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The cholinergic properties of some amino-acid esters and amides

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1. Several amino-acid esters and amides have been prepared and their toxicological and pharmacological properties have been investigated. Some of the quaternary esters and amides were lethal to mice at doses below 1 mg/kg; this lethality was usually associated with high nicotinic activity. None of the compounds showed high muscarinic activity.

2. The cause of death was asphyxia due to paralysis of the respiratory muscles, but at higher doses there was also failure of the medullary respiratory centre.

3. Administration of sub-lethal doses of nicotine to mice was shown to protect the animals from subsequent lethal doses of nicotine. The amino-acid esters did not share this property, nor was there any cross-protection between nicotine and these esters.

4. The possible nature of the nicotinic receptor is discussed with reference to these compounds and other known nicotinic agents. A tentative suggestion is made that nicotinic activity can result from a two-point interaction between the drug and the receptor.

As a result of work on the interaction of potent agonists with the post-ganglionic parasympathetic acetylcholine receptor (the muscarinic receptor) Beckett, Harper & Clitherow (1963) concluded that at least three sites on the receptor are involved. The two major sites, termed 1 and 2, were considered to interact with the quaternary nitrogen atom and the ester oxygen atom of acetylcholine respectively; the carbonyl oxygen atom of acetylcholine was considered to interact with a subsidiary site 3. More recently Bebbington & Brimblecombe (1965), using oxotremorine and related compounds, demonstrated that the interaction at site 3 has greater significance than was attached to it by Beckett *et al.* (1963); in particular the degree of interaction at this site determines to a large extent the activity of non-quaternary muscarinic agents.

In order to provide additional information on the relative spatial arrangements of sites 1, 2 and 3 an examination has been made of other compounds which bear structural similarities to acetylcholine. One such group of compounds includes the amino-acid esters (I) and amides (II) which have some groups in common with both acetylcholine and oxotremorine.

$$R_3\overset{+}{N}(CH_2)_nCO_2R' \,.\, X^- \qquad\qquad R_3\overset{+}{N}(CH_2)_nCONR'_2 \,.\, X^-$$

I II

The relative spatial arrangements of the different groups in these compounds can readily be varied and the effects of such variations on the pharmacological activity of these compounds can be examined.

The present paper describes the synthesis of these compounds (I and II) and gives an account of their pharmacological activity.

Methods

Chemical syntheses

Esters

The aminoacetates and aminobutyrates were prepared by reacting the chloroacetates and chlorobutyrates with the appropriate amines. With the chloroacetates the reaction was carried out at room temperature in benzene solution, but the chlorobutyrates required more vigorous conditions, namely sealed tube at 100° C for 4 hr (dimethylamino compounds) or refluxing benzene for 6 hr (pyrrolidino compounds). In all cases the products were purified by fractionation *in vacuo* after removal of amine hydrochloride by filtration. These compounds are listed in Table 1 and the corresponding quaternary salts, prepared from these tertiary amines and methyl iodide in acetone, are listed in Table 2.

The aminopropionates were prepared by the addition of secondary amines across the double bond of alkyl acrylates in benzene solution. The products were isolated

TABLE 1. *List of the amino-acid esters (general formula* $R'(CH_2)_nCO_2R$*) synthesized and tested pharmacologically*

No.	*R*	*R'*	*n*	b.p.	n_D^{25}	References
1	CH_3	$(CH_3)_2N$	1	38°/13 mm	1·4105	Terakawa (1954)
2	C_2H_5	$(CH_3)_2N$	1	55°/20 mm	1·4122	Viscontini & Meier (1950)
3	CH_3		1	76°/43 mm	1·4481	Matkovics, Foldeak & Porszasz (1960)
4	C_2H_5		1	77°/16 mm	1·4452	Matkovics, Foldeak & Porszasz (1960)
5	CH_3	$(CH_3)_2N$	2	60°/30 mm	1·4171	Nazarov & Kruglikova (1957)
6	C_2H_5	$(CH_3)_2N$	2	62°/15 mm	1·4167	Adamson (1949)
7	CH_3		2	101°/22 mm	1·4174	Matkovics, Foldeak, Porszasz & Sipos (1961)
8	C_2H_5		2	82°/8 mm	1·4470	Adamson (1949)
9	CH_3	$(CH_3)_2N$	3	59°/9 mm	1·4232	Willstätter (1902)
10	C_2H_5	$(CH_3)_2N$	3	56°/5 mm	1·4215	Adamson (1949)
11	CH_3		3	45–47°/15 mm		*
12	C_2H_5		4	67°/2 mm	1·4490	†

* Found: C, 63·2; H, 10·2; N, 8·0%. $C_9H_{17}NO_2$ requires C, 63·1; H, 10·0; N, 8·2%.
† Found: C, 65·4; H, 10·2; N, 7·9%. $C_{10}H_{19}NO_2$ requires C, 64·8; H, 10·3; N, 7·6%.

and purified by distillation *in vacuo*; the quaternary salts were obtained from the tertiary amines and methyl iodide in acetone solution. The compounds prepared are listed in Table 1 (tertiary bases) and Table 2 (quaternary salts).

