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Neuromuscular blocking activity of a new series of quaternary N-substituted choline esters

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Summary

1. The neuromuscular blocking activity of a new group of mono- and diquaternary N-substituted choline esters of several carboxylic acids has been evaluated in various whole animal preparations in the mouse, rat and cat.

2. A basic non-depolarizing mechanism was present in all but one compound —a depolarizing agent, which was studied for comparison. There was evidence of facilitatory activity and nicotinic stimulation in the monoquaternary compounds. These effects were diminished in the diquaternary compounds.

3. The presence of a bulky aromatic ring system on the nitrogen atom appeared to increase both neuromuscular blocking potency and facilitatory activity in the experimental animal. A similar relationship had previously been demonstrated *in vitro* in another study.

4. The duration of action, although short in most compounds, did not correlate well with previously determined *in vitro* hydrolysis rates, possibly because of species differences.

5. The general pharmacology of each compound appeared to depend considerably upon the structure of the choline moiety.

Introduction

All neuromuscular blocking agents currently in clinical use have inherent disadvantages and produce undesirable side effects. These include increased intra-ocular tension, cardiac arrhythmias, skeletal muscle injury and prolonged neuromuscular blockade, as reviewed in a previous paper (Kitz, Karis & Ginsburg, 1969). The ideal neuromuscular blocking agent should have a short duration of action (for example 5–10 min like suxamethonium) but should cause no depolarization of the postjunctional membrane (Bowman, 1962; Churchill-Davidson, 1958; Foldes, 1957). That is, it should produce a non-depolarizing block similar to that of (+)-tubocurarine and gallamine but of short duration. Its action should be specific enough to minimize undesirable side effects. An agent with such a brief action span need not require an antagonist. Nevertheless, the added possibility of pharma-

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cological reversal of the block, for example by the anticholinesterase agents commonly used to antagonize non-depolarizing type neuromuscular blockade, would be clinically advantageous.

Kitz, *et al.* (1969) have approached this problem by the design, synthesis, and *in vitro* testing of a new series of quaternary N-substituted choline esters of several carboxylic acids, and have advanced the 'bulky ester' concept. The rationale is that these compounds, like suxamethonium, may have brief durations of action, since they are potential substrates for the cholinesterases of the blood. If the nitrogen atoms of the choline fragment of the molecule were quaternized with large, space occupying alkyl groups and aromatic or alicyclic ring systems, the compounds might behave pharmacologically as 'pachycurares' as suggested by Bovet (1951) and therefore produce a non-depolarizing type block.

These concepts have been confirmed *in vitro* (Kitz, *et al.*, 1969). In the isolated frog sciatic nerve-sartorius muscle preparation, the neuromuscular blocking potency of some of the compounds equalled or surpassed that of several standard agents, for example gallamine, suxamethonium and hexafluorenium. In single-fibre microelectrode studies, compounds quaternized with ethyl, fluorenyl and *p*-nitrobenzyl groups produced no depolarization of the muscle end-plate. Some of them were hydrolysed at various rates *in vitro* by human plasma (pseudo) cholinesterase, and might therefore have a short duration of action in the intact animal.

It was apparent that in isolated test systems, this 'bulky ester' concept might prove feasible as a rationale for the development of a short acting non-depolarizing choline ester neuromuscular blocking agent for clinical use. Further pharmacological evaluation was necessary in the intact experimental animal. This paper presents the data obtained *in vivo* and correlates them with results previously noted *in vitro*.

Methods

Drugs

The new compounds were synthesized by Ginsburg (Kitz, Karis, & Ginsburg, 1969). They were dissolved at appropriate concentrations in 0·9% NaCl or in mixtures of 25–50% polyethylene glycol and 0·9% NaCl. The pH of the solutions, which were unbuffered, ranged between 5 and 6.

The compounds were administered intraperitoneally or intravenously to mice, rats, and cats. Several standard neuromuscular blocking agents were chosen as reference agents for control experiments in each species. These were the commercially available solutions of suxamethonium, (+)-tubocurarine, gallamine and hexafluorenium.

Acute toxicity

The intravenous and intraperitoneal LD50 and the intravenous ED50 were determined in conscious male CF-1S mice weighing 20–25 g. Groups of six mice received regularly varying single doses of one agent until one group had received the LD0 and one the LD100. There were always two or three other groups with LD values between 0 and 100. The ED50 was obtained using a similar procedure, and was defined as the intravenous dose at which 50% of the animals demonstrated

loss of the prehensile reflex, that is, were unable to hang suspended from a wire by their forepaws. The volume of solution administered varied from 0·1 to 0·3 ml. All survivors were observed for 1 week after injection. The intravenous and intraperitoneal LD50 and intravenous ED50 and standard errors were calculated according to the method of DeBeer (1945).

Neuromuscular blocking action

The compounds were initially studied in twitch-tension experiments performed in anaesthetized rats and cats. Three animals of each species were used for the evaluation of each new compound and each reference agent. Male Long-Evans rats weighing 250-350 g were anaesthetized with a mixture of α-chloralose (80 mg/kg) and sodium pentobarbitone (15 mg/kg) injected intraperitoneally. Cats of either sex weighing 2–4 kg received a mixture of α-chloralose (60 mg/kg) and sodium pentobarbitone (10 mg/kg) injected intraperitoneally. Tracheostomy was performed and respiration was controlled throughout the experiment with a Harvard small animal or rodent respirator, at a tidal volume of 15 ml/kg, and a frequency of twenty-five strokes/min for the cat, and fifty/min for the rat. The right common carotid artery was cannulated for recording of blood pressure via a Statham P23 D transducer coupled to a Sanborn 150 or Grass model 5 polygraph. Drugs were administered via a cannula in the right external jugular vein.

The right knee and foot were securely fixed and the sciatic nerve was exposed in the thigh, crushed centrally, and stimulated peripherally through shielded platinum wire electrodes with square-wave pulses from a Grass S-4 stimulator and S I U 5 stimulus isolation unit. The stimuli were of twice supramaximal strength and 0·2 ms duration at a frequency of 0·15 Hz. The resultant responses of the fast gastrocnemius muscle and the slow soleus muscle were recorded isometrically from the same leg, via two Grass FT-.03 force displacement transducers. The initial tension loads for gastrocnemius and soleus muscles were 50 and 7·5 g respectively in the rat and 100 and 50 g respectively in the cat. The muscles were bathed in liquid paraffin and their temperature, recorded by a Yellow Springs thermoprobe, was kept at $36 \pm 2°$ C by heat from a lamp.

After the preparation had stabilized, a control response of both muscles to a 2 s tetanic stimulus (250 Hz for the rat, 60 Hz for the cat) was recorded. These frequencies are within the optimum range for development of maximum tension in both muscles in both species (Close, 1964 ; Buller, Eccles & Eccles, 1960). When the preparation had again stabilized, a dose-response curve was constructed in each animal by giving increasing doses of one agent until a single dose produced 90–95% block of the gastrocnemius muscle twitch. Successive doses were not administered until the tetanus response had returned to the control level in both muscles. Each animal received only one new compound or reference agent. The solution volume for each dose was adjusted so that the animal received 1 ml/kg body weight.

