

PHYTOCHEMICAL AND PHARMACOLOGICAL STUDIES OF THE INDIAN DIOSCOREA HISPIDA

THESIS SUBMITTED TO
UNIVERSITY OF BOMBAY

FOR THE DEGREE OF
MASTER OF PHARMACY
IN
PHARMACOLOGY

SUBMITTED BY
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UNDER THE GUIDANCE OF
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DEPARTMENT OF PHARMACOLOGY
HAFFKINE INSTITUTE FOR TRAINING, RESEARCH & TESTING
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**A
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FACULTY OF TECHNOLOGY
In Pharmacology

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S e c t i o n I

I N T R O D U C T I O N

Dioscoreaceae consists of nine genera and over 650 species, of which about 600 species belong to cosmopolitan genus *dioscorea*. About 50 species are found in India¹; a large number among them occur in the wild state, a few species of them are popularly known as Yams for their edible large tubers. Some species of *Dioscorea* viz., *D. alata* Linn, *D. bulbifera* are cultivated for their excellent edible quality².

A few species of *Dioscorea* have attracted attention of the pharmaceutical industries on account of their steroidal sapogenin, diosgenin. Diosgenin is a starting material for the manufacture of cortisone and steroidal sex hormones. In India, diosgenin is mainly extracted from the wild species of *Dioscoreas* commonly *D. deltoidea* and *D. prazert*³. These are now in short supply because of gradual depletion. With increase in demand serious attempts have been made to cultivate them in India which has not been proved economic so far. The recent introduction of the Mexican species particularly *D. floribunda* and *D. composita* have done well in Bangalore and Assam, their yield has been reported to be poor in the North⁴.

The Yams of several species are soft, fleshy and edible. Tubers of some species contain tannins, biologically

active alkaloids which render them unpalatable or poisonous⁵. Dioscorea hispida is one of them. This species is a native of tropical Asia and occurs in the rain forest region from India to Formosa, Philippine Islands and New Guinea. It is found nearly throughout India, upto an attitude of 4,000 ft. in Sikkim, Himalayas and Khasi hills. It is rarely cultivated. About five varieties are recognised of which variety *daemona* occurs in India⁶.

The tubers of D. hispida form the chief famine food in the East in the uncultivated tract of Assam, Bihar, Bengal, Madhya Pradesh, Orissa and Deccan on account of their large yields, their growth of the surface of the soil and comparatively abundance; but considerable care is needed in preparing them for food. Those of its close ally D. dumetorum are a famine food in Africa, but selection in Africa has produced improved varieties; whereas no one has found the least sign of selection having been brought to bear by man on D. hispida.

As an indication of its poisonous nature, there is some use for it in Western India a Sanskrit name - "Marapashpoli" or deadly strangle cake and in those parts the Bhils poison tigers by placing pounded tubers in the carcasses of "Kills".

Dymock, Warden and Hooper⁷ pointed out that the tubers of D. hispida were so poisonous that a piece of an apple would poison a man death ensuing in six hours. They found out that it caused intense irritation in the mouth and throat, also a sense of suffocation followed by drowsiness and finally exhaustion. Others recorded that a poisoned man might become violent in the first stage and later died.

Greshoff⁸ stated that it was used in Java for stupefying fish.

In Bali, it was said that, thieves sometimes stupefied fowls with it, as a preliminary to stealing them.

In the Malay Peninsula it was quoted that, the tubers might sometimes be used for poisoning darts not as a principle poison but as an adjunct. The milky juice of the tubers used by Lepchas of Sikkim along with the juice of *Antiaris toxicaria* Lesch, as an arrow poison⁹.

Gimlette¹⁰ gave an account of criminal poisoning by its use, and in India liquor sellers have been known to be guilty of adding the juice of the tubers to the other liquor to produce thirst.

In preparing the tubers for food it is necessary to get rid of the poison, while preserving the starch etc. The people of the Bali roast the tuber and then remove its skin, next they smother it in ashes and leave it so for twenty four hours, at the end of this period they wash and crush it, using baskets in which it is submerged in a sea water for two days and two nights under a weight of stones; a washing in fresh water follows and subsequently a drying in the sun, The washing and drying may be repeated not one but twice until it is considered that the poison has been removed. To ascertain this a little of the pulp is held close to a hot fire, if it dries quietly it is considered safe for use; but if it writhes in drying it is not yet safe to use it. When the preparer is in doubt, he offers some of the preparation to a fowl and observes the effect after eating it on the fowl. If the fowl shows signs of dizziness the purification should be carried still further. Such a great care they take in preparing them for food as they are acrid and poisonous and when consumed in large quantities cause paralysis of the respiratory organ and even death.

The people of Amboina detoxify the tubers by kneading them with their hands and feet in sea water, and letting the pulp lie in small baskets under stones in the sea for two to three days and afterwards in fresh water

for another two days.

The tubers were resorted to by the people of Ceram, Celebes, the Suhe Islands and Bali. The use is made of them almost wherever the plant grows. It is used in Philippine islands, Borneo; Vorderman and Kilian have given some interesting information as to way in which the Madoerese fall back upon it when in need¹¹.

Ridley^{12,13} has mentioned its use in the Malay Peninsula that it is more or less cultivated about village. It is very commonly used in Siam, perhaps even in normal times, and during the great shortage of rice. It was abundantly dug in the East in 1917-1918. Every Burman knows that it can be turned to use.

In the Eastern Himalaya, the Lepchas of Sikkim use the tubers after seven days soaking in the running stream and subsequent cooking. It is also used in Gorakhpur District and State of Balrampur where the population is underdeveloped.

Mr. A. Smythies stated that enormous quantities were dug in the forest of Chanda District in the famine year, 1899-1900 and in the lean year forest having been

thrown upon for this purpose. All the hill tribes of India a little to the North of Chanda use it.- Korkus in the Melghat, Kathodis in the Bombay Ghats, the tribes of Chhota Nagpur and the Sontal, etc. In Chhota Nagpur, high caste folk when fasting is prescribed by their religion eat a meal made from it after removing the poisonous principles.

Dekkar¹⁴ showed that poison was distributed throughout the plant. In 1894, Boorsma¹⁵ assigned the poisonous properties to two alkaloids - Dioscorine and Dioscortine obtained from D.hirsuta blume . Schutte¹⁶ obtained the base in a crystalline form and mentioned that these two alkaloids were one and the same and he named it as Dioscorine. His work was criticised and it was advised to be repeated. On the basis of degradation experiments, Gorter¹⁷ suggested that the alkaloid belongs to tropane group. Leyva and Gutierrez¹⁸ isolated from the tubers of D.hispida, an alkaloid which gave the same colour reactions and possessed same toxicological properties towards mice and monkeys as dioscorine. It is generally considered that D.hirsuta, Blume and D.hispida Dennst, are synonymous. Pinder¹⁹ has repeated the work with slight modifications. He investigated the chemical and pharmacological properties related to the constitution of the alkaloid from D.hispida

and recorded that the alkaloid is identical with Dioscorine.

Schutte reported that Dioscorine produced paralysis of central nervous system and in general behave like picrotoxin. Gorter confirmed this noting that large doses were required. Leyva and Gutierrez noted similar toxicological effects with the alkaloid of D. hispida with addition of mydriatic effect, resembling that of atropine. There was therefore, no pharmacological data to indicate that the alkaloid of D. hispida investigated by Pinder was different from that Dioscorine investigated earlier.

The production of industrial starch and edible flour has been recently investigated. A clean white starch is easily prepared.

Such an inestimable value during the period of scarcity as a food, a great care is needed during its preparation and a huge demand of steroidal sapogenin and starch by the pharmaceutical industries have developed the interest in searching some more toxic principles having medicinal usefulness with the ancillary aim that these compounds may become a by-product of the above industries.

The bias among the former investigators regarding Dioscorine and its use as a arrow poison supported the interest with the impression that the tubers may contain some more toxic constituents having neuromuscular blocking activity.

S e c t i o n I I

L I T E R A T U R E R E V I E W

Anatomy and Physiology of Neuromuscular Junction :

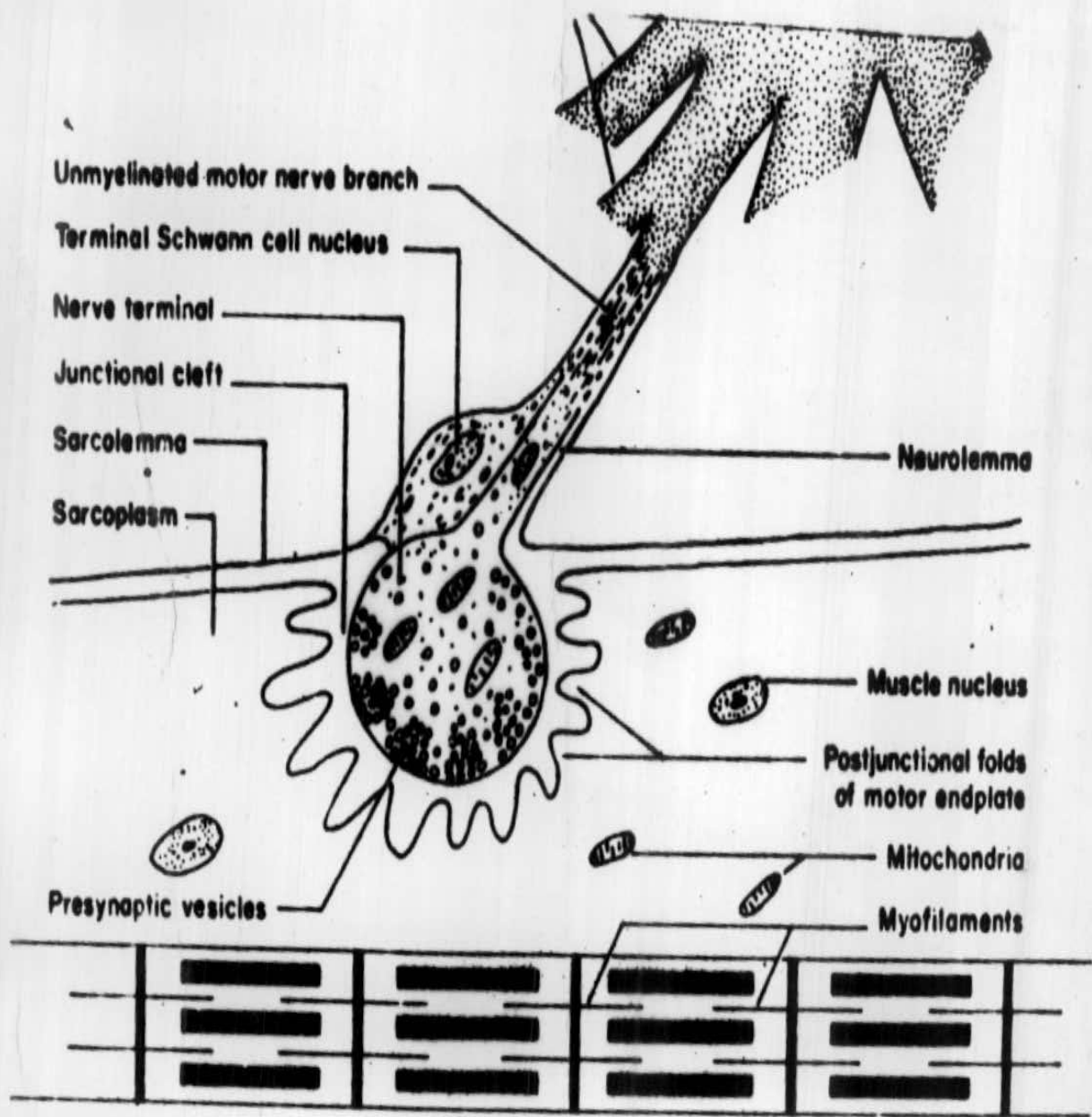
Neuromuscular conduction essentially constitutes a part of voluntary, efferent, peripheral nervous system. It involves conduction along a motor nerve and skeletal muscles adjoining it.

Anatomically there are two types of skeletal muscles.

(1) Multiple innervated skeletal muscles : These are characterized by irregularly shaped areas of sarcoplasm containing relatively small number of large and poorly defined myofibrils. Each muscle fibre receives along its length, many small nerve endings emanating from each of a number of different nerves and junctions having the appearance of a dense series of small grape-like bodies.

(2) Focally innervated skeletal muscles : These have uniformly distributed myofibrils. Each fibre is innervated either by a single nerve ending or by a strictly limited number of nerve endings. It possesses a discrete number of end-plates.

Generally multiple innervated fibres tend to produce a slow, graded contraction while focally innervated fibres



NEUROMUSCULAR JUNCTION

tend to produce a rapid all or none twitch response.

As the motor nerve axon approaches the bundle of muscle fibres, which it innervates, it undergoes extensive ramification and the high degree of myelination, provided by encircling Schwann cells disappears. Each branch of motor nerve proceeds to a muscle fibre with one motor nerve innervating 100 or so fibres. At innervation point the ultimate ramification of the nerve fibre penetrates the outer membrane of sarcolemma of the muscle fibre, while the terminal Schwann cell fuses with sarcolemma so that continuity is achieved between Schwann cell membrane or neurolemma and the sarcolemma, thus encapsulating the neuromuscular junction. The nerve-fibre itself terminates in a bulb-shaped extension fitting into an accommodating indentation of muscle fibre which is thrown into a number of folds and it is the specialized region of the muscle fibre membrane, which constitutes, muscle end-plate. There is no protoplasmic continuity between the nerve terminal and muscle fibre and the well defined space of approximately 500 to 1000 Å is termed as junctional cleft.

It is an established fact that the nerve-muscle transmission is exhibited via a neurohumoral transmitter acetylcholine. Consequently there is an organized system

for biosynthesis, release and destruction of acetylcholine. Acetylcholine is manufactured in motor nerve terminal cytoplasm from acetyl-coenzyme A of mitochondrial origin and choline derived from extracellular fluid by an active membrane mechanism. A cytoplasmic enzyme choline-o-acetyl transferase accelerates the synthesis and a little understood mechanisms load acetylcholine into synaptic vesicles. The vesicles are derived from the cell body by transport along the motor nerve. The vesicles fuse with the terminal membrane and empty their contents into the synaptic cleft. The vesicle membrane is then incorporated into the terminal membrane and latter recovered as a complex vesicle, which in turn sheds its coat to reveal a synaptic vesicle, refilled by acetylcholine.

An energy consuming process (the sodium pump) extrudes sodium ions accross the axolemma so that the concentration of these ions in the axoplasm is lower than outside. The freely diffusable ions distribute themselves on the two sides of the membrane in such a way that the concentration of potassium ions in the axoplasm is greater than that in the surrounding extracellular fluid. An opposite distribution is established for the chloride ions. Diffusible ions disposed in this way would tend to diffuse along their concentration gradients but this tendency is

offset by the fact that positively charged ions align themselves immediately outside the axolemma and negative ions take up a corresponding position on the inside of the membrane. The potential difference thus established is just sufficient to prevent the diffusion of the ions.

It should be clear that the size of the membrane potential is determined by the extent of the disparity between the concentrations of potassium (or chloride) on the two sides of the axolemma.

In mammalian nerves the resting potential lies between 60 and 90 millivolts. The resting potential is preserved until the nerve is stimulated.

Acetylcholine, released after nerve stimulation is believed to diffuse accross synaptic cleft.

It is assumed that the first step in reaction of acetylcholine with the sensitive surface of muscle end-plate is formation of a reversible complex with the receptor. In focally innervated muscle, the following sequences follow :-

1) Potential difference between inside and outside of the resting end-plate membrane, depending on selective permeability to K^+ and Cl^- is reduced. This produces a graded nonpropagated potential difference between outer surface of end-plate and rest of muscle membrane, constituting end-plate potential.

2) At a critical threshold value the end-plate potential induces local circuit currents of high density to flow in adjacent areas of the muscle fibre membrane serving to increase permeability to Na^+ .

3) Na^+ pass through the muscle fibre membrane along their concentration gradient to reverse the resting potential giving rise to self propagating "all or none" action potential, which triggers the contractile mechanism to bring about contraction.

4) During the refractory period (2 m. sec.) acetylcholine is hydrolysed, thus permitting repolarisation of the muscle end-plate. The remainder of the muscle fibre gets repolarised due to efflux of K^+ down their concentration gradient and extrusion of sodium ions via increased activity of "sodium pump" mechanism.

5) As the action potential traverses the membrane, there is rapid release of Ca^{++} from internal stores within the muscle and these ions pass into the immediate environment of the two contractile proteins, actin and myosin, the interaction resulting in shortening in muscle fibre. When the action potential has passed away, Ca^{++} ions are removed from the environment of actin and myosin and are once more sequestered in the store. As a result the contractile proteins become inactivated and the muscle returns to rest.

Though all conducting membranes are sensitive to acetylcholine, the degree of sensitivity of different membranes varies considerably. It is clear that the acetylcholine sensitive areas are confined to outer surface of end-plate.

The various locations of acetylcholine, biosynthesis, release and receptor occupation constitute sites of actions of drugs, active in blocking the neuromuscular conduction. The prime class under which these drugs could be classified are -

1. Presynaptic blockers.
2. Postsynaptic blockers.

The agents which act on the neuromuscular junction leading to interferences of events, before access of acetylcholine to its receptors constitute presynaptic neuromuscular blockers such a drug viz. presynaptic blocker finally reduces the amount of acetylcholine liberated after nerve stimulation. A presynaptic blockers, thus can be -

- 1) an acetylcholine discharge blocker e.g.
-Bungarotoxin,
- 2) acetylcholine synthetic inhibitor e.g. styrylpyridine derivatives,
- 3) choline uptake blocker e.g. hemicholinium.

Unlike this a neuromuscular blocker which interferes with receptor - acetylcholine combination and the consequent receptor activation constitute postsynaptic blockers. These are subclassified under two headings.

(a) Antidepolarising blockers : They actively prevent depolarisation of the postjunctional membrane. In animals with predominance of multiple innervated muscles, these blockers produce flaccid paralysis. This type of block can be reversed by anticholinesterase drugs like physostigmine, neostigmine, etc. Under the influence of increasing concentration of an antidepolarising neuro-

muscular blocker, the end-plate potentials rapidly diminish in size and duration till they are completely abolished.

The prototype action is provided by d-tubercurarine in this class.

(b) Depolarising blockers : These drugs, besides occupying acetylcholine receptors produce depolarisation at the motor end-plate.

Drugs in this category are actually agonists at the muscle receptor in that they effect depolarisation of the muscle similar that due to acetylcholine. In the case of these blocking agents, the depolarisation persists, prevents the action of acetylcholine. Thus, with this agent there is always a period of stimulation preceding the paralysis such as the initial fasciculations in cat.

Muscle innervated with multiple innervated fibres are more sensitive to depolarizing blockers and animals having predominant multiple innervated muscles viz. avian species, produce spastic paralysis with these type of drugs.

Here some questions arise such as - how a substance which imitates the normal transmitter, can produce a neuromuscular block and why the initial fasciculations fail to persist for the duration of the action of the drug.

There are different mechanisms by which depolarizing blockers execute neuromuscular paralysis -

1. the responsiveness of the end-plate region to acetylcholine is reduced ;
2. the ability of the end-plate to excite the immediately adjacent muscle membrane is impaired.

The analysis of the action of depolarising blocker in the cat showed that on one hand vigorous end-plate potentials could be evoked in its presence and on the other that the muscle membrane in the region of the end-plate has become electrically inexcitable. A similar state of affairs could be produced without drugs, by depolarising the end-plate electrically for a sufficient period.

It was, therefore, concluded that the block was due to the development of electrical inexcitability of

the region and that this occurs because of persistent depolarisation. Such a phenomenon is well known in the electrophysiology of nerve as "cathodal block".

Here, the next question comes why a prolonged excitation should produce an eventual irresponsiveness to electrical excitation.

Although the phenomenon is common with all excitable tissues, the answer to this is not yet clear. But it is known that there is a major change of permeability at the depolarised end-plate and that a large efflux of K^+ occurs. It is not surprising that when this efflux persists for minutes or even hours the muscle membrane in the vicinity loses its normal excitability. Efflux is over in a few milliseconds during the normal end-plate potential.

The paralytic effect of these drugs is antagonised by the competitive agents and is potentiated by the anticholinesterases.

Evidence obtained with radioactive labeled non-depolarising and depolarising drugs has brought out some differences in their sites of actions at the motor end-plate.

It has been suggested that a depression is present in the muscle membrane into which acetylcholine must enter to effect depolarisation. Antidepolarising blockers possibly block this phase physically, whereas the smaller depolarising type drugs enter the depression as acetylcholine does to effect their depolarisation and block. Their blocking property is due to persistence at the site. This would also explain why simpleonium compounds and most other active small compounds are depolarising blocking agent. Depolarising blockers may have intracellular representation in 2nd stage of neuromuscular block.

