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THE EFFECTS OF REPLACING ESTER BY AMIDE ON THE BIOLOGICAL PROPERTIES OF COMPOUNDS RELATED TO ACETYLCHOLINE

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1 Replacement of ester by amide in series of derivatives of diphenylacetic acid reduces the affinity for muscarine-sensitive acetylcholine receptors of the guinea-pig ileum from 40- to 100-fold. With similar series of phenylacetic acid the reduction is only 2- to 4-fold. In both series changes in the composition of the onium group produce similar changes in the affinity of amides and esters and it appears that the stiffness of the amide bond reduces the binding of the phenyl groups at the far end of the molecule from the onium atom.

2 Replacement of ester by amide in similar series of acetyl compounds reduces activity on the guinea-pig ileum over 1000-fold and on the frog rectus over 50-fold. Compounds with larger onium groups are antagonists on both preparations with log affinity constant around 3. The amides have similar affinity for electric eel acetylcholinesterase.

3 The amides are slightly bigger than the esters in solution and slightly more hydrophilic.

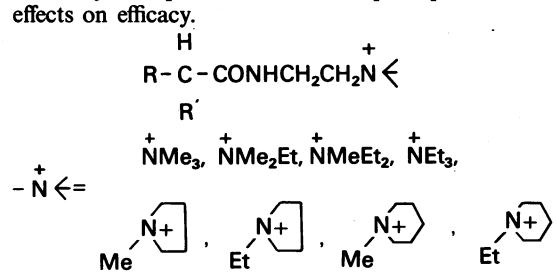
4 Replacement of ester by amide in acetylcholine reduces the proportion of *gauche* conformer about the C—C—bond from 100% to 39%.

5 The ability of acetylcholine to activate receptors is thought to depend on some degree of flexibility in the —CO—O— bond, though the hydration of the bond may also be important.

Introduction

The amide analogue of acetylcholine was synthesized by Hromatka, Kraupp & Skopalik (1953) but its pharmacological properties do not appear to have been reported, apart from a dose-response curve on rat intestine which appears in an illustration (Van Rossum, 1968) and indicates that it has about 0.01% of the activity of acetylcholine. It might be supposed that because the amide bond is stiffer than ester (Pauling, 1939; 1967; Gill, 1965), the compound is less able to fit the acetylcholine receptor, but it might also be less able to activate it. The effects of replacing ester by amide on affinity have therefore been observed directly by making the series of amides (I and II), which are antagonists, and measuring their affinity constants for the muscarine-sensitive acetylcholine receptors of the guinea-pig isolated ileum. Values for the corresponding esters are already known (Abramson, Barlow, Mustafa & Stephenson, 1969).

The series of acetylamides (III) and esters (IV) have been prepared and tested on the guinea-pig ileum, frog rectus and on acetylcholinesterase from electric eel. It is known from the work of Holton & Ing (1949) and Abramson (1964) that increasing the size of the onium group in acetylcholine decreases the activity and it was expected that some of the compounds in series III and IV would be partial agonists or antagonists, whose affinity for the receptors could be measured. By studying series it was therefore hoped that it would be possible to observe the effects of replacing ester by amide on the affinity of some of the acetyl compounds, and hence perhaps to assess effects on efficacy.



I R=R'=Ph
II R=Ph, R'=H
III R=R'=H
IV R=R'=H,—NH—=—O—

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If the effects of replacing —O— by —NH— on biological activity are due to changes in preferred conformation, associated with the greater stiffness of the amide bond, it might be possible to detect these from the n.m.r. spectra and these have been obtained and interpreted by Dr G.C.K. Roberts (National Institute for Medical Research, Mill Hill). It is possible, however, that changes in other chemical properties are involved, such as size or lipophilic character, and an attempt has been made to assess these from the apparent molal volumes and chromatographic properties of some of the compounds.

Methods

Compounds

The amides were prepared by adding the acid chlorides to an excess of the amine. The reaction was usually vigorous but was heated to ensure completion. The product was cooled, dissolved in a little water, made strongly alkaline with NaOH, and shaken with ether containing chloroform, (10% v/v) to extract the free base. The extract was dried with anhydrous magnesium sulphate and the solvent distilled off. The tertiary-amino ethylamides of diphenylacetic acid were solids and were recrystallized from combination of acetone, ethylacetate and petrol (b.p. 60–80°C). The other compounds were all distilled under reduced pressure. The phenylacetyl derivatives had b.p. 130°/0.25 mm (NMe_2), 160°/0.45 mm (NEt_2), 160°/0.7 mm (pyrrolidino), and 143°/0.15 mm (piperidino). The acetyl derivatives had b.p. 83°/0.4 mm (NMe_2), 92°/0.4 mm (NEt_2), 106°/0.35 mm (pyrrolidino) and 116°/0.3 mm (piperidino); Hromatka *et al.* (1953) recorded b.p. 117–8°/10 mm for the dimethylamino compound.

