease of concentration after adding a series of increasing concentration of acryloylcholine. In the first series the preparation was stimulated 1/10 sec, in the second series the preparation was stimulated 1/sec. The time scale is 30 sec.

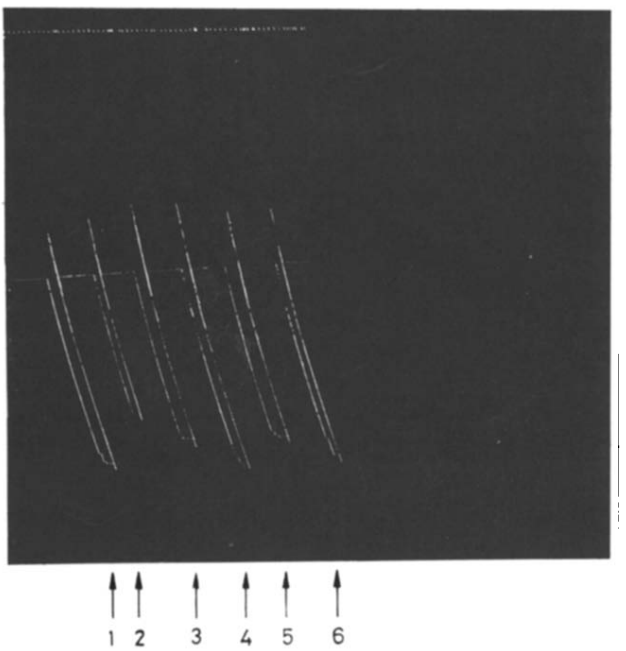


FIG. 5. The effect of acetylcholine (ACh), acryloylcholine (AcrCh) and propionylcholine (PrCh). (1) ACh 5×10^{-7} g/l; (2) AcrCh 3×10^{-7} g/l; (3) AcrCh 4×10^{-7} g/l; (4) AcrCh $5 = 10^{-7}$ g/l; (5) ACh 5×10^{-7} g/l; (6) PrCh 3×10^{-7} g/l.

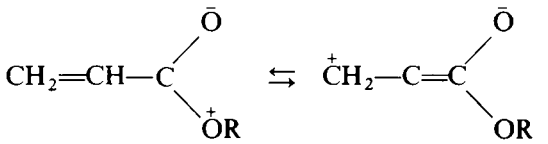
Acryloylcholine caused a similar contraction of the frog rectus abdominus muscle (Fig. 5) as acetylcholine and propionylcholine. The equipotent concentrations of the three substances were acetylcholine 0·5 μg/l, propionylcholine 0·3 μg/l and acryloylcholine 0·4 μg/l.

Injections of acryloylcholine into the femoral vein of rat was accompanied by a lowering of heart frequency. The effect was completely reversed if the animal survived. The lethal dose for intravenous injection was about 10 mg/kg. Subcutaneous injections of this dose did not produce any significant effects.

DISCUSSION

The allyl acid esters of choline constitute a new group of ChAc inhibitors. By comparing the inhibition by propionyl- and acryloylcholine esters (Table 2), it is evident that the double bond in the acylgroup is an absolute requirement. Substitution of the hydrogen atoms at carbon atoms C-2 and C-3 in the acyl group with methyl groups reduced the inhibitory power.

Acryloylcholine occurs in two resonance forms,



(a) (b)

but form (b) will dominate. In crotonylcholine and dimethylacryloylcholine the resonance structure (b) will be stabilized by inductive effect from the C-3 methyl groups.

This will, however, partially compensate the positive charge on C-3 carbon atoms. In addition steric factors may be important in modifying the inhibition.

In this context it is interesting to consider the other inhibitors of ChAc to look for any common denominator. The styrylpyridines were first reported to be reversible[2,4,22] and on prolonged incubation to be irreversible inhibitors[20] whereas the stilbazole derivatives,[3,4] the bromoacetonyl compound[5] and bromacetonyl-CoA[6] were all reported to be irreversible inhibitors of ChAc. Although some of these statements have been modified (R. E. Gibson, personal communication) they imply that the inhibitors bind very strongly to the enzyme. The halogenated acetylcholine analogues decompose rapidly and are therefore reversible inhibitors and cannot be directly compared with the other inhibitors. The styrylpyridines[1,2] and the stilbazoles[3,4] consist of a two ring structure separated by a double bond, one ring being an electron donor and the other an electron acceptor. The halogenated acetylcholine esters and the halogenated acetonyl compound will all have a transfer of electrons from the carbon atom to the halogen atom and to the oxygen atom. Thus all the known ChAc inhibitors contain an electron deficient and an electrondense group.

