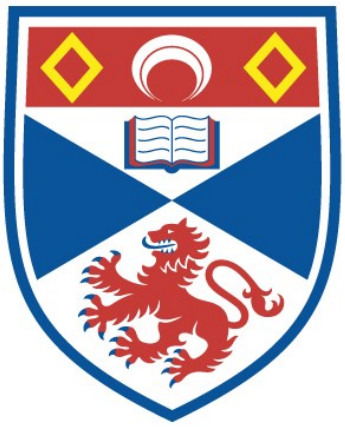
BIOCHEMICAL BASIS OF THE MUSCULAR ACTIVITY OF ERLANGIA CORDIFOLIA (S.MOORE) - (GATHUNA)

Njuguna John Mugo

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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BY

NJUGUNA JOHN MUGO

A THESIS SUBMITTED IN PART FULLFILMENT FOR THE DEGREE OF DOCTORATE OF PHILOSOPHY IN THE UNIVERSITY OF ST. ANDREWS

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I Certify that this work has been carried out in the Department of Biochemistry of the Faculty of Medicine of the University of Nairobi, Kenya.

Signed----------

Prof. Lodovico Sartorelli
The Chairman and Head of
the Department.

Date 10 Dec. 1976----------1976

This Thesis is my original work and has not been presented for a degree in any other University.

Signed ----------

Dr. N.J. Mugo, B.Sc.(Hons) St. And.,
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Date 10th December 1976.

This Thesis has been submitted for examination with my approval as University Supervisor.

Signed ----------

Professor G.R. Tristram

Date 29th December 1976

For my brother D. Ngugi Mugo who gave up his education so that his younger siblings may be educated.

A B S T R A C T

BIOCHEMICAL BASIS OF THE MUSCULAR ACTIVITY OF ERLANGIA CORDIFOLIA (S. MOORE)

by

Njuguna John Mugo

Water extract of the leaves of Erlangia cordifolia (S. Moore) - compositae (commonly known in Gikuyuland as Gathuna) has been traditionally used by the Gikuyu people of Kenya for centuries for the purpose of stimulating myometrial contractions in the process of parturition. The use of this extract has been called for when the progress of the birth process has been judged to be unsatisfactorily slow. The introduction to this work (Chapter 1) therefore surveys this use of the plant material and proceeds on to show the validity of using Erlangia material for the study of the process of muscle contraction. The section also includes a short discussion on the validity of the use of what could be termed a patho-

logical condition - that is, the obstetric 'lazy' uterus - for the purpose of studying a normal physiological process (muscle contraction) at the cellular and molecular levels. The Introduction ends with a discussion on the biochemical aspects of muscle contration that have to be investigated in order to ascertain fully what could be the biochemical basis of the activity of a compound or compounds that are said to affect the process of muscle contraction.

Chapter 2 explains the methods used to obtain the crude extract from Erlangia leaves and also a purified compound, cordifene, from the same source, that was found by the author of this work to have stimulatory activity on contracting muscle. The chapter also deals with the methods used to characterize cordifene chemically.

Chapter 3 describes the physiological experiments carried out to confirm the stimulatory activity of Erlangia extract and also of cordifene on the smooth muscle of the myometrium and that of the intestinal wall, in addition to similar effects on skeletal muscle. These experiments therefore confirmed the fact that the activity of Erlangia material has a common biochemical basis for all types of muscle at the molecular level.

Stimulation of muscle contraction can be brought about through biochemical effects on the nerve(s)

supplying the muscle or through direct effects on biochemical mechanisms occuring within the muscle cell itself. Chapter 4 of this work is therefore concerned with an investigation into the possibility of a chemical compound or compounds from Erlangia leaves that may be capable of influencing biochemical processes within the peripheral autonomic nervous system, as this system is known to be intricately involved in muscle contraction. Acetylcholine metabolism is important not only for the autonomic nervous system's biochemical role in muscle contraction: it is also important for the biochemical processes that take place in nervous impulse transmissions in general - with all the consequences that this has on all muscles, both voluntary and involuntary. For this reason, a possible effect of Erlangia material on the cholinesterase enzymes has been searched for.

Chapter 5 is concerned with a series of investigations into the different biochemical processes that occur in the muscle cell during the contractile activity and the mode of action by which Erlangia material might be influencing such processes. Availability of Ca^{2+} to the contractile proteins is obligatory for the process of muscle contraction in vivo. For this reason, an investigation was carried out into the possibility of Erlangia material having a role in Ca^{2+} translocation within the muscle cell.

The fuel for the contractile process is ATP which is hydrolysed by the myosin ATPase (E.C. 3.6.1.3) enzyme. It was therefore important to investigate the possibility of Erlangia material inducing increased myosin ATPase activity. And since ADP is a well known inhibitor of ATPase activity, an investigation was carried out to explore the possibility of increased removal of ADP from the enzyme nucleotide binding sites that might be induced by Erlangia material. The possibility of Erlangia material having activity on ADP metabolism in the muscle cell was also evaluated by investigating the possibility of the material inhibiting the uptake of the nucleotide by respiring mitochondria. The investigations in this section are concluded with an examination of the possibility of configurational changes occuring in myosin molecules as a result of a possible interaction in between the protein and Erlangia material. Configurational changes are known to accompany enzymic reactions and a part from this, the process of muscle contraction itself is one of the best examples of configurational changes occuring within a system of proteins.

Chapter 6 is a discussion based on the results of the investigations reported in the previous sections. Erlangia material was found to react with ADP forming a complex and, besides probably inducing increased

myosin ATPase activity, it was also found to induce marked configurational changes within the actomyosin molecule. The speed of contraction of muscle has already been related to myosin ATPase activity and since this work has related increased myosin ATPase activity to gross conformational changes occuring in the skeleton of the myosin molecule, it has been concluded that the increase in the muscle contractile activity that has been observed by the Gikuyu people of Kenya, as evidenced by accelerated labour, is related to the Erlangia-induced conformational changes occuring in the myometrial actomyosin molecules. Cordifene reproduced the physiological and biochemical results observed with the crude material of Erlangia cordifolia and for this reason, it was concluded that cordifene must be the active ingredient, or at least one of the major active ingredients, in Erlangia material that is involved in augmenting contractile activity in muscle.

ACKNOWLEDGEMENTS:

A famous German scientist, P. Willstaetter, once wrote that a scientist needs for success four Gs (Geduld, Geschick, Geld und Glueck) that is, patience, fate, money and luck respectively. It was certainly good fortune that the author came at an early stage of his investigations across such an extraordinary herb, Erlangia cordifolia (S. Moore) through the guidance of the prominent traditional medical practitioner, Mr. (or should I call him Doctor) Barnabas Kiriu of Kikuyu in Kenya, who led him to the secret held in the leaves of the herb. It was Mr. Kiriu who also instructed him on the traditional extraction and the uses of this particular extract and as such he is very grateful to Mr. Kiriu. To have joined the Department of Biochemistry of the University of St. Andrews, as an undergraduate, at the time of Professor G.R. Tristram was unforseen luck because not only did he prove to be a great source of inspiration during the author's undergraduate days but has continued to inspire the author even to the extent of persuading the University of St. Andrews to grant the concession allowing the whole of this research work for this distinction to be carried out six thousand miles away from the University Campus. This research work could not possibly have been carried out without this concession. As such the author will always be grateful to Professor Tristram and through him the University itself.

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For the production of this thesis the author is indebted to the Publishers of Scientific American and to Worth Publishers Incorporated for their courtesy in allowing him to use some illustrations from their publications.

As a result of investigations into possible uses of cordifene and its derivatives for the treatment of diseases, the author found it necessary, at a very early stage in the preparation of this thesis, to seek patent protection for all scientific findings connected with cordifene. In his efforts to obtain such patent rights, he has greatly indebted himselt to Mr. O. T. Ngwiri of Ontario, Canada, for Mr. Ngwiri's kindness in agreeing to act as the author's legal advisor and representative on patent matters covering the whole world, while at the same time post poning asking for his legal fees until a future date. In connection with patent protection, the author is also indebted to Professors D. Odhiambo and K. Thairu of the University of Nairobi for their efforts in facilitating the author's procurement of a grant from the University of Nairobi for paying the initial costs for the patent. The publication of this work in form of a thesis would have been impossible without the procurement of this patent protection.

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
ATPase	Myosin adenosine triphosphatase (E.C. 3.6.1.3.)
^{13}C	^{13}Carbon
Ca	Calcium
Ca^{2+}	Calcium ions
Ca^{2+} *ATPase*	Myosin ATPase with calcium ions as cofactor.
Carbachol	Carbamyl choline
C = O	Carbonyl group
DMPP	1, 1-Dimethyl-4-Phenyl Piperazinium Iodine
DNP	Dinitrophenol
DTNB	5, 5^1 - Dithiobis - (2 - Nitrobenzoic acid) or Ellman's reagent
EDTA	Ethylenediamine tetracetic acid
EGTA	1, 2 - bis (2 - bicarboxymethyl amino-ethoxy) - ethane)
Guanidine-HCl	Guanidine hydrochloride
^{1}H	Hydrogen
IR	Infrared
ITP	Inosine triphosphate
M	Molar
Mg^{2+}	Magnessium ions
Mg^{2+} *ATP*	Magnessium adenosine triphosphate complex

Mg^{2+} ATPase	Myosin ATPase with magnessium ions as cofactor
NEM	N - ethyl maleimide
NMR	Nuclear magnetic resonance
Pi	Inorganic phosphate
pMB	p - mercuribenzoate
SH	Sulphhydryl group
TCA	Trichloroacetic acid
UV	Ultraviolet
Σ	Molar absorption of light
λ	Wavelength
μl	Microlitre
μg	Microgram

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CHAPTER 1:

INTRODUCTION

1.1 Erlangia Cordifolia And The Traditional Gikuyu Obstetric Practice.

Erlangia Cordifolia (Oliv) (S. Moore) is locally known in the Gikuyuland as Gathuna. It grows in the Kenyan highlands that are inhabited by the Gikuyu people, as a common seasonal weed. Among the Gikuyu people the herb is well-known for its stimulant effect on the pregnant uterus and is widely used in the rural areas for the purpose of assisting mothers during parturition.

In the Gikuyu traditional medical practice, the therapeutic powers of this herb are deemed necessary when the mother has been in labour for some time and no visible evidence of progress in the labour can be detected. My interview with Traditional Medical Practitioners in the Gikuyuland has not yielded any information from which it could be unequivocally concluded that either an extract of this herb is administered to the mother as a result of failed second

stage of labour - that is, after the rupture of the membranes - or that the extract is administered to facilitate the first stage of labour. However, most Traditional Medical Practitioners indicated that the mother delivers her baby very shortly after the administration of the extract and as such it is probable that the extract is more commonly used for speeding up the second stage of labour than for speeding up the whole process of labour up to the birth of the child.

In all cases it is the ground terminal branches and leaves that are extracted with either cold or hot water and the extract so obtained administered orally to the patient during labour.

The plant (Fig. 1) is a small seasonal herb, 2-3 feet tall. It belongs to the genus compositae. The young leaves are green, becoming yellowish-brown in colour with age. The leaves are opposite, lanceolate in shape, short and do not vary greatly in size. The flowers are very small and purple. They are produced at the apex of the stem or the apex of the terminal branches.

1.2 Muscle Contraction As An Important Field of Study:

There are several reasons why the mechanism of muscle contraction should be understood. The most fundamental of these is the advancement of knowledge

for its own sake. Another good reason for wanting to understand the mechanism of muscle contraction is the hope that such an understanding may facilitate the understanding of muscle diseases especially those diseases whose pathologies are known to be expressions of inherited genes. Such diseases include dystrophia myotonica and the myopathies. In addition, the understanding of the expression of the genetic code in precise biochemical terms may, one hopes, lead to better therapeutic management of patients with such inherited pathologies.

The mechanism of muscular contraction is more than a biochemical problem and this is easily appreciated from the fact that a number of significant contributions to our understanding of this process has come from biophysicists and the electron microscopists. And, as Lehninger said in a lecture to the Institution of Electrical Engineers in London in 1969: "Actually, muscle should be looked upon as highly efficient and versatile machines, with remarkable engineering and performance characteristics when compared to man-made machines" (1).

To the biochemist, the process of muscle contraction holds a special place in his interests. This is because many of the activities of the cell that he studies are a reflection, to a large extent, of the behaviour of specific proteins in the cell and there is hardly a better demonstration of this generalization in biochemistry than that which the process of

muscle contraction provides. This is because the process can be explained primarily by the behavioural properties of the proteins myosin, actin, tropomyosin and troponin. The highly ordered structure that these proteins form in the muscle cell and their biochemical interactions primarily explain the process of muscle contraction.

1.3 Validity Of The Use Of Crude Plant And Muscle Extracts For Experimentation

In an analysis of a complex biochemical problem it is now a standard procedure to make an attempt to isolate the individual enzymes or the biologically active molecules themselves. Sometimes, however, it is sufficient to mince or ground the tissue. The mince and the 'breis' so obtained contain all the enzymes or the biologically active molecules of the original material, but in the case of the enzymes, the normal spatial relationships between them are destroyed by the disruption of the cellular architecture. More recently, homogenization which means grinding chopped tissue very finely presumably leads to more cell wall destruction and leads to more efficient release of the cellular components required. The aim of these methods is to avail the experimenter with cell-free extracts.

The usefulness of cell-free extracts is exemplified by yeast cell juice, which is obtained through

a process which involves the maceration of yeast cells with sand followed by squeezing the mass in a suitable press. Many of the enzymes extracted in this way require co-enzymes which can be removed by dialysis, and much of our present knowledge of the fermentation process has been gained by the use of dialysed yeast juice, often with supplementary tools in the form of selective inhibitors. Similarly the enzymes involved in glycolysis can be obtained in solution by acqueous extraction of minced muscle, for example. The usefulness of extracts can also be demonstrated by the history of the work on cytochrome oxidase and succinic dehydrogenase. For a long time, these important enzymes were found to be difficult to purify because of their insolubility in ice-cold water or isotonic potassium chloride solution and had to be studied in suitably fortified suspensions of finely minced or homogenized tissue.

In this work, extensive use of water extracts of the leaves of Erlangia cordifolia was made therefore, not only because this is the extract that the Gikuyu people had used since time immemorial but also because preliminary physiological tests had shown that the water extract increased muscle contraction in vitro.

Crystalline material obtained from the water, normal sodium bicarbonate solution and ethyl alcohol extracts of the leaves of Erlangia cordifolia were

also used in this work despite the fact that chemical analysis showed all of them to be mixed crystals. This was justified on the premises that valuable scientific information could be obtained as has been gathered while using, for example, protein crystals despite the fact that proteins in general are known to be very prone to the formation of mixed crystals while still being far from being chemically pure.

1.4 Validity Of A Study That Starts With A Pathological Condition In Order To Explain Or Understand The Normal.

Studies of the abnormal in order, in the process, to understand the normal have been found very useful indeed in biological sciences. Organisms which are intact but which are at the same time suffering from some pathological derangement of metabolism offer valuable experimental material for some scientific investigation. An example of this is to be found in albinos. Albinos are devoid of tyrosinase and may be used in studies of certain aspects of the metabolism of the aromatic amino acids. In an early attempt to decide as to whether amino acids undergo oxidative or hydrolytic deamination, cases of alcaptonuria were studied. It was found that when aromatic amino acids were excluded from the diet, homogentisic acid was no longer excreted. Bearing this fact in mind, it then became reasonable to

suppose that the administration of any intermediate metabolite that is found in between tyrosine and homogentisic acid would give rise to renewed excretion of homogentisic acid if it were to be administered to an alcaptonuric patient. The acid p-hydroxyphenylpyruvic acid, the product of oxidative hydroxylation of tyrosine proved conclusively, when it was administered to alcaptonuric patients, that deamination of tyrosine, and by inference, that of other amino acids, is an oxidative rather than a hydrolytic process (2).

In this type of studies, particularly important among the pathological conditions of which advantage has been taken is the state of diabetes. Spontaneous diabetes, induced by surgical removal of the pancreas or by injections of alloxan or of the diabetogenic hormone of the anterior pituitory, and the pseudo-diabetes induced by the injection of the drug phlorrhizin, have all been put to service especially in studies of the metabolism of fats and carbohydrates.

In view of the contribution of all these studies, the validity of a study of muscle contraction which is based, as its starting point, on an investigation of an abnormal state of muscular contraction - in this case, that of the myometrium at parturition - can then be appreciated.

1.5 Review of Literature

The striated pattern of muscle (Fig. 2) can be explained by the arrangement of the thick and thin filaments. The thicker of the two filaments as observed in the electronmicrographs of muscle contains all the myosin of muscle together with smaller amounts of other proteins whose function is little understood.

The thick filament (Fig. 3) is an assembly of myosin molecules. These are long rods with a double "head" at one end (Fig. 4).

The thin filament incorporate all the other three proteins involved in muscle contraction (Fig. 5). In intact muscle, the thin filaments are attached at one end to the Z line, a flat protein structure. In resting muscle, the successive Z lines are separated by a distance of about 2.2 microns and the thin filaments are aligned parallel to the long axis of the muscle fibre.

As shown in Fig. 6, the thick filaments interdigitate in the space between the thin filaments projecting from adjacent Z lines. The spacing in between the thick and the thin filaments is such that they can slide past each other without any hindrance.

Murray et al. (3) have demonstrated the chemical events of muscle contraction (Fig. 7). These authors have also shown that when ATP and a myosin

"head" combine, the resulting complex is somehow converted into a "charged" intermediate form which binds onto an actin molecule which forms part of a thin filament which has, in its turn, been activated by Ca^{2+} combining with the troponin molecule. When the two "charged" intermediates come together, the hydrolysis of ATP results followed by the release of ADP, inorganic phosphate and energy. It is this energy that powers muscle contraction. Myosin and actin remain attached to each other and it is this complex that is known as a "Rigor Complex". The "rigor complex" persists until a new ATP molecule binds to the myosin head when the myosin-ATP cycle is repeated.

Murray et. al. (3) go on to argue that whereas a "charged" thin filament will only combine with a "charged" thick filament (Fig. 8), an uncharged thin filament will likewise bind an uncharged myosin head. They continue to argue that as more and more myosin heads bind onto a thin filament which has no Ca^{2+} and therefore is "uncharged", a time comes when the thin filament becomes charged and that when this happens, then the thin filament can bind a "charged" myosin head and hydrolysis of ATP will then occur followed by contraction of muscle.

A rigor is that state of inextensibility that exists in muscle in the absence of Mg^{2+}ATP and not the development of tension that may occur when the

$Mg^{2+}ATP$ level in the muscle cell rapidly decreases (4). Tension development could be due to either calcium leakage into the cytoplasm of muscle cell or a decrease of the ATP concentration below the level required to maintain the resting state (4). This is because ATP is required to cause dissociation of the actomyosin complex (4) and it has been observed that to be adequate for relaxation the concentration of the nucleoside triphosphate must not be lower than that required for 80% of the maximal rate of its hydrolysis in the presence of calcium (5). It is plausible that since the removal of calcium from the cytoplasm back into the sarcoplasmic reticulum requires ATP (6), under conditions of very low ATP concentration some of Ca^{2+} in the cytoplasm are not pumped back and stay on in the cytoplasm. However, it could be that one of the prerequisites of tension development in muscle is the retention and accumulation of ADP within the myofibril. Chaplain observed this accumulation and came to the conclusion that tension in muscle appears to be mainly generated by the build-up of ADP (7).

There is a lot of evidence in literature that show that ADP binds on myosin. Morita (8) reported that the stoichiometry of ADP was one mole per one mole of heavy meromyosin at 0.06 M KCl at 25^{o}. Weber et. al. (4) say that free ADP and Pi are in

rapid equilibrium with the ADP and Pi in the Myosin-ADP-Pi complex and since Szent-Gyorgyi and Prior (9,10) observed that about half of the actin-bound ADP of superprecipitated actomyosin also exchanges with solvent ATP, it follows that there is probably a lot of ADP in the cytoplasm which can conceivably regulate the equilibrium in between the cytoplasmic ADP and the myofibrillar ADP in such a manner that there can be a lot of ADP which remains bound to some myosin molecules all the time. The evidence that ADP binds on myosin and the reported fact that ADP does not cause depolymerization of actomyosin (11) agrees well with Chaplain's (7) findings. Kiely and Martonosi's report (11) is all the more plausible because they found an affinity constant of ADP for myosin of 10^6 at very low ionic concentrations, the sort of ionic concentration likely to be found physiologically in muscle cells. It is therefore not surprising that well-washed myofibrils of rabbit skeletal muscle exhibit ADP as the main (70 to 80 per cent) nucleotide bound in them (12).

Abbot (13) has shown that stretching of a muscle effectively increases the number of cross-bridges formed in between the thin filament and the thick filament. This agrees well with the findings of Eisenberg et. al. that not all myosin heads capable of binding onto the thin filament do so at any one time (14). This then means that a chemical compound that could cause conformational changes on the

contractile proteins such that localized areas of these proteins decreased in size thereby effectively causing stretching in other parts along the length of the polymers, could also conceivably induce increased cross-bridge formation and therefore increased ATP hydrolysis. The effect of the chemical compound would then be to increase contractile activity in the muscle per unit time.

1.6 Discussion on Literature

During parturition, the myometrium retracts. This means that when the phase of relaxation follows the phase of contraction, the muscle does not resume its original length. At the same time there is a net increase in tension of the muscle at the end of the phase of relaxation. This could then mean that it is probable that after contraction, only a proportion of the ADP so formed in the myofibrils is released into the cytoplasm away from the myosin molecules.

Chaplain (15) found that the adenosine triphosphatase of insect actomyosin was activated by Ca^{2+} but that this activity was cooperatively inhibited by ADP. This was an echo of an earlier proposal by Blum et. al. (16) that the rate of ATP hydrolysis is limited by the release of ADP from the active site and that the activators of myosin ATPase bring about their rate acceleration by promoting the dissociation of the myosin-ADP complex. It therefore follows

that if an extract is to increase ATPase activity then it could probably do so through facilitating the removal of ADP from the myofibrils. This could be possible if the compound influenced the concentration of the cytoplasmic ADP in such a way that ADP in this compartment decreased because such a decrease would shift the myofibrillar ADP:cytoplasmic ADP equilibrium in the direction of decreasing myofibrillar ADP (4) and thereby make more active sites available for Mg^{2+}ATP binding.

Preliminary physiological experiments on effects of the extract of Erlangia cordifolia on muscle activity had shown a long lasting tension development caused by the extract. As pointed out above (4), increase in Ca^{2+} in the cytoplasm is reflected by tension development. Calcium release from its 'sockets' in the sarcoplasmic reticulum (Fig. 8) is brought about by the depolarisation of the cell membrane following a nerve impulse (1). A compound that increased muscular contractile activity through increasing the availability of calcium could then conceivably act through facilitating the nervous mechanism involved in the process of Ca^{2+} release from the sarcoplasmic reticulum.

This then means that a chemical compound that increases muscular activity - including that of the myometrium - could do this by either facilitating the availability of Ca^{2+} to the troponin molecules

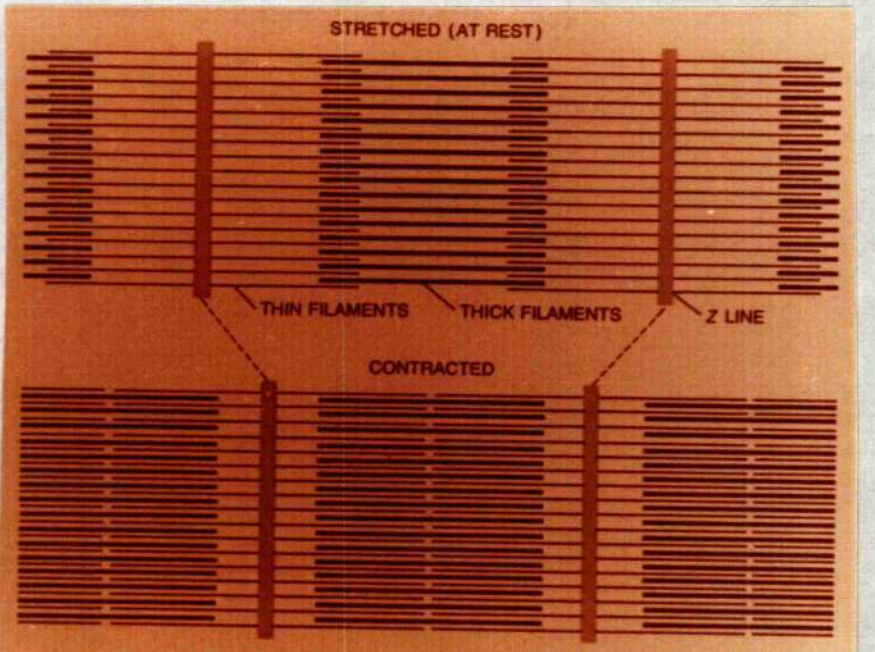
of the thin filaments or by removing ADP - a well known inhibitor of myosin adenosine triphosphate activity.

There is yet a third method by which a chemical acting directly on the muscle cell could bring about increased contractile activity and this is by having an activity on the myofibril such that the activity resulted in an increase in the number of cross-linkages formed at any given moment by the actin and the myosin residues of the thin and the thick filaments respectively. This is so because it would mean an increase in energy production for contractile activity (3) and would support the view of Eisenberg et. al. that not all "charged" myosin molecules on the thick filament complex with the thin filament (14).