The values obtained for the gastrocnemius muscle in each animal in the above procedure were plotted on log-probit paper and a straight line of visual best fit was drawn through these points. The point at which this dose-response curve crossed the 95% value (6·65 probits) was called the ' neuromuscular blocking dose ' for each individual animal. The dose-response curve was thus treated as an ' all or none ' phenomenon, with each dose plotted representing a percentage inhibition of the

maximum response, 100% blockade. The rationale for this type of treatment was strengthened by plotting the data arithmetically, in which case a sigmoid curve was obtained. Transferred to log-probit paper, the data gave a straight line. The construction of dose-response curves in this fashion has been discussed by DeBeer (1945) and Barlow (1964).

The duration of effect of the neuromuscular blocking dose from the time of injection to the point of 95% recovery of the gastrocnemius muscle twitch tension, was designated the T_{tw}. A 2 s tetanic stimulus was then given every 5–10 min until the tetanus had returned to the control level.

The time from administration of the neuromuscular blocking dose to the recovery of tetanus was designated the T_{tet}. If repeat doses of the compounds were given after the T_{tet}, there tended to be little cumulation of effect. The T_{tet} is therefore a good indication of the cumulation time of a compound.

In twenty cats, the mechanisms of action of compounds 11, 12, 15, 28 and 29 were delineated in the following preparations. (1) The tibialis anterior muscle was stimulated bilaterally as described by Bowman & Rand (1961). The rates of stimulation were 0·15 Hz for the right side and 1 Hz for the left. (2) Close-arterial injections were made into the tibialis anterior muscle by the method of Brown (1938) as modified by Blaber (1960). The initial tension for the tibialis anterior muscle was 50 g in both preparations. (3) Direct stimulation of the tibialis anterior was accomplished via steel needle electrodes inserted into the distal musculo-tendinous junction.

Effects on autonomic ganglia

The effects of compounds 11, 12, 15, and 29 were studied in the indirectly stimulated nictitating membrane of cats anaesthetized with chloralose and pentobarbitone. The resting tension on the membrane was 5 g. A 5 s train of stimuli (square wave pulses of 0·5 ms duration at a frequency of 20 Hz) was applied every 3 min to the cut peripheral end of the preganglionic fibres of the left superior cervical ganglion. The contractions of the membrane were recorded on a polygraph via a Grass FT-.03 transducer. The effects on the membrane of the previously established neuromuscular blocking dose of the new compounds were compared with standard doses of hexamethonium (4 mg/kg: Bowman & Rand, 1961).

Effects on vagal ganglia were evaluated by applying 10 s trains of stimuli to the cut peripheral end of the right vagus nerve. The depression of blood pressure obtained under control conditions was compared with that resulting after the administration of the neuromuscular blocking dose.

Results

Acute toxicity in mice

All values quoted refer to the cation. Results obtained in mice are presented in Table 1. The intravenous ED50 is given both in the form of mol/kg as well as in mg/kg to facilitate comparison of dosages with prior data derived from isolated preparations (Kitz *et al.*, 1969).

With the exception of triethylcholine, all the new compounds when administered intravenously produced the immediate onset of the following sequence of effects:

muscular weakness, ataxia, prostration, loss of traction and prehensile reflexes, dyspnoea, cyanosis, loss of righting reflex, and weak terminal convulsions. Death was apparently due to respiratory failure. The musculature was flaccid at death, and the heartbeat could usually be palpated in the chest after the respirations had ceased. Compound 12 as well as suxamethonium caused muscle fasciculation before the onset of the above effects.

Triethylcholine produced a more gradual onset of the above pattern. The rapidity of onset and the intensity of the effects produced could be increased by inducing the animals to exercise, for example by repeatedly forcing them to climb an inclined plane or to cling to a high-wire. These effects, including the greater potency on intraperitoneal than on intravenous administration, are qualitatively similar to those reported by Bowman & Rand (1961), although the dosage is slightly lower. The rate of recovery from the effects of triethylcholine was also much slower than that from all other compounds. The ED50 and LD50 values for the standard agents suxamethonium, tubocurarine, gallamine and hexafluorenium are in good agreement with previously published data (Macri, 1954 ; Lumb, 1963 ; Bowman, 1964).

All mice were observed for 1 week subsequent to drug administration. All survivors remained in good condition, continued to gain weight, and maintained normal dietary habits. There were no delayed deaths secondary to intravenous administration of the new compounds, all deaths occurring soon (within 3–15 min) after drug administration.

TABLE 1. *Toxicity of new quaternary N-substituted choline esters in mice*

Compound	LD50 i.p. ±S.E. (mg/kg)	LD50 i.v. ±S.E. (mg/kg)	ED50 i.v. ±S.E. (mg/kg)	ED50 i.v. ±S.E. (mol/kg)
Suxamethonium	2·08± 0·24	0·15±0·016	0·12±0·03	$4{\cdot}1\pm1{\cdot}0\times10^{-7}$
(+)-Tubocurarine	0·40± 0·01	0·11±0·014	0·09±0·02	$1{\cdot}7\pm0{\cdot}39\times10^{-7}$
Gallamine	4·20± 0·7	1·54±0·23	1·32±0·31	$2{\cdot}6\pm0{\cdot}61\times10^{-6}$
Hexafluorenium	8·40± 1·8	0·72±0·16	0·60±0·18	$1{\cdot}2\pm0{\cdot}36\times10^{-6}$
2	33 ± 7	29 ±2·0	23·2 ±4·1	$8{\cdot}8\pm1{\cdot}6\times10^{-5}$
3	58 ± 9	10·4 ±1·1	8·3 ±1·7	$2{\cdot}1\pm0{\cdot}43\times10^{-5}$
5	126 ±19	25·0 ±3·6	20·0 ±4·1	$4{\cdot}9\pm1{\cdot}0\times10^{-5}$
7	50 ± 7·8	27·7 ±4·0	22·2 ±3·7	$7{\cdot}1\pm1{\cdot}2\times10^{-5}$
9	25·0 ± 0·6	7·0 ±1·1	5·6 ±1·3	$1{\cdot}2\pm0{\cdot}29\times10^{-5}$
11	47·4 ± 7·0	35·8 ±4·3	28·6 ±3·5	$6{\cdot}4\pm0{\cdot}78\times10^{-5}$
12	67·9 ±22·2	8·3 ±1·3	6·6 ±1·7	$1{\cdot}8\pm0{\cdot}49\times10^{-5}$
13	25·0 ± 1·6	21·1 ±3·8	16·9 ±3·4	$3{\cdot}8\pm0{\cdot}76\times10^{-5}$
15	29·4 ± 4·5	13·4 ±1·5	10·7 ±1·8	$2{\cdot}6\pm0{\cdot}44\times10^{-5}$
17	29·8 ±14·9	28·9 ±1·7	23·1 ±2·0	$5{\cdot}2\pm0{\cdot}45\times10^{-5}$
19	59·5 ± 9·4	13·6 ±1·0	10·9 ±1·6	$2{\cdot}4\pm0{\cdot}35\times10^{-5}$
21	36·5 ± 8·8	3·1 ±0·7	2·5 ±0·7	$5{\cdot}5\pm1{\cdot}5\times10^{-6}$
23	175 ±16	4·2 ±1·3	3·6 ±0·9	$7{\cdot}9\pm2{\cdot}0\times10^{-6}$
25	55·4 ±16·8	20·2 ±1·7	16·2 ±2·0	$3{\cdot}9\pm0{\cdot}48\times10^{-5}$
26	38·7 ± 4·0	6·5 ±1·6	5·2 ±2·0	$1{\cdot}8\pm0{\cdot}68\times10^{-5}$
27	94·4 ± 5·6	6·2 ±0·9	5·0 ±1·1	$1{\cdot}5\pm0{\cdot}34\times10^{-5}$
Triethylcholine	27·6 ± 2·7	30·7 ±9·0	24·6 ±7·7	$1{\cdot}7\pm0{\cdot}53\times10^{-4}$
28	25·9 ± 3·0	4·9 ±0·4	3·9 ±0·3	$1{\cdot}5\pm0{\cdot}12\times10^{-5}$
29	62·4 ±10·1	4·8 ±0·4	3·8 ±0·5	$1{\cdot}3\pm0{\cdot}17\times10^{-5}$

Doses quoted refer to the cation. Drugs were injected intraperitoneally or intravenously. Structural formulae for the compounds are presented in Table 2. The ED50 is defined as the dose at which 50% of the animals demonstrated loss of the prehensile reflex, that is, were unable to hang suspended from a wire by their forepaws. The ED50 dose is presented also in the form mol cation /kg, for comparison with the data presented in Table 2 and in a previous paper (Kitz *et al.*, 1969).

