STUDIES ON THE PERIPHERAL AND CENTRAL PHARMACOLOGICAL ACTIVITY OF A SERIES OF SYNTHETIC NITROGENOUS STEROIDS

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ABSTRACT

SECTION A. EFFECTS OF NITROGENOUS STEROIDS ON THE CENTRAL NERVOUS SYSTEM.

A study has been made of the pharmacological properties of 26 nitrogenous steroids, with particular reference to their effects on the central nervous system. The results are discussed in terms of possible correlations between chemical structure and biological activity.

Twenty-three of the compounds under investigation were androstanes or pregnanes bearing a substituted amino group in the 2β - or 3α - position. Two of the remaining compounds carried an amino substituent at either the 6α- position (compound A18), or at the 17β - position (compound A25) whilst the material designated compound A7 was in fact an equilibrium mixture of 2α - morpholino-5α -androstane-3,17-dione and 2β -morpholino-5α - androstane-3,17-dione.

The compounds were investigated in mice for general anaesthetic and anti-Parkinsonian activity and also for their ability to induce loss of the righting reflex and to antagonize electrically- and chemically-induced seizures. In addition, the effects of the compounds on blood pressure and on neuromuscular and ganglionic transmission in the anaesthetized cat were investigated as were their effects on a polysynaptic

preparation of the spinalized cat.

Three of the compounds (A12, A17 and A24) on intravenous administration to mice, induced loss of the righting reflex and this effect was attributed to the possession of interneuronal blocking activity by these compounds. With respect to anticonvulsant activity, none of the compounds gave protection against electrically-induced seizures without the simultaneous presence of side effects such as excitation, sedation and/or loss of the righting reflex while, apart from one example (compound A24), no compound was active against leptazol-induced seizures. From these results it has been concluded that anticonvulsant activity is not a specific property of the compounds under investigation.

None of the compounds investigated produced a degree of anaesthesia sufficient for surgery nor did they antagonize the tremors induced by tremorine, the latter result suggesting the absence of anti-Parkinsonian activity.

It was observed that anticonvulsant activity and ability to induce loss of the righting reflex appeared to be associated more with compounds which have the nitrogenous radical in the 2β- position than with compounds which have the nitrogen function in the 3α- position and that the presence of additional nuclear substituents appeared to diminish the power to induce loss of the righting reflex.

SECTION B. EFFECTS OF NITROGENOUS STEROIDS AT THE NEUROMUSCULAR JUNCTION.

A total of ten 2β- and 3α- monoquaternary ammonium steroids of the androstane and pregnane types, as well as a single bisquaternary ammonium androstane have been investigated for neuromuscular blocking activity. The results are again discussed in terms of possible structure-action relationships.

The evaluation of the muscle relaxant activity of the compounds studied was carried out employing conventional techniques with the cat and the hen gastrocnemius muscle-sciatic nerve preparations, the rat phrenic nerve-diaphragm preparation and the frog rectus abdominis muscle preparation. Toxicity tests on mice were also conducted as were studies of the effects of the compounds on autonomic ganglia in the cat and the guinea pig and on respiratory musculature in the cat and the rabbit.

Applying the accepted criteria for the qualitative differentiation of depolarizing and non-depolarizing activity, all eleven compounds were shown to display typical non-depolarizing activity. In both the cat and the hen, the time of onset of maximum paralysis and the duration of block was significantly less than those for tubocurarine.

In all species examined, with the exception of the frog, the activity of the ten monoquaternary ammonium steroids as muscle relaxants proved very low and all were considerably less potent than tubocurarine. The most active compound (B1)

was only 1/16th as active on the cat as tubocurarine on a molar basis. Differences in potency between the various monoquaternary ammonium steroids have been interpreted as arising from variations in the nitrogen substituents rather than from alterations in hydrophilic to lipophilic balance.

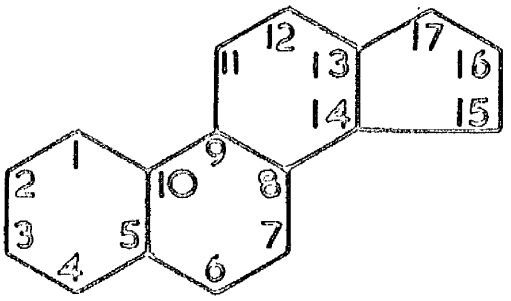
The one bisquaternary steroid under examination (compound B11) showed potency comparable to or greater than that of tubocurarine in all species, except the mouse. Consideration of the activity of this compound in terms of the activities of other steroidal bisquaternary ammonium salts already reported in the literature has been taken to indicate the relative unimportance of a "fixed" interonium distance in determining neuromuscular blocking activity. In contrast to the situation pertaining with the monoquaternary compounds which would seem to be without possible therapeutic application, the short-acting potent non-depolarizing properties of the bisquaternary ammonium compound, coupled with its apparent freedom from undesirable side effects, suggest that serious consideration of clinical trials for this compound is in order.

C O N T E N T S

CONVENTIONS FOR CITING REFERENCES

The conventions used in this thesis are those recommended in "Suggestions to Authors" (*J. Physiol.* (1966), **182**, 1 - 33). Where abbreviations did not appear in the Journal of Physiology, the *World List of Scientific Periodicals* (4th edn. 1963, London: Butterworths) was consulted.

GENERAL INTRODUCTION.



PERHYDROCYCLOPENTENOPHENANTHRENE.

GENERAL INTRODUCTION.

Steroids, which can be formally regarded as derivatives of the perhydrocyclopentenophenanthrene ring system - including compounds related to the parent nucleus by processes of ring enlargement, ring contraction and ring cleavage - are of virtually ubiquitous occurrence in both the plant and animal kingdoms (Fieser & Fieser, 1959; Deane, 1962; Holmes, Phillips & Chester Jones, 1963; Pincus, Thimann & Astwood, 1964). They constitute a most intriguing class of compounds and embrace many biologically-active members of particular physiological and pharmacological significance (see Woodbury, 1958; Villee & Engel, 1961; Applezweig, 1962; Bush, 1962; Deane, 1962; Sarett, Patchett & Steelman, 1963; Martini & Pecile, 1964; Dorfman, 1964, 1965) including subgroups as varied and important as the adrenocortical, male and female sex hormones, the cardiac glycosides, the bile acids, the anti-rachitic vitamins, certain saponins, the toad poisons and certain alkaloids.

Prior to 1952, the view was held that, with the exception of the oestrogens, structural requirements for the primary biological activities exhibited by steroids were extremely rigid and that no synthetic steroid would be capable of exhibiting greater potency than the natural steroid hormones since it was considered that the processes

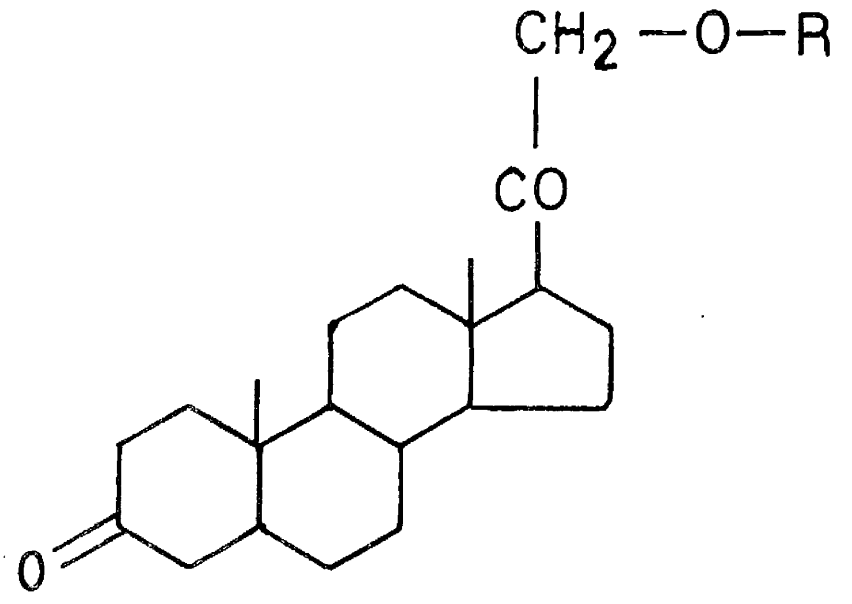
of evolution would ensure natural occurrence of the most effective compound. However, the demonstration that 9α-halogenated adrenocortical steroids, although still possessing powerful mineralocorticoid activity, were more potent anti-inflammatory agents than cortisone or hydrocortisone (Fried & Sabo, 1953, 1954), indicated the potential value of exploring the effects of structural alterations to steroidal molecules and as a result the preparation of steroid hormone analogues (Applezweig, 1962) became an intensively exploited field. A most important fact to emerge from these studies was that it was possible to obtain steroids in which various minor biological actions characteristic of natural steroid hormones were accentuated at the expense of the major physiological actions, and there are now available androgen analogues exhibiting reduced masculinizing but increased anabolic properties, progesterone-like compounds exhibiting general anaesthetic activity and oestrogen analogues having diminished feminizing but increased lipodiatic effects - several of which have come to assume an important place in modern clinical practice.

Included among the new synthetic steroids prepared as potential new non-hormonal therapeutic agents are many possessing nitrogen atoms, and indeed considerable interest has been shown in compounds of this type (Alauddin & Martin-Smith, 1962a, b; Martin-Smith & Sugrue, 1964). While

synthetic nitrogenous steroids exhibit a broad spectrum of biological activity (Martin-Smith & Sugrue, 1964), and many of them have been shown to possess potent anabolic (Matscher, Lupo & De Ruggieri, 1962; Arnold, Potts & Beyler, 1963), anti-inflammatory (Hirschmann and others, 1963; Steelman and others, 1963) and anti-hypercholesterolaemic activity (Counsell, Klimstra & Ranney, 1962; Ranney & Counsell, 1962a, b; Cantrall and others, 1963), their clinical application to date is not widespread, although there are indications that the full potentialities of this class of steroid have not yet been realized (Martin-Smith & Sugrue, 1964).

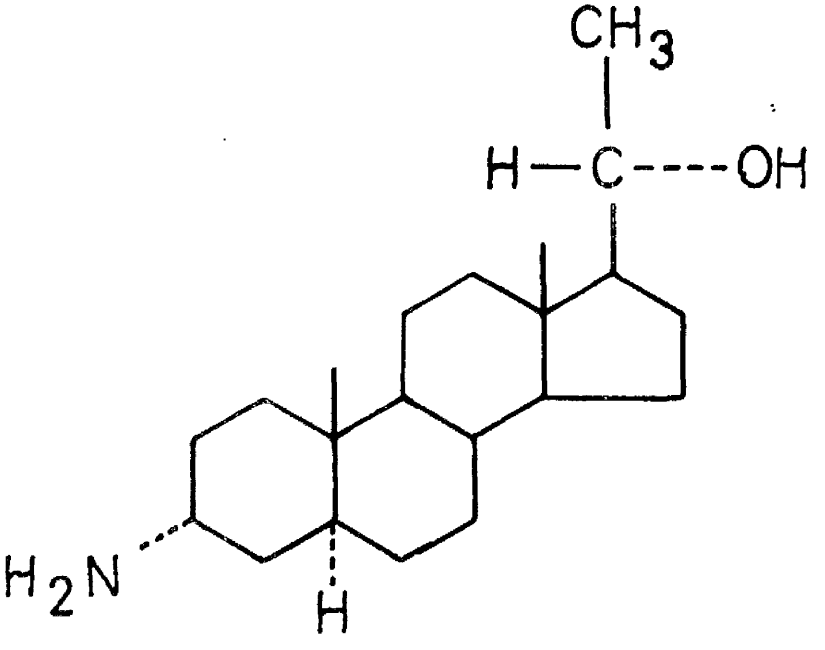
In particular, the depressant effects of nitrogenous steroids on the central nervous system (La Barre & Desmarez, 1959; Sugrue, 1963; Hewett, Savage, Lewis & Sugrue, 1964) and the potential of steroidal quaternary ammonium compounds in the field of neuromuscular pharmacology (May & Baker, 1963, 1965; Biggs, Davis & Wien, 1964; Alauddin, Caddy, Lewis, Martin-Smith & Sugrue, 1965) have attracted recent attention.

PURPOSE OF RESEARCH.



AMINO ESTERS OF 21-HYDROXYPREGNANEDIONE

R = Amino ester grouping



FUNTUMIDINE

SECTION A. EFFECTS OF NITROGENOUS STEROIDS ON THE CENTRAL NERVOUS SYSTEM.

With the discovery that adrenocortical and sex hormones possess sedative and hypnotic properties as well as convulsant or anticonvulsant activity, in addition to their hormonal actions, a search has been instituted for new synthetic steroids having potent central nervous system activity but devoid of any hormonal properties. As a result, several groups of nitrogenous steroids have been discovered which possess varying degrees of anticonvulsant, sedative and even general anaesthetic properties (Alauddin & Martin-Smith, 1962a, b; Martin-Smith & Sugrue, 1964, and references cited therein). These nitrogenous steroids include various amino esters of 21-hydroxypregnanedione which possess general anaesthetic activity (Figdor and others, 1957), the alkaloid funtumidine (3α-amino-20α-hydroxy-5α-pregnane) which is claimed to cause tranquillisation comparable to that of reserpine (La Barre & Desmarez, 1959) and several 2β- 6β- and 16β- morpholino steroids which display central nervous system depressant activity, as evidenced by induction of the loss of the righting reflex in the mouse (Hewett et al. 1964). However, despite the discovery of these compounds no nitrogenous steroid of clinical value as a central depressant has yet been found and it seems probable that the full potential of

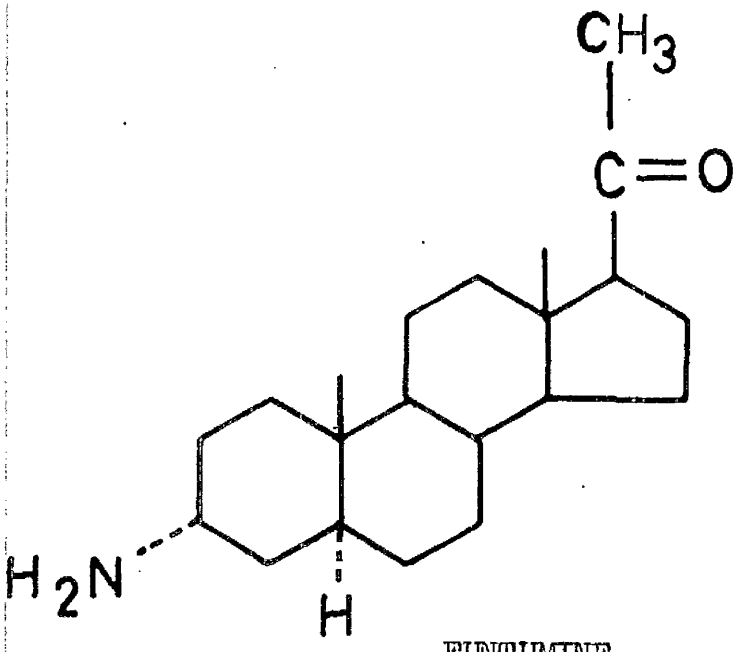
nitrogenous steroids with respect to central activity has still to be realized.

The present work described in this thesis represents part of the continuation of the search for a clinically useful nitrogenous steroidal central nervous system depressant - namely the determination of the pharmacological effects of a series of nitrogenous steroids on the central nervous system. These steroids were synthesised by Organon Laboratories Ltd. and kindly supplied by Dr. C.L.Hewett.

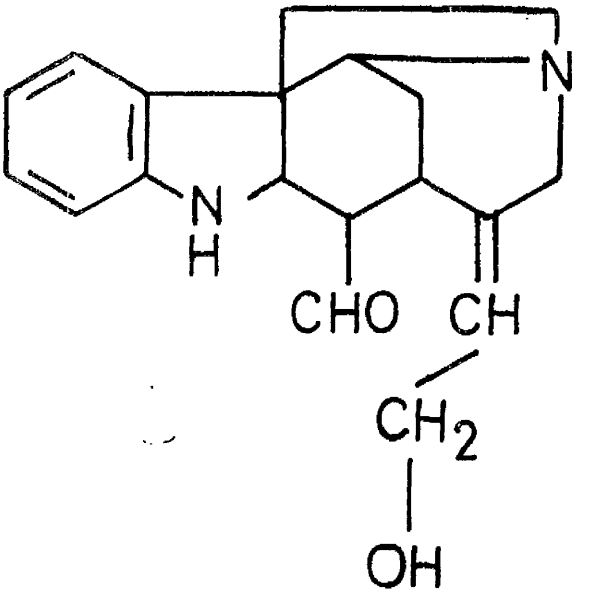
The results obtained in the present study are then discussed in terms of the structural requirements apparently necessary (in the light of present day knowledge) for a steroid to exhibit activity on the central nervous system.

SECTION B. EFFECTS OF NITROGENOUS STEROIDS AT THE NEUROMUSCULAR JUNCTION.

In recent times, owing to the potent neuromuscular blocking properties displayed by bisquaternary ammonium and trisquaternary ammonium compounds, coupled with the observation that certain monoquaternary ammonium compounds show marked cholinergic properties (Huguenard & Martin, 1950; Hey, 1952), there has been a virtual neglect of monoquaternary ammonium salts as potential muscle relaxants. Some recently published observations, however, have pointed



FUNTUMINE



WIELAND – GUMLICH ALDEHYDE

to the possibility of a profitable re-assessment of monoquaternary ammonium compounds, especially steroidal monoquaternary ammonium salts. For instance, the discovery that the trimethylammonium salts derived from the steroidal alkaloids, funtumine (3α-amino-20-oxo-5α-pregnane) and funtumidine (3α-amino-20α-hydroxy-5α-pregnane) exhibit weak non-depolarizing neuromuscular blocking activity (Blanpin & Bretaudeau, 1961; Blanpin & Pierre, 1961) demonstrates that muscle relaxant activity is certainly present in such compounds. Further, the possibility that the very potent bisquaternary compound C-toxiferine-I might be dissociating *in vivo* into two molecules of monoquaternary ammonium compound was suggested by the known *in vitro* hydrolysis in mild acid conditions of C-toxiferine-I into the metho-salt of the Wieland-Gumlich aldehyde (Battersby & Hodson, 1958, 1960) - and indeed some monoquaternary salts of *Strychnos*-type alkaloids are claimed to possess neuromuscular blocking potency comparable to that of tubocurarine (Karrer, Eugster & Waser, 1949). Finally, recent work with certain bisquaternary steroidal compounds (Kay & Baker, 1963, 1965) has cast doubt upon the validity of the classic two-point attachment theory of neuromuscular blockade and placed new emphasis on concepts such as the adumbration theory (Loewe & Harvey, 1952) with its postulate of a one-point attachment.

Accordingly, the present worker has investigated a series of 10 monoquaternary ammonium salts derived from 2β - and 3α- amino steroids of the androstane and pregnane types, which were kindly made available through the courtesy of Dr. C.L. Hewett of Organon Laboratories Ltd.

In addition, one bisquaternary ammonium steroid was also investigated in view of the recent interest in compounds of this type, which have been employed in attempts to elucidate the exact nature and properties of the cholinergic receptor (Alauddin et al.1965, and references cited therein). Such compounds have also aroused clinical interest (Mushin & Mapleson, 1964) since they possess, in animals, short-acting, non-depolarizing activity quantitatively similar to that of tubocurarine (Biggs et al. 1964).

S E C T I O N A

I. SURVEY OF THE EFFECTS OF STEROIDS ON THE CENTRAL NERVOUS SYSTEM.

A well-established minor pharmacological action associated with steroids is central nervous system activity. Not only is this property present in naturally-secreted steroid hormones of both the adrenocortical series and the sex hormone series, but it is also found in many synthetic non-hormonal steroids, including a number of nitrogenous derivatives.

Indeed, there is a large literature dealing with the central actions of steroids. Unfortunately, however, this is complex and frequently contradictory. Nevertheless, it would appear that sedative and hypnotic actions and convulsant or anticonvulsant actions are the major effects observed although very recently potent analgesic activity has been discovered in a synthetic steroid. In attempts to discover more about the underlying processes bringing about these phenomena, a number of studies have been made on the effects of steroids on brain excitability while attention has also been devoted to biochemical work, although the results from these studies have in fact done little to clarify the overall picture. The major features of central activity in steroids can be briefly summarised as follows:-

i) ADRENOCORTICOIDS.

This group would appear to have seen greater

investigation than any other. Perhaps this is not surprising in view of the natural role of the adrenocorticoids in controlling carbohydrate, fat and protein metabolism and in controlling the distribution and excretion of water and electrolytes throughout the body. Changes in brain excitability produced by both naturally-occurring adrenocorticoids and synthetic steroids having the ability to mimic these adrenocortical hormones have been assessed by their ability (a) to raise or lower the threshold at which electroshock seizures occur; (b) to protect against leptazol-induced convulsions; (c) to enhance or diminish seizures induced by auditory stimulation and (d) to alter the normal electroencephalogram pattern, but such tests give little indication of the exact locus of action of these compounds within the central nervous system. Certainly such experiments tell nothing of the fundamental mechanism by which the steroids exert their actions.

The effects produced by adrenocorticoids in the minimal electroshock seizure threshold (EST) test [which is defined (Woodbury & Davenport, 1952) as the smallest amount of current, in milliamperes (mA), delivered for a fixed period of time (0.2 seconds) through corneal electrodes, required to elicit detectable convulsions in experimental animals] can in general be correlated with the glucocorticoid or mineralocorticoid activity they possess. Thus, in intact rats, the glucocorticoids cortisone,

hydrocortisone, prednisone, prednisolone and fludrocortisone have been shown to decrease the EST, with the potency of the three synthetic derivatives being approximately 3 - 4 times, on a weight basis, that of cortisone and hydrocortisone (Mansor, Holtkamp, Heming & Christian, 1956). On the other hand, the mineralocorticoid desoxycorticosterone would seem to increase the EST of normal rats although corticosterone, the major secretory product of the adrenal cortex of this species (Bush, 1953), like adrenocorticotrophic hormone (ACTH), appears to have little or no effect (Woodbury & Sayers, 1950; Woodbury, 1952, 1954; Timiras, Woodbury & Goodman, 1954; Woodbury, Timiras & Vernadakis, 1957). Nevertheless, despite having little or no direct effect on the EST in intact rats, corticosterone, like aldosterone (Woodbury et al. 1957), would appear to have the ability to prevent both the increase in the EST caused by desoxycorticosterone and the decrease in the EST produced by hydrocortisone.

Adrenalectomy of rats with its resultant abolition of corticosterone biosynthesis has been found to decrease the EST, this effect being reversed both by saline and by desoxycorticosterone (Davenport, 1949; Timiras et al. 1954). Indeed, the ability of desoxycorticosterone to increase the EST becomes more pronounced in adrenalectomized rats as does the ability of hydrocortisone

to decrease the EST (Woodbury and others, 1951; Woodbury, 1954).

Despite suggestions to the contrary, (Hoagland, 1954), changes in brain excitability in adrenalectomized animals would not seem attributable to the reduced cerebral blood flow and oxygen consumption imposed by adrenalectomy since administration of saline to adrenalectomized rats still prevents a decrease in the EST in the presence of a reduction in cerebral blood flow and oxygen consumption (Woodbury, 1958).

In the light of the above facts, Woodbury (1954) has suggested that corticosterone may have a regulatory role in the rat serving to normalize an increased or decreased brain excitability. The same author (Woodbury, 1958) has also attempted to explain ability to interfere with the excitability of the central nervous system by postulating that adrenocortical hormones modify brain excitability by affecting brain sodium ion transport and gamma-aminobutyric acid (GABA) metabolism (vide infra).

In contrast to its ability to lower the EST as previously described, adrenalectomy (like adrenocortical hypofunction) would not seem to increase the susceptibility of rodents to leptazol-induced convulsions (Torda & Wolff, 1952a) and may even elevate the leptazol seizure threshold (Little & Conrad, 1960).

A contradictory and confusing picture, which may

be explicable in terms of dosage phenomena and/or duration of drug administration, of the effects of adrenocortical hormones and ACTH on the sensitivity of experimental animals to leptazol has emerged. Thus on the one hand daily administration of ACTH (6 - 8 mg/kg for 3 to 4 days) has been claimed to decrease the susceptibility of intact rats to leptazol while on the other hand, a single injection (1 - 8 mg/kg) of ACTH has been stated to increase the susceptibility of these animals to leptazol (Torda & Wolff, 1951, 1952a). Desoxycorticosterone appears to act in an opposite manner, acute administration (25 mg/kg) having been claimed to increase (Koch, 1959) and prolonged administration (0.5 mg daily for 8 days) to decrease (Swinyard, Schiffman & Goodman, 1955) the leptazol threshold. If one accepts the proposed (Woodbury, 1958) correlation between changes in the EST produced by desoxycorticosterone and fluctuations in the brain sodium ratio (the ratio of the concentration of extracellular to intracellular sodium ions in the brain), leptazol could conceivably be acting by annulling the anticonvulsant effect of this steroid either by interfering with the "sodium pump" mechanism for the extrusion of intracellular brain sodium ions or by facilitating the entry of sodium ions into brain cells (Swinyard et al. 1955).

In the audiogenic seizure test, in which the experimental animal is exposed to a sound stimulus in the

90 - 120 decibells range (Bevan, 1955), a characteristic seizure pattern is observed (Werboff, Hedlund & Havlena, 1963). The adrenocortical hormones have the ability to antagonize the different phases of the seizures produced by this method and so have been assayed for central activity in this way. Use of this test, however, has given rise to conflicting reports of the activity of cortisone (Ginsberg & Roberts, 1951; Vicari, Tracy & Jongbloed, 1952). Desoxycorticosterone is claimed to decrease the susceptibility of rats to audiogenic seizures (Colfer, 1947) while ACTH is stated to have no effect in this test (Hurder & Sanders, 1953).

Interpretation of the observed changes in the electroencephalogram (e.g.g.) induced by adrenocortical steroids in pathogenic conditions is very difficult. In patients with collagen diseases, cortisone or ACTH have been reported to restore abnormal e.e.g. patterns (characterized by high voltage, slow waves of 3 - 7/sec) to normal (Friedlander & Rottger, 1951; Pine, Engel & Schwartz, 1951) while in subjects with previously normal records, administration of these compounds can induce slowing of the alpha rhythm from 12 - 13/sec to 7 - 8/sec (Hoefer & Glaser, 1950; Debré, Mozziconacci & Nekhorocheff, 1952; Glaser, Kornfield & Knight, 1955). In this connection it is of interest that the e.e.g. patterns of normal,

non-hospitalized subjects during cortisone or ACTH administration did not differ from those obtained during the control period when no hormone was being administered (Friedman & Engel, 1956).

Desoxycorticosterone, in contrast to its ability to normalize the EST of adrenalectomized rats (Davenport, 1949), does not restore to normal the e.e.g. of these animals (Bergen, 1951) nor that of Addisonian patients (Hoffman, Lewis & Thorn, 1942).

Experimental studies attempting an assessment of the convulsant and anticonvulsant effects of adrenocorticoids in animals are few. However, convulsions have been observed following administration of ACTH to rabbits (Pincus, Natelson & Lugovoy, 1951) or administration of cortisone to various other rodents (Hicks, 1953).

Clinical observations are more numerous although the picture emerging is somewhat confusing. Thus, on the one hand, convulsive episodes, not of hypoglycaemic origin (Storrie, 1953), seem a not uncommon feature of Addison's disease (Engel & Margolin, 1941) in which adrenal hypotrophy decreases production of adrenocorticoids while, on the other hand, administration of cortisone and ACTH has been observed to result in generalized convulsions and even status epilepticus when employed therapeutically (Elkinton and others, 1949; Astwood, Raben, Payne & Cleroux, 1950;

Dorfman, Apter, Smull, Bergenstal & Richter, 1951; Irons, Ayer, Brown & Armstrong, 1951; Wayne, 1954). These last observations are also of interest in the light of the fact that ACTH is without effect or only slightly raises the EST in rats (Woodbury, 1954), but an explanation for the apparent anomaly may lie in the fact that in man ACTH liberates mainly hydrocortisone (Sweat, Abbott, Jeffries & Bliss, 1953), which will increase brain excitability, while in the rat ACTH liberates corticosterone which is without appreciable influence on brain excitability (Woodbury, 1954).

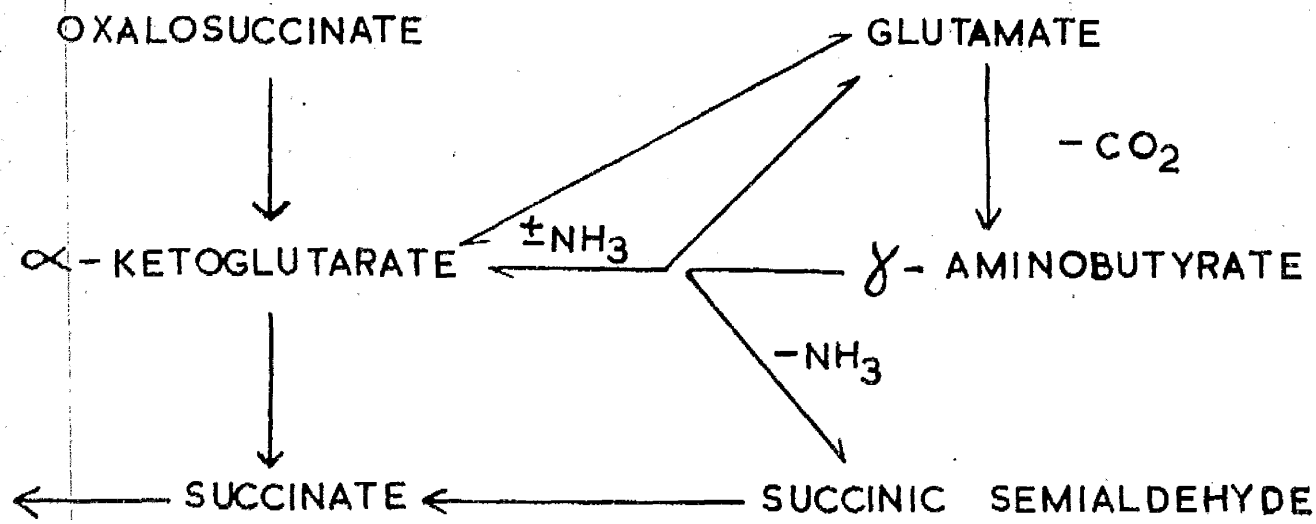
The first intimation of the existence of sedative and hypnotic activity in steroids of the adrenocorticoid group came from the work of Selye (1941a, b, 1942a) who showed that desoxycorticosterone and dehydrocorticosterone possessed appreciable central depressant activity. Cortisone was only weakly depressant (Selye, 1942a). Subsequent studies (Figdor and others, 1957; Atkinson, Davis, Pratt, Sharpe & Tomich, 1965) have confirmed the sedative and hypnotic activity of the naturally-occurring adrenocorticoids as well as many of their synthetic, non-hormonal, analogues.

Theories as to the Mechanism of Action of Adrenocorticoids in the Central Nervous System.

While various steroids including

desoxycorticosterone inhibit brain respiration *in vitro* (Eisenberg, Gordan & Elliot, 1949a,b; Gordan, Bentinck & Eisenberg, 1951), there is little evidence to indicate that this property represents the basic mechanism by which these compounds exert their central action (Eiduson, Geller, Yuwiler & Eiduson, 1964). Likewise, attempts to interpret the central nervous system activity of various steroids in terms of interference with possible central transmitters such as acetylcholine (Crossland, 1960; Eccles, 1962) and 5-hydroxytryptamine (5-HT) (Erspamer, 1961; Shore, 1962; Garattini & Valzelli, 1965) have met with little definitive support (Torda & Wolff, 1944, 1952b,c; Kato, 1960; Takahashi, Nasu, Tamura & Kariya, 1961; Giarman & Pepeu, 1962).

On the other hand, the postulated association between the effects of adrenocortical hormones on brain excitability, as measured by the EST levels, and the metabolism of gamma-aminobutyric acid (GABA), a possible central nervous system transmitter (Elliot & Jasper, 1958; Roberts, 1960), has received a measure of experimental support (Woodbury & Vernadakis, 1958). Thus, adrenalectomy and the administration of hydrocortisone to adrenalectomized rats both decrease brain GABA levels and increase brain excitability whereas administration of desoxycorticosterone to adrenalectomized rats markedly


OXALOSUCCINATE
GLUTAMATE
$-CO_2$
α-KETOGLUTARATE
$\pm NH_3$
γ-AMINOBUTYRATE
$-NH_3$
SUCCINATE
SUCCINIC SEMIALDEHYDE

increases brain GABA levels and decreases brain excitability. Since in intact rats, neither desoxycorticosterone nor hydrocortisone significantly alters brain GABA concentrations it is possible that hydrocortisone and adrenalectomy could reduce brain GABA levels by inducing the removal of glutamic acid from the main route of GABA metabolism and facilitating its entry via α - ketoglutarate into the tricarboxylic acid cycle (see diagram opposite), whilst desoxycorticosterone could increase GABA levels by preventing the entrance of glutamic acid into the tricarboxylic acid cycle, thus making it available for conversion to GABA (Vernadakis & Woodbury, 1960).

As might be expected from the normal function of adrenocorticoids, evidence has accrued that adrenocortical hormones interfere with brain electrolyte metabolism. Both adrenalectomy (Davenport, 1949; Timiras et al.1954) and hydrocortisone administration (Woodbury et al. 1957), to intact and adrenalectomized rats, decrease the brain sodium ratio (i.e., increase the intracellular brain sodium ion concentration), as measured photometrically (Davenport, 1949; Timiras et al. 1954), and increase brain excitability. In analogous situations, desoxycorticosterone, in both intact and adrenalectomized rats, increases the brain sodium ratio (i.e., decreases the intracellular brain sodium ion concentration) and

decreases brain excitability (Woodbury & Davenport, 1949; Woodbury et al. 1957).

In the light of the above observations on the effects of adrenocortical steroids on both brain sodium ratio and brain GABA concentration, Woodbury (1958) has presented an integrated explanation of the way in which adrenocorticoids modify brain excitability in terms of basic metabolic phenomena. Thus he considers that the propensity of the glucocorticoid hydrocortisone to increase brain excitability stems directly from its dual function of decreasing brain sodium ratio and of decreasing brain GABA levels while the ability of the mineralocorticoid desoxycorticosterone to decrease brain excitability is a direct result of its action in increasing both brain sodium ratio and brain GABA levels. The lower susceptibilities of the brain of the intact rat as compared with that of the adrenalectomized rat to an increase in excitability on administration of hydrocortisone or to a decrease in excitability on administration of desoxycorticosterone can then be rationalised in terms of the modifying influence of corticosterone. Thus in the intact rat, the release of corticosterone is considered to block any changes in brain GABA concentrations and so excitability changes stem mainly if not solely from changes in sodium ratios. In the adrenalectomized rat, where corticosterone secretion has been abolished, since brain

excitability changes will result from a summation of changes in both sodium ratios and GABA levels, more pronounced effects should be observed, as is indeed the case.

ii) SEX HORMONES.

In addition to their role in sexual function, the oestrogens obviously have the ability to induce changes in brain excitability as indicated by comparative data with mature and ovariectomized female rats and mature male rats. Thus intact, mature, female rats have been reported to exhibit lower seizure thresholds of excitability than intact males (Woolley, Timiras, Rosenzweig, Krech & Bennett, 1961) or ovariectomized females of the same age and strain (Woolley & Timiras, 1962b). Again, variations in oestrogen levels (as determined by daily vaginal smears) during the oestrus cycle apparently parallel alterations in brain seizure thresholds, with the EST being highest during diocstrus and lowest during oestrus (Woolley, Timiras, Srebnik & Silva, 1961). Daily administration of oestradiol to rats of either sex (0.04 - 5.0 mg/kg) has also been observed (Woolley & Timiras, 1962a) to lower markedly the EST and to give rise to a convulsant effect, characterized by tonic flexor phases of a shorter duration than those of untreated control animals, on the maximal electroshock seizure (MES) pattern which can be employed (Woolley & Timiras, 1962b,c) as

a measure of the ability of steroids to interfere with the characteristic pattern of electroshock convulsions elicited in control animals by the passage of a fixed, high density current of 150 mA through corneal electrodes.

In contrast to oestradiol, which uniformly exerts a direct excitatory effect on brain function, the male sex hormone, testosterone, appears able to increase or decrease brain excitability in the rat depending on the type of seizure studied, the age of the animal and the dose of hormone administered (Woolley & Timiras, 1962c). For example, testosterone in daily doses of 8.0 mg/kg has been reported to increase the EST and to exert an anticonvulsant effect on the MES pattern of 10 week old rats. However, by the time the rats have reached 13 weeks of age, continuous administration of the same daily dose of the hormone is stated to result in a change from anticonvulsant action on the MES pattern to a convulsant action in spite of which the EST remains increased (Woolley & Timiras, 1962c).

Progesterone (5.0 mg/kg/day) has been stated to produce an initial rise in the EST in female (but not in male) rats, an effect subsequently replaced, after 20 days, by a moderate convulsant effect in both sexes (Woolley & Timiras, 1962a). It has been suggested (Woolley & Timiras, 1962a) that the initial anticonvulsant effect

of progesterone in the female may be due to a rapid metabolism in this sex (Yates, Herbst & Urquhart, 1958) with certain reduction products exerting a more potent depressant effect than the parent compound (Figdor and others, 1957) although this suggestion may be difficult to reconcile with the established biogenetic pathway from progesterone via androsterone to oestrogens (Clayton, 1965).

Sparse attention has been paid to the effects of sex hormones on leptazol-induced seizures. Of the compounds examined, progesterone (100 mg/kg, Selye, 1942b) and testosterone, (50 mg/kg, Koch, 1959) have been claimed to antagonize such convulsions in the rat.

On the other hand, the effects of sex hormones on audiogenic seizures have been more thoroughly investigated (Werboff & Corcoran, 1961; Werboff et al. 1963, 1964). In these studies, the animals were classified, before testing, as seizure-resistant or seizure-susceptible, depending upon their response to an audiogenic seizure stimulus. Seizure-resistant and seizure-susceptible animals were considered to have a seizure incidence of 0% and 100% respectively, and the results obtained for the action of sex hormones for each group were then analysed separately. For the seizure-resistant group, the seizure incidence, after hormonal administration and/or castration, was expressed as a percentage and

compared for significance with the pre-treatment seizure incidence of 0% by means of a z-test (Edwards, 1950). The results for the seizure-susceptible animals were analysed similarly. The seizure incidence after treatment was compared with the pre-experimental level of 100% and tested for significance as previously indicated.

Oestradiol, in low doses (0.02 - 0.08 mg/kg), increases the incidence of audiogenic seizures in intact or gonadectomized male and female rats, irrespective of their initial susceptibility to seizures. This result is in agreement with the results of experiments employing the EST as the index of brain excitability (Woolley & Timiras, 1962a,b). Testosterone (2.5 - 10.0 mg/kg) and progesterone (1.25 - 5.0 mg/kg), however, may produce convulsant or anticonvulsant effects depending on the sex of the animal and its susceptibility to induced seizures. For example, progesterone, in seizure-resistant female rats, increases the incidence of audiogenic seizures while, in seizure susceptible females it reduces seizure incidence (Werboff et al. 1964).

It would seem that of the sex hormones only oestrogens have been investigated for their ability to interfere with the e.e.g. During menstruation (Duesser de Barenne & Gibbs, 1942) or in pregnancy when

progesterone levels are relatively high and oestrogen levels relatively low (Gibbs & Reid, 1942) there is a decrease in the electrical activity of the brain characterized by a slowing of the normal alpha rhythm of the cortex. Intravenous injection of oestrogens has an activating effect on the e.e.g. of epileptic women (Logothetis, Harner, Morrell & Torres, 1959) and on both normal and epileptic rabbits often resulting in seizures and even death in status epilepticus (Logothetis & Harner, 1960).

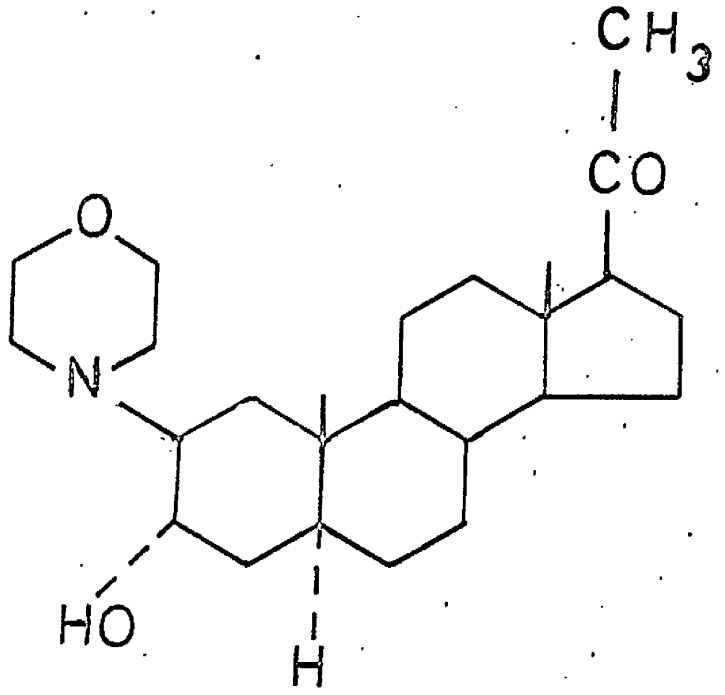
With regard to the convulsant activity of oestrogens, clinical studies have shown that there may be an increase in the incidence and severity of attacks in epileptics during the immediate premenstrual period (Ansell & Clark, 1956; Laidlaw, 1956). These changes occur concomitantly with an increased oestrogenic production (Logothetis et al.1959), refuting the view that the catamenial exacerbation of seizures is a progesterone-withdrawing phenomenon (Laidlaw, 1956).

In contrast to the convulsant activity of the oestrogens, it has been claimed (Selye, 1941b,1942a) that androsterone, epiandrosterone and progesterone possess considerable sedative and hypnotic activity. In attempts to elucidate the possible biochemical mechanisms underlying this central depressant action, it has been

shown (Wade & Jones, 1956) that progesterone reduces brain levels of adenosinetriphosphate (ATP), the breakdown of which is generally accepted to be the most important source of cellular energy (Krebs & Kornberg, 1957) and which is implicated in numerous physiological processes, including muscular contraction (Weber & Portzehl, 1954; Morales, Botts, Blum & Hill, 1955) and nerve impulse transmission (Huennekens & Whiteley, 1960). However, a reduction in the amount of available ATP cannot entirely account for the central activity of depressant steroids since other derivatives e.g., pregnenolone sodium succinate, while inhibiting oxidative phosphorylation, have no anaesthetic activity (Truitt, Bell & Krantz, 1956).

iii) NITROGENOUS STEROIDS WITH EFFECTS ON THE CENTRAL NERVOUS SYSTEM.

Certain groups of nitrogenous steroids appear to possess anticonvulsant and sedative properties (see inter alia Martin-Smith & Sugrue, 1964; Overbeek & Bonta, 1964). These include various steroidal monoximes (Babcock, 1958; Wechter, Schroeter & Buhler, 1961) and dioximes (Babcock & Wechter, 1962, Wechter, 1962; Upjohn, 1962a, b), some androstane-16β,17β-dicarboxylic acid imides (Crabbe, 1963), a number of 17β-acetamido-androstane derivatives (de Ruggieri, Ferrari & Gandolfi, 1963),

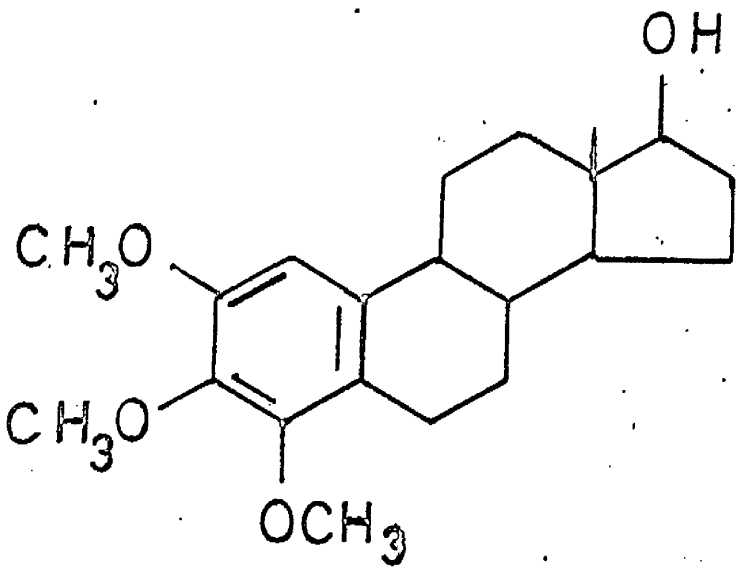


2β-MORPHOLINO-3α-HYDROXY-5α-PREGNAN-20-ONE

certain 3β-(aminomethoxy)-5-androsten-17-ones (Upjohn, 1964) and 2,16-bis (aminomethylene)-secoandrostanes (Knox & Kincl, 1963) as well as certain amino acid esters of 21-hydroxypregnanedione. These last compounds were prepared during structural modification studies to the hydroxydione molecule (Figdor and others, 1957) but although a number of the amino acetates possessed potent anaesthetic activity, their high toxicity precludes their clinical use.

The claim that the steroidal alkaloid funtumidine (3α-amino-20α-hydroxy-5α-pregnane) possessed sedative properties quantitatively similar to those of reserpine (La Barre & Desmarez, 1959) stimulated the preparation of a series of amino steroids structurally related to the former (Etablissements Clin-Byla, 1961a, 1964; Schmitt and others, 1962a,b; Birkenmeyer, Lednicer, Kagan & Magerlein, 1965). 3α-Amino-5β-pregnan-20-one, 3β-amino-5α-pregnan-20-one and 3β-amino-5β-pregnan-20-one are claimed to possess central nervous system depressant activity (Etablissements Clin-Byla, 1961a,b).

Several 2β-, 6β- and 16β-morpholino steroids also depress the central nervous system as evidenced by the induction of loss of the righting reflex in the mouse. Moreover, 2β-morpholino-3α-hydroxy-5α-pregnan-20-one protects mice from leptazol-induced seizures at a dose one



2,3,4-TRIMETHOXYOESTRA-1,3,5 (10)-TRIEN-17β-OL

third of that necessary to produce loss of righting reflex (Sugrue, 1963; Hewett et al. 1964). In addition, several of the compounds are active in protecting mice from electrically-induced seizures but unfortunately this anticonvulsant property is accompanied by simultaneous sedation and/or loss of righting reflex (Sugrue, 1963), effects which would appear to prohibit the clinical use of these compounds. Indeed, at present none of the nitrogenous steroids with depressant effects on the central nervous system would appear to be of therapeutic value.

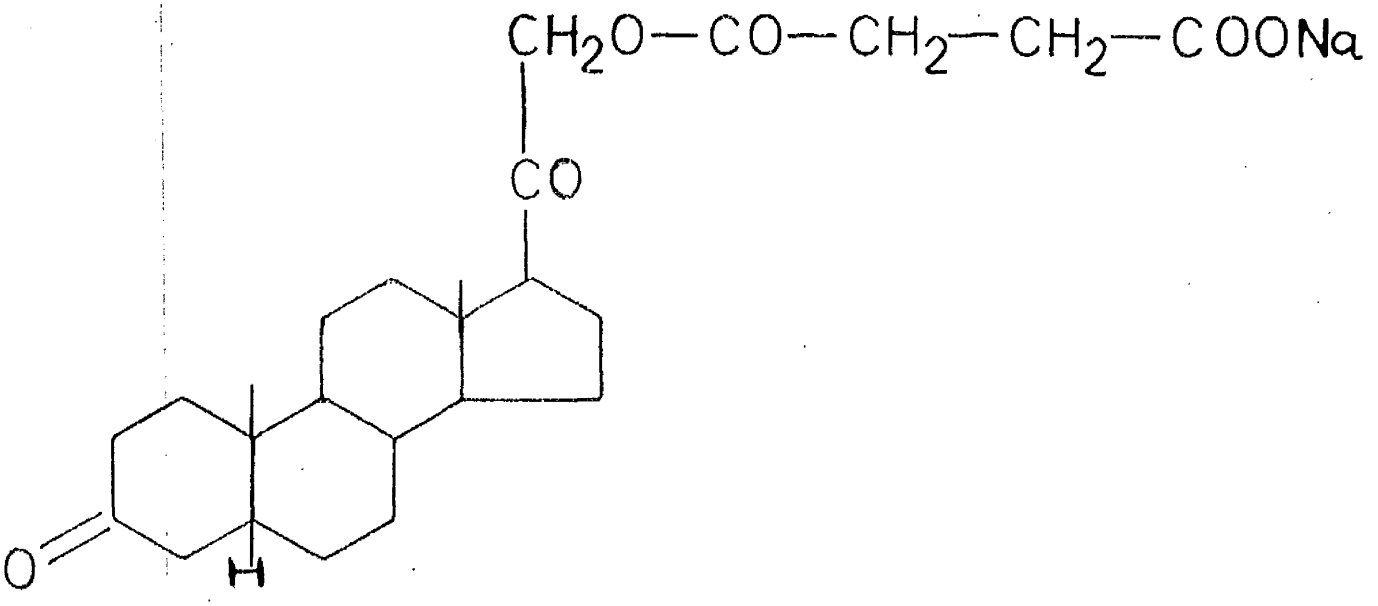
iv) STEROIDS WITH ANALGESIC ACTIVITY.