In our investigation on the biochemical basis of the action of Erlangia cordifolia extract on myometrial contractile activity, it was important to isolate a compound from the plant that either facilitated Ca^{2+} entry into the muscle cell cytoplasm or facilitated the decrease of ADP concentration in the cytoplasm so that the cytoplasmic ADP:myofibrillar ADP equilibrium would shift in the direction favouring the release of more ADP from the myosin molecules. Actin acts as a cofactor for myosin ATPase activity (17) and this means therefore that configurational changes involved in the contractile activity of muscle are found in the thick filament. A compound,

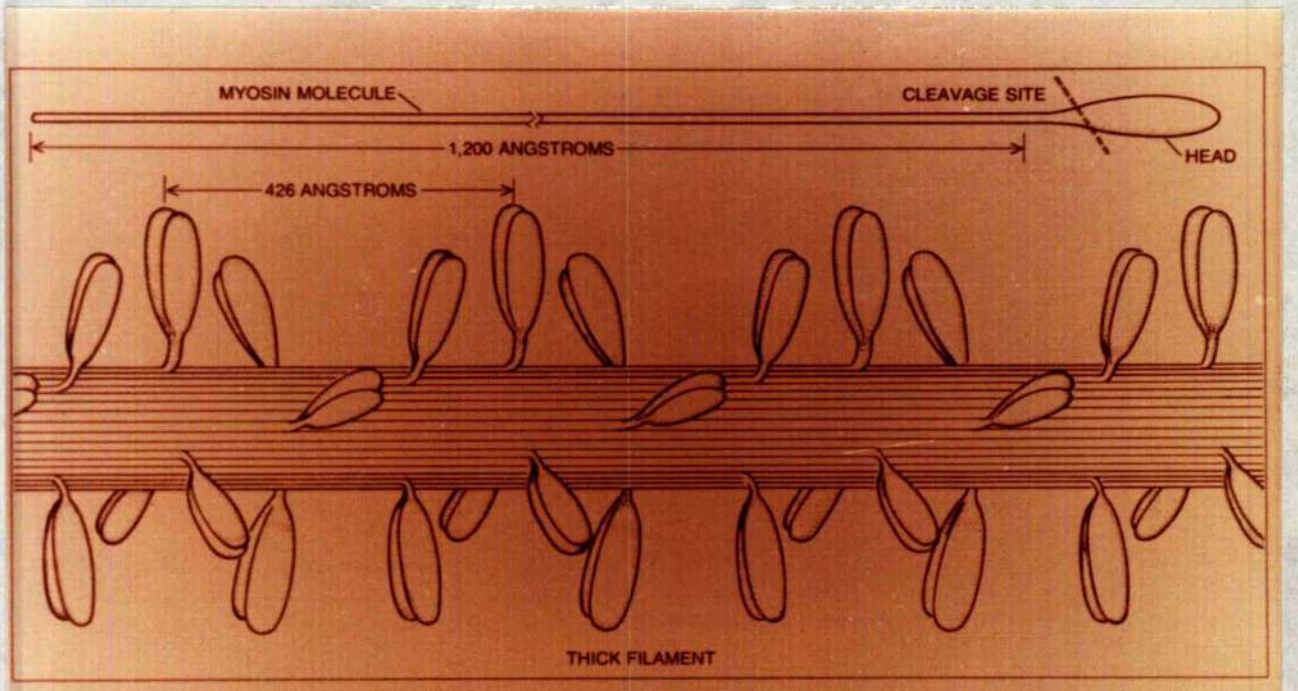
therefore, that caused configurational changes in myosin which resulted in a decrease in the size of the molecule - if it could be found in Erlangia cordifolia - could effectively explain the increased ATP hydrolysis by the myofibril observed with the crude extract from the plant because such localized shortening of the myosin polymer (18) would result in stretching in the other parts of the polymer thereby increasing cross-linkage formation in between the thick and the thin filaments (13). This then indicates the significance of the isolation of cordifene from the leaves of Erlangia cordifolia. Not only does cordifene bind ADP in vitro, it also causes gross configurational changes in the actomyosin molecule which result in the contraction of the molecule.

Fig. 1: A Bundle of the Herb, Erlangia cordifolia (S. Moore) - Compositae, showing twigs, leaves and flowers.



STRIATED PATTERN of muscle fiber is explained by the arrangement of thick and thin filaments. The diagram shows one sarcomere (the unit of muscle between two *Z* lines) and parts of the two adjacent ones, at a scale about the same as the top micrograph on the opposite page. Cross bridges swivel in opposite directions on each side of a central bare region, imparting the relative motion that causes a resting muscle (*top*) to contract (*bottom*).

Fig. 2: From "The Cooperative Action of Muscle Proteins" by J. M. Murrary and A. Weber. By courtesy of the Publisher, Scientific American Inc. (Copyright © Feb. 1974).



THICK FILAMENT is an assembly of myosin molecules, long rods with a double "head" at one end. The head has an active site where the chemical events involved in muscle contraction take place; purified heads are prepared for experiments by cleaving the molecule with an enzyme. In thick filaments myosins are bundled into a sheaf about 1.5 microns long with heads projecting in groups of three. Filament drawing includes pertinent aspects of a new model developed by John M. Squire of Imperial College London.

Fig. 3: From "The Cooperative Action" of Muscle Proteins" by J. M. Murrary and A. Weber. By courtesy of the Publisher, Scientific American Inc. (Copyright © Feb. 1974).

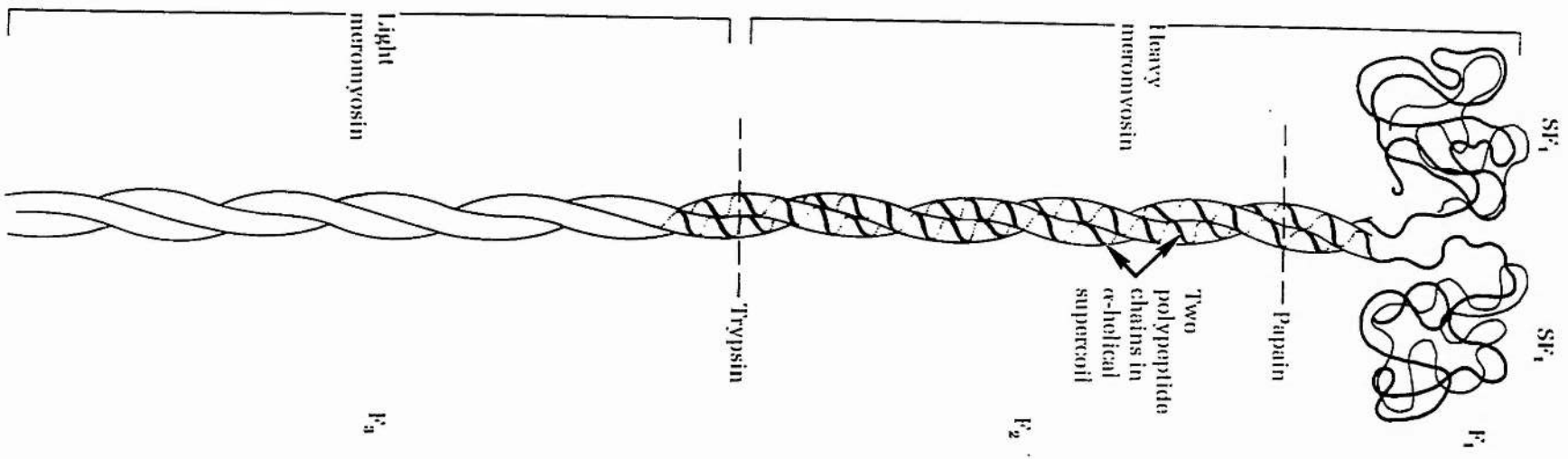
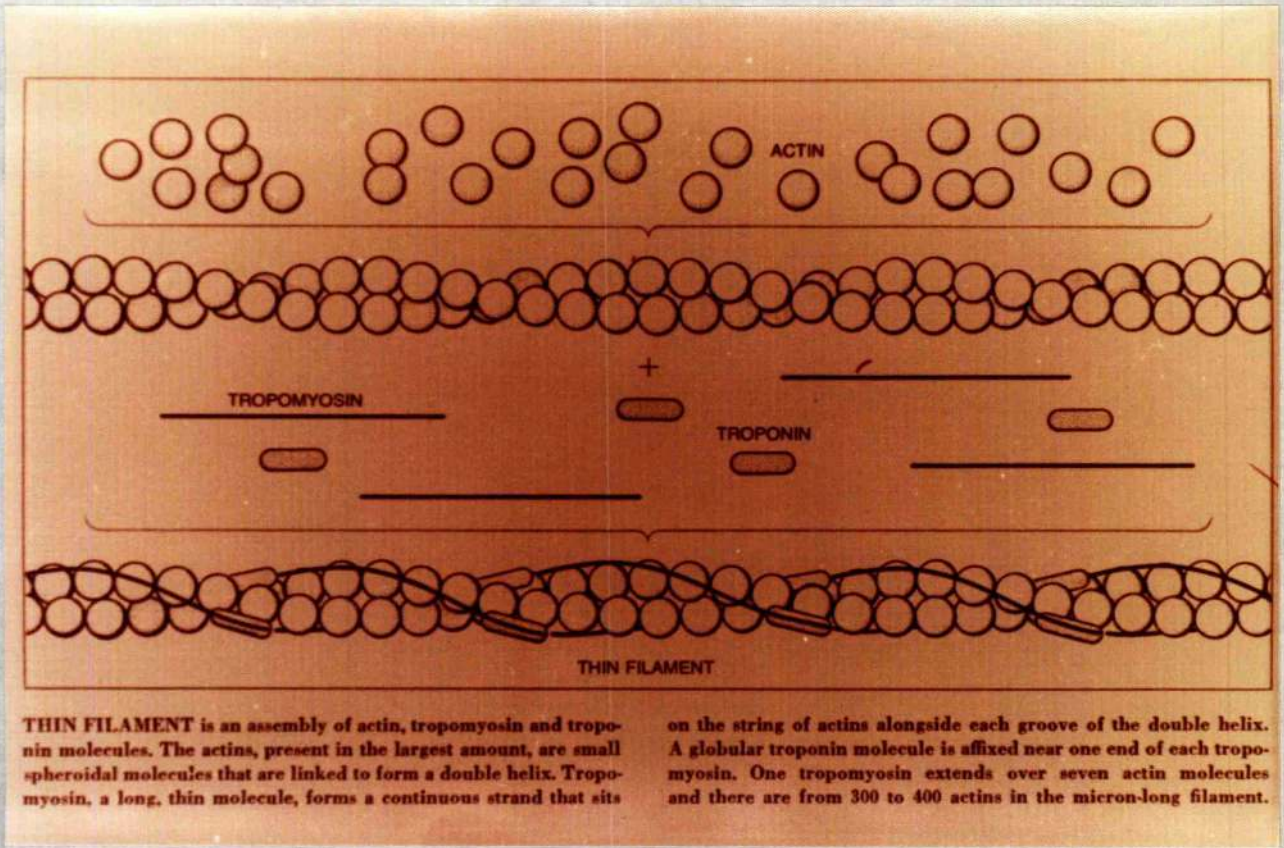


Fig. 4 Schematic representation of the myosin molecule showing its globular head and long tail and the points of enzymatic fragmentation. From Biochemistry by A.L. Lehninger. By courtesy of the Publishers, Worth Publishers Inc., New York, N.Y. U.S.A. (Copyright © 1970).



THIN FILAMENT is an assembly of actin, tropomyosin and troponin molecules. The actins, present in the largest amount, are small spheroidal molecules that are linked to form a double helix. Tropomyosin, a long, thin molecule, forms a continuous strand that sits on the string of actins alongside each groove of the double helix. A globular troponin molecule is affixed near one end of each tropomyosin. One tropomyosin extends over seven actin molecules and there are from 300 to 400 actins in the micron-long filament.

Fig. 5: From "The cooperative action of muscle proteins" by J. M. Murray and A. Weber. By courtesy of the Publisher, Scientific American Inc. (Copyright © Feb. 1974).

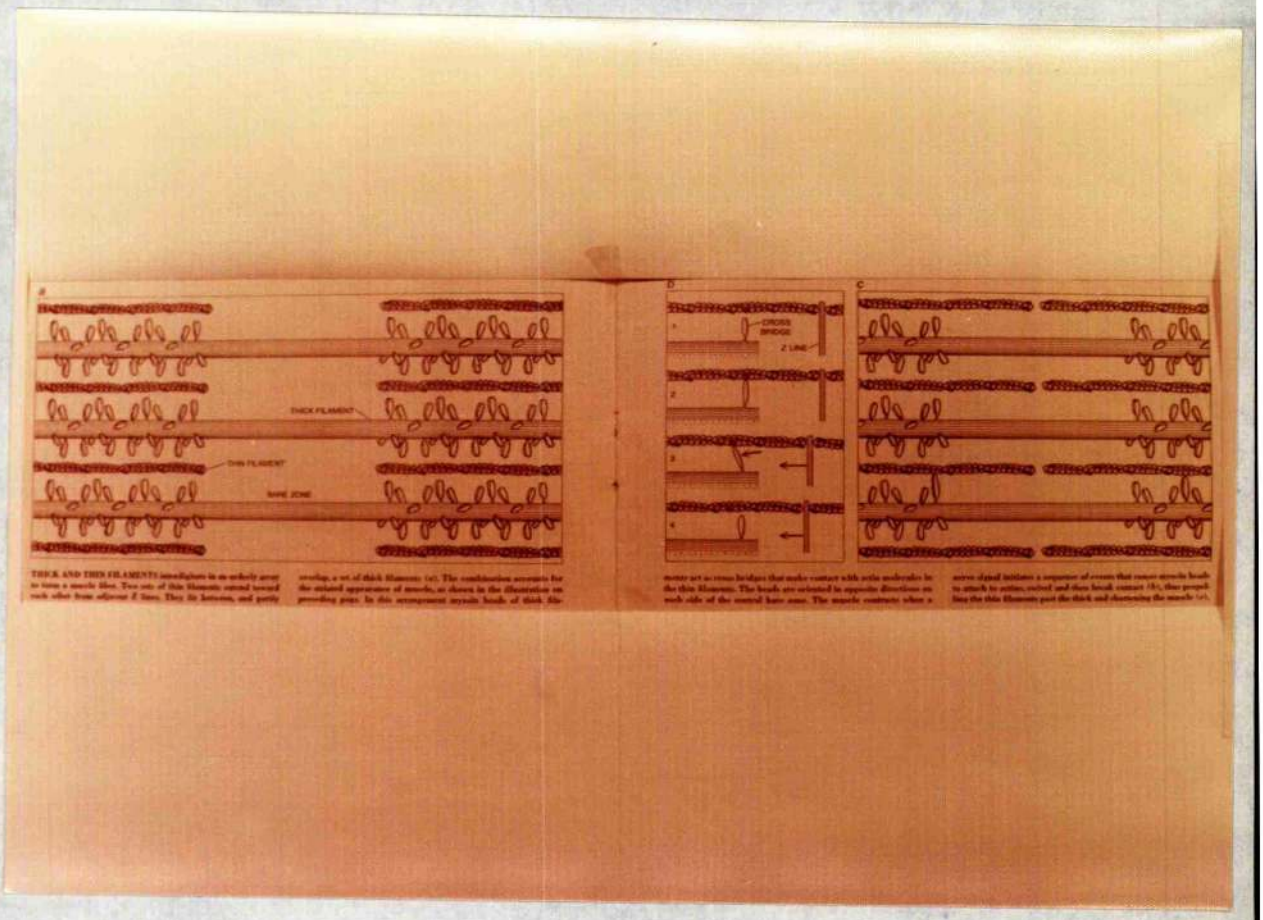
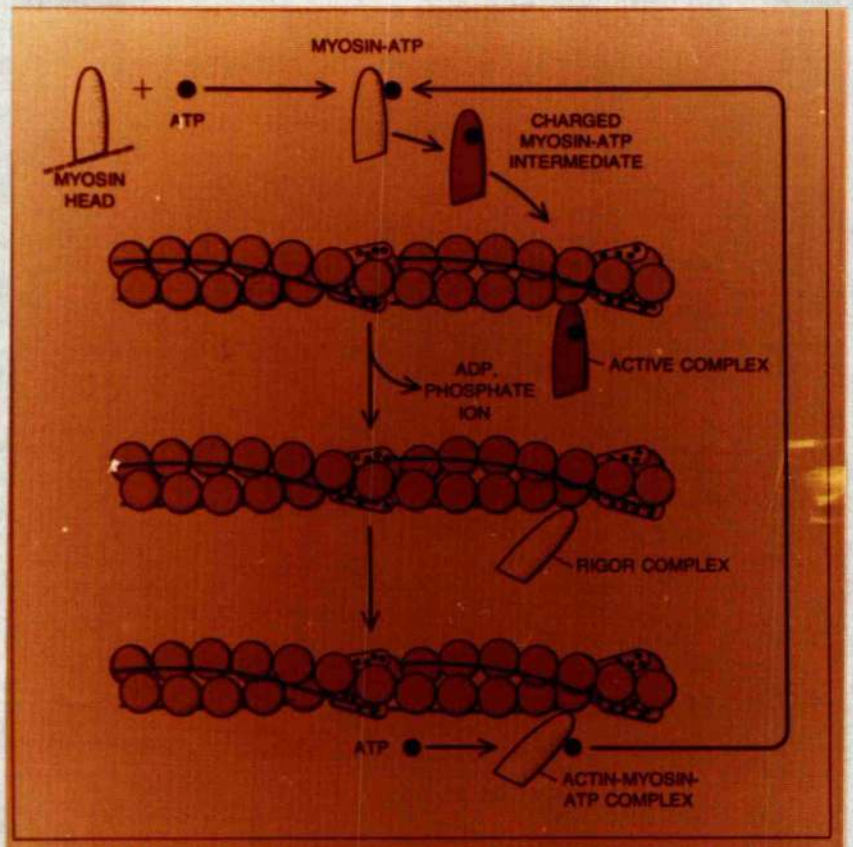
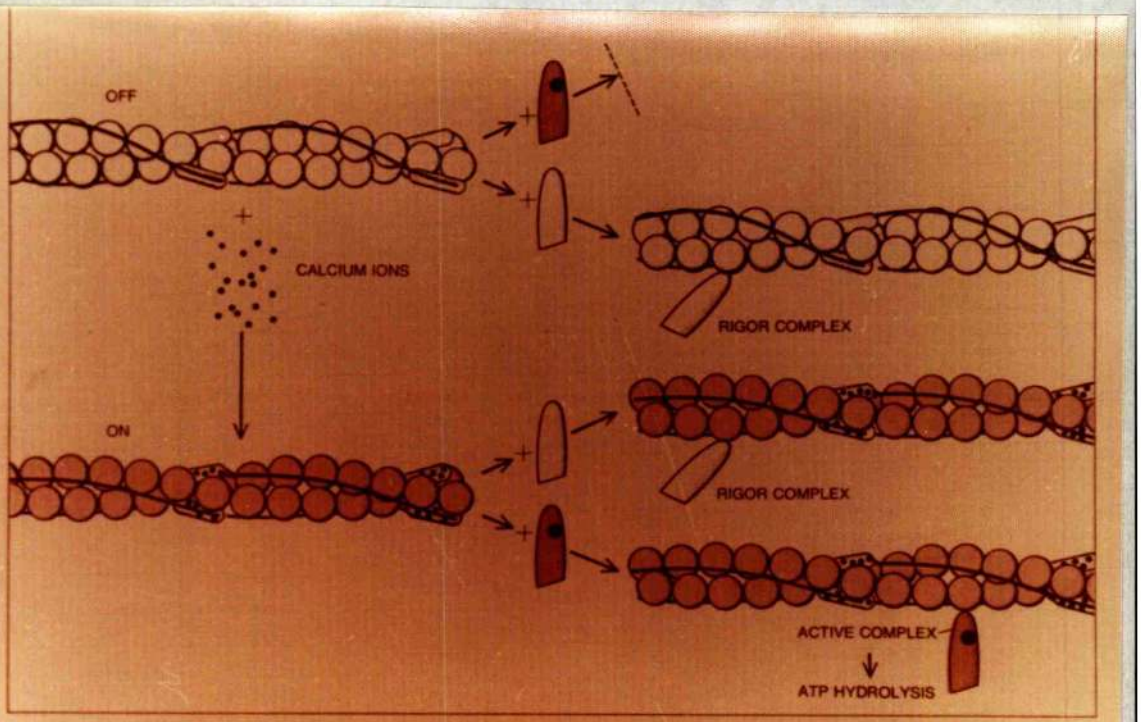


Fig. 6: From "The cooperative action of muscle proteins" by J. M. Murray and A. Weber. By courtesy of the Publisher, Scientific American Inc. (Copyright © Feb. 1974).



CHEMICAL EVENTS of the muscle-contraction cycle are outlined as they take place in the soluble experimental system described by the authors. A myosin head combines with a molecule of adenosine triphosphate (ATP). The myosin-ATP is somehow raised to a "charged" intermediate form that binds to an actin molecule of the thin filament. The combination, the "active complex," undergoes hydrolysis: the ATP splits into adenosine diphosphate (ADP) and inorganic phosphate and energy is released (which in intact muscle powers contraction). The resulting "rigor complex" persists until a new ATP molecule binds to the myosin head; the myosin-ATP is recycled, recharged and once again undergoes hydrolysis.

Fig. 7: From "The cooperative action of muscle proteins" by J. M. Murray and A. Weber. By courtesy of the Publisher, Scientific American Inc. (Copyright © Feb. 1974).



REGULATION OF MUSCLE CONTRACTION is accomplished primarily by calcium ions, which change the thin filament from an "off" to an "on" state, apparently by binding to troponin. When the thin filament is turned off (*top left*), a charged myosin-ATP intermediate cannot combine with it; when the filament has been turned on by calcium (*bottom left*), an active complex can be formed (*bottom right*) and hydrolysis proceeds. Formation of rigor complexes, however, is not sensitive to calcium control. Whether the thin filament is on or off, a myosin head can combine with an actin in the filament to form a rigor complex (*middle right*).

Fig. 8: From "The cooperative action of muscle proteins" by J. M. Murray and A. Weber. By courtesy of the Publisher, Scientific American Inc. (Copyright © Feb. 1974).

CHAPTER 2:

ISOLATION OF MATERIALS FROM THE LEAVES OF ERLANGIA CORDIFOLIA.

2.1 PREPARATION OF CRUDE EXTRACT OF ERLANGIA CORDIFOLIA MATERIAL

2.1.1 INTRODUCTION

The author's interviews with a number of Traditional Medical Practitioners in Gikuyuland had revealed that it was the terminal branches and the leaves of Erlangia cordifolia that were used therapeutically. This material was extracted with either cold water or hot water and it was the extract so obtained that was administered orally to the patient.

2.1.2 MATERIALS AND METHOD:

Fresh Erlangia cordifolia material (twigs and leaves) was first cleaned with ordinary tap water then rinsed with distilled water. The leaves were then ground with a blender (Fischer Scientific, Zeitweg, Zurich, Switzerland). If the leaves were

going to be extracted with water no further preparation of the leaves was carried out. However, for the ethyl alcohol and methyl alcohol extractions, the ground material was dried in an oven with a hot air current at 40-50°C for approximately one whole day.

The ground material was then placed in a glass flask and to the glass flask was added the extracting solvent. These solvents were cold distilled water, cold methyl alcohol (Reagent grade) and cold ethyl alcohol (Reagent grade). The extraction was also carried out with boiling solvents and in this case a refluxing column (Fig. 9), was attached to the glass flask in which the extraction was carried out. The heating of the solvents was carried out electrically by placing an electric heater below the extraction flask.

In every case the extracts were strained through glass wool and the residue discarded. The extract was then filtered using Whatman paper No. 1 (Balston Ltd., England). The extract was then concentrated by using a rotary evaporator (Rotavapor R, "Buchi Laboratoriums Technik, AG CH 9230 Flawil, Shweiz, Switzerland) the extract being heated to a temperature that allowed a reasonable distillation of the solvent. In the case of the water extract the temperature to which the extract was heated was 70°C. For ethyl alcohol and methyl alcohol extracts, the

temperature used was 50°C. The concentrate was then placed in a clean glass beaker under a hood in which a slow current of air was allowed to run over the beaker. After a week, crystallization was judged to be complete. The supernatant was decanted off and the white crystals washed with a mixture of acetone and isoprophyl alcohol (laboratory reagent grade). The wet crystals in the beaker were then placed back in the stream of cold air under the hood and allowed to dry.

2.1.3 CHEMICAL ANALYSIS OF THE EXTRACT

The crystals obtained were all white, needle-like and mainly rhombic in shape although other shapes were also observed. An attempt to obtain a melting point, by using a sealed capillary tube, in all of them failed. None of them could be melted at any temperature below 360°C. At 220°C, sublimation of the crystals occured, a white sublimate being obtained which left a black material behind. Burning the material with a Bunsen flame left a black material behind indicating that the crystals were inorganic in nature. All crystals were found to be extremely soluble in water.

Elemental analysis of the material indicated that it contained Ca (15.80%) Carbon (17.56%), hydrogen (6.32%). No other inorganic elements could be detected by the Atomic Absorption spectrophotometer

(SP 90 Atomic Absorption Spectrophotometer, Pye Unicam Ltd., Cambridge, England).

2.2 EXTRACTION OF CORDIFENE

2.2.1 INTRODUCTION

Preliminary work on this author's efforts to obtain a crystalline material that would have the expected biological activity, though successful, had only yielded mixed crystals which were inorganic. Chemically a more thorough effort had to be made to try and isolate an organic compound from the plant material that would exhibit the activity observed while using the mixed inorganic crystalline material.

2.2.2 MATERIALS AND EQUIPMENT

Blender (Fischer Scientific, Zeitweg, Zurich, Switzerland).

Rotary evaporator (Rotavapor R. Buchi Laboratoriums Technik, AG CH 9230 Flawil Schweiz, Switzerland).

Methyl alcohol (laboratory reagent grade)

n-Hexane (analytical grade)

Benzene (" ")

Diethyl ether (" ")

Anahydrous sodium sulphate (analytical Reagent)

10% Sulphuric acid (laboratory reagent grade)

Large Chrototographic plates

Florisil column (1 x 40 cm)

UV Spectrophotometer (S.P. 1800 ultraviolet spectrophotometer, Pye Unicam Ltd., Cambridge, England).

IR Spectrophotometer (Infrared Spectrophotometer, Pye Unicam Ltd., Cambridge, England).

NMR - ^{13}C, ^{1}H Megahead Nuclear Magnetic Resonance Spectrophotmeter.

Mass Spectrophotometer

Sargent automatic Recorder (Sargent Recorder model SRG E.H. Sargent and Co. Chicago, U.S.A.)

2.2.3 EXTRACTION PROCEDURE:

A weighed amount of fresh green leaves of E. cordifolia was put in methyl alcohol (laboratory reagent grade) and macerated in a blender to very small bits and the mass was left to stand at room temperature for three days after which the extract was filtered. The filtrate was concentrated in vacuo using a rotary evaporator. The concentrate was then stirred with:-

a) n-hexane (analytical grade) to extract the n-hexane soluble fractions).
b) Benzene (analytical grade) to extract the benzene soluble fraction).
c) Diethyl ether (analytical grade) to extract the ether soluble fraction).
d) Chloroform (analytical grade) to extract the chloroform soluble fraction).

Thin layer chromatography using silica gel plates and 10% ether in benzene (analytical grade) showed that most of the organic material were concentrated in the benzene fraction. Very little amounts of material was extracted by the other solvents from the methyl alcohol extract.

The benzene extract was then dried using anhydrous sodium sulphate and then concentrated in vacuo. For thin layer chromatography plates precoated with a fluorescent dye were used. The plates were visualized using ultraviolet light and then sprayed with 10% sulphuric acid before heating them in the oven. The disappearance of the compounds on heating confirmed their organic nature.