TABLE 2. *List of the quaternary amino-acid esters (general formula* $\overset{+}{R}'(CH_2)_nCO_2R \,.\, I^-$*) synthesized and tested pharmacologically*

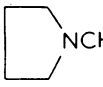
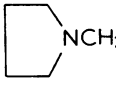
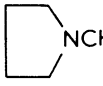
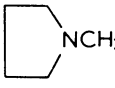
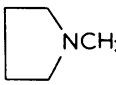
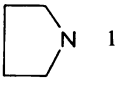
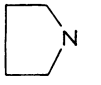
No.	*R*	$\overset{+}{R}$	*n*	m.p.	Recrystallization solvent	Analysis: Calculated C	Calculated H	Calculated N	Found C	Found H	Found N
13	CH_3	$(CH_3)_3N$	1	152–154°	Ethanol	27·8	5·5	5·4	27·9	5·7	5·1
14	C_2H_5	$(CH_3)_3N$	1	176–177°	Ethanol	30·8	6·0	5·1	30·6	5·9	5·3
15	CH_3		1	72–73°	Acetone	33·7	5·7	4·9	34·1	5·8	4·8
16	C_2H_5		1	45–47°	Acetone	36·1	6·1	4·7	36·4	6·2	4·7
17	CH_3	$(CH_3)_3N$	2	208–209°	Ethanol	30·8	5·9	5·1	31·3	6·1	5·4
18	C_2H_5	$(CH_3)_3N$	2	188°	Ethanol	33·5	6·3	4·9	33·9	6·5	4·8
19	CH_3		2	167°	Acetone	36·1	6·1	4·7	36·0	6·2	4·7
20	C_2H_5		2	86–89°	Acetone	38·4	6·4	4·5	38·2	6·5	4·4
21	C_2H_5	$(CH_3)_2NC_2H_5$	2	101–102°	Acetone/Ethyl acetate	35·9	6·7	4·7	35·7	7·0	4·9
22	C_2H_5	$(C_2H_5)_2NCH_3$	2	83–84°	Acetone/Ethyl acetate	38·1	7·0	4·5	38·3	7·2	5·0
23	n-C_4H_9	$(CH_3)_3N$	2	144–145°	Acetone	38·1	7·0	4·5	38·3	7·1	4·4
24	CH_3	$(CH_3)_3N$	3	130–131°	Ethanol/Acetone	33·5	6·3	4·9	33·6	6·6	5·0
25	C_2H_5	$(CH_3)_3N$	3	156–158°	Ethanol/Acetone	35·9	6·7	4·6	35·8	6·7	4·5
26	C_2H_5		3	132–133°	Ethanol/Acetone	40·2	7·0	4·3	40·4	6·8	4·3

TABLE 3. *List of the amino-acid amides (general formula* $R'(CH_2)_nCONR_2$*) synthesized and tested pharmacologically*

No.	*R*	*R'*	*n*	Method and solvent	b.p.	n_D^{25}	References
27	CH_3		1	A. Benzene/potassium carbonate	66°/0·2 mm	1·4795	
*	CH_3	$(CH_3)_2N$	1	A. Ether	93°/20 mm	1·4505	Marini-Bettolo & Cavalla (1954)
28	CH_3		2	A. Dimethylformamide/ potassium carbonate	100°/0·2 mm	1·4805	Solov'ev & Skoldinov (1962)
*	CH_3	$(CH_3)_2N$	2	B. Sealed tube	64°/12 mm	1·4585	Erickson (1952)
*	CH_3	$(CH_3)_2N$	4	B. Sealed tube	100°/0·2 mm	1·4609	Solov'ev & Skoldinov (1963)

The compounds marked * were reaction intermediates and were not tested.

Amides

The tertiary amino amides were prepared from haloacylamides and the appropriate secondary amines in a refluxing solvent (method A) or in a sealed tube at 100° C in the absence of a solvent (method B). Dimethylaminoacetdimethylamide was prepared directly from chloroacetyl chloride and excess of dimethylamine in benzene. In all cases the products were isolated and purified by fractionation *in vacuo* after removal of amine hydrochloride by filtration. The compounds prepared are listed in Table 3. The quaternary salts listed in Table 4 were prepared either from the tertiary bases and methyl iodide in acetone (method C) or alternatively from the haloacylamides and the appropriate tertiary amine in refluxing ethanol (method D).