TABLE 2. *Neuromuscular blocking potency and duration of action of quaternary N-substituted choline esters in rats and cats under chloralose-pentobarbitone anaesthesia*

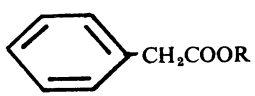
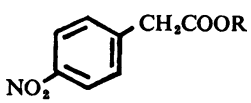
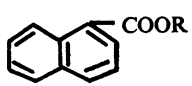
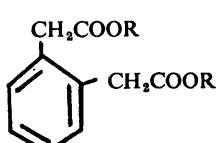
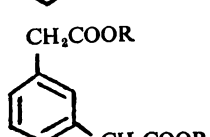
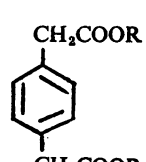
Compound	Structural formula	R	X^-	Neuromuscular blocking dose Rat mg/kg	Rat mol/kg	Cat mg/kg	Cat mol/kg	Duration of action—rat T_{tw} (min)	Duration of action—rat T_{tet} (min)	Duration of action—cat T_{tw} (min)	Duration of action—cat T_{tet} (min)
SDC	Suxamethonium		2 Cl^-	0·1 –0·15	$3·0–4·6\times10^{-7}$	0·03–0·04	$0·9 –1·2\times10^{-7}$	8–12	15–20	5–9	12–15
(+)-TC	(+)-Tubocararine		2 Cl^-	0·04–0·07	$0·6–1·1\times10^{-7}$	0·2 –0·4	$3·15–6·3\times10^{-7}$	21–30	40–60	25–36	75–90
GAL	Gallamine		3 I^-	2·5 –3·5	$4·8–6·7\times10^{-6}$	0·8 –1·0	$1·6 –1·9\times10^{-6}$	20–28	35–54	24–33	60–80
HFL	Hexafluorenium		2 Br^-	No block		0·4 –0·6	$0·8 –1·2\times10^{-6}$			21–28	30–40
2		R_2	I^-	50 –60	$1·75–2·1\times10^{-4}$			9–12	26–30		
3		R_4	Br^-	10·0–12·5	$2·5 –3·1\times10^{-5}$			3–4	5–7		
5		R_5	Br^-	15–17	$3·6 –4·1\times10^{-5}$			8–11	12–15		
7		R_3	I^-	No block							
9		R_2	2 I^-	11–13	$2·5 –2·9\times10^{-5}$	3·5 –3·9	$7·8 –8·7\times10^{-6}$	13–15	40–52	7–10	11–14
11		R_2	2 I^-	35–45	$0·78–1·0\times10^{-4}$	3·0 –3·5	$6·6 –7·8\times10^{-6}$	9–13	14–18	4–5	5–7
12		R_1	2 I^-	7–9	$1·6 –2·0\times10^{-5}$	0·8 –1·2	$2·2 –3·3\times10^{-6}$	12–14	13–15	6–9	9–12
13		R_2	2 I^-	50–60	$1·1 –1·3\times10^{-4}$	9·5 –11·0	$2·1 –2·4\times10^{-5}$	45–60	50–65	18–22	20–26

TABLE 2 *Continued*

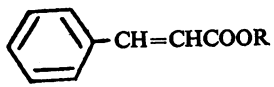
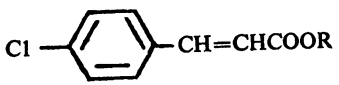
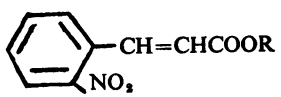
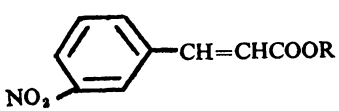
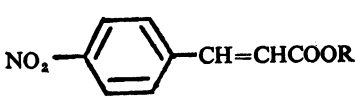
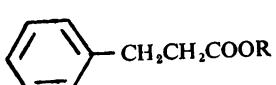
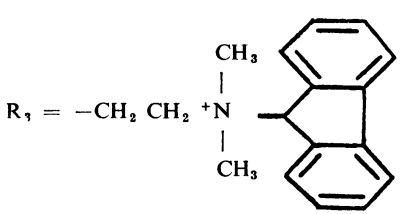
Compound	Structural formula	R	X^-	Neuromuscular blocking dose Rat mg/kg	Rat mol/kg	Cat mg/kg	Cat mol/kg	Duration of action—rat T_{tw} (min)	Duration of action—rat T_{tet} (min)	Duration of action—cat T_{tw} (min)	Duration of action—cat T_{tet} (min)
15		R_4	Br^-	11·0–12·6	$2·7 - 3·1 \times 10^{-5}$	5·0 –6·8	$1·2 - 1·7 \times 10^{-5}$	2–3	3–5	8–10	15–20
17		R_4	Br^-	19–22	$4·2 - 4·9 \times 10^{-5}$			3–4	5–7		
19		R_4	Br^-	9–11	$2·0 - 2·4 \times 10^{-5}$			2–3	8–10		
21		R_4	Tos^-	3·8–4·4	$8·3 - 9·6 \times 10^{-6}$			2–3	8–10		
23		R_4	Tos^-	4·3–5·5	$0·94–1·2 \times 10^{-5}$			4–5	8–10		
25		R_4	Br^-	18–22	$4·4 - 5·3 \times 10^{-5}$			1·5–2·0	2–3		
26		R_3	Br^-	7–9	$2·7 - 3·4 \times 10^{-5}$			8–10	10–12		
27	CH_3COOR	R_4	Br^-	9–12	$2·7 - 3·7 \times 10^{-5}$			8–11	12–14		
*	Triethylcholine	R_2	I^-	70–80	$4·9 - 5·5 \times 10^{-4}$	25 –35	$1·7 - 2·4 \times 10^{-4}$	60–90	70–95	120–180	150–200
28	HO-R	R_3	Br^-	6–8	$2·4 - 3·1 \times 10^{-5}$	4·0 –4·6	$1·6 - 1·8 \times 10^{-5}$	5–6	7–8	7–9	10–12
29		R_4	Br^-	13–16	$4·6 - 5·7 \times 10^{-5}$	3·9 –4·7	$1·4 - 1·7 \times 10^{-5}$	6–7	10–12	10–13	12–15