The concentrate was then introduced on a florisil column. Benzene and diethyl ether (in the ratio of 4:1 respectively) was then used for elution and afforded a solution which, on evaporation in air, gave white crystals of a pure compound which this author named CORDIFENE.

2.2.4 CHEMICAL CHARACTERIZATION:

1. MELTING POINT:

When placed in a sealed glass tubing, Cordifene was found to have a melting point of 200-201^{o}C.

2. INTRARED SPECTROSCOPY

Cordifene was dissolved in chloroform (Spectro-

scopy grade) and using an infrared spectrophotometer (Pye Unicam Ltd., Cambridge, England) an infrared spectrum (Fig. 10) was obtained. The major characteristics of the spectrum were:-

a) Vmax at 1,780 cm^{-1} indicating a C=O group of the compound's lactone group.

b) A peak at 1,720 cm^{-1} indicating another C=O grouping.

3. ULTRAVIOLET SPECTROSCOPY

Cordifene was dissolved in absolute ethyl alcohol (analytical grade) and using an SP. 1800 Ultraviolet Spectrophotometer (SP. 1800 Ultraviolet Spectrophotometer, Pye Unicam Ltd., Cambridge, England) and quartz cells, an ultraviolet spectrum (Fig. 11) was afforded whose characteristics were:-

$$\lambda \text{ max} = 213$$

$$\Sigma = 18,310$$

The ultraviolet absorption confirmed conjugation in the compound.

4. MOLECULAR WEIGHT DETERMINATION

A Molecular ion with a molecular weight of 376 was obtained using chemical ionisation mass spectrophotometry. This was obtained through the kindness of Prof. Koji Nakanishi of Columbia University, New York, U.S.A. in whose laboratory this molecular weight determination was carried out. Dr. Asafu Maradufu

of the International Centre for Insect Physiology and Ecology in Nairobi, Kenya, attempted to determine molecular weight using electron ionisation mass Spectrophometry to but his attempt failed because the molecule broke up into smaller molecules the biggest ion obtainable by this method having a molecular weight of 197.

5. MOLECULAR FORMULA:

A molecular formular of $C_{20}H_{24}O_7$ was obtained by the kindness of Prof. Nakanishi of Columbia University, New York, U.S.A. by the use of ^{13}Carbon and ^{1}Hydrogen Nuclear Magnetic Resonance spectroscopy (Fig. 12, 13a and 13b).

Cordifene (Fig. 14) is a stable white crystalline material which is soluble in 70% methyl alcohol).

2.2.5 CONCLUSION ON CHEMICAL ANALYSIS OF ERLANGIA CORDIFOLIA MATERIAL

Analysis of biologically active compounds in Erlangia cordifolia material has shown cordifene to be the main organic component. This conclusion was arrived at on the following grounds:-

1) Cordifene has the main biological activities elaborated by the crude extract - that is, increasing muscular contraction, enhancing ATPase activity, and binding ADP.
2. Cordifene was the major extractable compound

found in the methyl alcohol extract. Among the crude extractions, methyl alcohol extract was found to have the highest activity.

Cordifene, however, does not dissolve markedly in water but in 70% methyl alcohol while all the crude extracts were very soluble in water. Improvement in solubility of cordifene in aqueous solution is brought about by the addition of sodium hydroxide into the mixture. A 0.4 molar solution of sodium hydroxide dissolves cordifene well.

Gitler and Montal (19) showed that proteolipids (normally soluble in chloroform:methanol mixtures) have to be neutralized first before they can dissolve in completely or almost completely apolar solvents. By either protonation or adding monovalent or divalent cations into proteolipids (for example, phosphatidylserine, phosphatidylcholine and phosphatidylinositol) and even a complex molecule like cytochrome C, they could make these molecules dissolve in n-decane. This then means that alteration of the charge of a molecule can lead to a change in its solubility characteristics. Similarly it is possible to visualize the lactone of cordifene being opened up and with this change in the ionic configuration of the molecule a change in its solubility characteristic could ensue. Chances of this possibility taking place are suggested by three factors:-

a) That the crude extracts from Erlangia cordifolia material were all basic.

b) Alkalis open up the lactone rings easily.

c) Basic compounds occur frequently in plants.

These factors could contribute to the alkalinity required in the extraction media to ensure the opening up of the lactone ring and thereby ensure the solubility of the molecule in water leading to its extraction from the plant material. Cordifene's solubility in the water extract would therefore give credibility to the suggestion that it is probably the major contributor to increased contractility of muscle caused by the extract of Erlangia cordifolia.

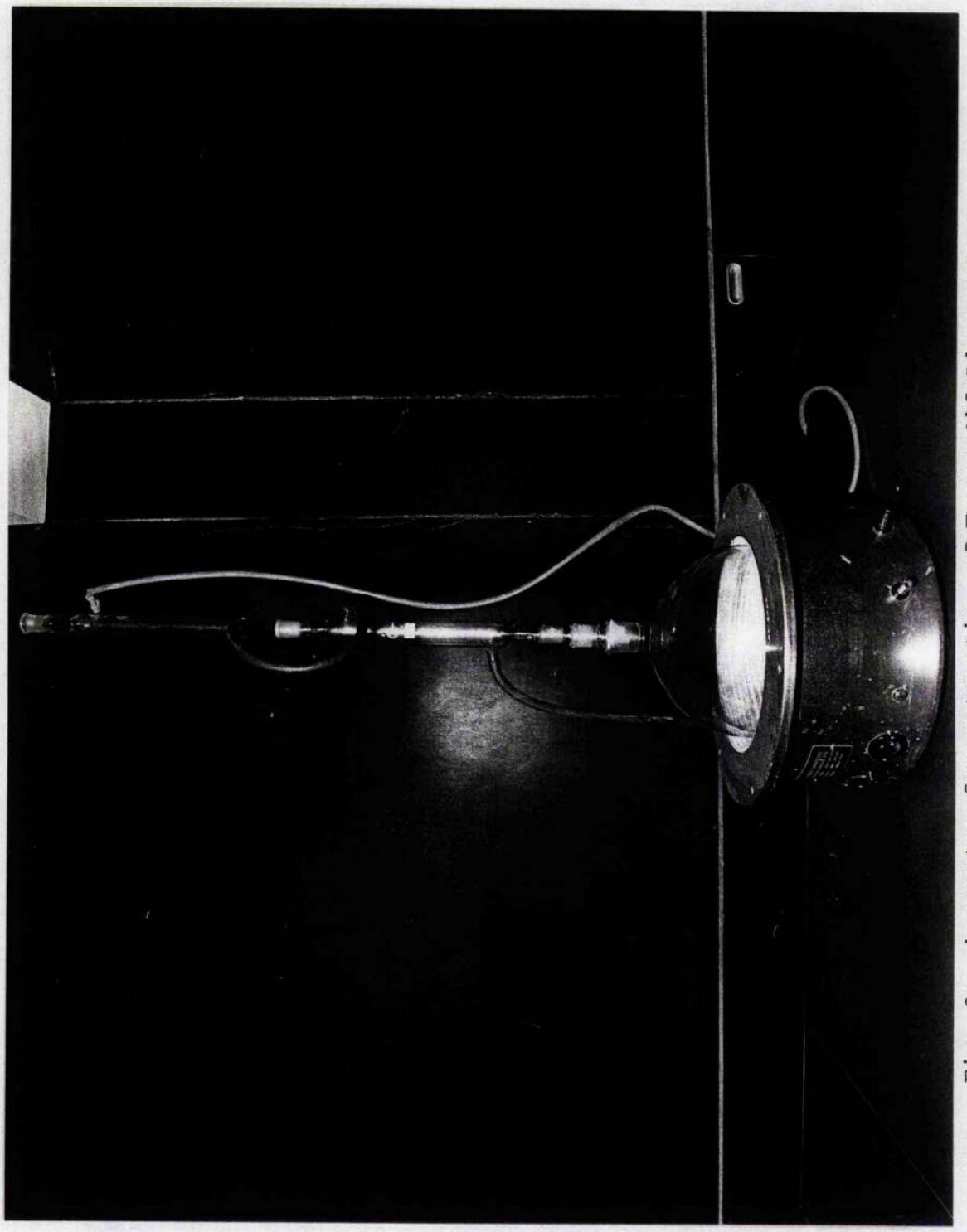


Fig. 9: Apparatus for extraction of E. cordifolia extract

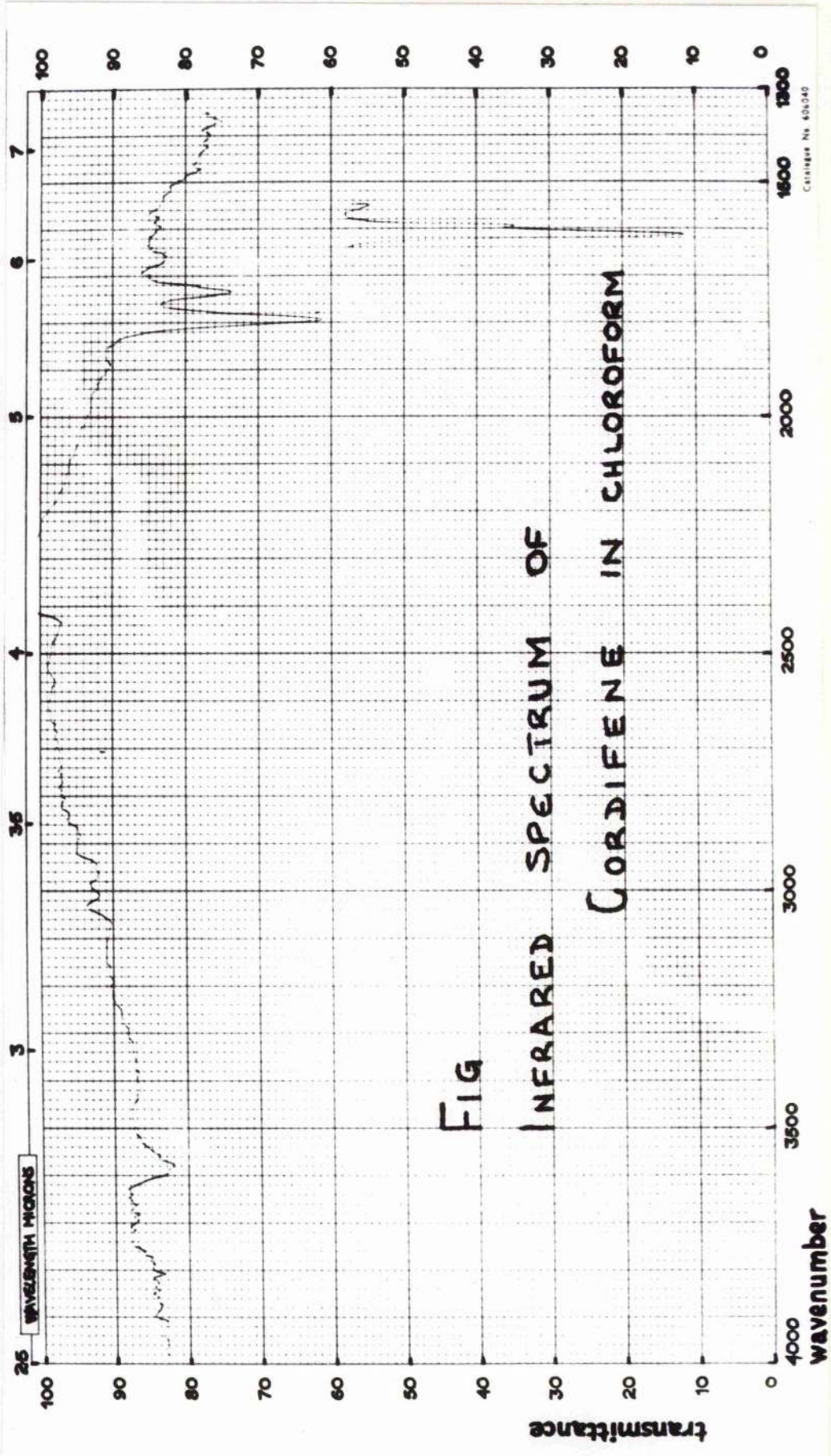


FIG
INFRARED SPECTRUM OF
CORDIFENE IN CHLOROFORM
transmittance
wavenumber
WAVELENGTH MICRONS

Fig. 10

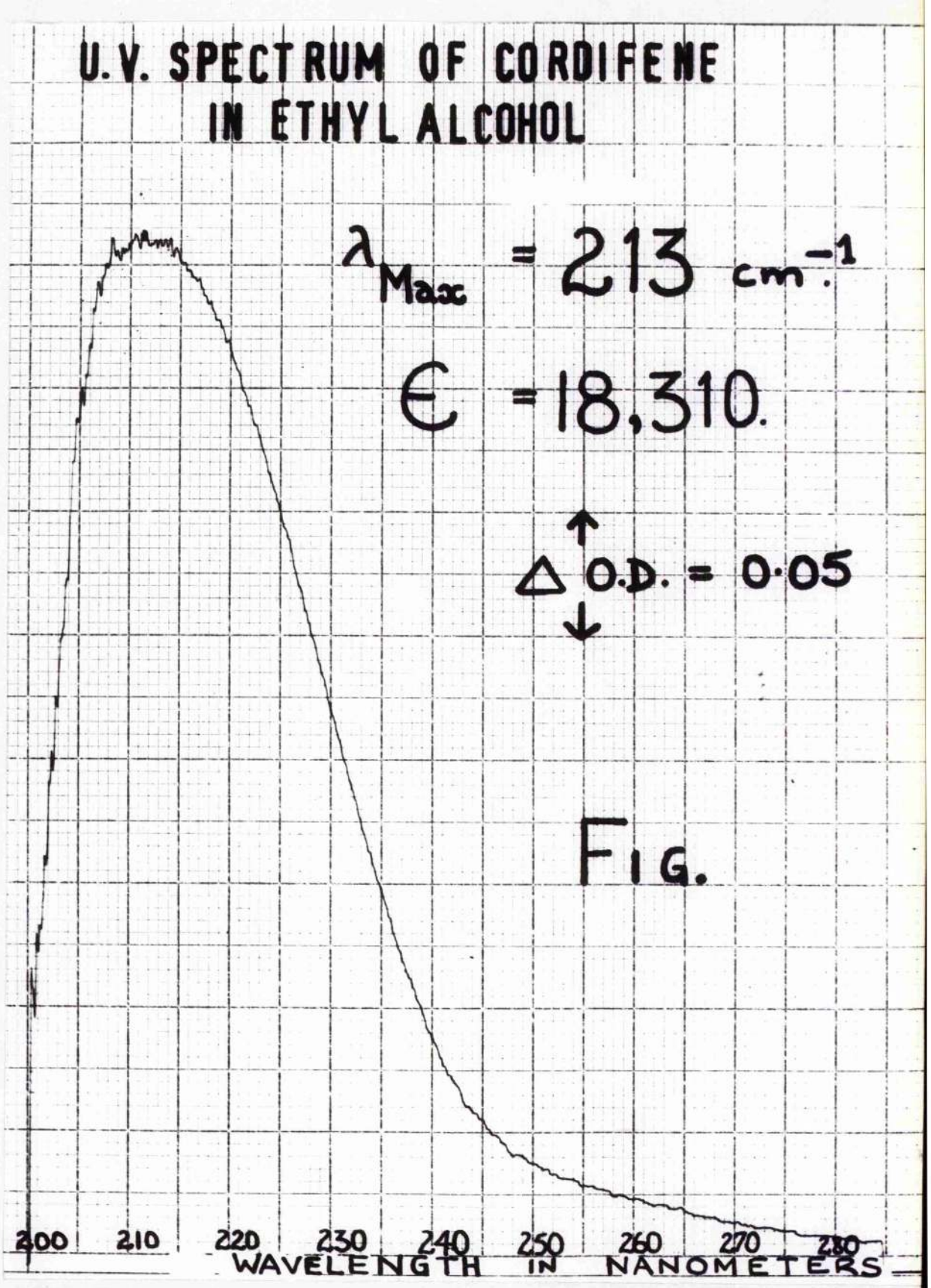

U.V. SPECTRUM OF CORDIFENE
IN ETHYL ALCOHOL
λ Max = 213 cm.-1
€ = 18,310.
Δ O.D. = 0·05
FIG.
200 210 220 230 240 250 260 270 280
WAVELENGTH IN NANOMETERS

Fig. 11

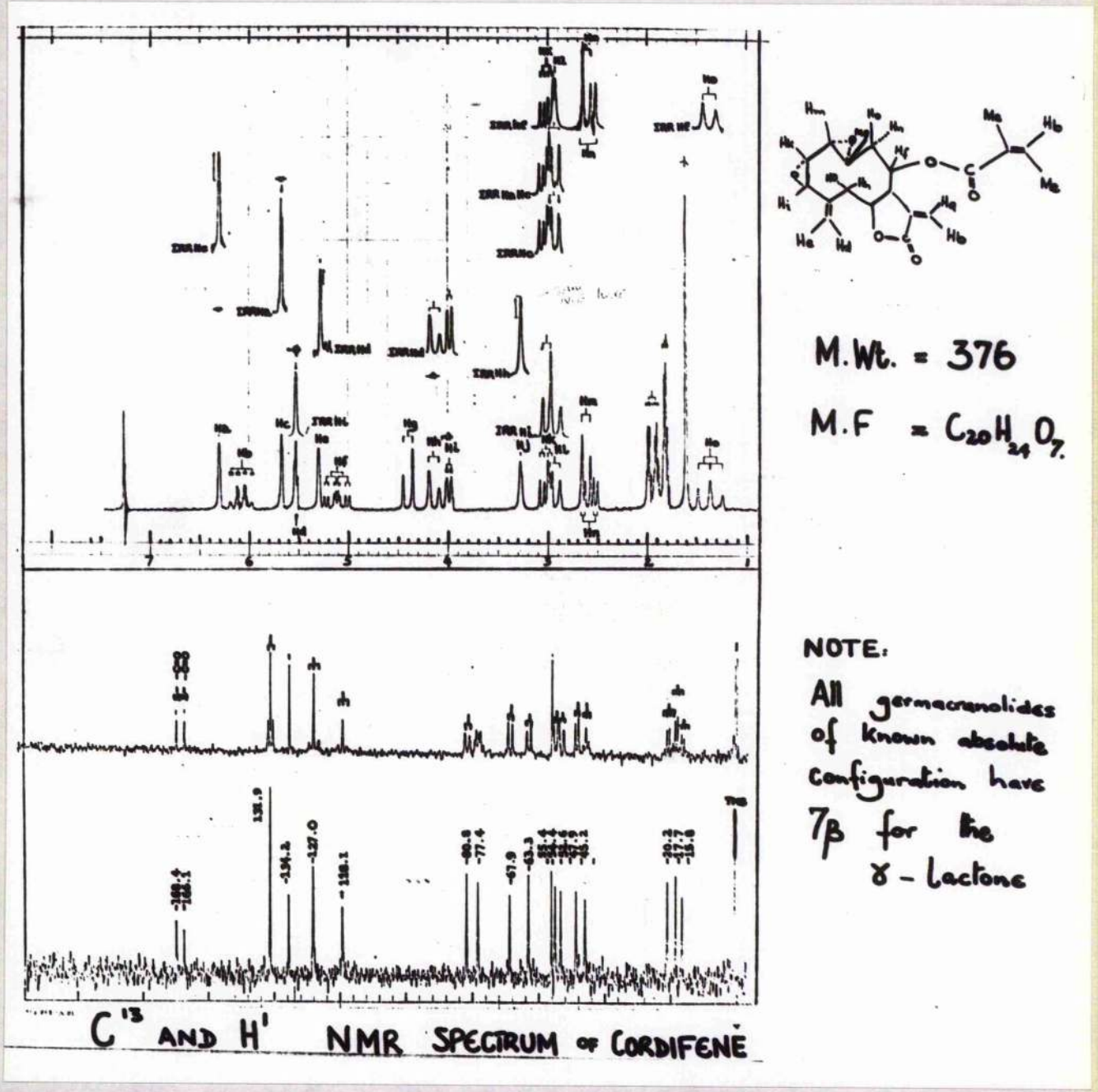


Fig. 12: ^{13}Carbon and ^{1}H nuclear magnetic resonace spectrum of cordifene

FIG.

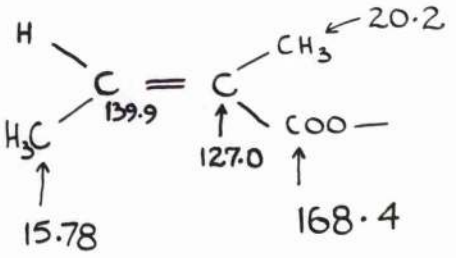
No 1				
1	15.78	$=C^{CH_3}$	3H	
2	17.71	$-\overset{\backslash}{\underset{/}{C}}-CH_3$	3H	
3	20.2	$=C^{CH_3}$	3H	
4	45.2	$-CH_2$ (H_n, H_o)	2H	
5	47.9	$-CH$	1H	
6	52.6	$-CH$	1H	
7	54.3	$-CH$	1H	
8	55.4	$-\overset{|}{\underset{|}{C}}-$	—	
9	63.3	$-CH-O$	1H	10
10	67.9	$-CH-O$	1H	10
11	77.4	$-CH_2-O$	2H	10
12	80.8	$-CH-O$	1H	10
13	118.1	$=C^{H}_{H}$	2H	
14.	127.0	$=C^{H}_{H}$	2H	
15.	127.0	$=C\diagup\diagdown$	—	
16.	134.2	$=C\diagup\diagdown$	—	
17.	139.9	$=C^{H}\diagup\diagdown$	1H	
18.	139.9	$=C\diagup\diagdown$	—	
19.	166.1	$C=O$	—	10
20.	168.4.	$C=O$	—	10
	TOTAL	C = 20	H = 24	O = 7~9 N ?

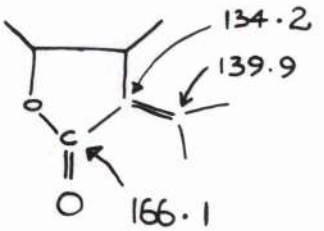
$C_{20}H_{24}O_8N = 392$
$C_{20}H_{24}O_7 = 376$
$C_{20}H_{24}O_6 = 360$
$C_{20}H_{24}O_7N = 390$
$C_{20}H_{24}O_6N = 374$
$C_{20}H_{24}O_6N_2 = 388$
$C_{20}H_{24}O_6N_3 = 402$

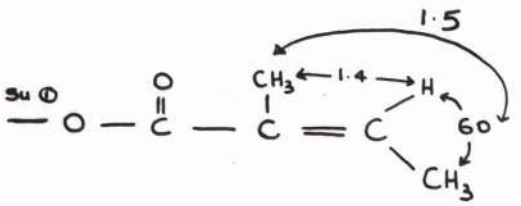
$C_{20} = 240$
$O_7 = 112$
$O_8 = 128$
$O_9 = 144$
$C_{20}H_{24} = 264$

Fig. 13: a) Partial interpretation of Fig. 12

No. 2





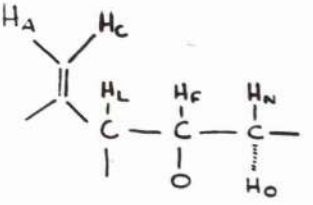


CH_3 = 1.81 = d, q.

CH_3 = 1.95 = d, ε

$=CH$ = 6.07 = ε ε

FIG.



$J_{HA.H_C}$ = ~0.4 ~0.2 Hz NOE

{Hc} HA = 35%

J_{HA, H_L} = 1.0 Hz

J_{HB, H_L} = 1.0 Hz

$J_{HL, Hf}$ = 3.9 Hz

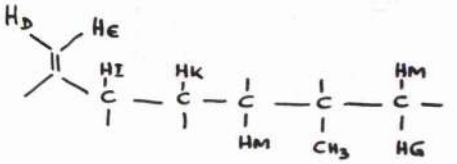
$J_{Hf, HN}$ = 9.6 Hz

$J_{Hf, HO}$ = 13.8 Hz

$J_{HO, HN}$ = 14 Hz

Noe {Hc} HA = 35% su ②

{HA} Hc = 25% su ②



{CH_3} H_H = NOE or J su ⑦, ⑧

{CH_3} H_K = NOE or J su ⑦, ⑧

{CH_3} H_G = NOE or J su ⑦, ⑧

{CH_3} H_H = NOE or J su ⑦, ⑧

J_{JI} = 0.8 Hz NOE ~9%

J_{DE} = 1.4 Hz

J_{DI} = 1.3 Hz } su ③

J_{EI} = 0.8 Hz

J_{IK} = 4.6 Hz

J_{KH} = 8 Hz

Fig. 13: b) Further interpretation of Fig. 12.