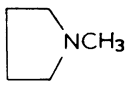
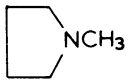
Biological methods

Toxicology

An estimate of the intravenous toxicity of each compound to female albino mice (18–25 g) was obtained by injecting groups of two animals per dose. Injections were made into a tail vein and a volume of 5 ml./kg was used. The LD50 was then determined using four groups of five animals for each compound. The ratio between doses was 1 : 1.2 and the LD50s were calculated using Thompson's (1947) method of moving averages employing the tables calculated by Weil (1952). LD50s were not determined for compounds which were not lethal at 50 mg/kg.

In other experiments attempts were made to protect mice against the lethal effects of some of these compounds by pretreatment with other drugs, the timing being selected to ensure that the premedicant was exerting its action when the test compounds were given. The drugs used were hexamethonium bromide, pempidine tartrate, decamethonium bromide, tubocurarine chloride, phentolamine and atropine sulphate, all administered by the intraperitoneal route 20 min before the test com-

TABLE 4. *List of the quaternary amino-acid amides (general formula* $\overset{+}{R}'(CH_2)_nCONR_2X^-$*) synthesized and tested pharmacologically*

No.	R	R′	n	X⁻	m.p.	Method and recrystallization solvent	Analysis: Calculated C	H	N	Found C	H	N
29	CH_3		1	I	166–167°	C. Acetone/ether	36·3	6·4	9·4	36·2	6·7	9·1
30	CH_3	$(CH_3)_3N$	1	I	229–231°	C. Ethanol	30·9	6·3	10·3	31·3	6·7	9·9
31	H	$(CH_3)_3N$	1	Cl	189–192°	D. Ethanol	39·4	8·6	18·4	39·7	8·9	18·2
32	CH_3		2	I	130°	C. Ethanol/acetone	38·5	6·8	9·0	38·5	7·1	8·9
33	CH_3	$(CH_3)_3N$	2	I	217–219°	C. Ethanol	33·6	6·7	9·7	33·9	7·3	9·8
34	H	$(CH_3)_3N$	2	Cl	180–181°	D. Ethanol	43·2	9·1	16·1	43·4	9·4	16·3
35	CH_3	$(CH_3)_3N$	3	I	165–166°	C. Ethanol/acetone	36·0	7·1	9·3	36·2	7·4	9·2
36	H	$(CH_3)_3N$	3	Cl	229–231°	D. Ethanol	46·5	9·5	15·5	46·4	9·7	15·2
37	CH_3	$(CH_3)_3N$	4	I	163–165°	C. Ethanol/acetone	38·2	7·4	8·9	38·2	7·8	9·0
38	H	$(CH_3)_3N$	4	Br^-	146–148°	D. Ethanol	40·2	8·0	11·7	40·6	8·3	11·7

pound ; nicotine hydrogen tartrate was also used but was given 40 min before the test compound.

In all the protection studies, parallel experiments were carried out in which nicotine tartrate was used in place of the amino-acid esters. In one such experiment 2-diethylaminoethyl diphenylpropylacetate (proadifen, SKF 525A) was given by the intraperitoneal route 80 min before the second intravenous dose of nicotine hydrogen tartrate.

Pharmacology

Isolated tissues

All the compounds were tested for their effects on the isolated guinea-pig ileum and on isolated chick semi-spinalis muscle. Measures were thus obtained of both muscarinic and nicotinic activity.

Approximately 2 cm of ileum was removed from a freshly killed guinea-pig at a point 5 cm from the ileo-caecal junction. This was suspended in a 5 ml. organ bath containing Ringer-Tyrode solution at 37° C. Hexamethonium bromide (500 μM) was included in the Ringer solution to block possible contractions due to ganglion stimulation. A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the solution. Contractions were recorded on a kymograph using an isotonic lever. Standard doses of 0.1 μM and 0.2 μM acetylcholine were used ; the doses of compounds being assayed were selected to give contractions approximately equivalent to those produced by the acetylcholine. Relative potencies were then calculated on the basis of four-point assays. Tests were carried out in all cases to check that the contractions were blocked by atropine sulphate (0.04 μM left in the bath for 1 min before adding the substance under test).