The tertiary bases were quaternized by adding methyl or ethyl iodide to their solutions in ethylmethylketone. The metho-salts were formed almost immediately but the reactions with ethyl iodide were left at least 24 h at room temperature to reach completion. When necessary the quaternary salts were forced out of solution by the addition of ether. They were recrystallized from combinations of ethylmethylketone, ethanol, ethyl acetate and ether and their m.ps and analyses are shown in Table 1. Two of the derivatives of phenylacetic acid, the ethyldimethylammonium (Me_2Et) and ethylpyrrolidinium compounds,

Table 1 Analyses and melting-points

Onium group	*Acetyl ester*	*Acetyl amide*	*Phenylacetyl amide*	*Diphenylacetyl amide*
$\overset{+}{N}Me_3$	161–163°	138.8–139.5°*	sinters	214.0–215.0°
		46.60 (46.63)	117.2–117.6° melts	29.93 (29.91)
			122.4–122.9°	
			36.40 (36.44)	
$\overset{+}{N}Me_2Et$	84–5°	98.4–99.4°*		185.9–186.5°
		44.15 (44.35)		28.94 (28.95)
$\overset{+}{N}MeEt_2$	65–6°	98.3–99.4°	sinters	168.9–169.8
		42.14 (42.28)	88–91° melts	28.03 (28.05)
			93.6–94.0	
			33.72 (33.72)	
$\overset{+}{N}Et_3$	119.5–120.0°	136.8–137.4°	132.5–133.5°	125.5–127.5°
		40.27 (40.39)	32.51 (32.51)	27.53 (27.21)
Methyl-pyrrolidinium	68.0–69.8°	93.0–95.4°	140.4–141.4°	195.0–196.0°
		42.49 (42.56)	33.99 (33.91)	27.92 (28.18)
Ethyl-pyrrolidinium	61–62°	84.4–85.6°		192.7–194.2°
		40.65 (40.65)		27.31 (27.33)
Methyl-piperidinium	107–8°	110.0–111.2°	133.6–134.4°	216.2–217.2°
		40.52 (40.65)	32.82 (32.68)	27.26 (27.33)
Ethyl-piperidinium	87–88°	78.7–80.5°	144.8–145.8°	174.9–176.1°
		38.80 (38.90)	31.70 (31.54)	57.72 (57.74 C)
				6.45 (6.53 H)
				5.66 (5.85 N)

Iodide analyses are gravimetric with samples of 50–250 mg; the microanalysis (C, H, N) was made by Mr M. West, Chemistry Department, University of Bristol. Theoretical values are shown in parentheses. Melting-points were measured with Mettler FP instruments, coupled to a potentiometric recorder and at a rate of heating of 0.2°C/min; values for the acetylesters are included for comparison. The asterisk indicates that Hromatka *et al.* (1953) recorded m.p. 137–8° and 95–7°C for these compounds.

could not be obtained crystalline, either as iodides or as bromides. It was necessary to make fresh supplies of the acetyl esters which were all prepared as iodides and their melting-points are included in Table 1 for comparison with the amides, which are also iodides.

Test preparations

The guinea-pig isolated ileum was set up at 37°C in oxygenated Tyrode solution containing hexamethonium (2.76×10^{-4} M) as described by Edinburgh Staff (1970). Responses were recorded isotonically and the load was about 0.5 gram. The activity of the agonists was expressed as the equipotent molar ratio relative to acetylcholine, estimated in 2 + 2 dose assays of Latin square design. The doses were added manually once every 2 min and allowed to act for 25 seconds. The affinity of the partial agonist, (acetamidoethyl)-*N*-methylpyrrolidinium, was estimated by the reciprocal plot method (Barlow, Scott & Stephenson, 1967) with responses to at least three doses of the partial agonist and control responses obtained with acetylcholine.

The affinities of the antagonists were measured as described previously (Abramson *et al.*, 1969; Barlow, Franks & Pearson, 1973). Carbachol was the agonist, allowed to act for 30 s and given once every 90 seconds. Experiments were also made at 29°C with some of the compounds to see if there were detectable differences in the effects of temperature on the affinity of different compounds, as had been found by Barlow, Berry, Glenton, Nikolaou & Soh (1976).

The frog rectus preparation was set up at room temperature (17–20°C) in aerated frog-Ringer solution as described by Edinburgh Staff (1970). Responses were recorded isotonically and the load was about 1 gram. Doses were made up in a 10 ml measure and poured onto the preparation. The activities of agonists were expressed as equipotent molar ratios relative to acetylcholine or carbachol but the results are complicated by the presence of cholinesterases in the tissue. For most of the esters (IV) the comparison was made with acetylcholine as standard and doses were allowed to act for 2 min and given once every 8 minutes. For the triethylammonium and ethylpiperidinium compounds, and for those of the amides which were agonists, it was necessary to allow doses to act for 5 min and to give them once every 16 minutes. Some comparisons of amides were made with acetylcholine as standard but most were made with carbachol, which was used as the agonist in all experiments for measuring the affinity of partial agonists and antagonists. In the assays of agonist activity, duplicate responses were obtained with at least two doses of each compound with several compounds tested on each preparation. The affinities of the triethylammonium and ethylpiperidinium esters, which were partial agonists, were estimated by the addition method (Stephenson, 1956). The affinities of the antagonists were calculated from the dose-ratio produced, with the antagonists given 2 min before the agonist, but because the compounds were so weak they were only tested in a single concentration (5 mM).

Electric eel acetylcholinesterase was bought from Sigma and all samples had the same batch number (125C-8000). Stock solutions were made in 0.9% w/v NaCl solution (saline) and stored in the refrigerator (4°C). All experiments with the enzyme were done at 25°.