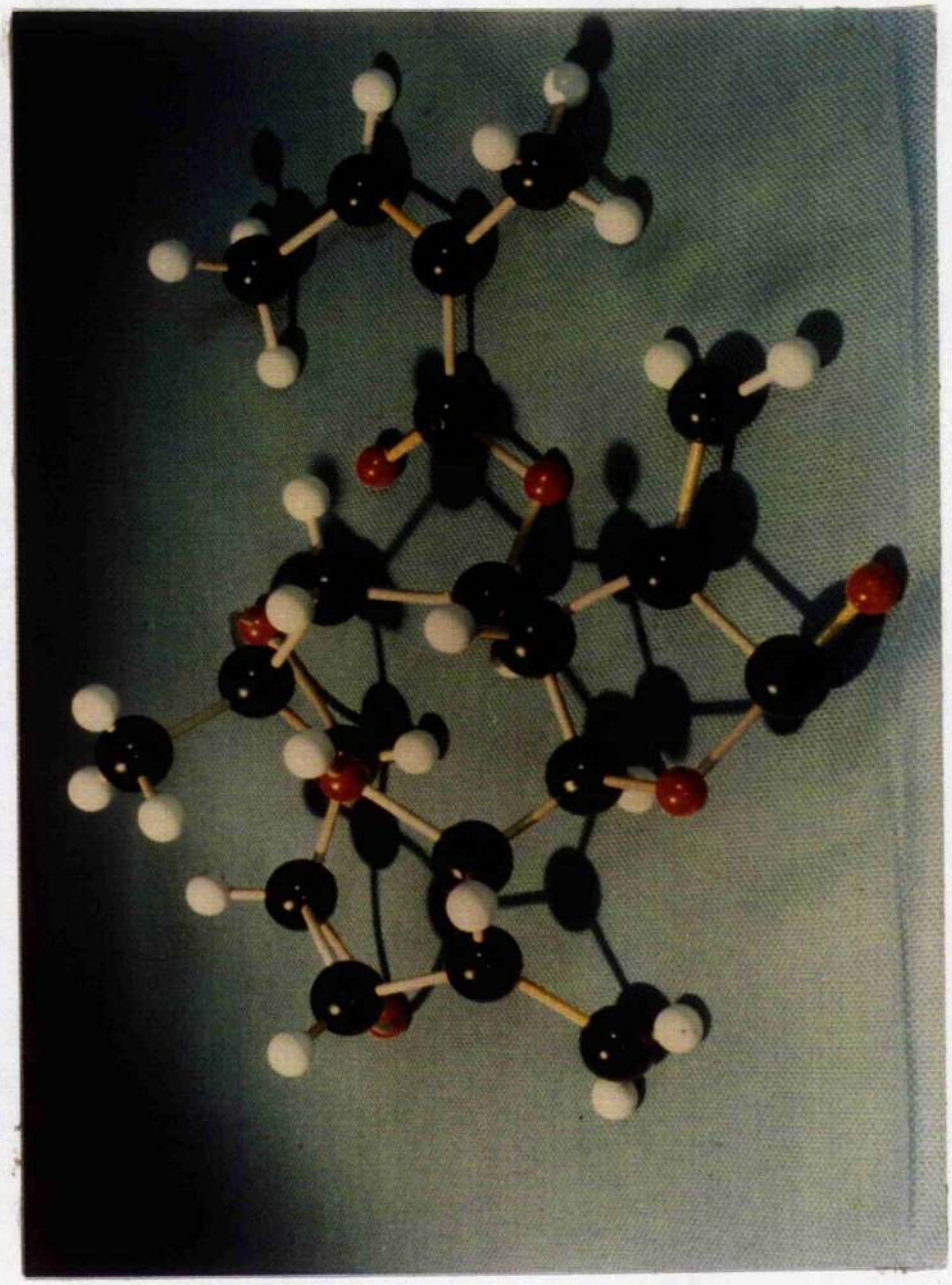


Fig. 14: The structure of cordifene

CHAPTER 3:

EFFECT OF ERLANGIA CORDIFOLIA MATERIAL ON MUSCLE

3.1 EFFECT OF ERLANGIA CORDIFOLIA MATERIAL ON THE SMOOTH MUSCLE OF THE MYOMETRIUM:

3.1.1 INTRODUCTION:

The author's interview with traditional medical practitioners had conclusively indicated that it was the pregnant woman in labour to whom the extract from Erlangia cordifolia was administered. As a starting point in the investigations on the effect of the extract on muscle contraction, the author had therefore to see whether he could induce increased contractions in rhythmically contracting myometrial strips. Guinea pig and albino rat myometrial strips were chosen for the purpose because these animals were readily available.

3.1.2 MATERIALS AND INSTRUMENTS:

A thermal regulated water jacketed glass tissue bath (scientific and research instruments Ltd.,

Croydon, Surrey, England).

Devices recorder - serial Number M 4 352 (Devices Sales Ltd., Welwyn Garden City, Hertfordshire, England).

Blender (Fischer Scientific, Zeitweg, Zurich, Switzerland).

Mixture of 95% O_2 and 5% CO_2 (East African Oxygen Ltd., Nairobi, Kenya).

Leaves and terminal branches of Erlangia cordifolia.

Kreb's solution. One litre of this solution contained 5.54 ml. Molar NaCl; 0.35 ml. Molar KCl; 0.29 ml. Molar $MgCl_2$, $MgSO_4 \cdot 7\ H_2O$; 0.28 ml. Molar $CaCl_2$; 2.1 ml. Molar $NaHCO_3$; 0.16 gm. anhydrous KH_2PO_4 and 2.1 gm. glucose.

Pregnant guinea pigs and pregnant albino rats (supplied by Medical Research Laboratories, Ministry of Health, Nairobi, Kenya).

Cordifene

Oxytocin.

3.1.3 EXPERIMENTAL PROCEDURE:

To one kilogram of fresh green leaves and terminal branches of Erlangia cordifolia was added two litres of distilled water and then blended with a blender and the mixture boiled for ten minutes after which the mixture was allowed to cool for four hours. The greenish yellow extract was then filtered.

A pregnant guinea pig or rat was sacrificed by

giving it a heavy blow on the head and the stomach wall was opened up as quickly as possible to extract the myometrium. A myometrium weighing about 20 gms was then immediately placed in a beaker containing Kreb's solution and equilibrated at 32°C. Into this solution containing the myometrium, a slow flow of the mixture of the oxygen and carbon dioxide gas mixture was introduced through a glass tube so as to keep the muscle alive. A suitable thin strip of the myometrium, about 0.75cm. wide and 2.5cm. long and weighing about one gram was then isolated from the myometrium and immediately placed in a glass petri dish containing Kreb's solution again equilibrated at 32°C. The strip was kept alive by constant application of the temperature - equilibrated Kreb's solution. The myometrial strip was then fixed onto the fixed point at the bottom of a glass tubing connected to the oxygen - carbon dioxide supply and the glass tubing with myometrial strip attached to it was then placed in the tissue bath containing Kreb's solution at 32°C. The other end of the myometrial strip was fixed onto the Devices recorder with a thin cotton string in such a way that contractions of the muscle would pull the string fixed onto the Device's recorder and this movement would then be detected by the recorder. The organ bath had an inflow at the top and an outflow at the bottom of the tissue compartment. This was the arrangement used throughout these recording experiments. The oxygen-carbon dio-

xide mixture was allowed to flow in at a steady rate of about two litres per minute. A volume of 20 ml. Kreb's solution in the tissue bath was the amount of Kreb's solution that bathed the tissue, always, in these experiments.

With most of the myometrial strips used, steady contractions of the muscle started immediately and automatically as soon as the strip was fixed (as described above) in the tissue bath. Where contractions did not start automatically, about 5 nanograms of oxytocin were added into the bathing solution to start off the contractions. If this failed it was then taken that the particular strip was dead and another strip was isolated and fixed as described above.

Contractions of the myometrial strip were recorded when the strip's intrinsic contractions were deemed to have become steady. After five minutes of recording of the steady intrinsic contractions, 0.1 ml. of Erlangia cordifolia solution was then added, using a micropipette, while the recording of the contractions was still going on. When a recording of the steady rate of contraction of the strip in the modified Kreb's solution had been done for at least five minutes, the modified Kreb's solution bathing the strip was then changed making sure that the replacing volume of Kreb's solution was always the same volume as that which was ran out of the

tissue bath. The procedure was repeated with steadily increasing amounts of the Erlangia cordifolia extract, the difference in between one dose of the extract and the next being always 0.1 ml. The reservoir of the Kreb's solution used for supplying fresh Kreb's solution was kept at room temperature and as such time had to be allowed for the fresh Kreb's solution to equilibrate to the water bath temperature of 32°C and the strip's intrinsic contractions in this fresh Kreb's solution to become steady for at least five minutes before the Erlangia cordifolia solution was introduced into the bath. No addition of the extract was made until the myometrial intrinsic contractions in the fresh Kreb's solution were deemed to have become steady and for at least five minutes. Then the recording of the contractions under the influence of the extract was then repeated followed by stopping the recording and running out the modified Kreb's solution in order to add fresh Kreb's solution in preparation for another cycle of recording.

In every case when this experiment was carried out the initial dose of the extract that was added to the 20 ml. of Kreb's solution using micropipettes and ordinary pipettes was always 0.1 ml.

The procedure was repeated using cordifene at a concentration of 5 mg. in 10 ml of 0.4 Molar NaOH. The tracings on the smoked paper were then fixed on the paper using a 1:1 solution of benzene and xylene.

3.1.4 RESULTS:

From Fig. 15A, it can be seen from the tracings that Erlangia cordifolia leaves and terminal branches contain a compound (or compounds) which not only increases the contractions in terms of magnitude but also the duration of each contraction is increased. The extract was also found to increase the magnitude of contractions and duration of the contractions in the non-pregnant uterus.

The results of stimulation due to cordifene (Fig. 15B) was a reproduction of what had been observed while using the crude extract.

3.2 EFFECT OF ERLANGIA CORDIFOLIA MATERIAL ON PERISTALSIS:

3.2.1 INTRODUCTION:

Peristalsis is a physiological phenomenon exhibited by the smooth muscles in the wall of the gastrointestinal system. If the effect of Erlangia cordifolia material was not just on the myometrium alone, and especially if this material simply augmented an already existing contraction wave, then one would expect the material to augment peristaltic contractions in the gut muscle. The series of experiments reported in here was therefore carried out to see whether Erlangia cordifolia material did in fact augment contraction waves in smooth muscles in general.

3.2.2 MATERIALS AND APPARATUS:

A thermal - regulated water jacketed glass tissue bath with the inflow at the top and the outflow at the bottom of it (Scientific & Research Instruments Ltd., Croydon, Surrey, England).

An electrically driven recording drum (C.F. Palmer (London) Ltd., England).

Smoking paper.

Glass micropipettes and pipettes.

Tyrode solution whose contents per litre were 8 gm. NaCl; 0.2 gm. $CaCl_2$; 0.2 gm. $MgCl_2$; 1.0 gm. $NaHCO_3$; 0.05 gm. KH_2PO_4 and 1.0 gm. of glucose.

Mixture of 95% O_2 and 5% Carbon dioxide (East African Oxygen Ltd., Nairobi, Kenya).

Erlangia cordifolia extract and cordifene.

3.2.3. EXPERIMENTAL PROCEDURE:

Preparation of Erlangia cordifolia extract and the cordifene solution were prepared as described in Section 3.1.3.

An adult guinea pig of about four months which had been starved for sixteen hours was sacrificed by giving it a heavy blow on the head with a piece of wood and the stomach wall opened up as quickly as possible so as to extract the small intestine. A small intestinal strip weighing about thirty grams was isolated and after being washed clean with warm Tyrode solution (at 37°C) was freed from its omentum

and placed in a small beaker containing the same physiological solution at 37°C. Into this beaker containing the myometrium a slow flow of the gas mixture of oxygen and carbon dioxide was introduced through a glass tube so as to keep the muscle alive. A suitable strip of the small intestine (duodenum, jejunum or ileum) about 4 cm. long and weighing about one gram was then isolated from the small intestinal specimen. The strip was then fixed onto the fixed point at the bottom of a glass tubing connected to the oxygen-carbon dioxide gas mixture source making sure that the tissue tubing remained patent. A thin cotton string was then fixed at the opposite end of the muscle strip again making sure that the muscle tubing retained its patency. The whole fixing process was carried out while the intestinal specimen was still bathed in the physiological solution at 37°C. The glass tubing onto which one end of the intestinal strip was fixed was then transferred into a physiological solution in the water jacketed glass tissue bath and the string attached to the lose end of the specimen was then attached to a lever that was designed to make a recording on the smoked paper on the drum (Fig. 16). The tissue bath was surrounded by water maintained at 37°C by a thermostatically controlled heating unit.

Recording of the intestinal strip's peristaltic activity was recorded kymographically. After tempera-

ture equilibration and when the peristaltic contractions had become reasonably uniform and therefore could be judged to be steady and constant, the strip was then treated with gradually increasing amounts of Erlangia cordifolia extract - always starting with 0.1 ml. of the extract and increasing the dose to be added by doubling the previous amount. An extract was added only when the contractions were judged to be constant.

The experiment was repeated using a solution of cordifene made by dissolving one mg. of cordifene in 10 ml. of 70% methyl alcohol and again with a solution of cordifene made by dissolving one gm. of cordifene in 20 ml. of 0.40 Molar NaOH. The tracings on the smoked paper were then fixed on the paper using a 1:1 solution of benzene and xylene.

3.2.4 RESULTS

Fig. 17 is a kymographic tracing showing the effect of crude Erlangia cordifolia material on peristalsis. Fig. 18 is a kymographic tracing showing the effects of cordifene on the peristalsis of the intestinal strip.

From the tracings, it can be seen that both Erlangia cordifolia crude material and cordifene increased both the tone and the amplitude of the intestinal contractions. Not only was the tone and the amplitude of the contractions increased, the increases were sustained for a long time.

3.3 EFFECT OF ERLANGIA CORDIFOLIA MATERIAL ON SKELETAL MUSCLE CONTRACTIONS:

3.3.1 INTRODUCTION:

Barany et al. (20) have shown that a number of substances which include urea, guanidine - HCl, ethylene glycol and ethyl alcohol stimulate Ca^{2+} - ATPase of chicken gizzard myosin and also activate Ca^{2+} - ATPase of rabbit uterus myosin but have no effect on the Ca^{2+} - ATPase of chicken breast myosin. Smooth muscle has an intrinsic mechanism that enables it to contract regularly in the form commonly referred to as peristalsis in the gastro-intestinal system. As such no extraneous stimulation is required to facilitate this contraction with a possible exception of the occasional uterine specimen.

Having found that Erlangia cordifolia increased the contractile activity of smooth muscle, and in view of the fact that some stimulants will have an effect on some types of muscles and not on others, it became desirable to find out whether the activity of E. cordifolia material was applicable to all types of muscles - that is, those with an intrinsic contractile mechanism for rhythmic contractions and those that had to be stimulated first before they could contract.

3.3.2 MATERIALS AND APPARATUS:

A kymograph recording drum, 200-250 volt, alternating current (C.F. Palmer (London) Ltd., England).

Electronic stimulator - Catalogue Number 418/8048/01. (C.F. Palmer (London) Ltd., England).

A phrenic nerve electrode - Catalogue Number 418/8095. (C.F. Palmer (London) Ltd., England).

Spring lever (C.F. Palmer (London) Ltd., England).

Seconds' timer - 200-250V., 50 Hz. (C.F. Palmer (London) Ltd., England).

Thermal regulated water-jacketed glass tissue bath (Scientific & Research Instruments Ltd., Croydon, Surrey, England).

Water bath: 200-250V., alternating current. (Scientific & Research Instruments Ltd., Croydon, Surrey, England).

95% O_2 and 5% CO_2 gas mixture (East African Oxygen Ltd., Nairobi, Kenya).

Kreb's solution consisting of 6.92 gm. NaCl; 0.354 gm. KCl; 0.294 gm. $MgSO_4$; 0.162 gm. KH_2PO_4; 2.10 gm. $NaHCO_3$; 2 gm. glucose and 0.282 gm. anhydrous $CaCl_2$.

White albino rats (supplied by the Medical Research Laboratories, Ministry of Health, Nairobi, Kenya).

Erlangia cordifolia material and a cordifene solution both prepared as in Section 3.1.3.

3.3.3 EXPERIMENTAL PROCEDURE:

The diaphragm of a white adult rat weighing about 250 gm. was isolated together with the phrenic nerve. During the isolation the diaphragm was kept alive by constant bathing in Kreb's solution. After isolation, the muscle with its nerve was connected to the kymograph electrode and this whole combination was then inserted in the organ bath which had been equilibrated at 37°C. The oxygen-carbon dioxide gas mixture was allowed to flow through the organ bath at a steady rate of about two litres per minute and when the nerve-muscle combination was deemed to have equilibrated at the constant temperature the end of the phrenic nerve farthest away from the muscle was then tied with a piece of thin cotton thread and connected to the recording lever. The stimulator electrodes were then connected to the apparatus following the instructions given by the manufacturers on the operation of the Electronic Square Wave Stimulator, H. 44. 1 - 10 Volts was chosen as the range of Amplitude variation.

The electric current was then switched on and muscular jerks allowed to equilibrate. The kymograph was then fixed in position and the impulse time recording system fixed (Fig. 19).

Recording was then commenced starting first with the recordings of muscular contractions of the diaphragm in pure Kreb's solution and these recordings

were continued until almost frictionless and constant values were achieved on the recorder before any Erlangia cordifolia extract could be added to the Kreb's solution bathing the muscle.

Erlangia cordifolia material was administered into the organ bath at constantly increasing doses starting with one ml. of the extract and increasing the amount during the next addition by one ml. at a time. Rinsing of the muscle with thermally-regulated Kreb's solution in between addition of the extract solution was strictly observed. After rinsing the muscle, the temperature of the bathing solution was allowed to equilibrate at $37^{o}C$ before the apparatus was switched on again and the procedure repeated - this time with a higher dose of the Erlangia cordifolia material.

The experiment was repeated using the solutions of cordifene. The experiment was again repeated using a Kreb's solution which was modified by non-addition of $CaCl_2$ during its preparation so as to deny the muscle Ca^{2+}. The smoked paper tracings were fixed on the paper using a 1:1 solution of benzene and xylene.

3.3.4 RESULTS:

Kymographic tracings of the contraction of skeletal muscle bathed in normal Kreb's solution and in Kreb's solution modified by:-

a) Erlangia cordifolia crude material is shown in Fig. 20.

b) Cordifene solution is shown in Fig. 21.

c) Lack of Ca^{2+} in the Kreb's solution is shown in Fig. 22.

From the tracings it can be seen that the Erlangia cordifolia material increased the amplitude of the contractions of the skeletal muscle as long as Ca^{2+} were present in the bathing solution. Cordifene had a similar effect. The increased amplitude of contractions was also maintained for a long time.

3.4 CONCLUSION ON KYMOGRAPHIC STUDIES ON MUSCLE CONTRACTION:

The results obtained through kymographic studies show unequivocably that there is a biochemical basis on the modification of the contractile activity of muscle that can be accredited to Erlangia cordifolia material as used by the traditional medical practitioners in the Gikuyuland in Kenya.

In the three types of muscle used to test the effect of Erlangia cordifolia material on the contractility of muscle, augmentation of contractions was observed. The effect of the material could not be artefactual as a result of volume or temperature changes in the bathing solution during the course of the experiments because the bathing solution's temperature was thermostatically regulated and the small volumes of either crude extract solution or cordifene

solution used were far too small compared with the bathing solution's volume to cause any appreciable changes in the overall volume of the bathing solution or its temperature.

It is difficult to say whether the appreciably large amounts of Ca^{2+} found in the crude extract of Erlangia cordifolia contributes markedly to the contractile process. A simple test with eriochrome T had shown that this element occured in the plant in the ionic form. But considering the small amounts of Erlangia cordifolia material used in these experiments, and considering that the optimal concentration of Ca^{2+} required for contraction - and when a small decrease in the available Ca^{2+} would not affect the contractile activity - is 2×10^{-2} Molar (21), then one must conclude that the Ca^{2+} contribution of the material is negligible for the purposes of muscle contraction. Another conclusion that can be drawn is that Erlangia cordifolia material does not have any effect on a contractile muscle system which is devoid of any Ca^{2+}.

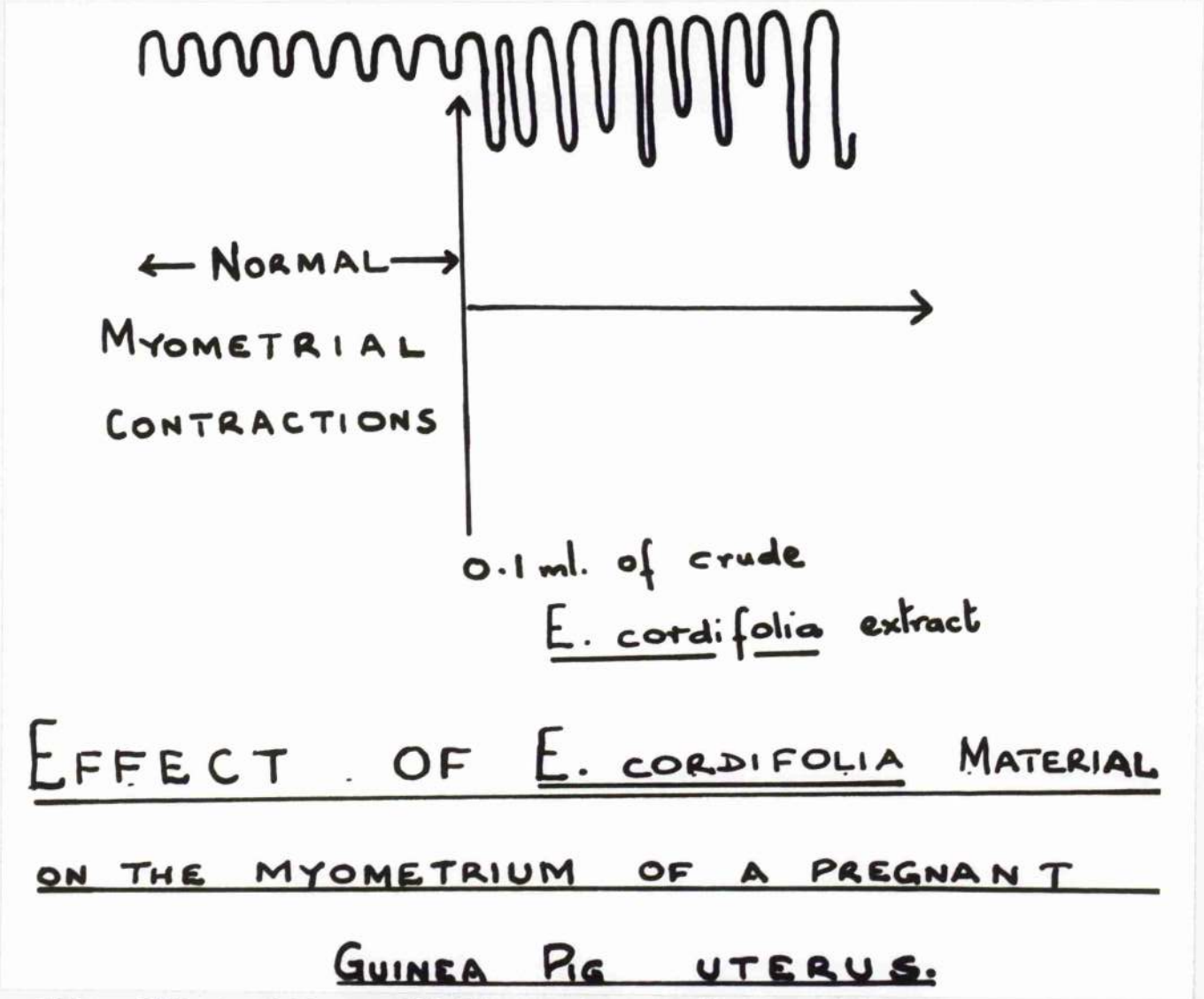


Fig. 15A: Effect of E. cordifolia crude material on the intrinsic contractile activity of the myometrium of a gravid guinea pig uterus.

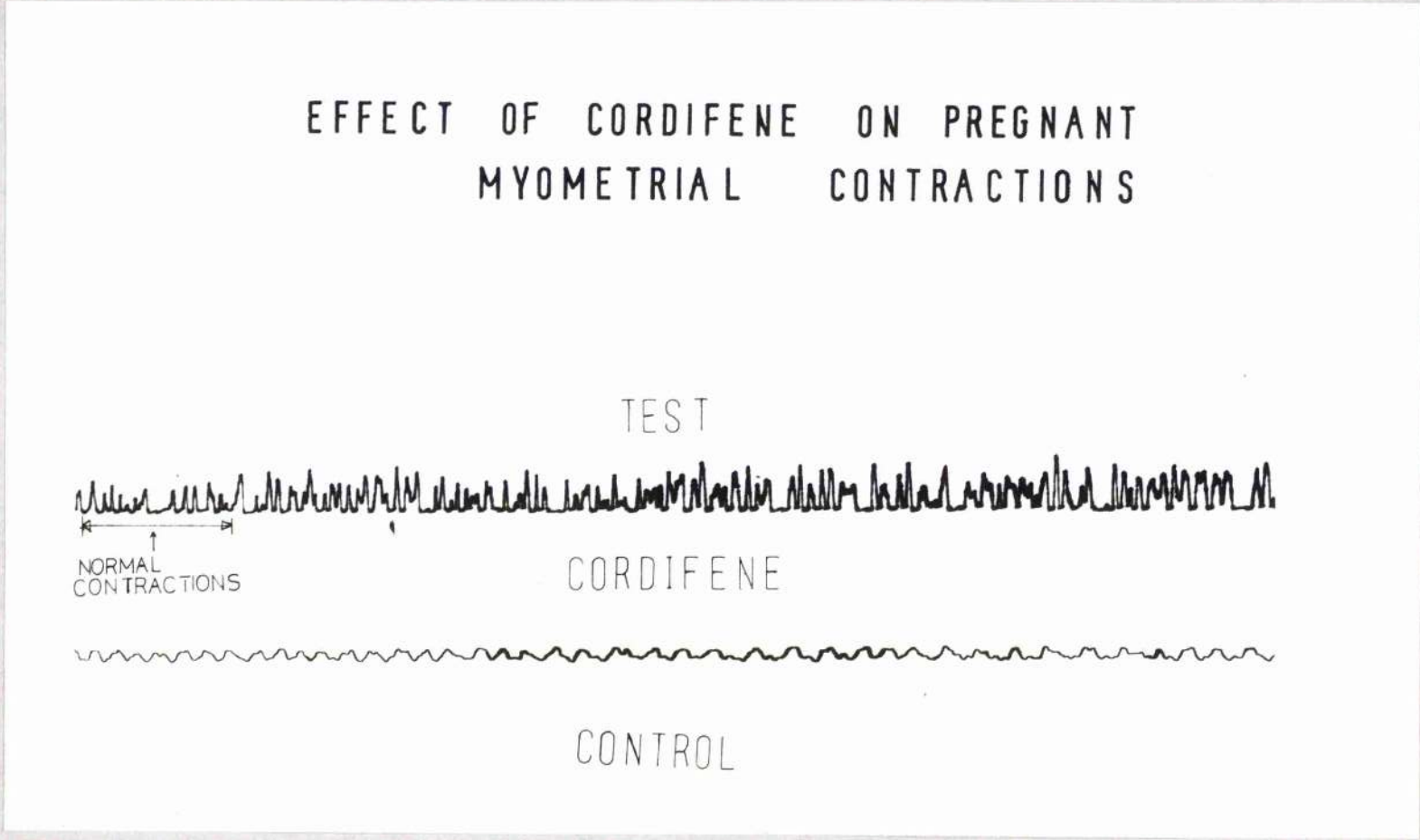


Fig. 15B: Effect of cordifene on the intrinsic contractile activity of the myometrium of a gravid guinea pig uterus

