J. Pharm. Pharmac., 1967, **19**, 502–508 Received February 2, 1967

Steroidal monoquaternary ammonium salts with non-depolarizing neuromuscular blocking activity

THE LATE J. J. LEWIS*, M. MARTIN-SMITH†, T. C. MUIR* AND H. H. ROSS‡

A series of ten 2β- or 3α-steroidal monoquaternary ammonium salts, having androstane or pregnane skeletons and related in structure to acetylcholine by possession of an oxygen function on the carbon atom next but one to the quaternary nitrogen atom, were investigated for neuromuscular blocking activity *in vivo* in the cat, hen and mouse and *in vitro* on the frog rectus muscle and on the rat phrenic nerve diaphragm preparation. All compounds displayed typical non-depolarizing activity, the duration of block being significantly less than that for (+)-tubocurarine in the cat and the hen. The potency of the salts was low with the most active compound, 3α-acetoxy-2β-piperidino-5α-androstan-17-one methobromide, being 1/16th as active on a molar basis in the cat as (+)-tubocurarine.

THE ability of monoquaternary ammonium salts to act as neuromuscular blocking agents has been known since the classic experiments of Crum Brown & Fraser (1869), but in recent years, owing to the much greater potency of bis-, tris- and tetra-onium compounds (*inter alia* Bovet, 1959; Barlow, 1960; Cavallito & Gray, 1960; de Reuck, 1962; Stenlake, 1963; Edwards, Lewis & Marren, 1966 and refs cited) coupled with the fact that monoquaternary ammonium compounds may show ganglion-blocking, anti-muscarinic or acetylcholine-like (e.g. Huguenard & Martin, 1950; Hey, 1952) properties, there has been a virtual neglect of investigations of new monoquaternary ammonium salts as potential neuromuscular blocking agents. Certain observations, however, seemed to us to point to the desirability of a re-assessment of monoquaternary ammonium compounds, especially steroidal monoquaternary ammonium salts.

Thus, indications of the possible presence of desirable neuromuscular blocking properties in polycyclic monoquaternary salts are to be found in the activities of certain compounds derived from strychnidine (Karrer, Eugster & Waser, 1949) and in the possibility that the potent bisquaternary compound C-toxiferine-I could be dissociating *in vivo* into two molecules of active monoquaternary ammonium compound as suggested by its known *in vitro* hydrolysis under mild acid conditions into the metho-salt of the Wieland-Gumlich aldehyde (Battersby & Hodson, 1958, 1960). Within the steroid group, it is known that the trimethylammonium salts derived from the alkaloids funtumine (3α-amino-5α-pregnan-20-one) and funtumidine (3α-amino-5α-pregnan-20α-ol) exhibit non-depolarizing neuromuscular blocking activity (Blanpin & Bretaudeau, 1961; Blanpin & Pierre, 1961), while recent work showing the presence of potent neuromuscular blocking activity in 3α,17β- and 3β,17α-bisquaternary ammonium androstanes, in which the quaternary heads lie on opposite sides of the

* From the Experimental Pharmacology Division, Institute of Physiology, The University, Glasgow, W.2.
† Department of Pharmacy, University of Strathclyde, Glasgow, C.1.
‡ Present Address: Department of Pharmacy, University of Aston in Birmingham, Gosta Green, Birmingham 4.

steroid nucleus (May & Baker, 1963, 1965), appears to cast doubt upon the validity of the classical two-point attachment theory of neuromuscular blockade (Barlow & Ing, 1948a,b; Paton & Zaimis, 1949) thus re-emphasizing concepts such as the adumbration theory (Loewe & Harvey, 1952) and suggesting that these compounds could be acting by way of a one-point attachment.