Some depolarising agents also show dual or mixed blocking activity. Drugs in this category are all depolarising agents but exhibit in addition, some of the characteristics of competitive blockade. The effects of such compounds are antagonised by anticholinesterases and augmented by antidepolarising agent

NEUROMUSCULAR BLOCKING AGENTS :

(A) Neuromuscular blocking agents from natural sources :

a) Curare and curare alkaloids :

The story of curare is one of the most fascinating in medicine; moreover it provides a very good example of how

the study of poisons has led to an understanding of physiology. Indeed, toxicology is the further of modern pharmacology and physiology. Magendie studied the toxicology and pharmacophysiology of the pure alkaloids newly derived from numerous crude drugs by Pelletier. His experiments with the then recently isolated alkaloids such as morphine, Veratrine, brucine, strychnine, and emetine were described in the Paris "Formulaire" of 1821. Magendie often used curare, the arrow poison obtained from South America, to immobilize his dogs, and the paralytic action of curare became well known to his pupil, Claude Bernard. The classical experiment in which Claude Bernard demonstrated that the point of action of curare is at the junctional tissue between the motor nerve and muscle. Natives of South America obtained curare from a number of different plant species and prepared it in several types of vessels, including pots, calabashes and bamboo tubes.

i) Tube curare : Clinically today d-tubocurarine, the active principle of tube curare, is widely used as a muscle relaxant during surgery, especially during prolonged procedures^{20,21,22}. It also has some use as an adjuvant in the treatment of tetanus. d-Tubocurarine dimethyl ether, which is one to three times as potent as the parent compound in man^{23,24}, is also used, though to

a lesser extent^{20,21}. Until recently, d-tubocurarine was assumed to be bisquaternary ammonium compound but one of the nitrogen atom constitutes a tertiary amine²⁵ (Figure-1). The structure of the dimethyl ether (identical with d-chondocurarine dimethyl ether) has not been definitely established today; however, from its degradation products when treated with sodium in liquid ammonia²⁶ an acceptable structure has been deduced²⁷.

The botanical source of the active principle of Bohm's tube curare, d-tubocurarine, was probably Chondrodendron tomentosum Ruiz and Pavon^{28,29}; today this is the source of the commercial product²¹. More than 20 other alkaloids have been isolated from Ch. tomentosum³⁰ but most of them do not exhibit curare-like activity. Those that do are bisquaternary alkaloids of the same basic structure as d-tubocurarine, differing only in their ether linkages³⁰. Methylation of the phenolic groups increases the activity of these active agents, whereas ethylation or butylation reduces or eliminates it²⁸. The isomer d-chondocurarine, which differs from d-tubocurarine only in the relative position of a phenolic and a methoxy group³¹ is from two to four times as potent³⁰.

The simple bisonium salt of d-tubocurarine with undissociated phenolic hydroxy groups is the active form of

the alkaloid^{32, 33, 34}. At low pH d-tubocurarine is primarily in the non-ionized form and its blocking activity is potentiated, but at high pH it loses activity.

ii) Pot curare : The inactive alkaloids extracted from the pot type of curare are phenolic tertiary bases^{28, 35} and the active ingredient is an amorphous phenolic quaternary base with high curare activity³⁵. The botanical origin of this curare was controversial for several years^{29, 35}, the alkaloids being obtained from several sources (Chondrodendron platyphyllum, Ch. microphyllum, Ch. candicans³⁵ and Ch. tomentosum²⁸). There was also the suggestion that Strychnos castelnote Wedd. might be a source in some samples³⁶. However, the active alkaloid(s) probably came from Ch. tomentosum^{28, 35} and the possibility exists that it was actually d-tubocurarine that gave pot curare its activity.

iii) Calabash curare : Of all the curares, calabash curare has enjoyed the longest popularity with the chemist. The alkaloids in this group comprise the most potent of the curare-like substances and have presented the investigator with the most taxing problems^{30, 37-40}. It is generally held that the botanical origin of the active ingredients of this curare is the Strychnos species,

especially S. toxifera Rob . Schomb³⁸. Marsh³⁷ has listed 24 species that are believed to have been used in curare; however, there are at least 41 quaternary bases in calabash curare, 30 of which have been isolated from S. toxifera⁴¹.

The alkaloids in calabash curare (from S. toxifera) fall into two distinct groups : (1) the C₁₉ and C₂₀ indole derivatives^{38,42}, which are all inactive biologically, and (2) the dimeric alkaloids with a C₃₈-C₄₀ skeleton. This latter group contains the active alkaloids, all of which have two quaternary nitrogens. Extensive chemical study of toxiferine I (Figure-2a), C-dihydrotoxiferine I (Figure-2b) and their related alkaloids^{39,43} has shown that many of the alkaloids of this curare can be placed in groups, or families of alkaloids. There are at least five members in the C-dihydrotoxiferine I group, and five in the toxiferine I group⁴³. Many of the transformations within the groups are obtained under very mild oxidation³⁹, and this might explain the large number of different alkaloids that are found in extracts of the *Strychnos* species³⁸.

Calabash curare is many times more potent than d-tubocurarine. This potency is derived in part from the alkaloid toxiferine I, which is 6 to 8 times more potent than d-tubocurarine in man, upto 240 times in the frog, and from 12 to 20 times in lower mammals⁴⁴. The alkaloids

C-alkaloid E and C-alkaloid G are more potent than toxiferine-I in experimental animals but have not been tested in man. Toxiferine-I and curarine I of the C-dihydrotoxiferine I group have been used clinically on a very limited basis. Waser has been using ^{14}C -curarine to study acetylcholine receptors in the neuromuscular junction.

b) Erythrina alkaloids :

The extracts of the seeds of the South American *Erythrina* species contain alkaloids with curare form activity⁴⁵⁻⁴⁹. Over 50 species of *Erythrina* contain active alkaloids^{30,46, 50-52}. The fact that these alkaloids are tertiary bases and active orally stimulated their study, both in chemistry^{45-49,60,61} and as a clinical agents^{62,63}. They are, however, no longer used in the clinic²¹.

The *Erythrina* alkaloids can be divided into two structural groups, both being indole derivatives. The most potent of the alkaloids, β -erythroidine (Figure-3a) and dihydro- β -erythroidine (Figure-3b) belong to the "lactone alkaloids", whereas the remaining active compounds are aromatic, having a benzene ring in place of the lactone ring⁵². Hypaphorine, the betaine of tryptophan, which is found in all species of *Erythrina* studied, is inactive:

It has been suggested that there is a biogenetic relationship between the erythroidines, the lactone bases, and the aromatic alkaloids^{52,59}. Two molecules of 3,4-dihydroxyphenylalanine can combine to yield the indole nucleus common to both groups⁵².

Dihydro- β -erythroidine (Figure-3b) is about five times more potent than β -erythroidine (Figure-3a)⁴⁷ and one-fifth to one-tenth as potent as d-tubocurarine⁵⁰. Paralysis caused by the Erythrine alkaloids is reversed by anticholinesterases⁴⁷. Quaternization of the Erythrina alkaloids results in a reduction of their potency that is approximately hundred fold⁴⁷. When the Erythrina alkaloids or their demethoxy derivatives are treated with strong acid at 125°C, a rearrangement occurs, with aromatization of ring⁵⁹. This aporearrangement results in compounds that no longer act at the neuromuscular junction but exhibit a CNS-depressant action^{51,64}.

c) Tropane derivatives :

The parent compound atropine is isolated from the plant Atropa belladonna (Figure-4). A dose of atropine which is usually fatal to animals, shows some curare-like effect at the neuromuscular junction; whereas its quaternary metho salt, atropine methylnitrate, acts as a reversible,

curare-like blocking agent with about one-twentieth the activity of d-tubocurarine in frogs and one-sixtieth in rabbits⁶⁵. When two atropine molecules are joined together either by connecting the tropic acid hydroxy groups (O,O-pentamethylenediatropine dimethiodide) or the tertiary nitrogens (N,N-pentamethylenediatropine dimethiodide), curarizing compounds are obtained that are about one-half to one-third as potent as d-tubocurarine in rabbits⁶⁵.

When the decamethylene-bisatropinium compound, which has the same interatomic distance as d-tubocurarine, was tested⁶⁶ it was about five times more potent than the corresponding pentamethylene compound, more than 100 times more potent than atropine methylsulfate, and at least twice as potent as d-tubocurarine and decamethonium in rabbits. In addition it was readily antagonized by neostigmine. Just as the metho salt of atropine is a more potent atropine-like agent than atropine itself, so the decamethylene-bisatropinium compound is still more effective, being about twice as potent as the metho salts and of considerably longer duration.

Several other bisatropinium compounds have been synthesized and tested^{67,68}. In most of them the curare-like property appears to be dependent on the steric relationship between the hydroxy or ester group and the

quaternary nitrogen. The trans or α -configuration is the more potent form of 1,4-xylene-bis(benzoyl-tropinium dibromide). The β -acyloxy derivatives are less active than the α -acyloxy analogs of bisquaternary tropeines. An exception to this rule of the α -form being more active than the β -form is found with hexamethylene-1,6-bis(benzoyl-tropinium dibromide)⁶⁹. Here the β -form (cis) is the more effective⁶⁷.

Methyl quaternization of tropine esters by aralkyl groups diminished the atropine-like effects and alters ganglionic and curare-like effects⁶⁷. As the cationic head is made more bulky for example, by substitution of benzyl for methyl groups a compound changes from a depolarizing to a non-depolarizing curare like agent⁷⁶.

When two tropine esters are linked by $-(CH_2)_m-$ $OCO-(CH_2)_n-COO-(CH_2)_m-$ ($m = 2$ or 3 , $n = 0-6$), potent, short acting curare like compounds are formed. The effects of these compounds are easily reversed by neostigmine and potency is dependent on the character of the tropine ester and the values of n and m . A higher value for n favours shorter duration of action⁷⁰.

The tropane derivatives, although not used clinically, are of much interest since they afford the chemist and pharmacologist a tool with which to investigate the mechanism of action of compounds active at the neuromuscular junction^{65,66}.

d) Derivatives of Cinchona alkaloids :

Quinine obtained from different species of *Cinchona* (viz. *Cinchona succirubra*, *C. calisaya*, *C. officinale*) (Figure-5) possesses a very weak curare-like action⁷¹. When the nitrogen in the quinuclidine ring is quaternized by alkyl groups, orally effective curare-like compounds are obtained^{30, 62,72}. The *N,N'*-dialkyl derivatives are about half as active as the monoalkyl derivatives⁷³.

A study of the *N*-monoalkylquininium salts from methyl to hexyl showed that, based on the ratio of effective to lethal dose in rabbits, the ethochloride⁷⁴, *n*-amyl bromide, isoamyl chloride, and hexyl bromide⁷⁵ were the most promising agents for use against pentylenetetrazole induced convulsions. Their margin of safety, as judged by the above ratio, was better than that of curare or β -erythrooidine salts. They were shorter acting than curare and less potent. The amyl derivatives were the most effective of the monoalkyl derivatives, studied;

however, potency on an absolute basis, without regard to toxicity, followed the order *n*-propyl chloride, *n*-propyl bromide, ethochloride, *n*-amyl bromide, hexyl bromide, *n*-butyl chloride, iso-amyl chloride, methochloride and iso-propyl chloride. Curare was 4-times more potent than the *n*-propyl chloride and 14 times more potent than the isopropyl chloride salt.

N-Methyl and *N*-ethylquinidinium iodides, and cinchonidine methiodide and ethiodide, are more active but more toxic than the analogous quinine compounds. The cinchonine compounds are less active than the others⁷⁶.

The quinine derivatives have been tested clinically^{62,72} but, with the possible exception of their use in the prevention of fractures during convulsant-shock treatment^{49,62}

E) Pyridine alkaloids :

Curare-like activity has been reported for several of the monoquaternary pyridine alkaloid salts⁷⁷. The most effective of those investigated was *N*-benzyl-*N*-ethyl conitinium iodide, which was about one-fifth as potent as curare in frogs. Of the conitine obtained from conium maculatum (Figure-6) and conhydrine salts, the β -isomers are slightly more potent than the corresponding α -compounds. The nicotinium, obtained from Nicotina tabacum (Figure-7), salts

are very weak curarizing agents. It is of interest that, as with the quinine derivatives, when both nitrogens of the pyridine alkaloids are quaternized, the activity is less than in the monoquaternary compounds³⁰.

f) Isoquinoline alkaloids :

In contrast to some of the other alkaloids (cinchona and pyridine), the monoquaternary isoquinolinium alkaloids are very weak curare-like agents when compared with their bis-quaternary counterparts^{32,77-79}, d-tubocurarine itself being a member of this last group. Upon N-alkylation of canadine, two isomers are obtained. The β -isomer is ten times more potent than the α -isomer in frogs. The naturally occurring alkaloid canadine (Figure-8) that is extracted from the bark of Xanthoxylum brachyacanthum is α -1-canadine methochloride (N-methyl-1-canadinium). The β -isomer is obtained synthetically⁷⁹ and is about one-tenth as potent as d-tubocurarine in frogs; it contains a quaternary bridge nitrogen similar to that in the calabash curare alkaloids³⁰.

g) Strychnos alkaloids :

The quaternary salts of Strychnos alkaloids other than the curare alkaloids are in general all curarizing

agents³⁰. Their potency ranges from one-third that of d-tubocurarine for N-ethylstrychnidinium chloride to one twenty-fourth for N-methylstrychninium. N-Methylstrychnidinium chloride is about one-sixth as potent as d-tubocurarine, whereas the iodide is only one-fourteenth as active⁸⁰. The bruciniums (Figure-9b) are only very weak curarizing agents^{72,74}. Alkaloid Strychnine (Figure-9a) and brucine were isolated from Strychnos nuxvomica.

n) Yohimbine alkaloids :

The quaternary derivatives of the yohimbine alkaloid (Figure-10) isolated from Corynanthe johimbe show curarizing activity⁸¹. N,N-Dimethylhexahydrotetrahyrinium appears to be the least toxic of the compounds studied. The chloride salt has about one twenty-seventh and the iodide, about one thirty-eighth - the activity of d-tubocurarine. Although these compounds, like the strychnine derivatives have low toxicities in the dose ranges yielding good paralysis³⁰, they have not proven to be of any clinical value.

t) Petaline alkaloid :

The alkaloid petaline (Figure-11) obtained from

extracts of Leontice leontopetalum has been reported to exhibit weak neuromuscular blocking activity of anti-depolarizing type^{77b}.

j) Malouetine and its derivatives :

Malouetine (Figure-12) was isolated from Malouetia berquaertiana. It is one third as potent as tubocurarine intravenously in mice, while being of similar potency in producing neuromuscular blockade in the cat tibialis nerve muscle preparation. It is of antidepolarising mode of action since reversal is effected by neostigmine and muscle contracture is absent in avians. It produced marked hypotension and bradycardia hence not studied clinically.

k) Conessine and its derivatives :

Conessine (Figure-13) has been reported as a neuromuscular blocking agent in 1948 by Stephenson⁷⁶. Its N,N'-dimethyl conessine was found to be of comparable potency to d-tubocurarine. In the monkey there appeared to be a somewhat slower recovery rate than in the cat.

l) Paravallaridine and its derivatives :

Alkaloid paravallaridine (Figure-14) obtained from Paravallis microphylla showed curarizing action

of the nondepolarizing type which are reversed by neostigmine⁷⁷. Bisquaternary 16- -diacetate was found to be highly potent.

m) Pyrrolizidine alkaloids :

The tertiary pyrrolizidine alkaloids heliotrine (Figure-15) and lasiocarpine (Figure-16) were isolated from Heliotropium europaeum by Trautner and Neufeld (1949) and Heliotropium lasiocarpum by Menschikov (1932). These alkaloids caused a neuromuscular block that resembles decamethonium induced block in its effects⁸². The N-oxides of these alkaloids are inactive.

n) Miscellaneous alkaloids :

Curare-like activity has been reported for papaverinium and the quaternary salts of the morphine alkaloids.

Other naturally occurring substances have been investigated in some detail for their activity at the neuromuscular junction. Maltoxin, an aromatic, non-quaternary base from malt rootlets, has a decamethonium-like action⁸³. Murexine, the choline ester of urocanic acid [Urocanylcholine; -(4-imidazolyl)acrylylcholine-] (Figer-17), which is found in the hypobranchial body of

Murex trunculus and other mollusks, is a neuromuscular blocking agent also with decamethonium like properties^{84,85}.

Several derivatives of murexine were tested, but little improvement over the parent compound was found. Dihydro-murexine, which is more potent in animals, proved to be almost inactive in man. Imidazolebutyrylcholine and imidazolepropoxylcholine have a lesser degree of side effects than murexine but have not been tested clinically.

There has been great interest in the fish toxin tetrodotoxin⁸⁶, which is obtained from several tissues, especially the liver and ovary of various species of Tetraodontidae (Puffer fish, ocean sunfish, porcupine fish)⁸⁷. Tetrodotoxin is a complex iminoperhydroquinazoline. Several derivatives of tetrodotoxin have been prepared and tested, but all are very much weaker than the parent compound. Although the early workers believed that this toxin acted much like curare at the neuromuscular junction⁸⁷, it has since been reported that the block is potentiated by both d-tubocurarine and succinylcholine, and is not antagonized by neostigmine. It is probable that this agent acts on both the nerve and the muscle membrane at the neuromuscular junction.

(B) Synthetic Neuromuscular Blocking Agents :

(a) Simple onium salts :

In general, onium salts effect not only a curare-like paralysis at the neuromuscular junction but also show muscarinic activity at parasympathetic nerve endings, as well as nicotinic activity at sympathetic ganglia^{77,88-89}. The curare-like action of these salts as a class was predicted by Crum Brown and Fraser.

The most active curare-like onium salts are tetramethyl and trimethylalkylammoniums. In the latter series the butyl and amyl derivatives are the most active both being about twice as potent in mice as the tetramethylammonium salt. In frogs the paralyzing dose of tetramethyl ammonium is about 10 times that of d-tubocurarine; however, in this same animal toxicity is somewhat reduced with the onium ion. All of these compounds exhibit nicotinic and/or muscarinic activity and this makes them unsuitable for clinical use as neuromuscular blocking agents. As a matter of fact, tetraethylammonium was in clinical use for years as an antihypertensive agent because of its pronounced autonomic ganglion-blocking action; it has now been displaced by newer agents such as guanethidine. Although tetraethyl-

ammonium is such a potent nicotinic blocking agent, it is a weak curare-like compound. When the methyl groups of tetramethylammonium are successively replaced by ethyl groups, there is a decrease in activity, the trimethylethyl- being about one-third, the dimethyldiethyl - about one twenty-fifth, the methyltriethyl - and tetraethylammonium ions about one-eightieth as potent at the neuromuscular junction as the parent tetramethylammonium.

The successive replacement of the methyl groups with any alkyl groups reduces the muscarinic activity of the compound, the tetraalkyl compounds other than methyl showing no such activity at all. If the replacement group is propyl or butyl, only a slight loss of curare-like activity is seen, less than observed with ethyl replacement. When a phenyl alkyl group is substituted for one of the methyl groups as in phenethyltrimethylammonium, there are pronounced muscarinic and nicotinic effects; however, the presence of a methyl group to the nitrogen decreased both the nicotinic and muscarinic activities. It is of interest also that triethylbenzyl - and triethylphenethylammonium salts both are without muscarinic and nicotinic actions.

Neurine (vinyltrimethylammonium, $\text{CH}_2=\text{CHN}^+(\text{CH}_3)_3$) has some curare-like action but is very toxic and possesses

very pronounced muscarinic and nicotinic actions. Ethynyl-trimethylammonium ($\text{HC} \equiv \text{CN} (\text{CH}_3)_3$) appears to be the only unsaturated alkylammonium ion that has curare-like properties but does not produce either nicotinic or muscarinic effect.

Betaine (carboxymethyltrimethylammonium) itself is pharmacologically inert^{77,90,91}, but some of the substituted betaine esters (ethyl, butyl, amyl, or benzyl side chains to nitrogen) do have definite curare-like activity with little muscarinic or nicotinic effect⁹⁰.

In general the simple onium salts, although they produce a curare-like paralysis, act as depolarizing. They are also agonists at the receptors at which acetylcholine acts, since most have muscarinic and nicotinic effects. However, until recently most agents tested for curare-like activity were tested for potency in causing paralysis only, and not for their mode of action. From recent studies of several onium compounds⁹²⁻⁹⁴ it appears that, as the size of the cationic head is enlarged, the mode of action of these onium salts changes from the depolarizing type (agonistic), as exhibited by tetramethyl and tetraethylammonium, to the true curare-like competitive

type (antagonistic). This latter action is found in such ions as tetrabutylammonium.

b) Bis-onium compounds :

1) Galamine and its analogs : During tests of the neuromuscular blocking activity of ethers formed from choline and homologous amino alcohols with phenols and polyphenols the clinically useful galamine triethiodide (Figure-18) was discovered. This drug is about one-third as potent as d-tubocurarine in the rabbit⁹⁵, about three-fourth as active in cat⁹⁶⁻⁹⁹, and about one-third as active in man.