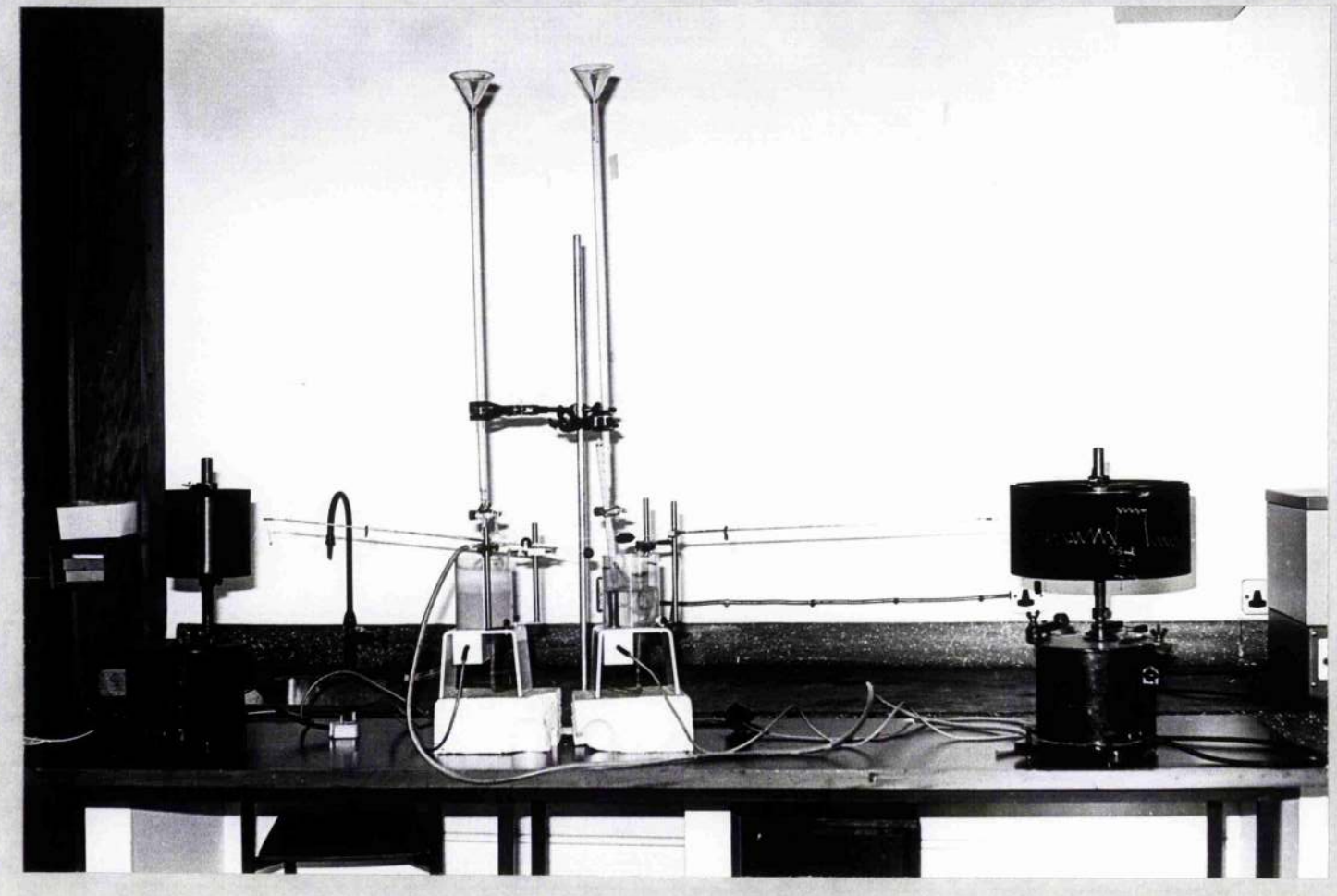


Fig. 16: Apparatus for recording peristaltic contractions of the ileum

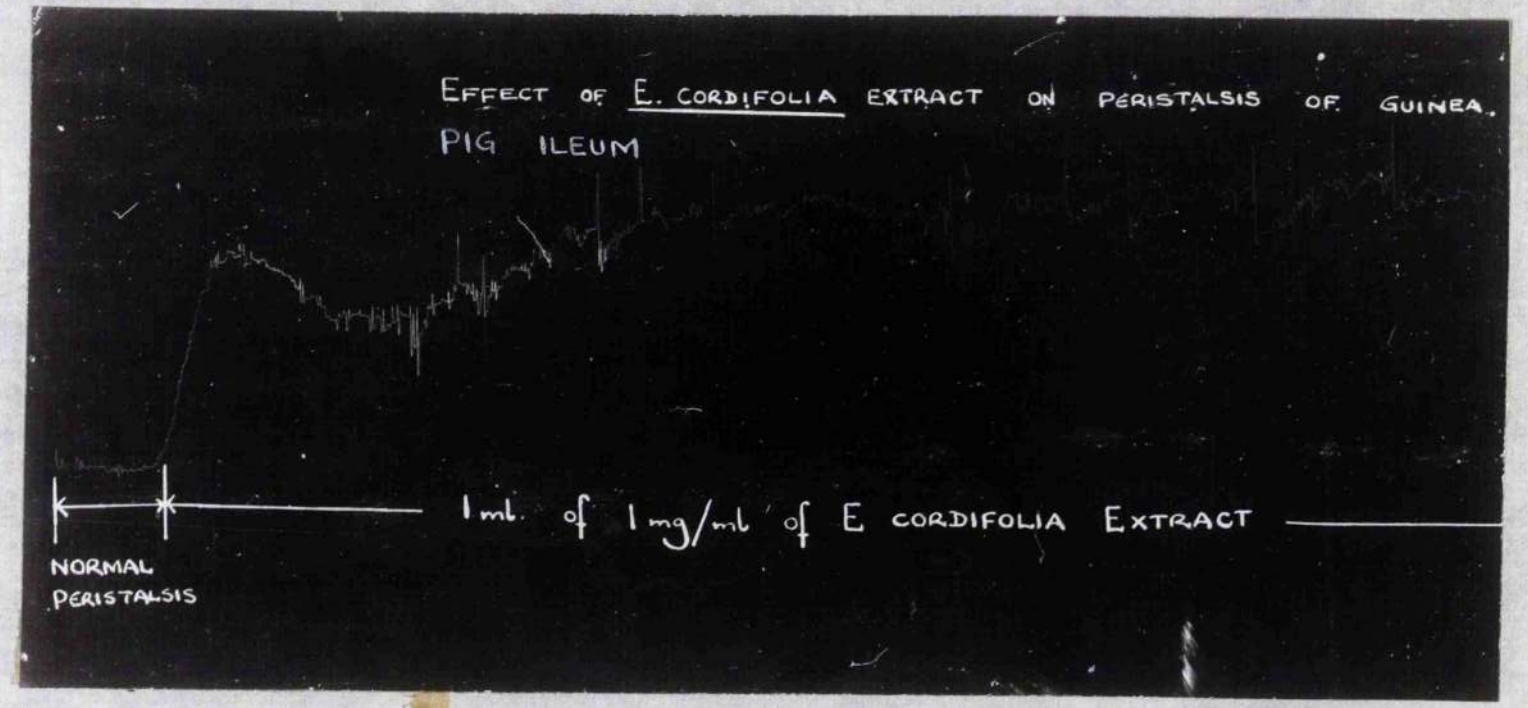


Fig. 17: Effect of crude E. cordifolia extract on the peristalsis of a guinea pig ileum

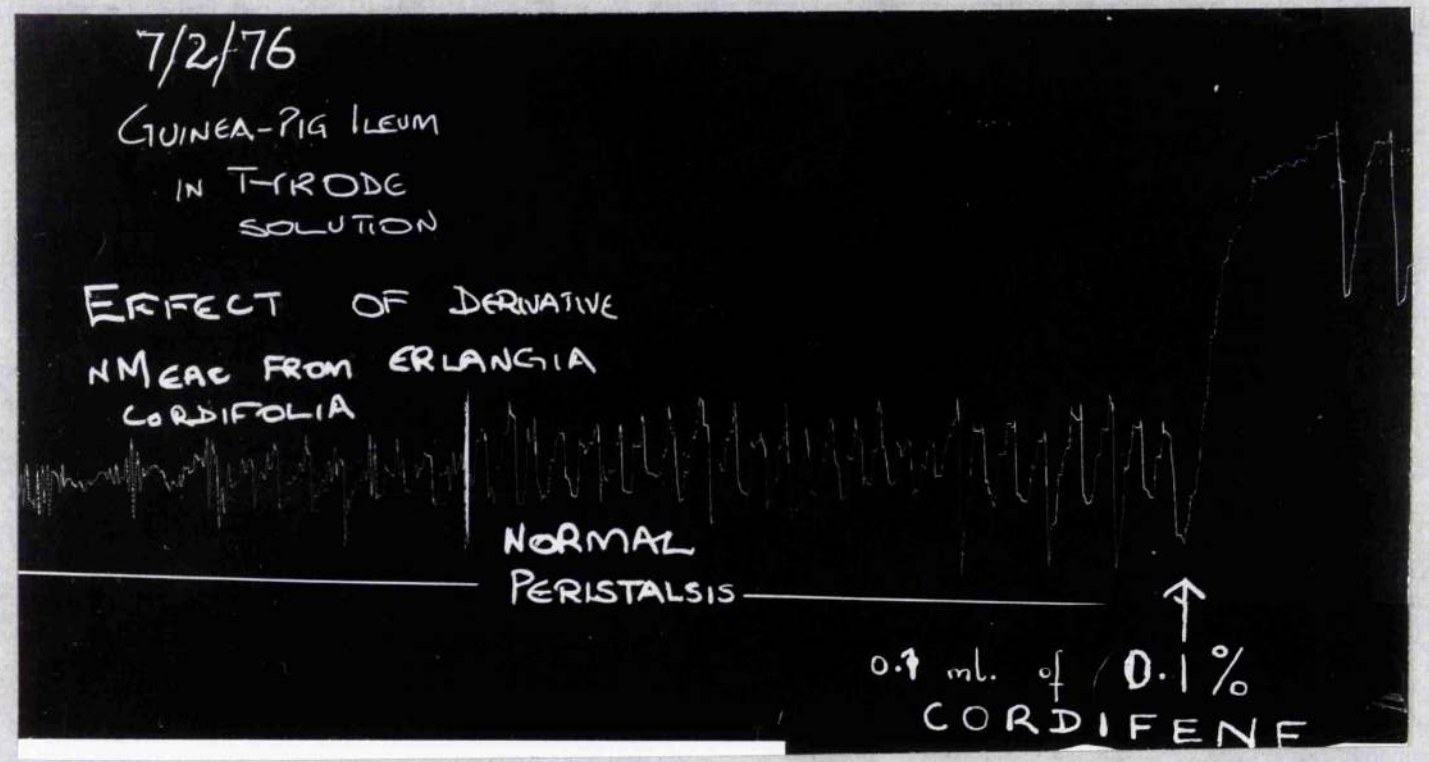


Fig. 18: Effect of cordifene on the peristalsis of a guinea pig ileum

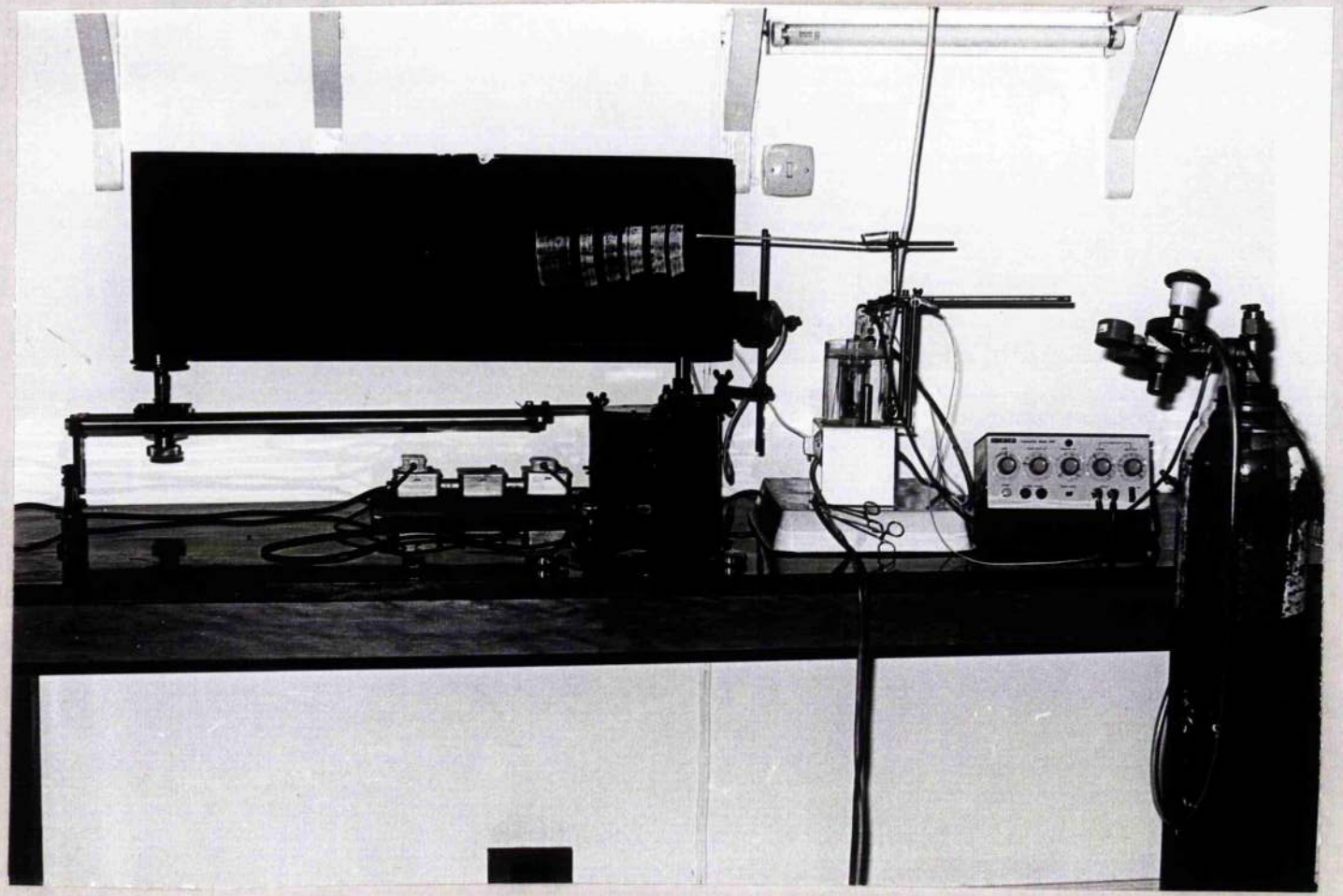


Fig. 19: Apparatus for recording the contraction of a diaphragm muscle

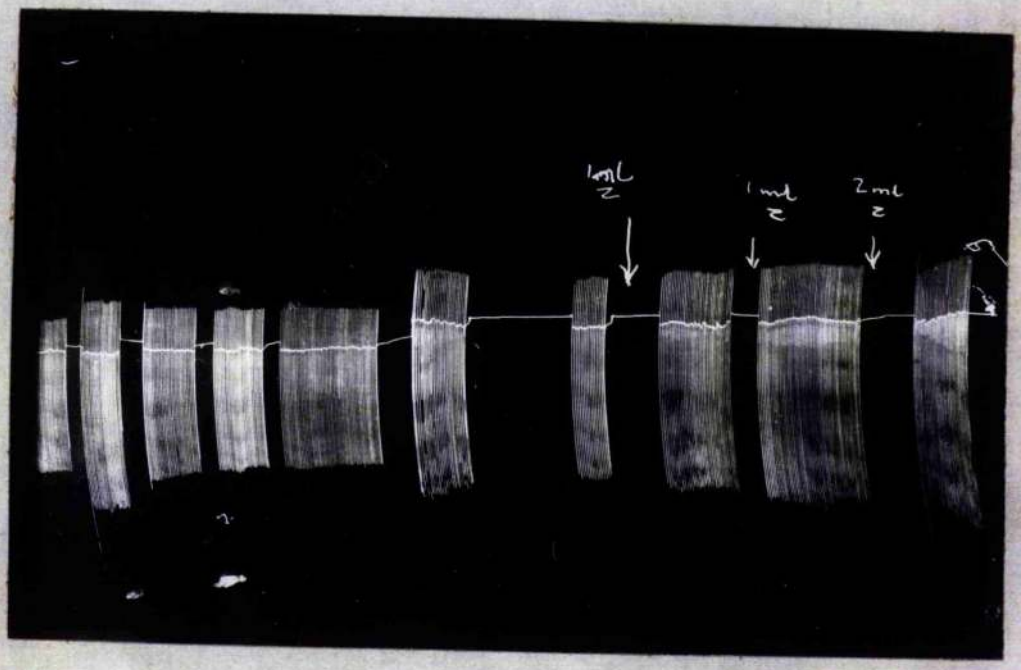


Fig. 20: Effect of crude E. cordifolia extract on the contraction of skeletal muscle of a rat diaphragm.

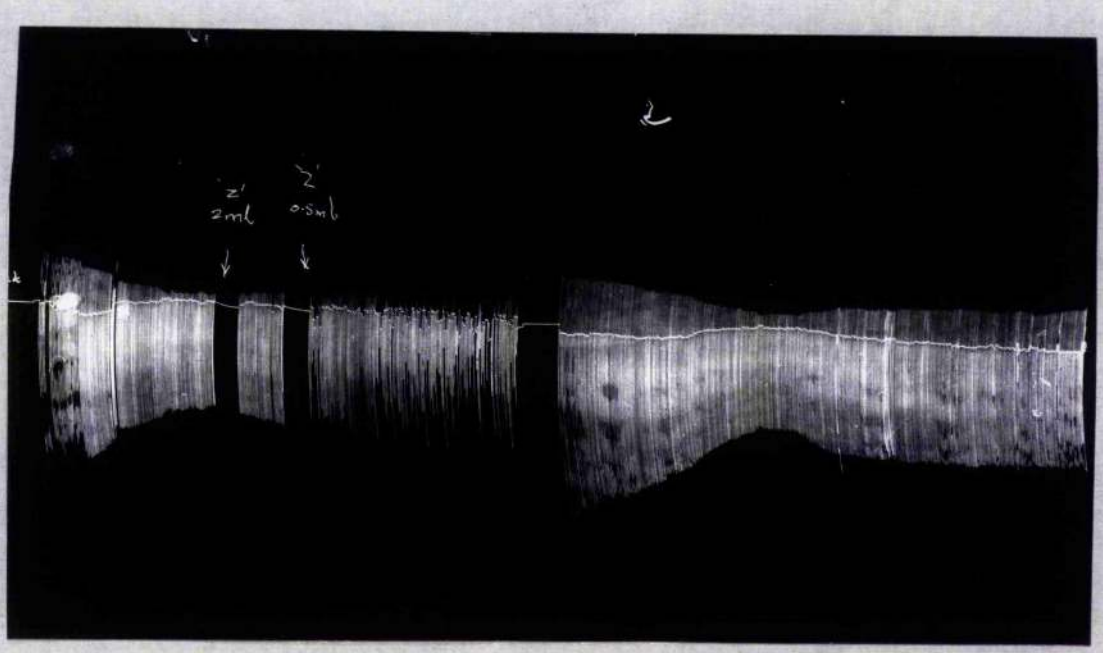


Fig. 21: Effect of cordifene on the contraction of skeletal muscle of a rat diaphragm.

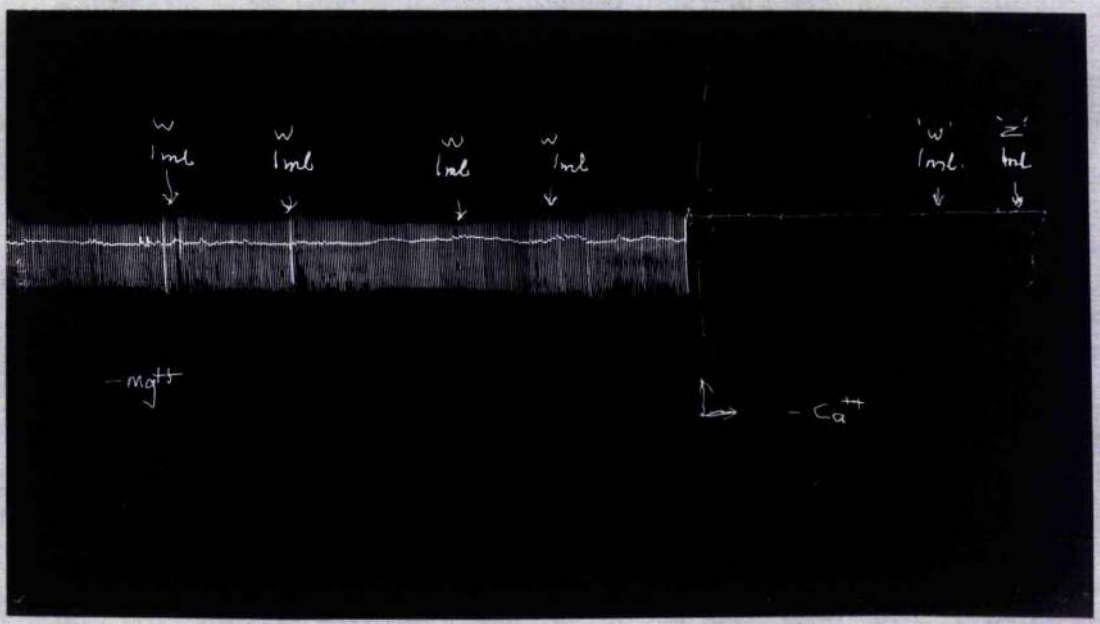


Fig. 22: Effect of crude E. cordifolia extract on the contraction of skeletal muscle of a rat diaphragm bathed in Kreb's solution which had no calcium ions.

C H A P T E R 4:

INVESTIGATION OF POSSIBLE BIOCHEMICAL INTERACTION IN BETWEEN ERLANGIA CORDIFOLIA MATERIAL AND MOLECULES WITHIN THE NERVOUS SYSTEM:

4.1 COMPARISON OF ERLANGIA CORDIFOLIA EXTRACT WITH CHEMICAL MEDIATORS WITHIN THE NERVOUS SYSTEM:

4.1.1 INTRODUCTION

There is a practical aspect related to the screening of new compounds of pharmacological interest that is provided by the mechanistic or theoretical side of pharmacology. This is so because deduction that may be made from comparing the activity of a new compound with those of known compounds may have immediate consequences for the experimenter. Such deductions which could be obtained by comparing a "pharmacological profile" of a new substance with those of known compounds may provide a plan for the next phase of experimentation on the mechanism of action of the substance.

In the case of Erlangia cordifolia extract, increased tone and contraction in muscle had been

established by physiological experiments on both smooth and striated muscles. The increased activity could be due to the increased rate of depolarization of the neuromuscular junction due to the stimulation of the nervous system (Fig. 23) supplying such a junction. The consequences of alteration of the nature of nervous supply to a muscle can be seen from the fact that when a nerve which normally innervates a white striated muscle is transplanted to a red striated muscle, the red muscle starts to behave like a white striated muscle (22). The action of the extract could also be histamine-like, meaning that it stimulates the muscle cell directly (23). Eliminating one or the other of these two mechanisms could establish the next line of investigation to be followed.

In the autonomic system a substrate can promote activity through one or more of a number of mechanisms. These are (23):-

1. Promotion of the release of the transmitter substances,
2. Augmentation of the sensitivity of the receptor site,
3. Prevention of the transformation of the transmitter substance,
4. Imitation of the transmitter substance which means that it must be recognized by the binding sites of the transmitter substance,

5. Promotion of the depolarization of the receptor site.

In order then that the author could confirm or refute a possible mechanism of action of Erlangia cordifolia material mediated through the autonomic nervous system, it was important to compare its activity with that of a number of known compounds that have effects on the autonomic system through one of the above mechanisms.

As Fig. 23 shows, the effect of a substance is mediated through a synapse. The first synapse in the peripheral autonomic system is the ganglia. Nicotine is the classic stimulant of the autonomic ganglia where in small doses, it will produce a temporary increase in the sensitivity of the ganglion cells. It achieves this by the depolarization of the receptor site (23). Another substance which has similar effect on ganglia is dimethylphenylpiperazinium whose mechanisms of action may probably be the augmentation of the sensitivity of the receptor. The respective mechanisms of these two compounds are found to occur in all peripheral autonomic ganglia.

In the parasympathetic system, the end-organ synapse has got acetylcholine as the transmitter. So, a compound that can stimulate an organ through an activity at the terminal synapse must have a muscarinic effect, that is, must stimulate the organ in the same way that acetylcholine does. Example of compounds that behave in this way are neostigmine

and carbamyl choline (carbachol). These two prevent the transformation of acetylcholine and by so doing prolong the life of the transmitter substance and hence its activity. Pilocarpine is another pharmacologic substance which acts at the end organ in the parasympathetic system by promoting the sensitivity of the end-plate. Hexamethonium is a ganglion-blocking agent in all autonomic nervous systems. Atropine, on the other hand, is a parasympathetic substance which affects the endorgans in the autonomic nervous flow. These last two substances reduce the sensitivity of the receptor cells. An attempt to see whether variation of the dose of Erlangia cordifolia extract would remove the blockage caused by these agents was investigated because this would have established an augmentation of the sensitivity of the receptor sites.

Adrenaline is known to cause relaxation of the non-pregnant cat uterus (24). For this reason, since adrenaline and nor-adrenaline are the terminal transmitters of the sympathetic autonomic system, it was not deemed necessary to investigate possible effects of Erlangia cordifolia material on the sympathetic system's terminal synapse. In addition, although the effect of sympathomimetics is easily demonstrated on the uterus, the effect of known sympathomimetic substances is so generalized that they have been described as the substances that cause the "fight or flight" syndrome - a clinical effect that the author could not elicit the description of from any of the

traditional medical practitioners that have used Erlangia cordifolia material that he investigated.

Ergometrine and ergotamine - alkaloids extracted from ergot or Secale cornutum (Ergot is a product of the fungus Claviceps purpurea) - have got activity in the plain muscles of the uterus and blood vessels but hardly any effect in the other viscera (25). Although Erlangia cordifolia material exhibited physiological effects similar to those of the ergot alkaloids, it however did exhibit properties like influence on skeletal and intestinal muscle contractions - properties that the ergot alkaloids do not exhibit. Although ergot alkaloids block adrenergic receptors, their main pharmacological effects do not depend on this action (25). These observations made any comparison of activity in between ergot alkaloids and the Erlangia cordifolia extract fruitless and as such it was not carried out.

To summarize then, the substances whose pharmacological activities were compared to that of Erlangia cordifolia extract were nicotine hydrogen tartarate, 1, 1 - Dimethyl - 4 - Phenyl piperazinium iodide (DMPP), neostigmine methylsulphate, carbamyl choline (carbachol) and pilocarpine hydrochloride. Erlangia cordifolia's possible antagonistic action against the effects of hexamethonium bromide and atropine sulphate were also investigated.

4.1.2 MATERIALS AND APPARATUS:

Nicotine hydrogen tartarate (BDH chemicals Ltd., Poole, England).