TABLE 1. NEUROMUSCULAR BLOCKING ACTIVITY OF STEROIDAL MONOQUATERNARY AMMONIUM SALTS IN DIFFERENT PREPARATIONS*

Compound		Molar potency ((+)-tubocurarine = 100)			
Code number	Chemical name	Cat gastrocnemius	Hen gastrocnemius	Frog rectus	Rat diaphragm
B1	3α-Acetoxy-2β-piperidino-5α-androstan-17-one methobromide	6	6	37·2	0·68
B2	2β,17β-Diacetoxy-3α-piperidino-5α-androstane methobromide	2	2·5	180	1·29
B3	2β-Acetoxy-3α-piperidino-5α-androstan-17-one methobromide	1·5	2·5	40·3	0·68
B4	3α-Acetoxy-2β-dimethylamino-5α-androstan-17-one methohydroxide	1·5	7·5	7·28	0·41
B5	2β-Dimethylamino-3α-hydroxy-16α-methyl-5α-pregnan-20-one methobromide	1·5	2	70·7	
B6	2β,17β-Diacetoxy-3α-piperidino-5α-androstane methohydroxide	2	6	34·3	0·68
B7	2β-Acetoxy-3α-piperidino-5α-androstan-17-one methohydroxide	1·5	1·5	131	
B8	3α-Acetoxy-2β-pyrrolidin-1′-yl-5α-androstan-17-one methobromide	1	6·5	22·7	0·69
B9	3α-Hydroxy-2β-piperidino-5α-pregnan-20-one methobromide	2	2	50·2	2·36
B10	3α-Acetoxy-2β-pyrrolidin-1′-yl-5α-pregnan-20-one methobromide	2	4	53·9	1·43

* The molar potencies quoted, relative to (+)-tubocurarine = 100, represent the average of three determinations for each preparation.

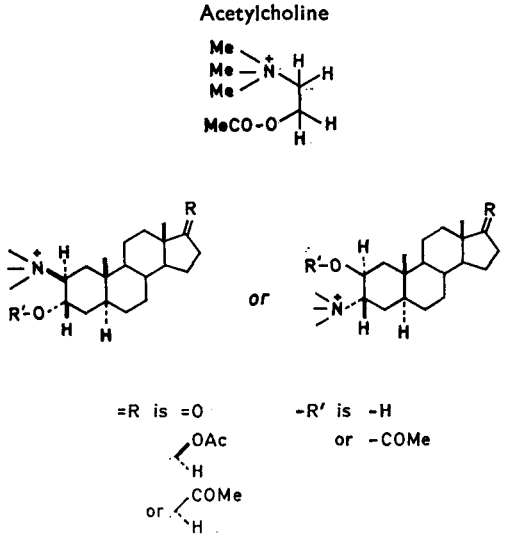

FIG. 1. General formulae of steroidal monoquaternary ammonium salts B1 to B10 showing their relationship to acetylcholine. In compounds B1, B2, B3, B6, B7, B9 the positively charged nitrogen atom is incorporated in a piperidine ring and in compounds B8 and B10 it is incorporated in a pyrrolidine ring.

In the light of these considerations and as an extension of recent interest in steroidal bisquaternary ammonium salts (May & Baker, 1963, 1965; Biggs, Davis & Wein, 1964; Khuong Huu-Lainé & Pinto-Scognamiglio, 1964; Mushin & Mapleson, 1964; Alauddin, Caddy & others, 1965) we investigated a series of ten monoquaternary ammonium salts derived from various 2β- and 3α-aminosteroids having androstane or pregnane skeletons and related in structure to acetylcholine through possession of an acetoxyl or hydroxyl substituent on the carbon atom next but one to the quaternary nitrogen atom. The general formulae of these compounds are in Fig. 1 and the individual structures are in Table 1.

Experimental

MATERIALS

The steroidal quaternary ammonium salts were kindly supplied by Organon Laboratories Ltd. Those bearing the nitrogen atom in the 2β-position were prepared as described in the patent literature (Organon Laboratories Ltd., 1966) and those bearing the nitrogen atom in the 3α-position were prepared by methods to be described by Hewett & Savage.

METHODS

Neuromuscular blocking activity

The neuromuscular blocking activity of the steroidal monoquaternary salts was evaluated using the following preparations.