In analogs of Figure-18 there was a decrease in curareform activity as ethyl groups were replaced successively with methyl groups. However, there was no significant loss of activity until at least two such substitutions were made. The tris-(trimethylammonium ethoxy) compound had a depolarizing action at the neuromuscular junction, there being a gradual change from true competitive action to a depolarizing one with successive methyl substitution⁹⁶. In a series of related N-substituted derivatives the trimethylammonium derivative was almost as active as gallamine triethiodide.

2) Succinylcholine : Succinylcholine (Figure-19)

has become prominent in the clinic as a short-acting muscle relaxant. It is not antagonized by neostigmine and is antagonistic to d-tubocurarine. It appears to play a dual role at the end plate, first depolarizing and subsequently setting up a competitive block to acetylcholine¹⁰⁰. It is about equipotent in man with d-tubocurarine, but it has about one-tenth the duration of action. This short duration is due to its susceptibility to hydrolysis by serum cholinesterase. Succinylcholine is a doubled acetylcholine molecule, and this fact may account for its rapid hydrolysis by cholinesterases.

3) Suxethonium : It has an ethyl group substituted for one methyl on each quaternary nitrogen atom, has a lower activity than succinylcholine. A second ethyl substitution produced a very marked reduction in activity. The sulfonium analog when tested in rabbits is about one-tenth as potent as succinylcholine; however, it has a longer duration of action.

4) Decamethonium : Decamethonium (Figure-20), one of the most potent neuromuscular blocking agents known, and one that subsequently stimulated the search for active agents, was discovered by two independent groups^{102,108}.

They studied simple bisquaternary ammonium salts for neuromuscular blocking activity, and observed the potent neuromuscular blocking action of this bisquaternary salts. Potency increased from the compound with two methylene groups in the linking chain upto the decamethylene compound; with from 10 to 18 methylene groups, potency decreased. The most active compound, decamethonium, was more than twice as potent as d-tubocurarine in the rabbit head-drop test. Augmentation of the contractions of the rat diaphragm muscle was observed at decamethonium doses that were lower than that needed for paralysis, and decamethonium was not antagonized by neostigmine¹⁰⁴. These observations have since been explained by the fact that this compound has an agonistic (depolarizing) as well as an antagonistic action at the end plate of the neuromuscular junction^{100,101,105,106}.

5) Carbolonium : Carbolonium (Figure-21) is a depolarizing agent with a marked tendency to produce a dual block in man if it is given in large doses¹⁰⁷.

6) Pancuronium : Pancuronium (Figure-22) is a member of a series of bisquaternary ammonium steroids that were synthesised in 1964. Extensive pharmacological and

clinical studies have shown that it is approximately five times as potent as d-tubocurarine as a competitive neuromuscular blocking agent, with minimal cardiovascular and little or no histamine releasing or hormonal actions¹⁰⁸.

Clinical uses of the Neuromuscular blocking Agents :

The neuromuscular blocking agents find their principle use in surgery. They promote complete muscle relaxation and permit major abdominal surgery to be carried out on patients who are only lightly anaesthetized and who are therefore spared the hazards which attend deep anaesthesia. They are also of value in orthopaedic surgery, enabling corrective manipulations to be carried out with ease.

The painful muscle spasms of tetanus can be reduced in intensity by neuromuscular blocking agents. The drugs have also been used in the treatment of status epilepticus and for attenuating the violent seizures which otherwise accompany electroconvulsive therapy. In these conditions it is useful to administer the muscle relaxant in conjunction with an appropriate anaesthetic. Antidepolarizing agents are sometimes used in diagnosis of myasthenia gravis.

STRUCTURE OF NEUROMUSCULAR BLOCKING
AGENTS.

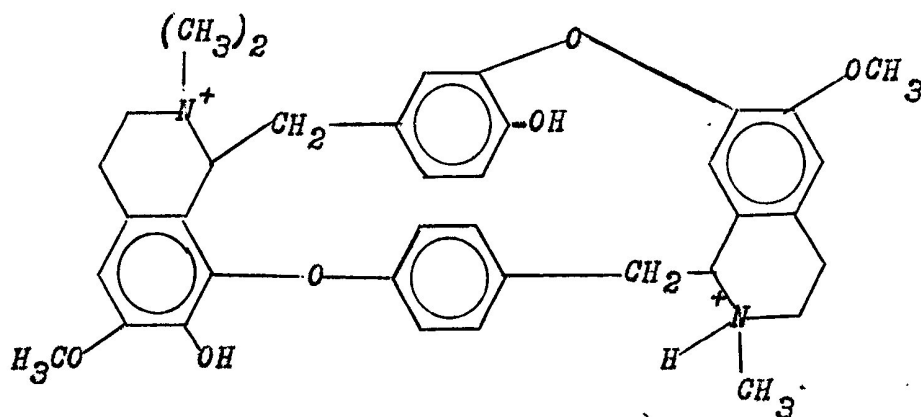
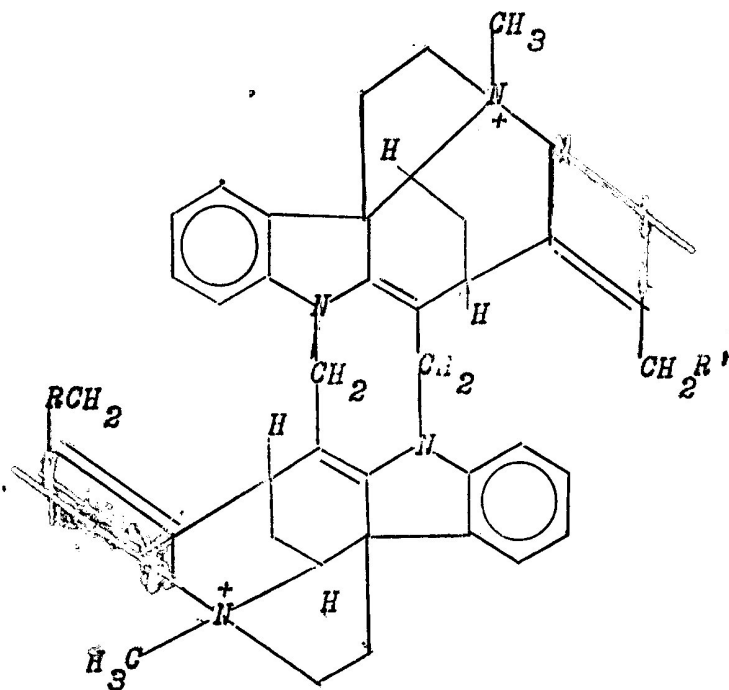
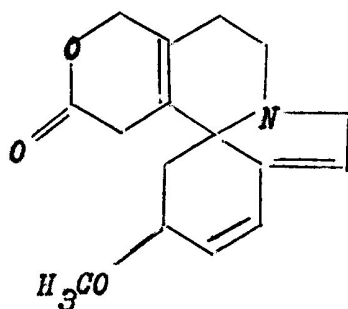
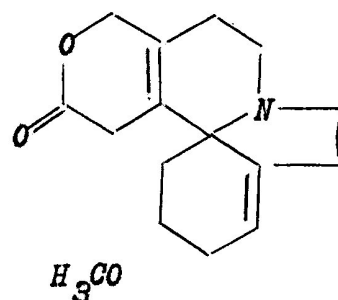
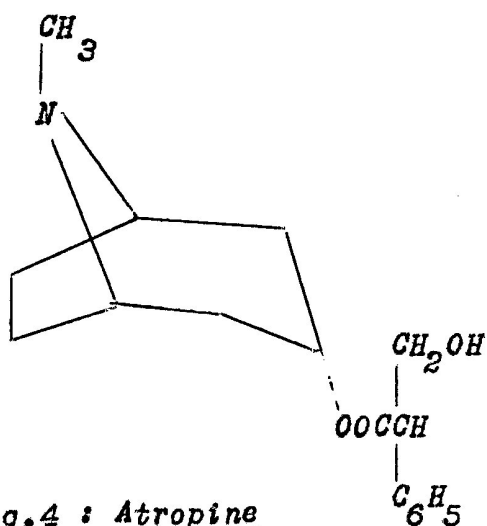
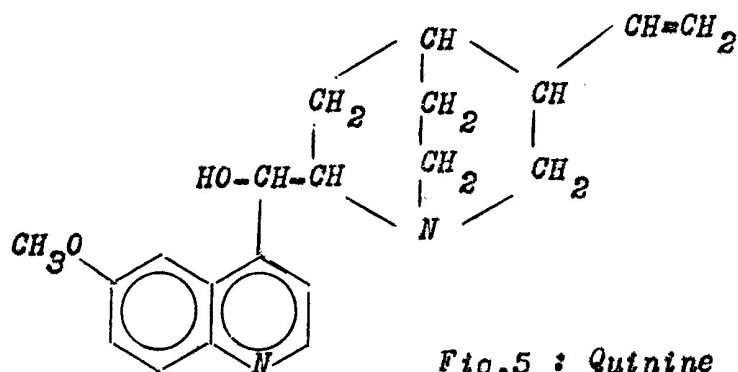


Fig. 1 : d-Tubocurarine



*Fig. 2 : (a) $R = R' = OH$ -Toxiferin I
(b) $R = R' = H$ -C-Dihydrotoxiferine I*

Fig. 3(a) : -Erythroidine.Fig. 3(b) : Dihydro-
-Erythroidine.Fig. 4 : AtropineFig. 5 : Quinine

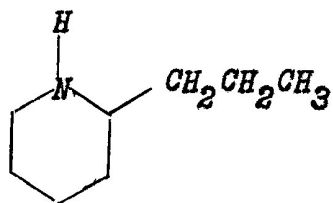


Fig. 6 : Conine from
Conium maculatum

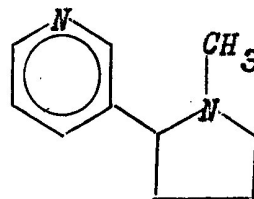


Fig. 7 : Nicotine from
Nicotiana tabacum

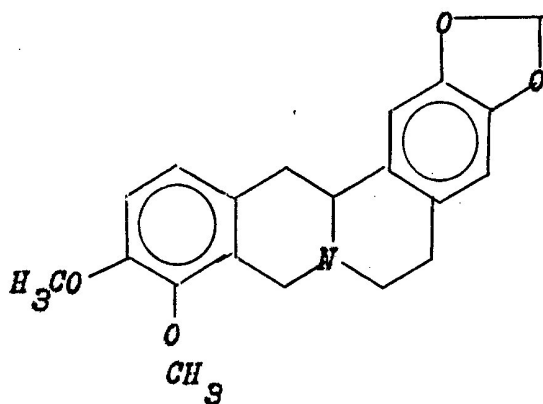
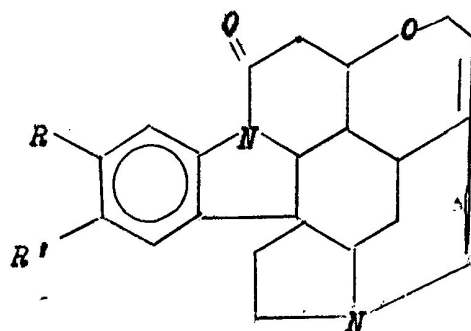


Fig. 8 : Canadine from Corydalis cava.



Strychnine

Strychnos nuxvomica

Fig. 9 : (a) $R' = R = H$ ---- Strychnine

(b) $R' = R = OCH_3$ ---- Brucine

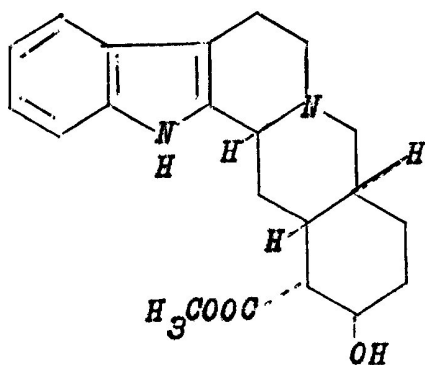


Fig. 10 : Yohimbine from *Corynanthe johimbe*

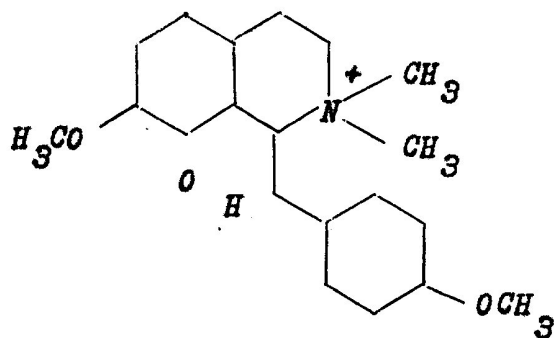


Fig. 11 : Petaline

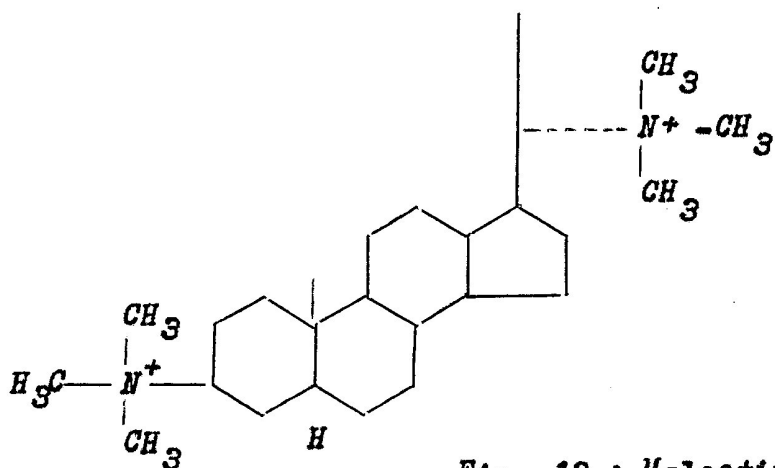


Fig. 12 : Maloetine

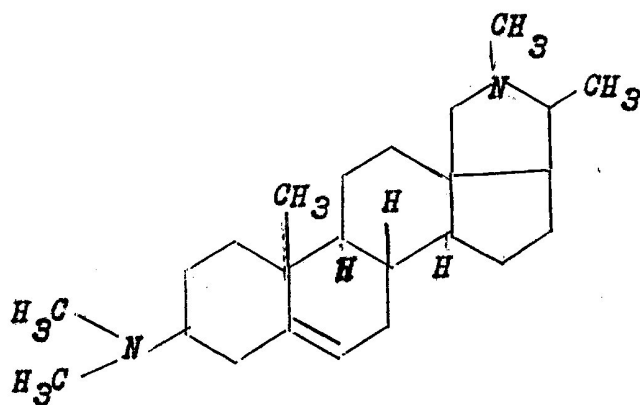


Fig. 13 : Conessine
from Holarrhena antidysenterica

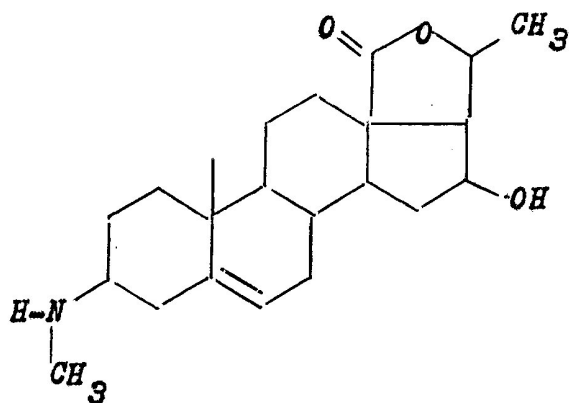


Fig. 14 : Paravallaridine from
Paravallaris microphylla.

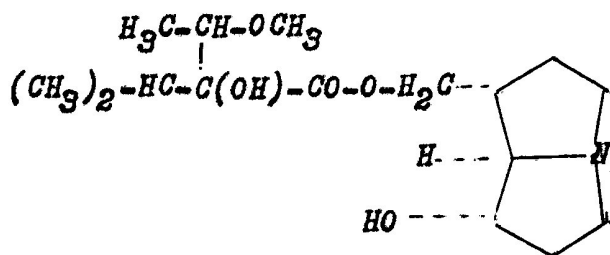


Fig. 15 : Heliotrine from Heliotropium europaeum

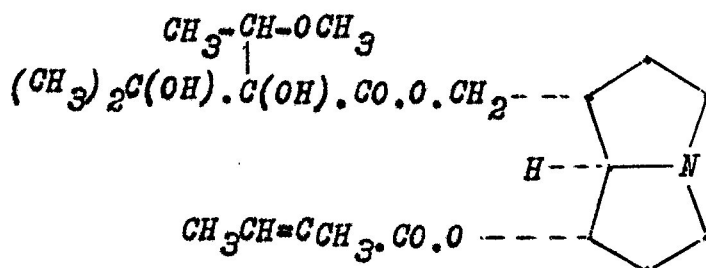


Fig. 16 : Lasiocarpine from *Heliotropium lasiocarpum* .

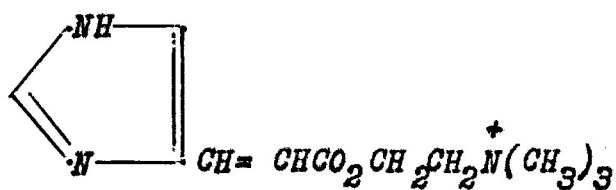


Fig. 17 : Murexine from *Murex trunculus* .

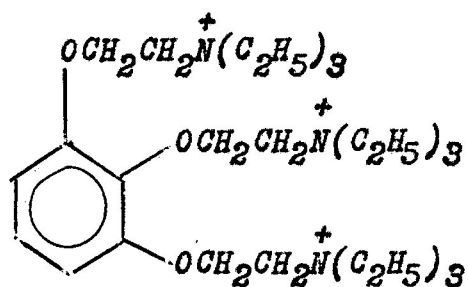


Fig. 18 : Gallamine .

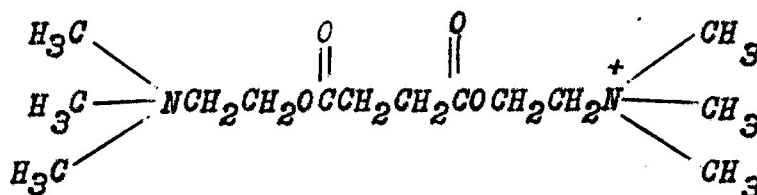


Fig. 19 : Succinylcholine .

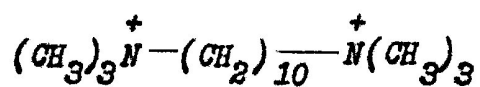


Fig. 20 : Decamethonium.

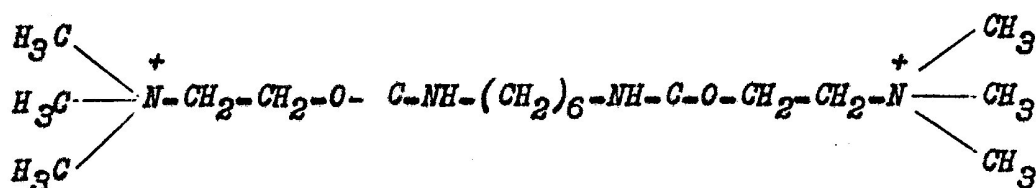


Fig. 21 : Carbolonium

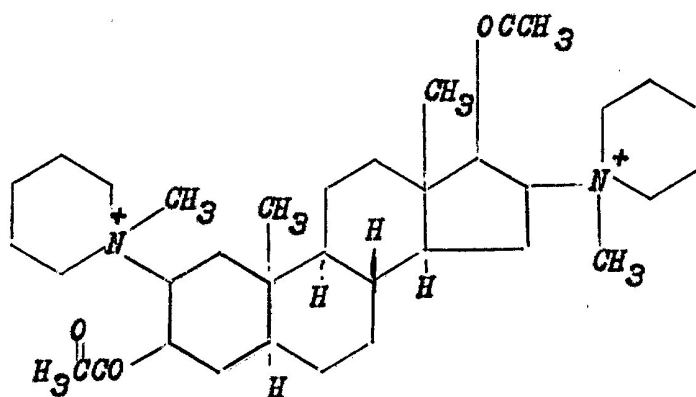


Fig. 22 : Pancuronium

S e c t i o n I I I

C H R O M A T O G R A P H Y

One of the problems continually facing phytochemist is the separation and purification of one or more natural compounds from a mixture of such compounds. One of the most convenient methods for achieving such separations is the use of chromatographic techniques. Such techniques can be used for the separation of large amounts or small amounts of material. The selection of a particular form of chromatography to achieve a separation is dependent on the material to be isolated, and often several chromatographic methods may be used sequentially to achieve the purification of a compound.

When chromatographic methods using column as a device fail to achieve the isolation of a compound from the mixture, the technique, thin layer chromatography of preparative grade is particularly useful.

Thin layer chromatography was described as long as 25 years ago but it has become of general value only in the last few years. In 1938, N.A. Ismatlov and M.S. Shraiber^{107a} applied the method to the separation and characterization of the extracts of medicinal plants.