Very recently, a new type of central action associated with the steroid nucleus has been uncovered, that of potent analgesic activity in a series of oestrane derivatives, the prototype of which is 2,3,4-trimethoxyoestra-1,3,5 (10)-trien-17β-ol (Axelrod, Rao & Baeder, 1966; Axelrod & Baeder, 1966). This compound appears to be devoid of oestrogenic activity and in the "rat-tail flick" method (D'Amour & Smith, 1941) it is claimed to be 40 and 160 times more potent than morphine sulphate and pethidine, respectively. It is reported to be successful in man in the control of post-operative pain and chronic pain due to malignancy (Axelrod & Baeder, 1966) and administration to cats and dogs of doses of 3-5 mg/kg allow abdominal surgery to be performed without

further medication. Toxicity is low and no undesirable side effects on the blood pressure, respiration or electrocardiogram of dogs were observed after intravenous administration.

v) PHARMACOLOGICAL OBSERVATIONS AND STRUCTURE-ACTION RELATIONSHIPS AMONG STEROIDS.

Following the pharmacological investigations which established the existence of general anaesthesia among hormonally-active steroids such as progesterone, desoxycorticosterone and androsterone (Selye, 1941a, b, 1942a), several attempts to correlate this effect with structural entities in the steroid nucleus have been made (Figdor and others, 1957; Witzel, 1959; Overbeek & Bonta, 1964; Atkinson et al. 1965). While considerably more steroidal compounds need to be investigated in this field before any closely defined structure-action relationships can be formulated, the broad generalizations have been made that central nervous system depressant activity is not limited to hormonally-active derivatives and that maximal activity is found in relatively simple, saturated steroids oxygenated at the extremities of the molecule i.e., at the 3 and 17 positions for androstanes and at the 3 and 20 or the 3,20 and 21 positions for pregnanes. Additional nuclear substitution or unsaturation normally reduces activity. Stereochemical



HYDROXYDIONE.

factors such as whether the steroid has an A/B trans or an A/B cis ring fusion, appear to exert little effect.

Within the steroids examined for general anaesthetic activity, those possessing a 5α- or 5β-saturated pregnane skeleton have proved to be the most active compounds (Overbeek & Bonta, 1964; P'An & Laubach, 1964). In particular, 21-hydroxy-5β-pregnane-3,20-dione sodium succinate (hydroxydione) (P'An and others, 1955) has achieved clinical recognition as a basal anaesthetic (Gordan, Guadagni, Picchi & Adams, 1956; Dow, 1961). In contrast to the studies made on pregnane derivatives, little attention appears to have been devoted to the synthesis and testing of androstane derivatives, despite the fact that both androsterone and epiandrosterone possess anaesthetic activity comparable to that of progesterone (Selye, 1942a).

II PRESENT STUDIES OF THE EFFECTS OF NITROGENOUS STEROIDS ON THE CENTRAL NERVOUS SYSTEM

a) EXPERIMENTAL METHODS.

Solubility.

Three of the compounds under investigation were readily soluble in deionized water. The remaining compounds were relatively insoluble in water and the soluble hydrochloride of each was prepared by adding to an accurately weighed quantity of the compounds the theoretically equivalent amount of N/10 hydrochloric acid plus 0.2 ml. excess. The mixture was then heated, if required, on the water bath to facilitate solution and made up to the required volume using deionized water. Clear, colourless solutions were obtained in all cases.

The pH of the drug solutions employed ranged from 2.5 to 4.5. Control solutions of acidified deionized water of a similar pH range were also prepared.

EXPERIMENTS USING MICE.

Solutions of the compounds were administered by slow intravenous injection into the dorsal tail vein of male albino mice (18g - 22g) using a 1 ml. graduated tuberculin syringe. In no instance was a volume greater than 0.4 ml. per 20g of mouse injected. An injection of similar pH and volume was also administered as a control.

Toxicity Tests.

Five to ten mice at three to four dose levels were used for each compound.

The mice were observed in various ways to measure the following effects:- increased or decreased spontaneous activity, excitation, convulsions, sedation, muscular paralysis, loss of righting, pinna and/or corneal reflexes and finally analgesia.

This observational technique gives an overall profile of the gross effects of a drug together with an indication of the onset, peak, duration, character and intensity of action.

Skeletal muscle paralysis was measured by placing the mice on a fine-mesh wire screen inclined at 60° to the horizontal (Thomson, 1946). Those mice which slid abruptly off the screen within 30 minutes after pretreatment with the drug were deemed to show a positive reaction.

Loss of the righting reflex was considered to have occurred if treated mice were unable to regain their balance when placed on their backs for one minute (Hine, Christensen, Murphy & Davis, 1949).

A fine nylon suture was used to confirm the presence of the pinna and corneal reflexes. The pinna reflex involves a twitch, tremor, or laying-back of the

ears when the external auditory meatus is stimulated using the nylon suture (Witkin, Spitaletta & Plummer, 1959). The reflex was considered to be abolished when no response could be elicited in either ear following treatment with the drugs under test. The corneal reflex was elicited by touching the cornea and conjunctiva and deemed absent when the suture, placed on the eyes, produced no closure of the lids. The ability of the compounds under test to exert an analgesic effect was detected by the absence of squeaking and biting in mice following pinching of their tails with a fine pair of forceps.

Compounds with a central stimulant action would be expected to produce increased motor activity, excitation and perhaps convulsions. Conversely, compounds possessing a hypnotic action would exhibit decreased mobility, muscular paralysis, loss of the righting reflex, disappearance of the corneal before abolition of the pinna reflex (Goodsell, Toman, Everett & Richards, 1954) and sedation.

Loss of the Righting Reflex.

During toxicity experiments on mice certain compounds produced a marked loss of the righting reflex at doses below the toxic range. Moreover, the pinna

reflex disappeared before the corneal reflex suggesting that interneuronal blocking activity might be a property of the compounds under investigation (Goodsell et al. 1954). However, these effects must be interpreted cautiously since a variety of pharmacological agents are known to produce loss of the righting reflex (O'Dell, 1960). Various investigators have used the loss of the righting reflex to compare the potencies of series of compounds suspected of possessing interneuronal blocking activity (Roszkowski, 1960; Burke, Papandrianos, Brannick & Hassert, 1961).

Consequently, an accurate determination of the dose causing loss of the righting reflex in 50% of the injected mice (ED50) was made. The dose killing 50% of the mice (LD50) on intravenous injection was also found.

Each compound was given at four dose levels:- one dose level producing zero loss, another 100% loss and two intermediate dose levels producing approximately 30% and 70% loss respectively of the righting reflex. The ED50 and LD50 values of the compounds producing a loss of the righting reflex were calculated using a graphic method (Miller & Tainter, 1944). The ED50 and LD50 of mephenesin, the most selective interneuronal blocking agent known (Domino, 1964), was also calculated.

Some of the compounds under investigation produced a more prolonged loss of the righting reflex than others. Employing the method used by P'An and his co-workers (1955) to estimate the duration of activity, one and a half times the ED50 dose of these compounds, provided that it was less than the the LD50, was administered to a group of ten mice and the duration of loss of the righting reflex observed.

Anticonvulsant Activity.

Adrenocortical hormones possess both convulsant and anticonvulsant properties. Desoxycorticosterone may raise the EST and cortisone and hydrocortisone may lower it in rats (Woodbury, 1954). Sex hormones can also alter the EST of rats, oestradiol lowering it (Woolley & Timiras, 1962a) and testosterone either raising or lowering it depending on several factors such as the dose of the hormone administered and the age of the animal (Woolley & Timiras, 1962c).

Furthermore, interneuronal blocking agents such as mephenesin have anticonvulsant actions against leptazol (Bastian, 1961), strychnine (Berger, 1949) and electrically-induced seizures (Domino, 1964). It was decided therefore to test the nitrogenous steroids for their ability to antagonize both electrically- and

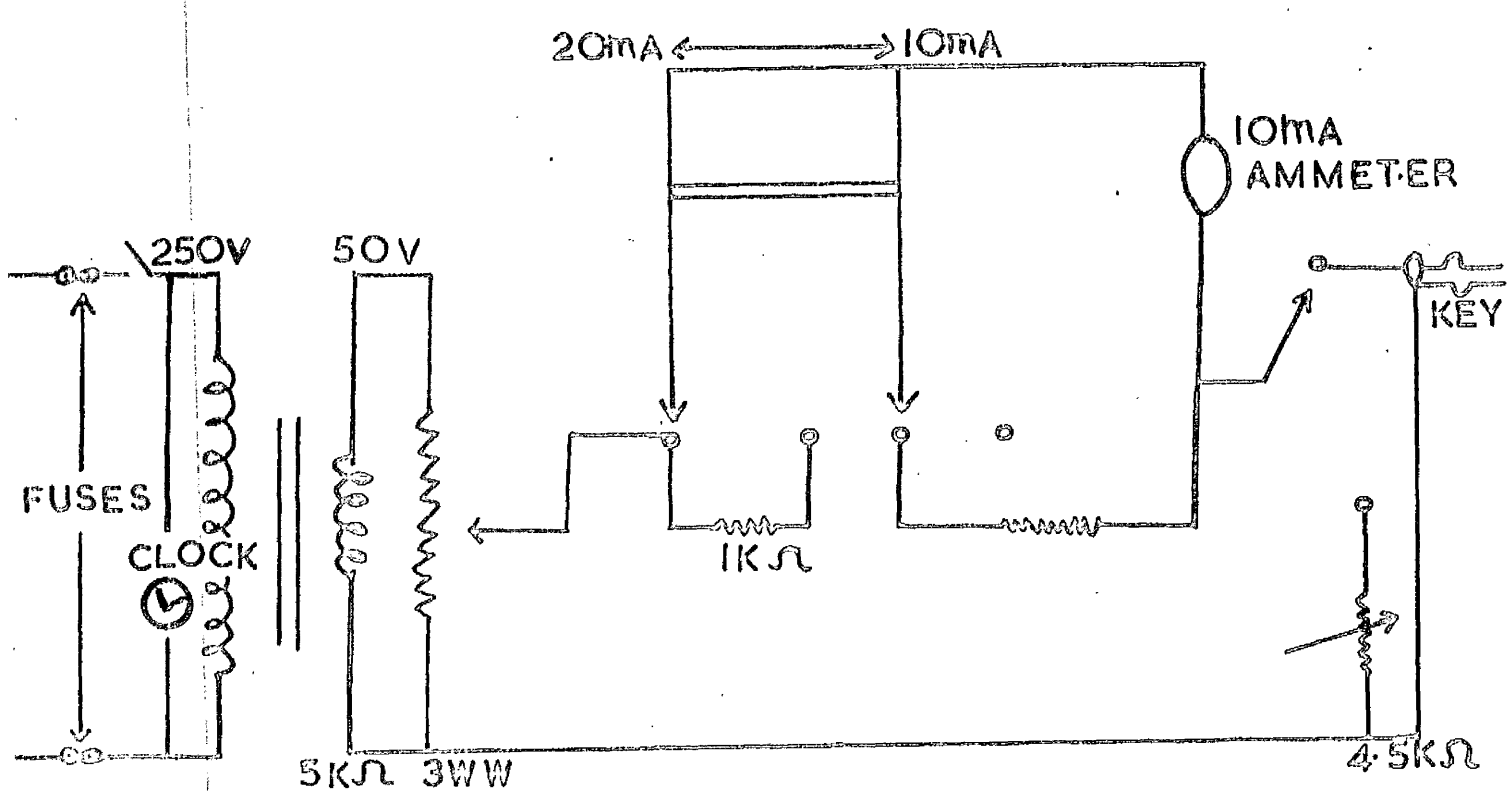


Fig. 2 Circuit diagram of apparatus used for inducing electroshock convulsions in mice.

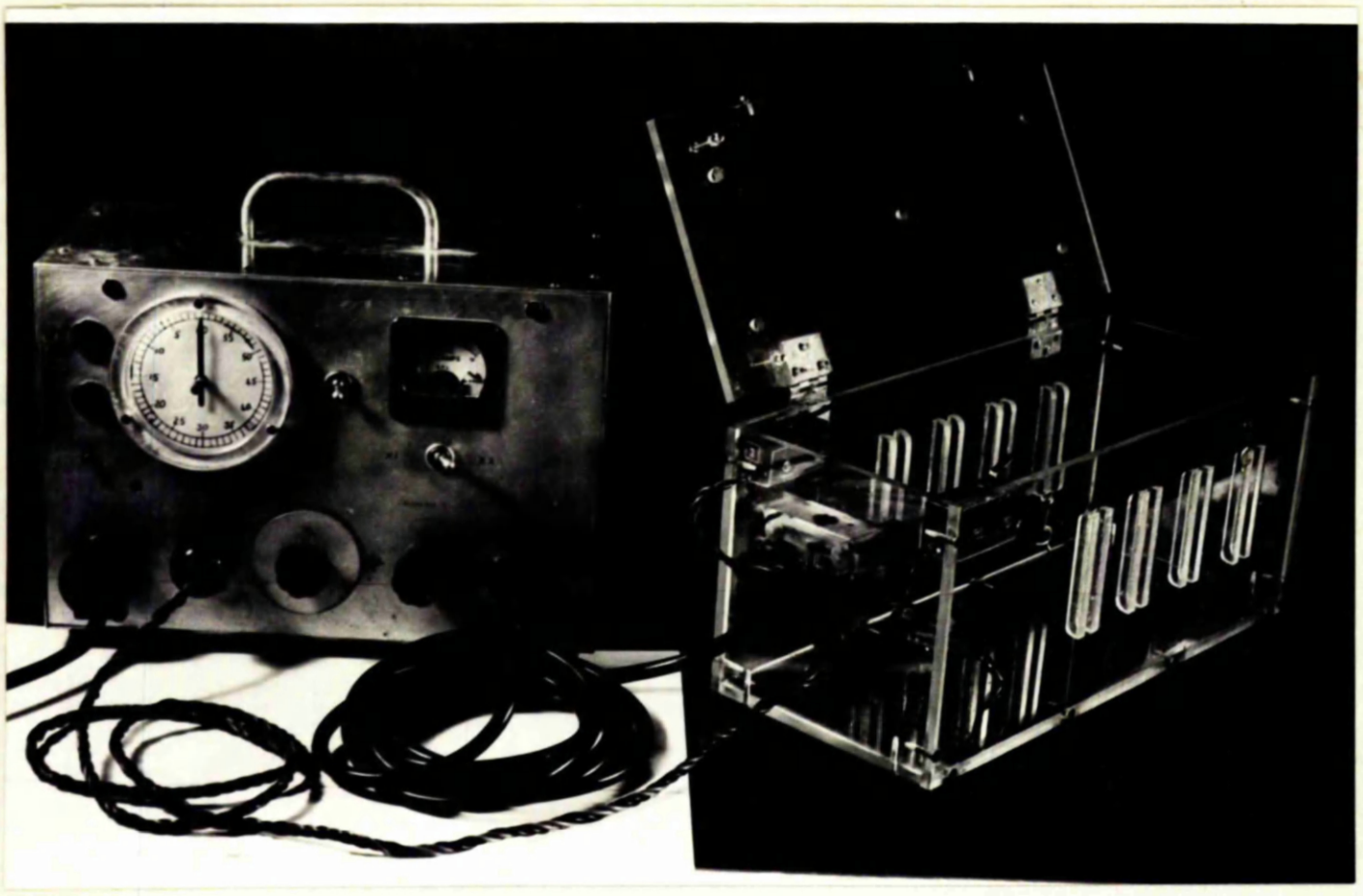


Fig. 1 Photograph of apparatus used for inducing electroshock convulsions in mice.

chemically-induced seizures.

Three dose levels were used for each compound and the anticonvulsant activity determined 5 and 20 minutes after intravenous injection. Where anticonvulsant activity was discovered at these times, the PD50 values were calculated by the method of Miller and Tainter (1944).

i) Protection Against Electrically-Induced Seizures.

The method used was based upon that of Ahmad and Lewis (1960) but ear-clip electrodes were employed (Hoyt & Rosvold, 1951). The circuit diagram and apparatus are shown on the opposite page (Figs. 1 and 2).

Each mouse was placed in a perspex box, one electrode clipped to each ear and the lid closed. A suprathreshold current of 20 mA applied for 5 seconds caused tonic extension of the hind limbs, the appearance of which was taken as the end-point. All mice to be used were tested 24 hours before the experiment and those which failed to convulse within five seconds were discarded. Eight mice were used at each dose level.

Sodium diphenylhydantoin and sodium phenobarbitone, compounds used in the treatment of major epilepsy, were compared with the protection afforded by the steroids under investigation.

The supramaximal electric shock method is the

most accurate means of indicating the usefulness of drugs in abolishing the seizures characteristic of clinical grand mal in that a high degree of correlation of effect exists between man and laboratory animal. Additionally, the method also favours drugs useful in the treatment of psychomotor epilepsy (Toman & Everett, 1964).

ii) Protection Against Leptazol-Induced Seizures.

The method (Berger, 1954) determined the ability of compounds to antagonize the convulsant effect of a pre-determined dose of leptazol.

Control mice were injected intraperitoneally with 120 mg/kg of leptazol. Within 30 minutes a sequence of excitement, myoclonic jerks, clonic seizures and death developed. The ability of the steroids under investigation to antagonize the convulsant action of this dose was assessed.

Ten mice were used at each dose level and the end-point of the assay taken as the appearance of persistent clonic convulsions. The mice were observed for 30 minutes following injection of the leptazol.

Sodium phenobarbitone and trimethadione, drugs used in the treatment of human minor epilepsy, were similarly compared. The leptazol test cannot be used with certainty to predict those drugs which will be

effective against human petit mal. It is not a specific test and drugs effective against grand mal can also show anticonvulsant activity (Toman & Everett, 1964).

Anti-Parkinsonian Activity.

Clinically useful anti-Parkinsonian drugs also possess anticonvulsant (Goodman & Gilman, 1965) and sedative activity (Lewis, 1964). The existence of sedative and anticonvulsant properties among the nitrogenous steroids justified their examination for anti-Parkinsonian activity.

The Tremorine Test.

Tremorine (1,4 dipyrrolidino-2-butyne), when administered to experimental animals, produces tremors, rigidity, asthenia and hypothermia in addition to a number of parasympathetic effects such as marked salivation, urination and defaecation, an overall picture closely resembling Parkinsonism in man (Everett, 1956). Drugs active clinically against Parkinson's disease have the ability to antagonize the tremorigenic and parasympathetic activity of tremorine (Farquharson & Johnston, 1959; Keranen, Zaratzian & Coleman, 1961; Friedman & Everett, 1964) and the antitremorine test is now used widely in the investigation of new compounds for potential

anti-Parkinsonian activity. The test can also be employed to detect drugs with central anticholinergic or central parasympathomimetic activity (Spencer, 1965). In addition, since it is believed that tremorine is converted in the liver to its active metabolite oxotremorine (Cho, Haslett & Jenden, 1961), the test can also be utilized to detect those compounds which prevent the appearance of tremors by inhibiting the metabolism of tremorine in the liver (Leslie & Maxwell, 1964).

Eight mice, pretreated with the test drug, were used at each of two dose levels. Fifteen minutes later tremorine (20 mg/kg) was injected intraperitoneally. This dose was found to be completely effective in producing tremors and salivation in untreated mice. The test mice were compared with control mice to assess the degree of protection afforded.

A points-scoring system was adopted in an attempt to assess the results semi-quantitatively. Viz:- complete protection - 0 points; almost complete protection - 1 point; moderate protection - 2 points; slight protection - 3 points; no protection - 4 points. Consequently, a compound affording complete protection to eight mice would score 0 points whereas a compound contributing no protection would score the maximum of

32 points. The mice were observed at 15, 30, 45, 60 and 90 minute intervals respectively after injection of tremorine and the percentage degree of protection at each of these times calculated. A comparison was made with the clinically used anti-Parkinsonian agent, atropine.

EXPERIMENTS USING CATS.

Cats of either sex (2 - 4kg) were used. Excluding the crossed extensor reflex preparation, for which ether was employed, anaesthesia was induced by intraperitoneal injection of sodium pentobarbitone (60 mg/kg). The trachea was cannulated to permit the application of artificial respiration when necessary, the amount of air entering or leaving the cannula being controlled by an adjustable sleeve. The external jugular vein on one side was exposed and a heparinized polythene cannula inserted. The cannula was connected to a 50 ml. burette containing saline by means of a piece of rubber tubing. The compounds under investigation were injected into the rubber tubing connecting the burette and the cannulated jugular vein. Each injection of drug was washed in using 3 ml. of normal saline.

To Record the Blood Pressure of the Anaesthetized Cat.

The common carotid artery, on the opposite side

of the neck from the cannulated jugular vein, was freed from the accompanying vago-sympathetic trunk and ligated as near the cephalad end as possible. A "bull-dog" clip was then placed round the artery about 3 cm distal to the ligature and a heparin-filled polythene cannula inserted into the artery with its pointed end towards the heart. The cannula was connected to a mercury manometer by rubber tubing filled with heparinized normal saline solution as an anticoagulant. The pressure in the mercury manometer was set at approximately 100 to 120 mm of mercury (i.e., the normal blood pressure of the cat). The "bull-dog" clip was then released and the blood pressure recorded on a moving smoked surface.

The Cat Gastrocnemius Muscle-Sciatic Nerve Preparation.

Skeletal muscle paralysis in animals can be produced by neuromuscular blocking agents, interneuronal blocking agents, hypnotics and other central nervous system depressants. The cat gastrocnemius muscle-sciatic nerve preparation (Bülbring & Burn, 1942) can detect the presence or absence of neuromuscular blocking activity. The production of muscular paralysis by the compounds under review warranted their further examination on this preparation to more accurately determine the nature of the paralysis produced.

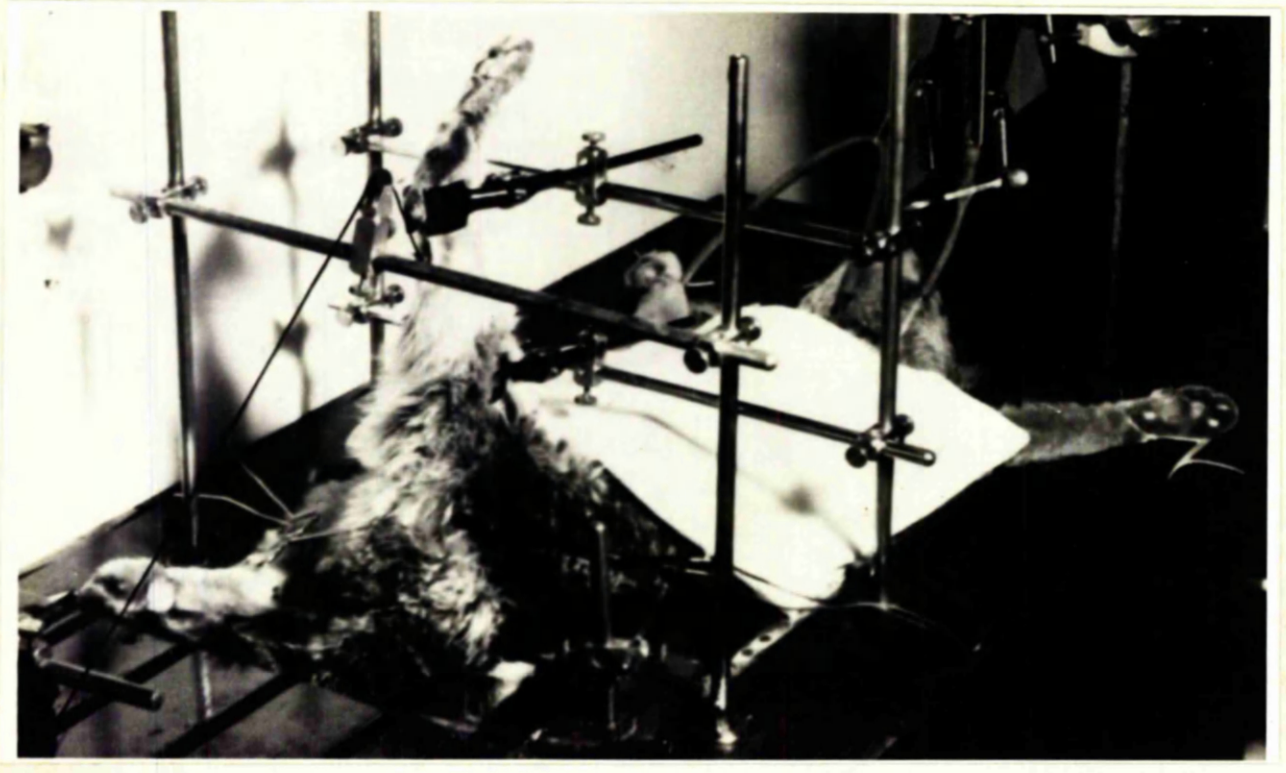


Fig. 3 The cat gastrocnemius muscle-sciatic nerve preparation.

When the trachea, as well as one of the external jugular veins and the carotid artery had been cannulated as previously described, one leg was selected for indirect stimulation of the gastrocnemius muscle via the sciatic nerve. The leg was held with its long axis perpendicular to the operating table and rigidly fixed by means of two clamps, one at the knee joint and the other at the ankle. The gastrocnemius muscle was partially dissected free from surrounding tissue and the Achilles tendon severed at a point near to its insertion into the calcaneus. A strong linen thread was then tied securely round the tendon and the free end of the thread connected via a system of pulleys to a Brown-Schuster spring-loaded, myograph lever, the writing point of which recorded the muscle contractions on a moving smoked surface (Fig. 3).

The skin covering the lateral aspect of the thigh was incised and the sciatic nerve located between the hamstring muscles. A pair of platinum electrodes was placed round the exposed nerve. The nerve was then stimulated supramaximally, using a Dobbie-McInnes square wave stimulator (6 to 8/min, 5 to 10 V, 1 to 2 msec). These values and the tension on the muscle (0.2 to 0.3 kg) remained constant during any one experiment.

The Cat Crossed Extensor Reflex Preparation.

The steroids under examination were tested on the cat crossed extensor reflex preparation which can be used to indicate the presence or absence of interneuronal blocking activity (Domino, 1964).

Anaesthesia was induced by ether inhalation and the cat was spinalized using the method described by Burn (1952). The anaesthetized cat was tied onto a warm operating table, the trachea cannulated and artificial respiration applied. Both common carotid arteries were freed from the accompanying vago-sympathetic trunks and tied off as near the cephalad end as possible. The cat was reversed, ventral side resting on the table and secured. The head was placed on a grooved wooden block to prevent obstruction of the rubber tubing connecting the tracheal cannula to the respiration pump. The skin of the dorsal region of the neck was divided down the midline by a scalpel from the top of the head to the level of the shoulders and the spinal cord freed from all connecting tissues. The vertebral arteries were occluded by tying them with a strong linen thread passed under the spinal cord at the level of the third cervical vertebra. The exposed cord was sectioned at the level of the second cervical vertebra by a pair of bone forceps. The brain was destroyed by a blunt probe pushed through the

foramen magnum. A small amount of bleeding occurred and this was arrested by the application of swabs of cotton wool soaked in hot, normal saline. Plasticine was used to plug the spinal column and a pad of saline-moistened cotton wool placed over the operated area. The skin at the back of the neck was sewn together and the cat turned over, ventral side up. One external jugular vein and one common carotid artery were then cannulated as previously described.

To obtain the crossed extensor reflex, the method employed was based upon that used by Liddell and Sherrington (1929).

The skin covering the patellar tendon of the left leg was removed, the tendon detached from the tibial tubercule and the patella freed from the articular attachments. By means of a needle, a strong linen thread was passed several times around and securely knotted to the detached patellar tendon. The leg was semiflexed at the hip and supported by a brass rod under the knee joint. The free end of the linen thread was attached via a system of pulleys to a Brown-Schuster spring-loaded, myograph lever, the writing point of which recorded on a moving smoked surface. The tension on the muscle (0.3 to 0.4 kg) remained constant during any one experiment.

The right leg was held in a vertical position by two clamps, one at the ankle and the other at the knee joint. The skin covering the lateral aspect of the thigh was parted and the sciatic nerve exposed. The nerve was crushed as low in the thigh as possible. A pair of platinum electrodes were then placed around the nerve as high in the thigh as possible and the nerve stimulated supramaximally by a Dobbie-McInnes square wave stimulator (6 to 8/min, 2 to 4 V, 1 to 3 msec). These values remained constant during any one experiment. To minimise fatigue of the preparation, 5 minutes stimulation periods alternated with 5 minutes rest.

The Cat Nictitating Membrane Preparation.

A marked and sustained fall in the blood pressure of the anaesthetized cat was observed with several of the compounds under test. To investigate the possibility that this effect was mediated by sympathetic ganglion blockade, the cat nictitating membrane preparation was used.

The head of the anaesthetized cat was kept in a rigid position during the experiment by tying the jaws securely to a brass rod passed between the teeth. The rod was clamped to two uprights fixed to either side of the operating table. The trachea, external jugular vein and common carotid artery were cannulated as previously

described. Using a fine needle, a linen thread was passed through the mid-point of the margin of the nictitating membrane of the right eye and securely knotted in place. The thread was then pulled forward and to the side to make an angle of about 30° with the long axis of the cat. It was then led over a system of pulleys and attached to a carefully balanced frontal-point writing lever. The contractions of the nictitating membrane were recorded on a moving smoked surface.

The right cervical sympathetic nerve was then carefully freed from both the right common carotid artery and the vagus and ligated at as low a point as possible in the neck. The cervical sympathetic chain was severed just above the ligature and placed on a pair of platinum electrodes. A piece of cotton wool soaked in normal saline kept the exposed part of the nerve moist.

The nerve was stimulated supramaximally for 15 seconds every 3 minutes by means of a Dobbie-McInnes square wave stimulator at a frequency of 800 to 1,200 per minute, at 8 to 15 V, the pulse width being 0.5 to 1.0 msec, all these values remaining constant for any one experiment. When standard reproducible contractions of the nictitating membrane were obtained to electrical stimulation of the sympathetic chain, the compounds under

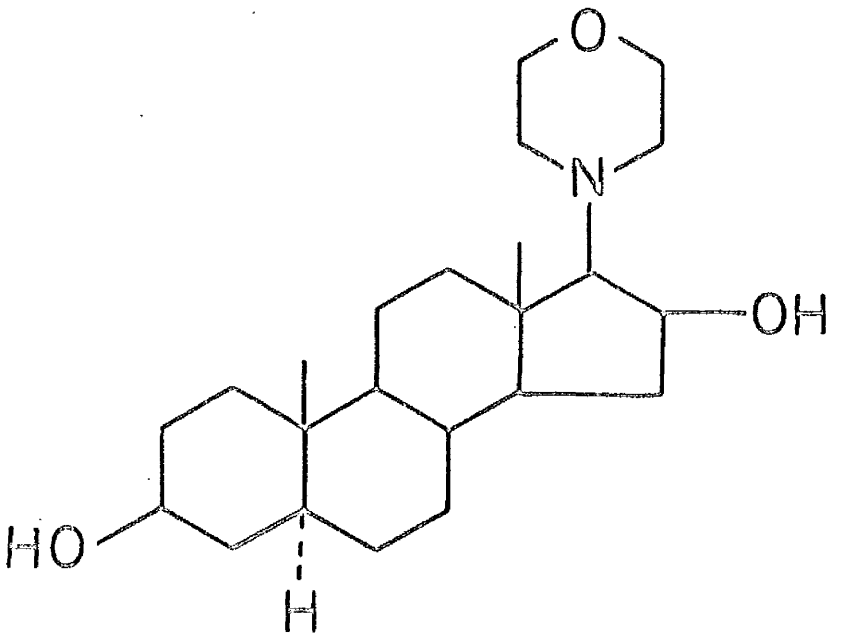
investigation were injected into the cannulated left external jugular vein one minute before the next period of stimulation.

The nature of the innervation of the nictitating membrane and its use in pharmacological analysis has been the subject of much controversy. The hypothesis that all postganglionic sympathetic fibres possess cholinergic innervation (Burn, 1962; Burn & Rand, 1962, 1965) has not been universally accepted (Bentley, 1962; Leaders, 1963, 1965; Boura & Green, 1965). Thus, the failure to demonstrate the presence of acetylcholinesterase in the smooth muscle cells of the membrane (Hellman & Thomson, 1961; Gardiner, Hellman & Thomson, 1962) coupled with the observation that in an *in vitro* (Gardiner & Thomson, 1961) and *in vivo* (Wilson & Long, 1959) membrane preparation hemicholinium was ineffective in preventing transmission confirm the validity of the use of this preparation in the analysis of agents acting at sympathetic synapses.

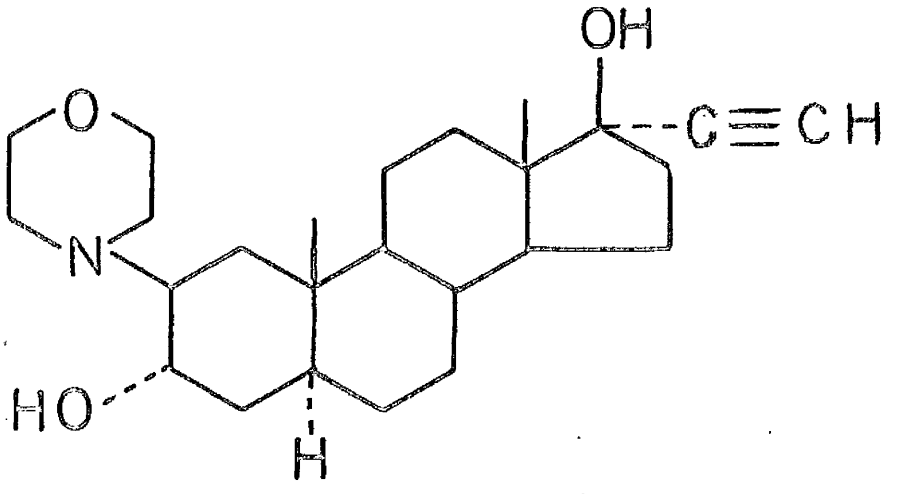
Stimulation of the cervical sympathetic nerve chain reaches the nictitating membrane across the synapses of ganglion cells in the superior cervical ganglion. If a drug under test possessed sympathetic ganglion blocking activity, a reduction in the height of the contraction of the nictitating membrane in response to

nerve stimulation would be expected.

R E S U L T S



Compound A25



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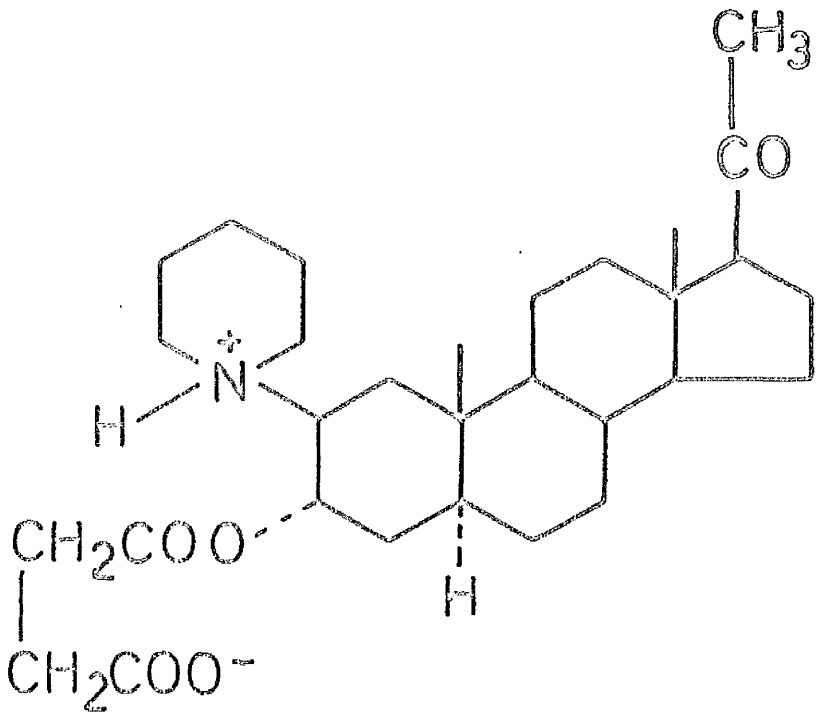

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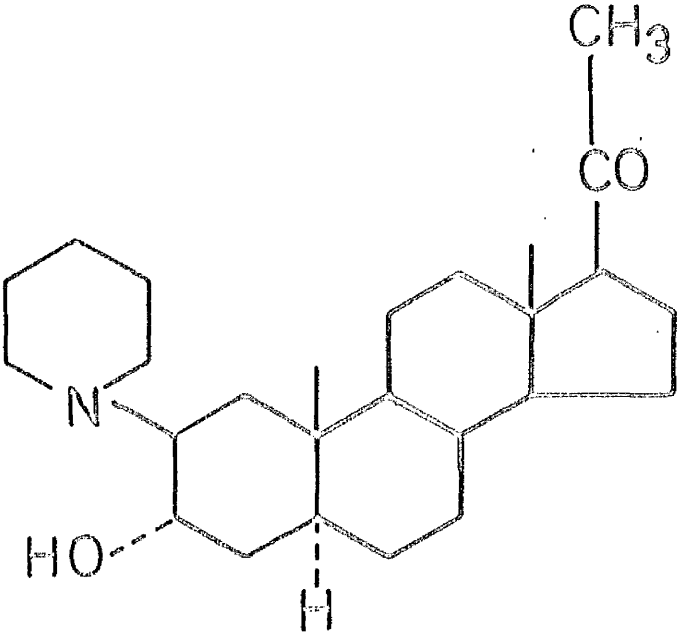

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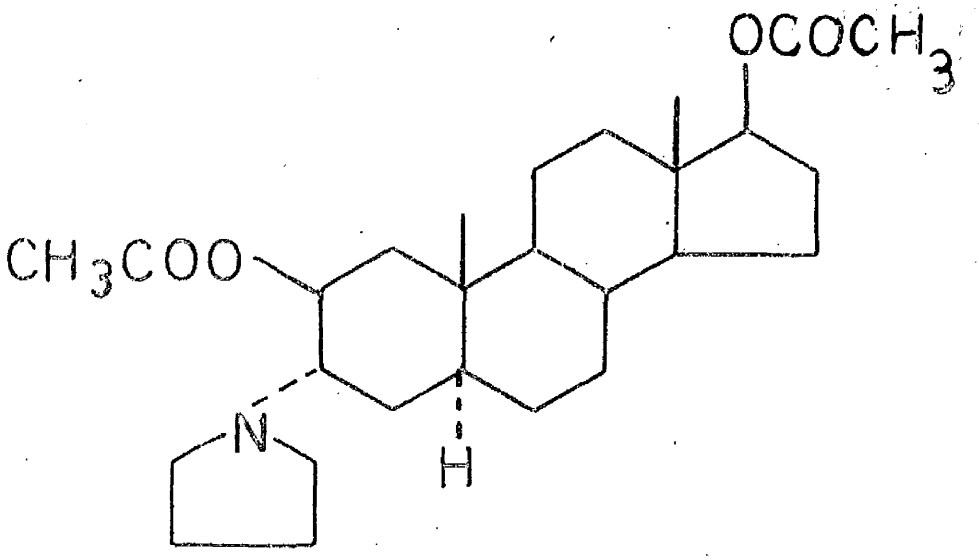

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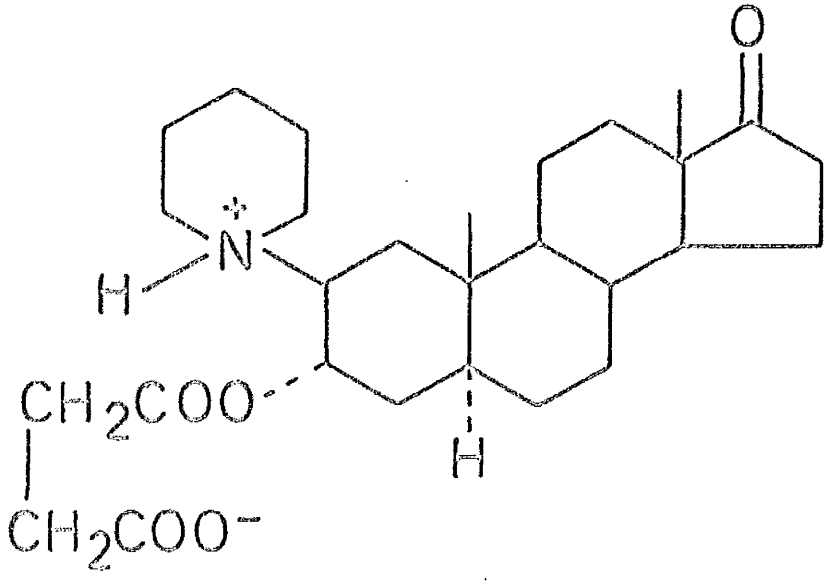

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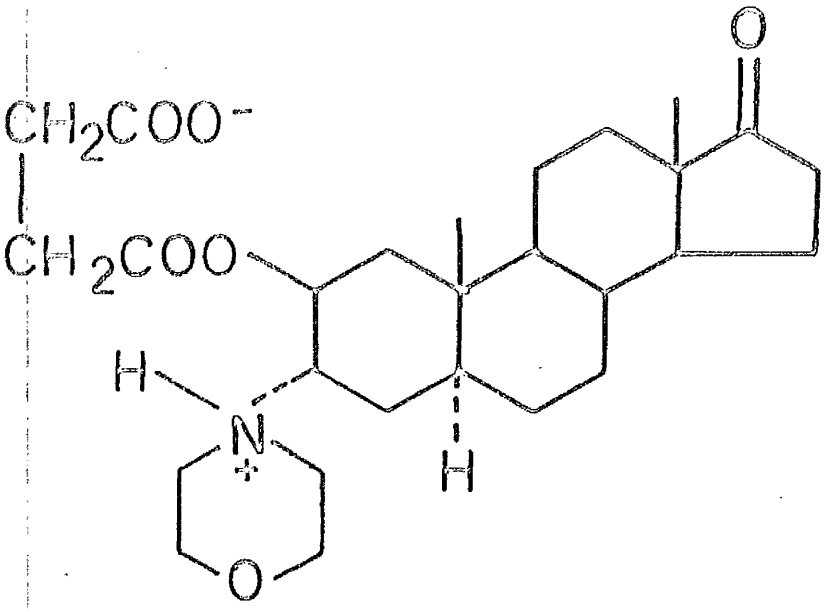

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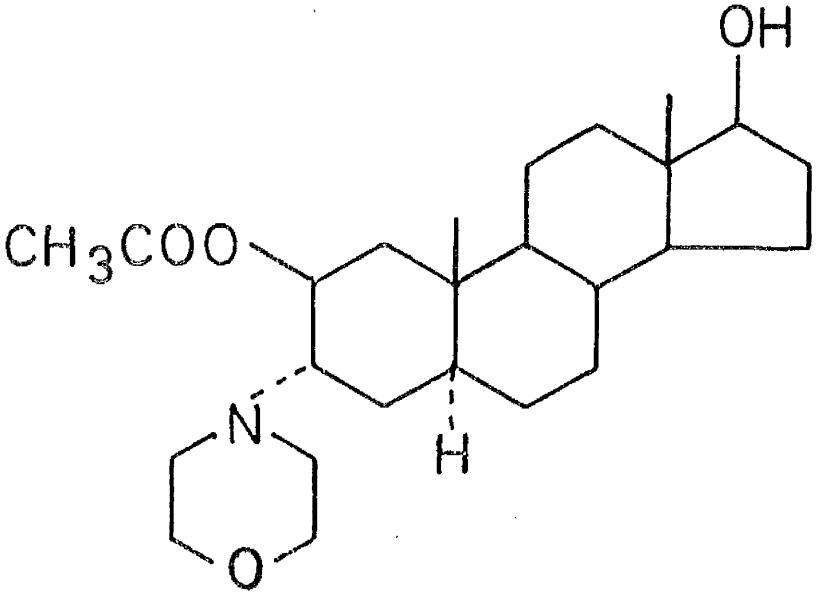

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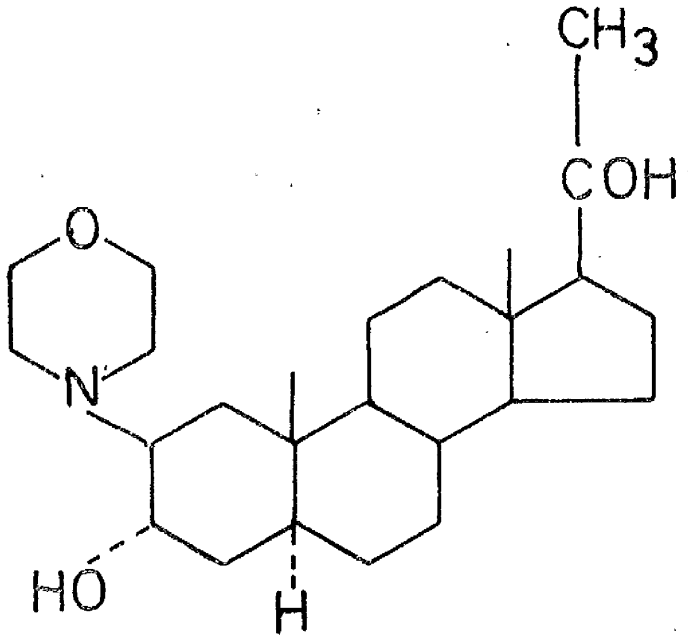

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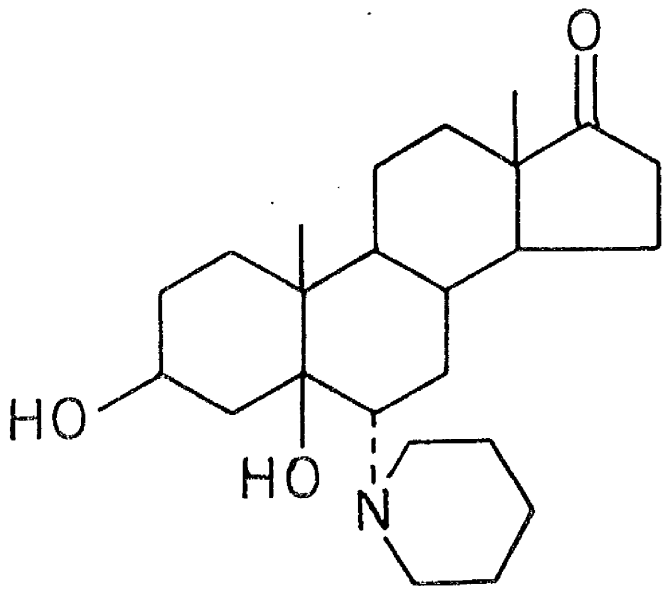

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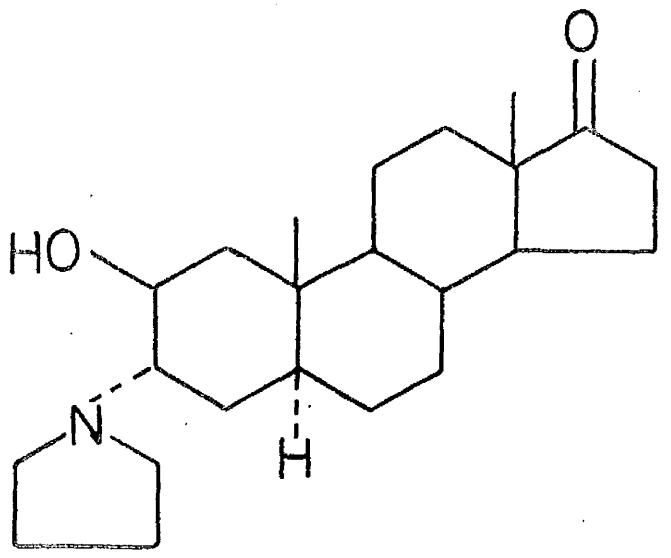

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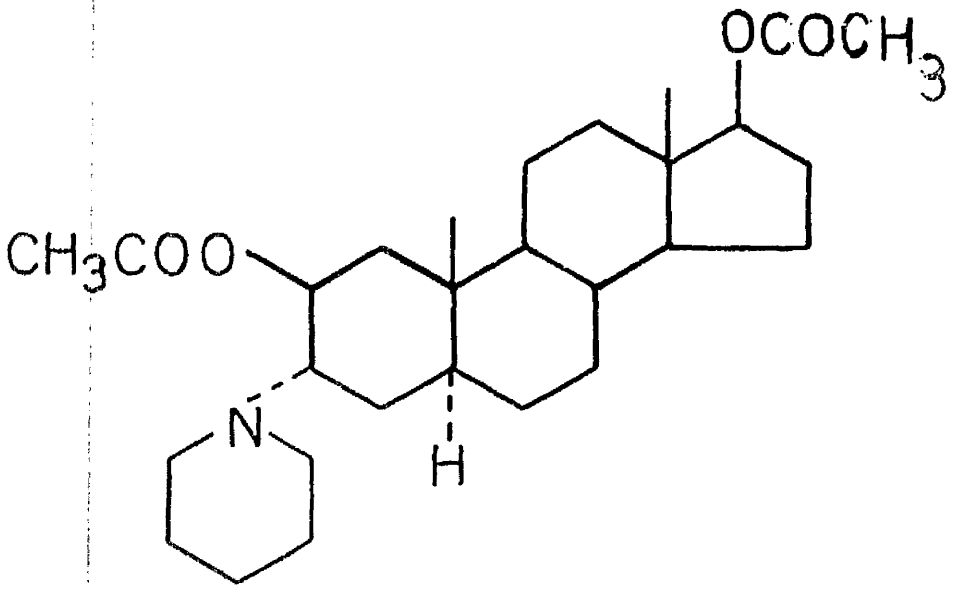

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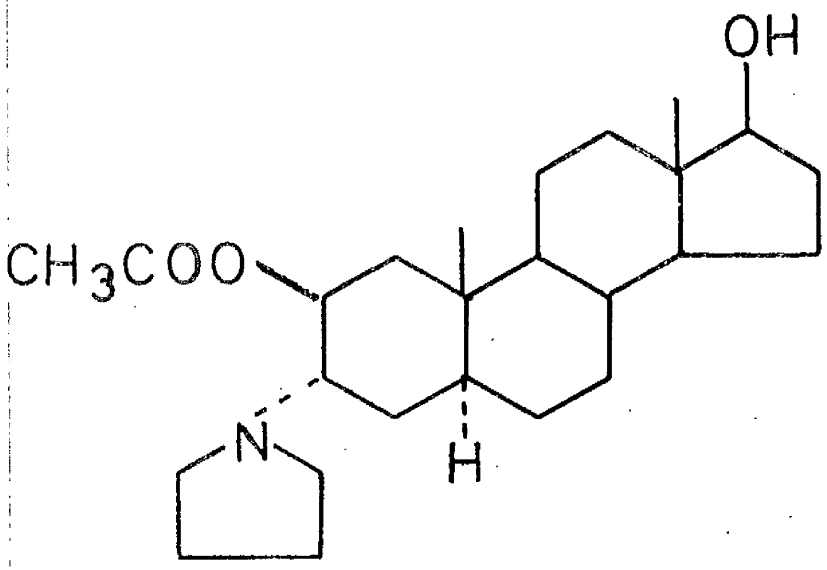