Cat gastrocnemius muscle—sciatic nerve preparation. Each compound was tested in three animals. The method was a modification of that described by Bülbring & Burn (1942). Cats of either sex weighing 2–4 kg were anaesthetized by intraperitoneal injection of pentobarbitone sodium (60 mg/kg). Contractions of the gastrocnemius muscle were elicited by supramaximal stimulation of the sciatic nerve at a frequency of 6–8/min, 5–10 V, pulse width 1–2 msec. These values and the tension on the muscle (0·2–0·3 kg) were constant during any one experiment. The contractions of the muscle were recorded on smoked paper with a Brown-Schuster, spring-loaded, myograph lever. Arterial blood pressure was recorded from a common carotid artery using a mercury manometer. Drugs were dissolved in 0·9% w/v sodium chloride solution and administered intravenously into an external jugular vein.

Hen gastrocnemius muscle—sciatic nerve preparation. Each compound was tested in three animals. The method used was similar to that described above for the cat except that the hens (1–2·5 kg) were anaesthetized by injection of phenobarbitone sodium (200 mg/kg as a 10% w/v solution in saline) into a wing vein. This dose was sufficient to maintain anaesthesia throughout the experiment.

Rat phrenic nerve—diaphragm preparation. Each compound was tested on three preparations. The method was based on that of Bülbring (1946). Adult rats of either sex (150–200 g) were killed and a triangular-shaped section of the diaphragm was dissected out together with its

accompanying phrenic nerve. The preparation was then attached to a Bell's electrode and placed in an organ bath at 29° containing double glucose Tyrode solution gassed with oxygen. The contractions of the diaphragm were recorded on a moving smoked paper using a light Starling heart lever. The frequency of stimulation of the nerve was 6 square pulses/min at 5–10 V, pulse width 0·5–2 msec. The drugs were added to the bath and allowed to act for 3 min. An interval of 15 min was allowed before addition of the next dose of a drug to permit the magnitude of contraction to return to normal.

Frog rectus abdominis muscle preparation. Each compound was tested on three preparations. The method was that of Garcia de Jalon (1947). Reproducible submaximal contractions of the rectus muscle were induced by 1·0–2·0 μg/ml of acetylcholine. A suitable time interval between each dose of acetylcholine was found to be approximately 3 or 4 min. The contractions were recorded for periods of 30–90 sec. Using the same time interval between each dose of acetylcholine, each drug was added 30–60 sec before the addition of acetylcholine on the same preparation. Each drug was then quantitatively compared with (+)-tubocurarine for its antagonism to acetylcholine.

Experiments with mice. The method used was similar to that employed by Thomson (1946) for the assay of insulin in mice. Groups of ten male albino mice (18–24 g) were injected intraperitoneally at different dose levels with the drugs under test and the mice placed on a fine-mesh wire screen inclined at an angle of 60° to the horizontal. That dose of drug at which five out of the ten mice slid abruptly off the screen within 30 min of the injection was considered to be the median paralysing dose (PD 50) and was expressed as mg/kg of body weight. Similarly, the dose of drug at which five out of the ten mice died within 30 min was taken as the median lethal dose (LD 50) and expressed as mg/kg of body weight.

Anticholinesterase Activity

The acetylcholinesterase preparation was obtained from rat brain by the method of Fenwick, Barron & Watson (1957). *In vitro* anti-acetylcholinesterase activity was determined manometrically by adaptation of the method of Ammon (1933) and expressed as a pI 50 value according to the method of Blaschko, Bülbring & Chou (1949).

Ganglion Blocking Activity

Cat nictitating membrane preparation—sympathetic ganglion blockade. Cats of either sex weighing from 2·0–4·0 kg were anaesthetized by intraperitoneal pentobarbitone sodium (60 mg/kg). After the preganglionic cervical sympathetic chain was severed (Lewis & Muir, 1960), contractions of the nictitating membrane were elicited by supramaximal stimulation of the peripheral end of the chain by means of square impulses at a frequency of 800–1200/min, 8–15 V, pulse width 0·5–1·0 msec. Stimulation was for a period of 15 sec every 3 min and the drugs under test were injected 1 min before the next period of stimulation.