The semi-spinalis muscle was removed from 3–10 day old chicks anaesthetized with ether. The preparation was used as described by Child & Zaimis (1960). Recordings of the contractions of the muscle were made in the manner described for the guinea-pig ileum. Nicotine hydrogen tartrate (2 and 3 μM) was used as the standard drug and again four-point assays were carried out. A check was always made that the contractions were blocked by hexamethonium (500 μM left in the bath for 1 min before adding the substance under test).

Some compounds were examined for their effects on neuromuscular transmission using the isolated chick biventer cervicis nerve-muscle preparation described by Ginsburg & Warriner (1960) which enables a distinction to be made between depolarizing and non-depolarizing neuromuscular blocking agents. Both types of drugs reduce the contractions of the muscle evoked by stimulation of the nerve but depolarizing drugs also cause a contracture of the muscle. Contractions were recorded on a smoked drum using a light semi-isometric lever.

Anaesthetized cat experiments

The effects of some of these compounds on the cardiovascular, respiratory and autonomic nervous systems of anaesthetized cats were investigated. The cats were anaesthetized with chloralose-urethane (2.5 ml./kg intraperitoneally of a solution containing chloralose 25 mg/ml. and urethane 250 mg/ml.). Blood pressure was recorded from a femoral artery using a Statham Physiological Transducer Model P23AA. Changes in tension of the nictitating membrane were recorded using an

E and M Myograph Type C (maximum sensitivity 5 g) with a resting tension on the membrane of 1 g. The respiratory rate was recorded using an Impedance Pneumograph (E and M). Subcutaneous needle electrodes on either side of the thorax were used and the electrocardiogram (e.c.g.) was also recorded from these electrodes. All signals were suitably amplified and recorded on a pen recorder (Physiograph "Six", E and M Instrument Company Inc.). The cat was also tracheotomized using a stainless steel tube and the larynx and oesophagus were reflected forwards to leave a cervical well which was filled with liquid paraffin. The cervical sympathetic trunk was dissected free of the vagus, cut at a point about 1.5 cm caudal to the superior cervical ganglion and placed on platinum stimulating electrodes. Supramaximal electrical stimulation was applied using an Attree stimulator delivering rectangular pulses of 4 msec duration at a frequency of 10/sec. Drugs were administered via a cannula in the femoral vein.

Anaesthetized rabbit experiments

Selected compounds were tested in anaesthetized rabbits to investigate their mode of action in causing death. The rabbits were anaesthetized with urethane (1.8 g/kg intravenously by an ear vein), the right phrenic nerve was exposed in the neck region, severed and the central end was placed on recording electrodes and the peripheral end was placed on stimulating electrodes. The compound action potentials detected by the recording electrodes were, after suitable amplification, displayed on a cathode ray oscilloscope. The signals were also recorded on magnetic tape for subsequent photography. Stimuli from an Attree stimulator (1.5 V, 400 msec) were delivered either once every 10 sec for single twitches or at 62 c/s for tetanus. Diaphragm movements were recorded using a modification of Head's diaphragmatic slip. The technique, originally described by Head (1889), involved making a midline incision exposing the xiphisternum, into which a hook was inserted. The slip muscles were carefully dissected away from the sternum, which was then cut away from the xiphisternum leaving the latter attached to the diaphragm by the slip muscles alone. The hook was attached to a myograph (E and M Type C) and the diaphragm movements recorded on a pen recorder (E and M Physiograph).

Results

The toxicity to mice of all the compounds studied, together with their effects on the isolated guinea-pig ileum and isolated chick semi-spinalis, are shown in Tables 5 and 6. None of the tertiary bases, whether ester or amide, was very toxic to mice and they showed little activity on the isolated tissues. Some of the quaternary salts, however, particularly the propionic acid derivatives, were lethal to mice at doses of less than 1 mg/kg. In general, high toxicity was associated with high nicotinic activity (measured on the chick semi-spinalis preparation), but this correlation between these two parameters did not hold for all compounds.

A detailed investigation was made of the toxicity of two of these compounds (17 and 18) both alone and after pre-treatment with various blocking agents, and the results are compared with those obtained with nicotine. The results are given in Table 7.

Pre-treatment with hexamethonium, a quaternary ganglion-blocking drug, raised the LD50 of nicotine approximately seven-fold. The LD50 of compound 18 was

increased about two and a half times, but there was no significant effect upon the LD50 of compound 17. Pempidine, a non-quaternary ganglion-blocking drug, was even more effective against nicotine, increasing the LD50 some twenty times ; it had no effect on the toxicity of compounds 17 and 18. Decamethonium, a quaternary depolarizing neuromuscular blocking agent, had no effect on the toxicity of nicotine ; it increased the toxicity of compound 18 about one and a half times and showed a similar tendency with compound 17. Following administration of tubocurarine, a quaternary non-depolarizing neuromuscular blocking agent, there was a tendency for the LD50s of all these compounds to the raised, but in no case was this change statistically significant. Phentolamine, an α-adrenoreceptor blocking agent, raised the LD50 of nicotine about three times and the LD50s of compounds 17 and 18 about one and a half times. Following administration of atropine, an anticholinergic drug, the LD50s of all three compounds were raised one and a half to two times.