In 1951 Kirchner, et al.¹⁰⁸ coated glass strips with a slurry of silicic acid mixed with starch as a binding

agent and succeeded in separating a number of terpenes by ascending chromatography. Little attention was paid to this development because of the great interest in paper chromatography at the time. It was generally recognized that lipophilic compounds were better separated by adsorption techniques, and attempts were made to adapt paper strips to adsorption processes by using acetylated cellulose and by impregnating the paper with alumina. These modifications were not too successful, and packed columns generally provided a better solution to these problems.

About 5 years later Stahl¹⁰⁹ in West Germany revived the technique of thin-layer chromatography (TLC) and demonstrated its usefulness in many different applications. He prepared 250 μ layers of silica gel on glass plates using plaster of Paris as a binder and standardized his equipment and coatings to the point where they were made available commercially.

The TLC technique offers considerable advantages over both column and paper chromatography. In column chromatography tailing and poor resolution occur unless the flow rate is well controlled. Among other reasons, this can occur because fluids flowing through a tube have a

greater flow velocity at the centre, due to frictional resistance at the sides. Additional problems arise from flow resistance and irregularities in the packing of the stationary phase itself.

In TLC these problems are minimized because the solvent flow occurs on a planar surface. Although many features of TLC are similar to paper chromatography, TLC is extremely more versatile because of the large number of materials available as stationary phase sorbents. Nor is spreading of solutes as severe because of the quantitative differences between the fibrous structure of paper and the particulate nature of TLC coatings. This permits lower solute detection thresholds in TLC and considerably shortened development time.

Sorbents and solvents : The availability of many sorbents in particle sizes suitable for thin-layer preparation makes many chromatographic principles adaptable to TLC. Absorption, partition, ion exchange, ligand-exchange, gel filtration, and electrophoresis may all be performed on thin layer plates. These techniques are listed in the Table below along with some commonly used sorbents and developing solvents.

T a b l eSorbents and solvents used in various TLC techniques.

<i>Technique</i>	<i>Sorbents</i>	<i>Solvents</i>
<i>Adsorption TLC</i>	<i>Silica gel</i> <i>Aluminium oxide</i> <i>Calcium phosphate</i> <i>Kieselguhr</i>	<i>Organic solvents</i> <i>usually as mixtures</i>
<i>Partition TLC</i>	<i>Cellulose</i> <i>Kieselguhr</i> <i>Silica gel</i> <i>Superfine sephader</i>	<i>Mixtures of organic</i> <i>and aqueous solvents</i>
<i>Reversed-phase partition TLC</i>	<i>Paraffin and Silicone</i> <i>oils impregnated on</i> <i>silica gel or</i> <i>Kieselguhr</i> <i>Acetylated-cellulose</i> <i>Polyamides</i>	<i>Mixtures of organic</i> <i>and aqueous solvents</i>
<i>Ion-exchange TLC</i>	<i>Modified ion-exchange resins</i> <i>DEAE-cellulose</i> <i>Acetylated-cellulose</i>	<i>Dilute acids, bases,</i> <i>and buffers.</i>
<i>Ligand-exchange TLC</i>	<i>Silica gel or</i> <i>kieselguhr</i> <i>impregnated with</i> <i>AgNO₃, tungstates,</i> <i>bisulfite</i>	<i>Organic or organic-</i> <i>aqueous mixtures</i>
<i>Gel-filtration TLC</i>	<i>Superfine sephader,</i> <i>G-25 to G-200</i>	<i>Dilute acids,</i> <i>bases, salts,</i> <i>buffers</i>
<i>Thin-layer electrophoresis</i>	<i>Silica gel</i> <i>Cellulose</i> <i>superfine Sephadex</i> <i>Agar</i>	<i>Aqueous buffers.</i>

Adsorption TLC is highly versatile and useful in the separation of a great many classes of compounds. It is the most commonly used form of TLC. The polarity of the substances to be separated dictates the combination of sorbent and solvent to be used.

Silica gel (silicic acid) is slightly acidic and therefore is best applied to the separation of neutral and acidic substances. Alumina (aluminium oxide), on the other hand, is basic and should be used for the separation of basic compounds. Alternatively, silica gel can be used for the separation of basic compounds if a dilute base (as ammonia) is added to the developing solvent. Silica gel and alumina are the most active (powerful) adsorbents; less active are calcium phosphate, calcium carbonate, and kieselguhr. The activity of silica gel or alumina can be reduced by mixing with kieselguhr. The most active sorbents are used with the most nonpolar compounds and the more inactive ones with polar molecules.

A third variable in selecting chromatographic conditions - the solvent system - gives a great flexibility to the method. An excellent and rapid manner for the selection of a solvent system is to place spots of the

sample on several locations on TLC plates prepared with various sorbents. A capillary filled with pure solvent is applied to the gel in the middle of the sample spot. The solvent develops a small, circular chromatogram with the migrating solutes arranged in concentric circles about the origin.

Using a series of polar to nonpolar solvents or solvent mixtures produces at least one sorbent-solvent combination where the solute of interest migrates about halfway between the origin and solvent front. This combination should yield good TLC separations. Cellulose is the most commonly employed sorbent for partition TLC. The same sorbents and procedures which are used in paper chromatography may be successfully applied to TLC. The low adsorptive power of kieselguhr also makes it easily adapted to this method. In addition, silica gel layers have been used for partition where the aqueous solvents decrease its adsorptive power. However, both adsorption and partition forces probably are involved.

The selection of gel-filtration and ion-exchange sorbents is made on the basis of considerations analogous to those required for column applications. The choice of thin-layer electrophoresis sorbents is usually made on the

basis of the substances to be separated. For example, silica gel layers have been used to separate amino acids, amines, phenols, and inorganic ions. Sephadex and agar gels have been applied to the separation of proteins, mucopolysaccharides, nucleic acids and viruses.

Preparation of Plates : To insure a physically stable layer which does not flake, binders may be added to the sorbents. Calcium sulfate, starch, and carboxymethylcellulose are commonly applied for this purpose. Acids or buffers also may be added to the sorbents, and fluorescent indicators may be incorporated to permit detection of ultraviolet-absorbing compounds. Commercially available sorbents are supplied either with or without binders and indicators.

A slurry of the sorbent mixture is made by the addition of water or in some cases an organic solvent. This is poured into a spreading device which is drawn across a series of 5 X 20 or 20 X 20 cm glass plates, depositing a uniform coating on them. Alternatively, reasonably good TLC plates may be made by placing two or three layers of adhesive tape along the edges of the glass plates, placing some slurry on each, and drawing a glass rod across them, supported by the tape. Gradient-

making applicators also are available.

Rather than coating a plate with one sorbent, two different sorbents may be applied simultaneously so that the layer is made of a gradient mixture of both. For example, silica gel and alumina may be used to prepare a pH gradient across the width of the plates. This may yield separations which otherwise would not be possible.

The binder usually sets within a few minutes of being poured and the plates are allowed to air dry in a level position for about an hour. Plates for adsorption TLC are activated by drying in an oven around 100°C for an hour. Additional heating is required for alumina plates. They are then stored over a desiccant until used.

Plates for reversed-phase TLC also must be thoroughly dried before impregnation with the stationary phase. This is best done by placing the plate into a shallow trough containing an organic solution of the stationary phase, and permitting the solution to migrate up the plate. Plates for normal-partition TLC may be used while still moist from preparation or moistened at a later time over boiling water. Gel-filtration and electrophoresis plates also must be moistened before use; better, they are

used while still moist from preparation. Commercially prepared plates containing binder are available with glass, plastic, or aluminium backing.

Development and Detection Techniques : Samples, which may range from a few micrograms to milligrams, are applied at points about 1.5 cm from one edge of the plate. For ascending development, the plate is placed into a rectangular-shaped tank with the developing solvent covering the bottom to a depth of about 0.5 cm. The inside of the jar is lined with filter paper to insure that the solvent will completely saturate the atmosphere. The cover is replaced and the solvent rises through the layer producing the chromatogram by elution development. The run is usually stopped when the solvent front transverses about $3/4$ of the plate. Rather than using a filter paper-lined jar, "sandwich-type" tanks may be used where the jar edges are very nearly in contact with the plate.

Descending techniques are less commonly used but may be of particular value in separating slowly moving compounds. Solvent is delivered to the plate by filter paper strips which saturate the layer. Solvent is permitted to drip from the end of the plate until solutes of interest are resolved or about to be eluted. The

descending technique is essential in gel-filtration TLC. Gradient development also is possible using special tanks which facilitate a continual change in the solvent composition.

Two-dimensional TLC is easily executed as is TLC fingerprinting. A variation of fingerprinting is possible on TLC where gel filtration is performed in one dimension and electrophoresis in the other.

After the solvent has evaporated, plates may be examined under ultraviolet light at 366 mμ for the presence of fluorescent compounds or at 254 mμ for ultraviolet-absorbing ones. In the latter case the compounds appear as dark spots against a highly fluorescent background which is produced by an indicator added to the sorbent. A great many chemical reagents may be applied to the layer by either spraying or dipping, just as in paper chromatography. Thin-layer plates are especially valuable where corrosive reagents are required, such as iodine vapors or sulfuric acid, which detect most organic compounds.

Radioactive compounds may be detected either directly by a scanning device which moves the plate under

a G-M tube and records the resulting increase in counting rate or by physically removing sections and determining the radioactivity present by liquid scintillation counting. The latter method gives great quantitative accuracy and permits the simultaneous detection of two or three isotopes.

A combination of this method with ultraviolet detection is used to characterize unknown drug metabolites. A solution of trace amounts of radioactive metabolites is mixed with reference compounds of known structure and chromatographed. The reference compounds are detected by liquid-scintillation spectrometry. Correspondence between radioactive and ultraviolet absorbing peaks indicates identity.

In case of preparative TLC, thick layers of sorbents may be prepared to separate several hundred milligrams on a single 20 X 20 cm plate. However, if the layer is much thicker than 2 mm, solvent flow becomes more column-like and the advantages of TLC are lost. Samples are uniformly streaked across the entire width of the plate and development produces a series of solute bands which may be eluted for additional characterization.

Recent advances in technology also make it possible to obtain infra-red, NMR and mass spectra data on very small samples. This may be eluted directly from TLC plates.

S e c t i o n I V

S P E C T R O S C O P Y A N D S T R U C T U R E

Determination of structure :

Phytochemist when he is confronted with an unknown compound, takes the various steps : determination of molecular weight and molecular formula : detection of presence - or absence - of certain functional groups, conversion into derivatives; synthesis by an unambiguous route in order to find the answer to the question : What is it ?

At every stage of structure determination from the isolation and purification of the unknown substance to its final comparison with an authentic sample - the use of instruments has, since last few decades revolutionized phytochemical practice. Instruments not only help a phytochemist to do what he does faster but, more important, let him do what could not be done at all before to analysis complicated mixture of closely related compounds, to describe the structure of the molecules in detail never imagined before.

The instruments most directly concerned with our primary interest, molecular structure, are the spectrometers - measurers of spectra.

The major advantages of these techniques are that usually they do not degrade the molecules studied, and that they are highly refined and automated. These advantages are also of importance to the pharmacologist. In addition, these techniques enable extremely small quantities of material to be studied, even in relatively impure systems.

The ultraviolet spectrum :

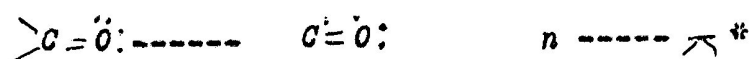
The ultraviolet spectrometers commonly used measure absorption of light in the visible and 'near' ultraviolet region, that is, in the 200-750 μ range. This light is of higher frequency (and greater energy) than infrared light and, when it is absorbed by a molecule, the changes it produces are, naturally, ones that require greater energy : changes in electronic states.

In a transition to a higher electronic level, a molecule can go from any of a number of sub-levels - corresponding to various vibrational and rotational states to any of a number of sub-levels; as a result, ultraviolet absorption bands are broad. Where an infrared spectrum shows many sharp peaks, a typical ultraviolet spectrum shows only a few broad humps. One can conveniently describe

such a spectrum in terms of the position of the top of the hump (λ_{max}) and the intensity of that absorption (ϵ_{max} , the extinction coefficient).

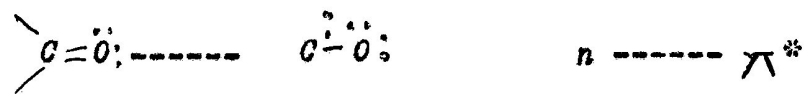
When one speaks of a molecule as being raised to a higher electronic level, one means that an electron has been changed from one orbital to another orbital of higher energy. This electron can be of any of the kinds one has encountered; a σ electron, a π electron, or an n electron (a non-bonding electron, that is, one of an unshared pair). A σ electron is held tightly, and a good deal of energy is required to excite it; energy corresponding to ultraviolet light of short wavelength, in a region - "far" ultraviolet - outside the range of the usual spectrometer. It is chiefly excitation of the comparatively loosely held n and π electrons that appear in the (near) ultraviolet spectrum, and, of these, only jumps to the lower - more stable - excited states.

The electronic transitions of most concern to the organic chemist are :- (a) $n \rightarrow \pi^*$, in which the electron of an unshared pair goes to an unstable (antibonding) orbital, as, for example,



and (b) $\pi \text{-----} \pi^*$, in which an electron goes from a stable

(bonding) orbital to an unstable orbital, as for example,



Conjugation of double bonds, however, lowers the energy required for the transition, and absorption moves to longer wavelengths, where it can be more conveniently measured. If there are enough double bonds in conjugation, absorption will move into the visible region, and the compound will be coloured.

The ultraviolet spectrum is not used primarily to show the presence of individual functional groups, but rather to show relationships between functional groups, chiefly conjugation; conjugation between two or more carbon-carbon double (or triple) bonds; between carbon-carbon and carbon-oxygen double bonds; between double bonds and an aromatic ring; and even the presence of an aromatic ring itself. It can, in addition, reveal the number and location of substituents attached to the carbons of the conjugated system.

The infrared spectrum :

Of all the properties of an organic compound, the one that, by itself, gives the most information about the compound's structure is its infrared spectrum.

A molecule is constantly vibrating; its bonds stretch (and contract), and bend with respect to each other. Changes in vibrations of a molecule are caused by absorption of infrared light; light lying beyond (lower frequency, longer wave-length, less energy) the red end of the visible spectrum.

Like the mass spectrum, an infrared spectrum is a highly characteristic property of an organic compound and can be used both to establish the identity of two compounds and to reveal the structure of a new compound.

The infrared spectra of a molecule are absolutely specific and since this allows the molecules to be characterised, the term "fingerprint" is used to describe the characteristic infrared pattern observed. It is not difficult to assign the absorption bands of simple molecules such as carbon dioxide and water, but phytochemists are usually interested in large complex molecules.

Certain bands which regularly appear near the same wavelength may be assigned to specific molecular groupings, in the same way as for chromophones which absorb in the ultraviolet region. These group frequencies are of great value in diagnostic work. When the bonding force

between two atoms increases i.e., the wavelength absorbed decreases. When the reduced mass $M_a - M_b / M_a + M_b$ (M = atomic weights of the atoms a and b involved in a bond increases, the vibration frequency decreases and hence the wavelength absorbed increases. For any given bond, bending vibrations occur at much lower frequencies than stretching vibrations.

The infrared spectrum helps to reveal the structure of a new compound by telling us what groups are present in or absent from the molecule. A particular group of atoms gives rise to characteristic absorption bands; that is to say, a particular group absorbs light of certain frequencies that are much the same from compound to compound. For example, the -OH group of alcohols absorbs strongly at $3200-3600\text{ cm}^{-1}$; the C=O group of ketones at 1710 cm^{-1} ; the $\text{-C}\equiv\text{N}$ group, at 2250 cm^{-1} ; the -CH_3 group at 1450 and 1375 cm^{-1} .

Interpretation of an infrared spectrum is not a simple matter. It is usually used in conjunction with such techniques as n.m.r. spectroscopy and mass spectrometry.

Nuclear magnetic resonance spectrum :

This technique is used for detecting atoms which

have nuclei that possess a magnetic moment. These are usually atoms containing an odd number of charges (protons) in their nuclei.

Since protons possess both spin and charge they behave like small magnets i.e., they have magnetic movements like electrons, but approximately 2000 times smaller. Thus, in the presence of a magnetic field a proton and other nuclei with a nuclear spin value of $1/2$ can exist in two states i.e., either in a low energy state with their nuclear spin aligned parallel with the field or in a higher energy state antiparallel to the field. In order to change from low energy state such nuclei must absorb the appropriate quantum of energy. In a magnetic field of several hundred millitesla (several thousand gauss) such nuclei absorb radiation in the radio-wave region of the electromagnetic spectrum, giving rise to the phenomenon known as nuclear magnetic resonance (n.m.r.). Most studies are conducted using the lightest isotope of hydrogen, ^1H . This nucleus possesses a nuclear spin value of $1/2$. Other nuclei common in molecules, such as ^{12}C , ^{16}O possess zero spin and hence lack a magnetic moment and do not give n.m.r. signals.

The frequency of the radio waves absorbed during n.m.r. depends on both the isotope being studied and the

magnitude of the applied magnetic field. These spectra are usually plotted as energy absorbed against the magnetic field strength applied (not wavelength or frequency).

The actual field seen by a proton in a particular compound depends upon its molecular environment since the applied field induces secondary fields by interacting with the bonding electrons adjacent to the proton. If this induced field opposes the applied field, a slightly higher field must be applied to make the nucleus resonate. Such nuclei are said to be shielded. The magnitude of the shielding decreases with increasing electron withdrawing power of nearby substituents. If the induced field augments the applied field, the nucleus is said to be deshielded and a smaller applied field is required to achieve resonance; such a displacement in the spectral line are known as chemical shifts. The presence of $^1\text{H-O-}$, $^1\text{H-CH}$, $^1\text{H-CH}_2$, etc. may be detected by the occurrence of particular peaks in the n.m.r. spectrum. In addition, the relative number of ^1H atoms in each type of chemical group within the sample is indicated by the intensity of the appropriate absorption peaks, i.e., by the areas under those peaks.

Nuclear magnetic resonance spectra are further complicated by interaction between like or different spins

through the bonding electrons. This spin-spin coupling may extend to nuclei which are 4-5 bonds apart. It is manifested as a splitting of the absorption peaks already separated by the chemical shift (hyperfine splitting). Intense magnetic fields are required to resolve the lines produced by this hyperfine splitting.

Like I.R., n.m.r. spectra are of great use in the study of chemical structures. The chemical shift yields qualitative information and the intensity of any peak yields quantitative information about groups present in the compound. In addition, the hyperfine structure yields information about adjacent nuclei and the magnitude of the hyperfine splitting yields information about geometrical relationships within the molecule. However, as with i.r. spectra, n. m. r. spectra are complex and require expert interpretation.

Mass spectrum : Mass spectrometry can sometimes provide all of the information required for the elucidation of a molecular structure. It is particularly valuable to phytochemists since it often enables a complete molecular structure to be deduced from only 10^{-6} to 10^{-9} g of material.

A mass spectrum may be shown as a plot of the abundance of ion against mass, and hence is not at all like the various types of electromagnetic spectra.

Unfortunately, unlike electromagnetic spectral techniques, mass spectrometry is a destructive technique, but fortunately, the amounts of material consumed are very small.

Principle : Mass spectrometry involves the production from a compound of an ionized form of the parent compound (parent molecular ion) and ionised fragments (fragment ions). These are all positively charged ions and are separated on the basis of their different mass/charge (m/c) values, and their masses may be determined to one part in 10^6 .