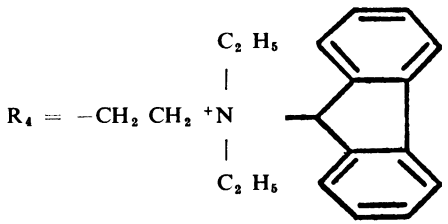
TABLE 2. *Continued*

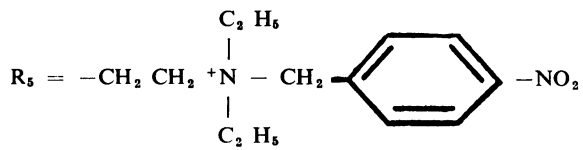
* = stimulus rate 1 Hz

$R_1 = -CH_2\,CH_2\,{}^{+}N(CH_3)_2\,CH_3$

$R_2 = -CH_2\,CH_2\,{}^{+}N(C_2H_5)_2\,C_2H_5$







Values quoted refer to the cation and represent the dose range obtained in three animals. All drugs were injected intravenously. The neuromuscular blocking dose is the dose necessary to cause 95% paralysis of the twitch of the gastrocnemius muscle. Data are presented in the form mol/kg as well as in mg/kg for better comparison with previously published data (Kitz *et al.*, 1969). T_{tw} is the duration of action in minutes from injection to 95% recovery of the twitch of the gastrocnemius muscle. T_{tet} is the duration of action from injection to the return of fully sustained two second tetanus at the control tension in the gastrocnemius muscle. T_{tw} and T_{tet} refer to the first period of block of the gastrocnemius muscle at the neuromuscular blocking dose in each experiment. For details, see text.

Neuromuscular blocking activity in anaesthetized rats and cats

Values obtained for the neuromuscular blocking dose, T_{tw} and T_{tet} are presented in Table 2, both in mg/kg and mol/kg as in Table 1. All values quoted refer to the cation, and represent the range obtained in three animals.

The compounds may be divided into three groups: the monoquaternaries, compounds 3, 5, 7 and 15 to 27; the diquaternaries, compounds 9, 11, 12 and 13; and the choline moieties, compounds 28, 29 and triethylcholine.

Initial screening in the rat and cat showed that all compounds except number 12 produced neuromuscular blockade of short to moderate duration without preblock augmentation of the twitches. The tetanus was usually poorly sustained, and post-tetanic facilitation was always present. However, edrophonium, in intravenous doses of 0·2–1·0 mg/kg, either had no effect on the block or intensified it. Compound 12, which depolarized the muscle end-plate *in vitro* (Kitz *et al.*, 1969) was studied for comparison.

The monoquaternaries all appeared to have considerable nicotinic stimulant activity. At low doses they generally caused tachycardia and hypertension; frequently the twitch response was augmented. Higher doses produced bradycardia, hypotension and neuromuscular blockade. These compounds were therefore eliminated from further consideration. Such effects are typical in monoquaternary choline esters (Brücke, 1956). These effects, particularly the stimulatory responses seen at low dosage, were much reduced in the diquaternaries and the choline moieties.

Mechanism of neuromuscular blockade

Compound 15, a typical monoquaternary, the diquaternaries, compounds 11 and 12, and the choline moieties, compounds 28, 29 and triethylcholine, were further studied in anaesthetized cats.

Compounds 28, 29 and triethylcholine

The pharmacology of these choline analogues should be established because these moieties represent some of the hydrolysis products of the ester compounds studied in this paper. The neuromuscular blocking action of several ester compounds depends largely on the nature of the choline moiety (Marshall, 1968; Dowd, Jennings, Marshall & Tracy, 1968). The pharmacology of triethylcholine and other analogues of choline has been studied by Bowman & Rand (1961, 1962) and Bowman, Hemsworth & Rand (1967). Choline and its analogues produce neuromuscular blockade by depolarizing, non-depolarizing, and prejunctional mechanisms, the action of each substance depending on the nature of the groups substituted on the nitrogen atom (Hutter, 1952; Bowman & Rand, 1961, 1962; Bowman *et al.*, 1967). The rate of stimulation has little effect on the depth and duration of block caused by depolarizing agents. Non-depolarizing agents, however, are much more effective in rapidly stimulated muscles, whereas choline analogues with prejunctional activity alone are able to cause a depression of the twitch only when rapid rates of stimulation are used (Bowman *et al.*, 1967).

Triethylcholine in doses of 10–40 mg/kg intravenously, produced neuromuscular blockade of slow onset in muscles stimulated indirectly at a rapid rate (1 Hz, Fig. 1). The maximum depth of block was reached in 20–30 minutes. Muscles stimu-

lated at a slow rate (0·15 Hz) were only very slightly affected. The soleus muscle was much less sensitive to the action of triethylcholine than was the tibialis anterior muscle. The block was completely antagonized by choline (3–6 mg/kg i.v.). During

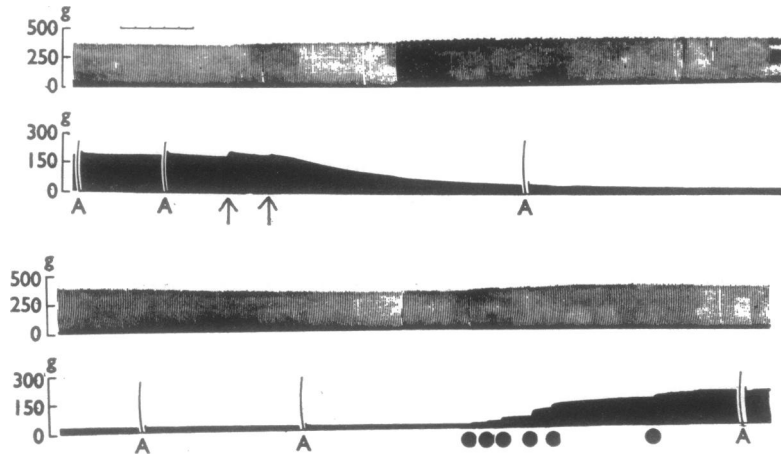


FIG. 1. Prejunctional neuromuscular blockade. Cat, 3·4 kg, bilateral tibialis anterior preparation. Continuous recording of maximal indirectly elicited twitches. Upper record of each pair: right tibialis, stimulus rate 0·15 Hz. Lower record: left tibialis, stimulus rate 1 Hz. Calibration scales are at left, time scale in minutes is at top. At A, acetylcholine (12 μg) was injected close-arterially. At arrows, a total of 30 mg/kg triethylcholine given intravenously produced gradual blockade of the rapidly stimulated muscle only, without affecting the acetylcholine-induced twitch. At dots, a total of 6 mg/kg choline intravenously completely reversed the block.