Peristaltic reflex of the isolated guinea-pig ileum—parasympathetic

ganglion blockade. The method employed was based on that of Trendelenburg (1917). Pieces of ileum about 7–10 cm long were removed from guinea-pigs of either sex (0·3–0·5 kg) and suspended in Tyrode solution at 30° gassed with oxygen. To prevent fatigue of the preparation, peristaltic movements were induced for 30 sec every 3 min. Drugs were added 30 sec before the initiation of peristalsis.

Results and discussion

The relative molar potencies (average of three determinations) of the compounds, as compared with (+)-tubocurarine = 100, in the cat, hen, frog and rat are shown in Table 1. Table 2 shows the PD 50, LD 50,

TABLE 2. PD 50 (± S.E.), LD 50 (±S.E.), THERAPEUTIC INDEX AND MOLAR POTENCY OF STEROIDAL MONOQUATERNARY AMMONIUM SALTS IN THE MOUSE. Observations from groups of ten animals

Code number	PD 50 mg/kg	LD 50 mg/kg	Therapeutic index LD 50/PD 50	Molar potency
B1	51 ± 3·45	64 ± 4·15	1·78	0·36
B2	46 ± 3·09	60 ± 3·30	1·30	0·47
B3	80 ± 4·49	82·3 ± 4·90	1·03	0·25
B4	136 ± 9·6	155 ± 9·00	1·14	0·11
B5	80·1 ± 4·39	102·8 ± 2·99	1·28	0·23
B6	64 ± 3·58	78·5 ± 4·09	1·23	0·31
B8	69·5 ± 2·39	71·8 ± 2·84	1·03	0·25
B9	70·2 ± 4·58	100·1 ± 4·23	1·43	0·24
B10	52 ± 2·33	59·5 ± 2·96	1·14	0·39
(+)-Tubocurarine	0·30 ± 0·03	0·54 ± 0·03	1·78	100

therapeutic index and molar potency of each compound in the mouse (with the exception of B7 of which insufficient quantities were available). Applying accepted criteria for the differentiation of depolarizing and non-depolarizing (cf. Paton & Zaimis, 1952) activities, all ten compounds were found to exhibit, on all preparations, typical non-depolarizing actions without any discernible depolarizing properties. Thus, in the cat, neuromuscular block produced by doses of 1–6 mg/kg of each steroid was intensified by (+)-tubocurarine (0·05–0·10 mg/kg) and in the hen, muscle contracture did not take place. In both the cat and the hen the block was quickly and completely reversed by neostigmine (0·02–0·10 mg/kg) and edrophonium (0·50–1·0 mg/kg). These results were supported by those obtained with the frog and rat preparations.

Although there is the possibility of an edge-on attachment of the β-acetoxy group and α-quaternary head to the receptor, the absence of depolarizing activity in all compounds incorporating a β-acetoxy-α-quaternary ammonium unit in their structure (i.e. all compounds except B5 and B9) might suggest that stereochemical factors imposed by the steroid nucleus are serving to prevent simultaneous access to both the anionic sites and the esteratic sites (e.g. Ing, 1949; Lands, 1951) of individual acetylcholine receptors, or that the quaternary ammonium groups are not permitted sufficiently close access to the anionic sites to initiate depolarization of the muscle endplate by whatever mechanism this initiation may occur (see Paton, 1961; Ariëns, 1964; Belleau, 1964, 1965).

Measurement of anticholinesterase activity revealed that the most

active of the steroids (compounds B6, B7 and B8) were more than 100 times less potent than eserine (pI50 values of 4·22 to 4·29; eserine, 6·54). It seems unlikely, however, in view of their non-depolarizing properties that they owe their neuromuscular blocking activity to an inhibition of acetylcholinesterase.