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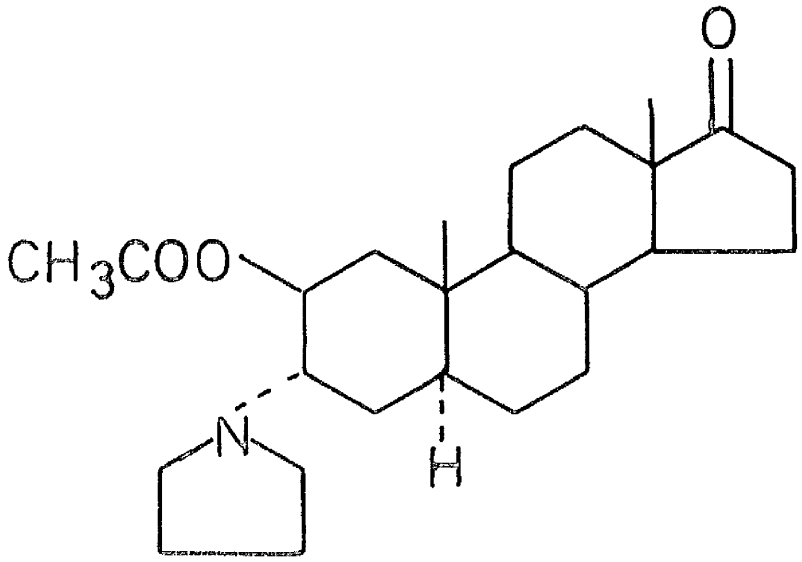

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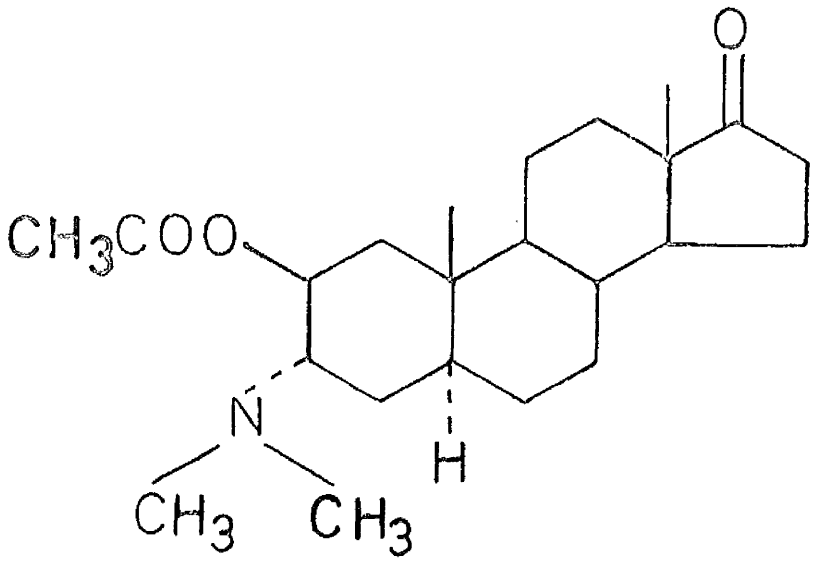

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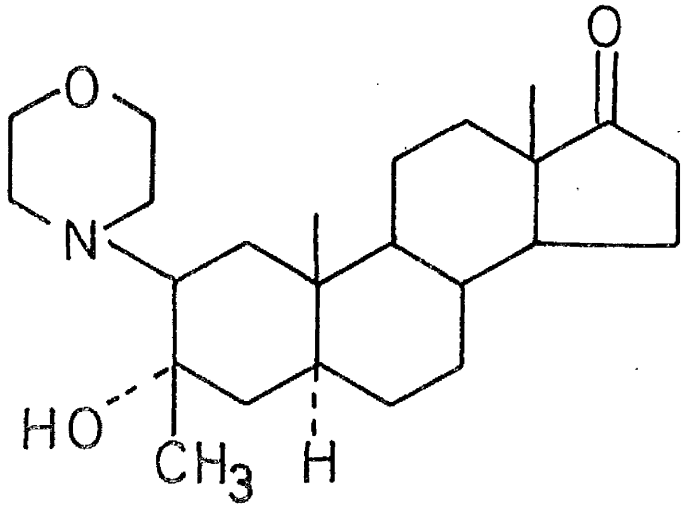
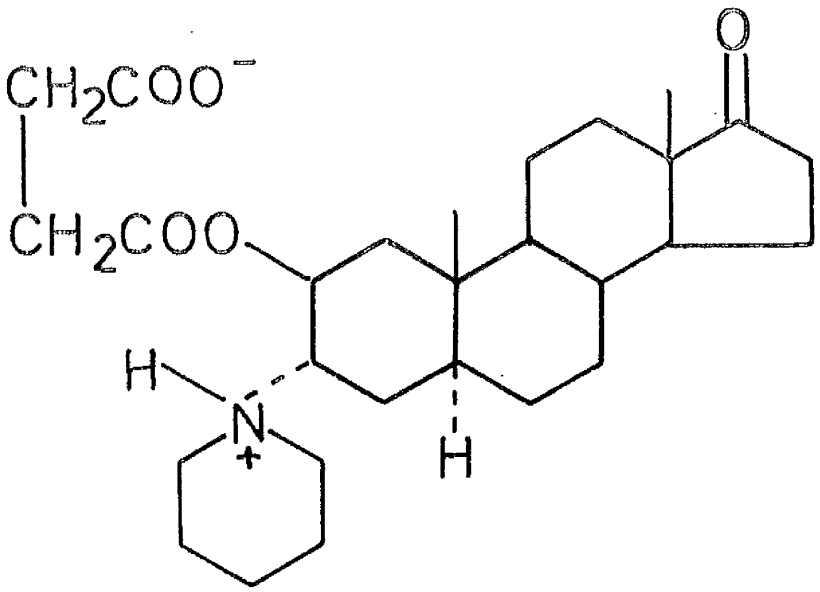


Compound A12



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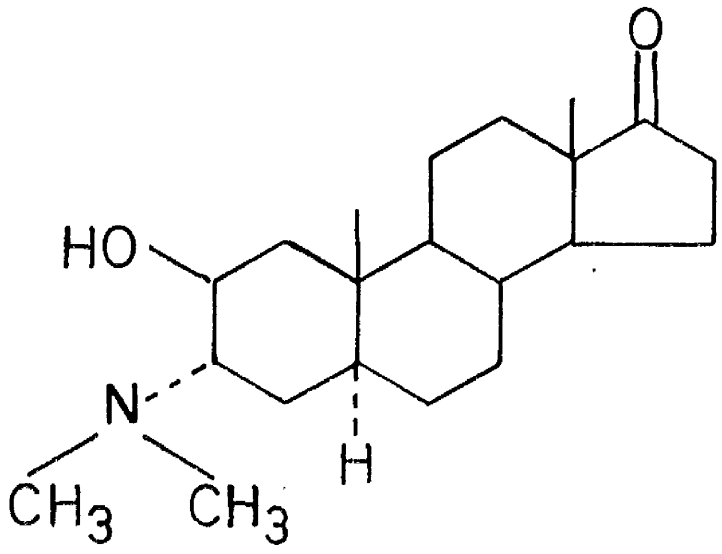

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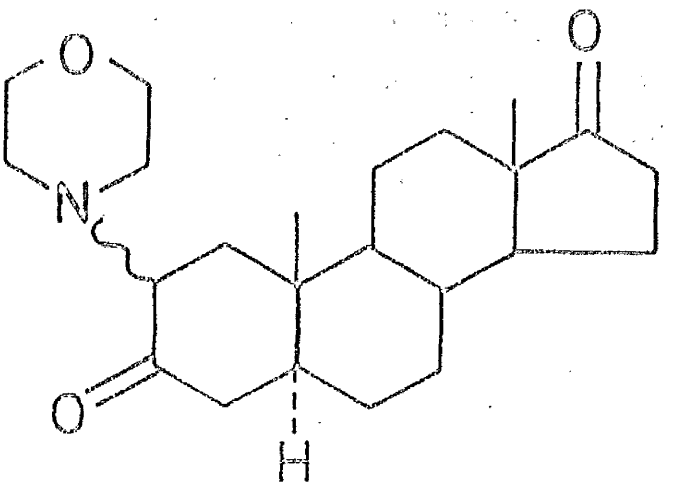

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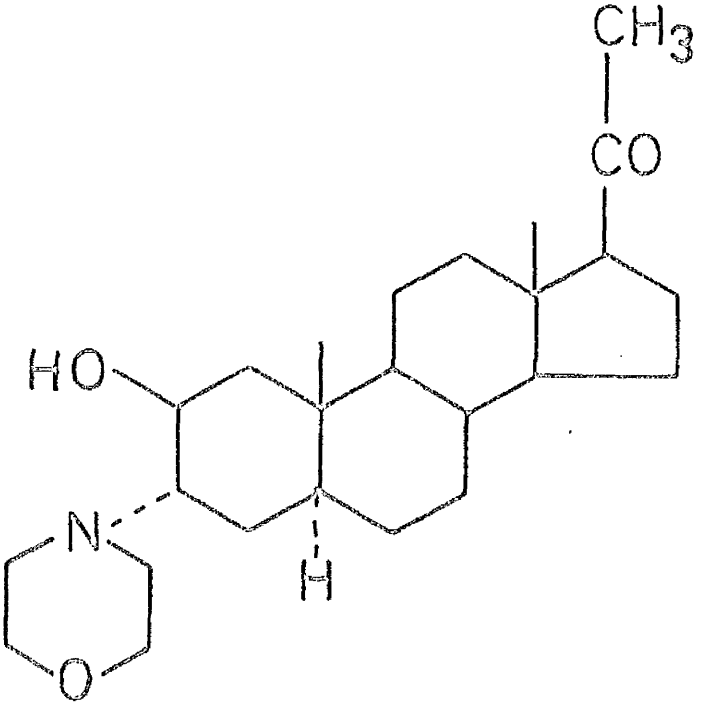

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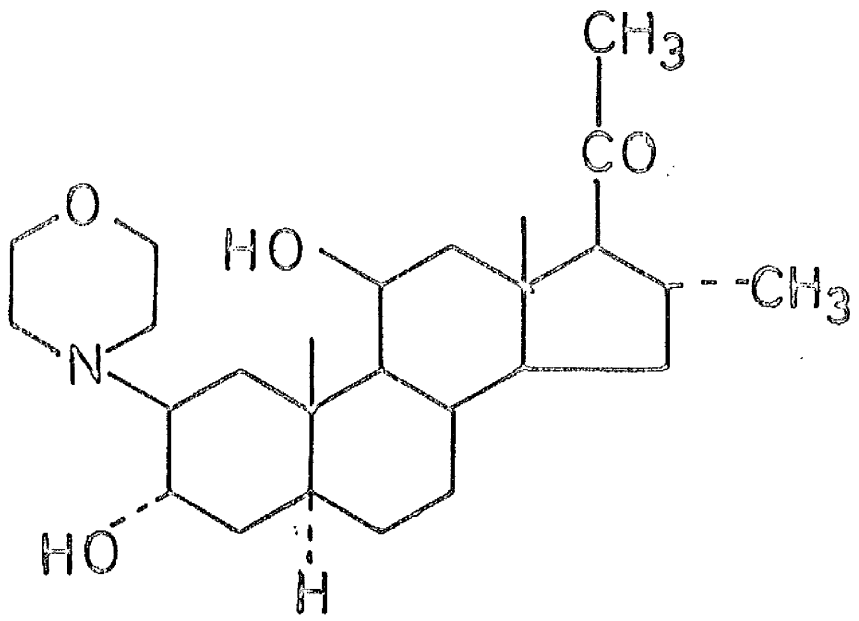

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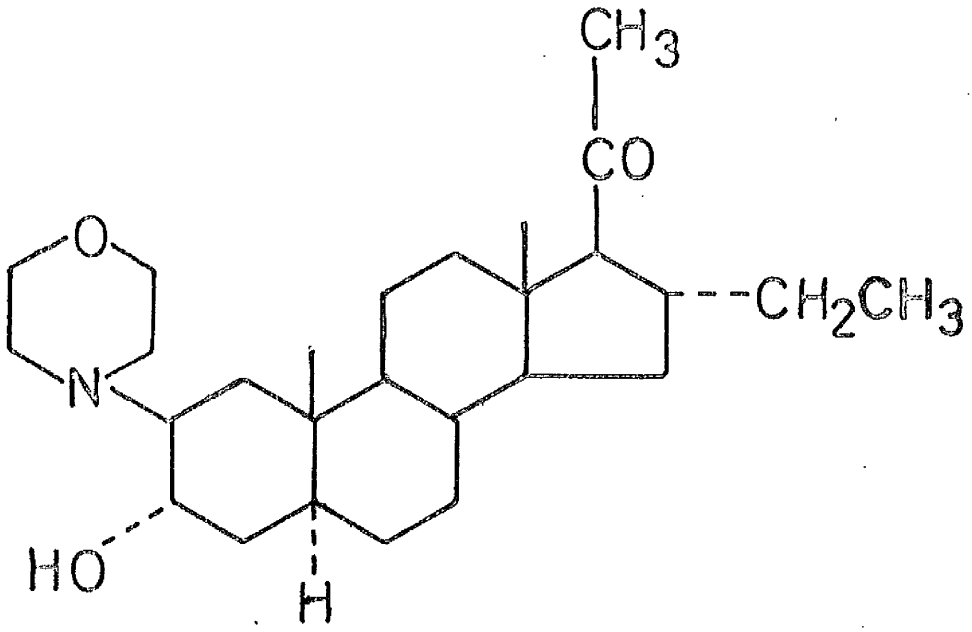

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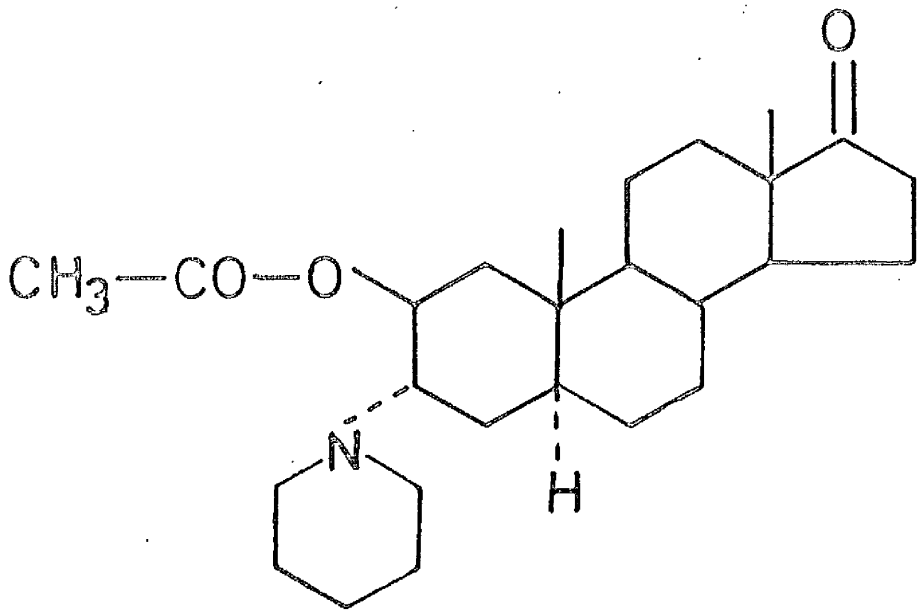

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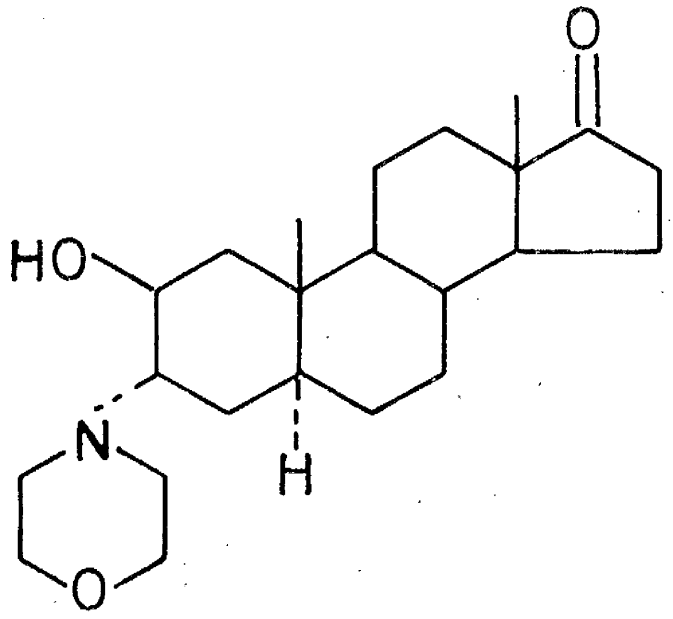
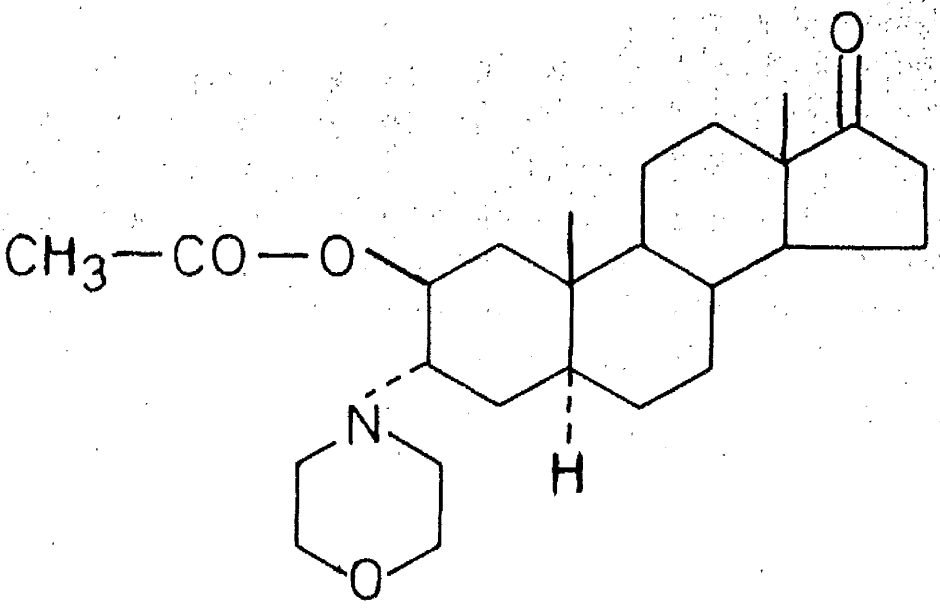
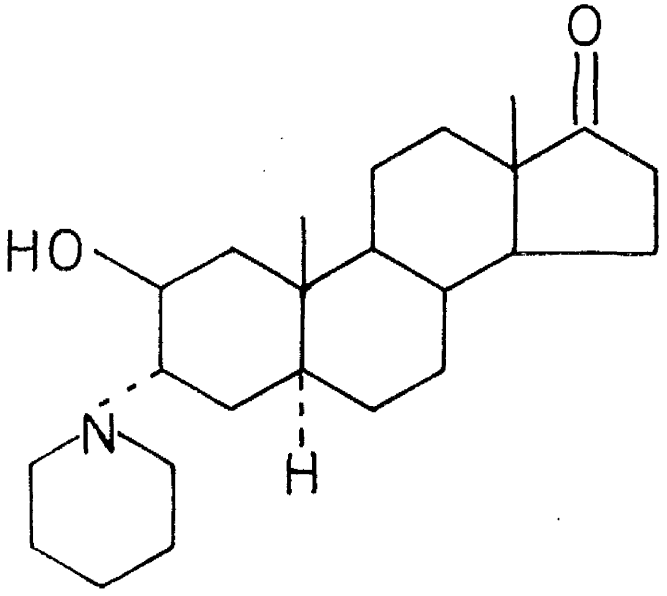


Compound A4

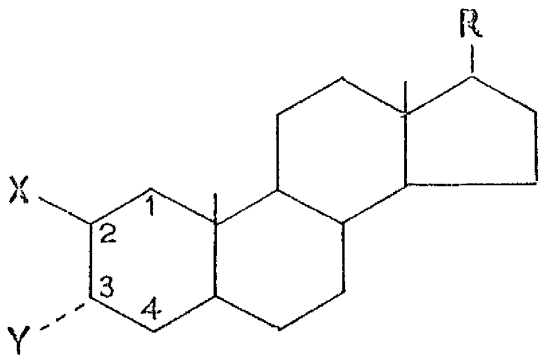


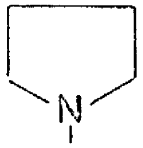
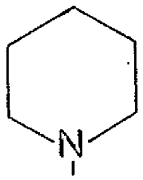
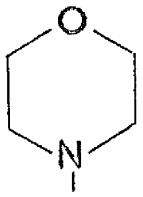
Compound A1

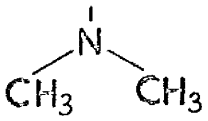
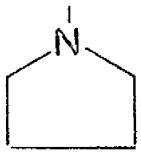
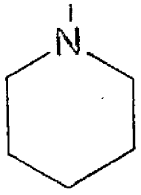
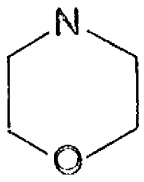


Compound A2

Table 1 Structural Formulae of Nitrogenous Steroids with Effects on the Central Nervous System.



where either X =  ,  or  , Y = OR′

or Y =  ,  ,  or  , X = OR′

R′ = H , CH_3CO or $CO(CH_2)_2COOH$

Fig. 4 General formula of twenty three of the twenty six nitrogenous steroids with effects on the central nervous system.

b) RESULTS.

Twenty three of the twenty six compounds under investigation were derived from 2β - and 3α-amino steroids of the androstane and pregnane types and fitted the general formula shown in Fig. 4. Compounds A5, A6 and A12 also possessed additional substituents at the 3, 11 or 16 positions of the steroid nucleus.

Two of the remaining three compounds possessed an amino substituent at either the 6α-position (compound A18) or at the 17β - position (compound A25) whilst the material designated compound A7 was in fact an equilibrium mixture of 2α-morpholino-5α-androstane-3,17-dione and 2β-morpholino-5α-androstane-3,17-dione (Savage, D.S., personal communication). The structural formulae of the compounds under investigation are shown in Table 1.

EXPERIMENTS USING MICE.

Toxicity Tests.

A positive biological response e.g., paralysis, convulsions or loss of the righting reflex is indicated by a plus sign, the intensity of effect being denoted by the number of plus symbols assigned:- four plus symbols indicated a very pronounced effect; three a marked effect; two a moderate effect; one a slight effect.

TABLE 2 (contd.)

Compound	Dose mg/kg	Increased Spontaneous Activity	Excitation	Convulsions	Decreased Spontaneous Activity	Sedation	Paralysis	Loss of Righting Reflex	Loss of Corneal Reflex	Loss of Pinna Reflex	Death
A20	50	+									
	75	+					±				20%
	87.5	+					+				20%
	100										100%
A21	50		±		+						
	75		+	+	+	+	++	+			20%
	100			+++	++		++	+			60%
	125										100%
A22	100										
	150		++	+							40%
	200										100%
A23	100										
	150				+						20%
	200	±		+	+						60%
	225										100%
A24	25		±		+		+	+		+	
	37.5				++		++	++			
	50				++	+	+++	+++		+	40%
	62.5				+++	++	+++	+++		++	40%
	75				+++	++	+++	+++		++	80%
A25	50	+	+	+	+						
	75	+	++	++	+		+	+			40%
	100										100%
A26	25				+		+				
	50			++	+		+	+			
	62.5			++	+		++	+			50%
	75										100%

TABLE 2 (contd.)

Compound	Dose mg/kg	Increased Spontaneous Activity	Excitation	Convulsions	Decreased Spontaneous Activity	Sedation	Paralysis	Loss of Righting Reflex	Loss of Corneal Reflex	Loss of Pinna Reflex	Death
A11	25				+						
	50		+	+	++		++				50%
	75										100%
A12	50				+			+		+	
	75				++	+	+	+		+	
	100				++	+	+	++		++	20%
	125				++	+	++	+++		+++	60%
	150										100%
A13	50		+	+	+		+				
	75		+	++	++	+	+++	+			40%
	100										100%
A14	25	±	+	++	+		+				
	50		+	++	++		++	++			40%
	75										100%
A15	50		+	++	+		++	+			
	62.5			+++			++	++			80%
	75										100%
A16	50	+	+	+			+				
	75		++	++	+		++				40%
	100										100%
A17	50										
	75				++		+	+		+	
	100				++	+	++	++		+	40%
	125			+	+++	++	++	+++		++	80%
A18	100						+			+	
	125		+	±	+		++	±		++	40%
	150										100%
A19	200		+		+					+	
	250				++	++	+	+		+	
	300				+++	++	++	+		++	40%
	400										100%

TABLE 2

TOXICITY TESTS ON MICE

Compound	Dose mg/kg	Increased Spontaneous Activity	Excitation	Convulsions	Decreased Spontaneous Activity	Sedation	Paralysis	Loss of Righting Reflex	Loss of Corneal Reflex	Loss of Pinna Reflex	Death
A1	50		+		+						
	75	±	+	+	+		+				
	100	+	+	+	+		+				
	150	++	++	++	+		++	+			20%
	175	++	++	+++	++		++	++			80%
A2	25		±	+	+	+	+	+			
	50	+	+	+	++	+	++	+			50%
	100										100%
A3	25	±			+		+				
	50	+	+	+	+		+				20%
	75										100%
A4	50				+		±				
	100			+	+		+	+			50%
	125										100%
A5	50										0%
	100		+		+	+	+	+			40%
	125		+	+	+		+	+			80%
	150										100%
A6	50										0%
	100			+	+		+	±			20%
	125		+	++	+		+	+			60%
	150										100%
A7	100				+		+			+	
	125				+	+	++	+		+	20%
	150				+	+	++	+		++	40%
	175										100%
A8	50		±		+	+	±	+			
	100		+	+	++	+	+	+			40%
	125										100%
A9	150		+								
	200	+	++								
	250	++	++				+				40%
	300	++	++				+++				80%
A10	25										
	50	+	+	+	++		+	+			80%
	75										100%

The compounds under investigation exhibited varying degrees of central nervous system activity including stimulation and depression (Table 2). Twelve of the amino steroids (compounds A1, A2, A3, A4, A8, A13, A14, A15, A16, A21, A25 and A26) on intravenous injection at doses (25 to 50 mg/kg) half to two-thirds below that of their toxic range, initially produced increased spontaneous activity, excitation and convulsions superseded by decreased movement, sedation and paralysis, none of the effects, however, being very striking. As the dose levels were increased into the lethal range, the effects observed became more intense in nature.

Compounds A12 (75 mg/kg), A17 (75 mg/kg), A19 (250 mg/kg) and A24 (37.5 mg/kg), however, these doses producing no fatalities in mice, possessed moderate sedative and paralyzing activity without previous stimulation of the central nervous system. Compounds A12, A17 and A24 also abolished righting and pinna reflexes but not the corneal reflex at doses (55 mg/kg, 79 mg/kg, and 28 mg/kg respectively) considerably below their LD50 (110 mg/kg, 98 mg/kg, and 49 mg/kg respectively).

None of the 26 compounds produced strychnine- or leptazol-like convulsions and no analgesia was observed at non-lethal doses. Death in all cases followed respiratory failure, usually preceded by convulsions.

TABLE 4 (continued)

Protective effect of compounds against Leptazol and Electrically induced Seizures in mice.

Compound	PD50 at 5 min mg/kg	PD50 at 20 min mg/kg	LD50 mg/kg
A25 Electroshock	0	0	75-100
Leptazol	0	0	
A26 Electroshock	33 ± 2.85	0	62.5
Leptazol	0	0	
Trimethadione Leptazol	327 ± 7.0	335 ± 9.3	
Sodium Diphenylhydantoin Electroshock	21 ± 1.25	20 ± 1.9	
Sodium Phenobarbitone Electroshock	30 ± 3.1	26 ± 2.6	
Leptazol	29 ± 2.2	27 ± 1.9	

TABLE 4 (continued)

Protective effect of compounds against Leptazol and Electrically induced Seizures in mice.

Compound	PD50 at 5 min mg/kg	PD50 at 20 min mg/kg	LD50 mg/kg
A19			
Electroshock	0	0	300-400
Leptazol	0	0	
A20			
Electroshock	0	0	87.5-100
Leptazol	0	0	
A21			
Electroshock	45 ± 4.5	64 ± 7.7	101 ± 11.3
Leptazol	0	0	
A22			
Electroshock	0	0	150
Leptazol	0	0	
A23			
Electroshock	0	0	175-200
Leptazol	0	0	
A24			
Electroshock	20.5 ± 2.5	47 ± 5.2	49 ± 4.4
Leptazol	46 ± 4.4	48 ± 6.7	

TABLE 4 (continued)

Protective effect of compounds against Leptazol and Electrically induced Seizures in mice.

Compound	PD50 at 5 min mg/kg	PD50 at 20 min mg/kg	LD50 mg/kg
A13 Electroshock	0	0	75-100
Leptazol	0	0	
A14 Electroshock	39.3 ± 4.4	0	46 ± 4.5
Leptazol	0	0	
A15 Electroshock	slight action	0	50-62.5
Leptazol	0	0	
A16 Electroshock	0	0	75
Leptazol	0	0	
A17 Electroshock	53 ± 6.5	81 ± 8.4	98 ± 3.6
Leptazol	0	0	
A18 Electroshock	0	0	125-150
Leptazol	0	0	

TABLE 4 (continued)

Protective effect of compounds against Leptazol and Electrically induced Seizures in mice.

Compound	PD50 at 5 min mg/kg	PD50 at 20 min mg/kg	LD50 mg/kg
A7 Electroshock	0	0	150-175
Leptazol	0	0	
A8 Electroshock	35.5 ± 5.7	0	102.3 ± 6.7
Leptazol	0	0	
A9 Electroshock	0	0	250
Leptazol	0	0	
A10 Electroshock	0	0	25-50
Leptazol	0	0	
A11 Electroshock	0	0	50
Leptazol	0	0	
A12 Electroshock	90 ± 6.25	0	110 ± 5.0
Leptazol	0	0	

TABLE 4

Protective effect of compounds against Leptazol and Electrically induced Seizures in mice.

Compound	PD50 at 5 min * mg/kg	PD50 at 20 min * mg/kg	LD50 mg/kg
A1 Electroshock	0	0	150-175
Leptazol	0	0	
A2 Electroshock	0	0	50
Leptazol	0	0	
A3 Electroshock	0	0	50-75
Leptazol	0	0	
A4 Electroshock	0	0	100
Leptazol	0	0	
A5 Electroshock	47.3 ± 3.8	86 ± 5.2	110.5 ± 5.0
Leptazol	0	0	
A6 Electroshock	108 ± 6.0	0	114 ± 6.3
Leptazol	0	0	

* = Time between injection of compound and electroshock
= Time between injection of compound and injection of Leptazol.
PD50 = That dose protecting 50% of the mice injected with test compound.

TABLE 3

Loss of righting reflex. LD50 and ED50. Values and duration of loss of righting reflex in mice of compounds A12, A17, A24 and mephenesin.

Compound	ED50 mg/Kg	Dose* mg/Kg	Duration** in min	LD50 mg/Kg	Therapeutic Index
A12	55 ± 4.2	82.5	8.8 ± 4.5	110 ± 5.0	2.0
A17	79 ± 4.7			98 ± 3.6	1.24
A24	28 ± 1.6	42.0	16.8 ± 8.1	49 ± 4.4	1.75
Mephen-esin	97 ± 8.2	145.0	9.8 ± 5.3	154 ± 11.0	1.59

* One and a half times the ED50

** Duration of loss of righting reflex.

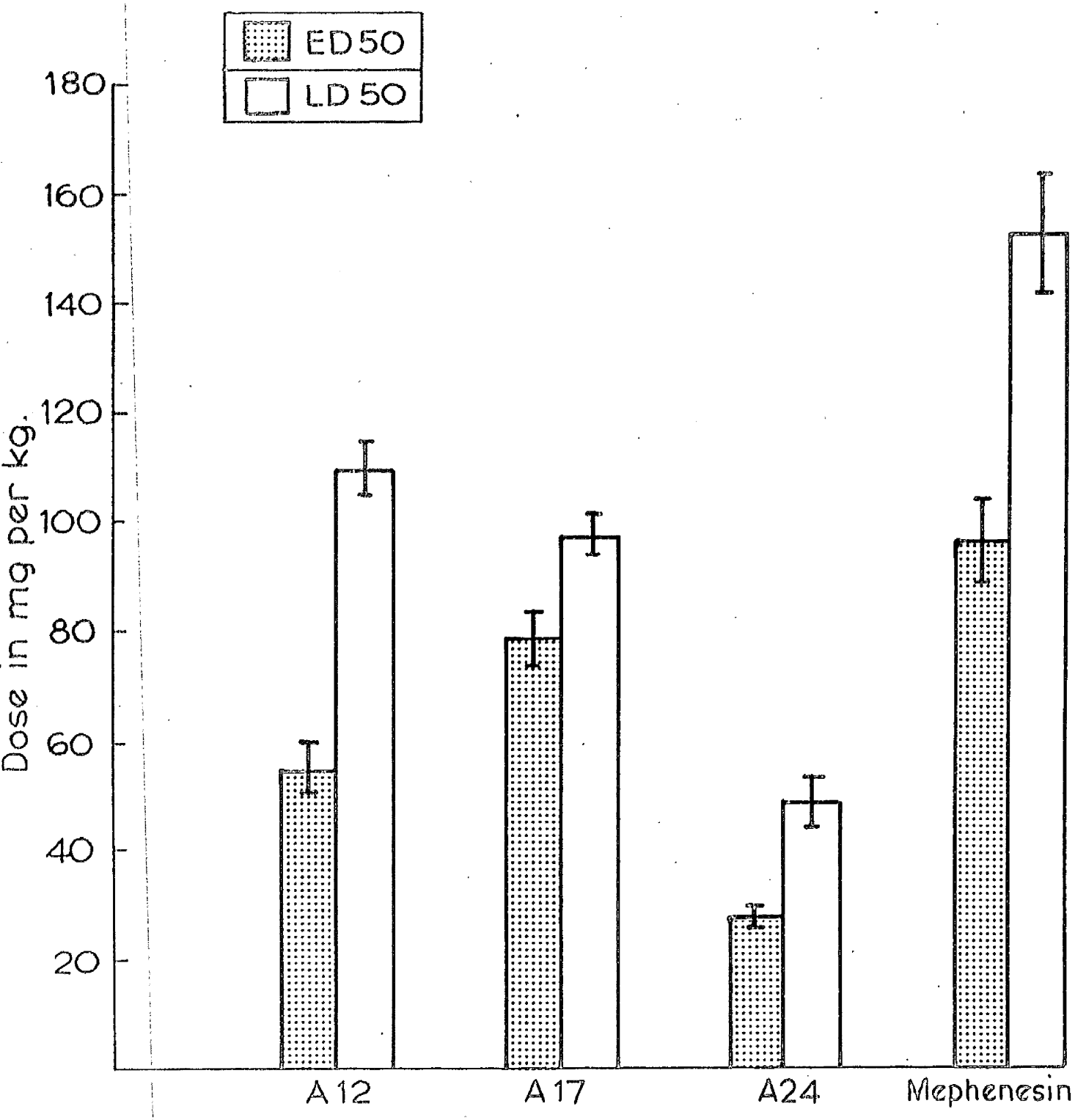


Fig. 5 Histogram of loss of righting reflex activity. LD50 and ED50 values of compounds A12, A17, A24 and mephenesin.

Loss of the Righting Reflex.

During toxicity tests on mice only compounds A12, A17, and A24 caused a moderate loss of the righting reflex at doses considerably below their toxic range. The ED50 was therefore determined for these compounds and also for mephenesin (Fig.5).

Compound A24 produced loss of the righting reflex of approximately twice the duration of that found for compound A12 or mephenesin when one and a half times the ED50 dose was administered to a group of ten mice (Table 3). On the other hand, the therapeutic index was lower than that of compound A12 but higher than that of mephenesin. The therapeutic indices of compounds A12 and A24 compared very favourably with that of mephenesin (Fig. 5).

Anticonvulsant Activity.

Table 4 shows the doses of the compounds required to protect 50% of the mice against electroshock and leptazol-induced seizures. Their LD50 values are also shown.

i) Protection Against Electrically-Induced Seizures.

Compounds A5, A6, A8, A12, A14, A17, A21, A24, and A26, at doses ranging from 20 to 108 mg/kg, were active in protecting 50% of the mice from electrically-induced

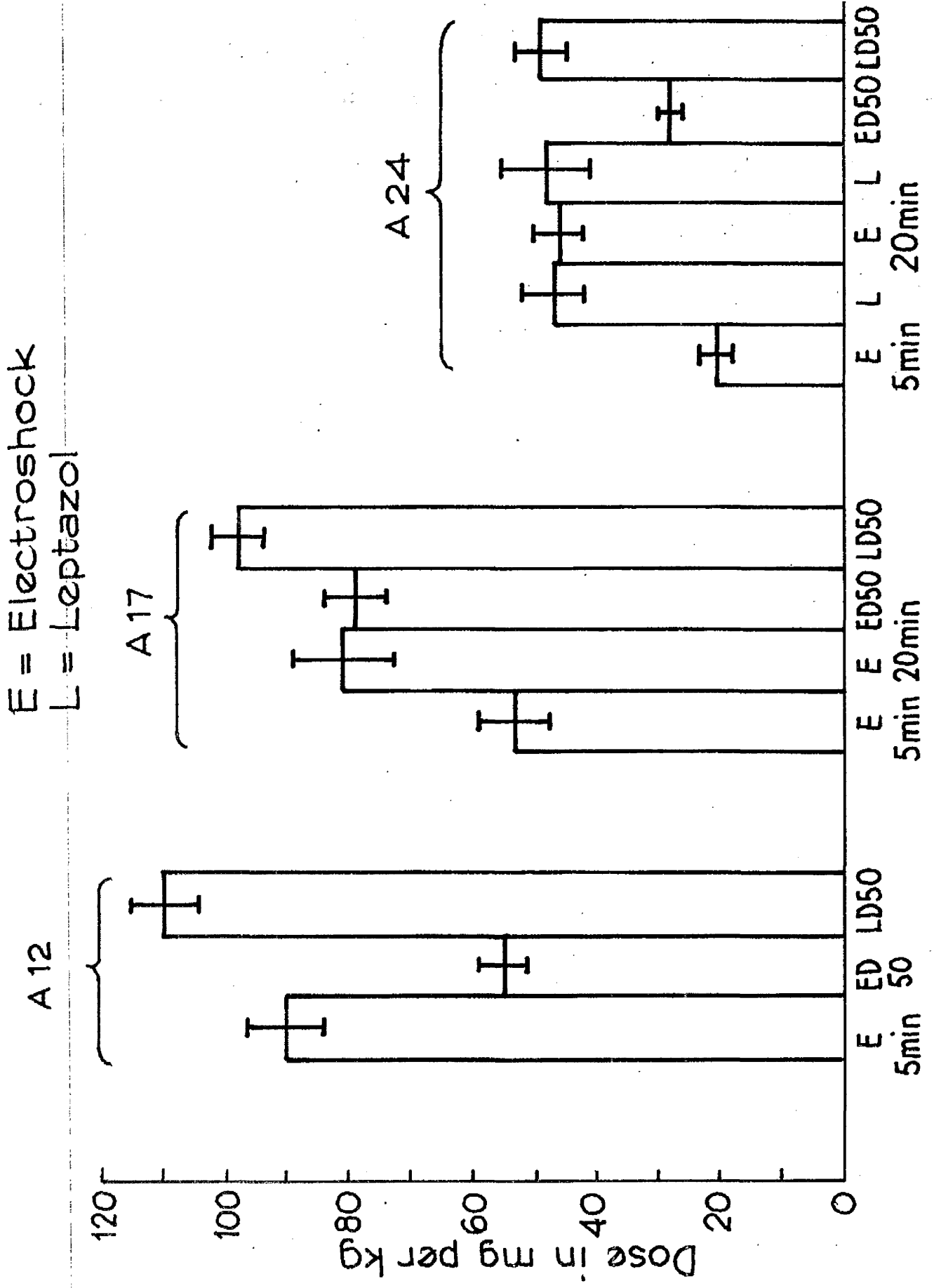


Fig. 8 Histogram of loss of righting reflex, anti-electroshock and anti-leptazol activities. ED50 values for loss of righting reflex, PD50 values for anti-electroshock and anti-leptazol activities and LD50 value for compounds A12, A17 and A24.

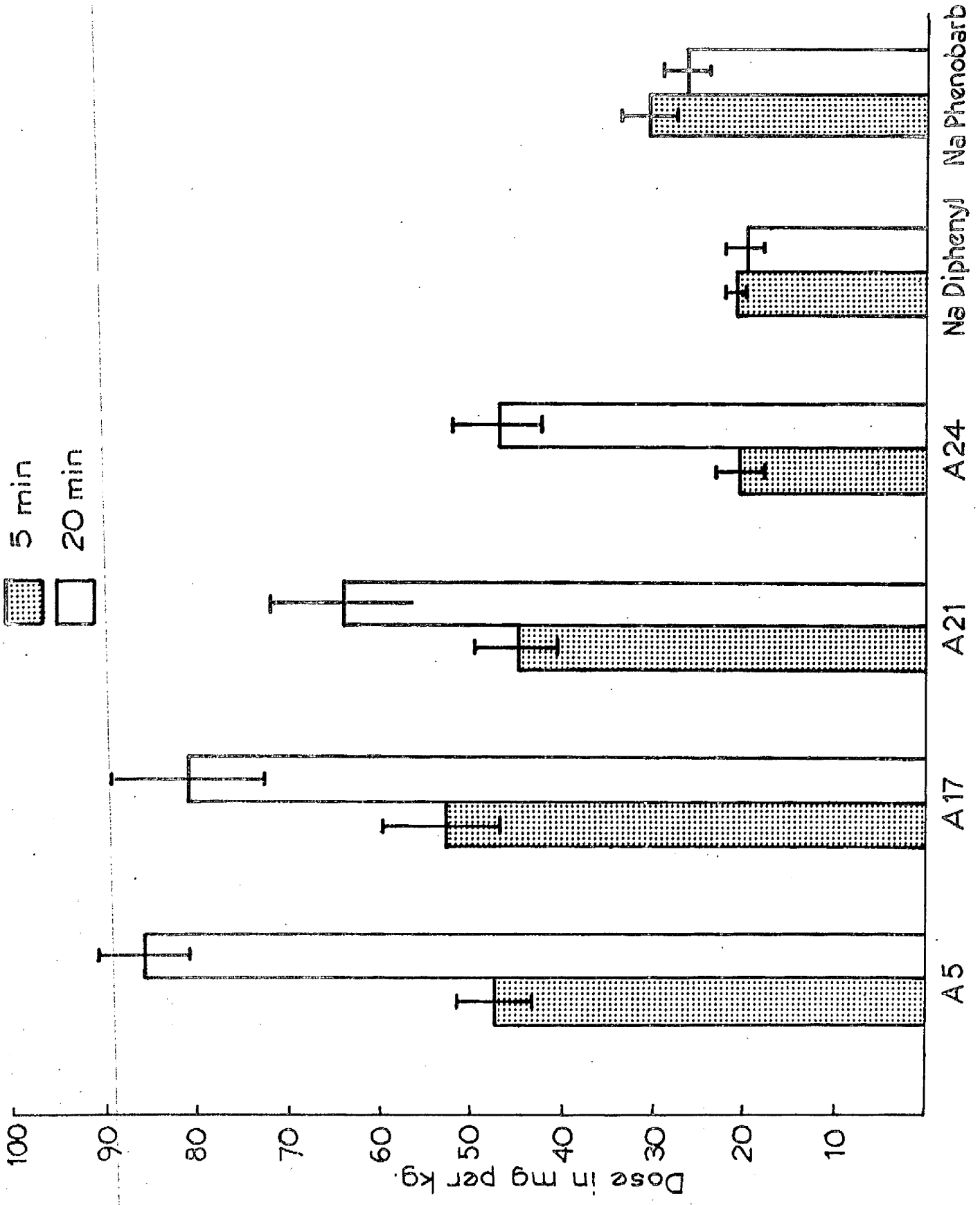


Fig. 6 Histogram of anti-electroshock activity. PD50 values at 5 min. and 20 min. for compounds A5, A17, A21, A24, sodium diphenylhydantoin and sodium phenobarbitone.

seizures 5 minutes after injection. However, when the period of time from injection to testing was increased to 20 minutes only compounds A5, A17 and A24 in doses (86 mg/kg, 81 mg/kg and 47 mg/kg respectively) close to their LD50 and compound A21 (64 mg/kg) were active in protecting mice from seizures (Fig. 6).

When the PD50 (5 min) values of the compounds were compared with their ED50 values for the loss of the righting reflex compound A17 was shown to have the most favourable index of activity (53 mg/kg v 79 mg/kg). However, the PD50 value of compound A17 increased to 81 mg/kg when the interval between testing and injection became 20 minutes (Fig.8).

It is obvious that marked anti-electroshock activity is not a specific property of the amino steroids under investigation since none showed anticonvulsant activity without the appearance of side effects such as excitation, sedation and/or loss of righting reflex. Figure 8 shows that a marked correlation exists between anti-electroshock activity and the ability to produce loss of the righting reflex. In contrast, the clinically useful anticonvulsant drugs sodium diphenylhydantoin and sodium phenobarbitone protected against electrically-induced seizures without the appearance of any visible side effects. Furthermore, their PD50 values at 5 and 20 minutes

TABLE 5

Anti-Parkinson Activity of compounds A5, A12, A17, A19, A21, A24 and atropine in mice.

Compound	Dose mg/kg	15 min*** Score*	15 min** %	30 min Score	30 min %	45 min Score	45 min %	60 min Score	60 min %	90 min Score	90 min %
A5	45	28	12.5	28	12.5	31	3	32	-	32	-
	90	28	12.5	27	16	30	6	32	-	31	3
A12	50	28	12.5	29	9.5	31	3	32	-	32	-
	100	24	25	27	16	30	6	28	12.5	32	-
A17	37.5	28	12.5	29	9.5	32	-	32	-	32	-
	75	22	31.25	29	9.5	32	-	32	-	32	-
A19	125	30	6	28	12.5	29	9.5	30	6	32	-
	250	24	25	26	18.75	30	6	30	6	30	6
A21	25	28	12.5	30	6	28	12.5	31	3	30	6
	50	27	15.5	30	6	31	3	32	-	32	-
A24	20	30	6	28	12.5	28	12.5	31	3	32	-
	40	22	31.25	27	15.5	27	15.5	30	6	30	6
Atropine	2.5	0	100	0	100	3	93.75	3	93.75	3	93.75
	5	0	100	0	100	0	100	0	100	0	100

* For description of Scoring System employed, see page

** Percentage Degree of Protection Afforded against Tremorine-induced Seizures.

*** Observation taken 15 min after injection of Tremorine.

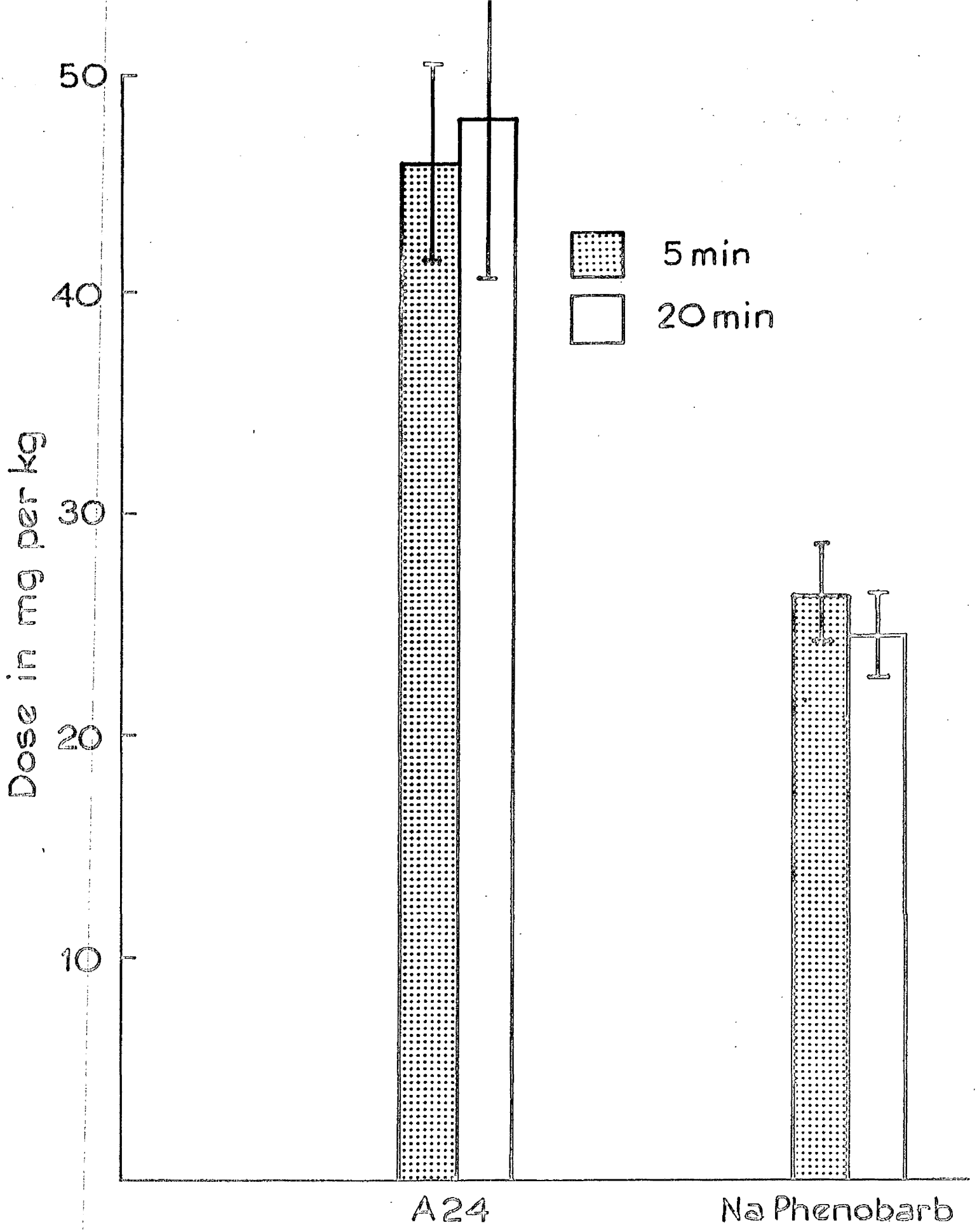


Fig. 7 Histogram of anti-leptazol activity. PD50 values at 5 min. and 20 min. for compounds A24 and sodium phenobarbitone.

were virtually the same.

ii) Protection Against Leptazol-Induced Seizures.