When the reaction was followed by electrometric titration, the stock solution of enzyme was diluted with a mixture of 0.14 M NaCl and 0.04 M $MgSO_4$. Belleau, Tani & Lie (1965) used a mixture containing 0.1 M NaCl and 0.04 M $MgCl_2$. The pH was recorded with a Metrohm E500 meter and combined electrode EA 121. It was kept at 7.35 by the addition of NaOH (0.1 M or 0.02 M; free from carbonate) from an E412 Dosimat with a 1 ml burette, reading in µl, activated by an E473 impulsomat. The solution was stirred magnetically, protected with a soda-lime tube, and the titration was carried out under nitrogen. The total volume was initially 20 ml. The velocity of the reaction, V, was calculated from the values of time and alkali added, and was corrected for the small amount of what appeared to be spontaneous hydrolysis. The substrate concentrations, S, were corrected for the increase in volume and loss due to reaction during the period in which the rate was measured. The substrate was tested, at least in duplicate, in four concentrations, in the range 0.1 to 0.5 mM and values of K_m and V_{max} were obtained by fitting the values of V and S to the expression $V = V_{max}S/(K_m + S)$ by the method of least squares.

In experiments with the spectrophotometric method (Ellman, Courtney, Andres & Featherstone, 1961) the reaction was performed in 0.1 M phosphate buffer, pH 8.1. The total volume was 3.2 ml and the concentrations of acetylthiocholine were 0.125, 0.25, 0.375 and 0.5 mM, each tested twice in any one experiment, and the values of K_m and V_{max} were calculated as above. In these experiments the very small amount of spontaneous hydrolysis was neglected.

In experiments with inhibitors the concentrations of substrate were increased and for each concentration of inhibitor the values of rate and substrate concentration were fitted to the hyperbola and apparent values of K_m and V_{max} were obtained. If the antagonism is competitive the 'apparent' K_m in the presence of a concentration I of inhibitor = $K_m(1 + I/K_i)$ and V_{max} should be unaltered. The ratio of the estimates of K_m in the presence and in the absence of inhibitor is, in fact, the dose-ratio and has been used to calculate K_i or the affinity constant $(1/K_i)$.

Apparent molal volumes

These were calculated from the densities of solutions of known composition by weight, measured with an Anton Paar Precision Density Meter, DMA 02D, as described previously (Lowe, MacGilp & Pritchard, 1973; Barlow & Franks, 1973). The temperature was 25.00 ± 0.01°C.

Values of R_M

Values of R_M (Bate-Smith & Westall, 1950) were calculated from chromatography of the compounds on Whatman No 1 paper with the solvent system butanol-ethanol-water (5:5:2 by volume). The spots were developed with the Dragendorff reagent (Thies & Reuther, 1954). Between 9 and 11 samples were run on each sheet, with any one compound tested at least in duplicate.

Results

The results of the experiments with the phenylacetyl and diphenylacetyl amides on the guinea-pig isolated ileum are shown in Table 2. With the derivatives of diphenylacetic acid the replacement of ester by amide reduced log K by from 1.6 to 2.0 units; with the derivatives of phenylacetic acid the difference was only from 0.2 to 0.7 log units. The composition of the onium group, however, appeared to produce similar effects on the affinity of the amides as it did on that of the corresponding esters, in spite of the big difference in affinity (Figure 1). This suggests that the onium part of the molecule is binding to the receptor in a similar way in esters and the corresponding amides and that the difference in affinity is due to the failure of the phenyl groups to contribute as much to the binding of the amides as they do to that of the esters. This effect, which could well be due to the greater stiffness of the amide bond, will be bigger in the compounds with two phenyl groups than in those with one.

With 8 of the compounds tested the value of log K at 29°C was higher than that at 37°C but with 3 it was lower, though the differences were all marginal. There is the suggestion, however, of a trend towards a reversal of the temperature effect with larger onium groups, because the compounds with apparently higher affinity at 37°C were those with

Table 2 Affinity for postganglionic acetylcholine receptors of the guinea-pig ileum

Onium	$\Delta\phi_V^0$	$PhCH_2CO$–			Ph_2CHCO–		
		Ester	Amide		Ester	Amide	
	cm^3/mol	37°	37°	29°	37°	37°	29°
$\overset{+}{N}Me_3$	0	4.533	4.290 ±0.028 (6)	4.342 ±0.039 (5)	7.159	5.492 ±0.040 (6)	5.610 ±0.023 (5)
$\overset{+}{N}Me_2Et$	15	5.093			7.578	5.709 ±0.021 (6)	5.741 ±0.017 (5)
Methyl-pyrrolidinium	19	5.084	4.741 ±0.015 (8)		7.440	5.569 ±0.038 (6)	5.659 ±0.023 (6)
$\overset{+}{N}MeEt_2$	30	5.379	4.977 ±0.028 (6)	5.093 ±0.023 (5)	7.584	5.577 ±0.054 (6)	5.631 ±0.014 (5)
Methyl-piperidinium	33.4	5.194	4.892 ±0.026 (7)		7.260	5.327 ±0.041 (6)	5.271 ±0.052 (5)
Ethyl-pyrrolidinium	34.4	5.568			7.558	5.547 ±0.038 (5)	5.605 ±0.023 (5)
$\overset{+}{N}Et_3$	44.5	5.785	5.113 ±0.017 (5)	5.189 ±0.018 (5)	7.367	5.421 ±0.045 (6)	5.321 ±0.052 (6)
Ethyl-piperidinium	48	5.525	4.831 ±0.004 (8)		7.015	5.199 ±0.042 (5)	5.167 ±0.022 (5)

Mean values of log K are shown with the standard error and number of results. Values for esters, included for comparison, are from Abramson *et al.* (1969). The onium groups are arranged in increasing size, estimated by $\Delta\phi_V^0$ the increment in apparent molal volume at infinite dilution calculated from results of Barlow *et al.* (1971).