Experiments with nicotine (see Table 8) showed that sub-lethal doses of this drug given 40 min before lethal doses protected mice against the lethal effects of these

TABLE 5. *Toxicity, muscarinic and nicotinic activity of amino-acid esters*

Tertiary bases				Quaternary salts			
Compound No.	LD50 i.v. mice (mg/kg)	Muscarinic activity. Guinea-pig ileum. Acetylcholine=1	Nicotinic activity. Chick semi-spinalis. Nicotine=1	Compound No.	LD50 i.v. mice (mg/kg)	Muscarinic activity. Guinea-pig ileum. Acetylcholine=1	Nicotinic activity. Chick semi-spinalis. Nicotine=1
1	>50	−ve	−ve	13	10	0·003	0·012
2	>50	−ve	−ve	14	>50	0·009	0·03
3	35	−ve	0·03	15	4·6	0·023	−ve
4	>50	−ve	0·04	16	6·5	0·003	0·045
5	>50	0·002	−ve	17	0·54	0·16	0·48
6	>50	−ve	−ve	18	0·43	0·03	0·32
7	25	−ve	0·008	19	1·8	0·005	0·09
8	35	−ve	−ve	20	1·6	−ve	0·09
9	>50	0·001	−ve	21	1·3	0·03	0·29
10	>50	−ve	−ve	22	20	−ve	0·009
11	15	0·011	−ve	23	0·29	−ve	0·24
12	>50	−ve	0·01	24	0·69	0·035	0·41
				25	2·08	0·066	0·38
				26	>50	−ve	0·067

−ve indicates <0·001 × acetylcholine or nicotine.

TABLE 6. *Toxicity, muscarinic and nicotinic activity of amino-acid amides*

Tertiary bases				Quaternary salts			
Compound No.	LD50 i.v. mice (mg/kg)	Muscarinic activity. Guinea-pig ileum. Acetylcholine=1	Nicotinic activity. Chick semi-spinalis. Nicotine=1	Compound No.	LD50 i.v. mice (mg/kg)	Muscarinic activity. Guinea-pig ileum. Acetylcholine	Nicotinic activity. Chick semi-spinalis. Nicotine
27	>50	−ve	−ve	29	>50	−ve	−ve
28	35	−ve	0·004	30	>50	−ve	−ve
				31	>50	−ve	−ve
				32	20	−ve	0·034
				33	37	−ve	0·04
				34	10	−ve	0·02
				35	2·1	−ve	0·38
				36	2·3	−ve	0·04
				37	6·0	−ve	0·004
				38	2·8	0·003	0·04

−ve indicates <0·001 × acetylcholine or nicotine.

TABLE 7. *Toxicity of nicotine, compound* 18 *and compound* 17, *administered intravenously to male albino mice*

Compound	LD50	Pretreatment with hexamethonium bromide 30 mg/kg i.p. 20 min before	Pretreatment with pempidine tartrate 23 mg/kg i.p. 20 min before	Pretreatment with decamethonium bromide 2 mg/kg i.p. 20 min before	Pretreatment with tubocurarine chloride 0·3 mg/kg i.p. 20 min before	Pretreatment with phentolamine methane sulphonate 25 mg/kg i.p. 30 min before	Pretreatment with atropine sulphate 17·4 mg/kg i.p. 30 min before
Nicotine (hydrogen tartrate)	1·6 (0·54–2·4)	10·7 (6·5–17·5)	32·2 (21·0–40·1)	1·9 (0·61–3·2)	2·5 (1·6–4·4)	4·6 (3·6–5·4)	2·8 (2·4–3·7)
Compound 18 (methiodide)	0·43 (0·39–0·47)	1·1 (0·89–1·3)	0·44 (0·37–0·52)	0·30 (0·26–0·36)	0·52 (0·41–0·68)	0·66 (0·55–0·78)	0·59 (0·52–0·66)
Compound 17 (methiodide)	0·54 (0·42–0·70)	0·78 (0·64–0·95)	0·36 (0·28–0·44)	0·38 (0·25–0·51)	0·81 (0·64–1·0)	0·82 (0·75–0·89)	1·17 (0·86–1·6)

All figures represent LD50s in mg/kg with 95% limits. Five groups of five animals each were used.

subsequent doses. In this way the LD50 of nicotine could be raised approximately eight and a half times. When the metabolic inhibitor proadifen (SKF 525A) was given a further 40 min before the sub-lethal doses of nicotine, the protective factor was decreased to about two and a half times. Compound 18 showed no such self-protecting properties, neither was there any cross-protection between nicotine and compound 18. The lethality of compound 18 was increased after pre-treatment with proadifen due, presumably, to inhibition of the normal detoxifying enzymes in the liver.