Only compound A24 was active against leptazol-induced seizures (Fig. 7) and this was at a dose approximately twice that necessary to protect 50% of the mice against electrically-induced seizures (Table 4). The PD50 value (5 min) and PD50 value (20 min) were both very close to the LD50 and much higher than the ED50 value for loss of righting reflex (Fig. 8).

In view of these findings, protection against leptazol-induced seizures would not appear to be an important feature of the pharmacological activity of the compounds under investigation.

Anti-Parkinsonian Activity.

Compounds A5, A12, A17, A19, A21 and A24 were selected for this test since during preceding experiments these appeared the most active in producing sedation, loss of righting reflex and/or protection against electrically-induced seizures.

The results in Table 5 indicate that none of the compounds in doses of from 20 to 250 mg/kg appear to possess the ability to abolish either the tremorigenic or parasympathetic effects of tremorine. Atropine sulphate, 2.5 mg/kg, injected intravenously, almost

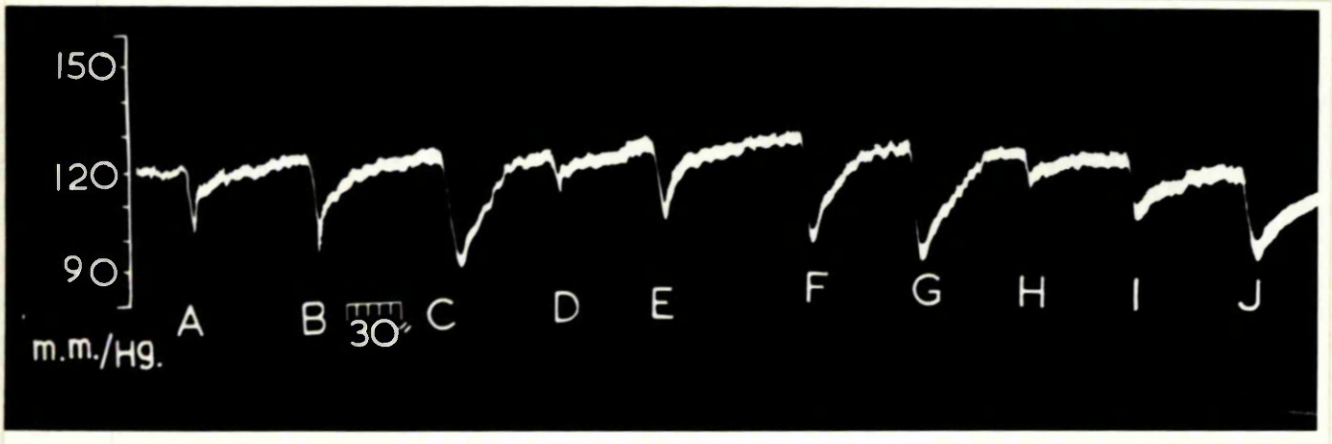


Fig. 10 Cat. Pentobarbitone anaesthesia. Blood pressure record from common carotid artery. Drugs administered intravenously. Vertical scale, on left hand side, indicates the blood pressure in mm of mercury.

At A, B and C, compound AlO 1, 2 and 4 mg/kg respectively.

At D, E, F and G, compound All 1, 2, 4 and 8 mg/kg respectively.

At H, I and J compound Al3 1, 2 and 4 mg/kg respectively.

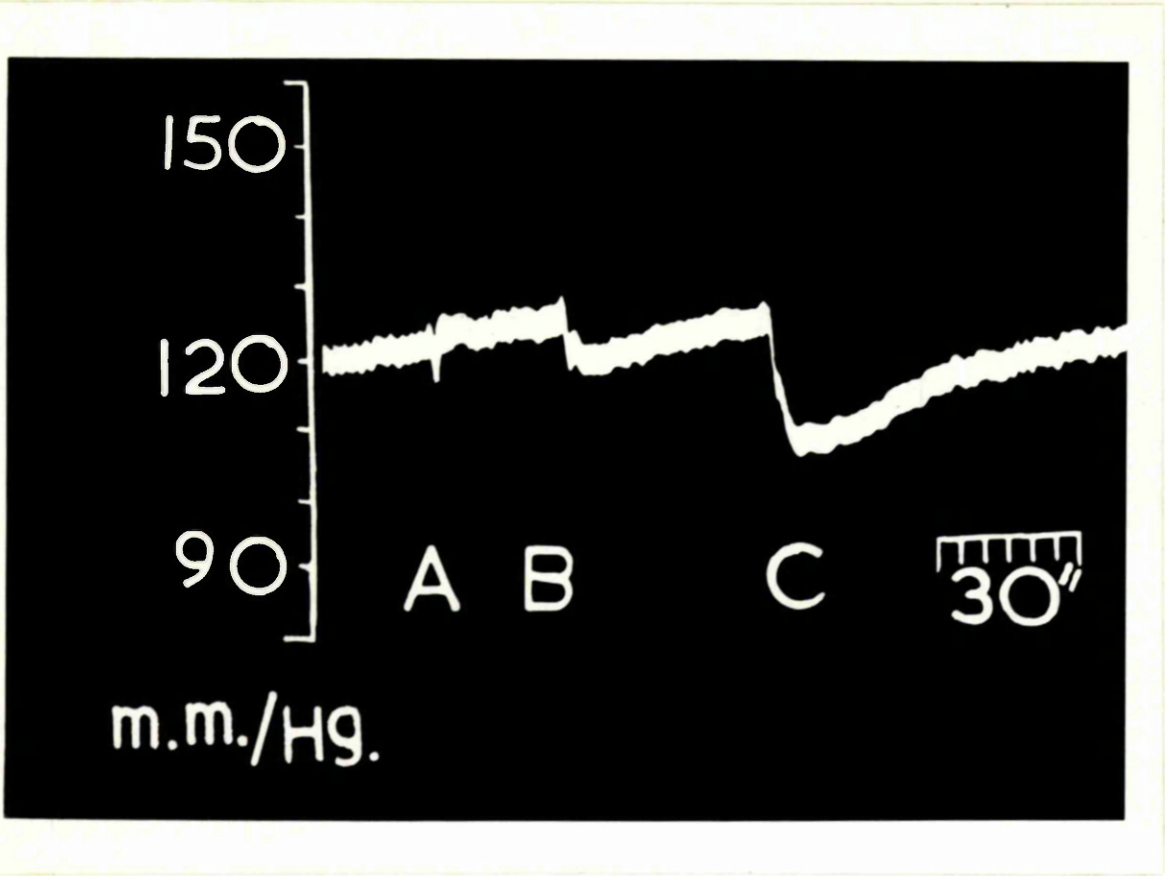


Fig. 9 Cat. Pentobarbitone anaesthesia. Blood pressure record from common carotid artery. Drugs administered intravenously. Vertical scale, on left hand side, indicates the blood pressure in mm of mercury.

At A, B and C, compound A18 1, 2 and 4 mg/kg respectively.

completely inhibited the tremors and completely antagonized the salivatory, urinary and defaecatory effects induced by tremorine. Moreover, it produced no noticeable side effects.

It appears unlikely that anti-Parkinsonian activity is a property of the steroids under investigation.

EXPERIMENTS USING CATS.

Effect on the Blood Pressure of the Pentobarbitone-Anaesthetized Cat.

All the compounds produced a depressor response on intravenous administration. Compounds A2, A3, A6, A12, A14, A16, A17, A18, A21 and A24 (all at a dosage of 4 mg/kg) had a moderate depressant effect, producing a fall of about 30 mm of mercury. Compounds A4, A5, A10, A11, A13 and A20 (4 - 5 mg/kg) had a more pronounced effect, each producing a fall of 40 to 60 mm. Figures 9 and 10 show the effects of compounds A10, A11, A13 and A18 on the blood pressure.

The Cat Nictitating Membrane Preparation.

Since compounds A4, A5, A10, A11, A13 and A20 had the most pronounced hypotensive effect, their potential sympathetic ganglion blocking activity was ascertained.

None of the compounds in doses of up to 10 mg/kg produced any reduction in the height of the contraction of

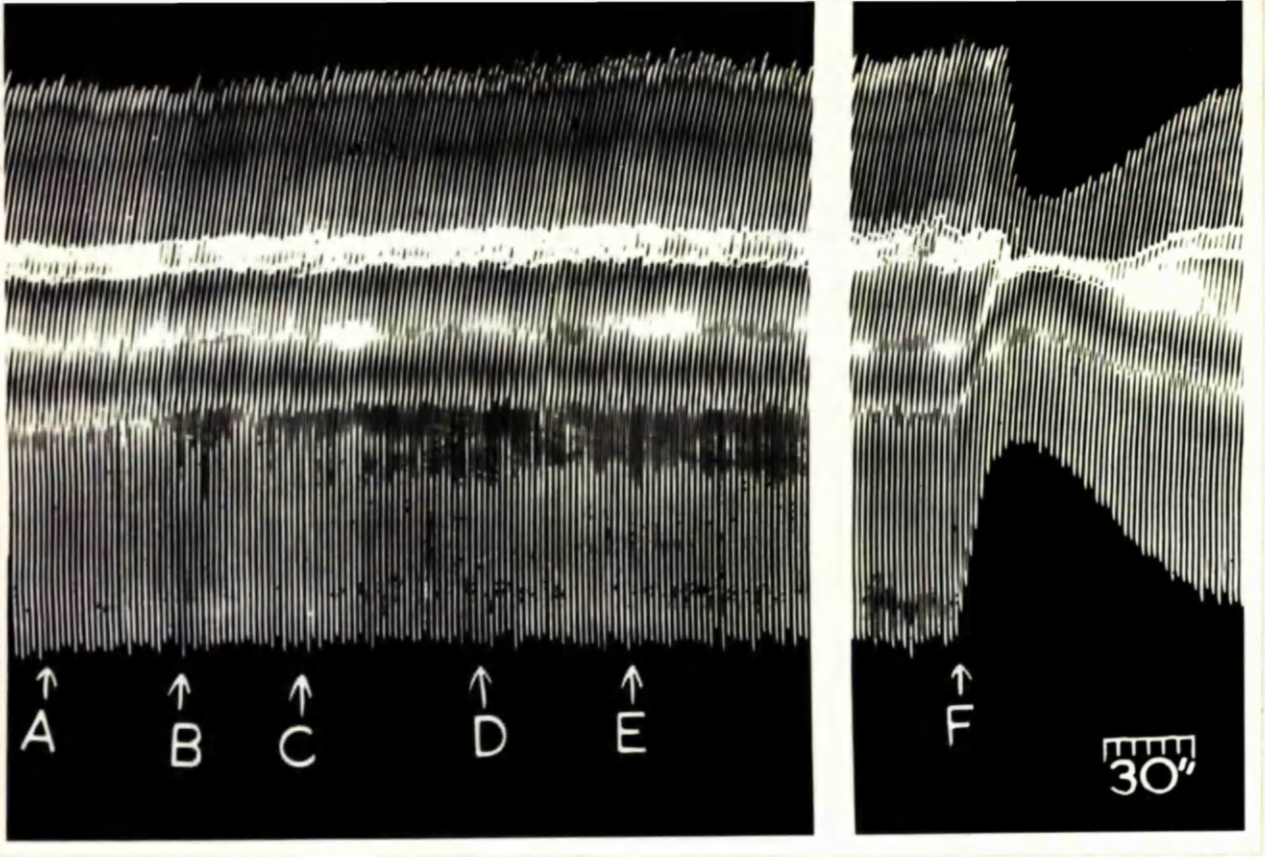


Fig. 14 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Indirect stimulation via the sciatic nerve at a frequency of 6/min, 6 V and 1 msec.

Drugs administered intravenously.

At A, B and C, compound A10 2, 4 and 8 mg/kg respectively.

At D and E, compound A11 5 and 10 mg/kg respectively.

At F, d-tubocurarine 0.10 mg/kg.

There was an interval of 7 min between the administration of E and F.

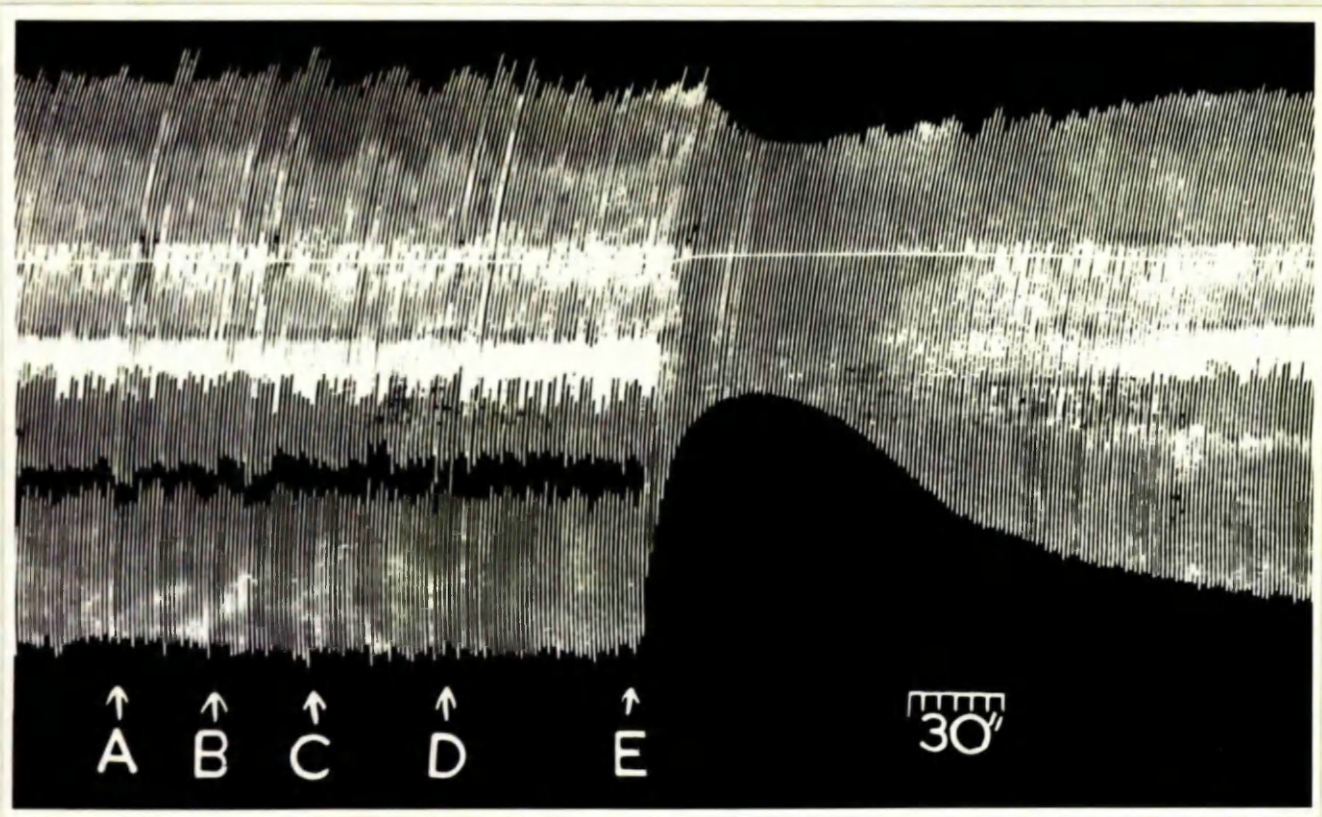


Fig. 13 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Indirect stimulation via the sciatic nerve at a frequency of 6/min, 5 V and 2 msec.

Drugs administered intravenously.

At A, B, C and D, compound A6 1, 2, 5 and 10 mg/kg respectively.

At E, d-tubocurarine 0.10 mg/kg.

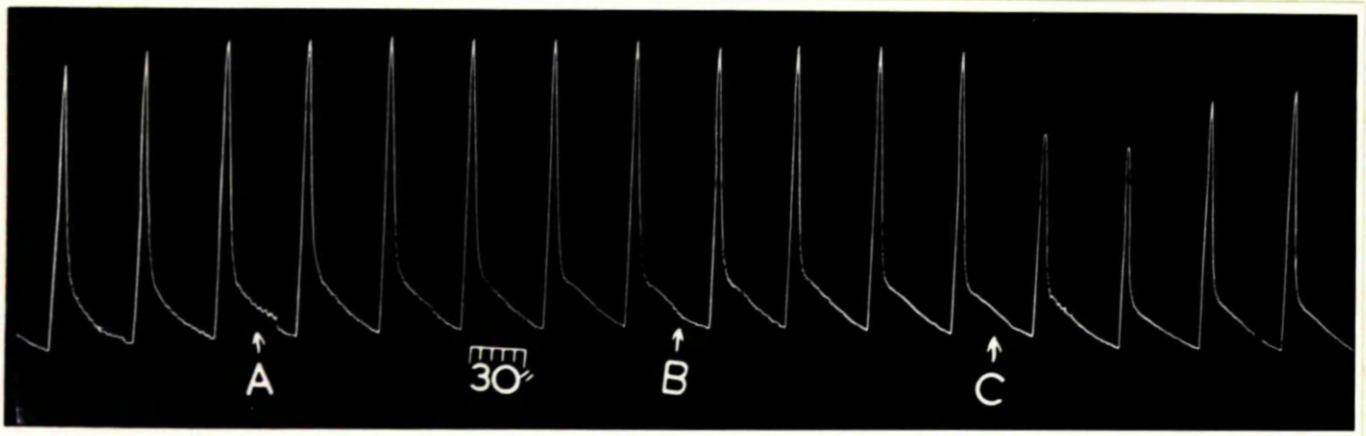


Fig. 12 Cat. Pentobarbitone anaesthesia. Contractions of the nictitating membrane elicited at 3 min intervals by preganglionic stimulation of the superior cervical nerve trunk at a frequency of 1200 impulses/min, 6 V and 0.5 msec for 15 sec. Drugs administered intravenously 1 min before stimulation.

At A and B, compound A5 5 and 10 mg/kg respectively.

At C, hexamethonium 0.5 mg/kg.

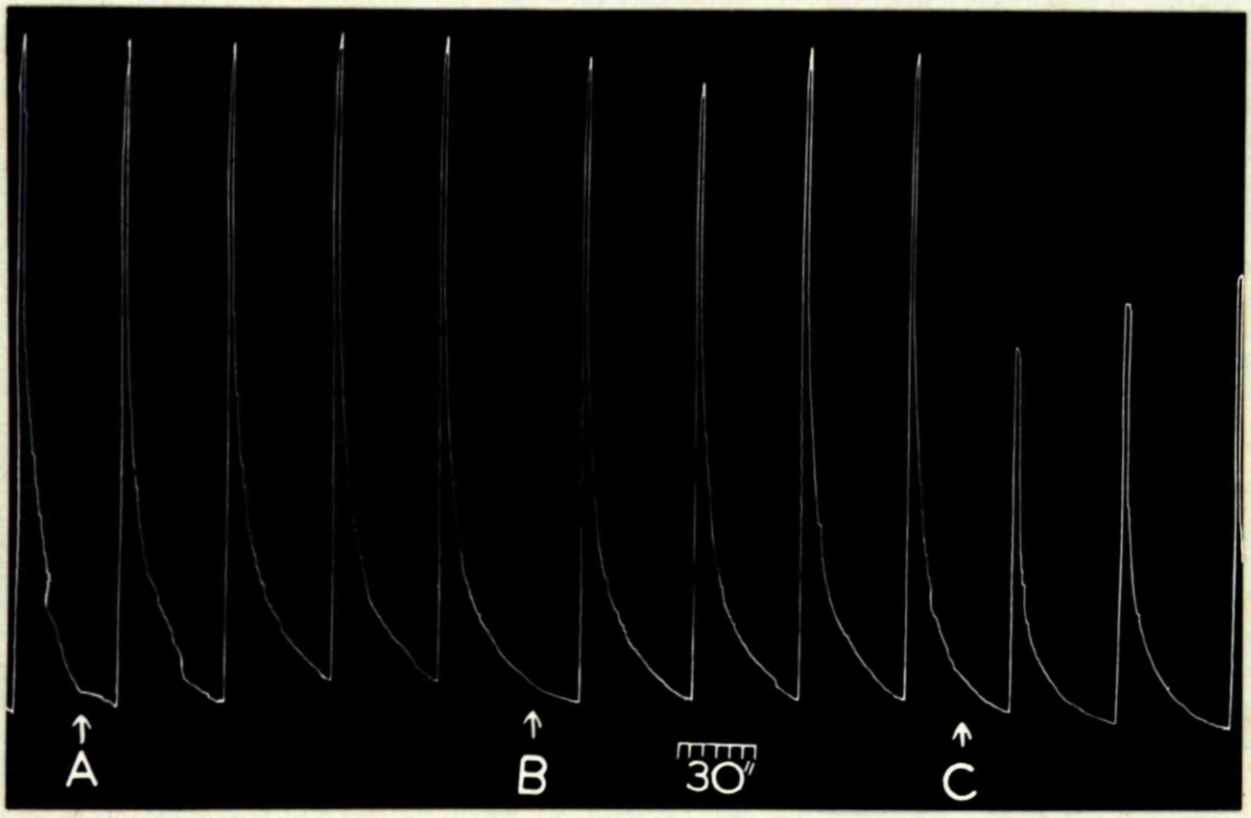


Fig. 11 Cat. Pentobarbitone anaesthesia. Contractio of the nictitating membrane elicited at 3 min. intervals by preganglionic stimulation of the superior cervical nerve trunk at a frequency c 1200 impulses/min, 8 V and 1.0 msec for 15 sec Drugs administered intravenously 1 min before stimulation.

At A and B, compound A20 4 and 8 mg/kg respectively.

At C, hexamethonium 0.5 mg/kg.

the nictitating membrane. In contrast, hexamethonium, 0.5 mg/kg, always markedly decreased the height of contraction (Figs. 11 and 12) in response to electrical stimulation.

In view of the absence of activity in this preparation, it seems unlikely that sympathetic ganglion blockade accounts for the hypotension observed with the steroids investigated.

Effect on the Gastrocnemius Muscle-Sciatic Nerve Preparation of the Anaesthetized Cat.

All the compounds were inactive on this preparation in doses up to 10 mg/kg (Figs. 13 and 14). In contrast, doses of 0.10 to 0.15 mg/kg of tubocurarine always produced marked neuromuscular paralysis.

Neuromuscular blockade, therefore, does not seem to be the cause of the paralysis and loss of righting reflex seen in mice during the toxicity tests.

Effect on the Crossed Extensor Preparation of the Spinalized Cat.

This preparation is used to identify compounds with interneuronal blocking activity. Only compounds A12, A17, and A24 (20 mg/kg) produced an inhibition of the crossed extensor reflex preparation. They were all less active on the preparation than mephenesin which at a dose

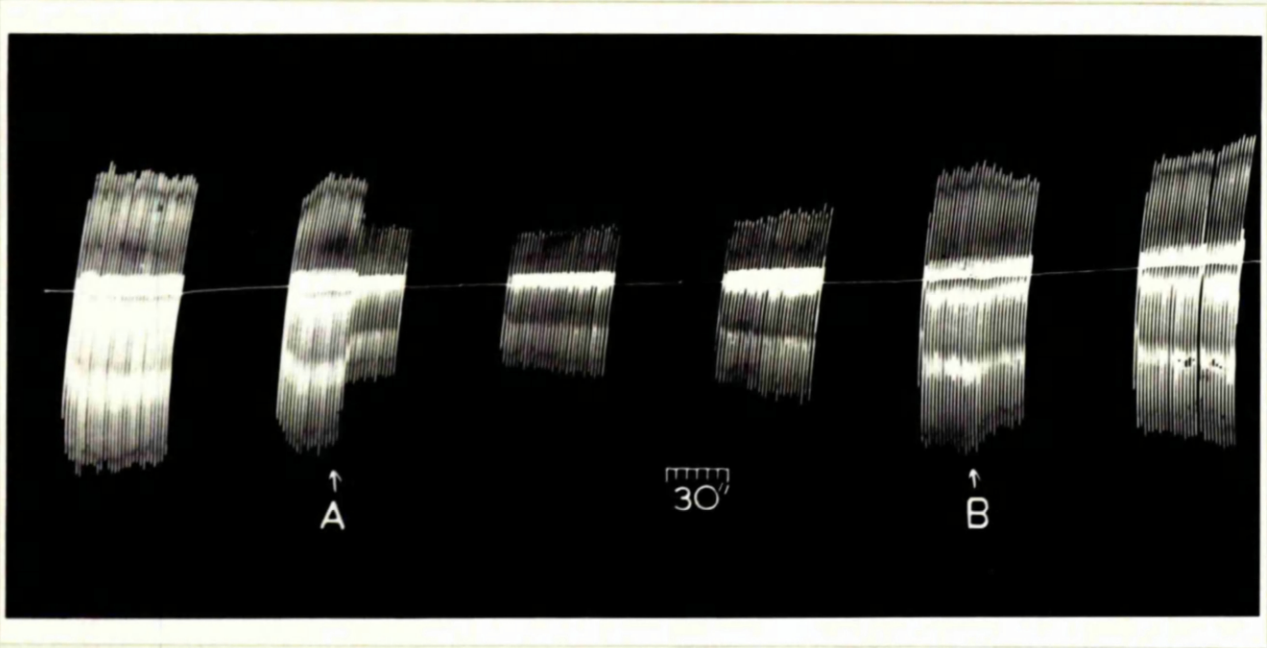


Fig. 16 Spinalized cat. Crossed extensor reflex preparation. Contraction downwards. Indirect stimulation via contralateral sciatic nerve at a frequency of 6/min, 3 V and 2 msec. Drugs administered intravenously.

At A, mephenesin 30 mg/kg.

At B, compound A12 20 mg/kg.

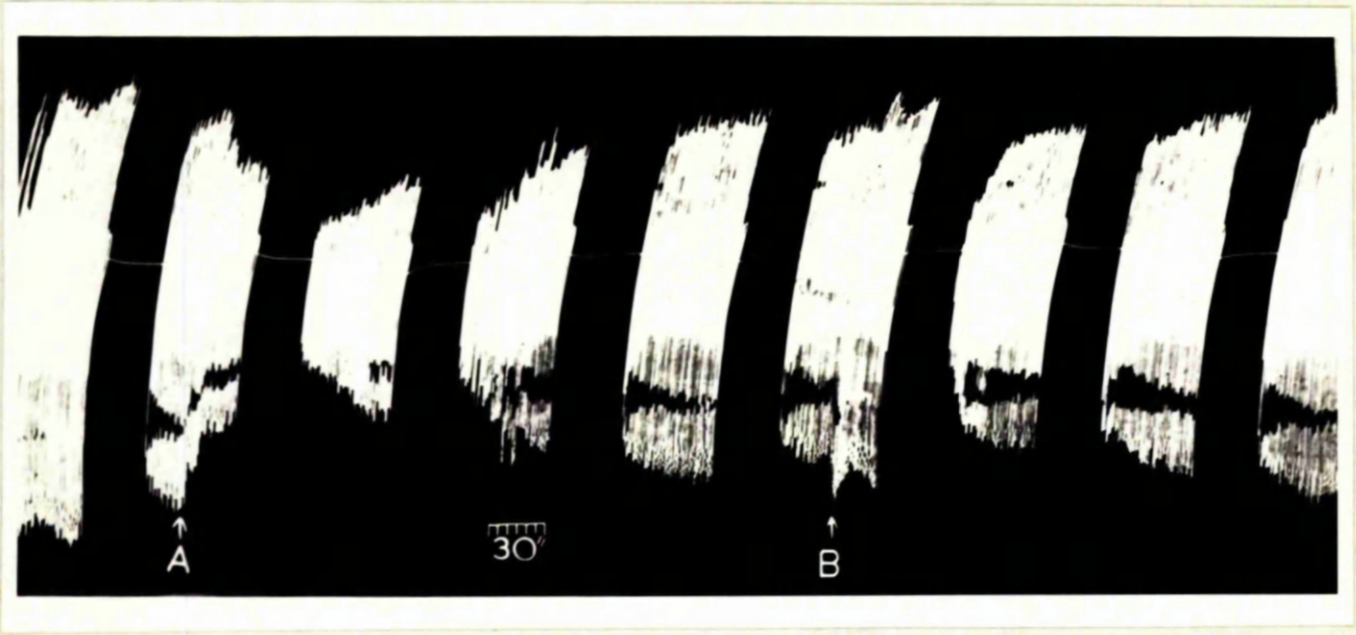


Fig. 15 Spinalized Cat. Crossed extensor reflex preparation. Contraction downwards. Indirect stimulation via contralateral sciatic nerve at a frequency of 6/min, 3 V and 2 msec.

Drugs administered intravenously.

At A, mephenesin 30 mg/kg.

At B, compound A24 20 mg/kg.

of 30 mg/kg produced an inhibition of the reflex three times as great as that of 20 mg/kg of compounds A12 and A24 (Figs. 15 and 16).

D I S C U S S I O N

c) DISCUSSION.

None of the 26 amino steroids of the present investigation (Table 1) exhibited any potentially useful clinical properties as judged from the results of the pharmacological tests to which they were subjected.

Results from the acute toxicity tests showed that the compounds did not induce a degree of anaesthesia sufficient for surgery nor did they produce any marked stimulation of the central nervous system. Compounds A12, A17 and A24, however, did produce a marked loss of the righting reflex and showed anticonvulsant activity. Compounds A5, A6, A8, A14, A21 and A26 gave protection against electrically-induced seizures although in no case was anti-electroshock activity observed without the simultaneous presence of side effects such as excitation, sedation and/or loss of the righting reflex. This would suggest that anticonvulsant activity is not a specific property of the amino steroids under investigation. Apart from compound A24, there was no amino steroid active against leptazol-induced seizures.

Loss of the righting reflex can be produced by a variety of pharmacological agents such as anaesthetics, interneuronal blocking agents, neuromuscular blocking agents and other central nervous system depressants (O'Dell, 1960) and it is not possible employing solely

observational techniques to conclude by what primary mechanism of action a drug produces this effect. However the absence of any effect on the cat gastrocnemius muscle-sciatic nerve preparation by the amino steroids used in the present work would indicate that loss of the righting reflex was not due to neuromuscular blocking activity. Moreover, during the toxicity experiments, no general anaesthetic effect was apparent, as judged by the fact that the reflex response to painful stimuli was not abolished by any of the compounds. However, administration of compounds A12, A17 or A24 to mice caused the pinna reflex to be abolished before the corneal reflex, suggesting that the loss of the righting reflex might be due to the possession of interneuronal blocking activity by the steroids being tested (Goodsell et al. 1954). This view was further substantiated by the finding that compounds A12, A17 and A24 had the ability to inhibit the crossed extensor reflex in the cat, a property typically possessed by interneuronal blocking agents e.g., mephenesin (Domino, 1964).

None of the amino steroids antagonized the tremors induced by tremorine (1,4-dipyrrolidino-2-butyne) (Everett, 1956). This observation would indicate that the compounds do not possess either central anticholinergic or central sympathomimetic activity (Spencer, 1965) and

that they do not inhibit the conversion of tremorine into its active metabolite oxotremorine in the liver (Leslie & Maxwell, 1964). In view of the negative results obtained in the tremorine test, it is unlikely that the compounds would be of any value in the treatment of Parkinson's disease.

Compounds A12, A17 and A24 induced a fall in blood pressure not only in intact but also in spinalized cats. There are many factors, both central and peripheral, which may contribute to a compound producing a fall in blood pressure on administration to experimental animals. Since in the spinalized animal, the brain has been destroyed, it is unlikely that the hypotensive activity of the compounds under investigation is due to any great extent to a depressant effect on the vasomotor centres of the brain stem and the medulla. Moreover, the hypotension does not seem to be attributable to sympathetic ganglion blocking activity (see page 52) and the fall in blood pressure may well be mediated via a direct action on the heart and/or the blood vessels.

In order to reach their site of action in the central nervous system the amino steroids have to cross the blood/brain barrier. It is generally believed that this barrier behaves as a selective lipoid membrane (Mark and others, 1957, 1958; Mayer, Maickel & Brodie, 1959).

Ionized substances, such as quaternary ammonium compounds, penetrate the blood/brain barrier much more slowly than do lipid soluble un-ionized compounds (Mayer & Bain, 1956; Levine 1959; Rall & Zubrod, 1960; Hansson & Schmiterlöw, 1961). In the latter, the rate of penetration is roughly parallel to the lipid-to-water partition coefficient of the un-ionized molecules (Mayer et al. 1959). With the exception of the esterified amino steroids (compounds A9, A19, A22 and A23), it seems likely that the compounds under investigation exist in the blood as lipid soluble un-ionized molecules which can readily penetrate the blood/brain barrier. Unfortunately, complete verification of this proposal was not possible due to the unfavourable solubility characteristics of the amino steroids preventing measurement of their pKa values from which, employing the Henderson-Haselbach equation, the percentage of amino-steroid which is un-ionized at biological pH can be calculated. The postulate nevertheless seems acceptable in view of the observation that the loss of the righting reflex induced by compounds A12, A17 and A24 is almost immediate following intravenous administration, a fact which also suggests that it is the compound per se and not a metabolite which possesses activity. The highly ionized, hydrophilic hemisuccinate steroid esters

(compounds A9, A19, A22 and A23), on the other hand, would be unlikely to cross the blood/brain barrier *per se*. It might be expected that *in vivo* hydrolysis of these compounds to the un-ionized lipid soluble parent amino steroids would take place through the action of the non-specific esterases of the serum, as was observed for other steroidal hemisuccinate esters by Figdor and others (1957). That this may not be the case for the hemisuccinate esters under investigation is demonstrated by the absence of central nervous system depressant activity in compound A23 (the hemisuccinate ester of compound A24), although A24 itself induces loss of the righting reflex. It would therefore seem that the hemisuccinate function of compound A23 may not be suffering *in vivo* cleavage to any great extent.

One interesting fact is apparent from Table 4 (opposite page 50); in those compounds showing protection against electroshock convulsions, the PD50 (20 min) is usually of the order of twice the dose of the PD50 (5 min). This might suggest that the compounds concerned are extremely rapidly deactivated in the body. If such were to be the case it might afford an added explanation as to why compound A23 is inactive since the rate of *in vivo* hydrolysis to compound A24 might be sufficiently slow with respect to the rate of inactivation

of compound A24 that a concentration of the latter necessary to produce an effect cannot be achieved. A study of the metabolism of these compounds would be of considerable interest but it was considered that such work was outwith the scope of this thesis.

The biological actions of the amino steroids reflect the properties of a group of structurally specific compounds since their potency and spectrum of activity can be vastly altered by small changes in chemical structure (see pages 48 and 49). It appears reasonable to assume, therefore, that the amino steroids react with hypothetical receptor sites which possess a geometry and electrical charge distribution complementary to those of the active drug molecule.

Unfortunately, owing to the limited number of compounds available, no broad generalizations on structure-action relationships would appear to be feasible. However, it was observed that anticonvulsant activity and loss of the righting reflex occurred more often with compounds having the nitrogen function in the 2 β - position than with compounds having the nitrogenous radical in the 3 α - position. Exceptions to this generalisation are, however, provided by compounds A8, A14 and A21 with respect to anti-electroshock activity, and possibly by compounds A15 and A19 which induce mild loss

of the righting reflex.

It is of some interest that Sugrue (1963) also found anticonvulsant activity and induction of loss of the righting reflex to be associated with other amino steroids containing a N-heterocycle in the 2β- position as a result of which he proposed that a β- face attachment of the steroid molecule to the receptor surface is operative. However, the present results with compounds A8, A14, A15, A19 and A21 would appear to make this hypothesis untenable.

A comparison of the structural features of compounds A5, A6 and A17 shows that all three compounds possess a 2β-morpholino group and the same basic pregnane skeleton. The least substituted member of these three compounds is A17 and it is also considerably more active than compound A5 in producing loss of the righting reflex, while compound A6 is inactive in this respect. It would thus appear, from this limited observation, that additional nuclear substitution in the amino steroids under investigation is detrimental to loss of the righting reflex activity. It is worthy of note that this conclusion is in agreement with the work of Figdor and his co-workers (1957) who also found that nuclear substitution decreased the central nervous system depressant activity of a series of water-soluble steroids.

S E C T I O N B

I. SURVEY OF THE EFFECTS OF NITROGENOUS STEROIDS AT THE NEUROMUSCULAR JUNCTION.

Interest in the pharmacology of drugs acting at the skeletal muscle--motor nerve junction has been sustained in recent years (Bovet, Bovet--Nitti & Martini-Bettolo, 1959; Lewis, 1960; Bowman, 1962; Stenlake, 1963; Taylor & Nedergaard, 1965; Thesleff & Quastel, 1965). Although the exact nature and properties of the cholinergic nicotinic receptor remain unknown, the recent chemical and pharmacological research devoted to rigid bisquaternary steroidal derivatives has been particularly relevant (May & Baker, 1963, 1965; Khuong Huu-Lainé & Pinto-Scognamiglio, 1964; Biggs et al. 1964; Martin-Smith & Sugrue, 1964; Alauddin et al. 1965), permitting an investigation of basic pharmacological problems of the receptor, unhampered by the changing of molecular conformation characteristic of non-rigid molecules. Compounds of this nature have also aroused clinical interest (Mushin & Mapleson, 1964) since they possess short-acting potent non-depolarizing properties in animals (see references cited).

In order to understand the complex mechanism of action of neuromuscular blocking agents, it is first essential to consider the anatomical features of the neuromuscular synapse and the physiological events which

occur there during neuromuscular transmission.

THE NEUROMUSCULAR JUNCTION.

i) Anatomical Features.

The anatomical features of the neuromuscular junction have been well defined, mainly as a result of light microscope (Coutcaux, 1947, 1955, 1958) and electron microscope studies (for reviews see Robertson, 1956; Andersson-Cedergren, 1959; and the monograph by Zacks, 1964).

As the motor nerve approaches the skeletal muscle surface, the nerve fibre loses its myelin sheath and divides into numerous terminal branchlets which lie in grooves or troughs - synaptic troughs - indenting the sarcolemma or muscle fibre surface. There is no direct protoplasmic continuity between the nerve and muscle fibre. Within the synaptic troughs, the sarcoplasm of the muscle fibre is thrown into deep infoldings - "junctional folds" (Robertson, 1956). The intervening gap (150 - 600 Å) between the nerve terminal and the muscle surface is called the synaptic cleft.

Anatomical features of the neuromuscular synapse have been correlated with their physiological function. Within the pre-synaptic nerve terminals, there is an accumulation of minute (ca. 500 Å diameter) secretory granules, "synaptic vesicles" (de Robertis & Bennett, 1955), which it is believed may be implicated in

the storage of acetylcholine (de Robertis & Bennett, 1955; Robertson, 1956). Histological and centrifugation studies have confirmed that acetylcholine and choline acetylase, the enzyme responsible for the biosynthesis of acetylcholine, occur together in the microsomal, or small granule fraction, of nerve tissue homogenates (Hebb, 1963). On the other hand, acetylcholinesterase, the specific enzyme responsible for the hydrolytic destruction of acetylcholine, is located mainly in the lamellae of the post-synaptic junctional folds (Couteaux & Taxi, 1952). Lower concentrations of the enzyme also occur within the pre-synaptic nerve terminals (Koelle & Steiner, 1956; Koelle, 1963).

ii) Chemical Transmission.

After travelling the length of the axon from the central nervous system, the nerve impulse from a motor nerve is believed to be transmitted to the skeletal muscle through the intercession of acetylcholine (Dale, Feldberg & Vogt, 1936; Katz, 1962; Eccles, 1964) which in consequence performs as a neurohormone.

The concept of chemical transmission via neurohormones was first applied to the sympathetic (Elliot, 1905) and subsequently to the parasympathetic (Loewi, 1921; Loewi & Navratil, 1926) branches of the autonomic nervous system.

While the essential role of acetylcholine in neuromuscular transmission is now generally accepted, the precise mechanism by which it fulfils this function remains in dispute. In contrast to the usually accepted view (del Castillo & Katz, 1956; Katz, 1962) which maintains that acetylcholine is released only pre-synaptically at nerve terminals by a multistage process and diffuses across the synaptic cleft (see page 67), Nachmansohn and his associates (Nachmansohn, 1959, 1963a) claim that acetylcholine plays an essential role in the propagation of the nervous impulse along the entire nerve fibre through the generation of bioelectric potentials. It is then postulated that at the nerve terminals these currents are of sufficient magnitude to bridge the synaptic cleft and mobilize acetylcholine post-synaptically thus generating the end-plate potential (for discussion of this controversy see Koelle, 1963; Nachmansohn, 1963a; Ehrenpreis, 1964).

iii) Biosynthesis and Storage of Acetylcholine.

The biosynthesis of acetylcholine involves acetylation of choline (Nachmansohn, 1946; Hebb, 1963), the energy for the conversion being supplied by adenosinetriphosphate (ATP)(Nachmansohn & Machado, 1943). In the first step it would seem that coenzyme A is acetylated in the presence of acetylkinase while the

second stage involves the choline acetylase-mediated transfer of an acetyl group from acetylcoenzyme A to choline.

It seems that intracellular choline stores may be inadequate for the synthesis of acetylcholine in amounts sufficient for all conditions of nervous activity and accordingly it has been suggested that there may be a specific choline carrier mechanism responsible for the transport of extracellular choline, derived from the breakdown of previously utilized transmitter, to intracellular sites for acetylation (MacIntosh, 1959, 1963).

The synthesis of acetylcholine is believed to take place mainly in the nerve terminals, as evidenced by the high concentrations of choline acetylase localized there (MacIntosh, 1963). The fact that choline acetylase is not abundant in areas away from the neuromuscular junction (Hebb, 1963) has been taken as providing support for this suggestion.

Acetylcholine is stored both inside ("bound") and outside ("free") the synaptic vesicles (MacIntosh, 1963) in the form of a physiologically inactive protein-bound complex (Hebb, 1963).

iv) Liberation of Acetylcholine.

While the release of acetylcholine in quantities

sufficient to produce muscular contraction only occurs on the advent of a nervous impulse, there is a continual spontaneous release of acetylcholine in multimolecular quanta from the nerve endings at rest (del Castillo & Katz, 1956; Katz, 1958a). These latter amounts produce miniature end-plate potentials which are, however, insufficient to initiate a propagated muscle action potential (Fatt & Katz, 1952; Katz, 1962).

A mechanism coupling the electrical impulse from the axon and the physical rupture of the vesicles may operate in the physiological release of acetylcholine. The entry of calcium ions into the nerve axoplasm during development of the action potential and their extrusion from the nerve axoplasm during rest (Harvey & MacIntosh, 1940; Birks & MacIntosh, 1957; Hodgkin & Keynes, 1957) has served to implicate this ion in the release of acetylcholine.

Evidence from experiments in which the external ionic environment was altered indicates that a mutual antagonism exists between calcium and magnesium ions. Thus, high exogenous concentrations of magnesium ions or low concentrations of calcium ions reduce the amount of acetylcholine liberated (del Castillo & Engbaek, 1953, 1954) while large concentrations of calcium ions increase the quantity of transmitter released (del Castillo & Stark, 1952).

Experimentally, release of acetylcholine from the storage particles can be achieved through use of high concentrations of K^+, Cs^+, Rb^+ or NH_4^+ (Brown & Feldberg, 1936; Feldberg, 1945), by freezing or thawing nerve-muscle preparations (Hebb, 1963) and by addition of drugs destroying nerve membranes e.g., the lecithinase of cobra venom (Gautrelet & Corteggiani, 1939; Braganca & Quastel, 1952). Acetylcholine may also be released in exchange for the uptake of choline esters (e.g., carbachol) into synaptic vesicles (McKinstry, Koenig, Koelle & Koelle, 1963).

v) Transmitter Function of Acetylcholine.

When acetylcholine is released it reduces the potential difference across the membrane of the muscle motor end-plate through what is regarded as an interaction with specific receptors on the external surface of the end-plate (del Castillo & Katz, 1955; Katz, 1958b). Unlike the remainder of the muscle membrane, the end-plate is highly sensitive to the action of acetylcholine (Buchthal & Linhard, 1942; Kuffler, 1943, 1945) and has a relatively higher concentration of acetylcholinesterase than other tissues (Couteaux, 1955; Koelle, 1963). If sufficient transmitter is released, the quantal depolarization which follows - the end-plate potential - exceeds the threshold level of stimulation of the adjacent

muscle and initiates in it a potential change (the action potential)(Nastuk, 1953). Unlike the end-plate potential, the action potential is propagated over the muscle fibre and in turn gives rise to the contraction of the muscle.

Following the interaction between acetylcholine and its receptors and underlying the changes in membrane potential there is a rapid and indiscriminate increase in the permeability of the muscle end-plate to various inorganic ions, especially Na^+ and K^+, on both sides of the membrane (Hodgkin, 1951; Nastuk, 1959; Katz, 1959). This induces a short circuit of the end-plate membrane thus producing a high local current-density. By this means, an impulse is transferred from the minute nerve endings to the vastly greater surface of the muscle fibre (Fatt & Katz, 1951; Nastuk, 1953; del Castillo & Katz, 1956). When the end-plate potential reaches the threshold value it gives rise to local ionic currents of sufficient intensity to increase the sodium ion permeability of neighbouring portions of the muscle membrane and thus initiate the action potential. The potential change across the end-plate membrane is a simple depolarization thus differing from the reversal of potential which results from the specific increase in sodium ion permeability occurring during the action

potential (Katz, 1962).

Acetylcholine released from the nerve endings is rapidly hydrolysed within a few milliseconds mainly by the action of acetylcholinesterase (Marnay & Nachmansohn, 1937; Nachmansohn & Rothenberg, 1945) thus permitting the end-plate to return quickly to its polarized condition ready to respond to further quanta of transmitter.

METHODS OF PRODUCING NEUROMUSCULAR BLOCK.

Any substance interfering with the normal physiological sequence of events at the neuromuscular junction may relax skeletal muscle. Into this category fall substances interfering with the synthesis, release or destruction of acetylcholine as well as those which impair its effect by blocking its access to its specific post-junctional receptors.

The relaxation of skeletal muscle may also be produced centrally by interneuronal blocking agents which interfere with the conduction of nerve impulses in polysynaptic pathways in the spinal cord.

Interference with the combination of acetylcholine with its receptors on the post-synaptic muscle membrane is the mechanism by which act all the clinically useful, peripherally acting, muscle relaxants and for the purpose of this thesis these will be specifically referred to as neuromuscular blocking agents.

i) Inhibition of Transmitter Synthesis.

While no substance is known which is a specific inhibitor of choline acetylase *in vivo*, the biosynthesis of acetylcholine itself may be prevented by the hemicholiniums (Schueler, 1955). These compounds are believed to interfere with the specific choline carrier mechanism thought to be responsible for the transport of choline from extracellular to intracellular sites for acetylation (Gardiner, 1957; Reitzel & Long, 1959a,b; Schueler, 1960). On the other hand, block by triethylcholine may be due to its mistaken uptake and acetylation in place of choline with the subsequent liberation of an inactive transmitter (Bowman & Rand, 1961; Bowman, Hemsworth & Rand, 1962).

ii) Prevention of Acetylcholine Release.

Block of conduction in terminal motor nerve endings may follow high frequency tetanic stimulation which leads to a reduction in the output of acetylcholine (Brooks & Thies, 1962; Otsuka, Endo & Nonomura, 1962) with concomitant decrease in the size of the end-plate potential. Procaine and other local anaesthetics, as well as high concentrations of magnesium ions or low concentrations of calcium ions (del Castillo & Engbaek, 1953, 1954; MacIntosh, 1959), may also modify acetylcholine release by inhibiting permeability changes necessary for

the conduction of nerve impulses (Shanes, 1958). On the other hand, the ability to block axonal conduction may be independent of the presence of acetylcholine. Inhibition of conduction in nerve fibres by fish toxins arises, it is claimed, from a specific block of the increase of sodium ion permeability associated with excitation (Kao & Nishiyama, 1965).

Alternatively, the ability of botulinum toxin to block the release of acetylcholine (Burgen, Dickens & Zatman, 1949; Brooks, 1954, 1956) does not affect either nerve conduction (Guyton & MacDonald, 1947), or the structure of the motor nerve terminals, the acetylcholine content of the synaptic vesicles remaining unaltered (Thesleff, 1960). The toxin may thus act selectively on the mechanism responsible for the release of acetylcholine (Thesleff, 1960).

iii) Inhibition of Transmitter Destruction.