Thus all the inhibitors including acryloylcholine could inhibit ChAc similarly. They could possibly bind to the enzyme with the positive group resulting either from polarization of the double bond or electron attraction of the neighbouring halogen atom. A nucleophilic sulphydryl or an imidazol group on the enzyme could both participate in such a binding. Both groups[20,21] have been suggested as candidates for

the active site of the enzyme. A similar explanation was offered by Baker and Gibson[4] for the mechanism of the inhibition of ChAc by stilbazoles. Also binding of the inhibitor to the enzyme by π-bonds supplied through the double bond is possible for the styrylpyridines, stilbazoles and acryloylcholine, but not for the halogenated compounds. On the other hand it has been suggested that the inhibition by strylpyridine could be explained by a charge transfer complex formation between the inhibitor and the enzyme.[2] This explanation is unlikely for acryloylcholine.

The inhibition of rat brain ChAc by acryloylcholine followed uncompetitive kinetics. The same is the case for the halogenated choline esters.[24] The styrylpyridines[22] and stilbazoles derivatives (R. E. Gibson, personal communication) showed noncompetitive kinetics. High concentration of acetyl-CoA, choline and NaCl did not affect the degree of inhibition of ChAc by acryloylcholine.

The inhibition of ChAc by CoA and acryloylcholine was almost additive indicating that these inhibitors inhibited the enzyme at different sites. Acryloylcholine was found to be a better substrate for buturylcholinesterase than AChE. The inhibition, unlike that of ChAc, showed lack of specificity for the acyl group and was readily reversible.

The neuromuscular block in rat diaphragm by acryloylcholine after DFP inhibition of AChE, was probably not caused by ChAc inhibition. Possibly there are biological barriers that prevent acryloylcholine from entering presynaptic terminals. The neuromuscular block of acryloylcholine has been observed before.[23] The effect of acryloylcholine on the frog rectus abdominus muscle was slightly higher than previously reported.[23] This may be due to slight differences in techniques since the effect was reported to vary considerably.

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REFERENCES

1. C. J. CAVALLITO, H. S. YUN, T. KAPLAN, J. C. SMITH and F. F. FOLDES, *J. med. Chem.* **13**, 122 (1970).
2. R. C. ALLEN, G. L. CARLSON and C. J. CAVALLITO, *J. med. Chem.* **13**, 909 (1970).
3. B. R. BAKER and R. E. GIBSON, *J. med. Chem.* **14**, 315 (1971).
4. B. R. BAKER and R. E. GIBSON, *J. med. Chem.* **15**, 639 (1972).
5. D. MORRIS and D. S. GREWAAL, *Life Sci.* **8**, 511 (1969).
6. J. F. A. CHASE and P. K. TUBBS, *Biochem. J.* **100**, 47 (1966).
7. B. O. PERSSON, L. LARSSON, J. SCHUBERT and B. SÖREBO, *Acta. chem. scand.* **21**, 2283 (1967).
8. V. P. WHITTAKER, *Biochem. Pharmac.* **1**, 342 (1958).
9. M. J. KEYL, I. A. MICHAELSON and V. P. WHITTAKER, *J. Physiol., Lond.* **139**, 434 (1957).
10. K. B. AUGUSTINSSON and M. GRAHN, *Acta. physiol. scand.* **27**, 10 (1953).
11. D. MALTHE-SØRENSSEN and F. FONNUM, *Biochem. J.* **127**, 229 (1972).
12. F. FONNUM and D. MALTHE-SØRENSSEN, *J. Neurochem.* **20**, 1351 (1973).
13. R. ANDERSEN, K. LAAKE and F. FONNUM, *Comp. Biochem. Physiol.* **42B**, 429 (1972).
14. F. FONNUM, *Biochem. J.* **113**, 291 (1969).
15. F. FONNUM, *Biochem. J.* **109**, 389 (1968).
16. F. FONNUM, *Biochem. J.* **103**, 262 (1967).
17. G. L. ELLMAN, K. D. COURTNEY, N. ANDERS JR. and R. M. FEATHERSTONE, *Biochem. Pharmac.* **7**, 88 (1961).
18. J. A. BARSTAD, *Arch. int. Pharmacodyn.* **107**, 4 (1956).
19. R. B. REISBERG, *Yale J. biol. Med.* **29**, 403 (1957).
20. H. L. WHITE and C. J. CAVALLITO, *Biochem. biophys. Acta* **206**, 343 (1970).
21. H. L. WHITE and C. J. CAVALLITO, *Biochem. biophys. Acta* **206**, 242 (1970).
22. J. C. SMITH, C. J. CAVALLITO and F. F. FOLDES, *Biochem. Pharmac.* **16**, 2438 (1967).
23. V. P. WHITTAKER, *Ann. N.Y. Acad. Sic.* **90**, 695 (1960).
24. D. MORRIS and D. S. GREWAAL, *Eur. J. Biochem.* **22**, 563 (1971).