The actions of some of the amino-acid esters were studied on the chick biventer-cervicis nerve-muscle preparation. Both a contracture of the muscle and a diminution in the height of contraction produced by nerve stimulation were observed with compound 18 (5 μM). Neostigmine increased the contraction. These are effects typically produced by depolarizing neuromuscular blocking agents.

The effects of some of these esters on an anaesthetized cat preparation were studied and a typical tracing is shown in Fig. 1. Compound 18 (20 μg/kg) produced a fall in blood pressure followed by a rise. There was a small contraction of the nictitating membrane but respiration was unimpaired. The cat was then

TABLE 8. *Comparison of the toxicities of nicotine and Compound* 18

Compound	LD50	Nicotine (0·8 mg/kg i.v.) given 40 min before	Compound 18 (0·22 mg/kg i.v.) given 40 min before	SKF 525A (50 mg/kg i.p.) given 40 min before	SKF 525A (50 mg/kg given 80 min before) +nicotine (0·8 mg/kg i.v.) given 40 min before
Nicotine (hydrogen tartrate)	1·6 (0·54–2·4)	13·8 (9·8–35·5)	1·7 (1·4–2·1)	3·1 (2·4–4·0)	4·2 (3·1–5·8)
Compound 18 (methiodide)	0·43 (0·39–0·47)	0·40 (0·33–0·47)	0·55 (0·42–0·74)	0·19 (0·15–0·23)	—

All figures represent LD50s (i.v. mice) in mg/kg with 95% limits. In all tests five groups of five animals each were used.

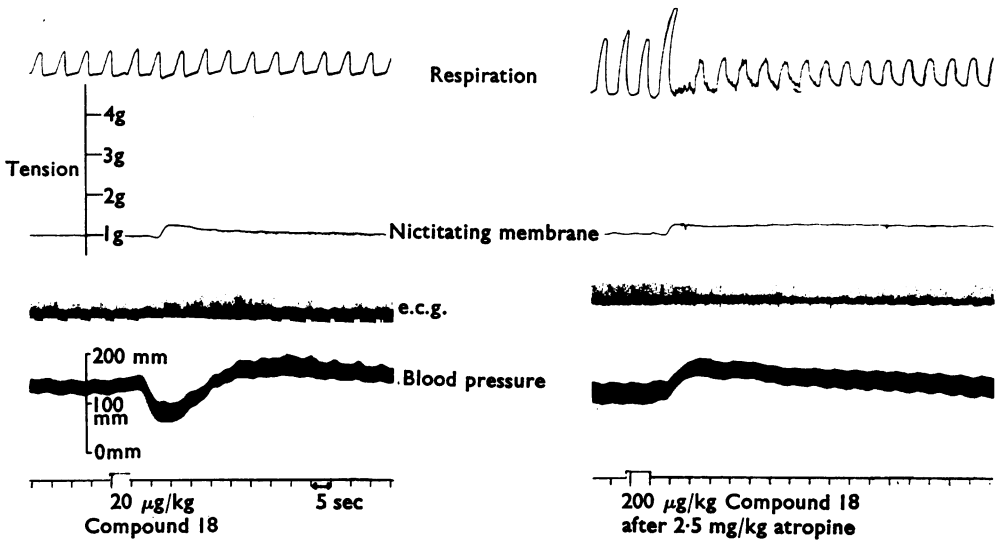


FIG. 1. Effect of compound 18 on anaesthetized cat preparation.

atropinized (2.5 mg/kg) and given compound 18 (200 μg/kg). A rise in blood pressure occurred, accompanied by a small contraction of the nictitating membrane and some depression of respiration. It is concluded that compound 18 had both muscarinic and nicotinic activity and supports the results obtained from the experiments on isolated tissues.