In both the cat and the hen, the time taken to reach maximal paralysis and the duration of the block were significantly less than those for (+)-tubocurarine. Moreover, as would be expected of non-depolarizing compounds (Zaimis, 1959), the potency and duration of action observed in the hen was in general comparable to that in the cat. There was, however, considerable variation in potency in other species. Thus, the sensitivity of the mouse (PD 50 values ranging from 46 $\pm$ 3·09 mg/kg to 136 $\pm$ 9·6 mg/kg) to the compounds was approximately four times (compound B8) to 17 times (compound B1) less than it was in the cat. On the other hand, the compounds were appreciably more active on the frog rectus abdominis muscle preparation in which their potency was approximately five times (compound B4) to 90 times (compound B2) as great as in the cat.

In addition to their neuromuscular blocking activity, as might have been expected (cf. Cavallito & Gray, 1960), all the monoquaternary compounds blocked both sympathetic and parasympathetic ganglia and produced a fall in the blood pressure of the pentobarbitone-anaesthetized cat. A given dose of any of the steroidal quaternary ammonium salts in each case gave greater neuromuscular blockade than sympathetic ganglion blockade where these effects were expressed as a percentage of the maximum blockade.

With the exception of compounds B2 and B7 as tested on the frog, the potency of the monoquaternary steroids as neuromuscular blocking agents was low and all were appreciably less potent than (+)-tubocurarine. The most active—compound B1—was 1/16th as active in the cat as (+)-tubocurarine on a molar basis while the least active—compound B8—was 1/100th as active. Since these two compounds are very similar in chemical constitution, differing only in the replacement of the 2β-*N*-methylpiperidinium grouping (in compound B1) by a 2β-*N*-methylpyrrolidinium grouping (in compound B8), it is probable that the variation in potency is directly ascribable to substituent effects with respect to the nitrogen atom with their attendant minor changes in the charge density, rather than to any critical change in hydrophilic to lipophilic balance (Cavallito, 1959). Further emphasis on the importance of factors other than hydrophilic to lipophilic balance in determining neuromuscular blocking activity is provided in the present work by the demonstration that compound B3 (the reversed analogue of compound B1 with respect to the acetoxyl and quaternary ammonium functions) is only 1/4th as active as compound B1.

Acknowledgments. The authors cordially thank Drs. W. R. Buckett, C. L. Hewett and D. S. Savage of Organon Laboratories Ltd., Newhouse, Lanarkshire, for providing the compounds studied and for valuable advice in the course of the work.

References

Alauddin, M., Caddy, B., Lewis, J. J., Martin-Smith, M. & Sugrue, M. F. (1965). *J. Pharm. Pharmac.*, **17**, 55–59.

Ammon, R. (1933). *Pflügers Arch. ges. Physiol.*, **233**, 486–491.

Ariëns, E. J. (1964). *Molecular Pharmacology. The Mode of Action of Biologically Active Compounds*, vol. 1, New York & London: Academic Press.

Barlow, R. B. (1960). In *Biochemical Society Symposia No.* 19, *Steric Aspects of the Chemistry and Biochemistry of Natural Products*, pp. 46–66, Cambridge University Press.

Barlow, R. B. & Ing, H. R. (1948a). *Nature, Lond.*, **161**, 718.

Barlow, R. B. & Ing, H. R. (1948b). *Br. J. Pharmac. Chemother.*, **3**, 298–304.

Battersby, A. R. & Hodson, H. F. (1958). *Proc. chem. Soc.*, 287–288.

Battersby, A. R. & Hodson, H. F. (1960). *J. chem. Soc.*, 736–741.

Belleau, B. (1964). *J. mednl Chem.*, **7**, 776–784.

Belleau, B. (1965). In *Advances in Drug Research*, vol. 2, pp. 89–126, editors Harper, N. J. & Simmonds, A. B., London: Academic Press.

Biggs, R. S., Davis, M. & Wein, R. (1964). *Experientia*, **20**, 119–120.

Blanpin, O. & Bretaudeau, J. (1961). *C.r. Séanc. Soc. Biol.*, **155**, 878–883.