The accumulation and persistence of acetylcholine in the presence of sufficiently high concentrations of anticholinesterases e.g., neostigmine and edrophonium may produce a prolonged depolarization of the motor end-plate resulting in block (Thesleff & Quastel, 1965). At the same time postponed transmitter hydrolysis may result in inadequate choline supplies for pre-synaptic acetylcholine synthesis (Perry, 1953).

iv) Drugs which Block the Actions of Acetylcholine at the Post-synaptic Membrane - Neuromuscular Blocking Agents.

While the terminology used to describe the different mechanisms of action of neuromuscular blocking agents is often confusing and misused (Bowman, 1962), these compounds can be conveniently classified into two main groups on the basis of whether or not they cause post-synaptic depolarization as first delineated by Paton & Zaimis (1952) and modified by Foldes (1954) and Zaimis (1959).

a) Non-depolarizing drugs, e.g., tubocurarine. These compounds act by reducing or preventing the depolarizing effect of acetylcholine.

b) Depolarizing drugs. e.g., decamethonium. These compounds mimic the action of acetylcholine, producing a depolarization of the end-plate region, for at least some of the period of their duration of action.

The type of block produced by the depolarizing compounds depends to a large extent on the properties of the muscle end-plates of the species employed. In contrast, the non-depolarizing compounds display a qualitatively uniform mode of action in all species (Taylor & Nedergaard, 1965), except perhaps the lizard, where the muscle end-plate seems very sensitive to depolarization

(Foldes, 1959).

In an early classification (Bovet, 1951), neuromuscular blocking agents were classed as "pachycurares" which possess bulky molecules and broadly corresponded to the non-depolarizing compounds and as "leptocurares" which possess long thin molecules and broadly corresponded to depolarizing compounds. However factors such as species variation and the "dual" mode of action of the depolarizing compounds (vide infra) make this classification unsatisfactory. For instance, nicotine (classed as a pachycurare) exerts a depolarizing action in the cat (Paton, 1951a) and tridecamethonium (classed as a leptocurare) has a non-depolarizing action in the chicken (Zaimis, 1952,1953).

a) Non-depolarizing Neuromuscular Blocking Agents.

These progressively reduce the amplitude and duration of the end-plate potential which may, depending upon the concentration of drug present, completely disappear (Fatt & Katz, 1951; Thesleff, 1955). When the end-plate potential falls below the level necessary to excite the adjacent muscle membrane, neuromuscular block results (Eccles, Katz & Kuffler, 1941). During paralysis by tubocurarine, the output of acetylcholine from nerve terminals is not significantly affected (Dale, Feldberg & Vogt, 1936), nervous conduction is unimpaired and the

skeletal muscle retains its sensitivity to direct electrical stimulation and iontophoretic application of ions (Fatt & Katz, 1951; del Castillo & Katz, 1957).

Block produced by non-depolarizing agents is antagonized by any procedure which increases the local concentration of acetylcholine e.g., anticholinesterases (Riker, 1953) or the repetitive stimulation of the motor nerve (Thesleff, 1955). Conversely, a reduction in the amount of transmitter released, either as the result of drug action e.g., the hemicholiniums or the application of potassium ions enhances this type of block.

b) Depolarizing Neuromuscular Blocking Agents.

These produce initially an acetylcholine-like depolarization of the end-plate region which is followed by a reduction in the sensitivity of the end-plate to the transmitter. The block produced by depolarizing agents has thus been divided into two distinct components. The first stage, phase I block (Jenden, Kamijo & Taylor, 1951, 1954), is characterized by a block of rapid onset and relatively short duration during which the muscle membrane is depolarized. This block may be preceded by muscular fasciculations or even a sustained contracture. The continuous presence of depolarizing drugs gradually decreases the sensitivity of the end-plate and phase I block is followed by membrane repolarization (Thesleff,

1955, 1958), in spite of the presence of the depolarizing agent. Phase II of the block may be more prolonged than phase I and qualitatively resembles that produced by non-depolarizing compounds in being reversed by anticholinesterases and potentiated by tubocurarine (Taylor & Nedergaard, 1965). Phase I and phase II blocks are therefore mutually antagonistic. This "dual" (Zaimis, 1953) or "biphasic" block (Jenden et al. 1954) is characteristic of all the depolarizing agents so far investigated and appears to occur in all types of voluntary muscle, including human voluntary muscle (Zaimis, 1959; Foldes, 1959).

It has been suggested (Taylor & Nedergaard, 1965) that the production of a phase II block by depolarizing compounds may depend upon their entry into the muscle fibres at the end-plate region - a suggestion in good agreement with certain autoradiographic experiments by Waser (1963). Tubocurarine, on the other hand, while not entering the muscle fibres, appears able to block the penetration of depolarizing compounds since it has been shown to prevent the onset and development of phase II block by decamethonium (Nedergaard & Taylor, 1963).

SPECIES VARIATION AND NEUROMUSCULAR BLOCK.

One of the most important factors in the

investigation of neuromuscular block is the choice of a suitable animal species. The problem of choosing animal species whose qualitative and quantitative responses to neuromuscular blocking agents resemble those in man is complicated by the variation in the kind and degree of the response not only of different species to the same drug but also of different muscles within the same species (Zaimis, 1959).

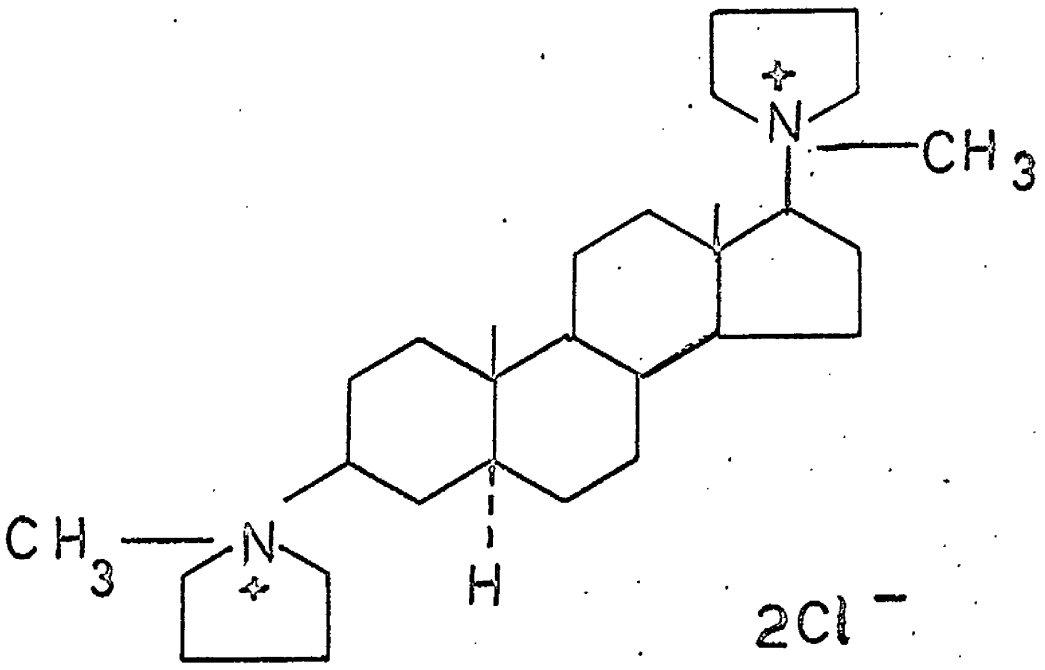
A comparison of the relative sensitivities of different animal species to depolarizing and non-depolarizing drugs has been made (Zaimis, 1959). With respect to non-depolarizing drugs there is little variation in sensitivity between species. Thus, the most sensitive species is only approximately five times as susceptible to tubocurarine as the least sensitive (rat > hare = monkey > rabbit > man > dog > cat = hen). On the other hand, the response to the depolarizing drug decamethonium shows a marked species variation, the most sensitive species being approximately 100 times as susceptible to decamethonium as the least sensitive (hen > cat > man > rabbit = dog > monkey > hare > rat). The rat is relatively sensitive to non-depolarizing agents but markedly insensitive to depolarizing compounds. The hen, on the other hand, is relatively insensitive to non-depolarizing compounds but sensitive to depolarizing

agents.

The variation in response between mammalian species (e.g., the cat) and avian species to depolarizing drugs is particularly striking. In mammals, there is a brief period of muscle fasciculation and potentiation of twitch height before the onset of block. The paralysis is flaccid in nature. In birds inhibition of the twitch height by depolarizing agents is accompanied by a contracture or shortening of the muscle which produces a spastic paralysis (Buttle & Zaimis, 1949; Paton & Zaimis, 1952).

In addition to quantitative differences in behaviour to depolarizing neuromuscular blocking agents between species there are also qualitative differences (Foldes, 1959). Thus, in avia, amphibia and some mammals e.g., cat and man, phase I depolarizing block is relatively prolonged and phase II block develops only slowly and may be difficult to detect. In the monkey and dog, however, phase I block is relatively brief (Foldes, 1959).

An attempt to correlate the observed clinical effects of some neuromuscular blocking agents e.g., tubocurarine, gallamine, decamethonium and suxamethonium with their activity in a number of species has led to the conclusion that no one animal species can be used to



3β,17β-DIPYRROLIDIN-1'-YL-5α-ANDROSTANE

BISMETHOCHLORIDE

predict the potency of these compounds in man (Hoppe, 1955). However, except for suxamethonium, the potency of which in man resembled most closely that in the rabbit, the most accurate estimates of potency were obtained from the cat and dog (Hoppe, 1955). On the other hand, the non-depolarizing steroidal bisquaternary ammonium compound, 3β,17β-dipyrrolidin-1'-yl-5α-androstane bismethochloride, in the hen, cat and rabbit, exhibited a duration of action similar to that of suxamethonium (Biggs et al.1964) and only in the monkey, as in man, was its recovery rate much slower being similar to that of gallamine (Mushin & Mapleson, 1964).

THEORIES OF DRUG-RECEPTOR INTERACTION.

Neuromuscular blocking agents are considered to exert their activity by their ability to combine with the acetylcholine receptors on the post-synaptic muscle end-plate. Drugs which act in this way through combination with specific receptors which impose severe limitations in stereochemistry and charge distribution on the drug molecule are known as "specific" drugs in contrast to "non-specific" drugs whose effects appear to be largely dependent upon their physical properties (Beckett, 1956; Beckett, Casy, Harper & Phillips, 1956; Ing, 1959).

The concept of receptors was first postulated

by Ehrlich (see Albert, 1965) and implied a complementarity of fit between the two entities i.e., drug and receptor, mediated by chemical forces. The development and general acceptance of this concept may be traced through the lock and key analogy of Fischer (1894) and in the work of Langley (1906), Clark (1937), Gaddum (1937) and Ing (1936). Thus, while current ideas concerning the nature of receptors may be somewhat removed from those of Ehrlich, the receptor theory is inherent in and fundamental to many of the more recent attempts to explain drug action including the work of Stephenson (1956), Ariëns (1954, 1964), Paton (1961) and Belleau (1964).

An extension of the receptor theory is the principle of biological antagonism which contends that a drug may compete with a natural substrate, or even another drug of similar chemical structure, for the same receptor. The substrate analogue like the substrate itself may be capable of producing a positive response and different degrees of partial agonism exist (Schild, 1954; Ariëns, van Rossum & Simonis, 1957; Gaddum, 1957). On the other hand the analogue may be incapable of evoking a positive response, and through passive occupancy of the receptors, antagonize the action of the substrate. The subject of biological antagonism has been treated mathematically using formulae in which the

measured biological response has been related either to physical adsorption laws or to laws governing enzyme kinetics. All mathematical relationships derived to explain drug action have been developed fundamentally from the law of mass action, underlining the importance of the chemical reaction as a model of drug behaviour.

Earlier attempts, including the work of Freundlich (see Clark, 1937), Langmuir (1916, 1917, 1918), Clark (1937) and Gaddum (1937), to describe mathematically pharmacological activity assumed that the degree of biological response was a linear function of the receptors occupied by the drug. The validity of this assumption has been questioned (Stephenson, 1956; Schild, 1957; Ariens *et al.* 1957) and the view put forward that in order to produce a maximal effect only a comparatively small proportion of the receptors require to be occupied. The remaining "spare" receptors may constitute 95% of the available total (Stephenson, 1956).

Until recently, the view that pharmacological activity lay in the ability of a drug to statically occupy the appropriate receptor site was unquestioned and the studies of Clark (1937), Gaddum (1937), Stephenson (1956) and Ariens (1964) have been collectively termed "occupation" theories. An interesting alternative view has recently been proposed (Paton, 1961; Paton & Waud, 1962)

in which it is claimed that the activity of a drug is more accurately related to the rate with which combination with the receptor takes place rather than to mere occupation of the site itself. This theory is again based on observations occurring during chemical reactions (Croxatto & Huidobro, 1956) and emphasizes the importance of the moment of encounter between drug and receptor. Thus, the rate of dissociation of the drug-receptor complex i.e., the rate at which receptors become available for further interaction is decisive in distinguishing between agonistic and antagonistic drugs. While it has not been possible to experimentally verify the rate theory, it may be used to classify muscle relaxants. Thus, depolarizing compounds can be visualized as possessing a high dissociation rate while non-depolarizing compounds may be regarded as possessing a low dissociation rate.

In the studies of Ariëns and his colleagues (Ariëns, 1964), drug-receptor interaction was related to two basic concepts derived from a consideration of the chemical bonds uniting the drug and the receptor. The term affinity defined the ability of a drug to enter into complex formation with a given receptor and intrinsic activity was defined as a measure of the power of the drug-receptor complex to evoke a positive biological

response. On the basis of these two concepts, three principal groups of muscle relaxants are recognized; the depolarizers or cholinomimetics having a high intrinsic activity e.g., decamethonium, the non-depolarizers or cholinolytics having a low intrinsic activity e.g., tubocurarine, and the non-competitors e.g., prodeconium which have an affinity for receptors other than those occupied by acetylcholine. The last group have been further subdivided into compounds which block the synthesis of acetylcholine (e.g., the hemicholiniums), inhibit its release (e.g., botulinum toxin) and prevent its destruction (e.g., neostigmine).

Variations in the molecular configuration or concentration of a drug may induce changes in intrinsic activity and affinity. This classification has thus been used to accommodate those compounds, the mode of action of which had hitherto been unconsidered, whose activity lies somewhere between those with a high intrinsic activity and those with a low intrinsic activity.

Recently, Belleau (1964), relying on analogies drawn from the field of enzyme kinetics, has postulated that true antagonists of acetylcholine react with the muscarinic cholinergic receptor, which is assumed to be a protein with acetylcholinesterase-like properties, to produce various conformational perturbations in the tertiary

structure of the receptor, which are incapable of eliciting any response. Such compounds, by reason of their molecular dimensions, interact with the hydrophobic periphery extending beyond the specific acetylcholine-binding site and produce a non-specific conformational perturbation of the receptor. One could postulate that non-depolarizing agents e.g., tubocurarine may interact with the receptor surface in such a manner. Acetylcholine and its agonists all interact only with the acetylcholine-binding portions of the receptor surface to produce an unique conformational perturbation of the delicately arranged non-polar residues of the receptor protein. It is possible that depolarizing compounds e.g., decamethonium may react with the receptor surface in such a manner to produce a response. Those compounds which possess side-chains which do not protrude too deeply into the hydrophobic periphery of the acetylcholine site may produce an equilibrium mixture of specific and non-specific conformational perturbations. Such compounds are partial agonists. [For discussion of proposed theories of drug-receptor interaction see reviews by Belleau (1964), Furchgott (1964) and Ing (1964)].

NATURE OF THE NICOTINIC CHOLINERGIC RECEPTOR.

Present-day knowledge concerning the nature of

receptors is far from complete in spite of numerous attempts to isolate and characterize them. Early workers conceived of a "periterminal network" (Boeke, 1929) or a molecular side chain of cellular substances (Langley, 1906) although a more recent view (Cavallito & Gray, 1960) suggests that the receptor surface might consist of a lattice of anionic groups arranged in a crystal-shaped pattern. The receptor may not be a discrete physical entity and has been envisaged as a sphere of influence bounded and defined by enzymes, coenzymes and metallic ions (Martin-Smith & Reid, 1959). The view that the cholinergic receptor may be structurally identical with acetylcholinesterase, first suggested by Roepke in 1937 has recently been repeated in a modified form. In his treatment of drug-receptor interaction, Belleau (1964) suggests that the receptor might be acetylated enzyme and have its origin in a biochemical pool of acetylcholinesterase. On the assumption that the receptor is a discrete physical entity possessing definite spatial and electrical properties, a number of attempts to isolate and characterize it have been made. In general, these have involved biochemical experiments in which the substance believed to contain the receptor was bound with radioactive neuromuscular blocking agents to form complexes. Experimental evidence indicating

strong, but probably non-specific binding, of radioactive gallamine to an acidic mucopolysaccharide led to claims for having isolated the receptor (Chagas, 1959). These claims were disputed by evidence suggesting that the receptor was in fact a protein (Ehrenpreis, 1960). Subsequent studies, however, showing that the postulated receptor substance had high avidity for non-specific substances such as procaine and low avidity for compounds like decamethonium and acetylcholine itself led Ehrenpreis (1963) to renounce his original claims although Nachmansohn (1963b) still believes that the protein fraction isolated may be the cholinergic receptor.

While the precise physical nature of the receptor substance thus remains unknown, attempts to localize and estimate the number of receptors at the cholinergic neuromuscular junction have been more successful. Thus, elegant autoradiographic studies of the uptake of radioactive C-curarine by mouse diaphragm have shown that the end-plate region lies in a circular band round the central tendon of the diaphragm (Waser, 1959, 1960). In addition, the work of del Castillo & Katz (1955) indicates that the cholinergic receptors are isolated on the exterior of the end-plate membrane since injection of acetylcholine or carbachol into the interior of a muscle fibre does not cause depolarization of the end-plate.

Using auto-radiographic techniques, Waser (1960, 1963) has estimated that the number of curarine molecules bound to one end-plate is 2.8×10^6 for the minimal lethal dose. This figure is in good agreement with calculations that 9×10^6 molecules of acetylcholine are needed for one depolarization of one end-plate of the rat diaphragm (Krnjević & Miledi, 1958) and it has also been shown that a minimum of 6.6×10^6 molecules of acetylcholine are released at the end-plate by a single nerve impulse (Krnjević & Mitchell, 1960). From the above observations, Waser (1959, 1960) has concluded that when the minimal paralyzing dose of curarine is applied to the end-plate less than 10^6 receptors will remain free and from this he estimated that the total number of nicotinic cholinergic receptors in one end-plate is probably about 10^7.

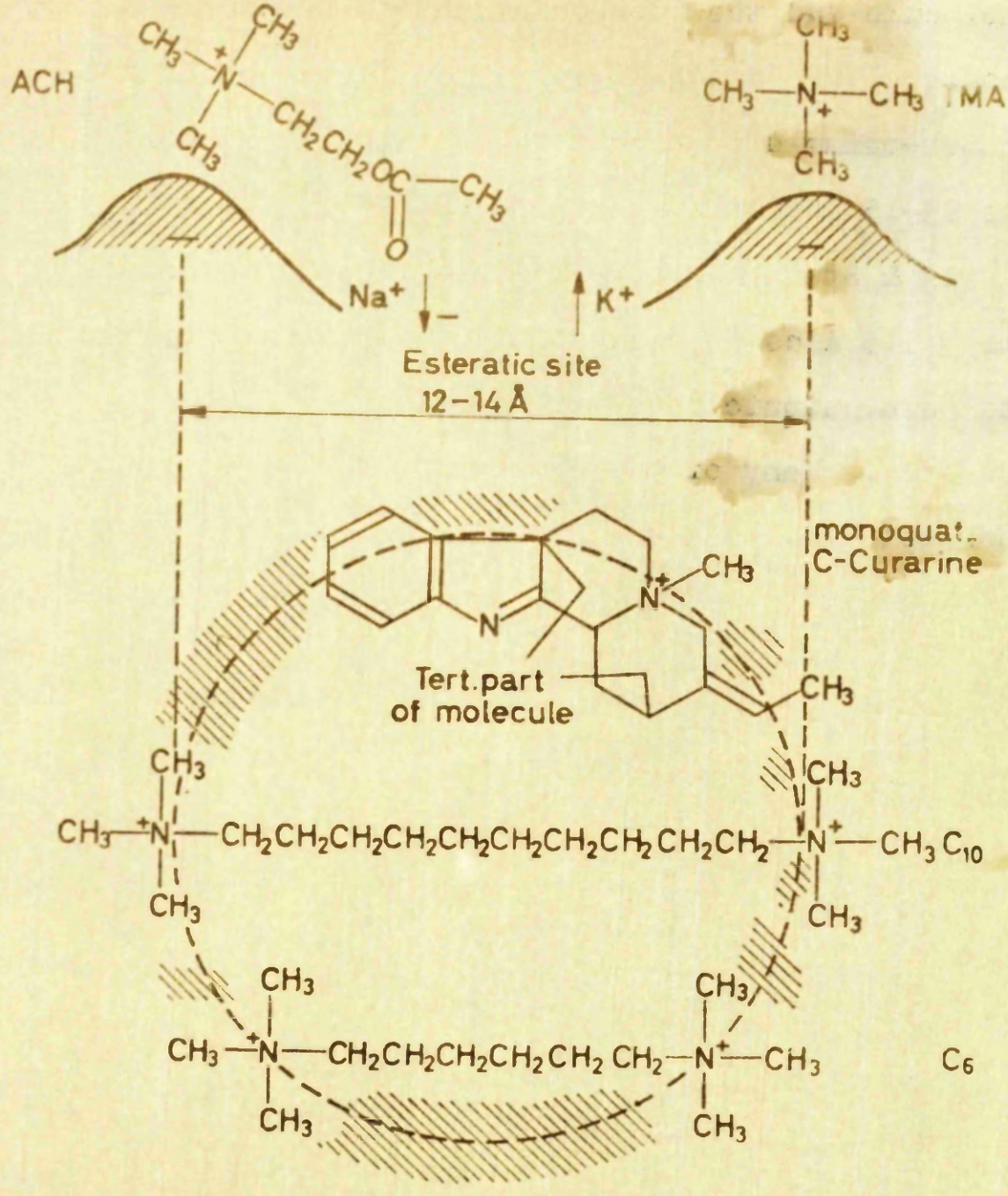
In addition to the physiological aspect, the chemical contribution to the problem of the nature of the nicotinic receptor has also been vigorously maintained on the assumption that the receptor will have molecular features and electrical characteristics complementary to those of the most active drug molecules. This approach has provided many of the clinically most useful neuromuscular blocking agents and has made it possible to correlate biological activity at the skeletal nerve-muscle junction with certain structural features.

In the field of neuromuscular pharmacology, interest has been centred on the quaternary ammonium ion present in the vast majority, although not all (Folkers & Major, 1937; Doulofeu, 1959; Domino, 1964) of the muscle relaxants. Evidence of the importance of the quaternary ammonium function in muscle relaxant activity was first provided by the demonstration (Crum Brown & Fraser, 1869a, b) that while a number of tertiary alkaloids including thebaine, brucine and atropine showed a wide variety of pharmacological properties, their quaternary salts all exhibited a common property of relaxing skeletal muscle. Subsequent investigations (for reviews see Craig, 1948; Cavallito & Gray, 1960) have confirmed the importance of the quaternary ammonium function for muscle relaxant activity. Monoquaternary compounds, however, have not proved clinically useful as muscle relaxants on account of their potent cholinergic properties (Hey, 1952; Cavallito & Gray, 1960). On the other hand, the presence of two quaternary ammonium functions in the molecule of tubocurarine (isolated in 1935 by King) together with the successful development of techniques for its clinical use (Bennett, 1941; Griffith & Johnson, 1942) provided a stimulus for the preparation and study of a large number of drugs each possessing two quaternary ammonium groups (Barlow & Ing, 1948a, b; Paton & Zaimis, 1948a, b; Bovet, 1951).

The higher potency of bisquaternary compounds compared with the corresponding monoquaternaries (Paton & Zaimis, 1949) led to the postulate that activity in the former group could be attributed to the interaction of optimally spaced quaternary ammonium centres (approximately 13-15 Å apart) with similarly spaced receptor groups on the motor end-plate. The complex formed by such "bivalent" molecules (Barlow, 1955) would be twice as active, it was claimed, as that formed by a corresponding monoquaternary compound. A logical extension of studies using bisquaternary salts were those involving tris-, tetra-, and polyonium compounds undertaken in the hope that a higher degree of activity than that found in bisquaternary salts would be derived by their several cationic centres reacting with a similar number of receptor sites (Edwards, Lewis, Stenlake & Stothers, 1959; Lewis, McPhail, Muir & Stenlake, 1961; Lewis, Martin-Smith & Muir, 1963).

While there was considerable evidence in support of the so-called equidistant concept in that the most active member of several series of compounds possessed an inter-onium distance comparable to that found in tubocurarine (10-15 Å), it soon became evident that a one-dimensional approach omitted several important considerations. Thus, although the vast majority of the compounds were non-rigid and contained a polymethylene chain, no attempt

was made to use rigid molecules. Inter-onium distance in non-rigid entities has no fixed value on account of conformational isomerism, which arises from the freedom of rotation about single C-C bonds in the polymethylene chain (Cavallito, Gray & Spinner, 1954). Moreover, in chemical media at least, ion pairing between a di-cation and one anion may occur. Hence, two onium centres may bond to a single anionic receptor (see Cavallito & Gray, 1960). There is no reason to assume, therefore, that the thermodynamically most stable molecular conformation participates in drug-receptor interaction. The difficulty of assessing the precise molecular conformation taking part in any particular drug-receptor combination has been emphasized further by differences between conductimetric measurements (Rice, 1956, 1958) and statistically calculated inter-onium distances (Gill, 1959). Additional physico-chemical studies have provided information on the behaviour of long chain, bisquaternary compounds in solutions. While the applicability of results employing purely chemical media to the biophase is speculative it is clear that long chain aliphatic compounds curve in solution and do not remain maximally extended (Brody & Fuoss, 1956). Elworthy (1963, 1964) has suggested that the reduction in chain length may represent an attempt by the molecule to minimise interfacial energy between water



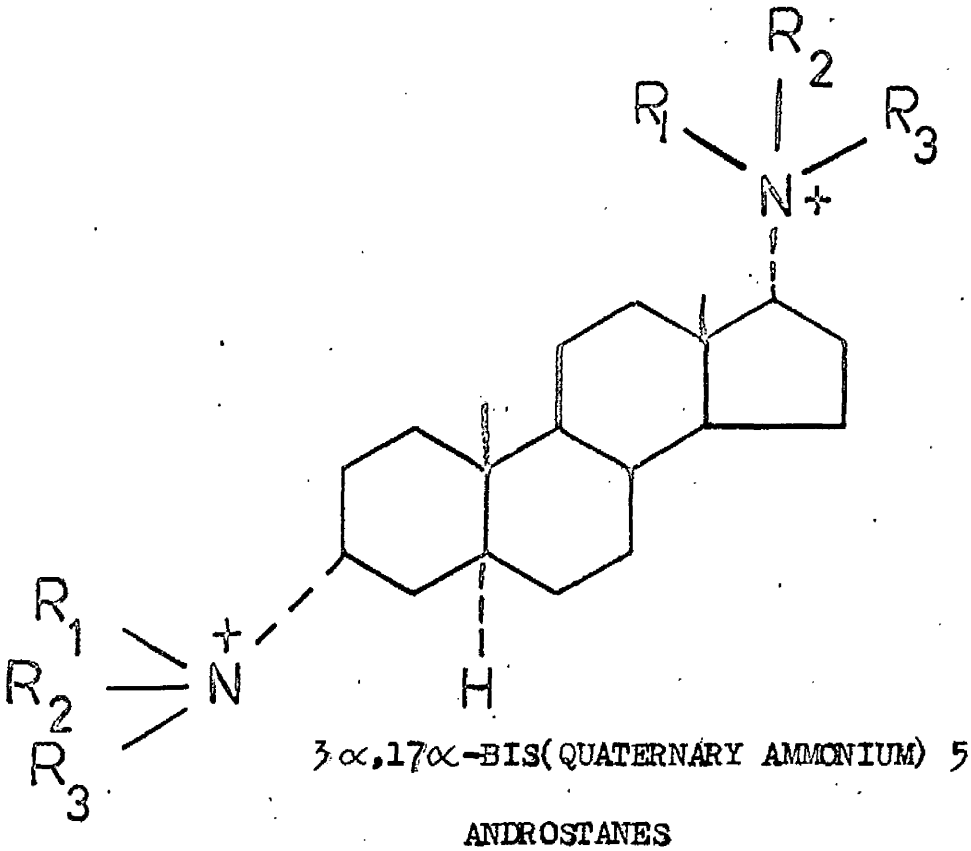
Model of a receptor pore in section and from above . Anionic wall occupied
depolarizing acetylcholine (ACH) or tetramethylammonium (TMA), at the bottom perme
tion of sodium or potassium ions. The pore can be blocked by one bisquaternary or t
monoquaternary curarine molecules, or by many smaller diamines such as hexamethoniu
(C_6). Depolarizing decamethonium (C_{10}) will leave enough room for ions to pass beside t
molecule.

(After Waser, 1959).

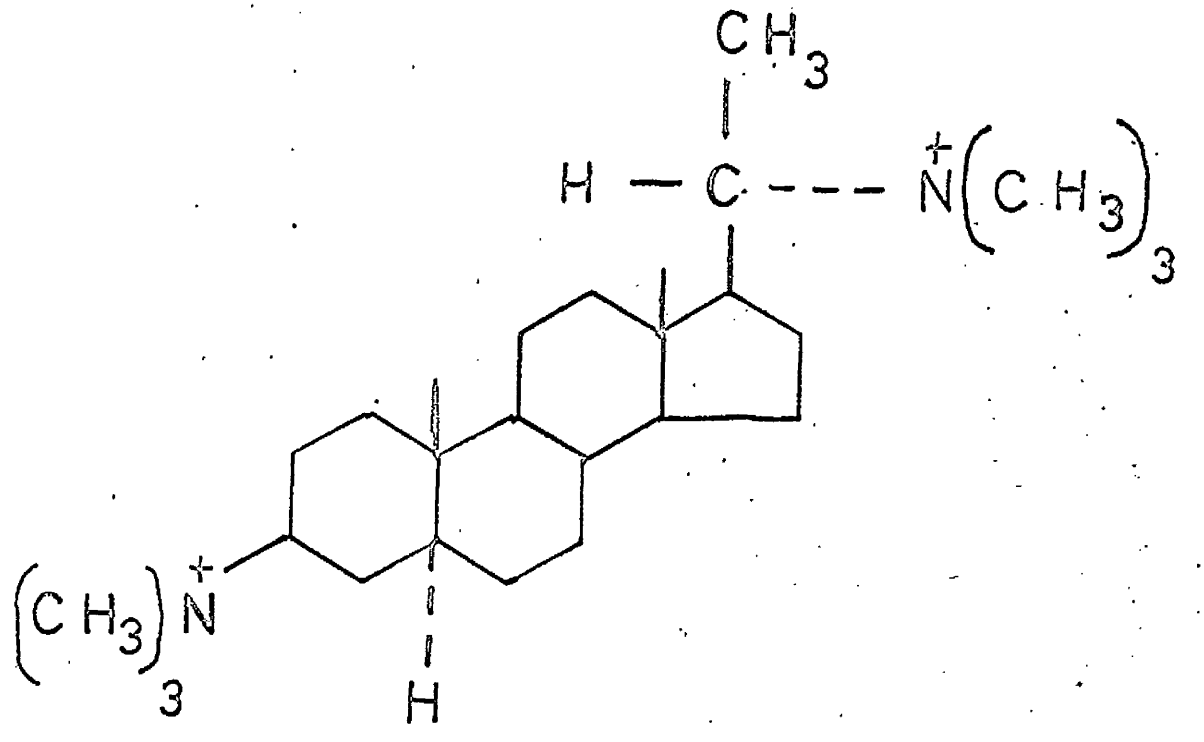
molecule and the hydrocarbon chain. Such considerations could clearly account for the differences in the estimated inter-onium distance in tubocurarine variously reported as 13-15 Å (Paton & Zaimis, 1949) or 9-12 Å (Carey, Edwards, Lewis & Stenlake, 1959). Elworthy (1964) has speculated that 9.5 Å as in decamethonium may be the critical distance between anionic receptor sites.

Many of the criticisms of the equidistant concept have been embodied in the adumbration hypothesis (Loewe & Harvey, 1952) which emphasized the need for a consideration of the overall structural characteristics of a drug in determining the degree of drug-receptor interaction. In this hypothesis, the adumbrating or umbrella-like structure of the bulk of the molecule shields the receptor and prevents a close approach of acetylcholine by steric interference. A similar view is inherent in Waser's pictorial representation of the receptor site in which he suggests that the receptor might be a "pore" of diameter 12-14 Å in the post-synaptic membrane with an anionic wall to which the depolarizing head of acetylcholine becomes attached and an esteratic site at the bottom for the binding of the ester groups of the transmitter (Waser, 1959, 1960).

The use of rigid molecules as a means of overcoming much of the valid criticism inherent in the use



3α,17α-BIS(QUATERNARY AMMONIUM) 5α-ANDROSTANES



MALOUETINE

of flexible molecules was a logical development in the problem of characterizing the cholinergic receptor. In this connection, recent attention has been devoted to the employment of the steroid nucleus as a supporting moiety upon which to append two quaternary ammonium groups in fixed spatial arrangements (May & Baker, 1963, 1965; Alauddin et al. 1965).

The presence of potent non-depolarizing neuromuscular blocking activity in a series of 3α,17α- bis (quaternary ammonium) 5α-androstanes (Alauddin et al.1965) and in stereoisomeric 3β,17β-, 3α,17β- and 3β,17α- compounds related to the former (May & Baker, 1963, 1965; Biggs et al.1964), each series having interonium distances which can be expected to differ from the 3α,17α- series, indicates the relative unimportance of a "fixed" interonium distance in determining neuromuscular blocking activity.

The activity of the 3β,17β-,3α,17β- and 3β,17α- bisquaternary salts indicate that, as with the malouétine (3β,20α-bistrimethylammonium-5α-pregnane) series (Khuong Huu-Lainé & Pinto-Scognamiglio, 1964) stereoisomerism in steroidal bisquaternary salts appears to have little effect on neuromuscular blocking activity. Moreover, the presence of high activity in stereoisomeric 3α,17β- and 3β,17α- bisquaternary ammonium androstanes (May & Baker, 1963, 1965), where the quaternary heads lie

on opposite sides of the steroid nucleus, again casts doubt upon the validity of the "two-point" attachment theory. It would appear therefore that new emphasis must be placed on concepts such as the Waser pore theory or the adumbration hypothesis with its postulate of a one-point attachment.

II PRESENT STUDIES OF THE EFFECTS OF NITROGENOUS STEROIDS AT THE NEUROMUSCULAR JUNCTION

a) EXPERIMENTAL METHODS.

Solubility.

The compounds were readily soluble in normal saline to give solutions of pH 3 to 8.7.

EXPERIMENTS USING CATS.

Cats of either sex (2 - 4 kg) were anaesthetized by intraperitoneal injection of sodium pentobarbitone (60 mg/kg). Cannulation of the trachea, one external jugular vein and one common carotid artery, to enable blood pressure to be recorded, was carried out as previously described (pages 38 and 39). Drugs were administered in saline via the external jugular vein.

The Cat Gastrocnemius Muscle-Sciatic Nerve Preparation.

Skeletal muscle consists of three main types:-

a) Red muscle characterized by slowly contracting fibres. e.g., soleus muscle.

b) White muscle characterized by rapidly contracting fibres. e.g., anterior tibialis.

These two muscles differ in their sensitivity to neuromuscular blocking agents. Whereas tibialis is very sensitive to decamethonium, soleus is relatively resistant, and whilst both muscles are sensitive to tubocurarine the soleus is the more sensitive (Paton & Zaimis, 1951a). Moreover, in tibialis, decamethonium

acts purely as a depolarizing substance while in soleus it may produce mixed block (Jewell & Zaimis, 1954).

c) Mixed muscle in which both red and white fibres are present.

The mixed gastrocnemius muscle was chosen for the present experiments since it appeared to offer the most accurate means of assessing the overall biological effect of muscle relaxant drugs on mammalian skeletal muscle with regard to their possible clinical use. This preparation previously described on page 39 may be used to investigate the qualitative effects of neuromuscular blocking agents and also semi-quantitatively to compare the potency of a new muscle relaxant with that of tubocurarine or decamethonium.

The Cat Nictitating Membrane Preparation.

The cat nictitating membrane preparation has already been described (see page 43) and it is used for the detection and estimation of sympathetic ganglion blockade.

Experiments on the Respiration of the Anaesthetized Cat and Rabbit.

In order to establish an accurate picture of the effects of neuromuscular blocking agents on the volume of air exchanged by the lungs, experiments were conducted using

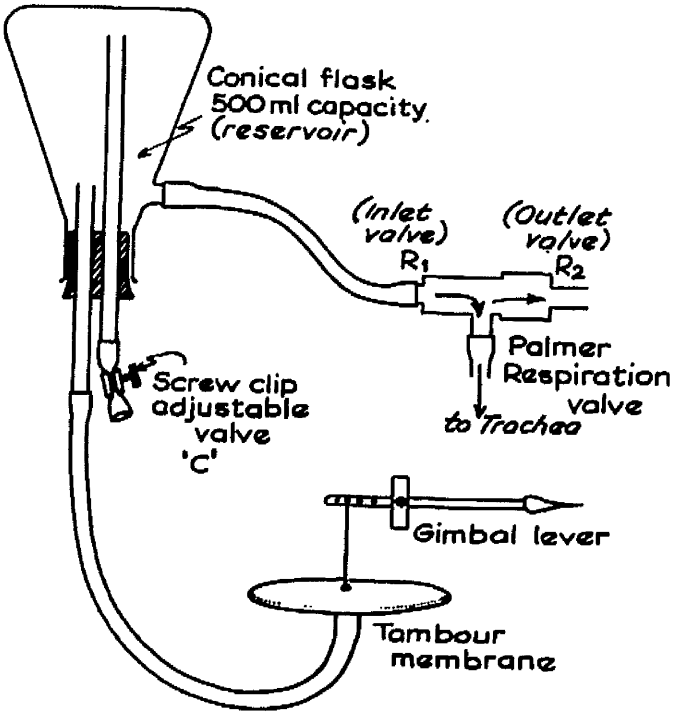


Fig. 17 Diagram of the apparatus used to record respiration, modified from that of Gaddum (1941).

approximate scale 1: 5

the method devised by Gaddum (1941). The cat and rabbit were chosen as the test species and the dose of the compounds required to produce respiratory paralysis in these animals determined. Cats (2 - 4 kg) were anaesthetized with sodium pentobarbitone (60 mg/kg) given intraperitoneally. Rabbits (1.5 - 4 kg) were anaesthetized by administration of a solution of urethane (4.5 mg/kg) into a marginal ear vein.

In both species, the trachea, one external jugular vein and one carotid artery on opposite sides of the neck, were cannulated.

The central limb of a Palmer's 'T' shaped respiratory valve was connected by rubber tubing to the tracheal cannula. The valve was then connected to a conical flask, acting as a reservoir, and leading to a tambour which recorded the respiratory movements of the animal (Fig. 17). A screw-clip valve (C) adjusted the air pressure in the reservoir to maintain the recording lever in a horizontal position. When the animal inspired, air was drawn from the reservoir faster than it entered through C and the consequent drop in pressure in the system was recorded by a depression in the tambour membrane and a rise in the lever. During expiration, the valve R, closed, air entered the reservoir at C and normal pressure conditions were restored. The

lever fell to its normal resting horizontal position. The expired air from the animal escaped by the outlet valve R_2. Alterations in the rate and depth of respiration of the animal could then be translated into changes in the frequency and extent of the lever movements. Prior to administration of the drug into the external jugular vein, a recording of normal respiration was obtained. Drugs, of known concentration, dissolved in normal saline, were administered via the external jugular vein at a constant rate of 0.3 ml/min, using a Palmer's slow infusion apparatus. Infusion of the drug was stopped when spontaneous respiration ceased and the animal was then artificially respired. The respiratory paralysing dose of the drug in both cats and rabbits was calculated from the rate and time taken for infusion of the drug and expressed in mg/kg.

EXPERIMENTS USING AVIAN SPECIES.

Non-depolarizing neuromuscular blocking agents produce a flaccid paralysis in avian species while depolarizing muscle relaxants cause a spastic paralysis (Buttle & Zaimis, 1949; Paton & Zaimis, 1952; Zaimis, 1953) which is characterized by contracture i.e., a shortening of the muscle which is independent of the degree of electrical stimulation applied. The contracture and inhibition of twitch height commence simultaneously but

the maximum degree of contracture usually precedes the onset of maximum paralysis (Crema, Scognamiglio & Bovet, 1959). These effects are readily demonstrable and the phenomenon is useful as a ready means of distinguishing between depolarizing and non-depolarizing compounds.

One explanation for the difference in the response of the two species may lie in the observation that the amount of acetylcholine released in response to a single supramaximal shock applied to the sciatic nerve of avian species only just attained the threshold level necessary to excite about 75% of the gastrocnemius muscle fibres, while for the remaining fibres it was subliminal (Brown & Harvey, 1938a,b). In mammalian muscle, on the other hand, it is generally accepted that the amount of acetylcholine released by a single supramaximal nerve shock elicits a synchronous contraction of all the muscle fibres (Blaber & Bowman, 1962).

The Chicken Gastrocnemius Muscle-Sciatic Nerve Preparation.

The method used was similar to that of Polikan, Smith and Unna (1954). Leghorn chickens, of either sex (1-2.5 kg) were anaesthetized by injection of a 10% sodium phenobarbitone solution in saline into a wing vein (200 mg/kg). This dose of barbiturate was generally found to be sufficient to maintain anaesthesia throughout the experiment. The trachea and an external jugular vein

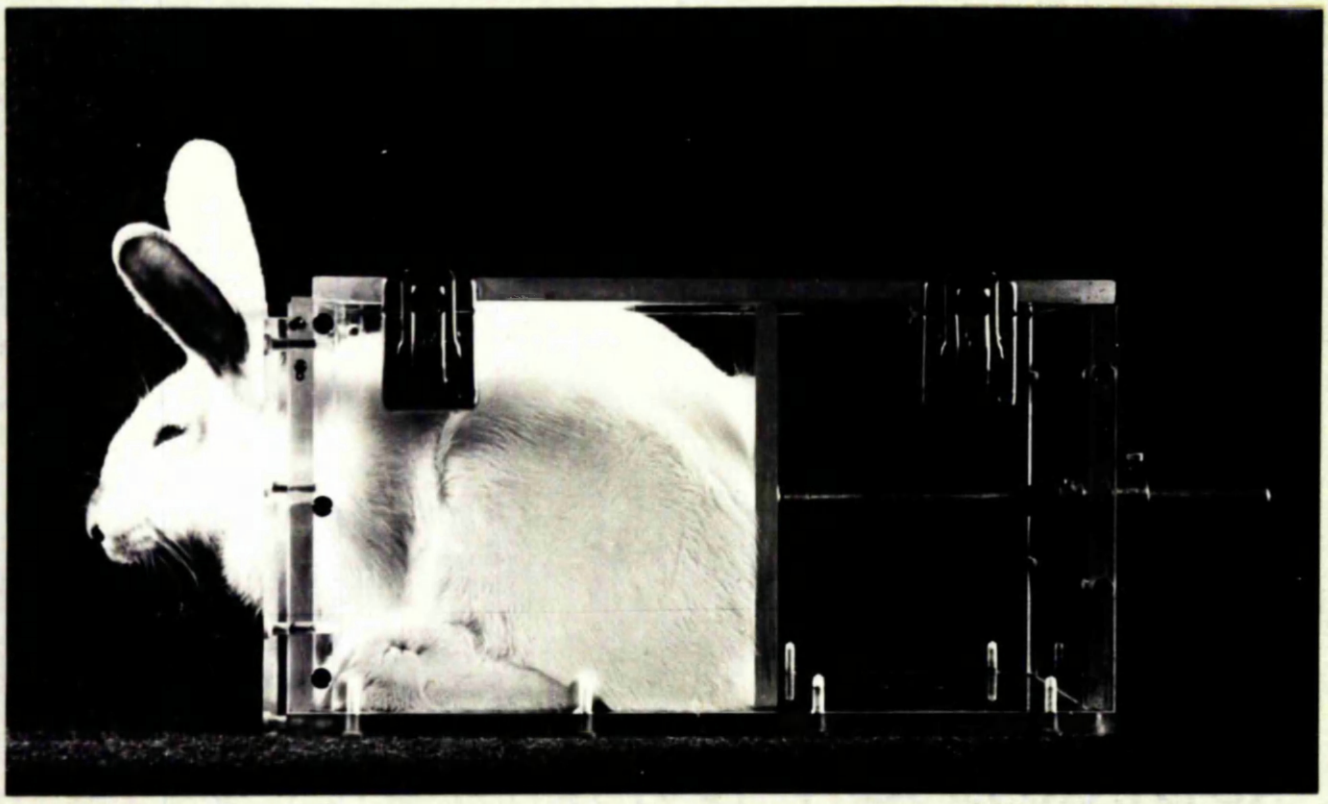


Fig. 19 Rabbit 'bleeding' box with animal prior to commencement of experiment.

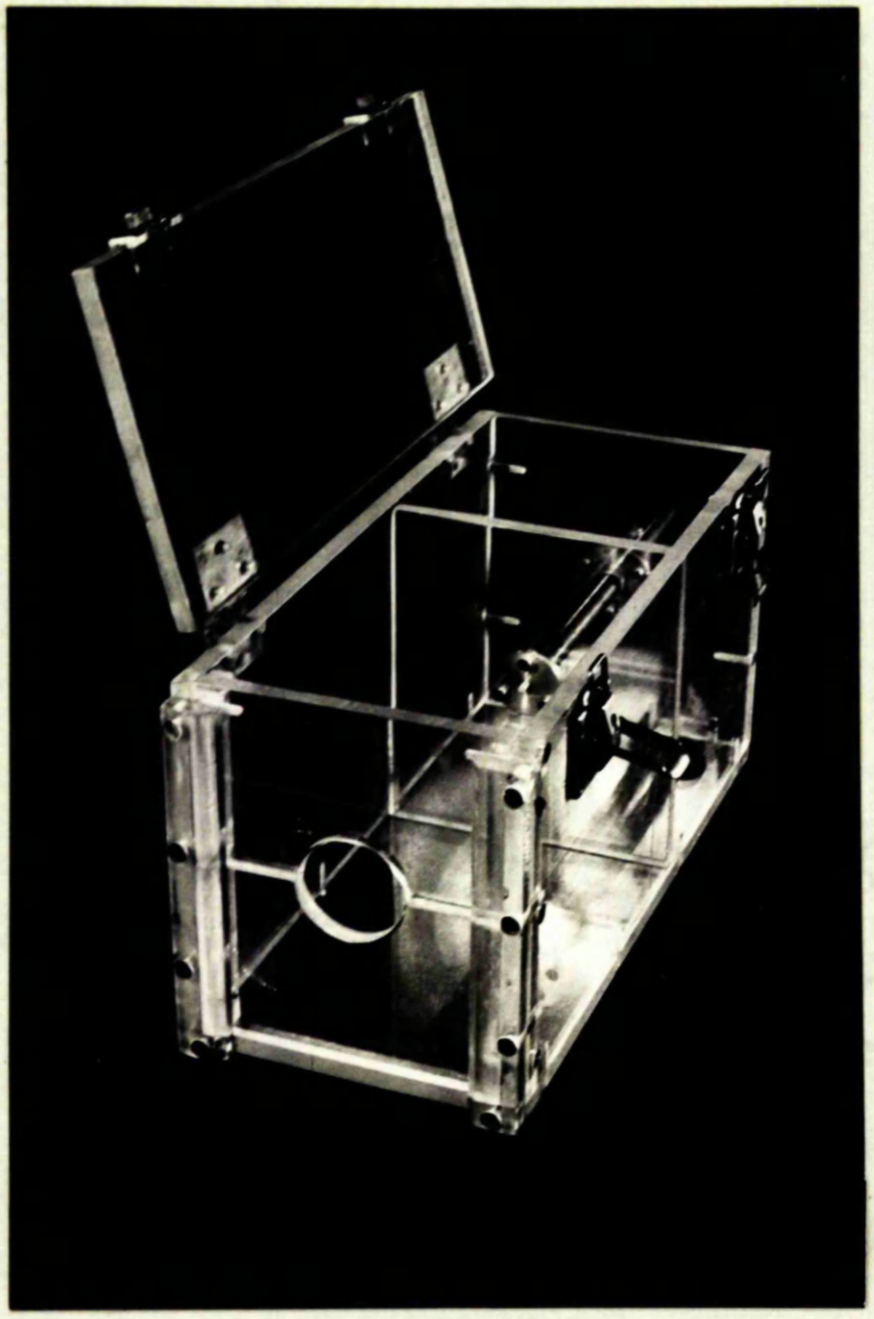


Fig. 18 Rabbit 'bleeding' box.

were cannulated. The tendon of the gastrocnemius muscle of one leg was carefully isolated and cut, as previously described for the cat gastrocnemius muscle-sciatic nerve experiment, and attached over pulleys to a Brown-Schuster myograph lever by a strong linen thread sewn through the cut end. The sciatic nerve was exposed and the distal end stimulated supramaximally using a Dobbie-McInnes square wave stimulator (6/min, 5 to 10 V, 2 to 3 msec).

The contractions were recorded on a smoked surface. Drugs were administered via the external jugular vein.

The Rabbit Head Drop Method.

The Estimation of the Head Drop Dose (HDD).

A modification (Varney, Linegar & Holaday, 1949) of the method outlined by Bennett (1941) was employed. Groups of nine rabbits of either sex (1.5 - 3 kg) were used for each drug. Each rabbit was placed in an individual perspex "bleeding" box (Fig.18) with its head protruding through the opening at the front (Fig. 19). Drugs under test, diluted in normal saline, were administered through a marginal ear vein at a constant rate of 0.3 ml/min using a Palmer's slow infusion apparatus. The rabbit remained quiet until just before the end-point of the experiment when there was usually a brief period of restlessness. Intravenous infusion was stopped only

when the neck muscles were fully relaxed and toneless and a light tap on the muzzle produced no raising of the animal's head. The reading on the timing device of the slow injection apparatus was then noted and the difference between this and the original reading used to calculate the volume of drug injected. The head drop dose (HDD) was calculated and expressed in mg/kg.