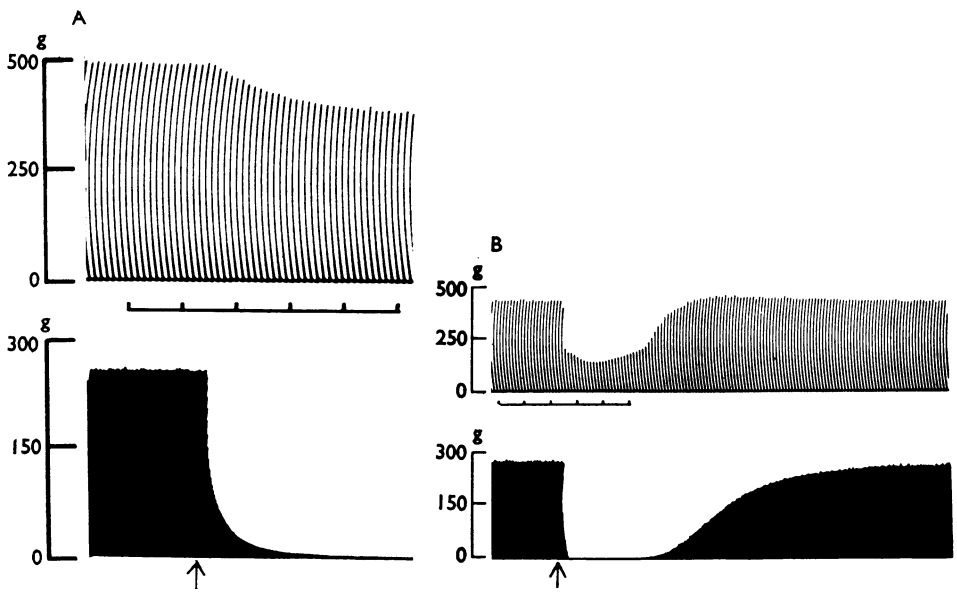


FIG. 2. Non-depolarizing blockade. Panels A and B, cats, 2·4 and 2·6 kg, bilateral tibialis anterior preparation. Maximal twitches were indirectly elicited at 0·15 and 1 Hz in the right and left tibialis (upper and lower records, respectively). Time scale is in minutes, calibration scales are at left. All compounds were administered intravenously. Panel A, at arrow, 0·1 mg/kg (+)-tubocurarine. Panel B, at arrow, 2·5 mg/kg compound 29. Non-depolarizing agents produced a much greater degree of block in rapidly stimulated muscles than in muscles stimulated at slow rates.

the block, twitches elicited by the administration of acetylcholine close-arterially were unaffected. Neostigmine in intravenous doses of 0·02–0·1 mg/kg only slightly antagonized the block. These results indicate the prejunctional nature of the block produced by triethylcholine and are similar to those of Bowman & Rand (1961).

Compounds 28 and 29, in contrast to triethylcholine, caused neuromuscular blockade of rapid onset in muscles stimulated indirectly at both slow and rapid rates (Fig. 2B), although the depth and duration of block were always greater in the rapidly stimulated muscles.

Choline caused only a transient augmentation of the twitch of rapidly stimulated muscles when administered during the block. There was no depression of the twitch on direct stimulation of the muscle during partial blockade.

Compounds 28 and 29 showed similar potency and pharmacological behaviour in the cat, doses of 4–4·5 mg/kg intravenously producing neuromuscular blockade

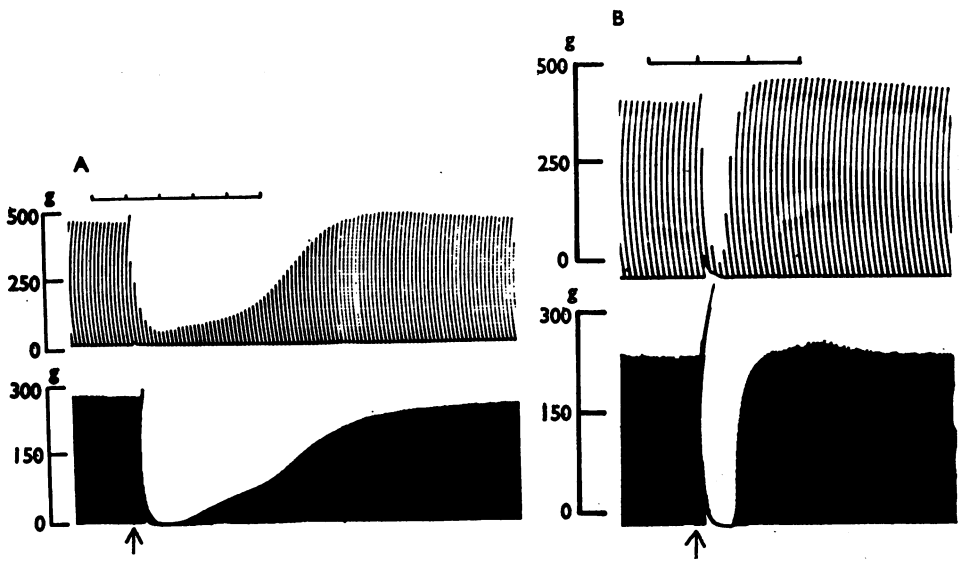


FIG. 3. Depolarizing blockade. Panels A and B, cats, 3·1 and 2·0 kg, bilateral tibialis anterior preparation. Maximal twitches were indirectly elicited at 0·15 and 1 Hz in the right and left tibialis (upper and lower records, respectively). Time scale is in minutes, calibration is at left. All compounds were administered intravenously. Panel A, at arrow, 0·015 mg/kg suxamethonium. Panel B, at arrow, 0·8 mg/kg compound 12. There was little difference in the degree of block produced by depolarizing agents in slowly and rapidly stimulated muscles.

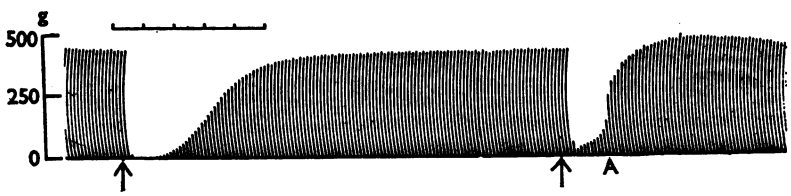


FIG. 4. Neuromuscular blocking activity of the N-fluorenyl choline moiety, compound 29. Maximal twitches of the tibialis anterior muscle were indirectly elicited at 0·15 Hz. Time scale is in minutes, calibration scale is at left. All injections were made close-arterially. Compound 29 had basically postjunctional non-depolarizing activity. Thus, the block produced by compound 29, 0·6 mg/kg at arrows, was not preceded by facilitated twitches or contractures. At A, acetylcholine 150 μg reversed the block.

lasting approximately 10 minutes. There was no preblock augmentation of the twitches on either intravenous or close-arterial administration. The tetanus during partial block was poorly sustained, and post-tetanic facilitation was present. Small doses of compounds 28 and 29 (1–2 mg/kg i.v.) deepened partial neuromuscular blockade caused by tubocurarine, gallamine and hexafluorenium. Doses of up to 1·0 mg/kg close-arterially did not cause a contraction in the unstimulated titialis anterior muscle. Similar doses blocked the twitch induced by acetylcholine (10–20 μg c.a.). Large doses of acetylcholine (100–200 μg c.a.) reversed the block (Fig. 4). These doses are of the order necessary to antagonize blockade produced by tubocurarine (Wilson & Wright, 1937). KCl (15–20 mg c.a.) also reversed the block. The depolarizing neuromuscular blocking agents suxamethonium and decamethonium (C-10) exerted a weak antagonistic effect only on close-arterial administration in very low doses (0·05–0·1 μg), whereas larger doses deepened the block. Similar results were obtained when the anticholinesterase agents edrophonium and neostigmine were administered close-arterially during partial blockade by compounds 28 and 29: low doses (edrophonium, 10–20 μg or neostigmine, 1–2 μg c.a.) weakly antagonized the block, whereas larger doses intensified it. It was not possible to antagonize neuromuscular blockade produced by compounds 28 and 29 by intravenous administration of depolarizing neuromuscular blocking agents or anticholinesterase agents. The above pattern of interaction of compounds 28 and 29 with anticholinesterase agents may be explained by the moderate degree of anticholinesterase activity which these compounds have. Their K_I values for binding to acetylcholinesterase are $8{\cdot}6 \times 10^{-6}$ and $1{\cdot}2 \times 10^{-5}$ M/l, respectively (Kitz, Karis & Ginsburg, 1969).