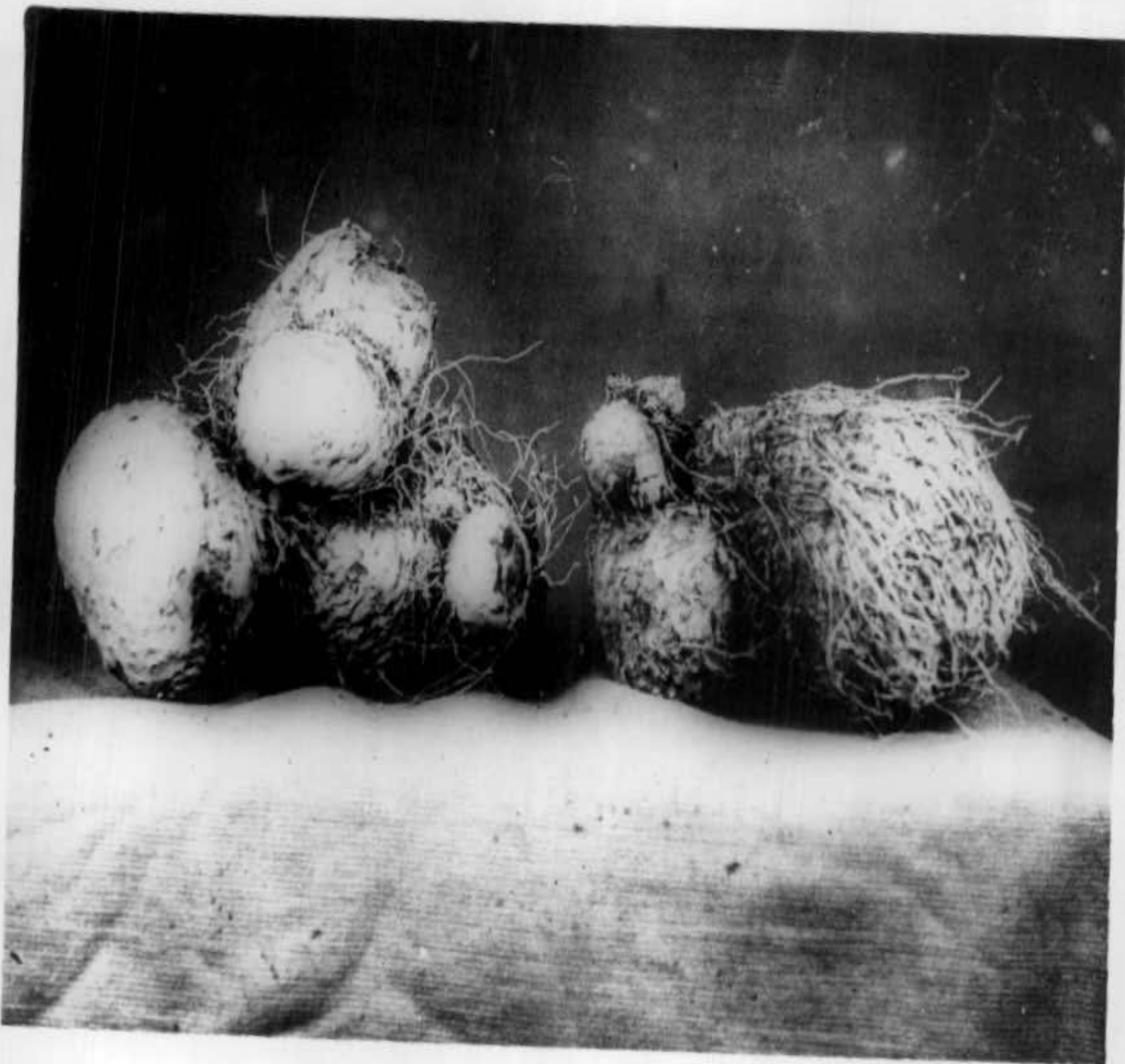
The majority of ions produced during the initial ionisation procedure have a single positive charge, i.e., one electron is removed from the molecule or fragment. Thus, the ions produced differ only in their mass. Occasionally, however, molecules lose more than one electron and multicharged - ions are produced. When two electrons have been lost, the ion exhibits an apparent mass to charge ratio one half that of the corresponding single charged ion. When producing ions by electronic bombardment the degree of fragmentation of molecule depends on the energy of the

bombarding electrons. At low energies ($1-2 \times 10^{-12}$) only one electron is removed from the molecule. The resulting positively charged molecule ion has a m/c value corresponding to the molecular weights of the parent compound. At the usual operating electron beam energy of 10^{-17} J, the molecules are cleaved into positively charged fragments of different masses. The way in which compound fragments to give a mass spectrum is characteristic of the compound being analysed and is called the cracking pattern. From this pattern the structure of the molecules can be deduced.

The mass spectra show a series of peaks or lines corresponding to the m/e values of the positive ions produced from the compound. The height of the peaks corresponds to the relative abundance of the ions. The parent ion is the peak with the greatest mass.

S e c t i o n V

P E Y T O C H E M I S T R Y



TUBERS OF DIOSCOREA HISPIDA VARIETY DAEMONA

MATERIALS AND METHODS :

Identification : The tuber (Figure-1) was identified as Dioscorea hispida Dennst variety DAEMONA (Roxb) on the basis of botanical characteristics with the help of Prof. P. V. Bole, St. Xavier College, Bombay.

Collection, Drying and Storage : The fresh tubers of Dioscorea hispida (6 gunny bags) were collected from Dapoli District of Konkan area, in the month of December, 1975. The tubers were washed to make them free from soil and cut into slices by the sharp steel knife. The slices were dried at ambient temperature (31°C) for overnight and milled to a coarse powder in a grinding machine. The semidried powder was subjected to complete drying at 31°C in shed. The dried powder was stored in a wooden drum in a dry place. Some fresh yams were also stored in the cool shed under dry earth without deteriorating the tubers.

Extraction : The fine coarse powder (60 to 100 mesh) was wetted with 500 ml of solvent and imbibed for four hours in a closed container. Imbibed powder was packed uniformly in a Soxhlet apparatus and macerated for overnight.

Next day more solvent was added and was exhaustively extracted with petroleum ether (40-60°, 3 litres), ether (3 litres), chloroform (2.5 litres) and ethyl alcohol 90% (2.5 litres) in succession. The solvent from the extracts were removed under reduced pressure. The marc after Soxhlet extraction was macerated with chloroform water for 24 hours. It was concentrated under vacuum evaporator and dried in a vacuum desiccator.

Recovered solvents were reused for extraction and all five extracts were subjected to preliminary pharmacological studies. This solvent system was replaced by methanol alone and the large quantity methanolic extract was prepared, methanol removed by distillation and crude extract was stored in an amber coloured bottle. This extract was further subjected to the isolation of total alkaloid.

Isolation of total alkaloid present in the tubers :

Hundred grammes of methanolic crude extract almost free from methanol was diluted with two litres of chilled water and made alkaline to pH 10.5 to 11.0 by adding slowly and carefully the strong ammonia with constant stirring by means of magnetic stirrer for 15 minutes. Aliquot portion

of chilled preparation was transferred to a two litre capacity separating funnel and was shaken with 500 ml of chloroform. When the liquids were shaken in the separating funnel, emulsification occurred between the chloroform and the aqueous solution, making it exceedingly difficult or impossible to separate the liquid. The formation of emulsion could frequently be avoided by rotating the separating funnel slowly and occasionally inverting in place of shaking vigorously. Emulsion occurred during this procedure was broken by the following processes :

1. addition of more of the chloroform,
2. gentle stirring with the glass rod, and
3. drawing of the separated solvent as rapidly as it was separated.

Chloroform washings were given till final wash contain negligible quantity of alkaloid.

All chloroform washings were pulled together and chloroform was recovered by distillation. The traces of chloroform from the residue were best removed by treating the residue with few ml of ethanol and evaporating the solution to dryness again. The crude alkaloid was subjected to preparative thin layer chromatography.

Preparative Thin Layer Chromatography : The

method of the preparative thin layer chromatography with some modifications was used for isolation of the alkaloids.

A glass sheet of 45 X 45 cm. was covered by a uniform thin layer of an adsorbent silica gel G containing calcium sulphate as a binding agent which facilitated the adsorbent sticking to the plate. The plate was prepared by spreading a slurry of an adsorbent in water (125 g of silica gel in 250 ml of glass distilled water) over it by means of clean uniform glass rod starting at one end and moving progressively to the other and then allowed to dry for an overnight at room temperature and activated in the next morning by heating in an electric oven at 100°C for one hour. Drying served to remove water and to leave a coating of thickness 2 mm of adsorbent on the plate. The plate was allowed to cool gradually (2 hours) and used for separation immediately.

The crude alkaloid was applied to the plate by a tuberculin syringe having needle (26 gauge) at least four centimeters from the edge. The chloroform was removed from the sample, by use of an air blower allowing further material, till total material had become 100 mgs, to be

applied to the band. Care was taken to ensure that the adsorbent did not flake off the sample application band.

Separation took place in a chromatography glass tank (50 X 55 X 15 cms.) which contained the developing solvent methanol and chloroform in proportion of 10 to 90 to a depth of 3 cms. This solvent system was found to be the best among (1) benzene + ethanol (absolute) in different proportions; (2) benzene + petroleum ether + methanol (40+40+20); (3) Petroleum ether + Acetone + Methanol (20+70+10); and (4) Chloroform + Methanol in different proportions - 99:1, 90:10, 75:25, 50:50, 25:75, 10:90 and 1:99. This was allowed to stand for at least 6 hours with a cover of glass plate over the top of the tank to ensure that the atmosphere within the tank became saturated with solvent vapour. Unless this was done, irregular running of the solvent with poor separation was observed. When equilibrium was occurred the cover-plate was removed and the plate was placed vertically in the tank so that it stood in the solvent. The cover plate was removed after 10 hours i.e., after reaching the solvent to the top, the plate was carefully taken out of the tank and dried in an electric oven at 60°C to remove all the vapours of the developing solvent.

Detection of alkaloids : In a fume-hood the plate was gently and evenly sprayed with Dragendorff reagent by atomizer. The alkaloids were named as Fraction I, II, III and IV from bottom to top.

Recovery of samples : Fractionwise the relevant area of silica gel were scrapped off the plate with a spatula and collected in the amber coloured bottles.

Thus, twenty-five plates were prepared and the material was collected in the respective bottles. After collecting sufficient amount of scrapped material, it was subjected to the following procedure in order to elute the maximum amount of free base from the adsorbed alkaloidal complex.

Hundred grammes of silica gel was treated with 500 ml of chloroform with constant stirring on a magnetic stirrer till dispersed uniformly. Dilute ammonia was added slowly until yellow colour of silica gel was completely disappeared. The mixture was filtered through whatman paper and chloroform elute was collected. Alkaloid was obtained from the elute by evaporating the solvent in a flash evaporator. This procedure was repeated for all fractions.

These were further subjected to thin layer chromatography in order to see whether the fractions thus collected of the alkaloids were single or a mixture of alkaloids.

Identification of single spot on thin layer chromatography :

Preparation of thin layer plates : The glass plates were coated (20 X 20 cms) with a thin layer of silica gel (250 μ thickness) with the help of movable spreader. The plates were allowed to dry at room temperature and activated in a oven at 110°C for 30 minutes.

Application of test solutions : About 10 mg of each fraction was dissolved in 10 ml of methanol and approximately 10 μ ml of the solution was applied as a single spot 2 cm from one end of the glass plate and 2.5 cm away from each other with the help of lamda pipette.

Separation chamber and development of chromatogram : The glass chamber of 21 X 21 X 9 cms. specially made for TLC was filled with different developing solvent system to a depth of about 1 cm. After 3 hours

the spotted glass plate was placed carefully in the chamber and covered with the lead. When the solvent front had moved for about 15 to 16 cms, the plate was taken out of the chamber and dried at room temperature. The plate was then sprayed with Dragendorff reagent and the number of spots whether single or mixture were confirmed.

Solvents used :

Benzene + Ethyl acetate (70 + 30)

Chloroform + Methanol (90 + 10)

Chloroform + Methanol + acetic acid (120 + 20 + 2)

Chloroform + Methanol + Ammonium hydroxide (120+20+2).

Crystallization : Attempts were made to crystallise Fraction II and Fraction III in different solvent systems such as methanol, methanol-chloroform mixture, methanol-petroleum ether, ether, ethanol.

Fraction II was purified by recrystallisation in methanol-chloroform (25:75). Fraction III was purified by preparing its hydrochloride and base repeatedly.

Same procedure was repeated for Fraction I and Fraction IV.

Melting point : Melting point of crystallised Fraction II was studied on an electrically heated melting point apparatus till it reproduced sharp and constant melting point.

Elemental analysis : The following test was performed for the detection of nitrogen, halogens and sulphur.

Sodium fusion test : (Lassalagne's test)

The short tube of hard glass (6-7 cm X 1 cm) was taken, to it a dry clean piece of sodium was dropped and heated it. When fused, a little of substance dropped over it and heated the tube at first gently. Further small quantity of the substance was added and then heated to redness for three minutes. The tube was broken in distilled water in a dish. Concentrated by boiling and filtered. The filtrate was divided into portions and tested as follows :

Test for nitrogen :

(a) 10 ml of the filtrate added to 5 ml of saturated ferrous sulphate and one drop of 5% sodium hydroxide, boiled for two minutes, added two drops of saturated solution

of ferric chloride, cooled and acidified with concentrated hydrochloric acid.

(b) Test for sulphur : To the 2 ml of the filtrate, added one drop of 5% sodium hydroxide solution followed by four drops of fresh sodium nitroprusside solution (1%).

(c) Test for halides : 5.0 ml of filtrate was acidified with dilute sulphuric acid, boiled for 5 minutes and then added a few drops of dilute nitric acid and silver nitrate.

Micro-analysis :

Ultraviolet spectra : The ultraviolet spectrum of alkaloid A and alkaloid B were recorded by means of Ultraviolet Visible, Perkin-Elmer, 402.

<u>Specifications</u>	<u>Alkaloid-A</u>	<u>Alkaloid-B</u>
Concentration	0.1 mg/ml	0.1 mg/ml
Solvent	Chloroform	Chloroform
Range	200 to 400 mμ	200 to 400 mμ
Cell	1 cm breadth	1 cm breadth.

Infrared spectra : The infrared spectra of alkaloid A and alkaloid B were recorded by means of Perkin-Elmer Grating Infra-red Spectrophotometer, Model 257.

<u>Specifications</u>	<u>Alkaloid-A</u>	<u>Alkaloid-B</u>
Preparation of sample	KBr Disc	Solution in chloroform
Concentration	2 mg/disc	1 mg/ml
Scan speed	Maximum	Maximum
Slit width	Normal	Normal
Wavenumber	625-4000 cm^{-1}	625-4000 cm^{-1} .

Nuclear magnetic Resonance : The nuclear magnetic resonance of alkaloid A and alkaloid B were recorded by means of varian 60 MHz N.M.R.

<u>Specifications</u>	<u>Alkaloid-A</u>	<u>Alkaloid-B</u>
Sweep offset (H_3)	500	500
Spectrum amplitude	16	25
Integral amplitude	415	415
Spinning rate (RPS)	60	60
Sweep time (Seconds)	250	250
Sweep width (H_3)	500	500
Filter	1	1
Rf power level	0.05	0.075
Solvent	Carbon tetra-chloride	Carbon tetra-chloride + Chloroform.

Tests for lactone ring :

1. Freshly prepared 1% alkaline sodium nitroprusside solution was added to the 5 mg of alkaloid-A and alkaloid-B. Changes in colour were noted.

2. One mg of potassium iodate (Anala^R) and one drop of concentrated sulphuric acid were added to the 5 mg of each alkaloid taken on a white tile. Colour reactions were recorded.

Mass spectra : Mass spectra of alkaloid-A and alkaloid-B were taken.

<u>Specifications</u>	<u>Alkaloid-A</u>	<u>Alkaloid-B</u>
Temperature of inlet	130	70
Energy	70	70
Ionising current	20	20

RESULTS :

Exhaustive extraction of 4000 g of dried powder with solvents - petroleum ether, ether, chloroform, ethyl alcohol (90%) in succession and maceration with chloroform water produced corresponding crude extracts weighing 4 g, 3.5 g, 10 g, 25 g and 60 g, respectively. Preliminary pharmacological studies (on chicken and rat phrenic nerve diaphragm) indicated the presence of potent depolarising type of neuromuscular blocking agent in all the above extracts. Preliminary phytochemical studies suggested that all the extracts may contain an alkaloid as one of the major constituent on the following chemical reactions.

- (a) Pink colour to Dragendorff reagent.
- (b) Yellow colour to Wagner reagent, and
- (c) White precipitate to Mayer's reagent.

Petroleum ether contains steroid in addition to the alkaloid.

This procedure of sequential extraction was a time consuming, hence solvent system was modified so that complete extraction of an active constituent i.e., n.m.b. agent could be possible. Methanol alone proved to be the best. Aqueous extract after Soxhlet extraction with

methanol did not show a neuromuscular blocking activity and also failed to produce colour test to Dragondorff reagent and Wagner reagent and white precipitate to Mayer's reagent.

Methanolic extract : 400 g. extract was obtained from 4 kg. and dried powder. The extract was brownish, resinous semisolid. It was less soluble in water, ethyl alcohol, ethyl acetate and sparingly soluble in carbon tetrachloride, benzene and petroleum.

Crude alkaloid : Total yield was 10% of the methanolic extract. It is hygroscopic, deteriorable at higher temperature (more than 250°C).

Preparative thin layer chromatography : The crude alkaloid was resolved into four main fractions. These were located by the Dragondorff reagent as the pink areas. It indicated that Fraction-II and Fraction-III were the major ones, while Fraction-I and Fraction IV were the minor alkaloids.

Recovery of the alkaloids :

Total crude alkaloid loaded : 2.5 gms.

<u>Fraction</u>	<u>Fractions obtained in mg.</u>	<u>% Composition.</u>
Fraction-I	75	3.0
Fraction-II	187.5	7.5
Fraction-III	187.5	7.5
Fraction-IV	100.0	4.0

Thin layer chromatography :

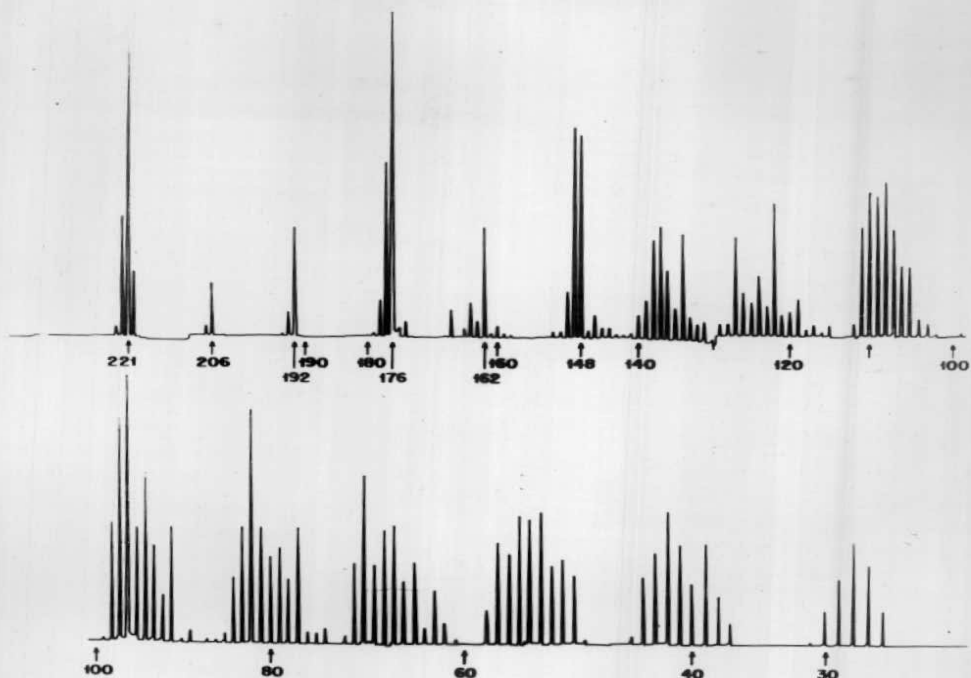
<u>Fraction</u>	<u>No. of the spots.</u>
Fraction-I	3 to 4
Fraction-II	single
Fraction-III	single
Fraction-IV	2

Physical studies :

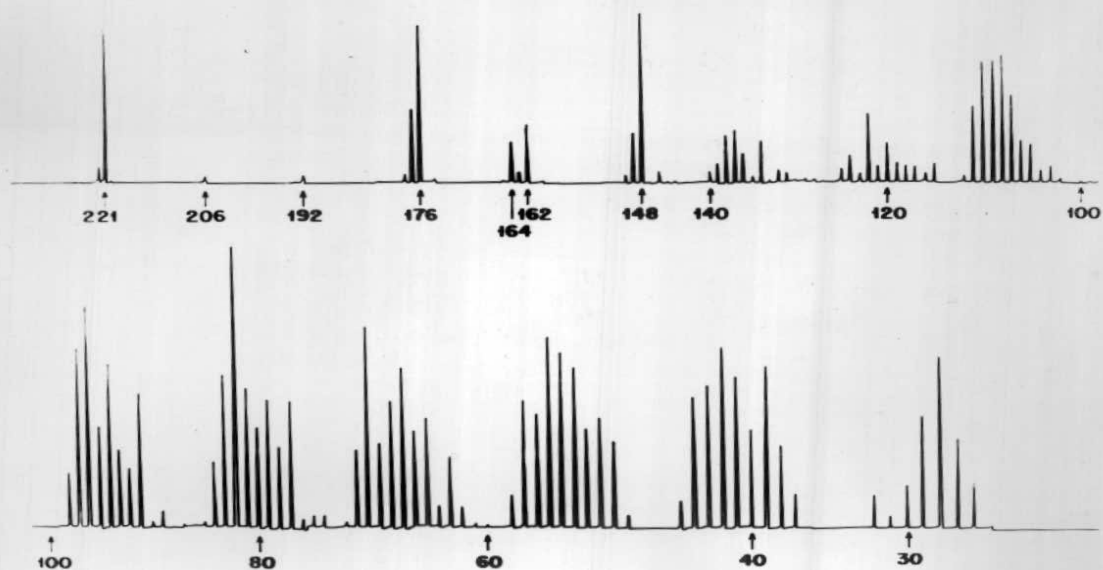
Fraction-I : The material was not separated in pure form as the amounts were low.