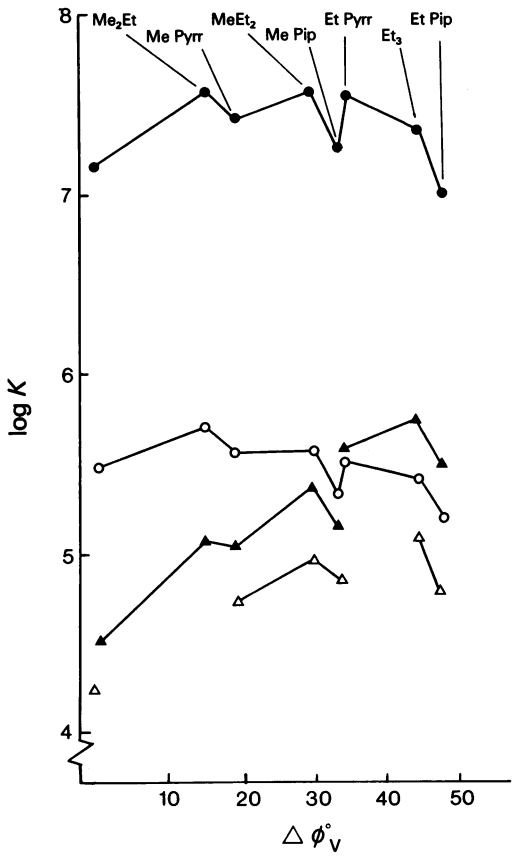


Figure 1 Effect of the composition of the onium group on the affinity of esters (●) and amides (○) of diphenylacetic acid and of esters (▲) and amides (△) of phenylacetic acid. Values of log K for the postganglionic muscarine-sensitive receptors of the guinea-pig ileum at 37°C are plotted against the increment in the size of the onium group, assessed from the increase in the apparent molal volume at infinite dilution calculated from the results of Barlow *et al.* (1971). Note that the lines joining the esters and amides of diphenylacetic acid are parallel, in spite of the big difference in log K. The line joining the esters of phenylacetic acid has been broken to avoid confusion, and the results with the 6 analogous amides appear also to be parallel to it, though there are smaller differences in affinity than with the derivatives of diphenylacetic acid. The errors attached to the estimates are less than 0.1 log units, i.e. about twice the size of the symbols.

the triethylammonium, methylpiperidinium and ethylpiperidinium groups.

The results obtained with the acetyl amides (III) on the guinea-pig ileum are shown in Table 3, together with values of the acetyl esters (IV). The methylpyrrolidinium ester has also been studied by Cho, Jenden & Lamb (1972) and by Schwarzenfeld & Whittaker (1977), who obtained equipotent molar ratios of 2.6 and 3.6 respectively. There is great variation in the results obtained with the weaker compounds, particularly when the equipotent molar ratio is greater than 1000:1, but there was no convincing evidence that any of the esters was a partial agonist. In contrast only two of the acetyl amides were agonists, one was a partial agonist and the rest were antagonists. There was no detectable difference between the affinity at 37°C and that at 29°C in the three instances studied. The activity of the amide analogue of acetylcholine on this preparation appears to be similar to that on rat intestine (Van Rossum, 1968).

The results obtained with the frog rectus are shown in Table 4. Schwarzenfeld & Whittaker (1977) obtained an equipotent molar ratio of 15 for the methylpyrrolidinium ester. The acetyl esters with triethylammonium and ethylpiperidinium groups were partial agonists when compared with carbachol but this was not apparent when they were compared with acetylcholine because the dose-response curve for acetylcholine is greatly flattened by the effects of the cholinesterase present in the tissue. It was possible to obtain an equipotent molar ratio for the triethylammonium compound relative to acetylcholine (1280 ± 240, 4 estimates, comparable with 5000 reported by Holton & Ing, 1949) but this has little meaning. Three of the amides were agonists and the rest were all tested as antagonists, though the methylpiperidinium compound usually produced a detectable but very small contracture of the preparation. The activity of the amide analogue relative to acetylcholine was much greater on this preparation than on the guinea-pig ileum. This was only partly because the acetylcholine is hydrolysed by cholinesterases in the tissue. The equipotent molar ratio relative to carbachol is not very different from that relative to acetylcholine.

The results obtained with the acetyl esters and acetylcholinesterase are shown in Table 5. There is a big variation in the estimates of K_m which is probably due to the difficulty of making experiments in a suitable range of substrate concentrations. According to Wilson (1971) the K_m for acetylcholine and this enzyme is 0.092 mM, and even the most dilute of the substrate concentrations tested (0.1 or 0.125 mM) is greater than this. With 20 ml of 0.1 mM solution there are only 2 μmol of substrate present and the reaction can only be followed for a very short time, unless the amount of enzyme present is very small. It is therefore likely that the rate with the more dilute solutions will be under-estimated and this will lead to an overestimate of the value of K_m. Although the value of K_m with the smaller amounts of enzyme, 0.13 mM, is close to that obtained by Wilson (1971), the value with the larger amounts, 0.20 mM is much