Compound 11

In the cat, compound 11 showed typical non-depolarizing behaviour. When administered intravenously or close-arterially, neuromuscular blockade resulted without any preblock twitch augmentation or fasciculations. Doses of up to 3 mg/kg close-arterially did not induce any muscular activity. There was considerable fade of tetanus, and post-tetanic antagonism of the block was present (Fig. 5A). The twitch response to close-arterial acetylcholine was abolished (Fig. 5B). Large doses of acetylcholine (100–200 μg) reversed the block. Partial blockade produced by compound 11 was antagonized by other depolarizing agents given intravenously or close-arterially, for example, suxamethonium, decamethonium and compound 12 (Fig. 7B). In the bilateral tibialis preparation, the rapidly stimulated muscle sustained a much greater degree of block.

The duration of action of compound 11 was very brief: intravenous doses of 3·2–3·9 mg/kg produced 95% block of the twitch with 95% recovery occurring within approximately 5 minutes. The low T_{tet} of 5–7 min indicates a very low cumulation time. Anticholinesterase agents administered during partial blockade either had no effect or deepened the block. Pretreatment with these agents greatly prolonged the block (Fig. 5C).

Compound 11, a diester, is hydrolysed *in vitro* by pseudocholinesterase at 3·3 times the rate of suxamethonium (Kitz *et al.*, 1969). This would account for its brief duration of action. Inhibition of the enzyme by anticholinesterase agents might therefore explain their prolongation of the action of compound 11, and possibly that of other hydrolysable ester compounds in this paper. The interaction

of anticholinesterase agents with non-depolarizing esters which are hydrolysed by plasma cholinesterase then would appear to depend on the resultant effect of inhibition of both true and plasma cholinesterase.

One of the hydrolysis products of compound 11 is triethylcholine. Large doses of compound 11 (30–60 mg/kg) produced a pattern of block in rapidly stimulated muscles which recovered in two stages: an initial rapid stage followed by a very gradual second stage. The second phase of the block was completely reversible by choline (5 mg/kg i.v.) (Fig. 6A). Since the compound is hydrolysed, this would indicate that the second phase of the block is primarily due to triethylcholine. This contrasts with the results of Marshall (1968) and Dowd *et al.* (1968) with other esters, where the second phase of the block was attributed to the entire compound. A dose of 30–60 mg/kg of compound 11 would release 20–40 mg/kg of triethylcholine on complete hydrolysis. This is well within the range of activity of triethylcholine in the cat, as was also reported by Bowman & Rand (1961).

When 90–95% paralysis of the slowly stimulated muscle was maintained in the bilateral tibialis preparation by continuous intravenous infusion of compound 11, results were obtained which strongly suggest that this compound is rapidly hydrolysed *in vivo* and releases triethylcholine (Fig. 6B). The onset of blockade in both

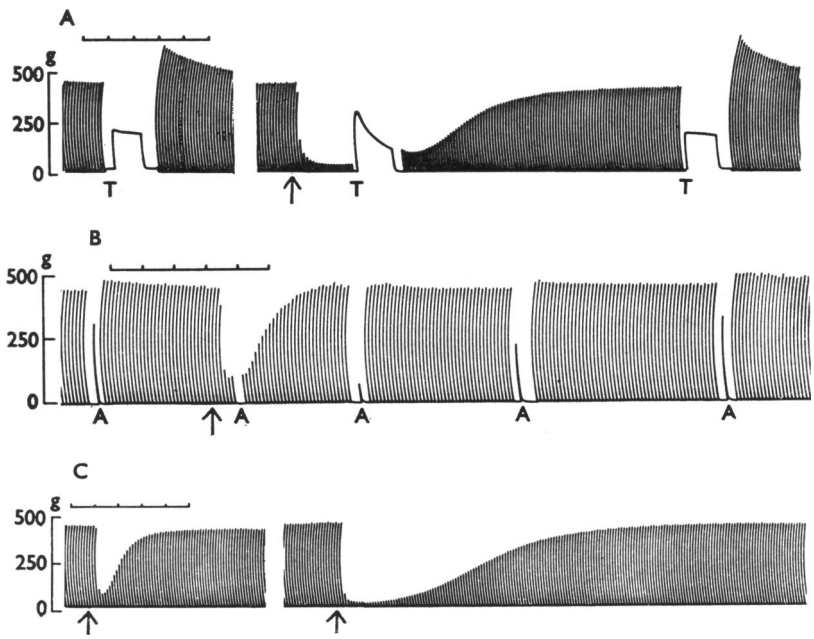


FIG. 5. Neuromuscular blocking activity of compound 11. Maximal twitches of the tibialis anterior or gastrocnemius muscles were elicited indirectly at 0·15 Hz. Time scales are in minutes, calibration scales are at left. Panel A, cat, 2·4 kg. At arrow, 3·5 mg/kg compound 11 was given intravenously. At T, tetanic stimulation at 60 Hz was maintained for 5 seconds. Tetanus during the block was poorly sustained and post-tetanic facilitation was present. Tetani during the control period and after recovery were recorded at 1/5 twitch sensitivity. Panel B, cat, 2·5 kg. At arrow, compound 11, 3·9 mg/kg, was given intravenously. At A, acetylcholine 15 μg was injected close-arterially. The acetylcholine induced twitch was abolished during the block, and recovered more slowly than the indirectly elicited twitch. Panel C, cat, 2·4 kg. At arrows, compound 11, 0·4 mg/kg, was given close-arterially. The interval between doses was 40 minutes. Twenty minutes before the second dose, neostigmine (0·1 mg/kg) was administered intravenously. Thus, although compound 11 showed non-depolarizing activity, its action was prolonged by pretreatment with anticholinesterase agents.

muscles was prompt. When the infusion was stopped after 20 min, a total dose of 65 mg/kg of compound 11 having been administered, the slowly stimulated muscle recovered quickly. The rapidly stimulated muscle, however, did not recover in two stages as it did when large single intravenous doses of compound 11 had been administered. Instead, a very gradual increase in twitch tension ensued, beginning at about the time when the slowly stimulated muscle had completely recovered. This pattern of slow recovery in the rapidly stimulated muscle was abruptly terminated by choline (6 mg/kg i.v.), twitch tension quickly returning