Fraction-II : It is named as alkaloid-A, is a odorless, yellowish white crystalline compound having melting point 89°C sharp. It is insoluble in water, soluble in methanol, chloroform and acetone.

MASS SPECTRUM OF COMPOUND-A



MASS SPECTRUM OF COMPOUND-B



Fraction-III : It is named as alkaloid-B, is a semisolid yellowish brown could not be crystallized, characteristic odour, soluble in water, methanol, chloroform, acetone.

Fraction-IV : Brown viscous semisolid.

Elemental analysis :

	<u>Nitrogen</u>	<u>Sulphur</u>	<u>Halides</u>
Fraction-I	+	-	-
Fraction-II	+	-	-
Fraction-III	+	-	-
Fraction-IV	+	-	-

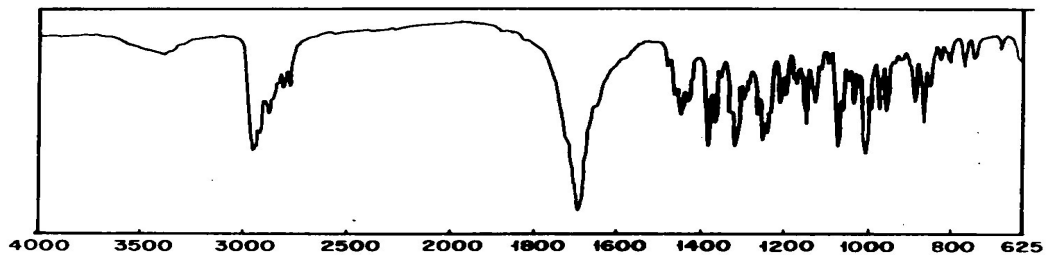
Micro-analysis :

<u>Elements</u>	<u>Alkaloid-A</u>	<u>Alkaloid-B</u>	<u>Calculated</u>
Carbon (%)	70.10	70.3	70.6
Hydrogen (%)	8.82	8.7	8.6
Nitrogen (%)	6.28	6.25	6.3
Oxygen (%)	14.8	14.75	14.5

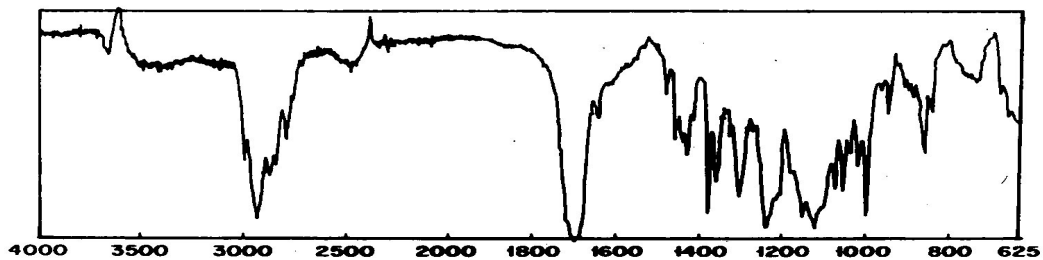
Mass spectra indicate that both alkaloids are having the same molecular weight i.e. 221. Molecular formula on the basis of microanalysis and mass spectra can be suggested as $C_{13}H_{19}O_2N$.

INFRA-RED SPECTRA

SAMPLE - A

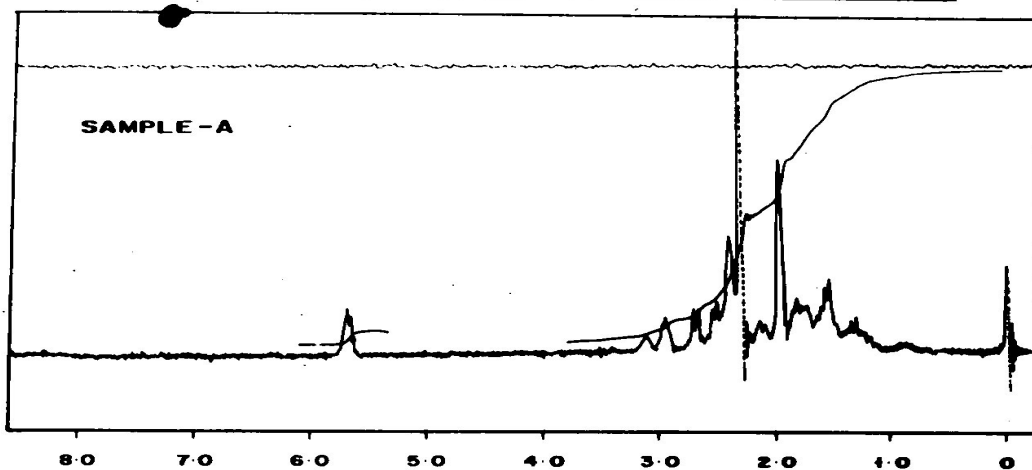


SAMPLE - B

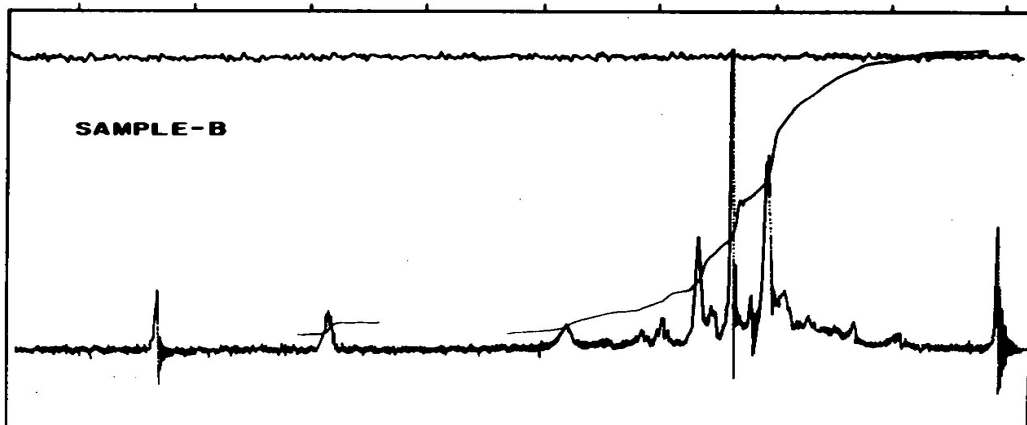


NUCLEAR MAGNETIC RESONANCE SPECTRA

SAMPLE - A



SAMPLE - B

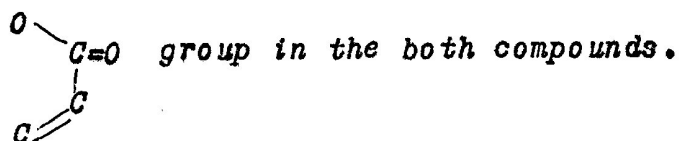


Spectronic studies :

Ultraviolet spectra : Ultraviolet spectra of alkaloids-A and B showed the single absorption maxima at 2470°A indicating the presence of an ethylenic bond conjugated with carbonyl group of a lactone ring.

Infrared spectra : Infrared spectra of A and B alkaloids showed the carbon-carbon stretching at 1690°A indicates the presence of six membered lactone ring.

Nuclear magnetic Resonance : Nuclear magnetic resonance of both compounds indicate the presence of



Both alkaloids produced reddish violet colour with alkaline sodium nitroprusside and violet colour with potassium iodate and sulphuric acid indicated the presence of lactone ring.

Reagents used :

Dragondorff's Reagent :

Stock solution : A mixture of 25 ml acetic acid, 2.6 g basic bismuth carbonate and 7.0 g sodium iodide is

boiled for few minutes. The copious precipitate of sodium acetate is filtered off through a sintered glass filter, after about 12 hours 20 ml of the clear red-brown filtrate are mixed with 80 ml of ethyl acetate and 0.5 ml water is added. This solution was stored in ambered colour bottle.

Spray Reagent : A mixture is made of 10 ml of stock solution, 100 ml acetic acid and 240 ml ethyl acetate.

Wagner reagent : 1.0 g iodine and ³10 g of potassium iodide are dissolved in 50 ml water by warming and 2 ml acetic acid added. The solution is made upto 100 ml with water. $(2.0 \cdot I_2 \rightarrow 6 \text{ g m l}^{-1} \text{ in } 100 \text{ ml})$

✓ Mayer's reagent : 1.355 g of Mercuric chloride dissolved in 60 ml of water, and 5 g of potassium iodide dissolved in ¹⁰20 ml of water, mixed and diluted with water to 100 ml.

S e c t i o n VI

P H A R M A C O L O G Y

MATERIALS AND METHODS :

The pharmacological studies of the crude extract in vivo and in vitro preparations were carried out.

In vivo effects : In these studies the crude alkaloid in the form of suspension was administered to different experimental animals and the effects were noted.

In vitro effects : From the observations of in vivo effects, one gets an indication of the type of action produced and accordingly different tissues were selectively studied for the drug action. Since the major effect was on the neuromuscular junction as a primary site of action, following tissues were selected for further study :-

- (1) Isolated rat phrenic nerve diaphragm preparation,
- (2) Frog rectus abdominis muscle preparation,
- (3) Isolated chick biventer cervicis muscle preparation.

In vivo studies : White male leghorn (*Gallus-gallus*) chicks weighing about 25 to 30 gms and 1-5 days old were injected with a crude alkaloid through the neck

vein in doses of 10 to 15 ug per bird. Crude alkaloid was also administered orally in dose range of 1 to 2 mg per bird.

Kasauli mice (either sex) weighing 18 to 20 gms were administered crude alkaloid in doses of 100 to 150 ug per animal intravenously and 200 mg per kg orally.

Albino rats (either sex) weighing 150 to 200 gms were administered crude alkaloid in doses of 1 mg to 2 mg per animal by intravenous route and 200 mgs/kg by oral route.

Guinea-pigs (either sex) weighing 200 to 250 gms were administered crude alkaloid in dose of 3 mg/kg by intravenous route.

Rabbits (either sex) weighing 1.5 to 3 kg were administered crude alkaloid in dose of 2 to 3 mg/kg by intravenously and 40 to 50 mg/kg by orally.

Stray cats of either sex weighing 2 to 3.5 kg were administered crude alkaloid in doses of 1 mg per kg intravenously.

Monkeys (Muceucea mulata) of either sex weighing 3 to 4.5 kg were administered crude extract in doses of 3 mg/kg intravenously and 24 mg/kg orally.

LD₅₀ : White male leghorn chicks weighing 25 to 30 gms of 1 to 5 days old were selected and grouped with six birds in each group. Birds in each group were administered specific doses of crude extract intravenously through neck vein ranging from 10 to 100 ug so that no two groups with consecutive doses showed 0 % or 100% effects. at the end of 24 hours for each group. Percentage of surviving animals corresponding to the dose range was found out for each group and then LD₅₀ value was calculated by the method of Wilcoxin et al.¹¹⁰.

Cat tibialis anterior nerve muscle

preparation¹¹¹ : A cat weighing about 2.5 kg to 3.5 kg was anaesthetised with intraperitoneal injection of 40 mg/kg of pentobarbitone sodium, when the animal was anaesthetised, trachea was exposed and a metal canula was inserted into and firmly tied in the place. The animal was kept on the artificial respiration.

Femoral vein was canulated with a polythene canula. Another polythene canula was inserted into the

femoral artery upto the bifurcation of abdominal aorta (which was confirmed at the end of experiment by dissection). The other end of the canula was connected to a three way connection and the whole unit was filled in with saline, which was heparinized to prevent clotting. The lateral peroneal nerve in left side was dissected free and a pair of platinum electrodes were applied and the nerve protected with cotton soaked in liquid paraffin. The electrodes were firmly placed in contact with nerve.

The tendon of tibialis anterior was then freed from its attachment and the skin was incised from tibialis to the head of tibia. The transverse ligament of the ankle was cut and the muscle was freed and raised from underlying extensor longus digitorum. An incision was made from the bone knob along the front of leg nearly to the knee. The animal was fixed on the stand by drills through the lower end of tibia and condyles of the femur. The tendon was then dissected upto belly of tibialis anterior muscle and the tendon was attached to isometric myographic lever. The belly of muscle was freed from connective tissue and the exposed tissue was moistened with paraffin to minimize dehydration. The contractions of the muscle were recorded on stimulation with supramaximal voltage of about 10 volts at frequency 0.1 Hz and duration of stimulation 0.5 m.second.

The gastrocnemius sciatic muscle nerve

preparation of cat¹¹² : A cat weighing 2.5 to 3 kg was anaesthetized with chlorolose 40 mg/kg intraperitoneally. Animal was placed on its back on a table. The fur on the dorsal aspect of the thigh and lower leg was removed. The trachea and femoral vein were cannulated. A vertical incision was made from the knob of bone on the inside of the foot along the front of the leg nearly to the knee, to expose subcutaneous fat and muscle. All the vessels were carefully ligated and the gap between the two major groups of muscles were opened up gently to expose the sciatic nerve. The nerve supplying the gastrocnemius muscle was carefully isolated and a loose ligature placed under it. A steel pin was driven through the lower end of the femur to fix the knee joint. The muscle was freed of its surrounding structures and tendon of insertion located on the anterior aspect of the ankle. The tendon was ligated and the suture led over a pulley to be attached to the bar of a palmer isometric lever. For close intra-arterial injection of drugs, the femoral artery was cannulated. The cannula was tied securely in place. Bipolar electrode was placed on the nerve to gastrocnemius muscle stimulated at the rate of six shocks per minute at 15 volts by square wave pulses of about 0.5 millisecond duration.

In vitro studies :

Isolated rat phrenic nerve diaphragm

preparation¹¹³ : Adult albino rat weighing about 150 to 200 gms was killed and bled cut. Thorax was opened on left side and sternum and its frontal part was removed. A cut was made at frontal insertion of the diaphragm. Lung lobes were removed. A diaphragm along with phrenic nerve was now cut. The diaphragm was then cut in a fin-like manner and the preparation was fixed on the electrode, meant for the purpose. The nerve was placed on platinum electrodes and the preparation was then placed in Kreb's solution. The solution was bubbled with carbogen (95% oxygen plus 5% carbon dioxide) in a bath of about 30 ml maintained at a temperature of 37°C. Depending on the purpose of the experiment, the preparation was stimulated at a specific voltage, amplitude and pulse width as described below :

a) Normally the preparation was stimulated indirectly at a supramaximal voltage of 2 to 4 volts with 0.5 millisecond duration at a frequency of 0.2 Hz.

While studying the effect of crude alkaloid it was administered in dose range of 50 to 200 microgram/ml

and the time taken for complete blockade of responses to indirect stimulation was noted in each dose, respectively.

b) After complete block to the responses of indirect stimulation, direct muscle stimulation was recorded at 100 volts with 5 millisecond duration at a frequency of 0.2 Hz.

c) The effect of neostigmine at initial and final stages of the block were recorded at the doses of 2 to 4 ug/ml.

Frog rectus abdominis muscle preparation : Frog (Rana tigrina) weighing about 100 to 200 gms was used. The rectus abdominis muscle was isolated and isotonic contractions were recorded on a smoked drum.

The tension on the muscle 1.5 g.

Magnification of contraction 10-12 times

The recorded maximal contractions varied from 2 to 8 centimeters.

After administration of drug, contractions were recorded for 90 seconds. The drum was kept static during contractions and the muscle was washed twice or thrice.

for 10 to 15 minutes with Ringer's solution till it completely relaxed.

Dose response curve for acetylcholine in doses of 0.5 to 1 ug per ml were taken. Crude alkaloid was added to the bath. For every dose of crude alkaloid, dose response curve for acetyl-choline was repeated and percentage block was noted.

Isolated guinea-pig ileum : The adult guinea pig weighing about 200 to 300 gms was killed by a blow on head and bled out. The ileum was taken out. To a piece of ileum measuring about 2 to 4 centimeters, a thread was attached on both the sides and the preparation was then mounted in a bath of 10 ml capacity, filled with Tyrode solution. A thread attached to upper end of the piece was connected to a frontal writing lever and contractions recorded.

Dose response curve for acetylcholine were recorded.

After administration of crude alkaloid in the concentration of 50 to 100 ug/ml, the dose response curve was repeated and change, if any, noted.

The weight applied to the lever 0.5 gms.

Magnification of the lever..... 10-12 times.

The contractions were recorded for 30 seconds.

Isolated chick biventer cervicis muscle :

preparation¹¹⁴ : White leghorn chick weighing about 50 gms and of 15 to 21 days old were anaesthetised with urethane chloralose anaesthesia by intraperitoneally route (urethane 250 microgram/ml + chloralose 15 microgram/ml) by 0.1 ml of intraperitoneal injection. The back of the neck was plucked and the skin incised along the midline from the skull to below the neck, exposing the two biventer cervicis muscles on either side of the midline. A thread was tied around the upper belly of one of the muscles which was then cut free from its attachment to the skull. By pulling the thread gently, the muscle was separated from the underlying semispinalis cervicis muscle. The upper tendon and muscle was carefully removed together with the lower tendon.

The electrode for stimulation was prepared by using two platinum wires with coiled ends fixed on the other to an aeration tube. The lower end of the other to an

aeration tube. The lower end of the muscle was tied to the aeration tube and the muscle was smoothly passed through the coiled ends of platinum wires.

The tissue was placed in an organ bath filled with 30 to 50 ml of Kreb's solution, maintained at a constant temperature of 37°C . Carbogen gas (95 % oxygen plus 5% carbon dioxide) was bubbled into it. The upper end of the tendon was attached to a starling lever and the contractions recorded on a smoked drum. The preparation was stimulated at a frequency of 0.1 Hz at 20 volts with pulse width of 0.5 milliseconds.



SPASTIC PARALYSIS IN CHICK PRODUCED BY CRUDE ALKALOID
(10 ug/ i.v.)

R E S U L T S :

In vivo :

White male leghorn chicks : Birds went in spastic paralysis immediately after intravenous administration of crude alkaloid. Bird showed abrupt recovery within 10 minutes. Oral administration of alkaloid (crude) produced paralysis after 5 to 7 minutes. Effect lasted for about 1 hour.

At higher doses onset was quicker, the duration was more and at still higher dose animal died due to respiratory failure. The deep respiration was preceded by hind limb paralysis at lower doses where as at higher doses the respiratory failure resulted in death.

Kasauli mice : Depending on the dose of crude alkaloid, administered intravenously the animals showed muscle paralysis, and tremors. Respiratory rate was increased and cyanosis was observed. Animals died due to respiratory paralysis, while heart beats continued even after death for some time. The animals showed micturation and defaecation just after injection.

The same effects were observed on oral administration at doses of 200 mg per kg. Onset of action was after 10 minutes and effect lasted for 45 to 50 minutes.

Albino rats : The symptoms after administration of the crude alkaloid, intravenously and orally, in general, resembled to those observed in case of mice. The animal died due to marked convulsions. Lethal dose was 2 mg per animal intravenously and 40 mg orally.

Guinea pigs : At lower doses, it showed paralysis while at higher doses tremors followed by convulsions and death were observed. Lethal dose - 3 mg/kg.

Rabbits : Rabbit showed flaccid paralysis, salivation, tremors and convulsions at doses of 1.5 to 3 mg/kg. Animal survived till doses of 1.5 mg/kg. Recovery was seen after 3 to 4 hours.

Same effects were observed on oral administration at higher doses. Animal recovered completely from paralysis after 8 to 10 hours.

Cats : It showed paralysis at doses of 1 mg/kg. Deep respiration, relaxation of nictating membrane, heavy salivation, relaxation of limb muscles, micturation and convulsions were observed. Twitchings lasted for about 10 minutes after death.

Lethal dose - 1 mg/kg.