Table 3 Activity of acetyl esters and amides on the guinea-pig isolated ileum

	Ester 37°C			*Amide*	
	A	*B*	*H*	*37°C*	*29°C*
$\overset{+}{N}Me_3$ 1	1	1	1	*1450* ±396 (6)	
$\overset{+}{N}Me_2Et$		*2.84*	*2.5*	*7010* ±1050 (8)	
Methylpyrrolidinium					
3.94 ±0.26 (3)	*8.5*			3.33* ±0.03 (5)	
$\overset{+}{N}MeEt_2$		*380*	*700*	2.95 ±0.03 (3)	3.05 ±0.06 (3)
Methylpiperidinium					
3520 ±1660 (3)	*3410*			3.10 ±0.04 (3)	3.11 ±0.06 (3)
Ethylpyrrolidinium					
171 ±14 (3)	*217*			2.90 ±0.06 (4)	
$\overset{+}{N}Et_3$ *4025* ±388 (3)			*1700*	3.03 ±0.02 (4)	2.99 ±0.06 (3)
Ethylpiperidinium					
5620 ±1860 (3)	*15200*			2.92 ±0.04 (3)	

Numbers in italics show the equipotent molar ratios for agonists relative to acetylcholine; all other values are mean estimates of log affinity constant. The asterisk indicates that this compound was a partial agonist and log K was obtained by the method of Barlow, Scott & Stephenson (1967). Mean values are shown with the standard error and number of results. The column headed A indicates results of Abramson (1964), B indicates results of Barlow, Scott & Stephenson (1963). H indicates results of Holton & Ing (1949). The onium groups are arranged in increasing order of size as in Table 2.

Table 4 Activity of acetyl esters and amides on the frog rectus *(Rana temporaria)*

	Ester		*Amide*	
		H	*vs* ACh	*vs* carbachol
$\overset{+}{N}Me_3$	1	1	*51.4* ±9.5 (5)	*35.7* ±1.3 (5)
$\overset{+}{N}Me_2Et$	*5.12* ±0.21 (4)	*5*	*500* ±67 (5)	*483* ±46 (4)
Methylpyrrolidinium	*8.37* ±0.40 (4)		*252* ±45 (5)	*180* ±3.3 (4)
$\overset{+}{N}MeEt_2$	*142* ±1.7 (4)	*300*		2.624 ±0.021 (4)
Methylpiperidinium	*77.5* ±5.1 (4)			2.587 ±0.079 (6)
Ethylpyrrolidinium	*125* ±3.7 (5)			3.084 ±0.011 (4)
$\overset{+}{N}Et_3$	3.297* ±0.059 (4)	*5000*		2.736 ±0.021 (4)
Ethylpiperidinium	3.409* ±0.038 (4)			3.084 ±0.023 (4)

Numbers in italics show the equipotent molar ratios for agonists relative to acetylcholine or carbachol; all other values are mean estimates of log affinity constant. The asterisk indicates a partial agonist with log *K* estimated by the addition method of Stephenson (1956). Mean values are shown with the standard error and number of results. The column headed H indicates results of Holton & Ing (1949).

bigger and is probably less reliable. Because of the uncertainty attached to absolute values of K_m, the value for the compounds relative to acetylcholine was calculated in each set of experiments and means of these values are included in Table 5. The estimates of V_{max}, however, will not be affected by the difficulty of measuring the rates with low substrate concentrations and because the highest concentrations are much less than those associated with inhibition by excess substrate (around 10 mM), the estimates of relative V_{max} should be very reproducible.

Increasing the size of the onium group decreases V_{max} and apparently decreases K_m, although appreciable changes are only seen with the ethylpyrrolidinium, triethylammonium and ethylpiperidinium compounds. These findings are consistent with those obtained by Holton & Ing (1949) who observed a decrease in the rate of hydrolysis when methyl groups in acetylcholine were replaced by ethyl groups, though the effect was not large.

When Ellman's method is used the substrate is acetylthiocholine. This has a smaller K_m and higher V_{max} than acetylcholine and by electrometric titration the mean relative K_m was found to be 0.8 and the mean relative V_{max} was 1.2. It is possible to work with much smaller amounts of enzyme, even with the disadvantage of the smaller volume of solution (3.2 ml). The mean estimate of K_m for acetylthiocholine with this method was 0.099 mM.

Ellman's method cannot be used directly for the esters but they can be tested indirectly by observing their effects on the rate of hydrolysis of acetylthiocholine. Values of K_i obtained in this way for the triethylammonium ester were 0.165 and 0.166 mM and for the ethylpiperidinium ester they were 0.089 and 0.105 mM (each was tested at 0.25 and 0.5 mM). These confirm the impression, obtained from the results of the electrometric titrations, that increasing the size of the onium group increases affinity but the effects are not large.

The results obtained with the amides are shown in Table 6. There is reasonable agreement between the values obtained by electrometric titration and those obtained by Ellman's method even though the pH with the former was 7.35, compared with 8.1 with the latter. The effects of changing the onium group on the affinity of the amides are strikingly different from the effects on the affinity of the esters. Although the amide analogue of acetylcholine has less than one-tenth of its affinity, the amides with piperidinium groups have affinity very similar to that of the corresponding esters.