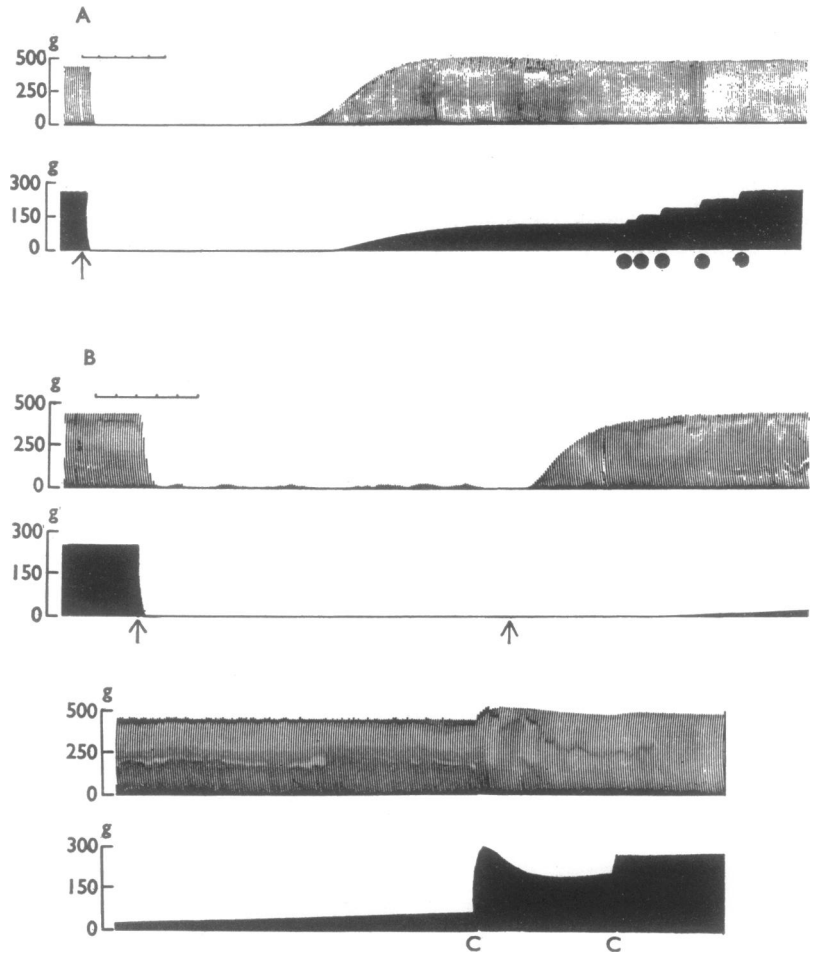


FIG. 6. Panels A and B, cats, 3·1 and 2·4 kg, bilateral tibialis anterior preparation. Maximal twitches were indirectly elicited at 0·15 and 1 Hz in the right and left tibialis (upper and lower records, respectively). Time scale is in minutes, calibration scales are at left. Panel A demonstrates the two-phase block produced by large single doses of compound 11. At arrow, compound 11, 60 mg/kg, was given intravenously. The second stage of the block was completely reversed by a total of 5 mg/kg choline intravenously at dots. In panel B, compound 11 was administered intravenously as a continuous infusion at the rate of (2–3 mg/kg)/min, begun at the first arrow. When the infusion was terminated at the second arrow, a total dose of 65 mg/kg compound 11 having been administered, the slowly stimulated muscle recovered quickly. Just when the slowly stimulated muscle had fully recovered, the twitch of the rapidly stimulated muscle began a very gradual pattern of return which was abruptly completed by two doses of choline (3 mg/kg) administered at C. Upper and lower pairs of records in panel B are continuous.

to the preblock level. A dose of 65 mg/kg of compound 11 would release about 40 mg/kg of triethylcholine, a highly active quantity in the cat.

It is therefore apparent that the initial action of compound 11 is non-depolarizing and postjunctional. A second, late, prejunctional action may be seen after large doses. The initial action is most likely due to the whole compound, whereas the second is probably due to the hydrolysis product, triethylcholine.

Compound 15

By most criteria, compound 15 produced neuromuscular blockade of the non-depolarizing type. The block was not preceded by facilitated twitches on intravenous or close-arterial administration. Tetanus during partial blockade was poorly maintained, and post-tetanic facilitation was present. When administered close-arterially, compound 15 did not cause a twitch in the unstimulated muscle. The acetylcholine-induced twitch was completely abolished. Small doses of compound 15 were additive with other non-depolarizing agents. In the bilateral tibialis preparation the rapidly stimulated muscle was more intensely paralysed. The block was antagonized by KCl, but not by suxamethonium, decamethonium or by anticholinesterase agents.

Compound 15 has moderate anticholinesterase properties. The K_I is $1{\cdot}9 \times 10^{-6}$ M/l. (Kitz *et al.*, 1969). Sub-blocking doses caused a slight (5–10%) augmentation of the indirectly elicited twitch, but did not antagonize partial paralysis caused by tubocurarine. Larger doses produced neuromuscular blockade followed by augmentation of the twitch after the block had recovered. Compound 15 is not susceptible to enzymatic hydrolysis *in vitro* (Kitz *et al.*, 1969). Its action in the cat was not prolonged by the prior administration of neostigmine as was that of compound 11. It therefore appears that the inability of anticholinesterase agents to antagonize the block produced by compound 15 may be due to its own moderate antagonism of the enzyme.

Compound 12

Compound 12 demonstrated the classical depolarizing effects in the cat first described by Paton & Zaimis (1951) with decamethonium: the blockade was preceded by generalized muscular fasciculation, contractures, and augmented twitches. Tetanus was well maintained and post-tetanic facilitation was absent. The depth and duration of block were similar whether a slow (0·15 Hz) or rapid (1 Hz) rate of stimulation was used (Fig. 3B). The unstimulated tibialis anterior muscle responded with a twitch when compound 12 was administered close-arterially.

Compound 12 antagonized neuromuscular blockade secondary to non-depolarizing agents, including compound 11. In fact, compounds 11 and 12 antagonized each other (Fig. 7, panels A and B). Other depolarizing agents, as well as anticholinesterase agents, deepened the block produced by compound 12.

Cardiovascular effects

The cardiovascular effects of the three groups of compounds varied considerably. The cardiovascular response obtained appeared to depend at least in part upon the action of the particular compound on autonomic ganglia. The results, presented

as approximate equipotent molar ratios (Barlow, 1964) with reference to hexamethonium 4 mg/kg, are listed in Table 3.

As previously mentioned, the monoquaternaries, as exemplified by compound 15, had chiefly facilitatory effects at low doses. At the neuromuscular blocking dose, however, compound 15 inhibited the contractions of the nictitating membrane of the cat, and blocked the vasodepressor response to stimulation of the peripheral right vagus nerve.

The nicotinic effects of the monoquaternary compounds upon autonomic ganglia, that is, stimulation followed by blockade of ganglionic transmission, were considerably modified in the diquaternaries. Compound 12 in doses up to the neuromuscular blocking dose produced only stimulation of the ganglia, evidenced by facilitation of the contractions of the nictitating membrane and a marked pressor response. These effects were blocked by hexamethonium. Diquaternary esters of choline, including suxamethonium and its higher homologues, have ganglionic stimulating properties (Brücke, 1956). Compound 11 displayed only weak antagonistic activity.

Compound 29, the choline moiety, was a pure antagonist. The blockade of vagal and sympathetic ganglia produced by this compound could be reversed by neostigmine or edrophonium. The mechanism therefore appears to be competitive in

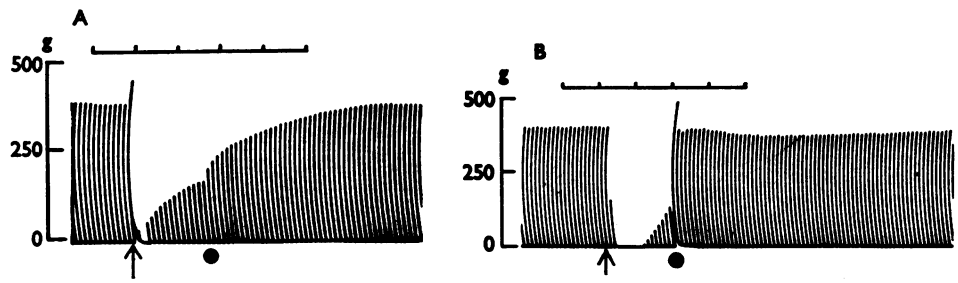


FIG. 7. Interaction of compounds 11 and 12. Panels A and B, cat, 2·8 kg. Maximal twitches of the tibialis anterior were elicited indirectly at 0·15 Hz. Time scales are in minutes, calibration scales are at left. All injections were made close-arterially. Compound 12, a depolarizing agent, and compound 11, a non-depolarizing agent, were mutually antagonistic. Thus, in panel A, compound 12 (0·2 mg/kg) at arrow, produced neuromuscular block preceded by a facilitated twitch and a contracture. The block was antagonized by compound 11, 0·3 mg/kg at dot. In panel B, the block produced by compound 11 (0·7 mg/kg) was reversed by compound 12 (0·1 mg/kg).