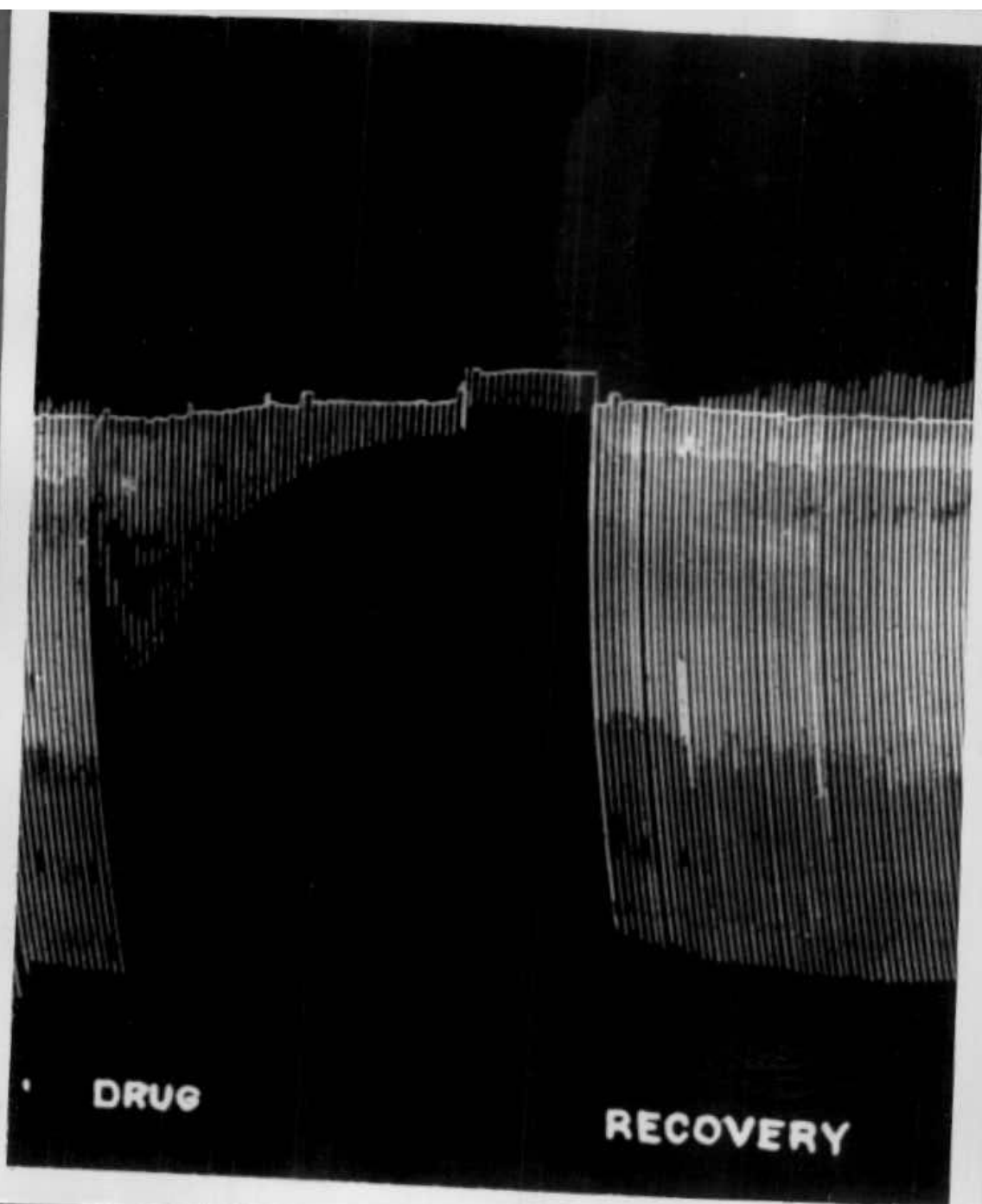
Monkey (Muceaca mulata) : Hind limb paralysis, deep respiration, tremors and convulsions were noted. At higher doses (3-4 mg/kg) convulsions followed the death.

Monkey survived upto 2 mg/kg intravenously. It did not exhibit any effect upto doses of 24 mg/kg on oral administration.

LD₅₀ (chick) intravenous route :

0.62 (0.51 to 0.73) milligrams/kg.

All these observations suggest that the absorption of neuromuscular blocking agent from gastrointestinal tract is better in rabbits than in the other animals. The relationship between intravenous and oral minimum effective dose is given in the Table .



CAT GASTROCNEMIUS MUSCLE PREPARATION
Drug: Crude Alkaloid, Close I.A. 1.5 mg.

Table

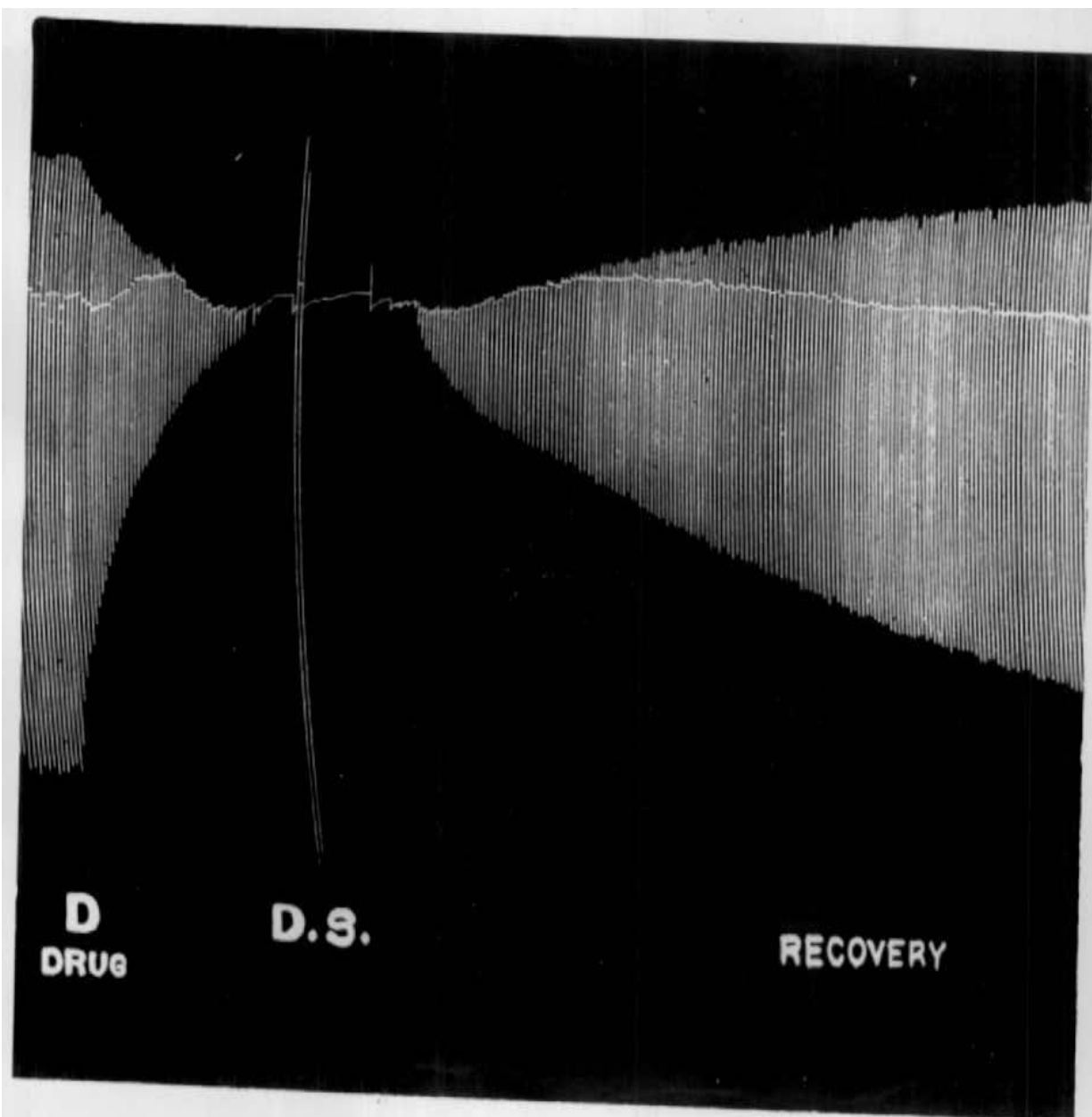
Relationship between intravenous and oral minimum effective dose in different species.

SPECIES	Minimum Effective Dose (mg/kg)		Ratio of i.v./oral dose
	Intravenous route	Oral route	
Chicks (Avian)	0.4	40	1 / 100
Mice and Rats (Rodent)	5.0	200	1 / 40
Rabbits (Mammals)	2.0	40	1 / 20

Cat tibialis anterior nerve muscle

preparation : The crude alkaloid at a dose of 1.5 mg given close intra-arterially in cat tibialis anterior nerve muscle preparation produced initial fasciculations followed by a progressive neuromuscular block. This effect was not antagonised by neostigmine but blockade was completely reversible by itself.

Cat gastrocnemius muscle preparation : When the drug was administered at doses of 1.5 mg/kg, close intra-



ISOLATED RAT PHRENIC NERVE DIAPHRAGM PREPARATION

D : Crude alkaloid (100 ug/ml)
D.S. : Direct Muscle Stimulation.

arterially it produced biphasic neuromuscular block, viz. a sudden block upto 50% followed by steady block which was completely reversible after two hours.

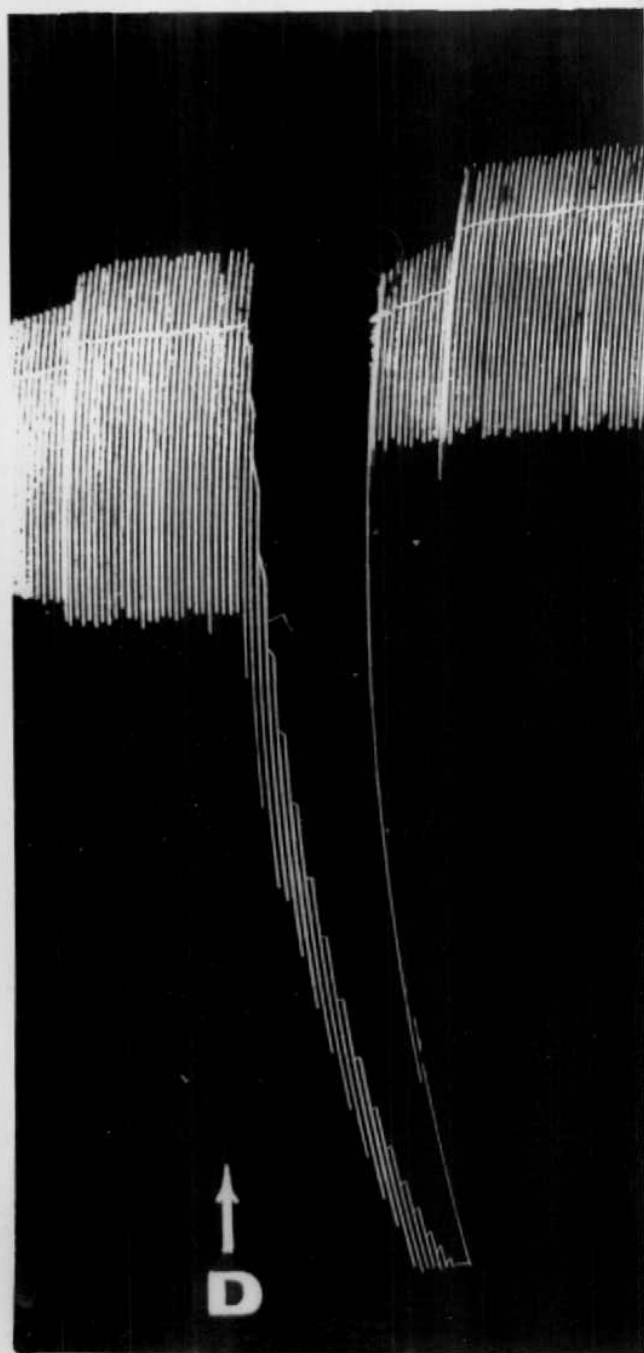
In vitro :

Isolated rat phrenic nerve diaphragm preparation :

It was observed that after administration of crude alkaloid 100 ug/ml the responses to indirect stimulation of the nerve muscle preparation progressively reduced. After complete blockade to responses indirect stimulation, the responses to direct muscle stimulation were not affected. The time taken for complete development of the block was dependent on the dose of crude alkaloid given. Higher the dose the quicker the block. Single washing of a blocked preparation showed complete recovery.

Antagonism to neostigmine : Neostigmine in the dose range of 2 to 4 $\mu\text{g/ml}$ temporarily antagonised the neuromuscular block produced by the crude alkaloid at the initial stages while deepened at the later stages.

Frog rectus abdominis muscle preparation : Crude alkaloid produced 40 to 80% block of the rectus abdominis muscle preparation of frog at doses of 15 to 60 $\mu\text{g/ml}$.



ISOLATED CHICK BIVENTER CERVICIS MUSCLE PREPARATION

Drug : Crude Alkaloid, 10 ug/ml.

It exhibited contractions by itself at higher doses than 100 $\mu\text{g/ml}$.

Isolated guinea-pig ileum : *On isolated guinea-pig ileum, the crude alkaloid produced a dose dependent contraction in dose range of 50 to 100 $\mu\text{g/ml}$.*

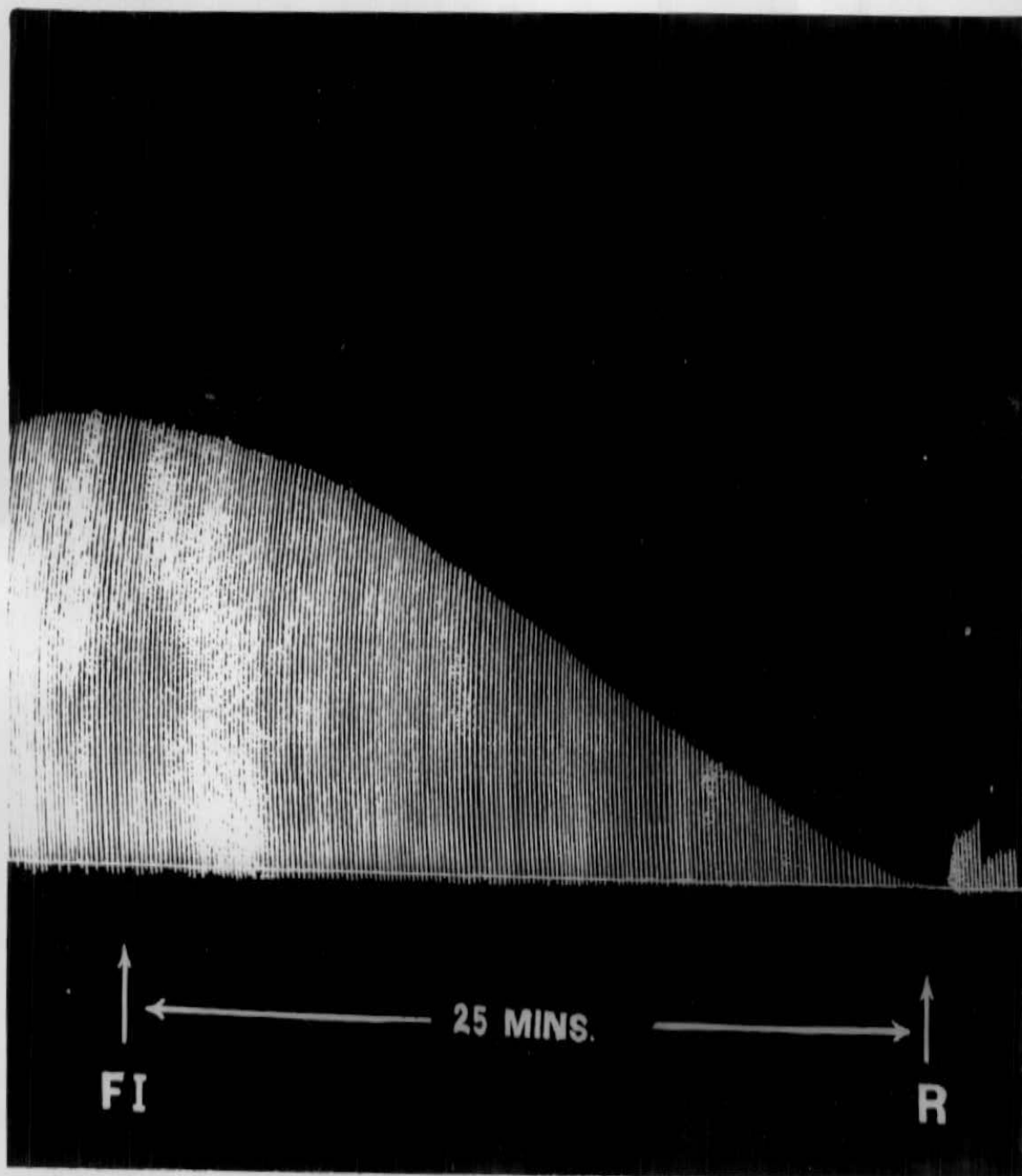
Isolated chick biventer cervicis muscle preparation : *The crude alkaloid produced sudden contraction of this preparation at doses of 1 to 10 $\mu\text{g/ml}$, followed by complete block to indirect stimulation. It was completely reversible after wash.*

Fraction - I

In vivo :

White male leghorn chicks : *It produced spastic paralysis at dose range of 0.5 μg to 5 μg intravenously. The onset was immediate and effect lasted for 5 to 7 minutes. Bird showed abrupt recovery.*

Higher doses upto 50 μg were required to produce complete paralysis by oral route. Paralysis was seen after 5 to 7 minutes. Effect remained for 10 to 20 minutes. Birds survived upto 50 μg .



CAT TIBIALIS ANTERIOR NERVE MUSCLE PREPARATION

F-I : Fraction I

R : After 4 hours

Albino mice : Mice showed flaccid paralysis on intravenous administration at dose of 10 μg /animal.

Gasping was observed at this dose.

At higher doses animal showed mild tremors and died due to respiratory failure.

Cat tibialis anterior nerve muscle preparation :

Fraction I produced complete block of tibialis anterior nerve muscle of cat at dose of 100 μg given close intra-arterially. This block was completely reversible by itself.

In vitro :

Isolated rat phrenic nerve diaphragm preparation :

The responses to indirect stimulation of the nerve muscle preparation were reduced after administration of Fraction-I at doses of 10 to 20 $\mu\text{g}/\text{ml}$.

Frog rectus abdominis muscle preparation : Fraction-I

antagonised the effect of acetylcholine at lower doses i.e., 1 to 2 $\mu\text{g}/\text{ml}$ on frog rectus abdominis muscle preparation. Itself contractions were produced at doses higher than 5 $\mu\text{g}/\text{ml}$.

Fraction - II (Alkaloid-A) :

In vivo :

White male leghorn chicks : Alkaloid-A did not exhibit paralysis in chicks upto doses of 1 mg/chick on intravenous administration.

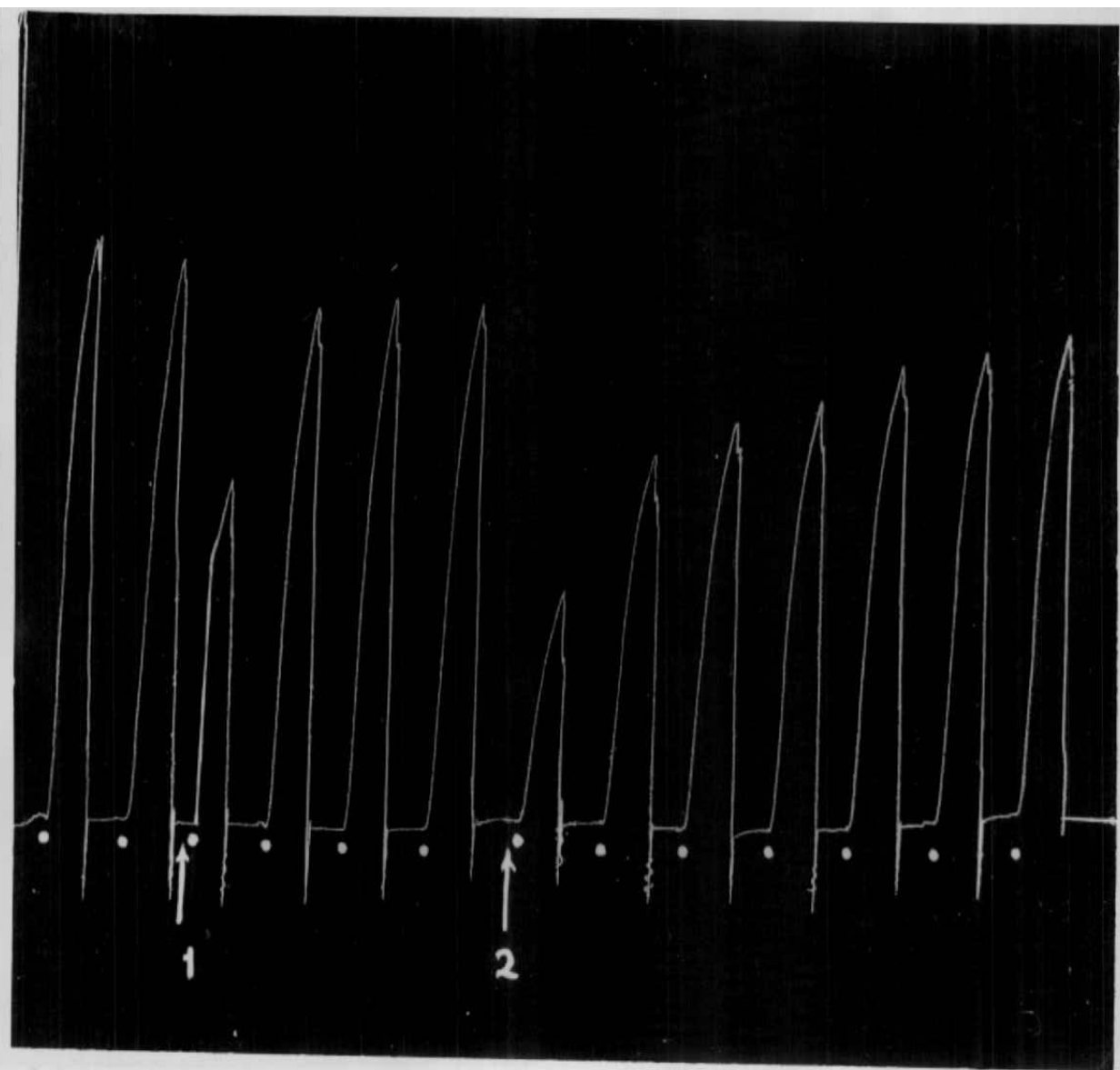
Cat tibialis anterior nerve muscle preparation :

It failed to produce block in tibialis anterior nerve muscle preparation of cat upto 2 mgs by close intra-arterially.