Pilocarpine hydrochloride (BDH chemicals Ltd., Poole, England).

Carbachol (May & Baker Ltd., Dagenham, England).

DMPP (1, 1 - Dimethyl-4-Phenyl Piperazinium iodide, K & K Laboratory, Plainview, N.Y., U.S.A.).

Neostigmine methylsulphate (Roche Products Ltd., Welwyn Garden City, England).

Atropine sulphate (Sigma chemicals, St. Louis, Missouri, U.S.A.).

Hexamethonium bromide (Sigma chemicals, St. Louis, Missouri, U.S.A.).

The solutions of these substances were made up as follows with distilled water:-

1. Nicotine (1 mg/ml)
2. Pilocarpine (4% solution)
3. Carbachol (1 mg/ml)
4. DMPP (10 nanograms/ml)
5. Neostigmine (0.5 mg/ml)
6. Atropine (0.5 mg/ml)
7. Hexamethonium (1 mg/ml).

The other materials and the apparatus used were as described in Section 3.2

4.1.3 EXPERIMENTAL PROCEDURE:

These experiments were carried out as described

in Section 3.2. The intestinal strip was allowed to contract in unmodified Tyrode solution and when the contractions were judged to be constant a steadily increasing dose of the known drug was added to the bathing solution, always starting with 0.1 ml until a good modification of peristalsis was observed. Once these good contractions had been recorded for three or so minutes uninterrupted, the solution of Erlangia cordifolia extract was then added in steadily increasing doses to the bathing solution containing the known drug. The additions of the extract were again always commenced with a dose of 0.1 ml of 1 mg/ml solution of the crystalline extract. This process, as described here, was carried out in the first series of experiments. In the second series of the experiments it was the Erlangia cordifolia extract solution which was added to the bathing solution first followed by the known drug, the addition of the two substances again being made in a stepwise fashion as described for the first series of experiments.

4.1.4 RESULTS:

Figs. 24, 25, 26, 27, 28 and Figs. 29 and 30 show the tracings obtained of the peristalses of an intestinal strip in the bathing solution modified as described in the legends accompanying the figures. The stimulation of contraction by the known substances was not fundamentally altered by Erlangia cordifolia

material suggesting that the known and the unknown substances acted through differing mechanisms on the contractile process. Erlangia cordifolia material failed completely to remove the nervous transmission block of hexamethonium and also that of atropine.

4.2 INVESTIGATION OF POSSIBLE IN VITRO ACTIVITY OF ERLANGIA CORDIFOLIA MATERIAL ON THE CHOLINESTERASE ENZYMES.

4.2.1 INTRODUCTION:

Investigation on possible in vivo activity of the Erlangia cordifolia material on the autonomic proximal ganglia (where the chemical mediator of the nervous impulse is known to be acetylcholine) had shown that the material did not affect the contractile process at this point. The material did not seem to affect the chemical mediation of the acetylcholine - mediated neuromuscular junctions. In muscle, acetylcholine is hydrolysed by the cholinesterase enzymes. It was therefore important to investigate as to whether the material had any effect on these enzymes that would lead to decreased activity of the enzymes and therefore a prolonged life for the chemical mediator. A prolonged life of acetylcholine within the muscle would lead to prolonged depolarization of the sarcoplasmic reticulum of the muscle cell and this

would, in turn, lead to prolonged muscular activity and delayed relaxation of the muscle. This would be of importance especially in the uterus where it is known that retraction occurs during labour - which means that by the end of the relaxation phase of this muscle's contractile mechanism some amount of contraction is left intact in the muscle.

In the following experiments, plasma cholinesterase activity (with the possibility of an effect on this activity by Erlangia cordifolia material) was measured by using acetylthiocholine as the substrate and the rate of production of acetic acid from this substrate as a measure of the rate of enzyme action. This is the procedure of Ellman et al. (26, 27).

4.2.2 REVIEW OF LITERATURE:

There are two divergent views concerning the role of acetylcholine in nervous function. The more widely held view holds that acetylcholine functions as a neurohumoral transmitter and only in certain types of nerves. Nachmansohn (28, and 29), on the other hand, has a body of evidence which supports the view that acetylcholine acts as an intracellular agent in both the transmission and the conduction and in all kinds of nerves. If, in deed, acetylcholine has such a wide implication in nervous mechanisms, then its rate of destruction in the body has great fundamental importance and implications for muscular contraction.

It is known that mammals possess both acetylcholinesterase and butyrylcholinesterase in the blood and all known anticholinesterases are capable of inhibiting both types of cholinesterase although some have a much higher affinity for one type than for the other (30). Both types of cholinesterase found in mammals hydrolyse choline esters far more rapidly than other esters at low substrate concentrations (31). This then means that in order to investigate fully the molecular basis of action of a substance that increases muscular contractility and tension, it is necessary to investigate the possibility of the substance having some anticholinesterase activity.

Since cordifene, one of the Erlangia cordifolia material constituents, is an ester, a competitive type of inhibition where cordifene would comp ete against acetylcholine for the binding sites of the enzyme was thought to be worthy of further investigation.

The consequences of inhibition of acetylcholinesterase are a potentiation of the effects of injected acetylcholine (which mimics nerve stimulation) and of nerve stimulation, and the spontaneous changes in the functional state of the effector cell which results from an accumulation of endogenous acetylcholine (30). Acetylcholine of non-nervous origin is found in the intestine among some other organs and the inhibition of butyrylcholinesterase which is found in this organ

leads to an increase in the tone and motility of the intestine (30).

Butyrylcholinesterase is predominantly found in blood plasma and it is conceivable that competitive inhibition of hydrolysis of acetylcholine will lead to potentiation of acetylcholine. Another mechanism by which acetylcholine could be potentiated by cordifene is by inactivating the enzyme. This is feasible from the fact that the α-methylene-γ-lactone grouping is well known as an enzyme-alkylating agent (32, 33) and alkylation of the enzyme may result in a reduction of its activity.

4.2.3 MATERIALS AND APPARATUS:

S.P. 1800 UV. Spectrophotometer (Pye Unicam Ltd., Cambridge, England).

Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y. U.S.A.).

Minor Centrifuge (Minor Centrifuge Serial No. AA 834, Measuring and Scientific Equipment Ltd., London, England).

DTNB - 5 - 5' Dithiobis - (2 - Nitrobenzoic acid) - also known as Ellman's reagent. (BDH, Poole, England).

Albino rats (supplied by the Medical Research Laboratories, Ministry of Health, Nairobi, Kenya).

0.2 M Phosphate buffer, pH 8.

Doubly de-ionized water.

In the preparation of the solutions for this

experiment, doubly de-ionized water was used. Doubly de-ionized water was also used throughout the experimental procedure.

A solution of 0.01 M DTNB was prepared by dissolving 19.80 mg. of DTNB in 0.2 M phosphate buffer pH 7.2 and making up the final volume to 5 ml. A solution of 0.2 M acetylthiocholine was made up by disolving 250 mg. of acetylthiocholine in water and the final volume being made up to 5 ml.

A sample of a blood serum was obtained by killing an adult rat on the day of the experiment. Blood was collected in a centrifuge tube and after allowing it to clot, the clotted blood was then centrifuged at 1000 rpm for 10 minutes and the resulting serum supernatant collected and diluted with an equal volume of the phosphate buffer.

1% Acetylcholine bromide solution (BDH, Poole, England).

4.2.4 EXPERIMENTAL PROCEDURE:

The procedure was based on that of Ellman and his colleagues (27). Four spectronic 20 tubes were thoroughly cleaned with tap water and rinsed with the doubly de-ionized water and then allowed to dry in air. Three of the tubes were used as the standards while the fourth was used for the Test. The whole procedure was carried out at room temperature.

Reagents were added into the tubes as shown in the table below:-

Reagent	Standards			Test
	I	II	III	
Phosphate buffer	1.50 ml.	1.50 ml	1.50 ml	1.50 ml.
Water	0.80 ml.	0.80 ml.	0.80 ml	0.80 ml.
Serum	10 µl	10 µl		10 µl
Acetylthio-choline		100 µl	100 µl	100 µl
DTNB	100 µl	100 µl	100 µl	100 µl
Erlangia cordifolia extract	100 µl		100 µl	100 µl
(or cordifene)	(75 µl.)		(75µl.)	(75µl.)

The micropipette used for the last addition into the tube was used for mixing up the contents rapidly while the spectrophotometer tube was in place. The wavelength used for the readings was 412 nanometers. The enzyme solution was always added lastly in the tube where applicable and in standard III where this did not apply the acetylthiocholine solution was the last addition. On making the last addition, the whole mixture was quickly stirred by blowing rapidly through the micropipette for about two seconds, and the reading at the end of the stirring taken as the zero time reading. Optical density readings were taken until there was no more change in the reading for at least five minutes.

The experiment was repeated using cordifene dissolved in 70% methanol. The concentration of the cordifene solution was 2 mg/ml.

On making up standard III, increased light absorption was observed on the addition of Erlangia cordifolia material or cordifene. Therefore, to ascertain a possible reaction in between cordifene and acetylthiocholine, the effect of U.V. absorption of acetylthiocholine by cordifene was tested. A U.V. spectrum in the far U.V. range was obtained using an SP 1800 UV. spectrophotometer (Fig. 31). (See the legends accompanying the Figure).

4.2.5 RESULTS:

Table I shows results of measurement of light absorption due to the development of yellow colour following a reaction in between the thiol (-SH) group detector, DTNB, and the thiol groups obtained from the hydrolysis of acetylthiocholine. Standard I measures the hydrolysis of serum's intrinsic thiol groups and standard 2 the hydrolysis of serum's intrinsic thiol groups in addition to the thiol groups from the hydrolysis of acetylthiocholine. Standard 3 measures the release of thiol groups in the absence of the enzyme and therefore the Test measures the effect of crude Erlangia material or of cordifene on the serum acetylcholinesterase enzymes.

Table 2 shows the optical density increases with time for standard 2 and the Test reaction media.

Crude E.cordifolia material afforded similar results.

4.2.6 CONCLUSION:

From Table 2, it can be seen that the increase in optical density in the two reaction media are comparable suggesting that the activity of the enzyme in the presence or absence of cordifene is the same. This then means that the instantaneous intense colour development observed in both Standard 3 and the Test reaction media (Table 1) is due to a reaction in between cordifene and acetylthiocholine, as confirmed by the results shown on Fig. 31, and it cannot be due to a reaction in between DTNB and cordifene as such an instantaneous intense colour development was not observed in Standard 1 where both cordifene and DTNB had been added.

The fact that the hydrolysis of acetylthiocholine is the same in the presence and absence of cordifene shows that cordifene has no cofactor nor inhibitory activity on the acetylcholinesterase enzymes. The fact that the shape of the contraction wave facilitated by cordifene (see Fig. 18) is different from that caused by any of the known anticholinesterase drugs suggests also that cordifene has no direct effect on the true acetylcholinesterase nor on the butyrylcholinesterase enzymes.

4.2.7 CONCLUSION ON THE INVESTIGATION OF POSSIBLE BIOCHEMICAL INTERACTION IN BETWEEN ERLANGIA CORDIFOLIA AND MOLECULES WITHIN THE NERVOUS SYSTEM:

Attempts to simulate the peripheral nervous system activity of pharmacologically active compounds showed that the activity of Erlangia cordifolia material could not be attributed to the stimulation of the nervous system. Equally the author's investigation into possible anticholinesterase activity by Erlangia cordifolia material showed that Erlangia cordifolia material had no anticholinesterase activity. The material was not found to contain any chemical compound that bound acetylcholine and as such its activity could not be attributed to possible binding to acetylcholine itself and consequently stimulating acetylcholine activity.

The experiments showed conclusively that Erlangia cordifolia did not have any activity on the peripheral nervous system and as such its mechanism for the stimulation of the contractile activity of muscle had to be located beyond the nerve supplying the muscle and therefore within the muscle cell itself. This then meant that the activity of Erlangia cordifolia resembled those of histamine and serotonin in the sense that these two compounds act on the muscle cell directly to cause their stimulation on it (23).

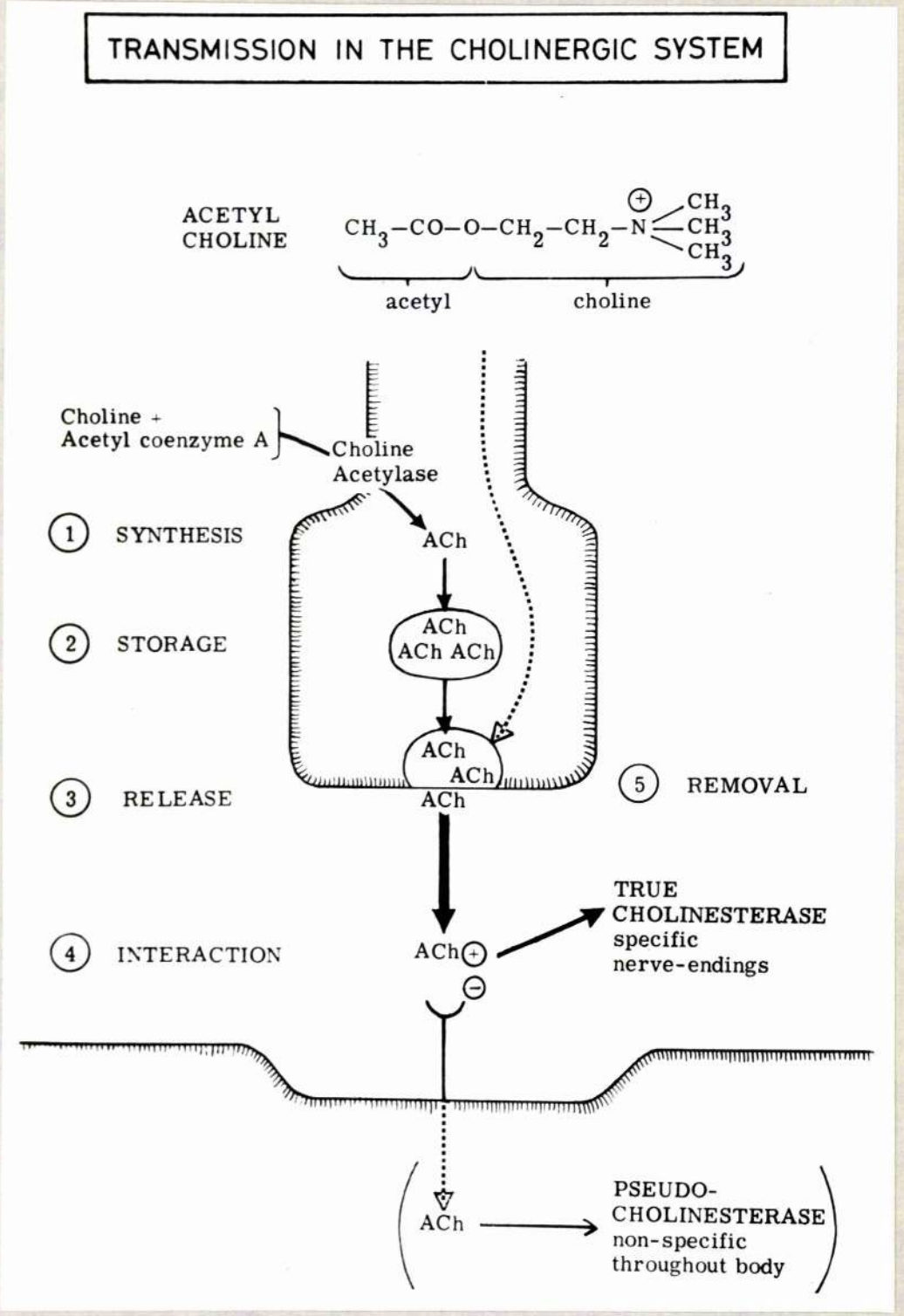


Fig. 23: Diagrammatic representation of transmission across a synapse in the autonomic nervous system.

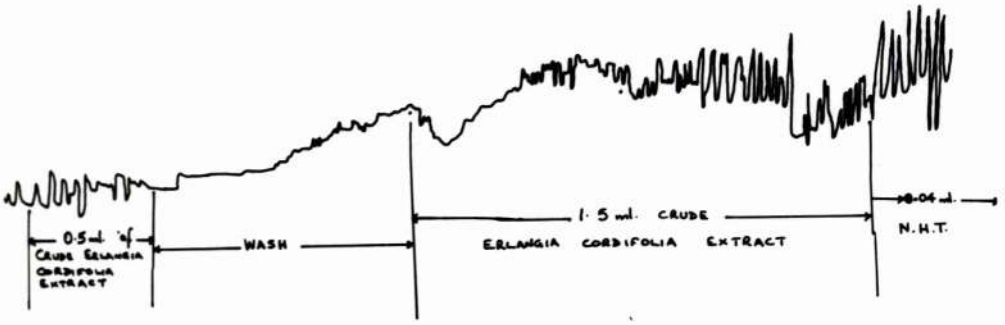


Fig. 24: The effect of E. cordifolia extract and Nicotine on the peristaltic activity of a guinea pig ileum.

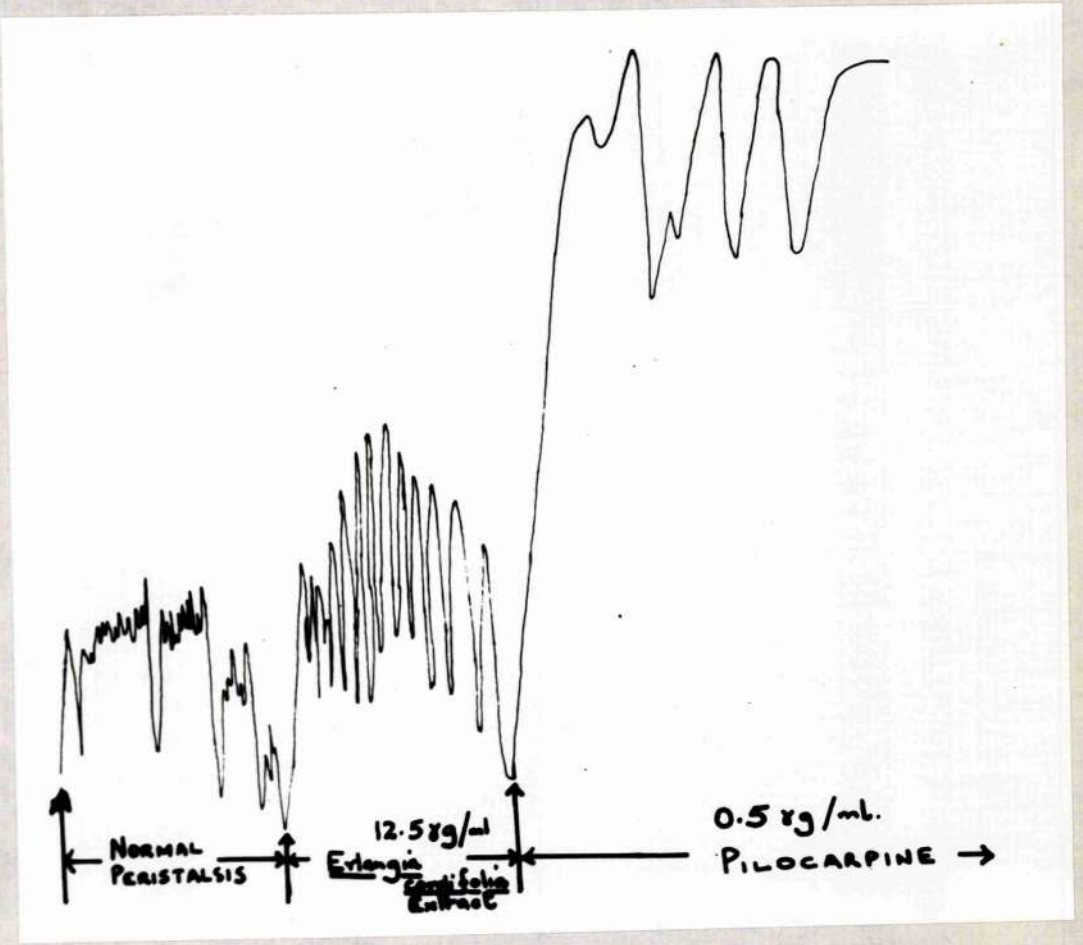


Fig. 25: Effect of E. cordifolia extract and pilocarpine on the peristaltic activity of a guinea pig ileum.

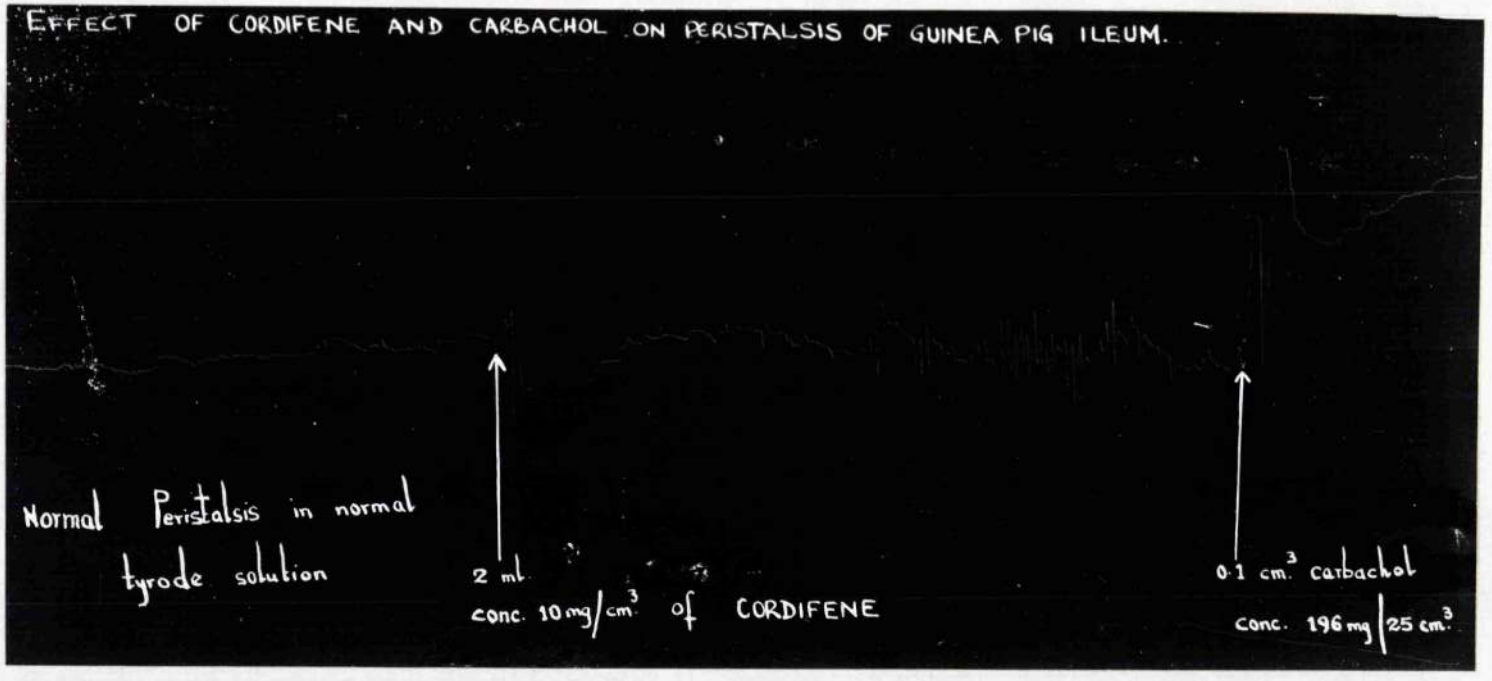


Fig. 26: The effect of cordifene and carbachol on peristaltic activity of a guinea pig ileum

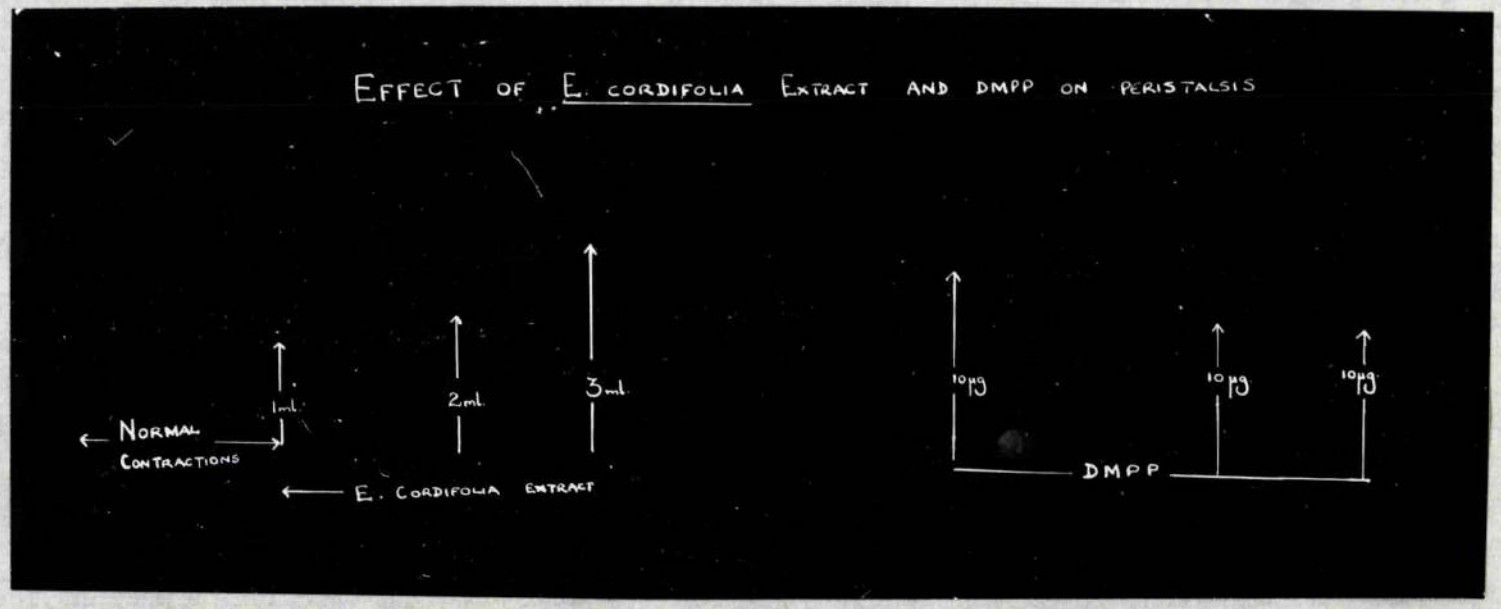


Fig. 27: The effect of DMPP and E. cordifolia extract on the peristaltic activity of a guinea pig ileum.