Blanpin, O. & Pierre, R. (1961). *Thérapie*, **16**, 179–183.

Blaschko, H., Bülbring, E. & Chou, T. C. (1949). *Br. J. Pharmac. Chemother.*, **4**, 29–32.

Bovet, D. (1959). In *Curare and Curare-Like Agents*, editors Bovet, D., Bovet-Nitti, F. & Marini-Bettolo, G. B., Amsterdam: Elsevier.

Bülbring, E. (1946). *Br. J. Pharmac. Chemother.*, **1**, 38–61.

Bülbring, E. & Burn, J. H. (1942). *J. Physiol., Lond.*, **101**, 224–235.

Cavallito, C. J. (1959). In *Curare and Curare-Like Agents*, editors Bovet, D., Bovet-Nitti, F. & Marini-Bettolo, G. B., pp. 288–303, Amsterdam: Elsevier.

Cavallito, C. J. & Gray, A. P. (1960). In *Fortschr. ArzneimittForsch.*, editor, Jucker, E., vol. 2, pp. 135–226, Basel: Birkhauser.

Crum Brown, A. & Fraser, T. R. (1869). *Trans. R. Soc., Edin.*, **25**, 151–203; 693–739.

Edwards, D., Lewis, J. J. & Marren, G. (1966). *J. Pharm. Pharmac.*, **18**, 670–676.

Fenwick, M. L., Barron, J. R. & Watson, W. A. (1957). *Biochem. J.*, **65**, 58–67.

Garcia de Jalon, P. D. (1947). *Q. Jl Pharm. Pharmac.*, **20**, 28–30.

Hey, P. (1952). *Br. J. Pharmac. Chemother.*, **7**, 117–129.

Huguenard, P. & Martin, C. (1950). *Anaesth. Analg.*, **7**, 336–346.

Ing, H. R. (1949). *Science, N.Y.*, **109**, 264–266.

Karrer, P., Eugster, C. H. & Waser, P. G. (1949). *Helv. chim. Acta*, **32**, 2381–2385.

Khuong Huu-Lainé, F. & Pinto-Scognamiglio, W. (1964). *Archs int. Pharmocodyn. Thér.*, **147**, 209–219.

Lands, A. M. (1951). *J. Pharmac. exp. Ther.*, **102**, 219–236.

Lewis, J. J. & Muir, T. C. (1960). *Lab. Pract.*, **9**, 712–715.

Loewe, S. & Harvey, S. C. (1952). *Arch. exp. Path. Pharmak.*, **214**, 214–226.

May & Baker Ltd. (1963). South African Patents 63/940, 63/941.

May & Baker Ltd. (1965). British Patent 1,003,681.

Mushin, W. W. & Mapleson, W. W. (1964). *Br. J. Anaesth.*, **36**, 761–768.

Organon Laboratories Ltd. (1966). British Patent 1,039,441.

Paton, W. D. M. (1961). *Proc. R. Soc.*, **B154**, 21–69.

Paton, W. D. M. & Zaimis, E. J. (1949). *Br. J. Pharmac. Chemother.*, **4**, 381–400.

Paton, W. D. M. & Zaimis, E. J. (1952). *Pharmac. Rev.*, **4**, 219–253.

de Reuck, A. V. S. (1962). *Ciba Foundation Study Group No.* 12, *Curare and Curare-Like Agents*, London: Churchill.

Stenlake, J. B. (1963). In *Progress in Medicinal Chemistry*, editors Ellis, G. P. & West, G. B., vol. 3, pp. 1–51, London: Butterworths.

Thomson, R. E. (1946). *Endocrinology*, **39**, 62.

Trendelenburg, P. (1917). *Arch. exp. Path. Pharmak.*, **81**, 55–129.

Zaimis, E. J. (1959). In *Curare and Curare-Like Agents*, editors Bovet, D., Bovet-Nitti, F. & Marini-Bettolo, G. B., pp. 191–203, Amsterdam: Elsevier.