A typical tracing from an experiment using the anaesthetized rabbit preparation is shown in Fig. 2. At a dose of 100 μg/kg, compound 18 produced an immediate, but short-lived, increase in respiration rate with an almost complete cessation of diaphragm movements. At this dose the action potentials recorded from the central end of the phrenic nerve were normal but tetany was not sustained in the diaphragm when the peripheral end of the phrenic nerve was stimulated. After compound 18 (250 μg/kg) the effects on the diaphragm were similar but recordings from the phrenic nerve showed a burst of asynchronous discharge (see Fig. 3). At a lethal dose of 500 μg/kg immediate and complete neuromuscular blocking occurred, but phrenic nerve discharges persisted for about 1 min after administration of the drug. It is concluded that the lethality of compound 18 is due to respiratory failure resulting from a peripheral blocking action at the neuromuscular junction; the central action—failure of the respiratory centre—appears to be a secondary phenomenon. Experiments with compounds 17 and 24 showed an identical pattern of events.

Discussion

The pharmacological studies on these amino-acid esters and amides revealed that although some of them showed muscarinic activity they were not very potent in this respect. Furthermore, in contrast to oxotremorine and some of its analogues, the tertiary bases were much less active than the quaternary salts. Examination of

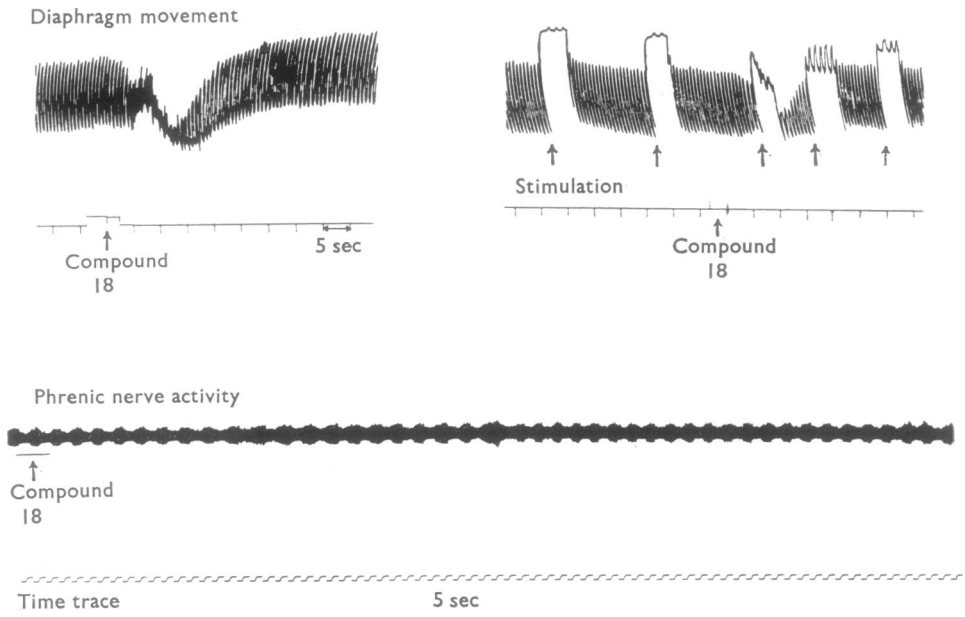


FIG. 2. Effects of compound 18 (199 μg/kg) on diaphragm movement and phrenic nerve activity of rabbit.

molecular models showed that among the acetates and propionates interactions were possible at sites 1 and 2 of the muscarinic receptor proposed by Bebbington & Brimblecombe (1965), the quaternary nitrogen atom interacting at site 1 and either the carbonyl oxygen or ester oxygen atom at site 2. No interaction at site 3 was possible. Models of the butyrates showed that if the quaternary nitrogen atom interacted at site 1 then the carbonyl oxygen atom could interact at either site 2 or site 3. In none of these esters was there any possibility of simultaneous interactions at sites 1, 2 and 3 of the proposed muscarinic receptor; this is presumably the reason for their low muscarinic activity, and for the low activity of the amides, to which the same observations apply.

Further general pharmacological studies revealed that these compounds were predominantly nicotinic in character. Only the quaternary salts showed appreciable nicotinic activity; the tertiary bases were either completely inactive or showed only weak activity. The high toxicity of some of these compounds was always associated with high nicotinic activity, but there was not always a direct numerical parallelism between these two parameters.