TABLE 3. *Ganglionic effects of compounds* 11, 12, 15, *and* 29

Compound	Dose mg/kg	Nictitating membrane response: Per cent of control	Nictitating membrane response: Blocking potency (approximate molar ratio, hexamethonium=1)	Vagal response per cent of control	Blood pressure response
Hexamethonium	4·0	5–35	1·0	0	Depressor
11	3·5	80–95	5–7	50–70	Depressor
12	1·2	130–150	–	NT	Pressor
15	6·5	50–65	3–4	0	Depressor
29	4·5	5–40	1·2–1·4	0	Depressor

The data listed were obtained in artificially ventilated cats anesthetized with chloralose and pentobarbitone. Each value represents the range obtained in three animals after intravenous administration of the neuromuscular blocking dose (see text). Doses quoted refer to the cation. NT=not tested. Blocking potencies are reported as approximate equipotent molar ratios (Barlow, 1964).

nature, and analogous to that of tetraethylammonium and triethylcholine (Bowman & Rand, 1961).

No cardiac arrhythmias following the administration of compounds 11, 12, 15 or 29 were noted on lead II of the electrocardiogram. Changes in heart rate were confined to simple increases or decreases in the sinus rate.

Discussion

Since the original work by Bovet (1951) and Bovet, Bovet-Nitti, Guarino, Longo & Fusco (1951), the neuromuscular blocking effects of esters of various carboxylic acids have been described. The subject has been reviewed by Brücke (1956) and Bovet-Nitti (1959). The most potent compounds in these early reports were esters of choline, which were depolarizing agents. More recently (Brittain, Collier & D'Arcy, 1961 ; Dowd *et al.*, 1968 ; Marshall, 1968) attempts have been made to produce esters which block by non-depolarizing mechanisms. The duration of action of many of these compounds has been short, but the potency has generally been low.

The two series of ester compounds described in this paper have shown non-depolarizing activity. The data in Tables 1 and 2 indicate that the structure-activity relationships reported *in vitro* by Kitz *et al.* (1969) also hold true in the experimental animal. The 'bulky ester' concept therefore appears to be valid at least in this series of compounds. Thus potency is increased by the substitution of large aromatic ring systems on the nitrogen atom (compounds 2 and 3 ; triethylcholine, 28 and 29) and by unsaturation in the chain (compounds 15 and 25). Such alterations increase the bulk and rigidity of the molecule, favouring a non-depolarizing mechanism and fitting Bovet's definition of 'pachycurares' (1951). Unfortunately, both the *in vitro* and *in vivo* studies have shown that these changes also increase a compound's ability to inhibit acetylcholinesterase. This is reminiscent of the facilitating and anticholinesterase properties of drugs such as benzoquinonium (Bowman, 1958), hexafluorenium (Foldes, Molloy, Zsigmond & Zwartz, 1960) and ambenonium and methoxyambenonium (Blaber, 1960 ; Blaber & Bowman, 1963). All these agents include an unsaturated ring system as one of the groups on the quaternary nitrogen atom, and they produce neuromuscular blockade of the non-depolarizing type.

The pharmacology of the choline analogues is considerably modified by the substitution of the bulky aromatic fluorenyl group for one of the ethyl groups on the nitrogen atom. Bowman & Rand (1961) reported ganglion blockade in the cat after doses of 30–50 mg/kg triethylcholine, whereas compound 29 is active in the range 3–5 mg/kg. The neuromuscular blocking potency of the triethylcholine moiety is also markedly increased by substitution of fluorenyl for ethyl. In addition, the mechanism of neuromuscular blockade produced by compound 29 differs from that of triethylcholine. The former blocks by a postjunctional non-depolarizing mechanism whereas the latter's effects are largely prejunctional (Bowman & Rand, 1961). Bowman & Rand (1962) and Bowman *et al.* (1967) reported postjunctional neuromuscular blocking effects in several choline analogues in which groups larger than ethyl were substituted on the nitrogen atom.

Therefore the pharmacology of the esters in this paper appears to depend considerably upon the structure of the choline moiety. Hypothetical compounds similar to number 11, esterified with triethylcholine or some other choline analogue

showing similar prejunctional activity, might be expected to produce late prejunctional neuromuscular blockade upon release of an appropriate amount of the choline fragment on hydrolysis. Agents esterified with compounds 28 or 29 or another choline analogue producing only postjunctional blockade should not cause this phenomenon, but a secondary postjunctional effect due to these choline fragments might appear if appropriate quantities accumulated upon hydrolysis. No such effect was demonstrated with compound 15, which is not subject to human plasma cholinesterase catalysed hydrolysis (Kitz *et al.*, 1969). The potential accumulation of pharmacologically active quantities of the choline moiety may therefore be important in the evaluation of new ester neuromuscular blocking agents. This would be a minor consideration, however, if the potency of the parent compound were much greater than that of its choline moiety, so that only relatively inactive amounts of the latter were released. Such a situation exists with suxamethonium, where the ratio of the potency of the whole compound to choline in the cat is approximately 1,000 to 1 (Hutter, 1952 ; Bowman, 1964).

Although the duration of action of most of the new compounds is short, there appears to be no relationship between the hydrolysis rates reported by Kitz *et al.* (1969) and the duration of action found *in vivo*. Hobbiger & Peck (1969) have shown that the plasma cholinesterase of different species hydrolyses representative substrates at varying rates, the order for suxamethonium being monkey>man> rat>dog>cat. Other substrates were generally hydrolysed faster by man than by the latter species. The duration of action of hydrolysable ester neuromuscular blocking agents in man therefore may not be predictable with accuracy from results obtained in the experimental animal, although it might be expected that such compounds having a short duration of action in the rat and cat would also have a short action span in man.

It is acknowledged (Bowman, 1964) that the cat is the species most closely mimicking man in its response to depolarizing and non-depolarizing agents, whereas the rat is relatively insensitive to most neuromuscular blocking agents and responds atypically to depolarizing agents (Zaimis, 1953 ; Thesleff, 1955 ; Bowman, 1964). In fact, Ireson, Ford & Loveday (1969) have shown that neuromuscular blockade produced by suxamethonium in rats may be antagonized by edrophonium. The well known species differences first noted by Zaimis (1953) indicate that the cat is most sensitive to depolarizing agents, whereas the rat is most sensitive to tubocurarine. For the above reasons, it was felt that the study of the new compounds in the rat and cat might give the broadest indications of their potency and mode of action.

The above noted species differences in sensitivity to neuromuscular blocking agents and in rates of hydrolysis of ester compounds make application of the data to man difficult. If a compound were hydrolysed *in vitro* at an appropriate rate by human plasma, however, and had shown reasonable potency, non-depolarizing activity, and short duration of action in the experimental animal, it might then be considered for trial in man if appropriate toxicological studies were negative. This paper and the previous study (Kitz *et al.*, 1969) have shown that three of the above requisites may be achieved in the diquaternary ester compounds tested in this paper. Several additional series of esters are currently being studied in an effort to increase potency and reduce anticholinesterase activity.

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