Although the ether oxygen atom (16) is slightly heavier than —NH— (15) the apparent molal volume

Table 5 Hydrolysis of esters, $CH_3COOCH_2CH_2NR_3$ by electric eel acetylcholinesterase

		Values relative to acetylcholine	
	K_m (*mM*)	K_m	V_{max}
$\overset{+}{N}Me_3$	0.20 ±0.02 (13)	1	1
$\overset{+}{N}Me_2Et$	0.23 ±0.01 (4)	1.01 ±0.07	0.88 ±0.03
Methylpyrrolidinium*	0.12 ±0.01 (4)	0.85 (1.20) ±0.04	0.81 (0.86) ±0.02
$\overset{+}{N}MeEt_2$	0.22 ±0.02 (4)	0.97 ±0.11	0.70 ±0.02
Methylpiperidinium	0.24 ±0.03 (4)	1.09 (1.09) ±0.14	0.65 (0.69) ±0.03
Ethylpyrrolidinium	0.15 ±0.01 (4)	0.69 (1.21) ±0.08	0.63 (0.80) ±0.03
$\overset{+}{N}Et_3$	0.14 ±0.03 (4)	0.62 ±0.12	0.33 ±0.02
Ethylpiperidinium	0.17 ±0.02 (4)	0.80 (0.33) ±0.15	0.42 (0.41) ±0.03

Results obtained by electrometric titration with 0.1 M NaOH were used to calculate K_m and V_{max} and their values relative to those for acetylcholine. Mean estimates are shown, ± the standard error, with the number of results in parentheses. The asterisk indicates that the methylpyrrolidinium compound was tested in a separate group of experiments for which the mean K_m obtained with acetylcholine was 0.14 mM. The values in parentheses are the averages of duplicates obtained with 0.02 M NaOH and much smaller amounts of enzyme for which the mean K_m for acetylcholine was 0.13 mM.

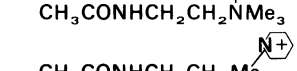
at infinite dilution for the amide analogue of acetylcholine was found to be slightly bigger, 177.5 ± 0.2 cm^3/mol compared with 175.7 ± 0.1 cm^3/mol for acetylcholine. The values for the triethylammonium compounds were 222.1 ± 0.2 cm^3/mol for the amide compared with 220.6 ± 0.6 cm^3/mol for the ester. All the compounds were iodides and the temperature was 25°C. The difference, though small, indicates that the ester and amide groups do not interact with water in the same way. This is to be expected because oxygen is more electronegative and can only act as a hydrogen acceptor, whereas the —NH— group is potentially a hydrogen donor. Such differences in interactions with water might affect the partitioning of the compounds and be apparent in the experiments with paper chromatography. The R_M value is directly proportional to the logarithm of the partition coefficient and the results (Table 7) indicate, however, that

Table 6 Affinity for acetylcholinesterase (electric eel)

	Amides		*Esters*
	Ellman's method	*Electrometric titration*	
$\overset{+}{N}Me_3$	2.808 ±0.073 (2)	2.888 ±0.119 (4)	4.0
$\overset{+}{N}Me_2Et$	3.023 ±0.113 (2)	2.967 ±0.066 (8)	4.0
Methylpyrrolidinium	3.004 ±0.014 (2)	2.833 ±0.104 (8)	4.06
$\overset{+}{N}MeEt_2$	3.393 ±0.032 (2)		4.01
Methylpiperidinium	3.786 ±0.008 (2)	3.642 ±0.056 (4)	3.97
Ethylpyrrolidinium	3.727 ±0.018 (2)		4.16
$\overset{+}{N}Et_3$	3.232 ±0.045 (5)	3.077 ±0.088 (8)	4.21
Ethylpiperidinium	4.541 ±0.038 (5)		4.10

Mean estimates of log affinity constant ($1/K_i$) for the acetylamides are shown with the standard error and number of results. Values for the esters have been calculated from the mean estimate of the K_m relative to acetylcholine (taken from Table 5) and assuming that log K for acetylcholine is 4.0, corresponding to K_m = 0.1 mM.

Table 7 Effects of groups on paper chromatography

	$\triangle R_M$
$CH_3CH_2CH_2CH_2CH_2NMe_3$	−0.36 (±0.01; 7)
$CH_3CH_2OCH_2CH_2\overset{+}{N}Me_3$	−0.23 (±0.02; 4)
$CH_3COCH_2CH_2CH_2\overset{+}{N}Me_3$	−0.04 (±0.02; 5)
$HOCH_2CH_2CH_2CH_2CH_2\overset{+}{N}Me_3$	−0.03 (±0.01; 4)
$CH_3COOCH_2CH_2\overset{+}{N}Me_3$	0
$CH_3CONHCH_2CH_2\overset{+}{N}Me_3$	0.05 (±0.01; 8)
	−0.23 (±0.02; 2)

Values are the means (±s.e. and number of estimates) of the difference in R_M between the compound and acetylcholine, run on the same paper, with the solvent system butanol:ethanol:water (5:5:2). A negative sign indicates that the compound runs faster than acetylcholine i.e. is more lipophilic. All compounds were iodides.

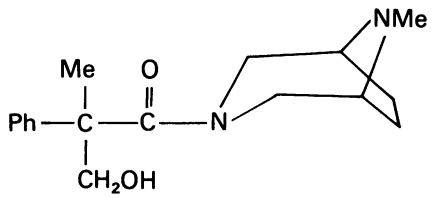
in conditions where the effects of methylene, ether and carbonyl groups are clearly seen, the replacement of ester by amide produces only a slight increase in hydrophilic character. The melting points of the compounds shown in Table 1 also suggest that the amides are not very different from the esters. Except with the trimethylammonium compounds the amides usually have slightly higher melting points than the corresponding esters.

The proton magnetic resonance spectrum (270 MHz) of the amide analogue of acetylcholine in D_2O resembles that of chlorocholine. The ratio of *trans* to *gauche* conformers around the —NH—C—C—$\overset{+}{N}$— bond was calculated from coupling constants as described by Partington, Feeney & Burgen (1972) who estimated the proportion of *trans* in chlorocholine to be 36%. For the amide analogue of acetylcholine the proportion of *trans* was calculated to be 39% (±6%; G.C.K. Roberts, personal communication). This is therefore slightly more stable than the *gauche* form; if they were equally stable the ratio of *trans* to *gauche* would be 1:2 (33%). In acetylcholine the *gauche* form predominates and the proportion of *trans* conformer is very small. If the stability of the *gauche* conformer depends primarily on electrostatic interaction between the onium group and the ether oxygen atom in the ester group, the replacement of —O— by the less electronegative —NH— group would be expected to decrease the proportion of the *gauche* conformer much as has been found.