This method of assay is best suited to compounds having a fairly rapid onset of action and a duration of effect comparable to that of tubocurarine. Also, by comparing the head drop dose to that producing respiratory paralysis in the rabbit a correlation can be effected between the respiratory and the neuromuscular paralyzing potency of the drugs under investigation.

EXPERIMENTS USING MICE.

Estimation of the Approximate Median Paralyzing Dose (PD50) and the Approximate Median Lethal Dose (LD50).

The method described is based upon that of Thomson (1946) for the assay of insulin in mice. Groups of ten male albino mice (18 - 24g) were injected intraperitoneally at different dose levels with solutions of the nitrogenous steroids, in normal saline. The mice were then placed on a fine-mesh wire screen inclined at an angle of 60° to the horizontal and those which developed

a typical skeletal muscle paralysis and slid abruptly off the screen within a period of 30 minutes after injection of the drug, were considered to show a positive reaction. The dose at which five out of ten injected mice slid off the screen was considered to be the approximate median paralyzing dose (PD50) and was expressed in mg/kg of body weight. In order to determine the approximate median lethal dose (LD50), the dose at which five out of ten mice died within half an hour was taken and expressed in terms of mg/kg of body weight.

EXPERIMENTS USING THE ISOLATED GUINEA PIG ILEUM.

Inhibition of the Peristaltic Reflex.

The peristaltic reflex is elicited by an increase in intraluminal pressure within the intestine and consists of two phases. Firstly, a preparatory phase which is associated with contractions of the longitudinal muscle of the intestine. These contractions are an inherent property of the muscle fibres and are not affected by drugs interfering with nervous structures in the intestinal wall. Secondly, an emptying phase, in which contractions of the circular muscle of the intestine take place and this is controlled by the autonomic nervous system, and is susceptible to drugs (Trendelenburg, 1917; Paton & Zaimis, 1949, 1951b).

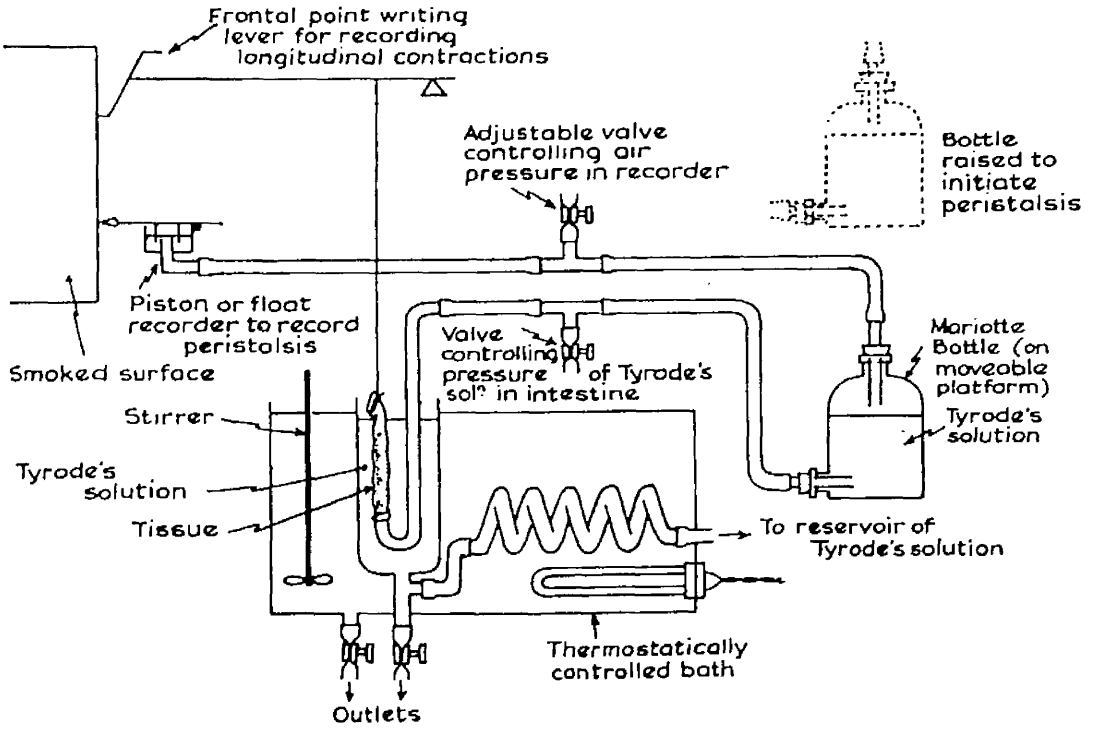


Fig. 20 Diagram of the apparatus used to record the longitudinal and peristaltic movements of the isolated guinea pig ileum. Modified from Trendelenburg (1917).

As the ganglia involved in the peristaltic reflex are probably parasympathetic in origin (Feldberg & Lin, 1949), inhibition of this reflex can be used to estimate the potency of drugs inducing parasympathetic ganglion blockade. The most commonly employed method of studying peristalsis is that of Trendelenburg (1917). Isolated guinea pig ileum was chosen because it has smaller pendular movements and less variability in tone than that of other mammalian species. Guinea pigs of either sex (0.3 - 0.5 kg), were killed and a piece of ileum (7 - 10 cm) removed from the region proximal to the ileocaecal junction. A piece of thread was tied round the oral end of the segment and the thread attached to a writing lever. The caudal end was connected to a glass "U" tube which led via a valve to a Mariotte bottle containing Tyrode solution which was placed on a movable platform (Fig. 20). The bottle could be raised or lowered as required to subject the intestine to a regulated, intraluminal hydrostatic pressure. Air bubbles were removed from the system and the neck of the bottle connected by rubber and glass tubing to a float volume recorder which led to a frontal-point lever writing on a smoked surface. A lever attached to the cephalad end of the preparation recorded, at the same time, the pendular movements of

the intestine. To prevent fatigue of the preparation, peristalsis was induced for only 30 seconds every 3 minutes. The temperature was thermostatically controlled at 30° C throughout each experiment. The drugs under investigation were added 30 seconds prior to the inducement of peristalsis and the effects on the peristaltic reflex and on the longitudinal contractions were recorded.

Experiments Using the Isolated Frog Rectus Abdominis Muscle.

The method of Chang and Gaddum (1933), modified for muscle relaxant drugs (Garcia de Jalon, 1947), was employed. This method can be used to estimate the potency and mode of action of new compounds under investigation.

An adult frog was killed, decapitated and pithed. It was then laid on its back on a cork-covered dissecting board and pinned down. The skin of the abdomen was removed, the rectus muscle exposed and dissected from its insertion into the pelvic girdle to the point of insertion into the cartilage of the pectoral girdle. One half of this muscle, obtained by longitudinal dissection at the midline, was used in each experiment. A loop of thread was sewn through the xiphisternum and a longer thread around the pubic

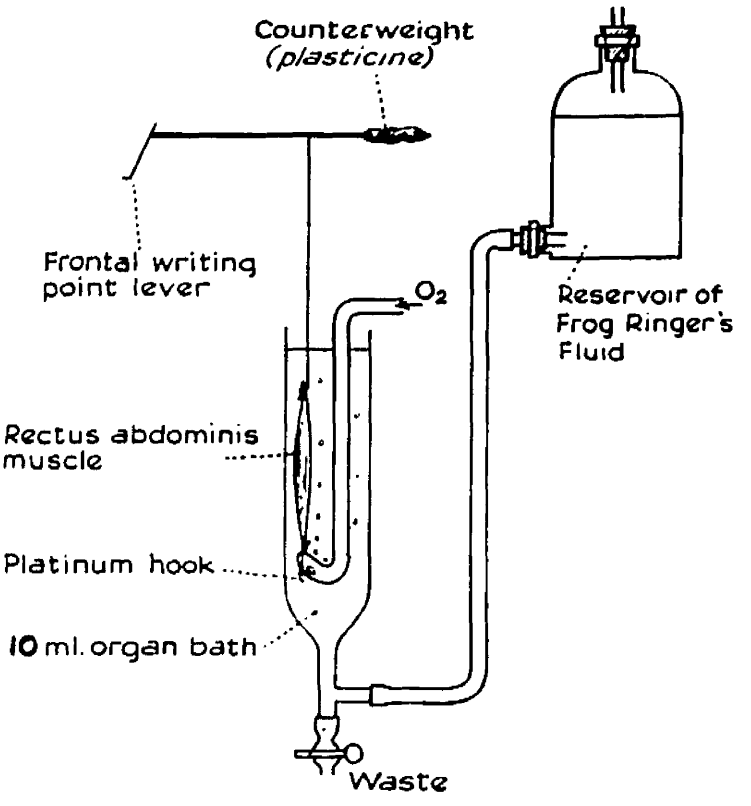


Fig. 21 Diagram of the apparatus used to record the effect of drugs on the isolated frog rectus abdominis muscle.

end. The muscle was then fixed, by means of the loop, to the base of a 10 ml organ bath filled with oxygenated Ringer's solution at room temperature. The long thread was then attached to a modified frontal-point writing lever (Fig. 21).

All drugs under investigation, dissolved in normal saline, were added to the bath using a 1 ml graduated tuberculin syringe. The concentrations of acetylcholine used to produce contractions of the muscle were of the order of 1.0 to 2.0 μg/ml. Two uniform submaximal contractions to the same dose of acetylcholine were obtained prior to the investigation of other drugs. A suitable time interval between each dose of acetylcholine was found to be approximately 3 or 4 minutes and the resulting contractions were recorded for periods of 30 to 90 seconds. Acetylcholine-inhibiting drugs were added from 30 to 60 seconds prior to the addition of acetylcholine and were quantitatively compared with tubocurarine as inhibitors of acetylcholine. The muscle was washed a fixed number of times with fresh frog Ringer's solution at the completion of each cycle. Several additions of acetylcholine were needed for complete recovery, the length of time taken depending on both the nature and the quantity of the drug added to the bath.

The Isolated Rat Phrenic Nerve-Diaphragm Preparation.

The method used is essentially that described by Bülbring (1946). Adult rats of either sex (150 - 200g) were killed and exsanguinated. The skin over the chest was removed and the thorax opened along the right side of the sternum. The frontal part of the left thoracic wall was then removed and the left phrenic nerve exposed. It was carefully freed in situ from fat and other tissues, taking care not to damage the nerve. The nerve was then ligated at a point three to four cm from its junction with the diaphragm and severed just above the ligature. A triangular-shaped section of the diaphragm was dissected out with a portion of the rib cage attached to its costal margin. A long thread was sewn through the apex of the diaphragm. The costal margin was attached to the base of a Bell's electrode by means of a platinum wire. The phrenic nerve was then carefully laid over a small groove in the Bell's electrode and the preparation placed in a 100 ml organ bath at 29°C containing double glucose Tyrode solution. The thread sewn through the apex of the diaphragm was attached to a light isotonic Starling heart lever which recorded the contractions on a moving smoked surface. The nerve was stimulated supramaximally using a Dobbie-McInnes square wave generator at 6/min, 5 to 10 V, the pulse width being 0.5 to 2 msec. These

values were kept constant for any one experiment. A sintered glass distribution tube was fixed at the bottom of the bath to provide a vigorous supply of oxygen.

The drugs under investigation, in solution in normal saline, were added to the bath using a 1 ml graduated tuberculin syringe and allowed to act for 3 minutes before the Tyrode solution was changed. An interval of 15 minutes was allowed prior to the addition of the next dose of a drug to allow the magnitude of contraction to return to normal.

This preparation is best suited to the detection and assay of curare-like substances. It is comparatively insensitive to decamethonium and other depolarizing muscle relaxants (Lewis & Muir, 1959).

Determination of Anticholinesterase Activity.

Anticholinesterase activity is a property of many quaternary ammonium compounds some of which also possess neuromuscular blocking activity (Riker, 1953; Todrick, 1954). The compounds under examination were tested for anticholinesterase activity in view of the possibility that this property, in some cases might be sufficiently great to contribute towards the neuromuscular block observed.

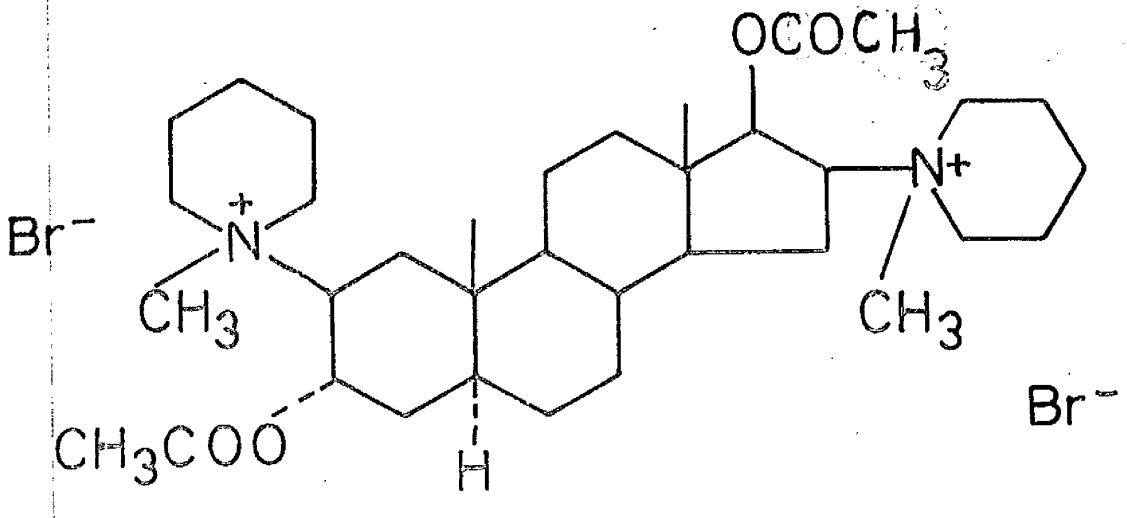
A cholinesterase-containing preparation from rat brain was made by the method of Fenwick, Barron and Watson

(1957) and *in vitro* anti-acetylcholinesterase activity determined manometrically by a modification of Ammon's method (1933). Rat brains were excised, rinsed free from blood in isotonic saline and weighed. The brains were homogenized using a Potter-Elvehjem homogenizer (2 mins, 1500 rev/min, room temperature) diluted with deionized water to give a 5% w/v suspension, and allowed to remain at room temperature for two hours. After centrifugation (10 min, 600 x g, room temperature), the acetylcholinesterase-containing supernatant was removed.

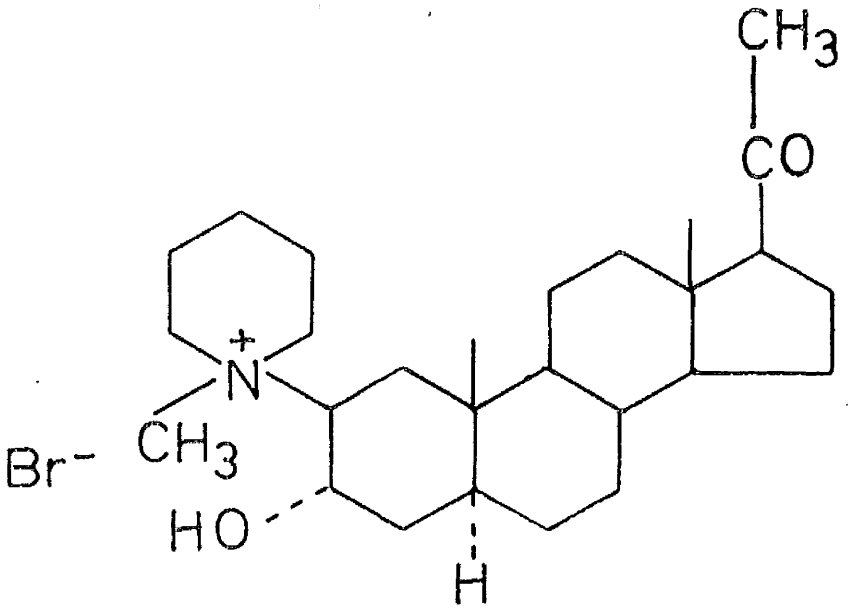
Each flask contained 2.0 ml of Krebs-Ringer bicarbonate solution (Umbreit, Burris & Stauffer, 1957) and 0.5 ml of enzyme preparation in the main compartment, and 0.5 ml of acetylcholine chloride solution (0.036M) in the sidearm. Drugs under investigation, dissolved in normal saline, were added (0.3 ml) to the main compartment. Different molar concentrations of each drug were employed. In order to minimise the effects of anaerobic respiration involving endogenous substrates, the flasks were incubated for 30 minutes with the stop cocks open before the acetylcholine was tipped into the main compartment. Measurements were then made of the volumes of carbon dioxide evolved in 30 minutes from the bicarbonate medium of acetic acid, produced as a result of the enzymic hydrolysis of acetylcholine.

Blank determinations, omitting substrate, were carried out to estimate the carbon dioxide liberated from the medium by anaerobically produced lactic acid. The residual acetylcholinesterase activity in each flask was then calculated. The inhibition of the evolution of carbon dioxide was used as a measure of the anti-acetylcholinesterase activity of each drug. The negative logarithm of the molar concentration of the drug producing an inhibition of 50% (PI50) was then calculated according to the method of Blaschko, Bülbring and Chou (1949).

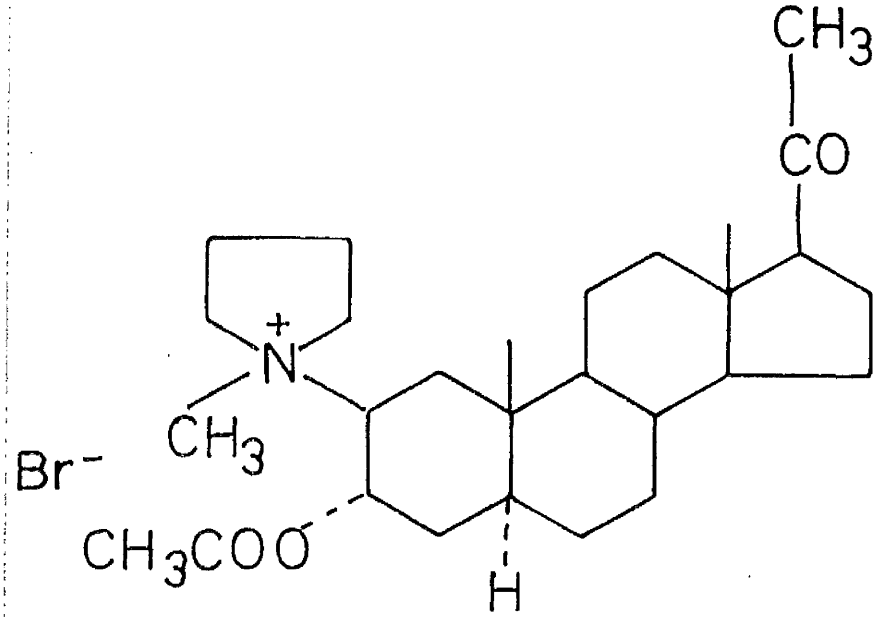
R E S U L T S



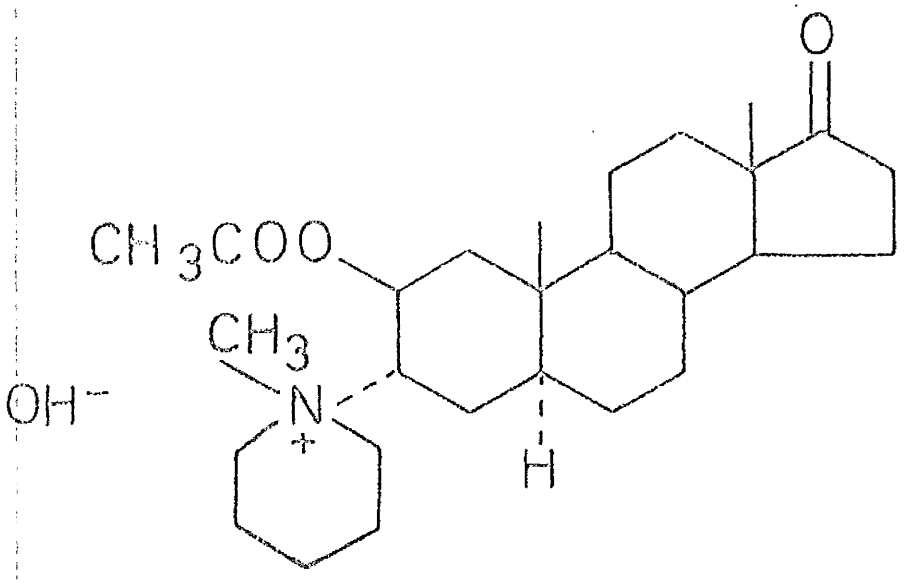
Compound B11



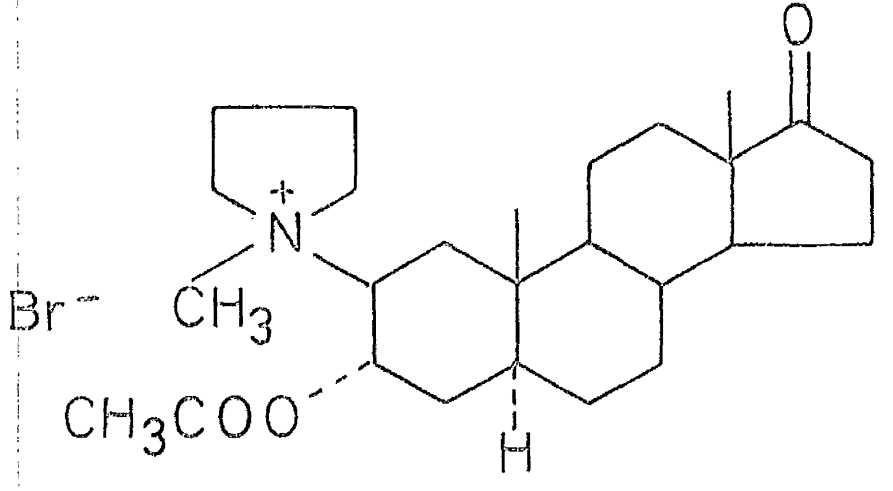
Compound B9



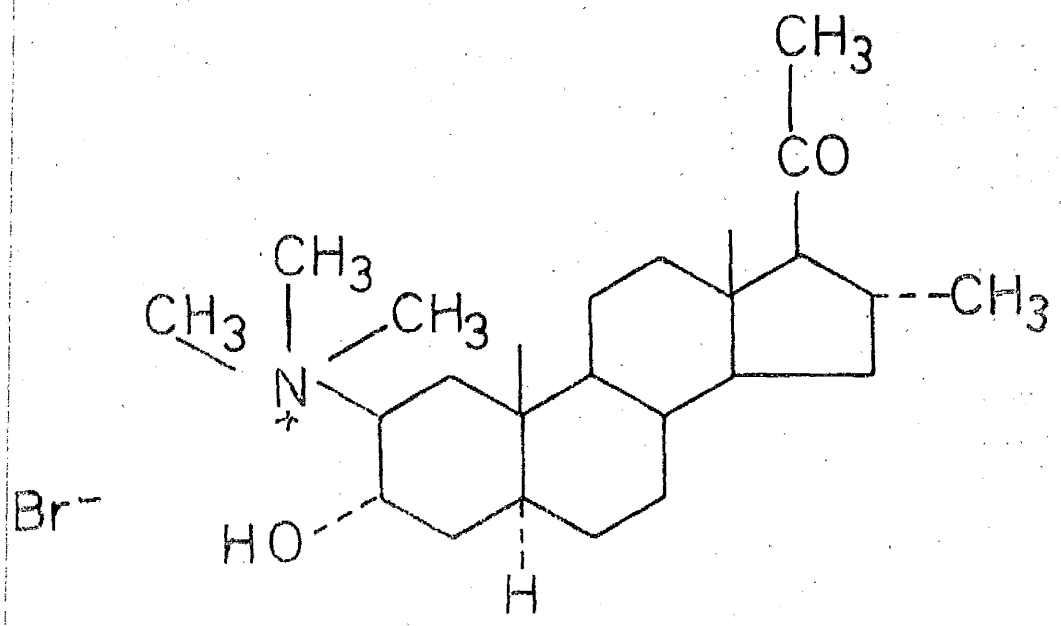
Compound B10



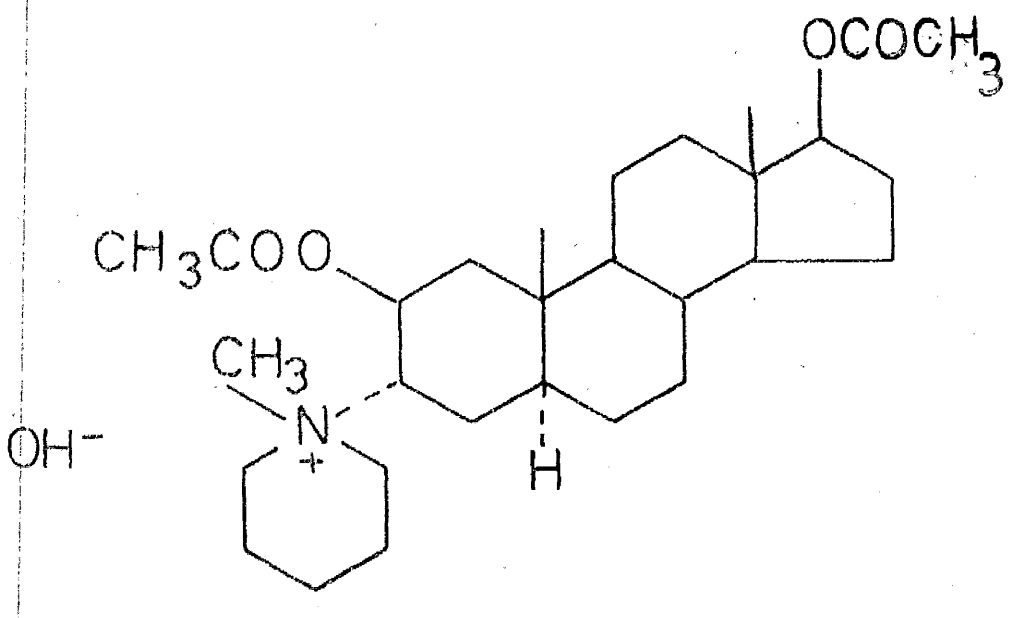
Compound B7



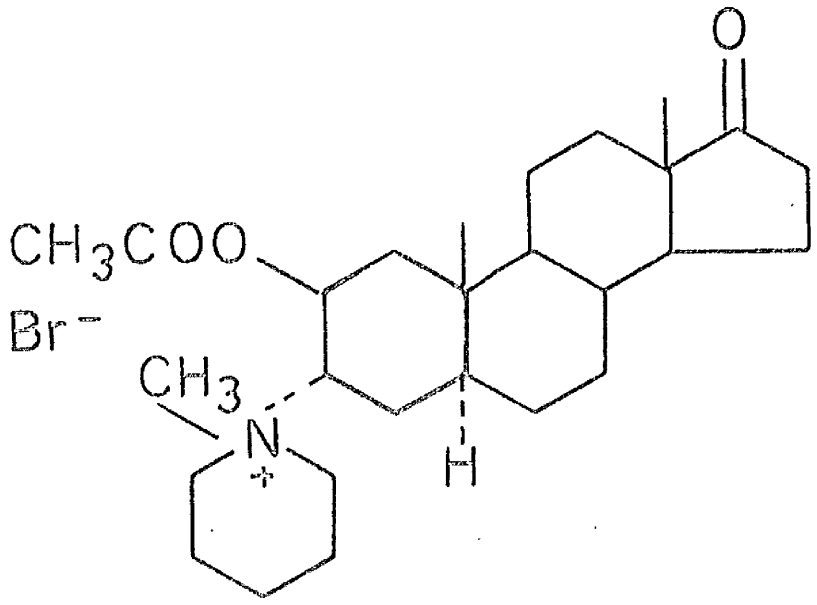
Compound B8



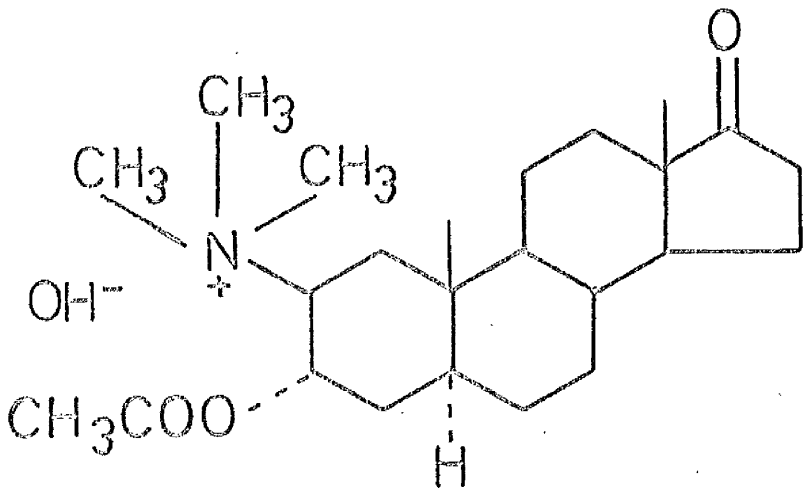
Compound B5



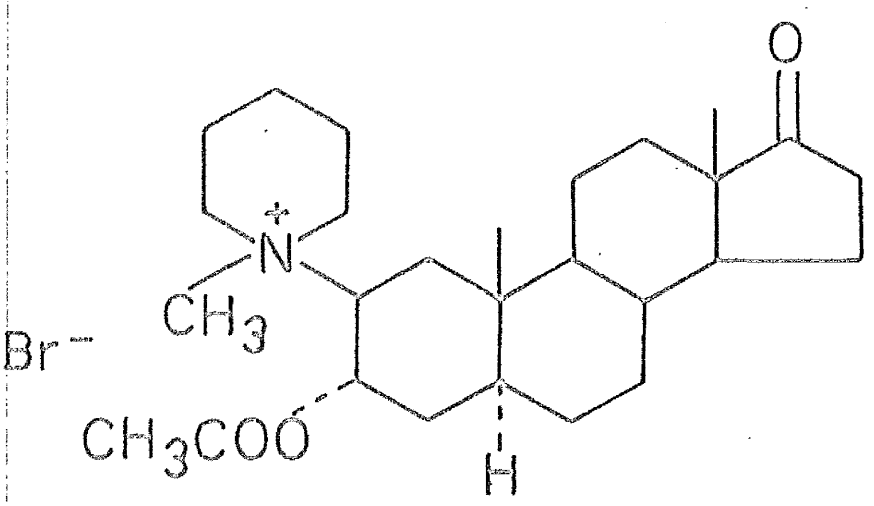
Compound B6



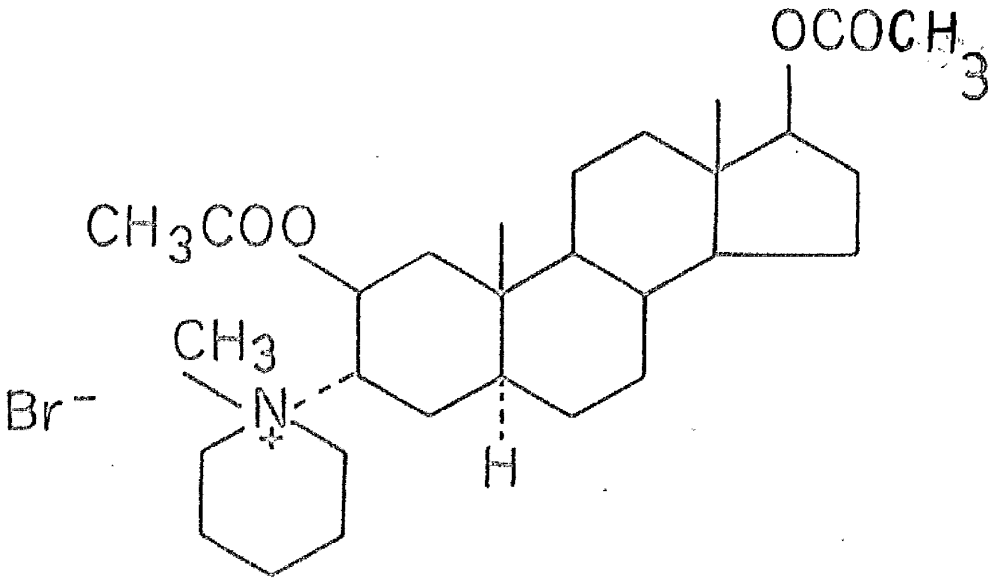
Compound B3



Compound B4



Compound B1



Compound B2

Table 6 Structural Formulae of Nitrogenous Steroids with Effects at the Neuromuscular Junction.

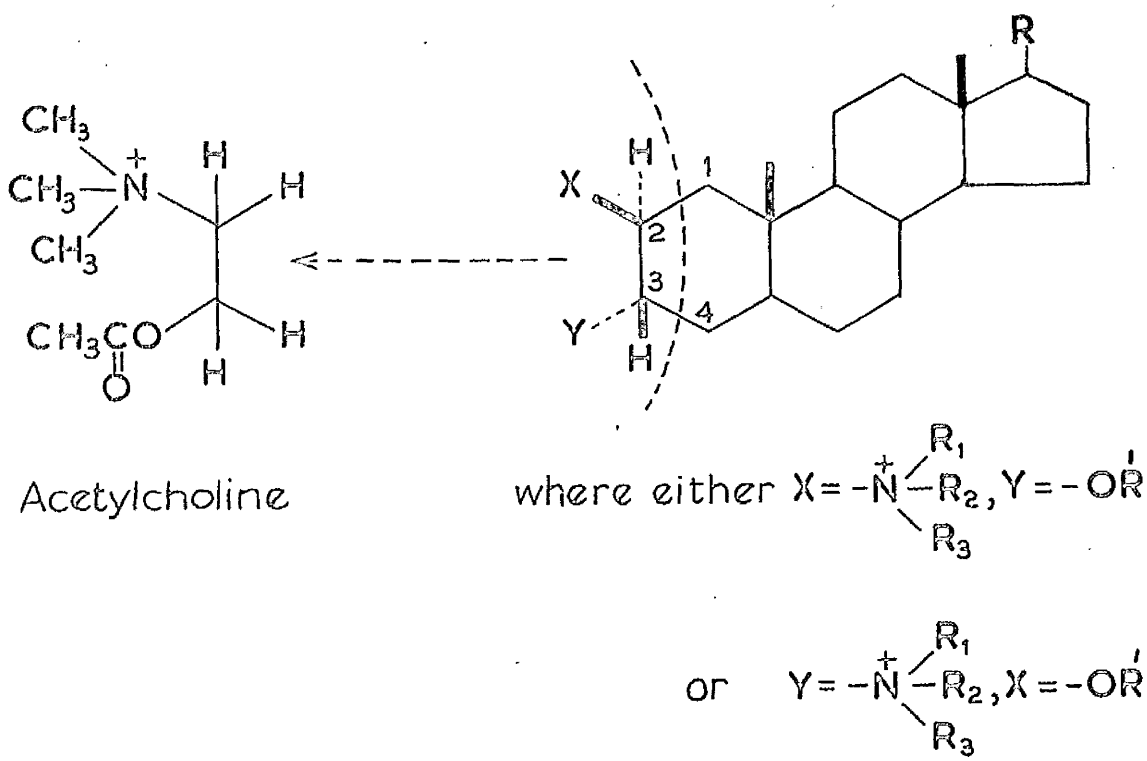


Fig. 22 General formula of compounds B1 to B10.

Eight out of the ten compounds studied, where R^1 = acetyl, incorporate an acetylcholine-like moiety in their molecular structure. This can be seen by comparing the structure of acetylcholine with the structural unit to the left of the dotted line in the steroid formula. R_1 and R_2 were not always discrete radicals and in most cases, except compounds B4 and B5, were residues of the same heterocyclic ring system, namely pyrrolidino or piperidino.

b) RESULTS.

Ten of the eleven compounds under investigation were monoquaternary ammonium salts derived from 2β- and 3α- amino steroids of the androstane and pregnane types. They fitted the general formula shown in Fig. 22. Their structural formulae are shown in Table 6.

The remaining compound, B11, was a 2β,16β - bisquaternary ammonium androstane (Table 6).

In a few instances, the scope of the pharmacological testing was limited by the amount of material available. The results are divided into two main headings:-

i) Investigation of neuromuscular block on various species using conventional techniques.

ii) Investigation of other effects including those on respiratory musculature and at autonomic ganglia.

During the experiments to investigate neuromuscular block the following accepted criteria were applied (Paton & Zaimis, 1952) in an attempt to qualitatively differentiate the compounds on the basis of their depolarizing or non-depolarizing properties:-

1) Depolarizing drugs produce muscular fasciculation and briefly increase the tension of the muscle twitch prior to its depression.

2) The reciprocal antagonism of tubocurarine and

decamethonium to the block produced by depolarizing and non-depolarizing agents respectively.

3) The ability of edrophonium and neostigmine to antagonize the block produced by non-depolarizing drugs and to potentiate, or only slightly antagonize, the block produced by depolarizing agents.

4) The activity of non-depolarizing drugs is intensified and prolonged by ether anaesthesia whereas depolarizing drugs are antagonized.

5) A tetanus is well maintained in muscles treated with depolarizing drugs but rapidly wanes in muscles treated with non-depolarizing muscle relaxants.

6) The induction of a spastic paralysis in avian muscle by depolarizing drugs as opposed to the flaccid paralysis produced by non-depolarizing compounds.

7) The ability of non-depolarizing drugs to antagonize the contracture produced by depolarizing compounds on the isolated frog rectus abdominis muscle.

i) INVESTIGATION OF NEUROMUSCULAR BLOCKING POTENCY USING CONVENTIONAL TECHNIQUES.

The Cat Gastrocnemius Muscle-Sciatic Nerve Preparation.

All the compounds, in the doses employed, produced complete or incomplete reversible block on this preparation. The ten monoquaternary steroids,

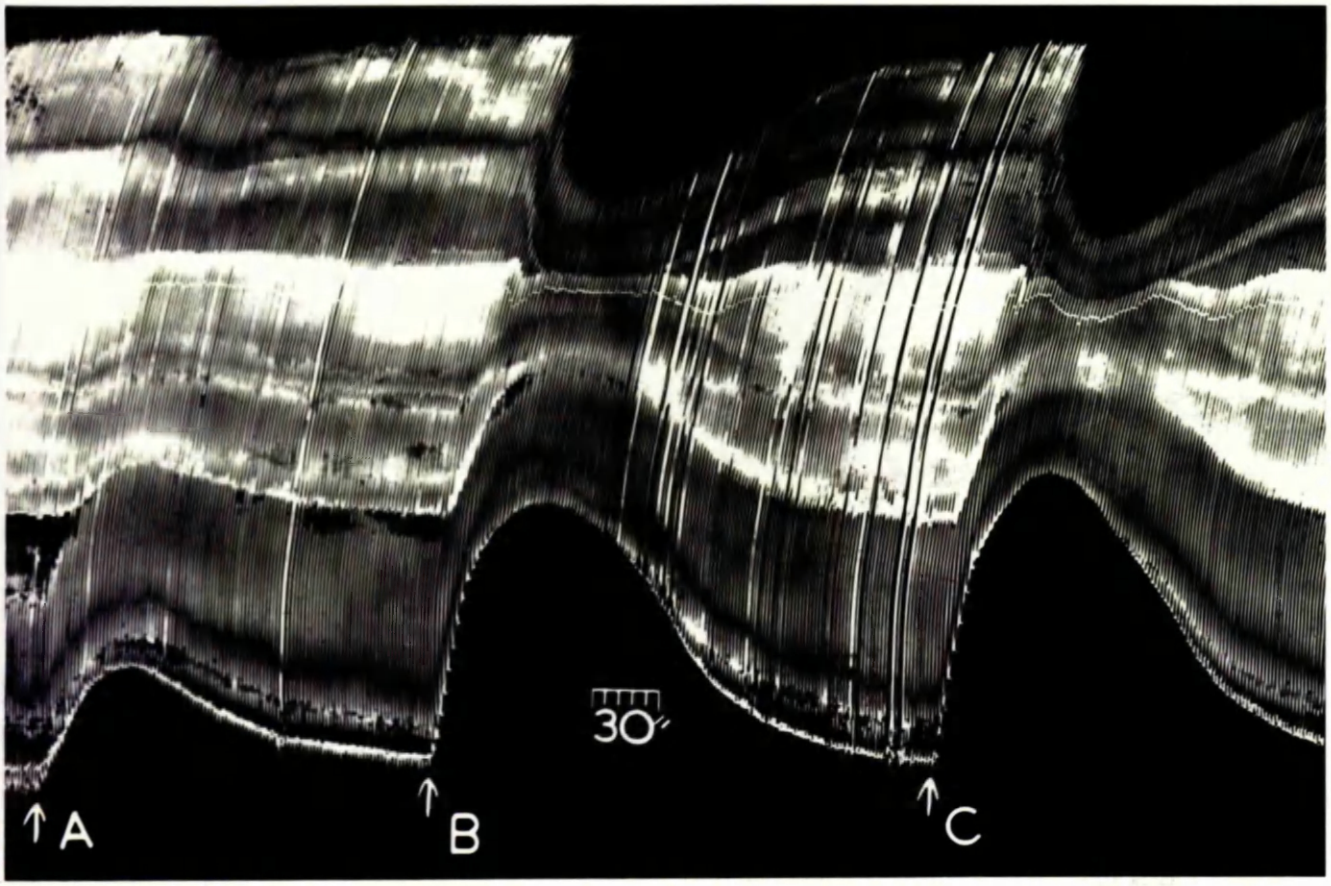


Fig. 24 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Indirect stimulation via the sciatic nerve. Drugs administered intravenously.

At A, B and C d-tubocurarine 0.150 mg/kg.

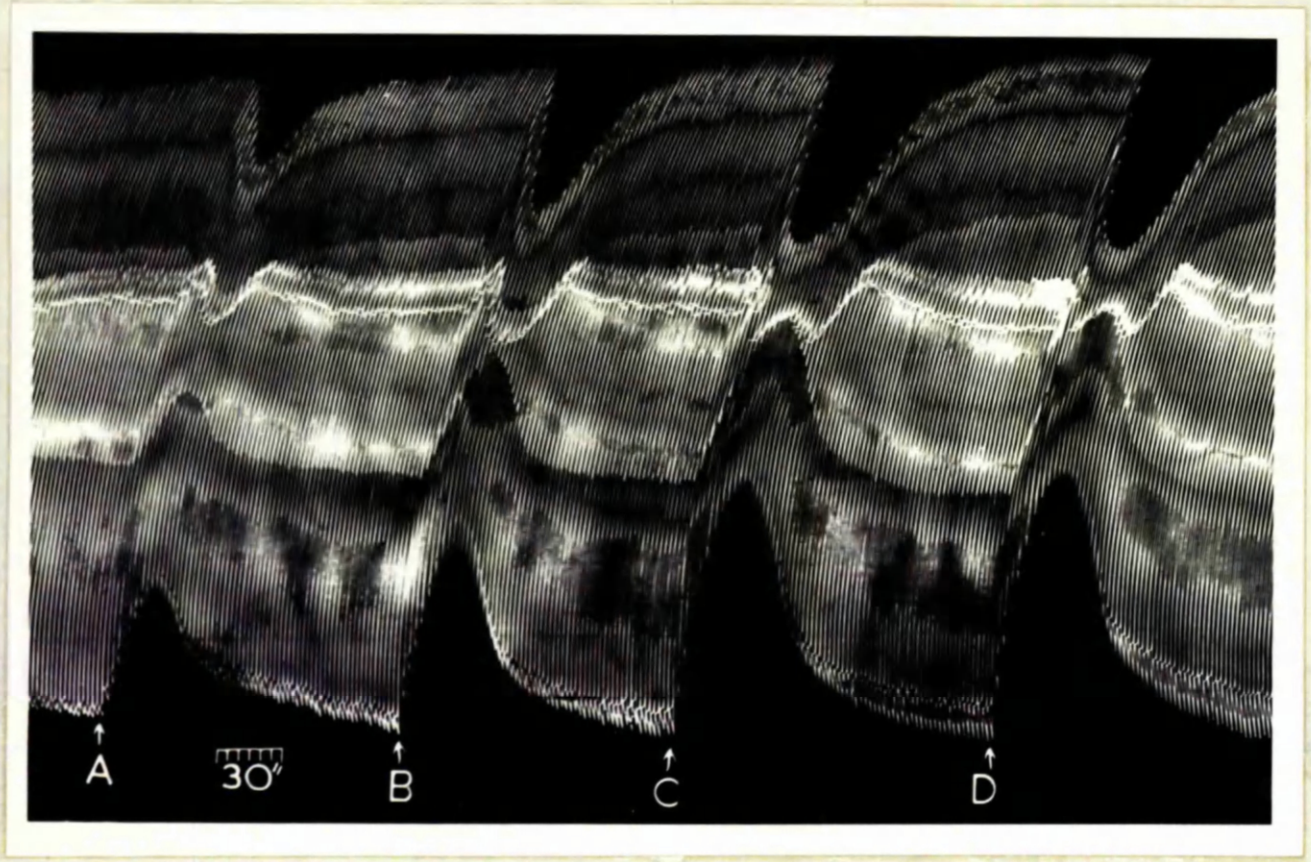


Fig. 23 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Indirect stimulation via the sciatic nerve. Drugs administered intravenously.

At A, B, C and D, compound B3 6 mg/kg.

TABLE 7

A comparison of the properties of compounds B1 to B11 on the cat gastrocnemius muscle-sciatic nerve preparation.

Compound	NEUROMUSCULAR BLOCKING ACTIVITY			Preliminary Excitation of Skeletal Muscle	EFFECT, on the block produced, of					Response of partially blocked muscle to indirect tetanus
	Time of onset of maximum paralysis (min)	Duration of paralysis (50% block approx.) (min)	Approx. Molar Potency (Tubocurarine = 100)		Tubocurarine (0.025-0.1 mg/kg)	Decamethonium (0.02-0.05 mg/kg)	Edrophonium (0.25-0.5 mg/kg)	Neostigmine (0.05-0.10 mg/kg)	Inhalation of ether vapour	
B1	1 - 2	12 - 17	6	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B2	1 - 2	13 - 18	2	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B3	2 - 3	14 - 20	1.5	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B4	1 - 2	18 - 23	1.5	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B5	2 - 3	15 - 20	1.5	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B6	1 - 2	12 - 17	2	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B7	1 - 2	17 - 22	1.5	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B8	2 - 3	18 - 23	1	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B9	1 - 2	11 - 16	2	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B10	2 - 3	14 - 19	2	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
B11	1 - 2	18 - 23	124	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained
dTc	2 - 5	25 - 35	100	None	Potentiation	Antagonism	Antagonism	Antagonism	Potentiation	Poorly sustained

dTc = Tubocurarine.

in doses of from 1 to 6 mg/kg, produced an approximately 50% reduction in the amplitude of the muscle twitch. They were appreciably less potent than tubocurarine, the most active, compound B1, being 1/16th as active as tubocurarine on a molar basis while the least active, compound B8, was 1/100th as active. In contrast, the bisquaternary compound, B11, possessed a potency slightly greater than that of tubocurarine on a molar basis (Table 7) and doses of 0.05 to 0.10 mg/kg produced an approximately 50% inhibition of the muscle twitch.

The duration and degree of neuromuscular block produced by all eleven compounds depended both on the magnitude of the dose employed and the number of doses administered. Neuromuscular block became more prolonged and intense following the second injection of the same dose than following the first. Similarly, the third injection of the same dose caused a more prolonged block than following the second. The effect of a fourth injection of a similar dose often did not significantly differ from that of the third dose. Similar effects were also observed using tubocurarine (Figs. 23 and 24).

In all compounds, the time of onset of maximum paralysis and the duration of block were significantly less than those for tubocurarine (Table 7).

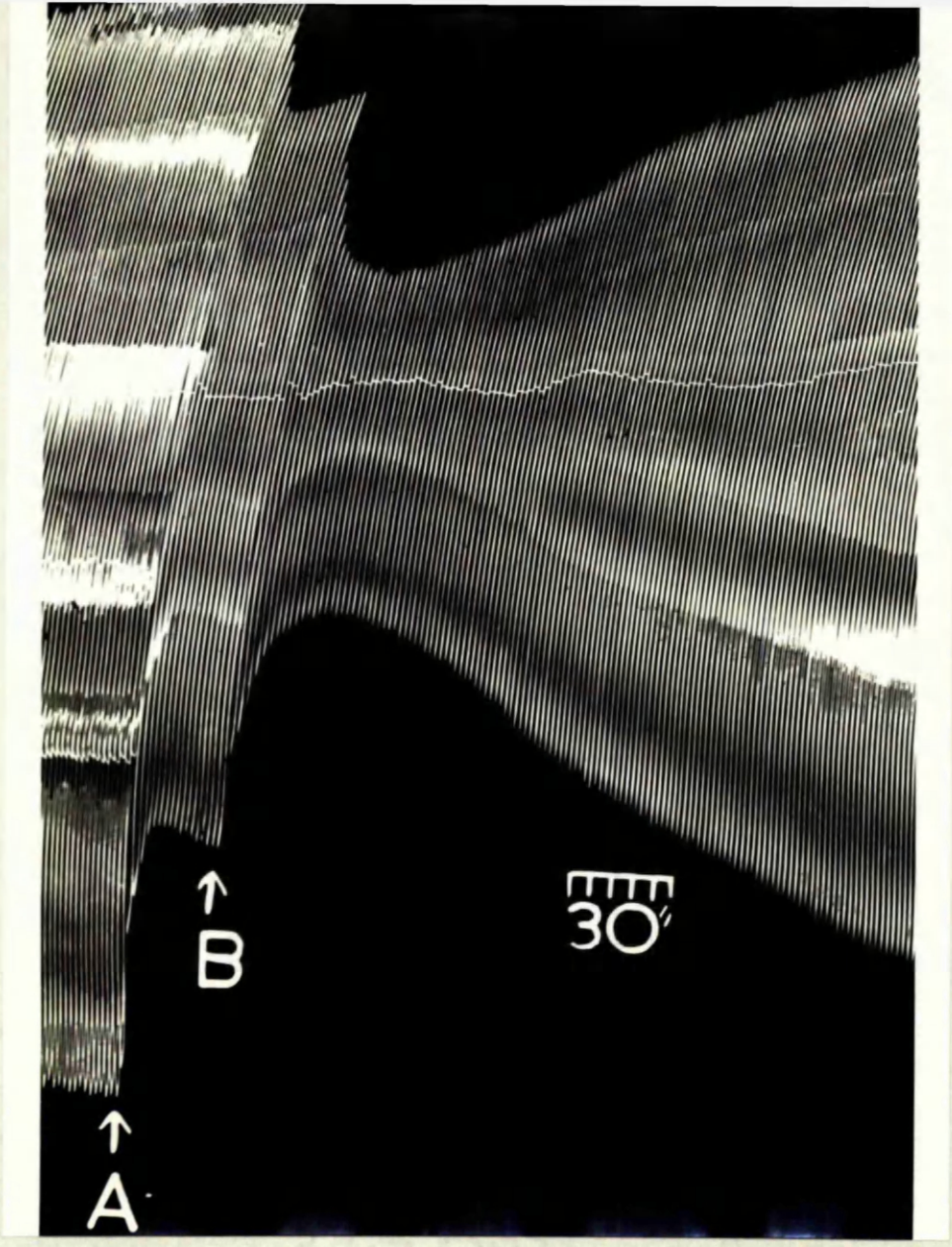


Fig. 26 Cat gastrocnemius muscle-sciatic nerve preparation.