In vitro pharmacological studies indicated that these compounds act as depolarizing neuromuscular blocking agents and the *in vivo* experiments on rabbits confirmed that it is this depolarizing action at the neuromuscular junction of the respiratory muscles which is the primary cause of death. Experiments carried out to assess the effects of various drugs on the toxicity of two of these esters (compounds 17 and 18), however, indicated that other actions may play some part in the lethality of these compounds. Thus the protection afforded by hexamethonium is probably due to its action in blocking the effects of the esters at autonomic ganglia. Phentolamine is presumably effective by virtue of its action at the post-ganglionic sympathetic endings, where it will antagonize effects resulting from over-activity of sympathetic ganglia. Atropine might be expected to counteract any effects resulting from muscarinic activity of the drugs, although the fact that it is also beneficial in protecting nicotine-poisoned mice is difficult to explain along these lines. Tubo-

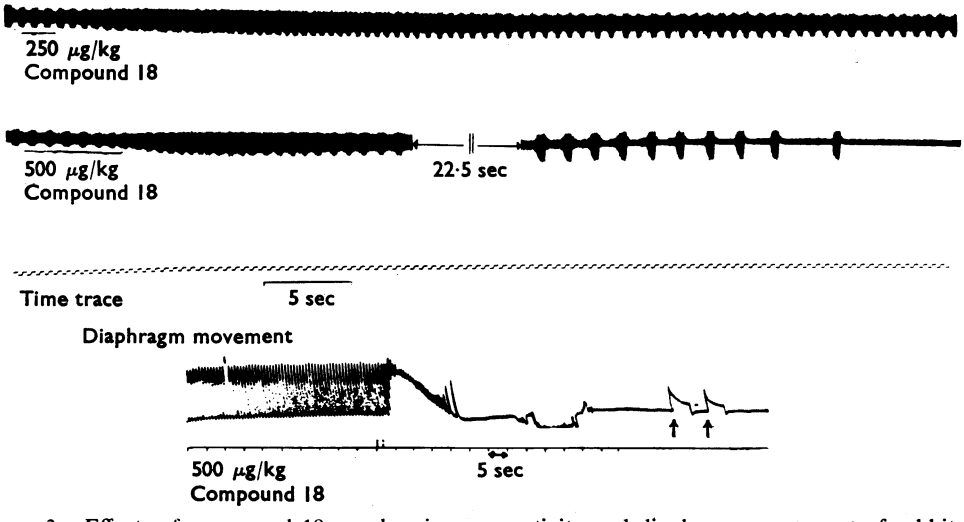


FIG. 3. Effects of compound 18 on phrenic nerve activity and diaphragm movement of rabbit.

curarine as a non-depolarizing type of neuromuscular blocking agent is without effect and decamethonium as a depolarizing type of neuromuscular blocking agent tends to increase the toxicity by acting additively with the amino-acid esters.

The results of the toxicological experiments indicate quite clearly that these esters are not acting in exactly the same way as nicotine. For example, although hexamethonium is effective in protecting mice from nicotine poisoning, pempidine is more effective (see Table 7). This would suggest that while the ganglionic effects of nicotine are important in the toxic syndrome, its central effects, which would be antagonized by the tertiary base pempidine but not by the quaternary salt hexamethonium, are even more important.

A finding of some interest is that sub-lethal doses of nicotine are effective in protecting animals from the lethal effects of subsequently administered toxic doses. The fact that this protection is markedly reduced in animals pre-treated with the metabolic inhibitor proadifen (SKF 525A) suggests that it is a metabolite of nicotine which is responsible for this protective action. No such self-protection was seen with compound 18 nor was there any cross-protection between nicotine and compound 18. These facts provide additional evidence for different modes of action of nicotine and the amino-acid ester.

The relatively high nicotine activity of some of these esters and amides is of interest. A comparison of molecular models of these compounds with those of standard nicotinic agents such as acetylcholine, carbachol and the 4-oxopentyl trimethylammonium ion showed that in every case a quaternary nitrogen atom was separated from a carbonyl oxygen atom by 4–4.5 Å. As a working hypothesis it seemed reasonable to assume, therefore, that the nicotinic receptor has at least two essential sites separated by 4–4.5 Å. There are two further points which should be considered, however, and which suggest that all the known facts cannot be accommodated by such a receptor model. In the first place, in the series of amino-acid esters and amides studied, although there was little difference in nicotinic activity between the propionates and butyrates (for example, compounds 18 and 25), the propionamide (compound 33) had only about one-tenth the nicotinic activity of the butyramide (compound 35). No explanation for this discrepancy can be offered at present.

Another drug which should be considered in any discussion of nicotinic activity is nicotine itself. This compound has a pyrrolidine nitrogen atom separated from a pyridine nitrogen atom by about 4 Å and could thus fit the proposed sites on the receptor. It is not clear, however, why nicotine, a tertiary base, should be such a potent nicotinic agent when the amino-acid esters and amides described in this report only show high nicotinic activity as the quaternary salts. Neither is it entirely satisfactory to postulate interaction of the pyridine nitrogen atom at that site on the receptor which interacts with the carbonyl oxygen atom of the other drugs considered.

Some of the points raised in this discussion indicate the need for further studies which are being planned.

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