The ^{13}C n.m.r. spectrum of the amide in D_2O shows a single signal for the NH—CH_2—CH_2 resonance, indicating the existence of only one form, presumably with the *trans* arrangement about the —CO—NH— bond. The amount of *cis* form present must be less than 5% (the limit of detection) and the difference in stability should be greater than 1.7 kcal/mole.

Discussion

The results obtained with the phenylacetyl and diphenylacetyl compounds on the guinea-pig ileum (Table 2; Figure 1) indicate that the replacement of ester by amide can reduce up to 100-fold the affinity of compounds in which a large part of the binding involves groups at the far end of the molecule from the onium atom. This is consistent with the suggestion (Barlow, 1973) that the greater stiffness of the amide bond causes the enantiomeric forms of the amide (V) to have a higher stereospecific index than the enantiomeric forms of hyoscyamine (1140 compared with 330) even though they have much lower affinity and break 'Pfeiffer's rule' (Pfeiffer, 1956).



V

However, the effects of replacing ester by amide on affinity are different in different series and in acetyl compounds the effects on affinity at the muscarine-sensitive acetylcholine receptor are difficult to assess because all the esters are agonists. Burgen, Hiley & Young (1974) obtained a value of log $K = 5.04$ for acetylcholine and receptors in the guinea-pig ileum and Van Rossum (1968) obtained a value of 4.8 for rat intestine. Most of the amides are partial agonists or antagonists with log K around 3 (Table 3) so it appears that affinity has been reduced up to about 100-fold. The change lowers the activity of acetylcholine and its ethyldimethylammonium analogue 1000-fold so it apparently reduces efficacy about 10-fold. This is consistent with the finding that most of the amides are antagonists, whereas the esters are agonists.

On the frog rectus it is possible to compare directly the affinities of the esters and amides with triethylammonium and ethylpiperidinium groups and the affinity is only lowered about 4-fold. There is again a reduction of efficacy, with five of the amides becoming antagonists. The results suggest that the effect on efficacy is very similar to that on the ileum. If this is reduced 10-fold and there is a 4-fold reduction in affinity, the equipotent molar ratio for the trimethylammonium compound should be 40 (Table 4).

With acetylcholinesterase the amides are not substrates so the effects of this structural change on 'efficacy' are profound. If the reciprocal of K_m can be used as a measure of the affinity of the esters, as has been assumed, the effects of the composition of the onium group on affinity do not appear to be large. There is only a small increase with triethylammonium and ethylpiperidinium and a moderate decrease in relative V_{max}. There are bigger changes in the affinity of the amides, related to shape as well as size. The increase is bigger, for instance, with the lop-sided methylpiperidinium and ethylpiperidinium groups, compared with the more spherical triethylammonium group (Figure 2). The difference between the structure-affinity relationships of the esters and amides suggests that the enzyme might be allosteric, with substrates and inhibitors binding to different sites. The inhibition is reversible but not strictly competitive; the inhibitors depress V_{max} but the Hill coeffi-

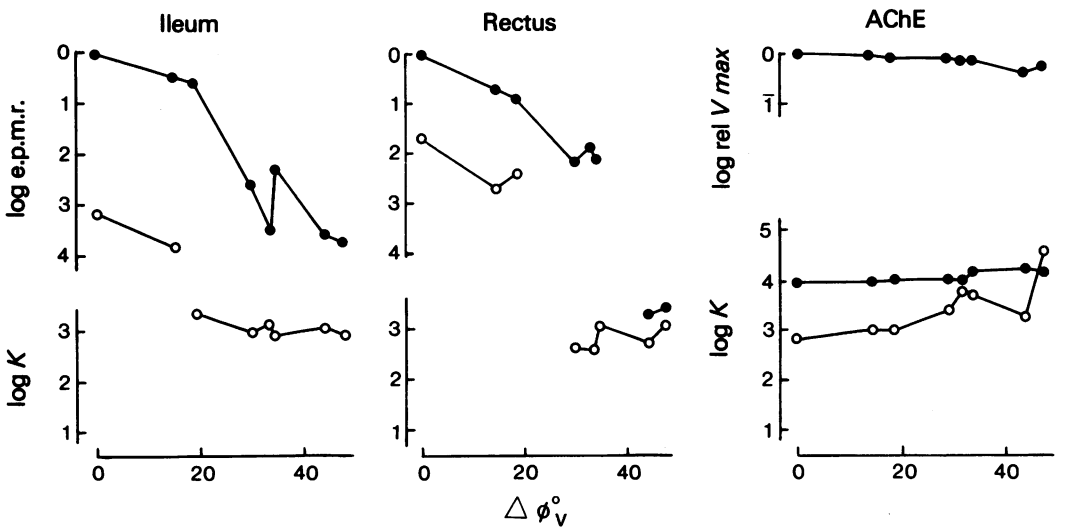


Figure 2 Effects of the composition of the onium group on the biological properties of acetic esters (●) and amides (○). Values of log affinity constant are shown for the partial agonists or antagonists and values of log equipotent molar ratios (e.p.m.r.) relative to acetylcholine are plotted against the increment in the size of the onium group, expressed as in Figure 1. Note the break in the scales of the ordinate. For acetylcholinesterase (AChE) the log of V_{max} relative to acetylcholine replaces the equipotent molar ratio. The errors in log K are less than 0.1 log units, about twice the size of the symbols; errors in log equipotent molar ratio are about the same for the more active compounds but much larger for the weaker ones (log e.p.m.r. > 3, see Tables 3 and 4). For the ileum the temperature was 37°C, for the rectus it was between 17 and 20°C, and for acetylcholinesterase it was 25°C.

cients are all close to unity (Figure 3). It is not clear, therefore, if the amides fail to act as substrates simply because their greater Π electron delocalization makes them less sensitive to attack by protons or if they become bound exclusively to a regulatory site, instead of to the active centre. The former might, in fact, contribute to the latter.