Pentobarbitone anaesthesia. Contraction downwards.

Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A. compound B4 3 mg/kg.

At B. d-tubocurarine 0.025 mg/kg.

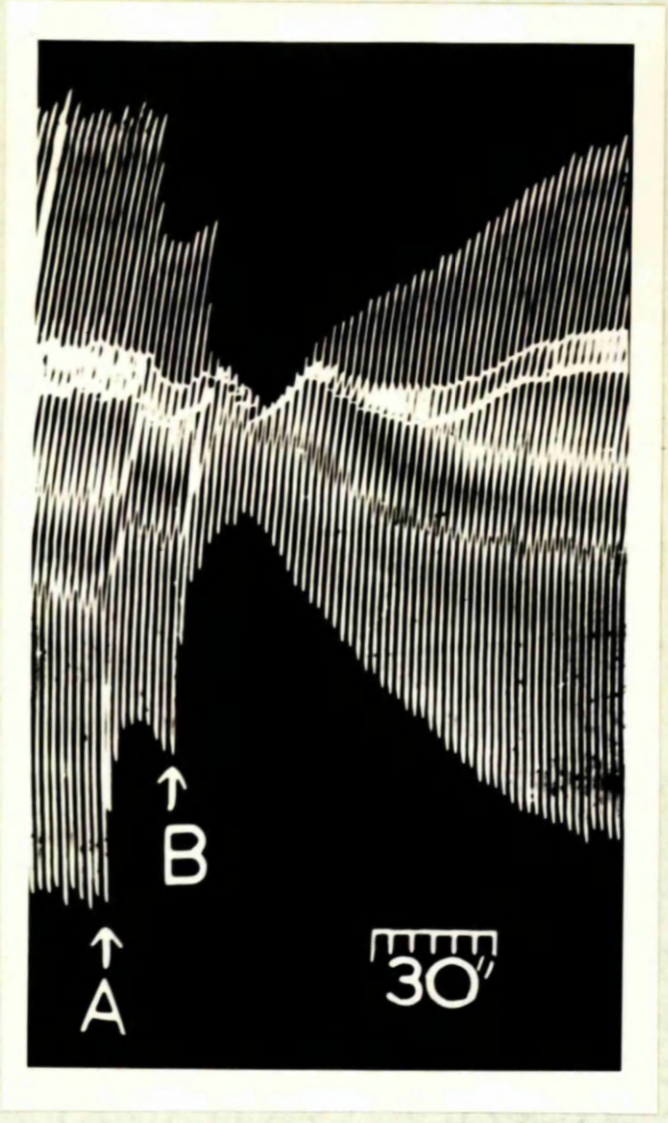


Fig. 25 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B1 0.5 mg/kg.

At B, d-tubocurarine 0.05 mg/kg.

None of the compounds appeared to produce any initial stimulant effects and there was no evidence of muscular twitching or fasciculation.

The Effect of Tubocurarine and Decamethonium on the Block Produced.

Muscle relaxants with dissimilar types of action generally antagonize each other whereas similarly acting drugs, given successively, produce an additive effect (Paton & Zaimis, 1952). These observations prompted an investigation of the effects of tubocurarine and decamethonium on the block produced by the compounds under test.

Investigations were carried out using a dose of compound and a dose of either tubocurarine or decamethonium selected to produce, when given alone, a measurable (25% - 40%), but not intense, depression of the twitch height.

Tubocurarine.

A dose of tubocurarine (0.05 - 0.10 mg/kg) given at the point of maximal depression of the twitch height produced by any of the compounds, enhanced the intensity of the neuromuscular block (Figs. 25 and 26). Thus, in Fig. 25, the dose of compound B1 (0.5 mg/kg) and the dose of tubocurarine (0.05 mg/kg) were each

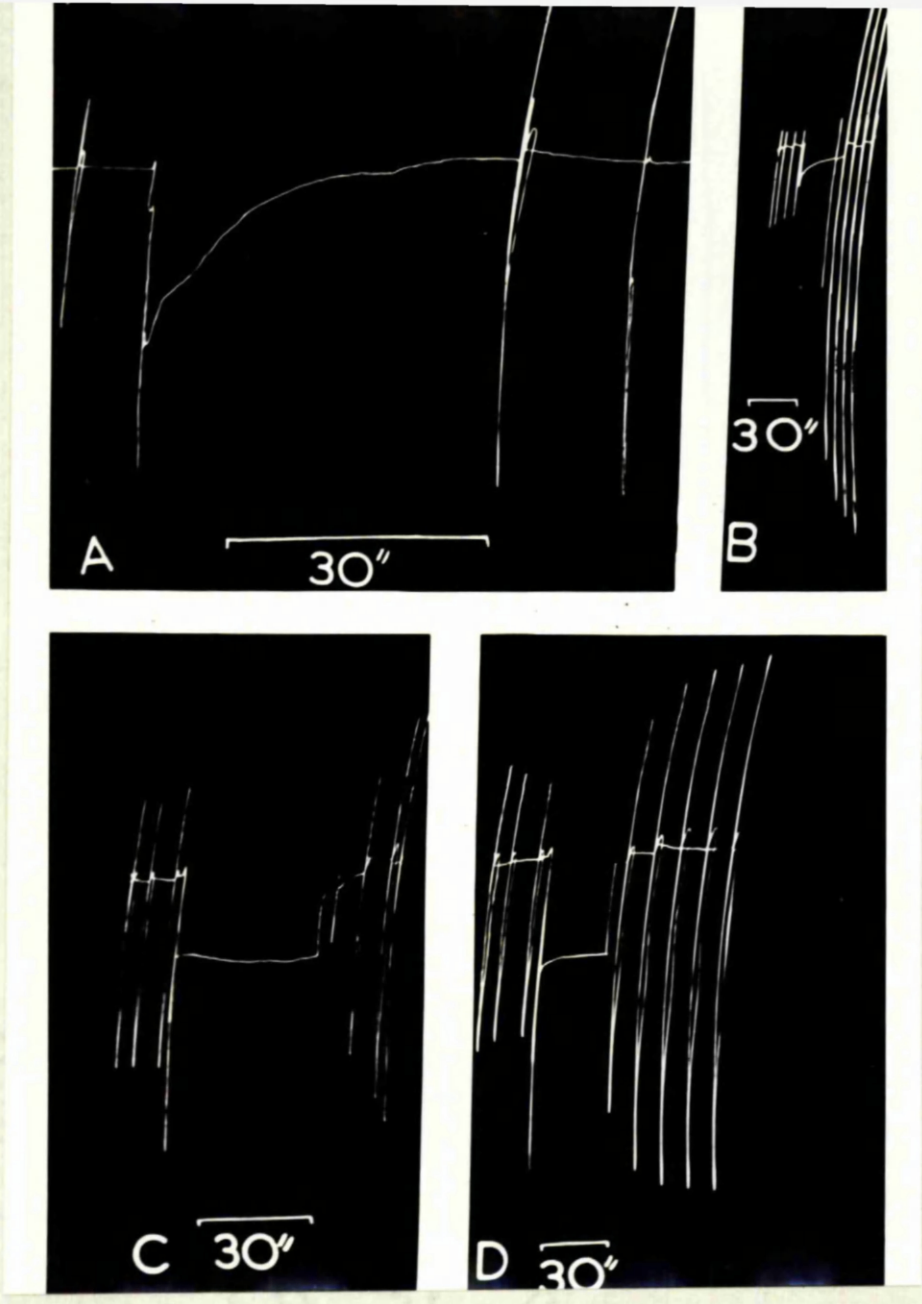


Fig. 28 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve (1200 impulses /min.) during partial block by:

A, compound B1 1.5 mg/kg.

B, d-tubocurarine 0.150 mg/kg.

C, decamethonium 0.07 mg/kg.

D, untreated muscle.

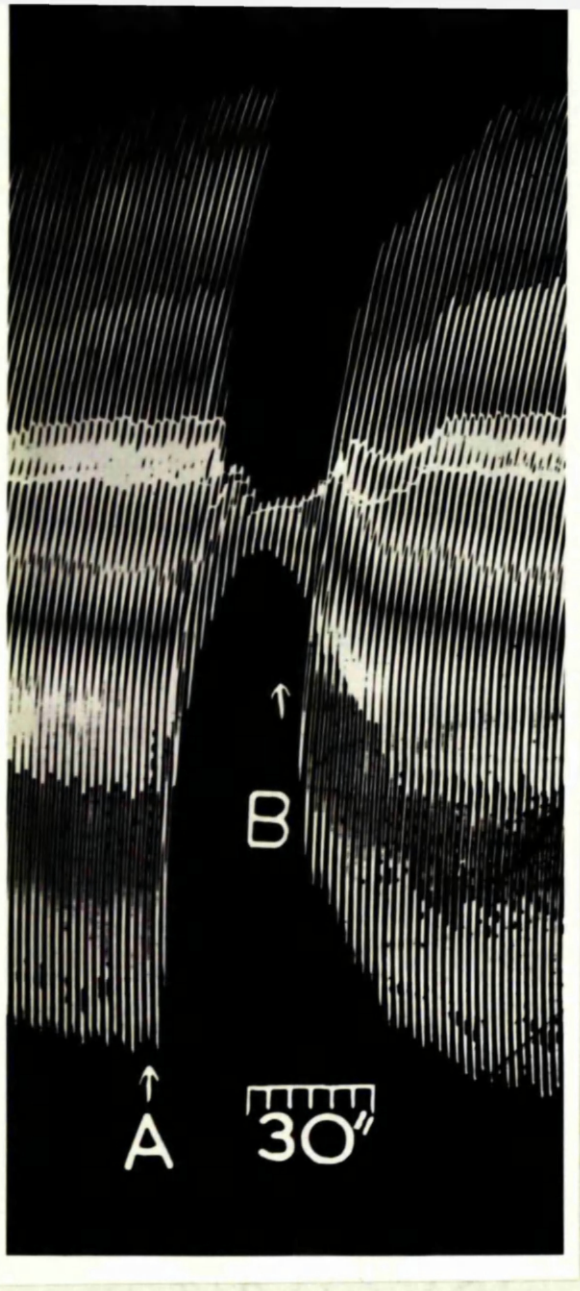


Fig. 27 Cat gastrocnemius muscle-sciatic nerve preparation.

Pentobarbitone anaesthesia. Contraction downwards.

Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B2 4 mg/kg.

At B, decamethonium 0.05 mg/kg.

selected to produce a neuromuscular block of about 33%. When the dose of compound B1 was followed by a dose of tubocurarine at the point of maximal depression, the degree of neuromuscular block was increased to approximately 80%.

Decamethonium.

Decamethonium (0.02 - 0.05 mg/kg) was injected at the point of maximal depression of the muscle twitch produced by the compounds. Immediately there was an increase in amplitude and, within approximately 5 minutes, a complete reversal of the block was achieved in all cases (Fig 27).

The investigations carried out using tubocurarine and decamethonium indicated that all the compounds under test were non-depolarizing in nature and no evidence of a depolarizing component was found.

Effect of Indirect Tetanization of the Partially Blocked Muscle.

All the compounds (1 - 7 mg/kg for compounds B1 to B10, 0.10 - 0.15 mg/kg for compound B11) produced a poorly sustained response of partially blocked muscle to indirect tetanization in a manner similar to that produced by tubocurarine (0.10 - 0.15 mg/kg)(Fig. 28) and different from the well-sustained tension of tetanized muscle partially blocked by decamethonium (0.05 - 0.10 mg/kg).

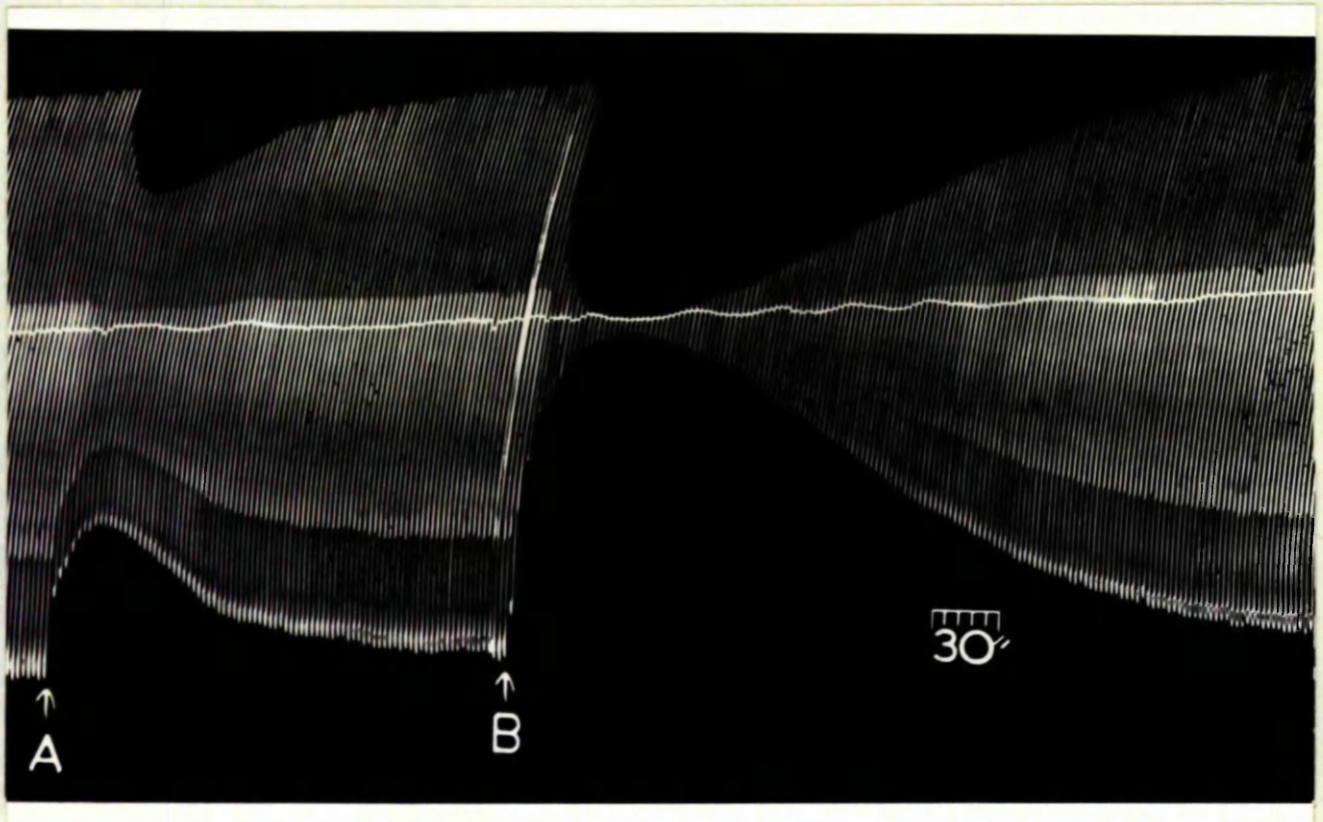


Fig. 29 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B8 4.5 mg/kg.

At B, compound B8 4.5 mg/kg during ether inhalation.

Effect of Ether Anaesthesia.

Ether anaesthesia has been shown to intensify the action of non-depolarizing neuromuscular blocking agents (Paton & Zaimis, 1952; Foldes, 1960) whereas the activity of depolarizing agents appears to be less affected, only a slight increase or reduction being observed (Paton, 1953).

A dose of drug was added to produce a neuromuscular block of about 25% to 50%. After recovery was complete, the same dose was repeated in the presence of ether vapour administered from a bottle connected to the artificial respiration pump. The administration of ether vapour was begun immediately after injection of the drug and continued for a period of 5 minutes. The neuromuscular blocking potency of all the compounds was intensified and prolonged by ether anaesthesia (Fig. 29). This furnishes further proof that the quaternary steroids under test possess non-depolarizing blocking properties.

Studies of Drug Antagonism.

Anticholinesterases, for example edrophonium and neostigmine, by virtue of their ability to intensify the action of acetylcholine, antagonize the block produced by non-depolarizing agents (Bülbring & Chou, 1947; Blaschko, Bülbring & Chou, 1949), but the block produced

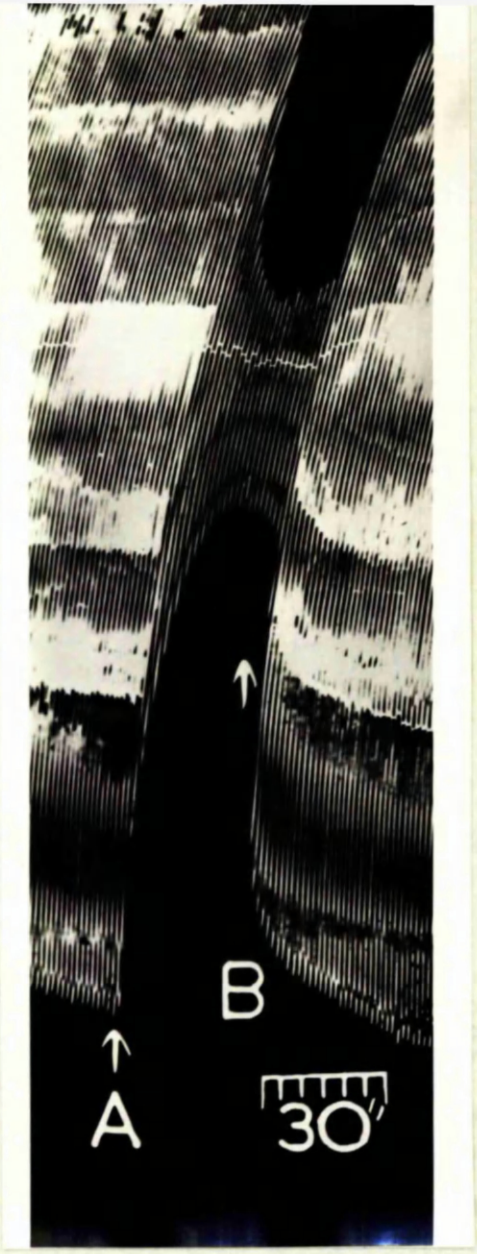


Fig. 31 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B9 4 mg/kg.

At B, neostigmine 0.05 mg/kg.

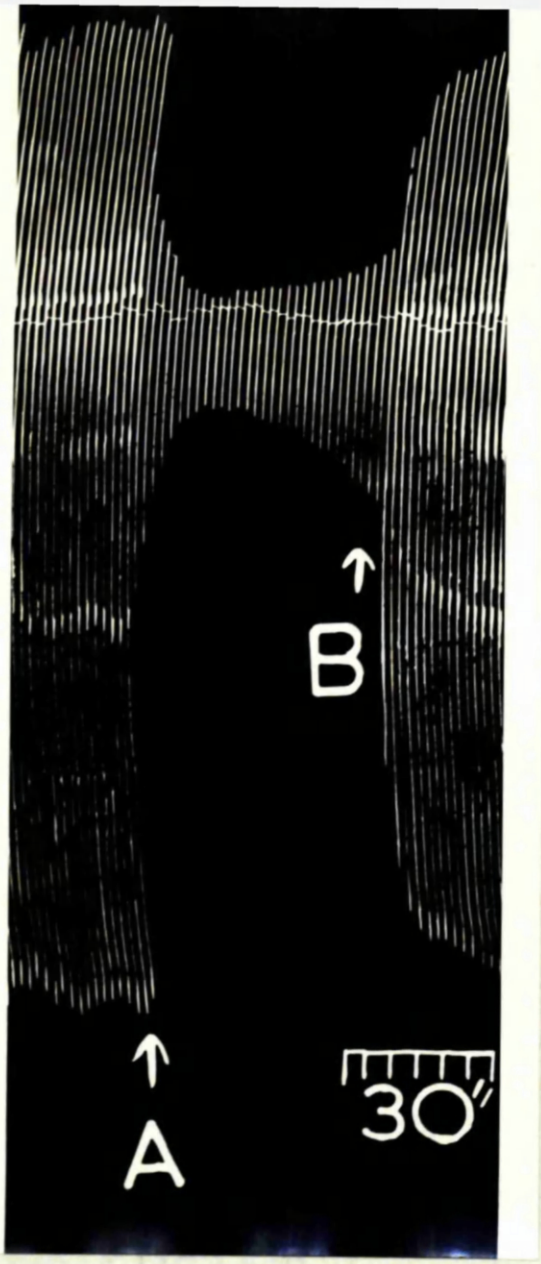


Fig. 30 Cat gastrocnemius muscle-sciatic nerve preparation. Pentobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B1 3 mg/kg.

At B, edrophonium 0.5 mg/kg.

by depolarizing agents may be potentiated or only slightly antagonized (Paton, 1951b). The anticholinesterases edrophonium and neostigmine were selected for studies to observe the qualitative nature of the block produced by the compounds under investigation. Each was injected into the cat at the point of maximal neuromuscular block.

Edrophonium.

Edrophonium (0.5 - 1.0 mg/kg) injected intravenously at the point of maximum depression of the twitch height, completely and rapidly reversed the neuromuscular block produced by all the compounds. A typical tracing is shown in Fig.30.

Neostigmine.

Neostigmine (0.02 - 0.10 mg/kg) produced results qualitatively similar to those obtained with edrophonium, although its duration of action was more prolonged. It completely and effectively antagonized all the compounds under test (Fig.31).

These observations with neostigmine and edrophonium again indicate that all the quaternary steroids are non-depolarizing compounds.

A summary of the quantitative and qualitative properties of the compounds on the cat gastrocnemius muscle-sciatic nerve preparation is given in Table 7.

TABLE 8

Individual and Mean Head Drop Doses in the Rabbit of compounds B1, B11 and Tubocurarine.

Compound	HEAD DROP DOSES (H.D.D.)		No. died / No. injected	Molar Potency (Tubocurarine = 100)
	INDIVIDUAL mg/kg	MEAN ± SEM mg/kg		
B1	13.3 13.8 13.3 13.1 14.2 9.7 13.7 19.3 14.7	13.9 ± 0.82	2/8	1.2
B11	0.36 0.22 0.25 0.18 0.25 0.20 0.21 0.26 0.25	0.24 ± 0.02	5/8	109
dTc	0.33 0.24 0.26 0.33 0.34 0.21 0.33 0.20 0.29	0.28 ± 0.02	4/8	100

dTc = tubocurarine.

When the preparation was giving a consistent inhibition, for example 50%, of the maximum twitch height, in response to a fixed dose of any of the compounds, a dose of tubocurarine was selected which produced a similar quantitative effect. The approximate molar potencies were then calculated with reference to tubocurarine according to the formula:- Molar Potency of New Compound =

$$\frac{\text{Molecular Weight of New Compound}}{\text{Molecular Weight of Reference Compound}} \times \frac{\text{Dose of Reference Compound Producing a Certain Effect}}{\text{Dose of New Compound Producing the Same Effect}} \times 100$$

The reference compound, tubocurarine, was arbitrarily assigned the value 100.

The Rabbit Head Drop Test.

Only compounds B1 and B11 were selected for this test, the former being the most potent monoquaternary steroid on the cat while the bisquaternary compound (B11), was more potent on the cat than tubocurarine. The head drop dose of tubocurarine was estimated for comparison and the results are shown in Table 8. In keeping with the observations on the cat, compound B11 was more potent on the rabbit than tubocurarine and its duration of effect was significantly less than for tubocurarine (10 min v 15 min for complete recovery). Compound B1, on the other hand, was only 1/6th as active on this preparation

TABLE 9

A comparison of the properties of compounds B1 to B11 and tubocurarine on the hen gastrocnemius muscle-sciatic nerve preparation.

Compound	NEUROMUSCULAR BLOCKING ACTIVITY			
	Time of onset of maximum paralysis (min)	Duration of paralysis (50% block approx) (min)	Approx Molar Potency (dTc = 100)	Type of Neuro-Muscular Block Produced
B1	1 - 3	15 - 20	6	Non-depolarizing
B2	3 - 4	18 - 23	2.5	Non-depolarizing
B3	2 - 3	13 - 18	2.5	Non-depolarizing
B4	1 - 3	12 - 17	7.5	Non-depolarizing
B5	4 - 5	22 - 27	2	Non-depolarizing
B6	2 - 3	12 - 17	6	Non-depolarizing
B7	2 - 3	15 - 20	1.5	Non-depolarizing
B8	2 - 3	13 - 18	6.5	Non-depolarizing
B9	1 - 2	11 - 16	2	Non-depolarizing
B10	2 - 3	13 - 18	4	Non-depolarizing
B11	2 - 3	15 - 20	94	Non-depolarizing
dTc	5 - 10	30 - 40	100	Non-depolarizing

dTc = Tubocurarine.

as it was on the cat gastrocnemius muscle-sciatic nerve preparation.

EXPERIMENTS USING AVIAN MUSCLE.

Hen Gastrocnemius Muscle-Sciatic Nerve Preparation.

This preparation can be used to differentiate between depolarizing and non-depolarizing drugs. It may also be used semi-quantitatively to compare the approximate potencies of new compounds with those of decamethonium and tubocurarine.

All the compounds investigated behaved qualitatively like tubocurarine and were devoid of contracture-inducing properties. An approximately 50% reduction in the amplitude of the twitch height was produced by doses of from 1 to 5 mg/kg of the ten monoquaternary compounds. Compound B11, however, produced approximately 50% inhibition of the muscle twitch with doses of 0.05 to 0.10 mg/kg. Compounds B2, B3, B5, B6 and B10 were all slightly more active than on the cat but compounds B4 and B8 increased activity by factors of 5 and 6 respectively. Their potency compared with tubocurarine, however, was still very low (Table 9). B11 was the most active compound, being approximately equipotent with tubocurarine. The duration and onset of action of all the eleven compounds was found to be approximately the same as when these compounds

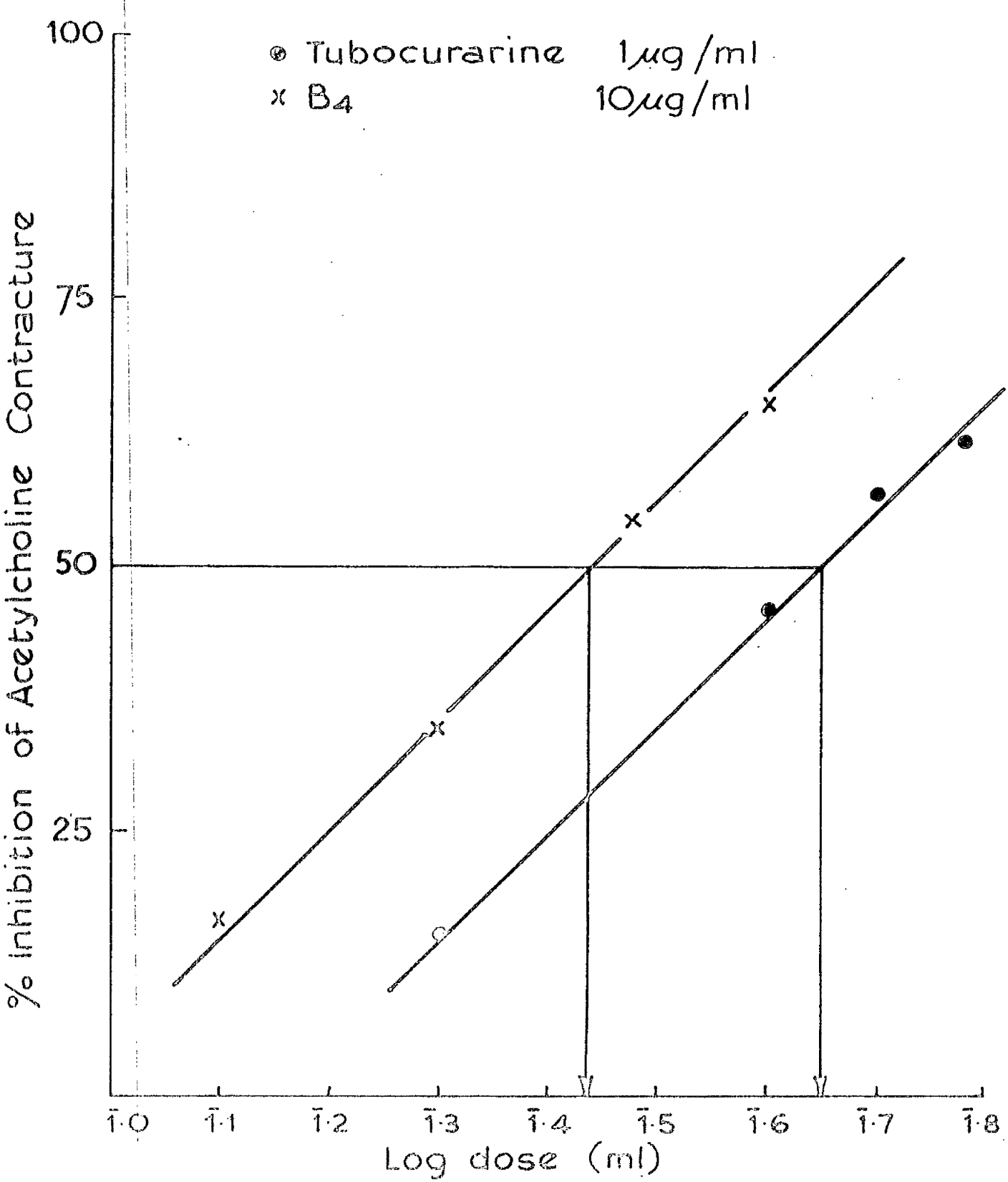


Fig. 36 Graph to show method of estimating the potency of compounds B1 to B11 on the frog rectus abdominis muscle.

TABLE 10

A comparison of the potency of compounds B1 to B11 with tubocurarine on the frog rectus abdominis muscle.

COMPOUND	APPROXIMATE MOLAR POTENCY TUBOCURARINE = 100
B1	37.2
B2	180
B3	40.3
B4	7.28
B5	70.7
B6	34.3
B7	131
B8	22.7
B9	50.2
B10	53.9
B11	671.7

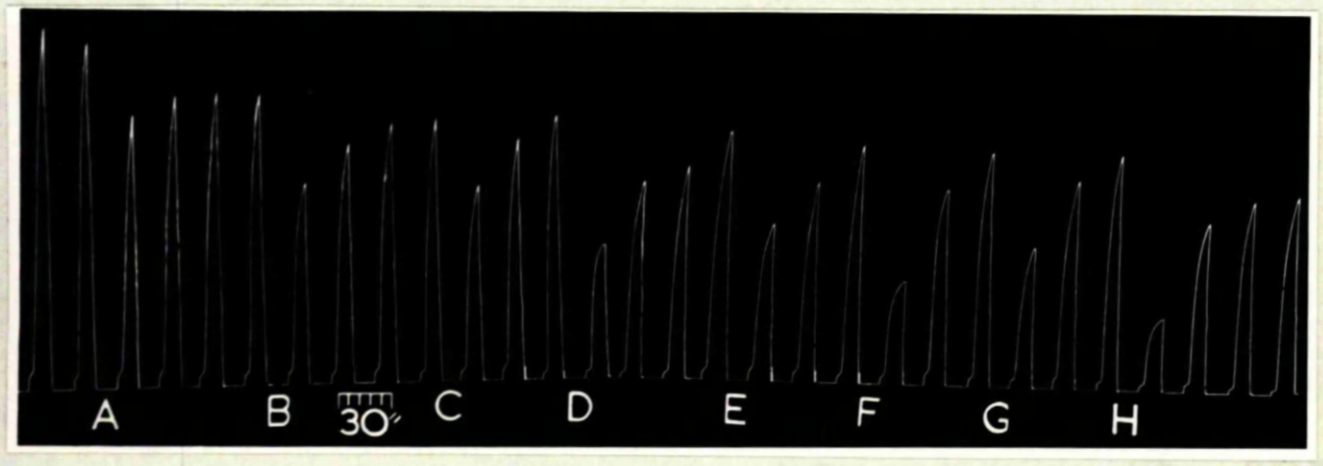


Fig. 35 Isolated frog rectus abdominis muscle.

All contractions were due to acetylcholine,

1 g/ml, acting for 30 seconds.

At A, C, E and G, d-tubocurarine 0.2,

0.4, 0.6 and 0.8 g/ml respectively.

At B, D, F and H, compound B2 0.2, 0.4,

0.6 and 0.8 g/ml respectively.

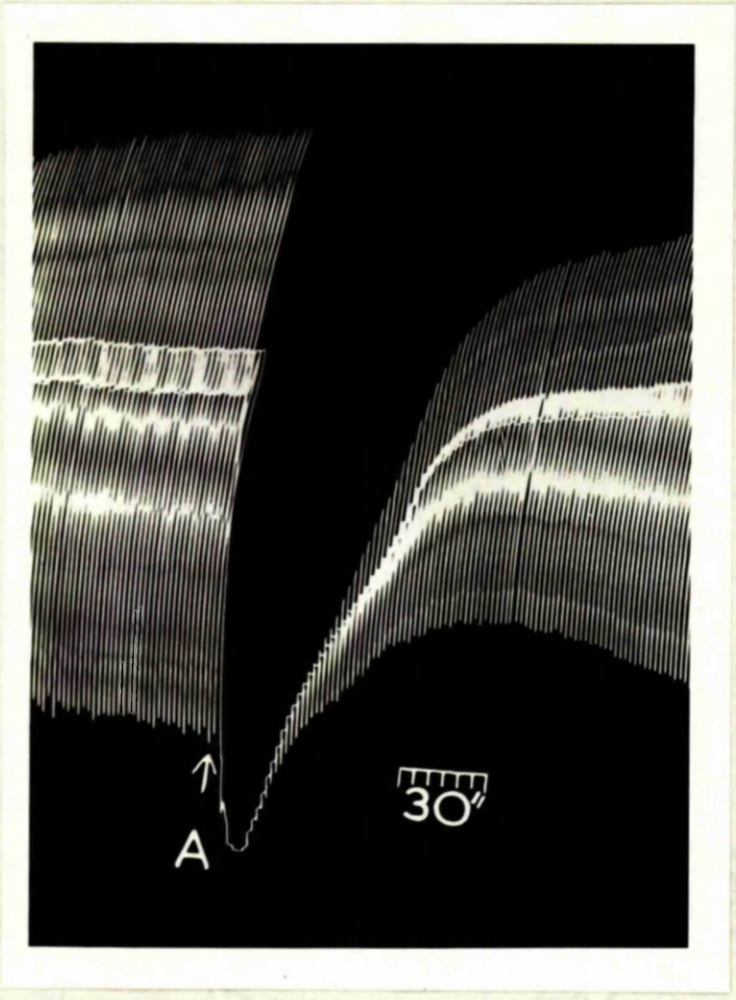


Fig. 34 Hen gastrocnemius muscle-sciatic nerve preparation. Phenobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, decamethonium 0.03 mg/kg.

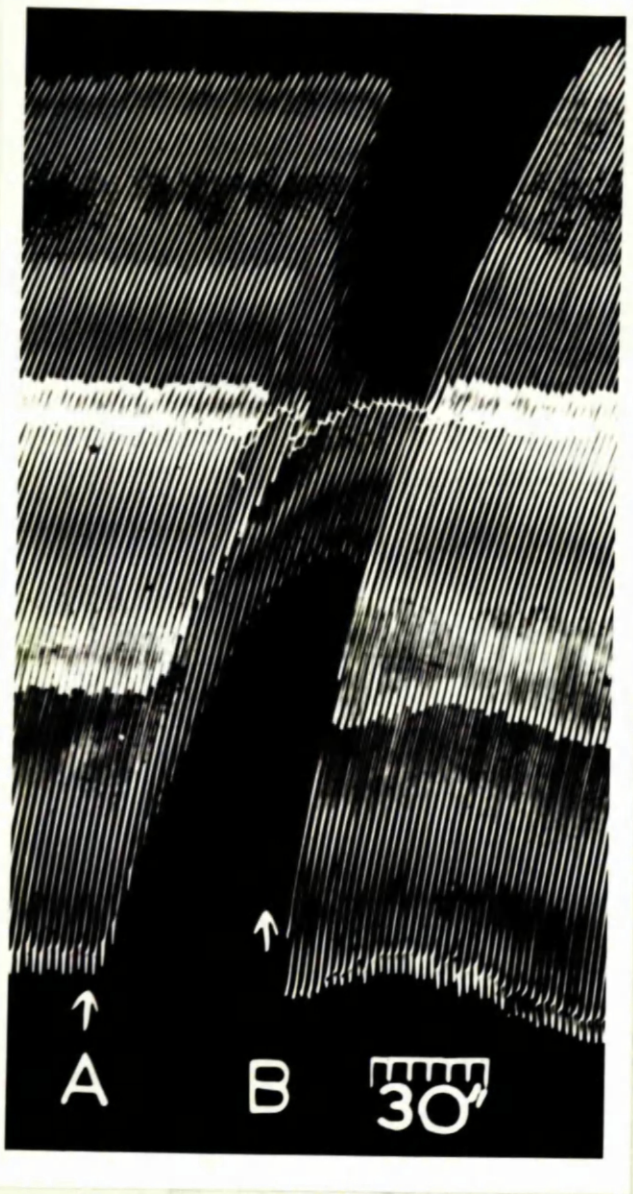


Fig. 33 Hen gastrocnemius muscle-sciatic nerve preparation. Phenobarbitone anaesthesia. Contractions downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B5 6 mg/kg.

At B, edrophonium 0.50 mg/kg.

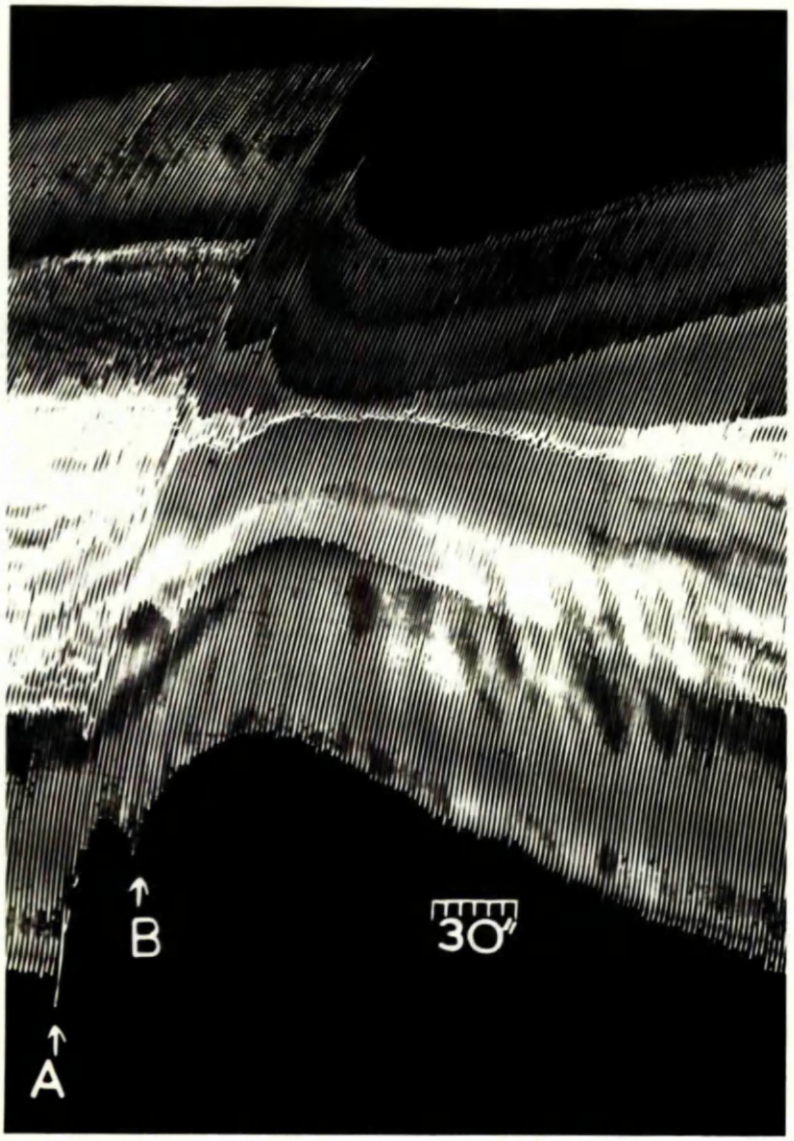


Fig. 32 Hen gastrocnemius muscle-sciatic nerve preparation. Phenobarbitone anaesthesia. Contraction downwards. Drugs administered intravenously. Indirect stimulation via the sciatic nerve.

At A, compound B4 2 mg/kg.

At B, d-tubocurarine 0.10 mg/kg.

were tested on a similar preparation in the cat.

All the compounds exhibited typical non-depolarizing activity on this preparation as shown by the absence of a muscular contractural effect, the potentiation of the block by tubocurarine (0.05 - 0.10 mg/kg) (Fig.32) and its complete and rapid reversal by edrophonium (0.5 - 1.0 mg/kg)(Fig.33). For comparison, the well-known contracture of avian muscle produced by a depolarizing compound, decamethonium, is shown in Fig.34.

Frog Rectus Abdominis Muscle Preparation.

None of the compounds investigated caused any direct contractural response in this preparation confirming their non-depolarizing nature. On the other hand, each acted qualitatively similarly, at doses of from 0.03 μg/ml to 6 μg/ml, to tubocurarine (0.1 - 2.0 μg/ml) and produced graded inhibitory effects to acetylcholine-induced contractions (1.0 - 2.0μg/ml)(Fig.35). The potency of each of these compounds as measured by the degree of inhibition of acetylcholine-induced contractions is shown in Table 10. Tubocurarine was similarly compared and the potency of each compound was determined graphically as shown in Fig.36.

The Rat Phrenic Nerve-Diaphragm Preparation.

All the monoquaternary compounds (0.05 - 0.25 mg/ml)

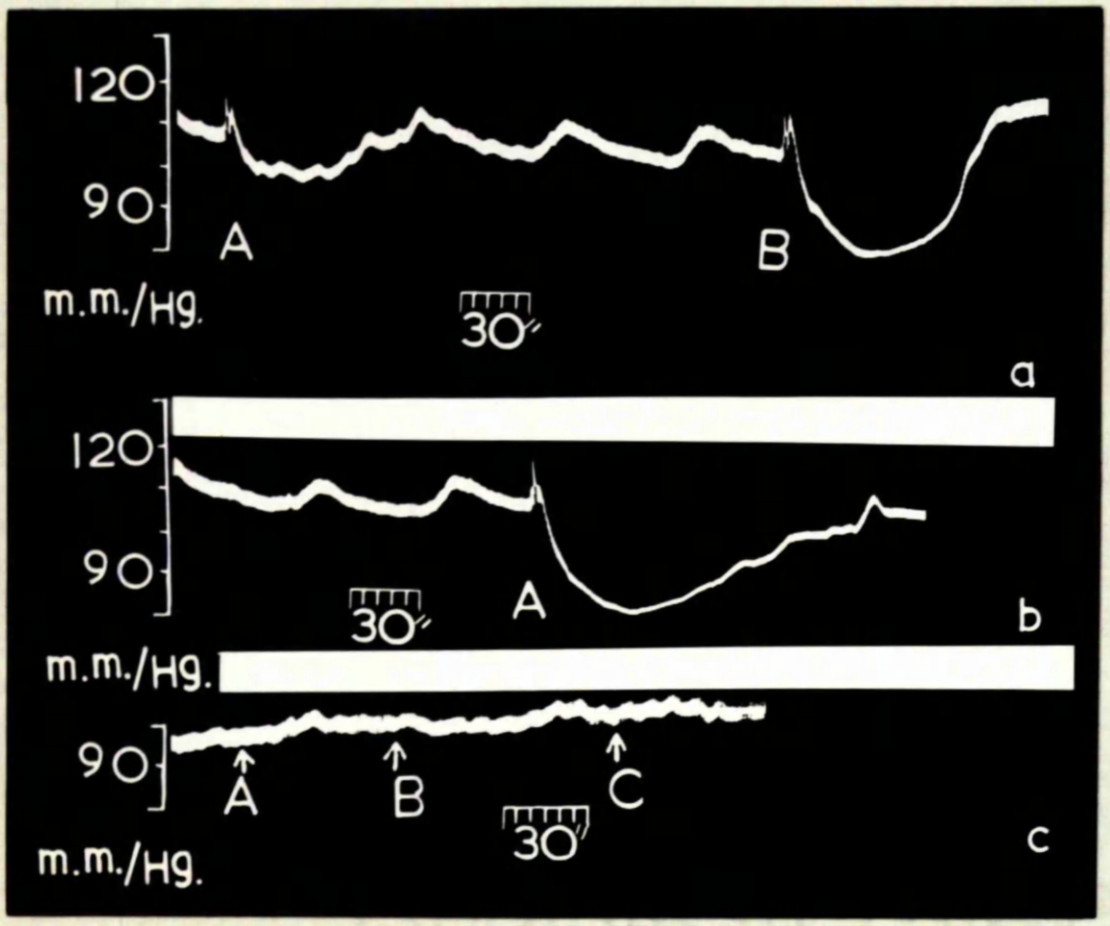


Fig. 38 Cat. Pentobarbitone anaesthesia. Blood pressure record from common carotid artery. Drugs administered intravenously. Vertical scale, on left hand side, indicates the blood pressure in mm of mercury.

(a) At A and B, compound B1 1 and 2.5 mg/kg respectively.

(b) At A, d-tubocurarine 0.50 mg/kg.

(c) At A, B and C, compound B11 0.25, 0.50 and 1.0 mg/kg respectively.

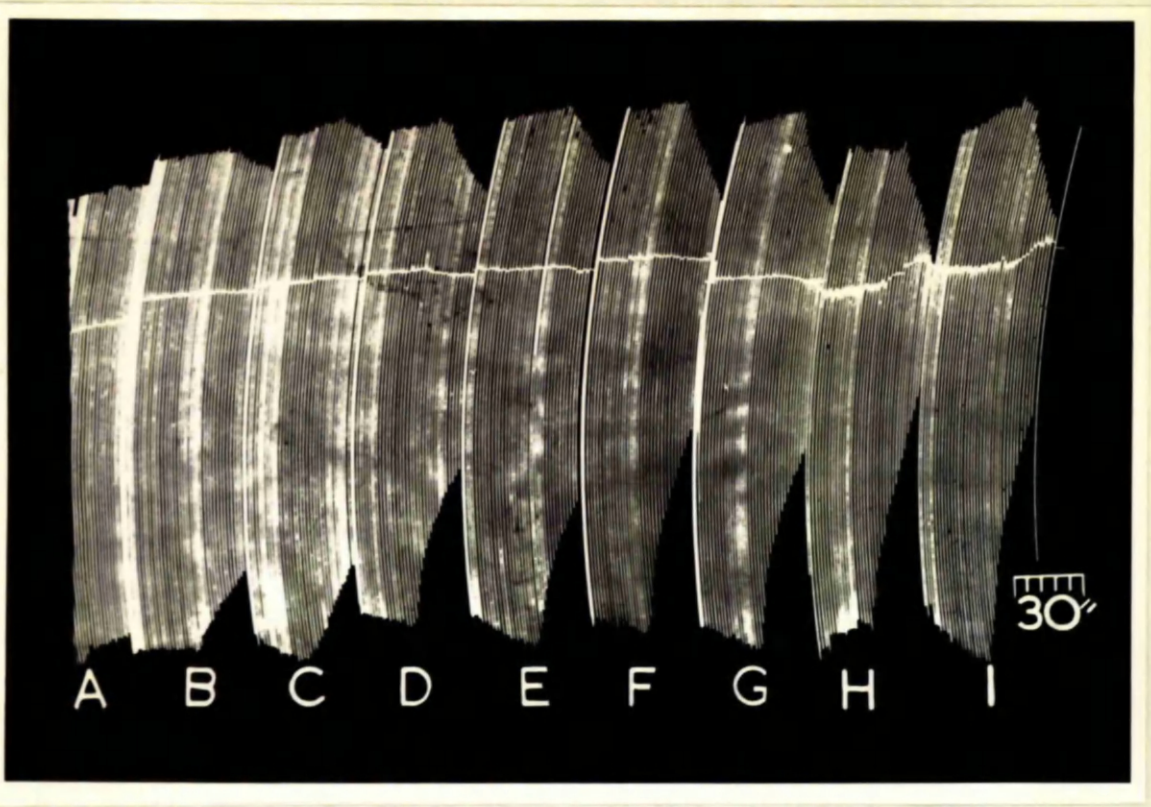


Fig. 37 Isolated rat phrenic nerve diaphragm preparation. Contractions downwards. Indirect stimulation via the phrenic nerve. Drugs allowed to act for 3 min. every 15 min.