In vitro :

Isolated rat phrenic nerve diaphragm : It was observed that Alkaloid-A blocked the responses to indirect stimulation of the nerve muscle preparation. It did not affect direct muscle stimulation after complete blockade to indirect stimulation. Anticholinesterase neostigmine at a dose of 2 μ g/ml partially antagonised the blocking effect of the drug. The block was completely reversible after washings.

Frog rectus abdominis muscle preparation : Alkaloid-A produced dose dependent inhibition of acetylcholine contractions of rectus abdominis muscle preparation of frog



FROG RECTUS ABDOMINIS MUSCLE PREPARATION

ACh : 100 ng/ml.

1 : Fraction III 100 μ g/ml

2 : Fraction III 200 μ g/ml.

in a dose range of 50 to 200 $\mu\text{g/ml}$.

Fraction - III (Alkaloid-B) :

In vivo :

White male leghorn chicks : Alkaloid-B exhibited hyper excitation in chicks at lower doses and spastic paralysis and excitation at higher doses. Paralysis remained for a few seconds, while excitation lasted for a long time and the animal died.

Cat tibialis anterior nerve muscle preparation :

Alkaloid-B failed to produce block in tibialis anterior nerve muscle preparation of cat upto 2 mgs.

In vitro :

Isolated rat phrenic nerve diaphragm preparation :

Alkaloid-B produced the complete block at doses of 200 $\mu\text{g/ml}$ on the rat phrenic nerve muscle preparation. The effects were partially reversed by neostigmine at a dose of 2 $\mu\text{g/ml}$. The block was completely reversible after washings.

Frog rectus abdominis muscle preparation : Alkaloid-B antagonised the effect of acetylcholine on frog rectus

abdominis preparation at a dose of 100 μ g/ml.

Fraction - IV :

In vivo :

White male leghorn chicks : The bird did not exhibit the paralysis upto 1 mg intravenously.

Cat tibialis anterior nerve muscle preparation :

It did not produce the neuromuscular blockade upto 2 mgs.

In vitro :

Isolated rat phrenic nerve diaphragm preparation :

It was observed that after administration of fraction-IV, 200 μ g/ml, the responses to indirect stimulation of the nerve muscle preparation progressively reduced. After complete blockade to responses to indirect stimulation, the responses to direct muscle stimulation were not affected

Neostigmine in the dose of 2 μ g/ml did not antagonise the neuromuscular block produced by the Fraction-IV. The block was reversed after washings.

Frog rectus abdominis muscle preparation : Fraction-

IV potentiated the effect of acetylcholine on the frog rectus abdominis muscle preparation at doses of 100-200 μ g/ml.

DRUGS AND CHEMICALS USED

1. *d*-Tubocurarine chloride (Burroughs Wellcome & Co.)
2. Succinylcholine chloride (Burroughs Wellcome & Co.)
3. Pecamethonium bromide (" " " ")
4. Neostigmine bromide (Roche)
5. Sodium chloride (B.D.H.)
6. Sodium bicarbonate (Sarabhai M. Chemicals)
7. Glucose (Pfizer)
8. Potassium chloride (B.D.H.)
9. Calcium chloride (B.D.H.)
10. Potassium dihydrogen phosphate (Sarabhai M. Chemicals)
11. Magnesium sulphate (Sarabhai M. Chemicals)
12. Magnesium chloride (Sarabhai M. Chemicals).

Constitution of Physiological Solutions :

KREB'S SOLUTION

No.	Ingradients	Quantity in grams/litre.
1.	Sodium chloride	6.9
2.	Sodium bicarbonate	2.1
3.	Glucose	2.0
4.	Potassium chloride	0.4
5.	Calcium chloride	0.28
6.	Potassium dihydrogen phosphate	0.16
7.	Magnesium sulphate	0.14

TYRODE SOLUTION

<i>No.</i>	<i>Ingredients</i>	<i>Quantity in grams/litre</i>
1.	<i>Sodium chloride</i>	8.00
2.	<i>Sodium bicarbonate</i>	1.00
3.	<i>Glucose</i>	2.00
4.	<i>Potassium chloride</i>	0.20
5.	<i>Calcium chloride</i>	0.20
6.	<i>Sodium dihydrogen phosphate</i>	0.05
7.	<i>Magnesium chloride</i>	0.01

FROG-RINGER SOLUTION

<i>No.</i>	<i>Ingredients</i>	<i>Quantity in grams/litre</i>
1.	<i>Sodium chloride</i>	6.40
2.	<i>Sodium bicarbonate</i>	0.35
3.	<i>Glucose</i>	0.70
4.	<i>Potassium chloride</i>	0.30
5.	<i>Calcium chloride</i>	0.17

S e c t i o n VII

D I S C U S S I O N

PHYTOCHEMISTRY :

Dragondorff's reagent (potassium-bismuth iodide), Wagner's reagent (potassium iodide with a solution of iodine), Mayer's reagent produce definite chemical compounds with alkaloids resulting into change in colour or formation of precipitate. All extractives and fractions obtained from preparative TLC produced change in colour and precipitation with these reagents as shown in the Table-1, suggest that all may contain alkaloid.

Isolation by preparative thin layer chromatography revealed the presence of minimum four alkaloids since crude alkaloid resolved into four fractions on the plate.

The thin-layer chromatographic studies of these fractions suggest that two fractions i.e., Fraction I and Fraction II are single, homogeneous alkaloids. It can be clearly said on the basis of average R_f value that is 0.33 and 0.55 for Fraction II and Fraction III in the developing solvent system that they are different. Fraction I and Fraction IV are found to mixture of three to four and two alkaloids, respectively (86 page). Average content of Fraction I, Fraction II, Fraction III and Fraction IV was found to be 0.03, 0.075, 0.075 and 0.04 % respectively.

From the above percent composition, it can be said that Fraction I and Fraction II are the major constituents while rest are minor.

Spectral and chemical studies of these alkaloids indicate the presence of lactone ring on the following evidences :-

1. U.V -- Single maximum at 2470°A indicate the presence of $\text{C}=\text{O}$ group in conjunction with ethylenic or lactone group.
2. I.R.-- Stretching at 1690°A indicate the presence of six membered ring.
3. H.M.R. -- Peaks between 72 and 73 indicates the presence of $-\overset{\text{O}}{\underset{\text{O}}{\text{C}}}-$ attached to ethylenic group.
4. Reddish violet colour to alkaline sodium nitroprusside and violet colouration to potassium iodate and sulphuric acid indicate the presence of lactone ring (page No. 83).

Mass spectra and microanalysis suggest the same molecular weight i.e. 221 for both the alkaloids - Fraction II and Fraction III . Their molecular formula can be suggested

Table - 1
Tests for Alkaloids

<i>Name of the Material</i>	<i>Dragendorff's Reagent</i>	<i>Wagner's Reagent</i>	<i>Mayer's Reagent</i>	<i>Inference</i>
<i>Petroleum ether</i>	<i>Pink</i>	<i>Yellow</i>	<i>White ppt.</i>	<i>Alkaloid present</i>
<i>Ether</i>	<i>Brownish pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Chloroform</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Ethyl alcohol</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Water</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Methanol</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Fraction I</i>	<i>Brown</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Fraction II</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Fraction III</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>
<i>Fraction IV</i>	<i>Pink</i>	<i>"</i>	<i>" "</i>	<i>" "</i>

as $C_{13}H_{19}O_2$ on the spectral and microanalysis studies.

UV, IR spectral and microanalysis suggest the similarities between dioscorine and the alkaloids which we have isolated. NMR and mass spectrum of Dioscorine is not available hence differences of Dioscorine from alkaloid isolated by us on the basis of these criteria is not evident. But dissimilarities in physical properties given in Table-2 are clearly suggestive of their individual existence.

Table - 2

Physical characteristics of alkaloids.

No.	Alkaloid	Colour and State	Solubility in water	M.p. ^{°C}
1.	Alkaloid-A	Pale yellowish, Crystalline	Insoluble	89
2.	Alkaloid-B	Brownish, semi- solid mass	Soluble	-
3.	Dioscorine	Greenish Yellow Crystalline	Soluble	54-55

In short, all these studies clarify that the alkaloids which we have isolated are similar to Dioscorine

which was isolated by the former workers but are not
Dioscorine.

PHARMACOLOGY :

Crude alkaloid contains an agent blocking the neuro-muscular transmission on the following evidences:-

(1) Spastic paralysis and deep respiration in chicks on i/v and oral administration with abrupt recovery.

(2) Flaccid paralysis and deep respiration in mice, rats, guinea pigs, rabbits, cats and monkeys on i/v administration.

(3) Block of response to indirect stimulation without affecting the direct muscle stimulation of isolated rat phrenic nerve diaphragm preparation and potentiation of neuromuscular block by neostigmine at later stages.

(4) Progressive neuromuscular blockade of cat tibialis anterior nerve muscle preparation and cat gastrocnemius muscle preparation with initial fasciculations and no antagonism by neostigmine and completely reversible by itself.

(5) Inhibition of responses of acetylcholine on frog rectus abdominis muscle preparation at lower doses and itself contraction at higher doses.

(6) Sudden contracture followed by progressive block on isolated chick biventer cervicis muscle preparation and completely reversible after mere washings.

All these observations suggest that the crude alkaloid contains neuromuscular blocking agent of depolarising type resembling to decamethonium. However, it differs from decamethonium in its absorption by oral route.

Death due to the respiratory failure is noted which is supposed to be the consequences of neuromuscular blockade. Heart beat continued for few minutes even after the death, in different animals indicating that the primary lethal effects are not at cardiovascular system.

Tremors and convulsions are observed in all animals except chicks. They may be indicative of the effect of central nervous system stimulation.

Salivation, micturation, lacrimation in all the animals and itself contraction of isolated guinea-pig ileum

indicate that the crude alkaloid may contain the parasympathetic agent.

Fraction I : Fraction I produced spastic paralysis in chicks, flaccid paralysis in mice, complete block of tibialis anterior nerve muscle preparation of cat, reduction of responses to indirect stimulation of rat phrenic nerve diaphragm preparation and inhibition of frog rectus abdominis preparation at average of 10 to 15 times. Lower doses indicate that it is the potent neuromuscular blocking agent of depolarizing type.

Fraction II : Fraction II blocks the responses to indirect stimulation of rat phrenic nerve diaphragm preparation without affecting direct muscle stimulation and partially is antagonised by neostigmine indicating the antidepolarising type of action. It inhibits the responses of acetylcholine of frog rectus abdominis muscle preparation without itself causing contraction. The block is reversible after washing. This suggest that the alkaloid A exhibit antidepolarising type of action.

Fraction III : Fraction III causes hyper excitation, defaecation in chicks. It causes tremors

and convulsions in mice and rats indicating the central nervous system effects. It causes spastic paralysis in chicks at very high doses which lasted for a fraction of second.

Fraction III causes the neuromuscular blockade of isolated rat phrenic nerve diaphragm preparation. It does not affect the direct muscle stimulation and block is partially reversed by neostigmine. It causes the dose dependent inhibition of frog rectus abdominis muscle preparation.

These observations suggest that the Fraction III has the mixed type of action

Fraction IV : Limited amount of fraction IV studies indicated that it had no activity in chicks upto doses of 1 mg. In vitro studies as compared to fraction I showed that fraction IV is 20-times less active in its capacity to induce neuromuscular blockade.

It seemed to be qualitatively different from the observations that neostigmine could not antagonise it while mere washings reversed the block.

It potentiated the effect of acetylcholine on frog rectus abdominis muscle preparation.

These observations i.e., potentiation of acetylcholine effect in presence of fraction IV and in vitro studies on rat phrenic nerve diaphragm preparation are indicating of the fact that it may have mixed type of neuromuscular blocking activity. However, this fraction contains two alkaloids each of which may possesses different types of activity.

Alkaloid Dioscorine is reported to have picrotoxic like action. Fraction III resembles to some aspects of alkaloid dioscorine. All other alkaloids exhibited the effects differing from Dioscorine.

Chemical studies suggest that the alkaloids losses tropane nucleus in the ring. The tropane derivatives are known to have antimuscarinic activity¹¹⁴. Our studies indicated that tropane derivatives investigated in present studies exhibit antinicotinic activity. Further purification and structure elucidation of these tropane derivatives and the studies of their structure activity relationship regarding antinicotinic activity may indicate a new tropane lead. The postulated new lead may yield a

new series of nicotinic receptor blocker. Also it may give a new insight into mechanism of neuromuscular blockade.

Since the compound is orally absorbed, it may find some use in spastic conditions as well as parkinsonism. For this clinical studies to assess its safety as well as utility are necessary.

Our studies indicate that Fraction I is orally absorbed and has got depolarising type of neuromuscular blocking activity. It is potent as compared to decamethonium.

To the best of our knowledge this is the first group of naturally occurring compound having depolarising type of neuromuscular blocking activity and also effective orally.

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S Y N O P S I S

S Y N O P S I S

Dioscorea consists of nine genera and over 650 species of which about 600 species belong to the cosmopolitan genus *Dioscorea*. About 50 species of *Dioscorea* are known to occur in India¹.

A few species of *Dioscorea* are largely cultivated since they have gained considerable economic importance as a source of the steroidal sapogenin diosgenin, which is used commercially as a precursor of the many medicinally active steroids.

Some wild species of *Dioscorea* are well known for their edible large tubers popularly known as Yams. *Dioscorea hispida* is one of them. It is distributed throughout India. In Konkan area, the people use the tubers as a famine food after processing. They wash the tubers, boil till it is softened and peeled. The softened mass is sliced and placed in a wicker basket and immersed in a running stream of water for 2-3 days. Finally they dry it and eat it after baking. Dymock, Warden and Hooper² pointed out that the tubers of the *D. hispida* were so poisonous that a piece of size of an apple would bring death in a man in 6 hours. It causes intense irritation in the mouth, throat,

also the sense of suffocation followed by drowsiness and finally exhaustion. Its juice is used as an arrow poison³.

The present studies were undertaken to isolate and characterize its toxic fractions causing neuromuscular block. Our ancillary aim was to find out the constituents other than steroidal saponin, diosgenin which may have importance as a by-product of the steroidal industries.

The study was divided into two parts :- 1) Phytochemistry ; 2) Pharmacology.

PHYTOCHEMISTRY :

The fresh tubers were collected from the Konkan area, milled to a coarse powder and dried under shade. The dried powder (4 kg) was subjected to sequential soxhlet extraction using solvents petroleum ether (6 lit., 40-60°), ether (6 lit.), chloroform (5 lit.), methanol (5 lit.) and water. The preliminary pharmacological studies were carried out and it was observed that the maximum neuromuscular blocking activity was obtained in methanolic extract. Hence methanolic extract was prepared in large quantity (1 kg) and was alkalined by strong ammonia and then taken into chloroform. The substance (100 g) thus obtained was a crude alkaloidal

mixture which was highly potent. The alkaloidal mixture (100 mg/plate) was further subjected to preparative thin layer chromatography (2 mm. thickness) with resolving media silica gel G in a solvent system chloroform : methanol (9:1). The alkaloidal fractions were located and eluted with chloroform. The fractions were named starting from bottom to top as I (3%), II (7.5 %), III (7.5 %) and IV (4.0%).

The melting point, microanalysis (C, H, O, N), infrared spectra nuclear magnetic resonance and mass spectra of pure alkaloids II and III were studied.

Fraction II is yellowish white needle shaped crystalline alkaloid having melting point 89°C. Fraction III is brownish semisolid alkaloid. The infra red, nuclear magnetic resonance and mass spectra of these compounds indicate the presence of tropane and unsaturated lactone ring. The micro-analysis and spectral studies suggest that both compounds having same molecular weight (221), are unsaturated and related to Dioscorine but are not Dioscorine.

PHARMACOLOGY :

The crude extract and the purified alkaloids were tested for their neuromuscular blocking activity both in in vivo and in vitro.

The extract was studied in albino mice weighing 18-22 gms. and young white leg horn male chicks weighing 25-30 gms. The extract was given intravenously. The effect of extract on oral administration was also studied.

Cats weighing between 2.5 to 3.5 kg were used for tibialis anterior nerve muscle preparation as described by Brown⁴.

The isolated phrenic nerve diaphragm preparation was set up as described by Bulbring⁵.

The rectus abdominis muscle of frog Rana tigrina was also used to characterise the fractions.

Crude extract in the dose of 200 mg/kg orally and 10 mg/kg i.v. exhibited paralysis in mice. The extract in a dose of 10 ug intravenously produced spastic paralysis in chicks. The onset of action was immediate and complete

recovery was observed within 10 minutes. Oral administration of extract produced paralysis at dose 40 mg/kg. The onset of action was about 5 to 7 minutes and effect lasted for about 1 hr.

At higher doses onset was quicker, the duration was more and animal died due to respiratory failure. The deep respiration was preceded by hind limb paralysis at lower doses whereas at higher doses the respiratory failure was observed leading to death.

The extract at a dose of 1.5 mg given close intra-arterially in cat tibialis anterior nerve muscle preparation produced initial fasciculations followed by a progressive neuromuscular block. This effect was not antagonised by neostigmine but blockade was completely reversible by itself.

On isolated phrenic nerve diaphragm preparation of rat the crude extract in the dose of 100 ug/ml. produced neuromuscular block without affecting the response to direct muscle stimulation. It was partially antagonised at initial stages while deepened at later stages by neostigmine in dose of 2 ug/ml.

The extract exhibited dose dependent contraction on the rectus abdominis muscle preparation of frog in a dose

range of 15-60 ug/ml.

Fraction I produced similar effect as crude extract at lower doses. It showed spastic paralysis in chicks in doses 0.5 ug i.v. and 50 ug orally.

Fraction II did not exhibit paralysis in chicks upto doses 1 mg/chick intravenously. It also failed to produce block in tibialis anterior nerve muscle preparation of cat upto 2 mg. On isolated phrenic nerve diaphragm preparation of rat it produced neuromuscular block at a dose of 200 ug/ml. Neostigmine partially antagonised blocking effect. ACh induced responses were antagonised by Fraction II (100 ug/ml) on frog rectus abdominis muscle preparation.

Fraction III exhibited similar effect to Fraction II, except excitation in chicks at lower doses and spastic paralysis and excitation at higher doses. Paralysis remained for few seconds, while excitation lasted for long time and animal died.

Fraction IV was inactive in chicks upto 1 mg intravenously and upto 2 mg in tibialis anterior nerve muscle preparation of cat. On rat phrenic nerve diaphragm

preparation the neuromuscular block produced at a dose of 200 ug/ml. It potentiated the effect of ACh on frog rectus abdominis muscle preparation.

DISCUSSION :

The present studies indicate that the methanolic extract of *Dioscorea-hispida* has a potent neuromuscular blocking activity than the other extracts. It is found to be an alkaloidal mixture which can be resolved in minimum four fractions. Fractions II and III are pure alkaloids. The crude extract as well as fraction I produced depolarising type of neuromuscular blocking activity with the following evidences :-

i) spastic paralysis in chicks; ii) initial fasciculations with progressive block in tibialis anterior nerve muscle preparation of cat; iii) potentiation of neuromuscular blocking activity by neostigmine and iv) contractions of the frog rectus abdominis muscle preparation.

The fractions II and III exhibited curare type of neuromuscular block in frog rectus abdominis muscle preparation and phrenic nerve diaphragm preparation of rat.

All these observations suggest that the plant contains a mixture of depolarising and antidepolarising type of neuromuscular blocking agents. To the best of our knowledge this is the first natural occurring group of compounds having depolarising action and also effective orally.

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STATEMENT UNDER UNIVERSITY RULE

No. 0.412 and 0.413

As required by the University Rule No. 0.412 and 0.413, I state that the work presented in this thesis is of original in nature and so far the best of my knowledge has not been submitted for any degree of this or any other University.

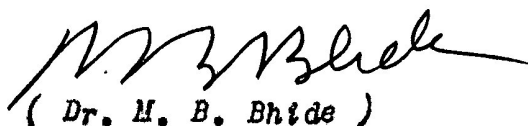
Whenever reference has been made to the work of others, it has been clearly indicated along with the source of information in the "References".



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