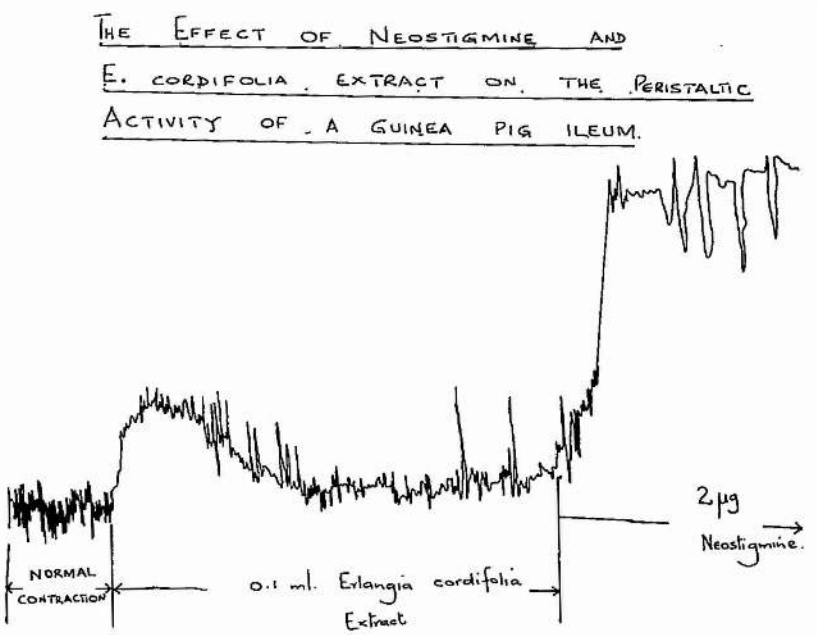


Fig. 28: The effect of neostigmine and E. cordifolia extract on the peristaltic activity of a guinea pig ileum.

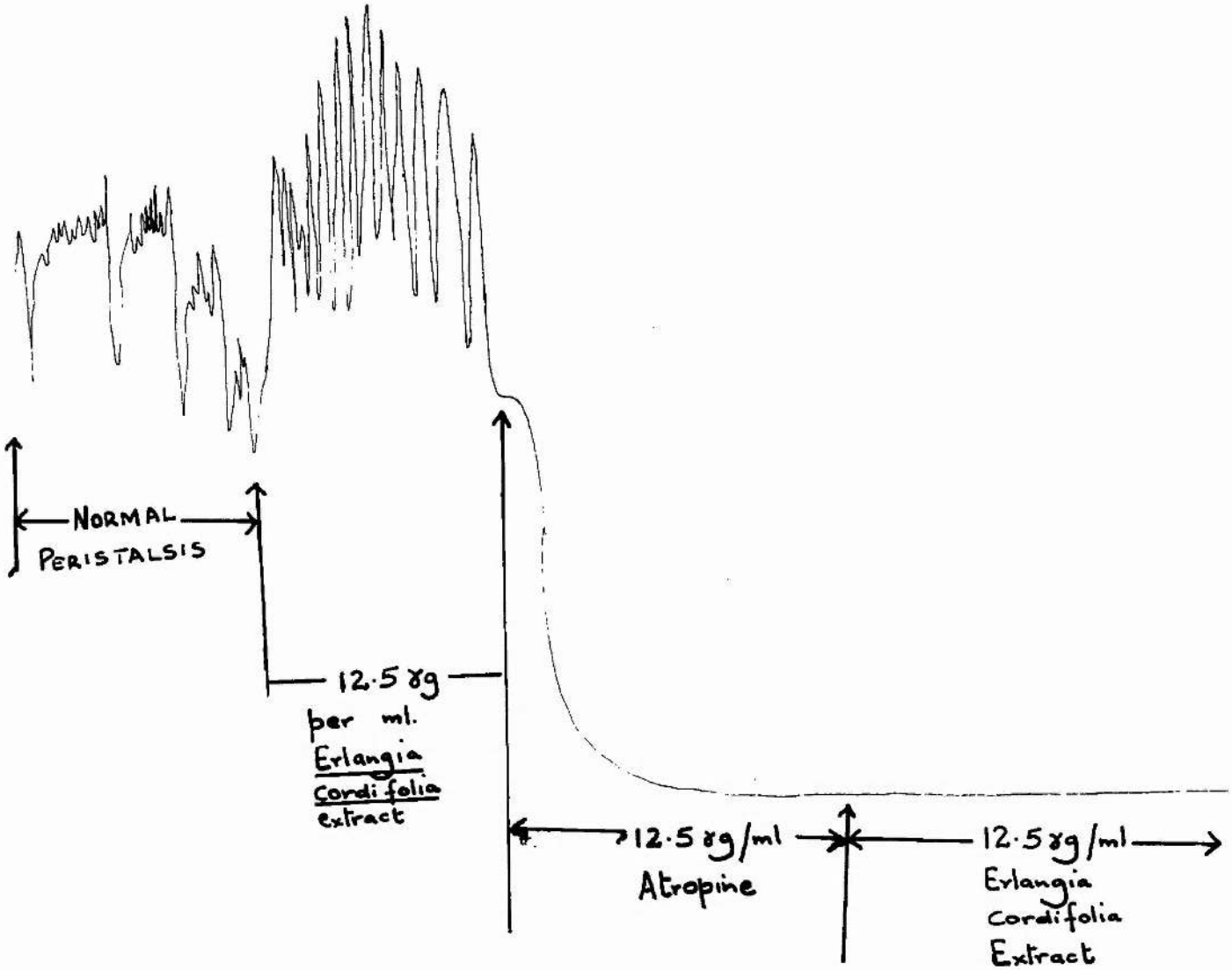


Fig. 29: The effect of atropine and E. cordifolia extract on the peristaltic activity of a guinea pig ileum.

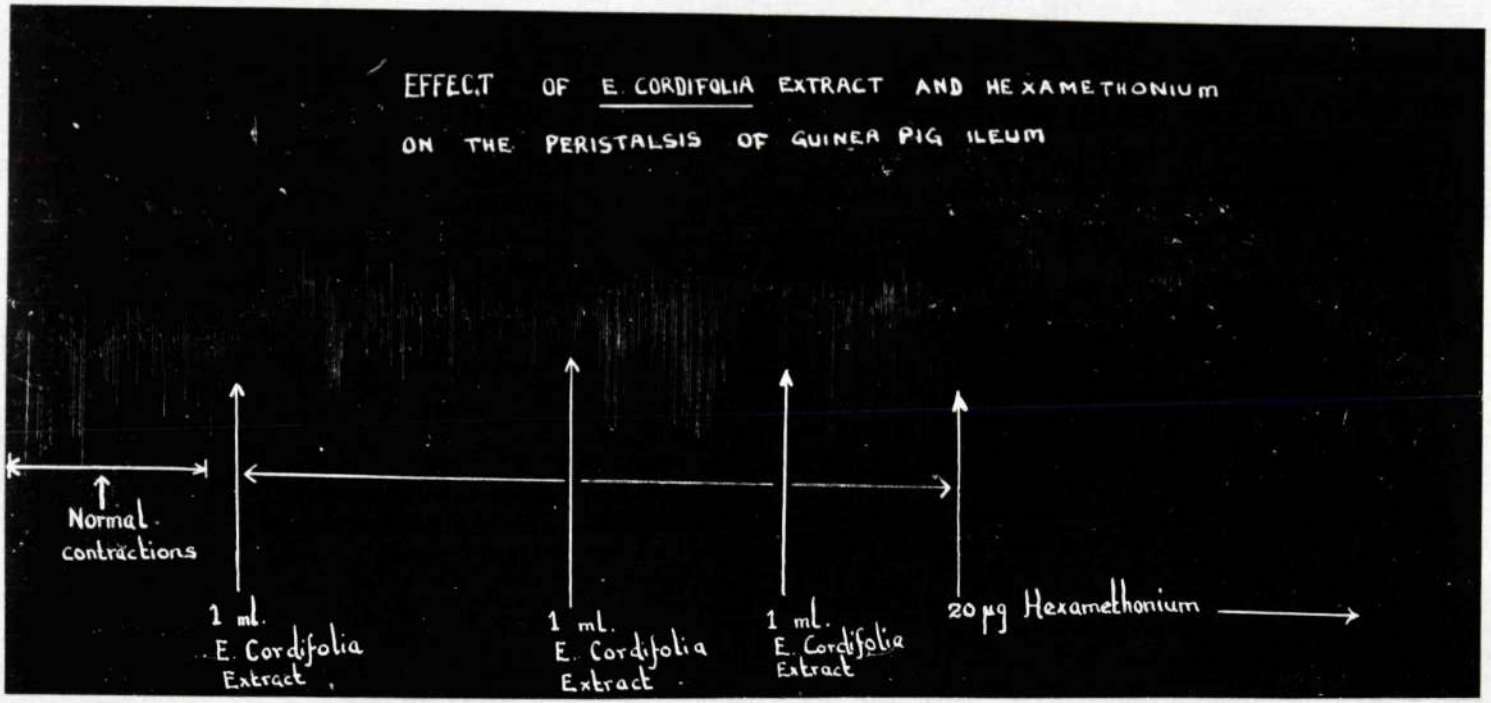


Fig. 30: The effect of E. cordifolia extract and hexamethonium on the peristaltic activity of a guinea pig ileum.

Fig. 31: In the test sample, 2.5 ml. of 100 µg/ml acetylthiocholine in methanol (analytical grade) were added into a 1 cm. silica cuvette and a similar volume of methanol was added into the reference cuvette. An U.V. spectral scan was then carried out at room temperature and the proc edure was repeated after the addition of 10 µl of a cordifene methanol solution (660 µg of cordifene/ml) into both cuvettes.

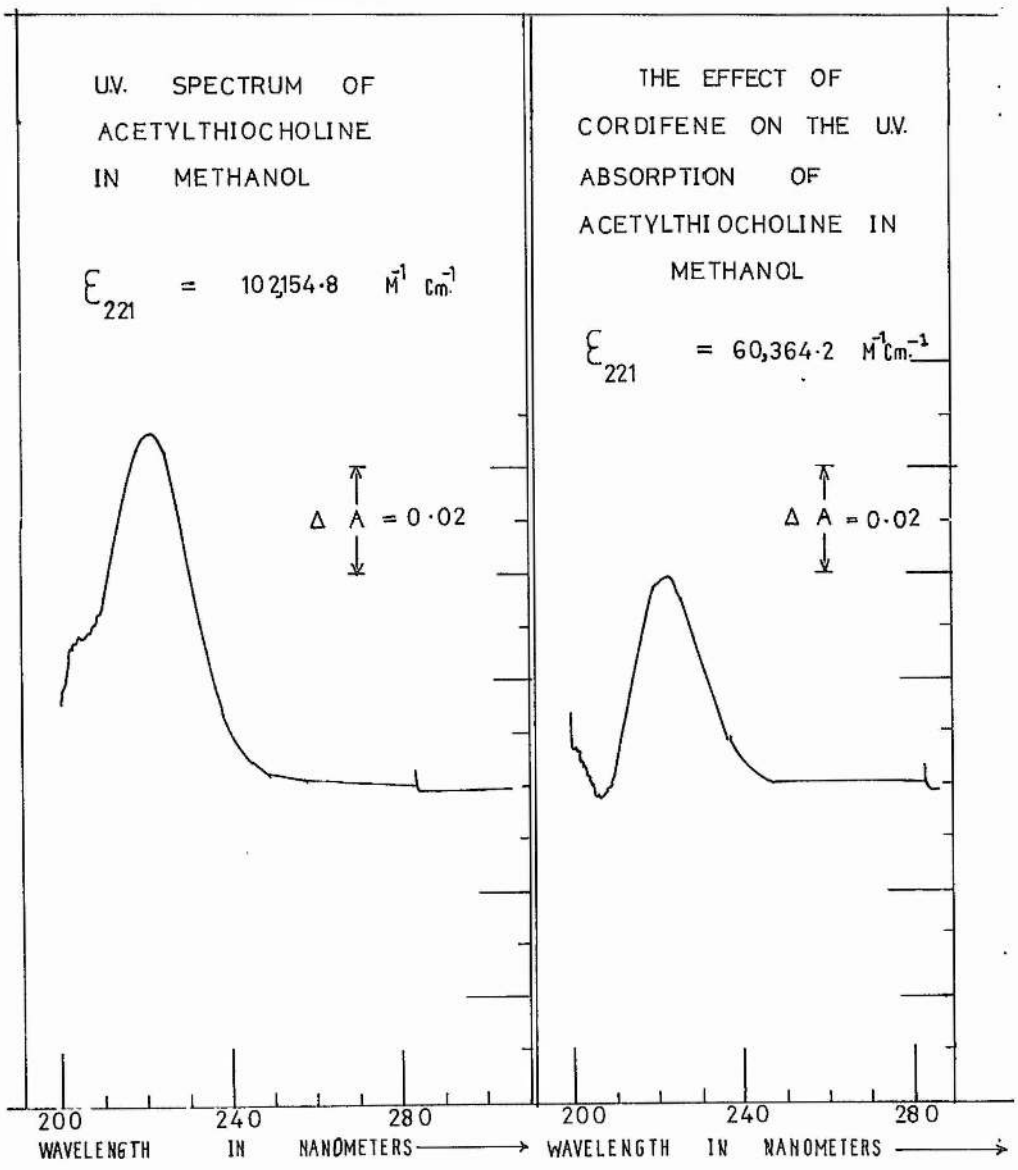

U.V. SPECTRUM OF ACETYLTHIOCHOLINE IN METHANOL
ε_{221} = 102,154·8 M^{-1} Cm^{-1}
Δ A = 0·02
200
240
280
WAVELENGTH IN NANOMETERS
THE EFFECT OF CORDIFENE ON THE U.V. ABSORPTION OF ACETYLTHIOCHOLINE IN METHANOL
ε_{221} = 60,364·2 $M^{-1}Cm^{-1}$
Δ A = 0·02
200
240
280
WAVELENGTH IN NANOMETERS

Fig. 31

C H A P T E R 5:

5.1 EFFECT OF CORDIFENE ON THE TRANSLOCATION OF Ca^{2+} BY MYOFIBRILS

5.1.1 INTRODUCTION:

That calcium ions are required in the process of muscle contraction is now a well established fact. An investigation on the biochemical basis of an observed effect on muscle contraction must therefore, of necessity, take into account a possible role on Ca^{2+} translocation by the agent causing the observed effect on muscle contraction. A series of experiments were therefore conducted with the view of investigating this kind of role for cordifene on muscle contraction.

5.1.2 LITERATURE REVIEW

Although the role of Ca^{2+} in the troponin - tropomyosin system in the activation of the thin filament seems to be well understood now, there are other observed effects on the contractile system that do not seem to be explicable on the basis of Ca^{2+} activity on

the troponin-tropomyosin system.

Endo (34) concluded that greater tension - producing ability in myofibrils at longer length (that is, stre tched fibres) with low Ca^{2+} must be a real property of the contractile system and is not facilitated by the sarcoplasmic reticulum. He also observed that the rate of tension development after a sudden increase in calcium ion concentration in the medium was very much dependent on the level of free calcium ion and that a stretched fibre developed tension faster than a slack fibre. This has also been observed by Julian (35) using fibres whose sarcoplasmic reticulum function had already been destroyed with a detergent. Little is known at present about the mechanism of stretch-induced increase in activation of the contractile system by calcium but it appears very unlikely that the amount of calcium bound to troponin increases with stretch.

Sugden and Nihei (36) have shown that both Ca^{2+} and Mg^{2+} can bind tightly to myosin, but that only the binding of Mg^{2+} shows a direct influence on the enzymic activity. They also suggested that since in the presence of Ca^{2+} ITPase and pMB - modified myosin ATPase become liable to the effect of Mg^{2+} then the tight binding of Ca^{2+} is 'indirectly' involved in the activation of the enzyme so that when the site for this tight binding of Ca^{2+} is occupied by Mg^{2+} the activation does not occur.

Daniel and Hartshorne (37) located sulphydryl groups that are essential for Ca^{2+} sensitivity on the heavy chains of the myosin molecule and also found that the inhibition of ATPase activity by troponin B or troponin B plus tropomyosin was also removed under conditions similar to those which eliminated Ca^{2+} sensitivity. For several years it has been known that sulphydryl reagents can remove the Ca^{2+} sensitive response of natural actomyosin (38) and this effect has been shown through increased ATPase and superprecipitation activities by such reagents.

5.1.3 MATERIALS AND APPARATUS:

The back skeletal muscles of New Zealand albino rabbits were used as the source of the myofibrils. The isolation procedure was as described in Hawk's Physiological Chemistry (39).

Radio-active calcium chloride ($^{45}CaCl_2$) - purchased from Radio-chemical centre, Amersham, England.

Scintillating mixture was made up as follows:- 2.5 - diphenyl-oxazole (PPO) 9 gm; 1,4-bis-2-(4-methyl-5-phenyl oxazolyl) benzene (POPOP) 0.6 gm; ethanol 300 ml; redistilled toluene to make up the volume to 1000 mls.

Relaxing solution. This contained 21.5 mM K_2SO_4, 12 mM Na_2SO_4, 20 mM Tris- 20 mM Maleate- NaOH pH 6.8, 4 mM ATP, 4 mM $MgSO_4$, 4 mM EGTA and 93 mM sucrose.

0.4 mM EGTA stock solution.

Packard Tri-carb Liquid Scintillating Spectrophotometer Model 3320 (Packard Instrument International S.A. Ltd., Downers Grove, Ill., U.S.A.).

The reaction medium consisted of relaxing medium, 0.07mM $^{45}CaCl_2$ - which means that the free $^{45}Ca^{2+}$ concentration was always slightly less than 5 µM (40), and 157µg. myofibrillar protein per ml.

5.1.4 METHOD:

The reaction medium consisted of 7 ml. of 157 µg. per ml. myofibril protein. To this medium a calculated amount of cordifene in 70% methanol was added to ensure a cordifene concentration of 2 µg. per ml. A calculated amount of labelled calcium chloride ($^{45}CaCl$) - EGTA buffer was then added to make the concentration of free $^{45}Ca^{2+}$ 55µM (calculation of Ca - EGTA equilibrium was carried out by the method of Tonomura et al. (40)). The reaction was then allowed to proceed for a period of five minutes before the protein was filtered through a millipore filter (pore size (ϕ) = 0.45 mµ) aided by sunction. The supernatant contained labelled calcium which failed to translocate into the protein. 0.1 ml. of supernatant was inoculated into 10 ml. of scintillating mixture in a glass counting vial. The filter paper containing the filtered protein was injected into another counting vial containing 10 ml. of scintillating mixture. This procedure was repeated using the protein mixture into which no cordifene

had been added. The counting was carried out using the Packard Tri-carb Liquid scintillating spectrophotometer model 3320.

5.1.5 RESULTS:

Table 3 shows the amount of label bound by the myofibril protein in the presence of and in the absence of cordifene. The presence of cordifene reduced the binding of calcium by almost a half.

5.1.6 CONCLUSION:

Cordifene does not facilitate the translocation of calcium from the cytoplasm into the contractile system of muscle.

5.2 EFFECT OF CRUDE ERLANGIA CORDIFOLIA EXTRACT AND OF CORDIFENE ON MYOFIBRILLAR ADENOSINE TRIPHOSPHATE (E.C. 3.6.1.3) ENZYME ACTIVITY.

5.2.1 INTRODUCTION:

It was Szent-Gyorgyi, Straub and their colleagues (9, 10) who clarified the effects of ATP on the contractile proteins. Chaplain in Oxford (15) found that when he incubated glycerinated muscle fibre bundles for a short time in a relaxing medium and then transferred such fibres from a relaxing to an activating solution, there was a sudden burst of high enzymic activity which then decayed to a steady - state level as the tension rose.

The author had already observed an increase in tension followed by increased peristaltic activity on the addition of Erlangia cordifolia extract into the Tyrode solution bathing an active intestinal strip. It was therefore important to investigate the effects, if any, of the extract on myofibrillar ATPase activity.

5.2.2 LITERATURE REVIEW:

Many compounds have been reported in literature as being capable of influencing myosin ATPase activity (41). There are different mechanisms proposed by means of which these agents are believed to bring about changes in the enzyme activity.

Ebashi et al. (42) have proposed a mechanism by means of which such activation of myosin ATPase could come about. They proposed that the activation of this enzyme in 0.6 M KCl is related to magnesium chelating ability of the reagents within a group of EDTA analogues. This is so because Offer (43) has shown that Mg^{2+} is firmly bound to myosin where it appears to exert an inhibitory influence.

The activation of Ca^{2+} ATPase has been shown to be brought about by several agents which include urea, guanine-HCl, ethanol and ethylene glycol (20). All these compounds are believed to influence the hydrogen and the hydrophobic bonds of the enzyme protein and Barany et al. (20) have proposed that

sodium dodecylsulphate should be included in this list. By affecting the hydrogen and the hydrophobic bonding of a molecule, these compounds can bring about a conformational change in the molecule. LiBr and KCl, on the other hand, are believed to bring about a conformational change in the myosin ATPase molecule by affecting the electrostatic bonds in the molecule (20).

The idea of a conformational change is made even more plausible by the work of Sekine and Kielley (44) who blocked one sulphydryl group of myosin ATPase with N-ethylmaleimide (NEM) and by so doing increased the enzyme's activity. They concluded that the activation of myosin ATPase by NEM is caused by conformational changes brought about by the reaction in between myosin and the NEM. Barany et al. (20) found that when the cysteine residues of myosin are exposed by the action of urea on the protein, iodoacetate reacted with their sulphydryl groups without affecting the ATPase activity. On the other hand, iodoacetamide reacted with sulphydryl groups and in the process reduced the activity of the enzyme. This finding lends strong support to the idea that a huge conformational change can bring about changes in the activity of myosin ATPase.

In view of the fact that α-methylene-γ-lactone compounds have been shown to alkylate sulphydryl groups and thereby bring changes in the activity of biologically active compounds (45) and since one of

the constituents of Erlangia cordifolia material - cordifene - is such a compound, the possibility of Erlangia cordifolia material having a direct effect on the myosin ATPase was thought to be distinctly plausible.

5.2.3. MATERIALS AND APPARATUS:

Actomyosin: stock solution of actomyosin were obtained from the red skeletal muscles of guinea pigs and albino rats using the method of Perry (46) with slight modifications. The modifications involved diluting the 0.5 M KCl in which the protein was dissolved to 0.28 M KCl followed by centrifugation of the solution at 10,000 rpm for 10 minutes. The precipitated actomyosin was then homogenized with 0.28 M KCl and then centrifuged at 10,000 rpm for 10 minutes twice over to separate actomyosin from myosin. The actomyosin precipitate which came down at the end of the second centrifugation of the 0.28 M KCl solution was then homogenized with 0.05 M KCl so as to dissolve other contaminating proteins and again centrifuged twice at 10,000 rpm for 10 minutes each time. The actomyosin precipitate was finally redissoved in 0.6 M KCl and stored at $4^{o}C$ until needed for use.

The concentration of the protein used in this series of experiments was 2 mg/ml.

Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y., USA.)

4% sodium bicarbonate (Laboratory reagent grade).

0.1% anhydrous calcium chloride (Laboratory reagent grade).

5 mM ATP - disodium dihydrogen salt (BDH chemical, Poole, England).

Erlangia cordifolia material at a concentration of 1 mg.%.

Cordifene at a concentration of 40 mg.% in 70% methyl alcohol.

20% Trichloroacetic acid (TCA) (Laboratory reagent grade, BDH, Poole, England).

Ammonium molybdate (Laboratory reagent grade, BDH, Poole, England).

Sodium sulphite (Analytical reagent, BDH, Poole, England).

Sodium metabisulphite (Laboratory reagent grade, BDH, Poole, England).

5N Sulphuric acid (Laboratory reagent grade, May & Baker Ltd., Dagenham, England).

ANS (1 - amino - 2 - Naphthol - 4 - sulphonic acid) (Laboratory reagent grade, BDH, Poole, England). The reagent was prepared by dissolving 0.25 mg of ANS and 14.25 mg. $NaHSO_3$ in 45 ml. of distilled water and to this solution was added 15 ml. of 10% sodium sulphite solution. After shaking thoroughly, the volume was made up to 100 ml. with distilled water.

5.2.4 EXPERIMENTAL PROCEDURE:

ATP hydrolysis by myosin ATPase was carried out as described by Hawk et al. (39) with the exception of the fact that the myosin was dissolved in 0.6 M KCl.

Three test tubes were thoroughly cleaned with soap and then rinsed carefully with doubly distilled water and then dried.

Solutions were added into the three tubes as shown in the table below in the cold room where a constant temperature of $4^{o}C$ was maintained.

Blank	Tested tube 1	Tested tube 2
$NaHCO_3$ solution 1ml	$NaHCO_3$ solution ..... 1ml.	$NaHCO_3$ solution ..... 1ml.
$CaCl_2$ solution ..... 0.5 ml	$CaCl_2$ solution ..... 0.5 ml.	$CaCl_2$ solution ..... 0.5 ml
Water..... 1 ml.	Water.... 1 ml.	Erlangia cordifolia extract .....0.5 ml
Actomyosin..0.5 ml.	Actomyosin..0.5ml.	Actomyosin...0.5ml.
	ATP.....0.5ml.	ATP.....0.5ml.
TCA.....0.5 ml.	TCA.....0.5ml.	TCA.....0.5ml.

The enzymic reaction in each test tube was timed and later stopped with trichloroacetic acid. The times allowed the enzymic reaction were 0, 10 minutes, 40 minutes, 50 minutes, 60 minutes up to 120 minutes.

On addition of trichloroacetic acid, the protein and the protein nucleotide complexes were precipitated. The filtrate was then removed and a 1 ml. aliquot of the filtrate taken and into it was added:-

1 ml Ammonium molybdate solution

1 ml sulphuric acid

0.4 ml. ANS.

The volume was then made up to 10 ml. with distilled water. This solution was allowed to stand for thirty minutes (47) at room temperature to allow for colour development. The colour developed was measured colorimentrically using a spectronic 20 spectrophotometer at 660 nanometers.

The procedure was repeated using cordifene in stead of the crude Erlangia cordifolia material.

5.2.5. RESULTS:

Actomyosin samples were found to liberate "intrinsic" inorganic phosphate which complexed with the ammonium molybdate to give a colour intensity whose absorbance was subtracted in every reading obtained for the hydrolysis of ATP by actomyosin (See Table 4 Column A) or by actomyosin in the presence of Erlangia cordifolia material (See Table 4 Column B) or by actomyosin in the presence of cordifene (See Table 4 Column C). From Table 4, it can be seen that hydrolysis of ATP in the presence of Erlangia cordifolia material is three times faster than in its absence and Fig. 32 shows that cordifene, at the concentration used, almost

halves the time required for the hydrolysis of ATP.