At A, B, D, F, and H, compound B11 1.0, 1.5, 2.0, 2.5 and 3.0μg/ml respectively.

At C, E, G and I, d-tubocurarine 1.0, 1.5, 2.0 and 2.5μg/ml respectively.

TABLE 11

A comparison of the Potency on the Rat Phrenic Nerve-Diaphragm Preparation of compounds B1 to B11 with tubocurarine.

COMPOUND	APPROXIMATE MOLAR POTENCY (TUBOCURARINE = 100)
B1	0.68
B2	1.29
B3	0.68
B4	0.41
B5 *	-
B6	0.68
B7 *	-
B8	0.69
B9	2.36
B10	1.43
B11	70.77

* Insufficient material.

were relatively insensitive on this preparation, the most potent, compound B9, being approximately 1/40th as active as tubocurarine (Table 11). A typical tracing is shown in Fig.37.

ii) RESULTS OF TESTS OTHER THAN THOSE DESIGNED TO INVESTIGATE NEUROMUSCULAR BLOCKING POTENCY.

Effect on the Blood Pressure of the Pentobarbitone-Anaesthetized Cat.

The ten monoquaternary compounds, on intravenous injection, caused a marked fall in the blood pressure at doses (1 - 6 mg/kg) at which they also produced partial or complete neuromuscular block. The hypotension observed was similar in duration and effect to that seen after the injection of tubocurarine (0.1 - 0.2 mg/kg) (Fig.38). The bisquaternary compound, B11, on the other hand, produced no significant rise or fall in blood pressure of the cat even when administered in doses 10 times that required to induce a neuromuscular block of 50% (Fig.38).

Estimation of Sympathetic Ganglion Blocking Activity.

The Cat Nictitating Membrane Preparation.

All ten monoquaternary compounds produced an approximately 50% inhibition of the height of contraction of the nictitating membrane at doses ranging from 4 to

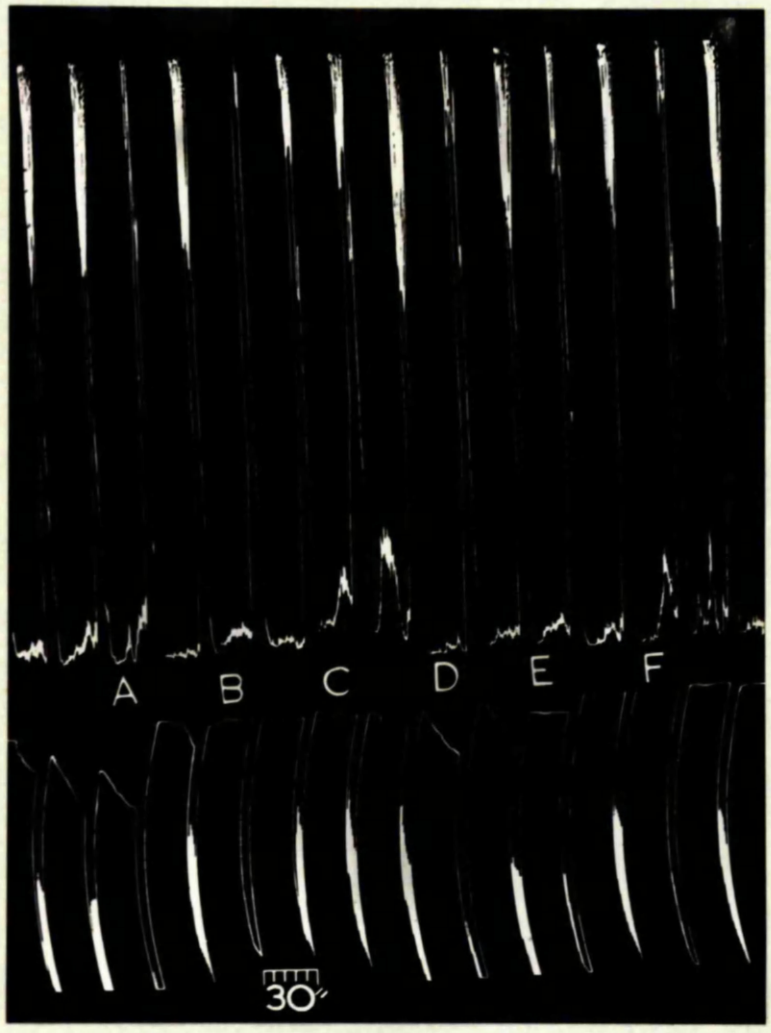


Fig. 42 Guinea pig ileum. The effects of drugs on contractions of the longitudinal muscle layers (upper trace) and on peristalsis (lower trace) recorded by Trendelenburg's method (1917). A 50 ml. organ bath was used.

At A, compound B6 0.125 mg.

At B, hexamethonium 0.25 mg.

At C, D, E and F, compound B10 0.25, 0.50, 0.75 and 1.0 mg.

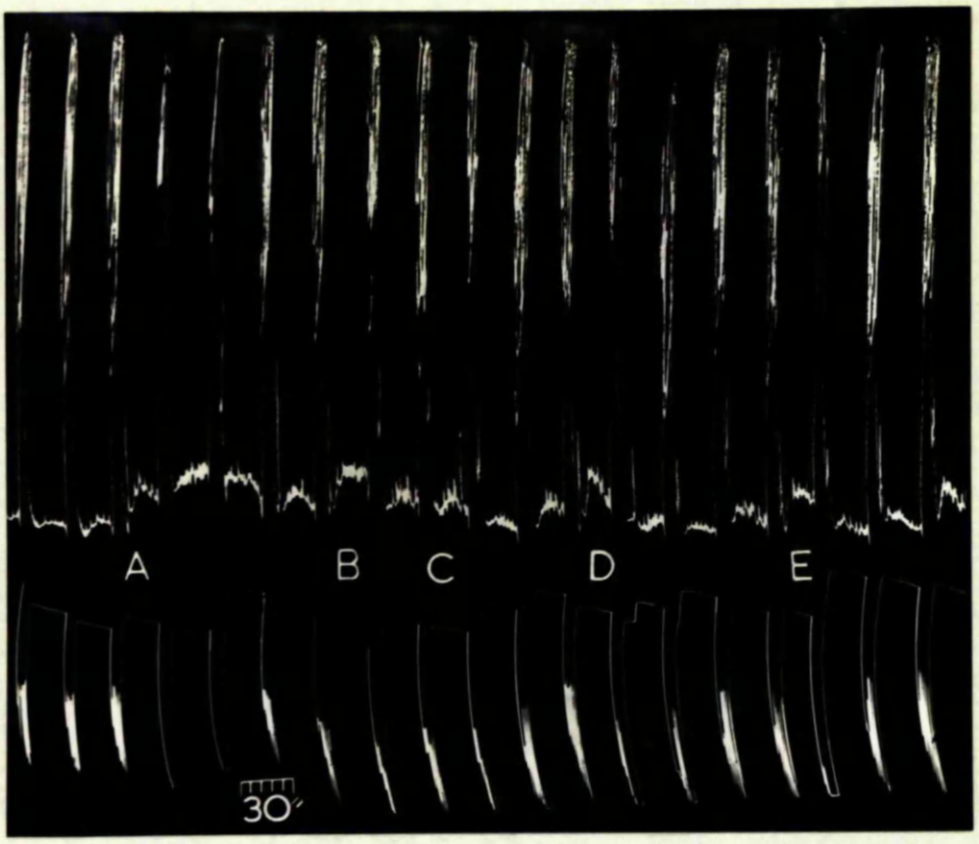


Fig. 41 Guinea pig ileum. The effects of drugs on contractions of the longitudinal muscle layers (upper trace) and on peristalsis (lower trace) recorded by Trendelenburg's method (1917). A 50 ml. organ bath was used.

At A, hexamethonium 0.5 mg.

At B, C, D and E, compound B1 0.5, 1.0, 2.0 and 3.0 mg.

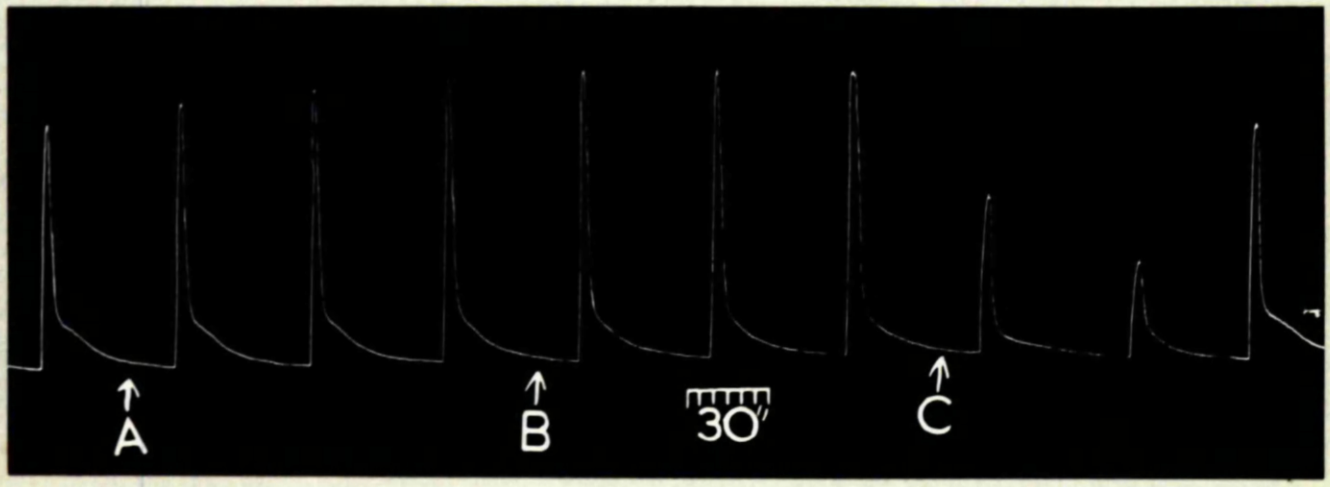


Fig. 40 Cat. Pentobarbitone anaesthesia. Contractions of the nictitating membrane elicited at 3 min intervals by preganglionic stimulation of the superior cervical nerve at a frequency of 1000 impulses/min, 8 V and 1.0 msec for 15 sec. Drugs administered intravenously 1 min before stimulation.

At A and B, compound B11 0.5 and 1.0 mg/kg respectively.

At C, d-tubocurarine 0.50 mg/kg.

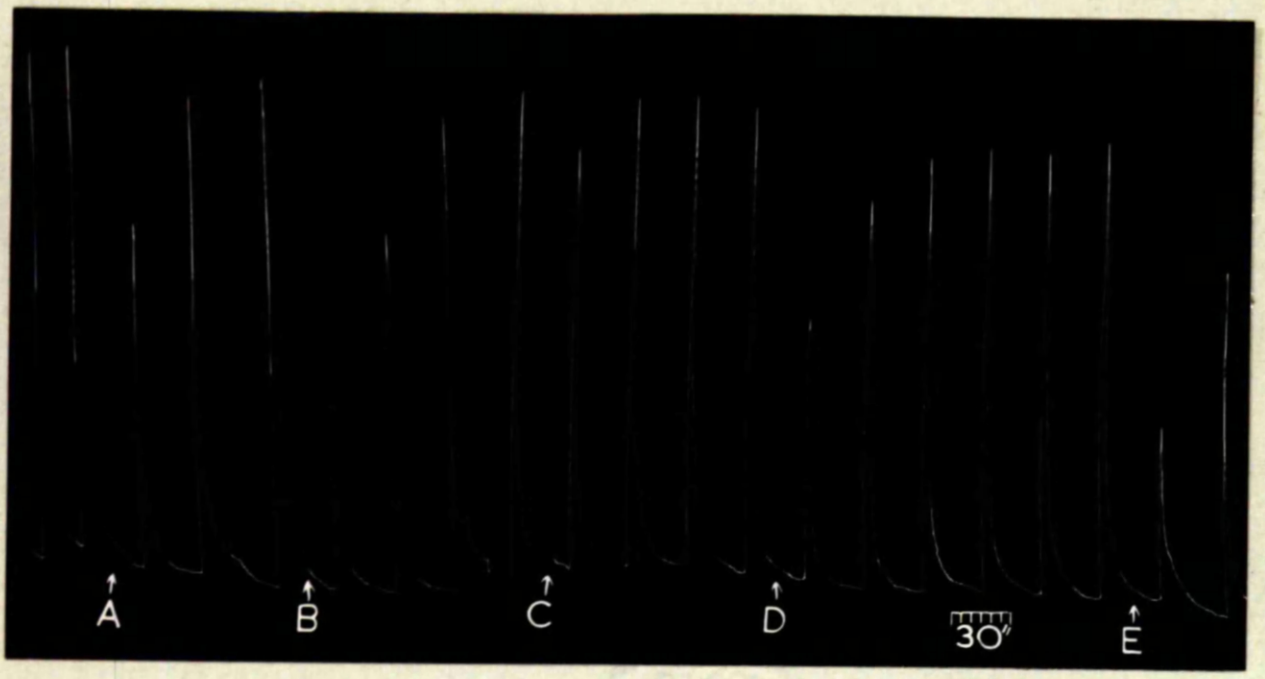


Fig. 39 Cat. Pentobarbitone anaesthesia.

Contraction of the nictitating membrane elicited at 3 min. intervals by preganglionic stimulation of the superior cervical nerve at a frequency of 1200 impulses/min, 8 V and 1.0 msec for 15 sec.

Drugs administered intravenously 1 min before stimulation.

At A and B, compound B10 4 and 8 mg/kg respectively.

At C and D, compound B1 2 and 4 mg/kg respectively.

At E, hexamethonium 0.5 mg/kg.

TABLE 12

A comparison of the Sympathetic Ganglion Blocking Activity of compounds B1 to B10 with tubocurarine.

COMPOUND	APPROXIMATE MOLAR POTENCY (TUBOCURARINE = 100)
B1	6.2
B2	4.7
B3	7.7
B4	3.2
B5	5.8
B6	8.0
B7	5.7
B8	3.9
B9	7.7
B10	6.5

8 mg/kg (Table 12). It seems likely, therefore, that the fall in blood pressure observed with the compounds can be attributed in some degree to sympathetic ganglion blockade.

Tubocurarine (0.50 mg/kg) and hexamethonium (0.50 mg/kg) also produced a 50% reduction in the height of contraction of the membrane (Figs.39 and 40).

Compound B11, which had no effect on blood pressure, produced no sympathetic ganglion blockade at doses 10 times that required to induce approximately 50% neuromuscular paralysis in the cat (Fig.40).

Guinea Pig Ileum. Parasympathetic Ganglion Blocking Activity.

All eleven compounds produced an inhibition of the peristaltic reflex at doses varying from 0.125 to 3 mg (Fig.41). Compound B6 (0.125 mg) possessed significant inhibitory activity on the Trendelenburg preparation and was twice as active as hexamethonium (0.250 mg)(Fig.42). Compounds B2, B3, B5, B7 and B9 also blocked peristaltic movements and were equipotent with hexamethonium in this respect.

EXPERIMENTS UPON MICE.

Estimation of the Approximate Median Paralyzing Dose (PD50).

Following intraperitoneal injection of the

TABLE 13

A comparison of the Potency and Toxicity of compounds B1 to B11 with tubocurarine in mice.

Compound	Approx. Mean Median Paralyzing Dose (PD50) mg/kg ± SEM	Approx. Mean Lethal Paralyzing Dose (LD50) mg/kg ± SEM	Therapeutic Index $\frac{LD50}{PD50}$	Approximate Molar Potency Tubocurarine = 100
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
B7 *	-	-	-	-
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 ± 2.53	59.5 ± 2.96	1.14	0.39
B11	1.22 ± 0.06	2.5 ± 0.22	2.05	23.3
dTc	0.30 ± 0.03	0.54 ± 0.03	1.78	100

* Insufficient material.

dTc = Tubocurarine.

compounds under test, the mice, after a brief period of excitability and restlessness, developed a typical flaccid paralysis and slid abruptly off the inclined screen. Recovery from paralysis was normally complete within 10 to 15 minutes. The inactivity of the monoquaternary compounds in inducing paralysis on this species was striking and the most potent compound, B2, was only approximately 1/200th as active as tubocurarine taken on a molar basis (Table 13). In contrast, the bisquaternary compound, B11, was much more potent than the monoquaternary derivatives being approximately one-quarter as active as tubocurarine on a molar basis.

Estimation of the Approximate Median Lethal Dose (LD50).

The median lethal dose was determined in a similar way to the median paralyzing dose. Intraperitoneal injection of a minimal lethal dose of the compounds caused rapid development of flaccid paralysis followed by respiratory failure. None of the compounds, except the bisquaternary steroid (B11), had a therapeutic index superior to that of tubocurarine (Table 13).

The results were calculated by the graphical method of Miller & Tainter (1944) and are shown in Table 13. The percentage paralyzed or killed in each group, at each dose level, was plotted on a logarithmic scale, as a probit

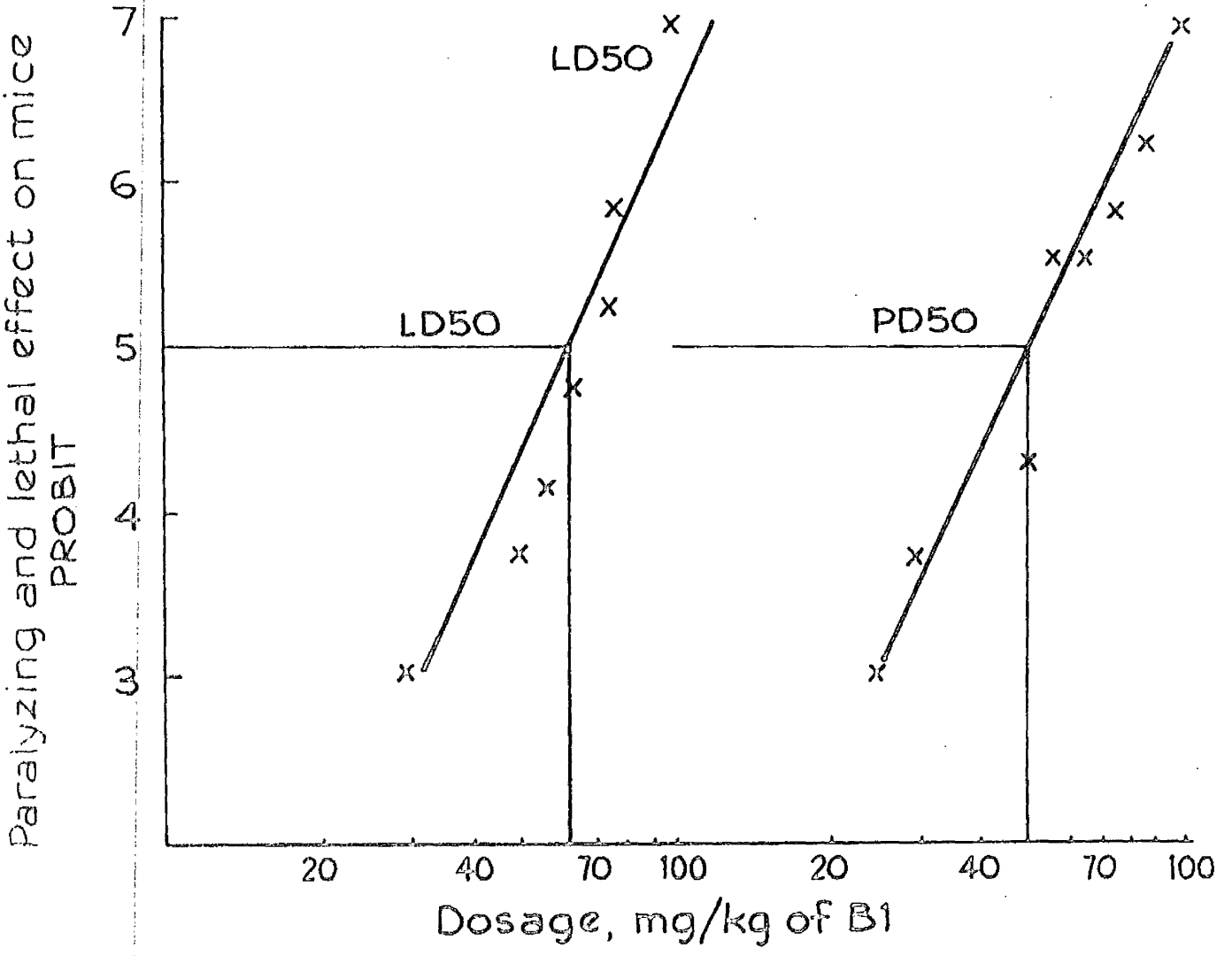


Fig. 43 Graph to show method of estimating LD50 and PD50 of compounds B1 to B11 (Miller & Tainter, 1944).

value against the dose (mg/kg) of drug producing the effect. The estimated PD50 was that dose corresponding to 50% (probit 5) and could be read directly from the graph in mg/kg. The median lethal dose (LD50) was determined in a similar fashion.

To estimate the standard error of the PD50 and LD50 the doses producing 16% and 84% (probits 4.0 and 6.0) of the effect were read from the graph (Fig.43). The difference between these values is the estimated increment necessary to increase the effects by two probits in this dose range (2S). The approximate average standard error of the mean (S.E.M.) was calculated from the formula, $S.E.M. = \frac{2S}{\sqrt{2N}}$.

The value N indicated the total number of animals in the groups which would be expected to show results between 6.7% and 93.3% (probits 3.50 and 6.50).

Experiments on the Respiration of the Anaesthetized Rabbit and Cat.

In view of the clinical importance of the action of muscle relaxants on the muscles controlling respiration, an investigation of the effect of the most potent monoquaternary compound, B1, and the potent bisquaternary compound, B11, upon respiration in the rabbit

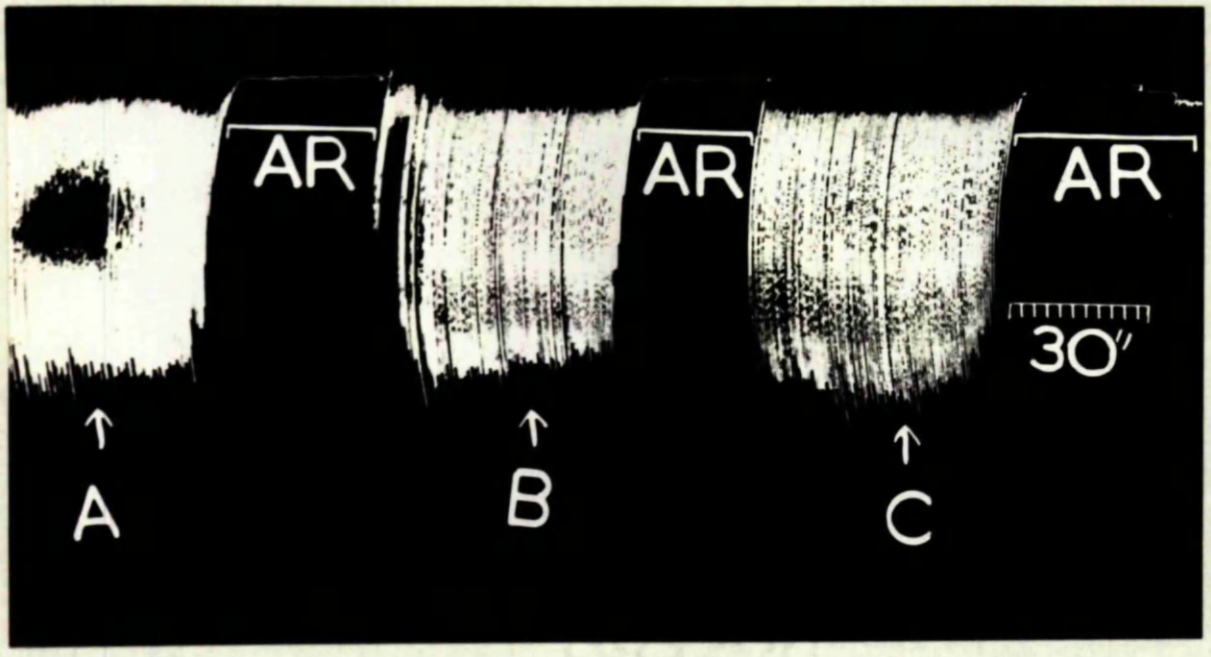


Fig. 45 Cat. Pentobarbitone anaesthesia. Record of respiratory movements using the method of Gaddum (1941).

At A, intravenous infusion of compound B11 0.093 mg/ml.

At B, intravenous infusion of d-tubocurarine 0.093 mg/ml.

At C, intravenous infusion of compound B1 1.86 mg/ml.

At AR, artificial respiration was given.

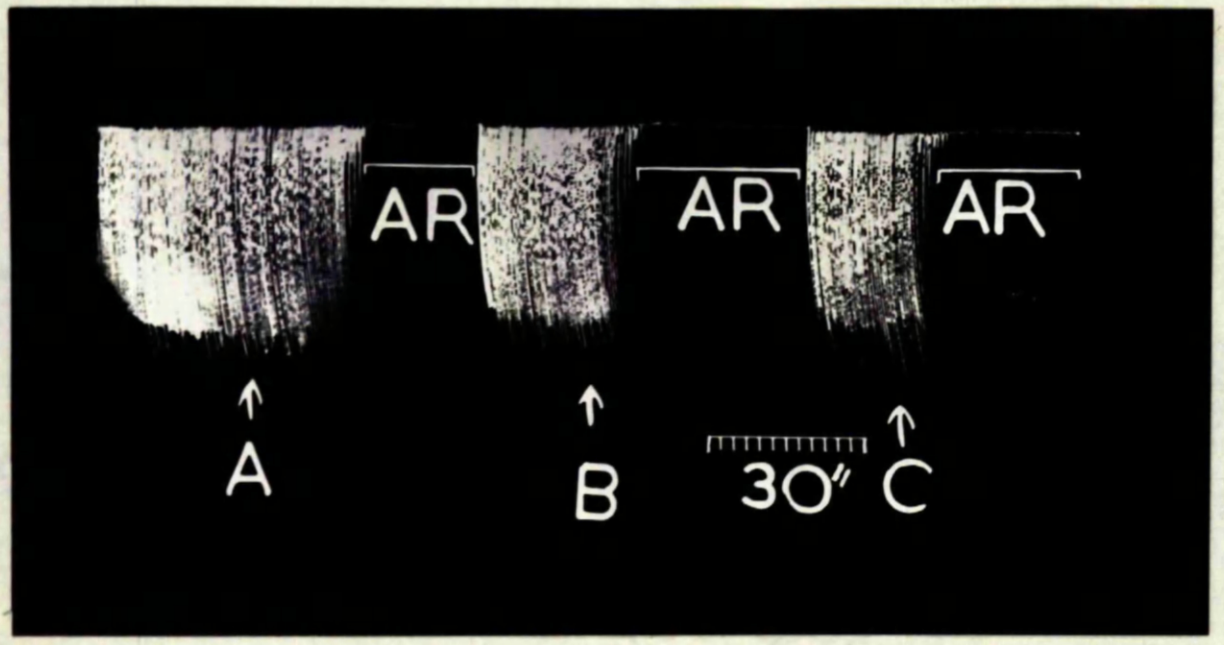


Fig. 44 Rabbit. Urethane anaesthesia. Recording of respiratory movements using the method of Gaddum (1941).

At A, intravenous infusion of compound B1 1.86 mg/ml.

At B, intravenous infusion of d-tubocurarine 0.093 mg/ml.

At C, intravenous infusion of compound B11 0.093 mg/ml.

At AR, artificial respiration was given.

TABLE 14

Individual and Mean Respiratory Paralyzing Doses in the Rabbit of compounds B1, B11 and tubocurarine.

Compound	Respiratory Paralyzing Dose		Molar Potency (Tubocurarine = 100)
	Individual mg/kg	Mean mg/kg	
B1	13.3, 14.3, 13.6	13.7	1.24
B11	0.22, 0.24, 0.24	0.23	102
dTc	0.23, 0.25, 0.30	0.26	100

Individual and Mean Respiratory Paralyzing Doses in the Cat of compounds B1, B11 and tubocurarine.

B1	2.17, 2.54 2.40	2.37	4
B11	0.18, 0.14, 0.16	0.160	98
dTc	0.17, 0.17 0.17	0.17	100

and cat were conducted.

Each drug was tested three times on different animals. The first dose administered which produced respiratory arrest was referred to as the respiratory paralyzing dose. Subsequent doses, less than the first, were not included.

The doses of compounds B1 and B11 required to produce respiratory paralysis in rabbits were similar to their head drop doses, compound B11 being approximately equipotent with tubocurarine while compound B1 was much less active (Table 14). Typical tracings are shown in Fig.44.

In cats, the doses of compounds B1 (2.37 mg/kg) and B11 (0.16 mg/kg) required to produce complete respiratory paralysis were less than those required to paralyze respiration in the rabbit (Table 14). Tracings of these results are shown in Fig.45.

Anticholinesterase Activity.

Measurement of anticholinesterase activity revealed that all compounds had PI50 values significantly less than that of eserine (Table 15). Compound B8 possessed the highest anticholinesterase activity of the compounds investigated, 4.29 compared to 6.54 for eserine. It seems unlikely, therefore, that the compounds can be exerting their action via inhibition of acetylcholinesterase.

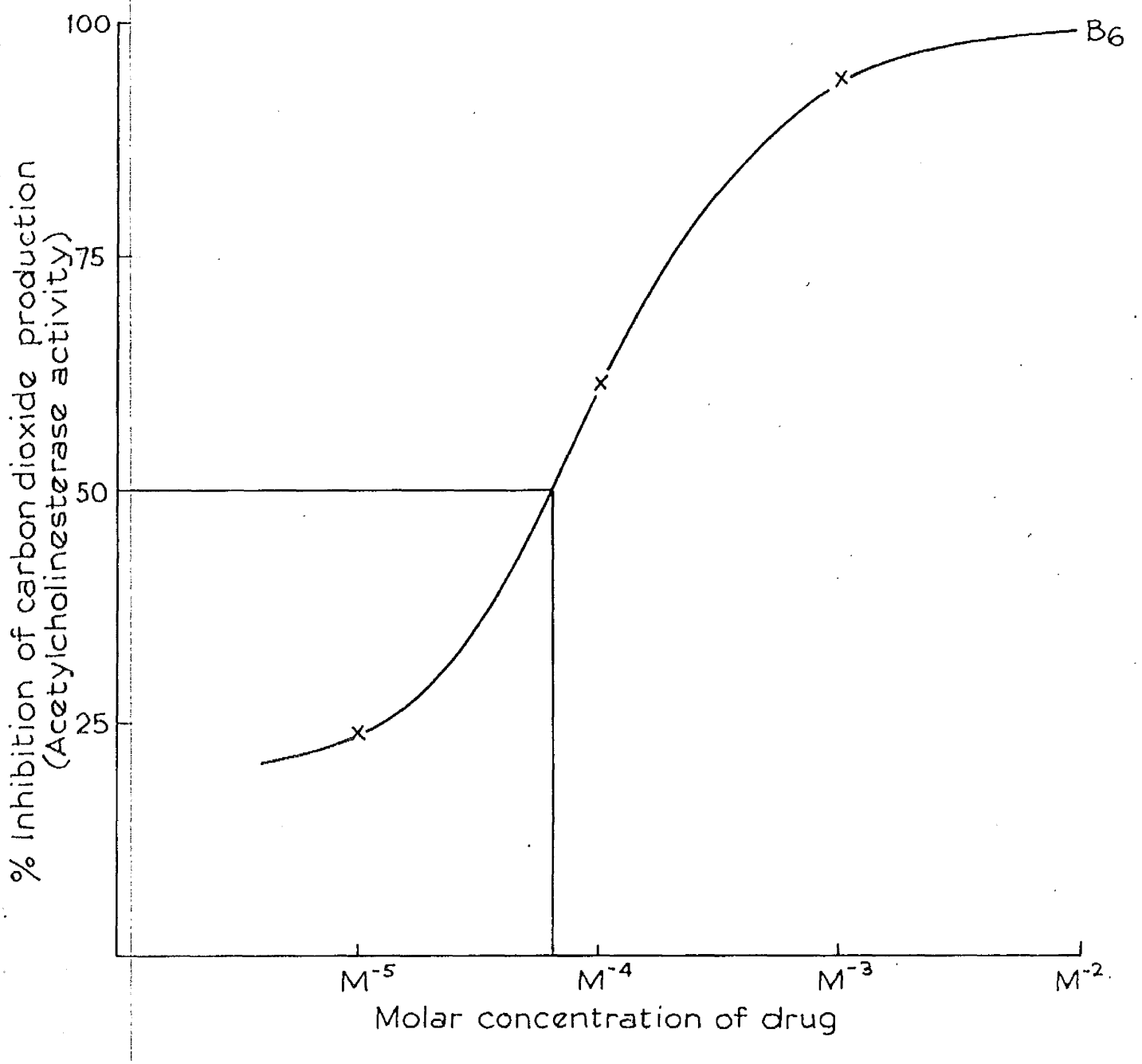


Fig. 46 Graph to show method of calculation of pI50 values for compounds B1 to B11 (Blaschko, Bülbring & Chou, 1949).

TABLE 15

The Anti-Cholinesterase Activity of compounds B1 to B11, eserine and tubocurarine.

Compound	PI 50 *
B1	3.98
B2	2.68
B3	3.28
B4	3.49
B5 **	-
B6	4.27
B7	4.22
B8	4.29
B9	3.48
B10	3.24
B11	3.13
dTc	2.58
eserine	6.54

*PI50 = The negative logarithm of the molar concentration of the drug producing a 50% inhibition of carbon dioxide production.

** Insufficient material.

An example of the method of calculation of the PI50 values is shown in Fig.46.

D I S C U S S I O N

c) DISCUSSION.

Each of the eleven compounds under investigation for which formulae are given in Fig.6 produced a relaxation of skeletal muscle and exhibited, on all preparations employed, typical non-depolarizing activity without any discernible depolarizing properties. Thus, in the cat, neuromuscular block was intensified by tubocurarine while 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 and edrophonium. Qualitatively, these results were supported by those obtained on the isolated frog rectus abdominis muscle. It is of some interest that depolarizing activity is absent in those monoquaternary steroids (compounds B1, B2, B3, B4, B6, B7, B8 and B10) incorporating the acetylcholine-like moiety and in the bisquaternary compound (B11), which is essentially decamethonium-like in so far as it consists solely of a hydrocarbon residue bearing two ammonium functions. This might suggest that the considerable bulk of the steroid nucleus is preventing the quaternary heads from fitting the anionic sites sufficiently well to disrupt the water molecules on the membrane of the receptor, in the manner postulated by Belleau (1964), to initiate ion transport and

depolarization of the muscle membrane. Instead, all compounds would appear to be forming complexes involving non-specific conformational perturbations in the receptor. In terms of the theories of Ariëns (1964) and Paton (1961), the compounds under investigation could also be visualized as possessing a low intrinsic activity or a low rate of dissociation from the receptor respectively.

Apart from the above indications of the non-depolarizing nature of the block, measurement of anticholinesterase activity, using enzyme prepared from rat brain, revealed that the most active of the steroids under test (compounds B6, B7 and B8) were more than 100 times less potent than eserine. It seems unlikely, in view of this considerable variation in potency, that the compounds under investigation can be exerting their actions via inhibition of acetylcholinesterase.

In both the cat and the hen, the time taken to obtain maximum 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 activity observed in the hen was in general comparable to that in the cat. There was, however, the anticipated variation in potency in other species. Thus, in mice, employing the inclined screen method to determine the paralysing dose, all the compounds showed

a PD50 of 10 to 15 times the dosage required to produce paralysis 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 5 (compound B4) to 90 times (compound B2) as great as it was in the cat.

In addition to their neuromuscular blocking activity, the ten monoquaternary compounds also caused inhibition of both sympathetic and parasympathetic ganglia, as might be expected of monoquaternary ammonium salts (Cavallito & Gray, 1960). The degree of sympathetic ganglion blockade in the cat in all cases was more pronounced than that of neuromuscular blockade in the same species on a mg/kg basis. In contrast to the monoquaternary compounds, the single bisquaternary ammonium steroid tested did not appear to possess sympathetic ganglion blocking activity even in doses 10 times that required to produce approximately 50% neuromuscular paralysis in the cat.

With the exception of the potency of compounds B2 and B7 on the frog rectus abdominis muscle preparation, 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 so similar in chemical constitution it is tempting to ascribe the variation in potency to steric effects about the nitrogen atom, although variation in the charge density of the nitrogen can not be overlooked. Replacement of a pyrrolidino ring by a piperidino ring in a molecule of this size would not be expected to produce a marked change in hydrophilic to lipophilic balance - a factor well known to influence neuromuscular blocking activity (Cavallito, 1959). Additional evidence that changes in potency in the monoquaternary steroids could indeed be directly attributed to variations in the nitrogen substituents is provided by results obtained with the 3α,17α- bisquaternary ammonium androstanes. Here, on going from the bistrimethylammonium compound to the bisdiethylmethylammonium compound, non-depolarizing potency increased only to fall off again in the bistriethylammonium compound (Alauddin et al. 1965). Since the relative rigidity of the steroid ring system would not be expected to permit any marked changes in the interonium distance with change of substituents on the nitrogen atoms, as is known to occur in the flexible polymethylene bisquaternary ammonium salts (Elworthy, 1964), the change in potency could be due to variations in the

nitrogen substituents, as appears to be the case for the monoquaternary compounds.

It is of interest that compound B3 (the reversed analogue of compound B1), in which the acetate function is on position 2 of the steroid nucleus and the nitrogen function is on position 3, is only 1/4th as active as compound B1. This again serves to emphasize the importance in the present series of steric factors rather than hydrophilic to lipophilic balance.

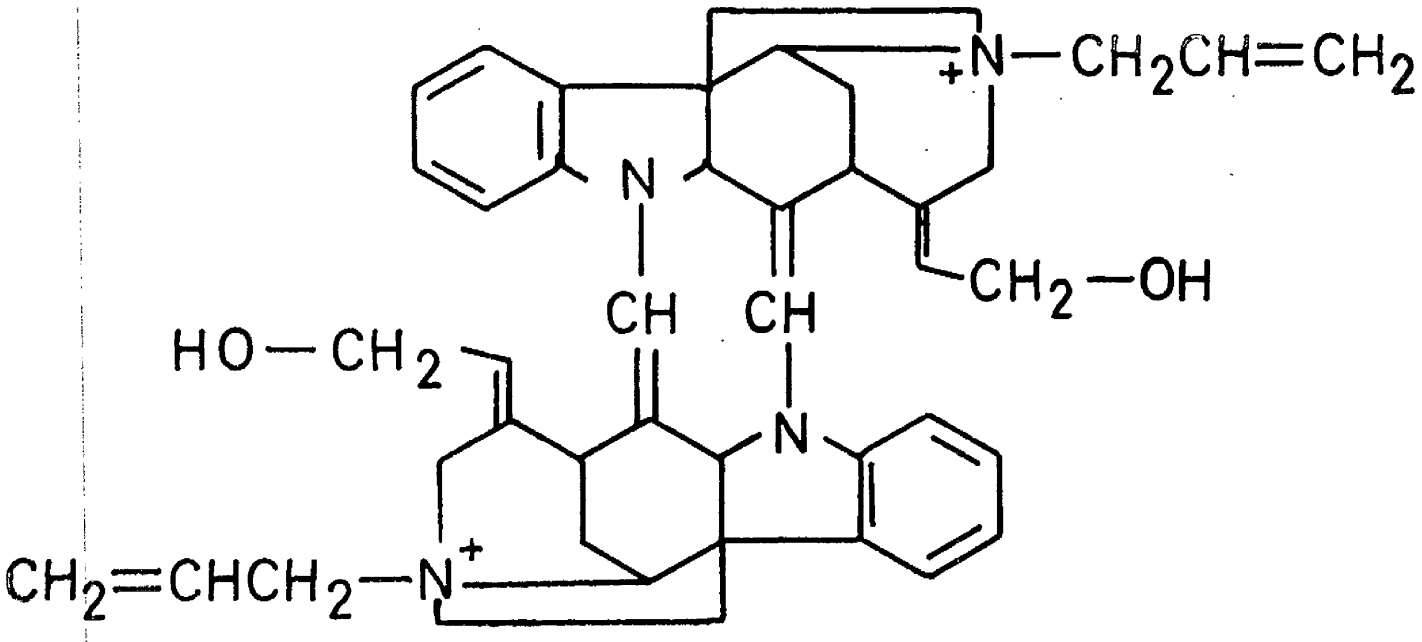
The one bisquaternary steroid under examination showed activity comparable to or greater than that of tubocurarine in all species, except the mouse. The potency of this compound which has an interonium distance of 9.3 - 9.7 Å according to the conformation adopted by ring A, thus differing from the 3β,17β - bisquaternary ammonium androstane derivatives which have interonium distances of from 10.5 - 11.2 Å (Biggs, Davis & Wein, 1964) provides further evidence that the original two-point attachment theory of neuromuscular blockade (Paton & Zaimis, 1949, 1951), involving anionic receptor sites separated by ca 14 Å, needs modification. A further fact incompatible with the original two-point attachment hypothesis is the existence of activity comparable to that of tubocurarine in 3α,17β - and 3β,17α - bis-quaternary ammonium androstanes where the quaternary

heads lie on opposite sides of the steroid nucleus (May & Baker, 1963, 1965; Biggs et al.1964). It would appear, therefore, that emphasis must be placed on factors such as accessibility to the site of action, lipophilic to hydrophilic balance, distribution between sites of action and sites of loss and probably other factors (Cavallito, 1959; Cavallito & Gray, 1960) as determinants of neuromuscular blocking activity. In addition, the high activity of the bisquaternary steroid (B11), in which both quaternary ammonium functions are in the β - configuration, makes it clear that the angular β - methyl groups on C-10 and C-13 of the steroid nucleus are not interfering with receptor interaction, as was also observed for the malouétine series (Khuong Huu-Lainé & Pinto-Scognamiglio, 1964) and for the 3β,17β-, 3α, 17β - and 3β,17α - bisquaternary ammonium androstanes (May & Baker, 1963, 1965; Biggs et al. 1964).

The present results with the ten monoquaternary compounds and the single bisquaternary steroid fully confirm the well-established fact (Cavallito & Gray, 1960) that bisquaternary compounds are more potent neuromuscular blocking agents than their related monoquaternary derivatives. If this is not due to an increased bonding to the receptor made possible by a 2-point attachment as the evidence just discussed would suggest, certainly the

addition of a second hydrophilic quaternary ammonium function to a monoquaternary compound would be expected to alter the hydrophilic to lipophilic balance of the molecule. Thus, the enhanced potency of bisquaternary compounds may be due in some measure to the molecules having attained an optimum compromise between lipophilic and hydrophilic bonding functions.

The need for at least two onium centres in the one molecule for the appearance of appreciable neuromuscular blocking activity also serves to place emphasis on the adumbration theory with its postulate of a one-point attachment in which the onium group not attached to the receptor repels incoming acetylcholine molecules (Loewe & Harvey, 1952). Certainly, as already discussed, the presence of high activity in the 3α,17β- and 3β, 17α- bisquaternary ammonium androstanes (May & Baker, 1963, 1965; Biggs et al. 1964) where the quaternary heads lie on opposite sides of the steroid nucleus make it difficult to envisage a true two-point attachment to the receptor. There is still, of course, the possibility of Waser's pore theory (Waser,1959,1962) being substantially correct, since a molecule of either the 3α,17β- compound or the 3β,17α- compound could slip inside the pore with one onium group attaching itself higher on the rim of the pore than the other.



DIALLYLNORTOXIFERINE

POSSIBLE CLINICAL APPLICATION OF THE COMPOUNDS INVESTIGATED.

The value of the three well established muscle relaxants in clinical use, namely tubocurarine, gallamine and suxamethonium as adjuncts to surgery and anaesthesia (Bowman, 1962) is somewhat marred by the fact that each of the three may produce undesirable side effects. Tubocurarine can cause both histamine release (Sniper, 1952) and ganglion blockade (Ottolenghi, 1959; Foldes, 1960), gallamine may induce tachycardia (Riker & Wescoe, 1951) while the muscle pain and irreversible nature of the block produced by suxamethonium and other depolarizing drugs are disadvantages to their use (Foldes, 1960). Hence the recent advent of diallylnortoxiferine which, in both pharmacological (Lissac, Herenberg, Vallois, Pocidalo & Liot, 1963; Bächtold, Fornasari & Hürlimann, 1964) and clinical studies (Waser & Harbeck, 1962; Lund & Stovner, 1962; Foldes, Brown, Lunn, Moore & Duncalf, 1963), has been shown to be a potent, easily reversible, short-acting muscle relaxant of the non-depolarizing type with high specificity and free from side effects.

All ten monoquaternary compounds blocked synaptic transmission in both sympathetic and parasympathetic ganglia. Possession of this property, together with their weak neuromuscular blocking activity in a number of species makes it very unlikely that these compounds would

be of any clinical value.

The bisquaternary compound (B11), on the other hand, did not appear to possess any marked ganglion blocking activity and its lack of a hypotensive effect suggested the absence of histamine-liberating properties. In addition, in both cat and hen, neuromuscular blocking potency was similar to that of tubocurarine while the time of onset of maximum paralysis and the duration of block was significantly less than for tubocurarine. Furthermore, the compound was no more potent than tubocurarine in inducing paralysis of the respiratory muscles of the cat and the rabbit. Thus, while great caution must be exercized in attempting to assess the potential clinical value of a compound from results obtained on animal preparations, as already indicated, the apparent absence of any serious side effects and the potent, short-acting, non-depolarizing nature of the block makes it possible that this steroidal bisquaternary compound, or perhaps a structurally related derivative, may be of some clinical use.

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APPENDIX

SECTION A. EFFECTS OF NITROGENOUS STEROIDS ON THE CENTRAL NERVOUS SYSTEM.

The code numbers and chemical formulae of the compounds which were investigated for central nervous system activity are shown in the table below. Their structural formulae are shown in Table I (opposite page 47).

Code Number	Chemical Formula
A1	3α-Morpholino-5α-androstan-2β-ol-17-one, 2β acetate.
A2	3α-Piperidino-5α-androstan-2β-ol-17-one.
A3	3α-Piperidino-5α-androstan-2β-ol-17-one, 2β acetate.
A4	3α-Morpholino-5α-androstan-2β-ol-17-one.
A5	2β-Morpholino-16α-methyl-5α-pregnane-3α,11β-diol-20-one.
A6	2β-Morpholino-16α-ethyl-5α-pregnan-3α-ol-20-one.
A7	2ε-Morpholino-5α-androstane-3,17-dione.
A8	3α-Morpholino-5α-pregnan-2β-ol-20-one.
A9	3α-Piperidino-5α-androstan-2β-ol-17-one, 2β succinate.
A10	3α-Dimethylamino-5α-androstan-2β-ol-17-one.

Code Number	Chemical Formula
A11	3α-Dimethylamino-5α-androstan-2β-ol-17-one, 2β acetate.
A12	2β-Morpholino-3β-methyl-5α-androstan-3α-ol-17-one.
A13	3α-Pyrrolidino-5α-androstane-2β,17β-diol, 2β acetate.
A14	3α-Pyrrolidino-5α-androstan-2β-ol-17-one, 2β acetate.
A15	3α-Pyrrolidino-5α-androstan-2β-ol-17-one.
A16	3α-Piperidino-5α-androstane-2β,17β-diol, 2β,17β diacetate.
A17	2β-Morpholino-5α-pregnane-3α,20β-diol.
A18	6α-Piperidino-androstan-3β,5β-diol-17-one.
A19	3α-Morpholino-5α-androstan-2β-ol-17-one, 2β succinate.
A20	3α-Morpholino-5α-androstane-2β,17β-diol, 2β acetate.
A21	3α-Pyrrolidino-5α-androstane-2β,17β-diol, 2β,17β diacetate.
A22	2β-Piperidino-5α-androstan-3α-ol-17-one, 3α succinate.

Code Number	Chemical Formula
A23	2β-Piperidino-5α-pregnan-3α-ol-20-one, 3α succinate.
A24	2β-Piperidino-5α-pregnan-3α-ol-20-one.
A25	17β-Morpholino-5α-androstane-3β,16β-diol.
A26	2β-Morpholino-17α-ethinyl-5α-androstan-3α,17β-diol.

SECTION B. EFFECTS OF NITROGENOUS STEROIDS AT THE NEUROMUSCULAR JUNCTION

The code numbers and chemical formulae of the steroids which were tested for neuromuscular blocking activity are shown in the table below. Their structural formulae are shown in Table 6 (opposite page 109).

Code Number	Chemical Formula
B1	2β-Piperidino-5α-androstan-3α-ol-17-one, 3α acetate methobromide.
B2	3α-Piperidino-5α-androstane-2β,17β-diol, 2β,17β diacetate methobromide.
B3	3α-Piperidino-5α-androstan-2β-ol-17-one, 2β acetate methobromide.
B4	N-(3α-Acetoxy-17-oxo-5α-androstan-2β-yl)-trimethyl ammonium hydroxide.

Code Number	Chemical Formula
B5	2β-Dimethylamino-16α-methyl-5α-pregnan-3α-ol-20-one methobromide.
B6	N-Methyl-N-(2β,17β-diacetoxy-17-oxo-5α-androstan-3α-yl)-piperidinium hydroxide.
B7	N-Methyl-N-(2β-acetoxy-17-oxo-5α-androstan-3α-yl)-piperidinium hydroxide.
B8	2β-Pyrrolidino-5α-androstan-3α-ol-17-one, 3α acetate methobromide.
B9	2β-Piperidino-5α-pregnan-3α-ol-20-one methobromide.
B10	2β-Pyrrolidino-5α-pregnan-3α-ol-20-one, 3α acetate methobromide.
B11	2β,16β-Dipiperidino-5α-androstane-3α, 17β-diol, 3α,17β diacetate dimethobromide.