If the receptors in the ileum and rectus also behave allosterically and efficacy can be regarded as selectivity (Colquhoun, 1973), the replacement of ester by amide produces a shift towards the inactive form in both systems, though this is not so extreme as is seen with acetylcholinesterase. Although allosteric behaviour by acetylcholine receptors is still arguable, it would explain very easily one of the most striking features of the results, viz. why changes in the structure of the onium group which produce modest changes in affinity (up to 10-fold, Figure 2) can alter the equipotent molar ratio by 3 orders of magnitude.

In the crystal the ester group of acetylcholine is planar (Baker, Chothia, Pauling & Petcher, 1971), probably because of its partial double-bond character. In the amide the group should also be planar and the energy required for interconversion of the two forms (*cis* and *trans*) should be bigger than with the ester group. This is not known exactly but the ^{13}C n.m.r. spectra, indicating the presence of only one conformer, suggest that the energy required is large. The main effect on conformation of replacing ester by amide in acetylcholine appears therefore to be the effect on the arrangement about the —C—C— bond, seen in the p.m.r. spectra. Is this reduction in the proportion of *gauche* conformer from 100% in acetylcholine to 39% in the amide sufficient to account for the 1000-fold drop in activity on the guinea-pig ileum? This seems unlikely. Although many muscarine-like compounds exist predominantly as the *gauche* conformers, the potent antagonist, benziloylcholine is also predominantly in this form and Partington, Feeney & Burgen, (1972) concluded 'there is no simple correlation between predominant conformations and the potency of action of the drugs at either the nicotinic or muscarinic receptor'. If, however, as they have considered, the form active at the receptor is in some intermediate conformation, it is possible that the thermodynamic price which must be paid to achieve this is much greater in the amides than in the esters because they are stiffer. This might account for the 10-fold drop in efficacy at both types of receptor. Unfortunately it has not been possible to assess the relative stiffness of ester and amide. Another possible reason for the lower activity of the amides is the difference in the arrangement of water molecules around the bond, suggested from the values of ϕ^{0}_{v}. At present, however, the extent to which water

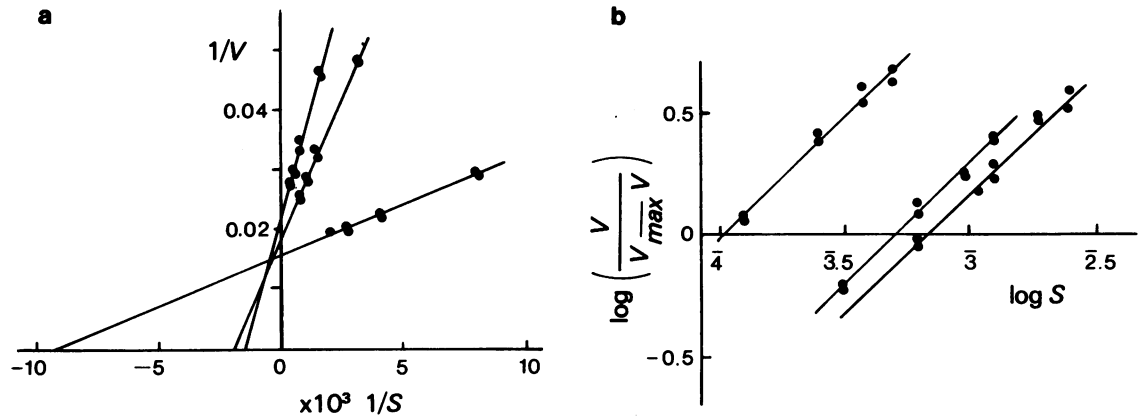


Figure 3(a) Inhibition of electric eel acetylcholinesterase by the amide analogue of acetylcholine. Values of $1/V$ (in units based on the change in absorbance with time) are plotted against $1/S$ (in litres/mol) for 4 concentrations of substrate in the absence of the inhibitor and in the presence of concentrations of 5 mM and 10 mM amide. Each concentration was tested in duplicate and the lines drawn have been fitted by least squares to each set of eight points. In some instances the points coincide and only one is visible. Note that the inhibition is reversible but the intercept on the ordinate increases with increasing concentration of inhibitor, i.e. V_{max} is reduced, which is not consistent with true competition. Temperature, 25°C. (b) The same results presented as Hill plots. The lines were fitted by the method of least squares and the slopes were 0.99, 1.00, and 1.00 for the controls and the reactions in the presence of 5 mM and 10 mM amide, respectively. The estimates of K_i, calculated from the intercepts on the abscissa scale, were 1.33 and 1.86 mM, compared with 1.31 and 1.84 obtained by the reciprocal plots shown in (a).

molecules are involved in drug-receptor interactions is not clear.

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