5.2.6 CONCLUSION:

It can be concluded that Erlangia cordifolia material has an activity on the myosin molecule itself or on the hydrolytic mechanism of ATP by the myosin ATPase.

5.3. A EFFECT OF ERLANGIA CORDIFOLIA EXTRACT ON THE BINDING OF ADP TO MYOSIN AND ON THE MYOSIN-ADP COMPLEX.

5.3.A.1 INTRODUCTION:

One of the mechanisms by which an enzyme's activity can be increased is by the removal of its product in cases where the product can combine with the enzyme protein. That ADP can combine with adenosine triphosphate and is, as such, a potent inhibitor of this enzyme is well known. The author therefore decided to explore the effect of cordifene and of crude Erlangia cordifolia extract on the interaction of myosin and ADP.

The experiments performed were of two types. The first series explored the possibility of a demonstrable effect of ADP or cordifene (or crude Erlangia cordifolia extract) or a combination of both on the UV absorption of myosin at a UV range where the UV absorption of the nucleotides is not pronounced (48). The second series explored the possibility of a

demonstrable effect of cordifene on adenosine diphosphate's UV absorption at a UV range where the absorption of the nucleotide is known to be pronounced.

5.3.A.2 LITERATURE REVIEW:

The fact that hydrolysis of ATP by myosin ATPase (E.C.3.6.1.3) leads to the formation of ADP and inorganic phosphate has been not only shown by kinetic studies (49,50) but also by the decay of the difference spectra induced on the myosin molecule by ATP finally the spectrum resembling that induced on the protein by ADP (8). Sartorelli et al. (51) were able to separate ADP from a nucleotide charcoal absorption following addition of ATP to myosin and subsequent precipitation of myosin from the reaction medium.

A widely held view is that ADP is a competitive inhibitor for ATP binding onto the myosin ATPase enzyme. From this it has therefore been suggested that ADP inhibits myosin ATPase activity and this effect has been shown in vitro in the case of pure myosin by Green et al. (52), and Nanninga (21). This view of these workers tends to confirm the view of Blum and Felauer (16) who proposed that the rate of ATP hydrolysis is limited by the release of ADP from the active site, and that activators of myosin ATPase effect rate acceleration by promoting the dissociation of the myosin-ADP complex.

It is also interesting that in support of this work, Taylor et al. (49) have discussed several lines of evidence which would suggest that the proposition of Blum and Felauer (16) was correct. Hotta and Bowen (53) have also presented evidence that ADP and phosphate generated by ATP hydrolysis are bound to myofibrils. Trentham et al. (54) have shown that the ADP and phosphate present in the enzyme-product complex are in rapid equilibrium with free ADP and phosphate and therefore rapidly interact with the enzymes such as glyceraldehyde dehydrogenase or pyruvate kinase. Chaplain (15) found that tension in glycerinated insect fibrillar muscle appears to be mainly generated by the build-up of ADP. He also found that the decrease in the myosin ATPase activity paralleled by the tension increase was constant and that this phenomenon was reversible. Chaplain's findings support the work of Imamura et al. (55) who found a lack of a rapid initial burst release of free ADP from a myosin-ATP system in the presence of 5mM $MgCl_2$ which lead to the inevitable conclusion that during the initial burst hydrolysis of ATP by myosin, the ADP then formed is bound to myosin. And, as Kiely and Martonosi (11) proposes, the ADP so bound to the myosin during the rapid initial phase might contribute to the establishment of the slower steady rate of ATP hydrolysis - a suggestion that agrees well with the findings that activators of myosin ATPase inhibit the binding of ADP.

Having therefore observed in earlier experiments an increase in tension in muscle and an increased myosin ATPase activity, an investigation as to a possible correlation in between these observations and inhibition of ADP binding onto myosin by Erlangia cordifolia extract was thought of as being of fundamental importance.

Compounds with unsaturated lactones have been found to react with amino groups (56) and as such the effects of cordifene on the UV absorption of ADP was investigated in an effort to confirm the interaction in between cordifene and ADP.

5.3.A.3 MATERIALS AND METHOD

(a) The effect of ADP and cordifene on UV Light Absorption of actomyosin

The experiment was carried out with myosin which had been prepared as described by Perry (46) but with the following modification:-

1. One portion of the protein was dialysed against 0.5 M KCl containing 1 mM $MgCl_2$ and 0.1 Tris-HCl (pH 7.7) for three hours so as to partially remove the nucleotide attached to it.
2. The other portion of the protein was similarly dialysed for twenty hours instead of three hours in an attempt to eliminate as much ADP from the protein as possible.

Spectrophotometric measurements were carried

out using a Unicam SP 1800 Ultraviolet Spectrophotometer (Pye Unicam Ltd., Cambridge, England) connected to a Unicam AR 25 Linear Recorder (Pye Unicam Ltd., Cambridge, England) at room temperature (25°C).

ADP (disodium dihydrogen salt) was purchased from BDH chemicals Ltd., Poole, England. All other reagents were laboratory reagent grade.

Two matched silica cells (1 cm. light-path length) were used. One was a reference cell while the other was a test cell. Additions, as shown in the legend to the Figures (Figs. 33, 34, 35, 36, 37, 38, 39 and 40), were made with glass pipettes when the amount to be added was more than 1 ml. or a micropipette when the amount to be added was less than one ml.

The scan was started as soon as possible after mixing the contents of the sample cell with a glass rod. This was to ensure minimum nucleotide alteration by the enzymes in the myosin system. The scan was repeated after five minutes to see whether any changes in the UV light absorption would occur with time.

The concentration of the protein was determined using the Biuret method. A molecular weight of myosin of 500,000 (57) was assumed and was used for calculating the molar absorption.

(b) Investigation of the possibility of cordifene-ADP complex formation.

To ascertain the effect of cordifene on the UV

absorption of ADP and a possible cordifene-ADP complex formation in solution, the experiment as described in Section 5.3.A above was repeated without the addition of myosin. The UV absorption spectra of ADP and those of ADP together with different concentrations of cordifene were obtained. The additions of solutions into the cuvettes were as shown on the legends accompanying Figure 43.

5.3.A.4 RESULTS

Table 5 and Table 6 are a summary of the effects of ADP and cordifene on the UV absorption of myosin as shown on Figs. 33,34,35,36,37,38,39 and 40. An examination of the Tables show that ADP and cordifene either singly or in combination cause an increase in the molar absorption of myosin. The effect is better appreciated when the absorption at 350 nanometers is examined. At this region, the absorption observed is mainly that of the protein as the nucleotides and cordifene have minimum absorption at this wavelength range (48).

A comparison of Fig. 41 and Fig. 42 show that ADP affect the molar absorption of cordifene and Fig. 43 show a diminution of the smaller peak of ADP absorption around 210 nanometres with increasing concentrations of cordifene. A comparison of the molar absorptions of myosin when in the one case only cordifene is added to the protein and in the other when both ADP and cordifene are present (see figs.

35 and 39; 36 and 40) show that ADP decreases the absorption of the protein caused by cordifene.

5.3.A.5 DISCUSSION:

The hypochromic effect of cordifene on UV absorption of ADP around 210 nanometres indicates a reaction in between the two compounds. This suggests that cordifene combines with free ADP that is released from the myosin heads.

The molar absorption of cordifene or a complex of cordifene and ADP is too small to explain the observed effects of these two compounds on the UV absorption of myosin, that is, if it were to be assumed that there is no interaction in between any of these two compounds (or their complex) with myosin and that therefore the light absorption was a summation of the individual absorption. This then means that the two compounds singly and in combination combine with myosin causing configurational changes in the protein's tertiary (and probably secondary) structure which results in the observed increases in the molar absorption of myosin.

A comparison of the effects of ADP on the molar absorption of a sample of myosin that has been dialysed for only three hours with that of a sample of myosin that has been dialysed for twenty hours shows that dialysis has an effect on the myosin-ADP complex. The decay of the spectrum of myosin-ATP complex to that induces by ADP on myosin has demonstrated that

myosin-ATP absorbs more intensely at 280 nanometres than does myosin-ADP complex (8). This then means that the addition of ADP to a system of myosin solution that is not completely devoid of nucleotides, as is the case with myosin which has been dialysed for only three hours, increases the binding of ADP to myosin (54) vis-a-vis that of ATP whose concentration is then far much lower compared to that of ADP. This then would lead to the quenching of the absorption of the protein at 280 nanometres. After twenty hours of dialysis, most of the nucleotides have already been removed from the protein and most of the ATP has already been hydrolysed to ADP. The intensity of absorption of the protein is therefore far much lower compared to the absorption of the protein after only three hours of dialysis. The addition of ADP to the protein at this stage can then only increase the intensity of absorption of the protein at 280 nanometres.

Table 5 and Table 6 show that myosin is still capable of being modified even after twenty hours suggesting that the protein is still intact biologically and has not disintegrated markedly. In view of the fact that West (48) has argued that a nucleotide bound to actin slows down its rate of disintegration with time, it is probable that a far much longer time of dialysis is required if myosin is to be ridden off its content of nucleotides and faster disintegration can set in. The lower intensities

of absorption observed with the older myosin can be explained partially, therefore, as caused by a decrease in the content of nucleotides in the protein and partially as a consequence of disintegration caused by ageing. The fact that the intensity of absorption induced by cordifene is less in the older myosin (compare Fig. 35 with Fig. 39) would tend to support the view that some disintegration takes place with time.

When the two modifiers are present together in the myosin solution, the intensity of absorption is less than when only cordifene is present alone (compare Fig. 36 with Fig. 35 or Fig. 40 with Fig. 39). Taking into account that the two modifiers individually induce an increase in the intensity of absorption of the protein and since the observed intensity of absorption when both modifiers are present in the protein solution is not a summation of their individual effects on the intensity of absorption, the observed results support the view that ADP combines with cordifene thereby decreasing the concentration of cordifene in the solution and therefore the intensity of absorption induced by this modifier on the protein.

5.3. B. THE EFFECT OF CORDIFENE ON THE ADP INDUCED RESPIRATION BY RAT LIVER MITOCHONDRIA.

5.3. B.1 INTRODUCTION:

The author's observation that cordifene affected

the UV absorption of ADP around 210 nanometres suggested that cordifene formed a complex with ADP. To confirm this suggestion, the author decided to examine the effect of cordifene on the ADP induced respiration by rat liver mitochondria. If cordifene could prevent respiration of mitochondria without affecting the respiratory capability of the protein, this would add more weight to the suggestion that cordifene binds ADP and that the disappearance of ADP effects on myosin ATPase (E.C. 3.6.1.3) was not due to increased mitochondrial ATPase activity and the activities of the other enzymes involved in ADP metabolism in mitochondria - a situation that might have accounted for increased dissociation of the myosin-ADP complex.

5.3.B.2 REVIEW OF LITERATURE:

It is well known that the conditions par excellence for the maximal consumption of oxygen by mitochondria are when the external medium bathing the mitochondria has got an adequate supply of respiratory substrate and a high concentration of both ADP and inorganic phosphate but a low concentration of ATP. When these conditions are reversed, that is, when the concentration of ATP in the external medium is high while those of ADP and inorganic phosphate are low, then mitochondria only show a very low respiratory rate. Of the three components that

determine the rate of respiration and therefore the rate at which oxygen is consumed, the ADP concentration is the most critical in setting the respiratory rate because of the extraordinarily high affinity of the mitochondria for ADP and maximal rates of respiration occur even when the concentration of ADP is as low as 0.2 mM (1).

5.3.B.3 MATERIALS AND METHODS:

Liver material was obtained from fully grown albino rats which had been purchased from the Animal Breeding House of the Public Health Laboratories of the Ministry of Health in Kenya. These livers were used as the source of the mitochondria. The isolation procedure for the mitochondria from the liver material was that of Schneider and Hogeboon (1950) (57).

ADP (disodium dihydrogen salt) was purchased from BDH chemicals Ltd., Poole, England. All other reagents were laboratory reagent grade.

The Respiratory Control Ratio (RCR) of the mitochondrial preparations was determined by the use of a Clark Oxygen electrode (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.) connected to an S.R.G. Sargent Recorder (Yellow Springs Instrument Co. Inc., Yellow Springs, Ohio, U.S.A.). The oxygen electrode was covered with a teflon membrane which was obtained from Yellow Springs Instru-

ment Co. Inc., Yellow Springs, Ohio, U.S.A. All additions as they were made are shown in legends to the figures and they were made with micropipettes wherever the volume of the material to be added was less than one cubic centimeter. A magnetic plea was used to keep the reaction mixture homogeneous.

Cordifene was dissolved in 70% methanol adding five milligrams of cordifene in one cubic centimeter of the alcohol solution and this solution was the source of cordifene for all the experiments.

5.3.B.4 RESULTS:

Oxidation of succinate by mitochondria is shown in Fig. 44. Respiratory control ratio of the mitochondria was 6 which means that the protein particles were coupled.

Fig. 45 shows that 60 µl. of the cordifene solution reduced the amount of oxygen uptake on addition of 5 µl. of ADP to the respiring mitochondria by a factor of two. When a further 5 µl. of ADP solution was added the mitochondria continued to respire and at the same rate as the control.

A similar trace to that shown in Fig. 45 was obtained when glutamate was used as the substrate for oxidation.

Attempts to study the stoichiometry of the complexation of ADP and cordifene by this procedure failed. The cause of this failure was taken to be due to the excessive amount of methanol in the reac-

tion medium. Methanol is known to cause such disastrous effects on the mitochondrial membranes that a leakage in the membrane results.

5.3.B.5. CONCLUSION:

The effects of cordifene on the production of ATP by mitochondria not only shows that cordifene does not affect the mitochondrial membranes adversely, it also shows that cordifene binds or complexes with some of the ADP added to the system such that the bound ADP cannot be phosphorylated to ATP. Its lack of adverse effects on the mitochondrial membranes and the fact that state 3 and state 4 respiration rates in Fig. 44 and Fig. 45 were the same, shows that cordifene has no effect on the mitochondrial adenosine triphosphatase.

5.4 EFFECT OF CORDIFENE ON "ACTIVATED" MYOSIN

5.4.1 INTRODUCTION:

Experiments on calcium uptake by muscle indicated that cordifene inhibited uptake of Ca^{2+} into the myofibril. Cordifene has also been shown to bind ADP. Being an α-methylene-γ-lactone compound, it would bind onto sulphydryl groups and also to amino groups. Cordifene has also got two epoxides on its structure which make it even more active as an alkylating agent. These structural features would confer such reactivity to the compound that it

can be expected to react very rapidly with some residues of a protein like myosin thereby causing minor or even major morphological changes on the protein. The following experiments were therefore carried out to investigate the possibility of morphological changes on myosin brought about by a reaction in between the protein and cordifene.

5.4.2 REVIEW OF LITERATURE:

The modification of the primary, secondary and tertiary structures of myosin have been found to take place by several workers using different chemical compounds. Sekine and Kielley (44) have shown that the primary structure of myosin can be modified by such reagents as N-methylmaleimide (NEM) which binds onto the sulphydryl groups.

Rainford et al. (58) have shown that it is unlikely that the mechanism of activation of myosin ATPase by DNP involves a reaction with SH groups or with any other known group. Levy et al. (59) observed that, in the presence of Mg^{2+}, high concentrations of DNP inhibit myosin ATPase and that this inhibition is prevented by ATP, ADP and PPi or tripolyphosphate and from these observations they suggested that conformational changes was the basis of their observations.

In 0.6 M KCl, myosin exists essentially as monodisperse protein molecules. Huxley (18) found that

at an ionic strength of 0.15 M a system of myosin filaments which was heterogeneous in length was formed and this system of filaments had several morphological features in common with the native thick filament of the myofibril.

Fumi Morita (8) has observed conformational changes in heavy meromyosin following hydrolysis of ATP by the protein.

Conformational changes have also been observed by Huxley and Brown (60) when actin binds onto myosin in the formation of rigor complexes.

The structure of cordifene incorporates two epoxides, a methylene group adjacent to a hydroxyl group which is close to an epoxide (see Fig. 14). All these chemical groupings are very reactive and can conceivably, especially owing to their arrangement on the molecule, contribute markedly to the biological reactivity of the compound. Epoxides are very reactive because of the ease with which the highly strained three membered ring can be opened up and they will undergo acid-catalyzed and base-catalyzed reactions (61).

Although little is known about the relation between structure and activity of compounds that are unsaturated lactones, their reactivity towards thiols and amines has been demonstrated and the presence of other reactive groups in their structure suggests that their activity may result from alkylation of nucleophilic centres in a biological system (56).

5.4.3 MATERIALS AND APPARATUS:

Skeletal muscle actomyosin from the back and leg muscles of guinea pig which had been supplied by the National Health Laboratories of the Ministry of Health, Kenya.

Cordifene dissolved in 70% methanol (5 mg/ml).

1% uranyl acetate solution

Carbon-coated grids

1% Ammonium acetate solution

Transmission Electron Microscope (Philips Transmission Electron Microscope, Electronic EM 201, Philips Scientific and Analytical Equipment, N.V. Philips, Gloeilampenfabrieken, Eindhoven, Netherlands).

The carbon-coated grids and the solutions of uranyl acetate and ammonium acetate were kindly supplied by Professor Benedetti of the Faculty of Science of the University of Paris. Professor Benedetti, who happened to be temporarily attached to the Department of Biochemistry of the University of Nairobi during an International course on Cell Organization which had been organized by International Cell Research Organization of the United Nations in April of this year, kindly helped the author to prepare the stained films for viewing and photographing with the Electron Microscope.

Actomyosin was prepared by the method of Perry as described in Section 5.2.3 of this work and then following modifications were made to the actomyosin solution

in 0.6 M KCl. To the actomyosin solution ATP (disodium dihydrogen salt - obtained from BDH, Poole, England) was added to make a final ATP concentration of 270 γg. per gram protein. The protein solution was then divided into two halves and to one half, a cordifene solution in 70% methanol was added so as to make the final cordifene concentration in the solution 0.56 mg. per gram protein. The cordifene containing actomyosin solution was then centrifuged and the supernatant discarded. The precipitate was then resuspended in 0.6 M KCl solution.

5.4.4 METHOD:

The actomyosin solutions to be used for making the film on the grid were, first of all, diluted five times with 1% ammonium acetate solution. A carbon-coated grid was then picked with a pair of forceps and onto the exposed carbon-coated surface of the grid was applied a drop of the diluted actomyosin suspension with a fine glass pipette without touching the grid with the glass tip. The grid was then kept covered under a watch glass for thirty seconds after which the excess fluid was withdrawn from it using a Whatman No. 1 filter paper leaving only enough fluid on the grid to make a wet film. The grid was then quickly washed at an angle with 1% aqueous uranyl acetate solution. The film was then dried.

The film, so negatively stained, was then inserted into the Electron Microscope viewing chamber

for viewing and photography.

5.4.5 RESULTS:

Figure 46 is a micrograph of the actomyosin molecules in absence of cordifene. Fig. 47 is a micrograph of the actomyosin molecules in the presence of cordifene. Comparison of the two figures shows that configurational changes occur when actomyosin is reacted with cordifene.

5.4.6 CONCLUSION:

Cordifene was dissolved in 70% methanol and to eliminate solvent effects (41) the cordifene treated actomyosin was centrifuged so as to be redissolved in 0.6 M KCl, a solution known to maintain myosin essentially in the monomeric state. In addition, the two solutions were equally diluted with ammonium acetate solution and this ruled out variability in ionic concentrations in between the two protein solutions (62) which would have suggested the differences observed in the films to be due to either polymerization or depolymerization of myosin filaments. Taking these precautions into account, the most plausible explanation for the observed shortening of actomyosin molecules in the presence of cordifene is the contraction of the skeleton of the protein molecule probably following ATP hydrolysis. However, a collaboration of this deduction is required and

this would only come from the finding of an antidote to cordifene, the reaction of which with the contracted protein molecules would permit the return of the conformation of the protein molecules depicted in Fig. 46.

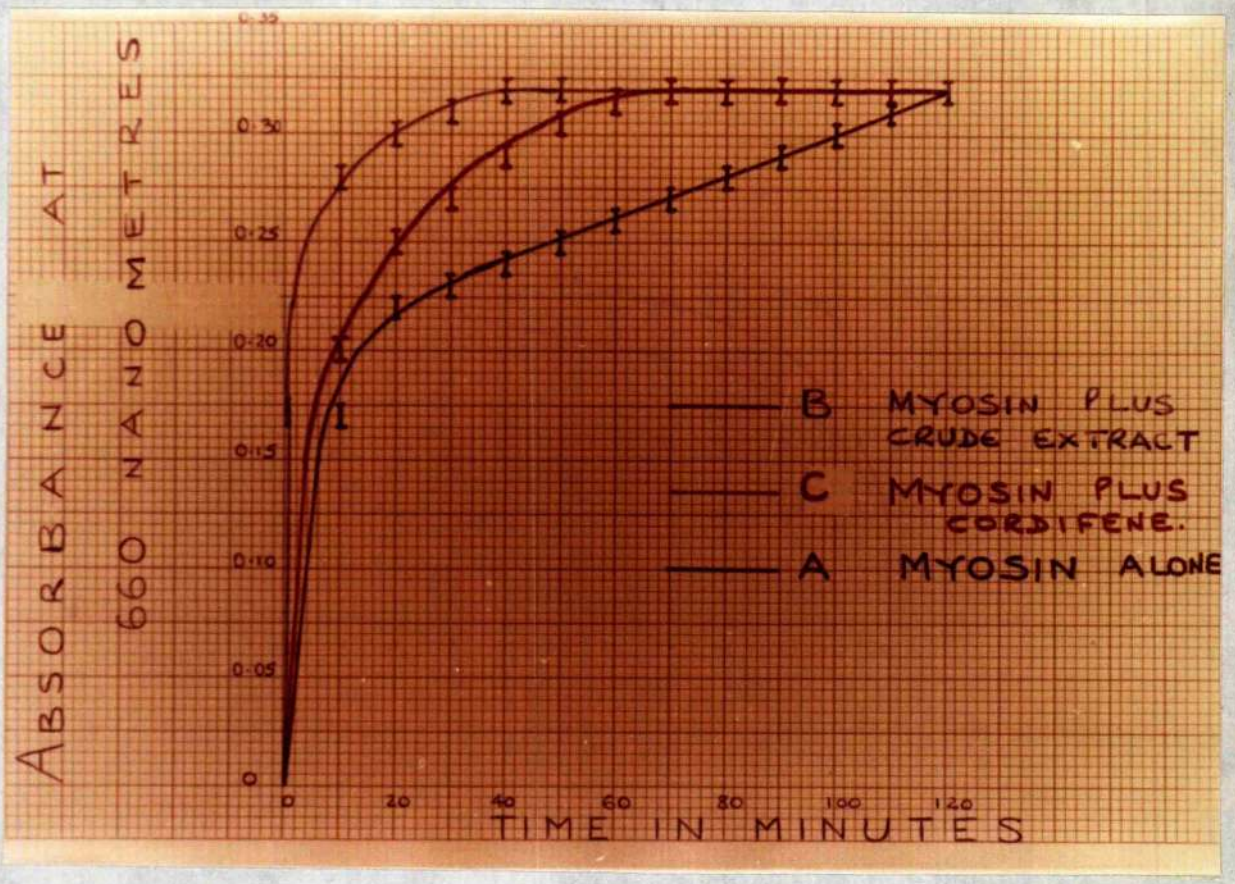


Fig. 32: Effect of E. cordifolia extract and of cordifene on the rate of ATP hydrolysis by myofibrillar ATPase (E.C.3.6. 1.3). The assay was carried out as described in sections 5.2.3 and 5.2.4.

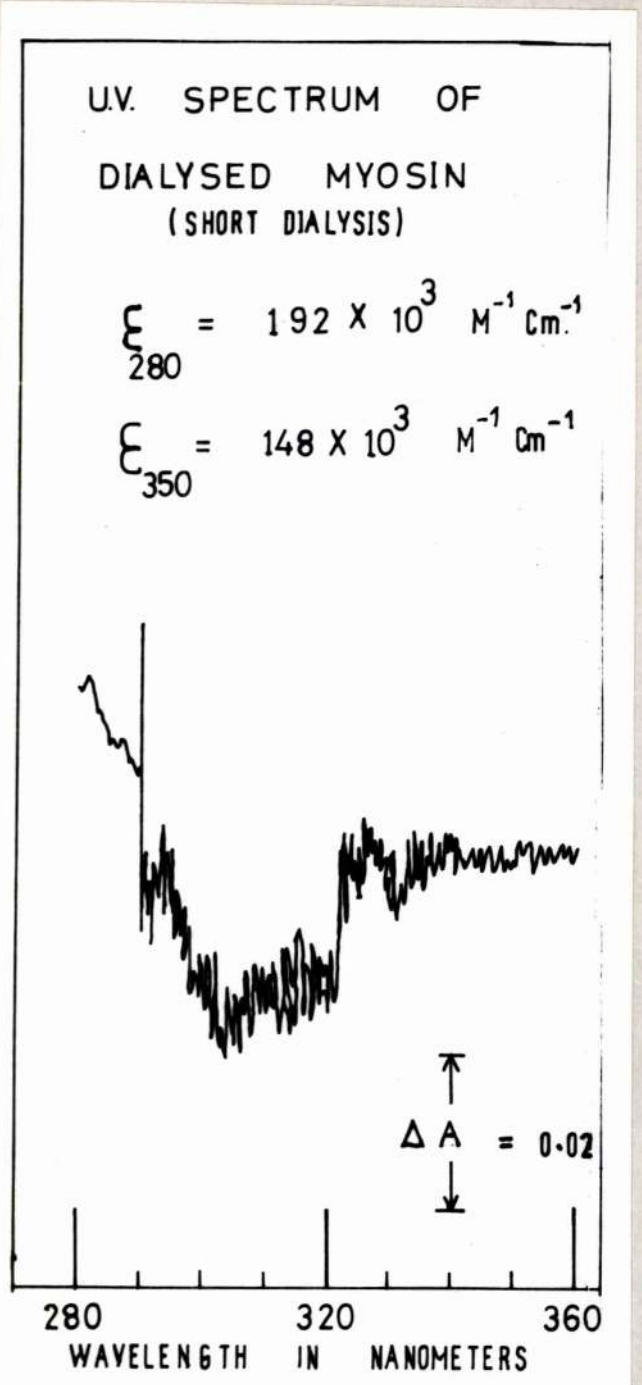


FIG.

Fig. 33: The reference cuvette contained 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80µg of myosin per ml, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

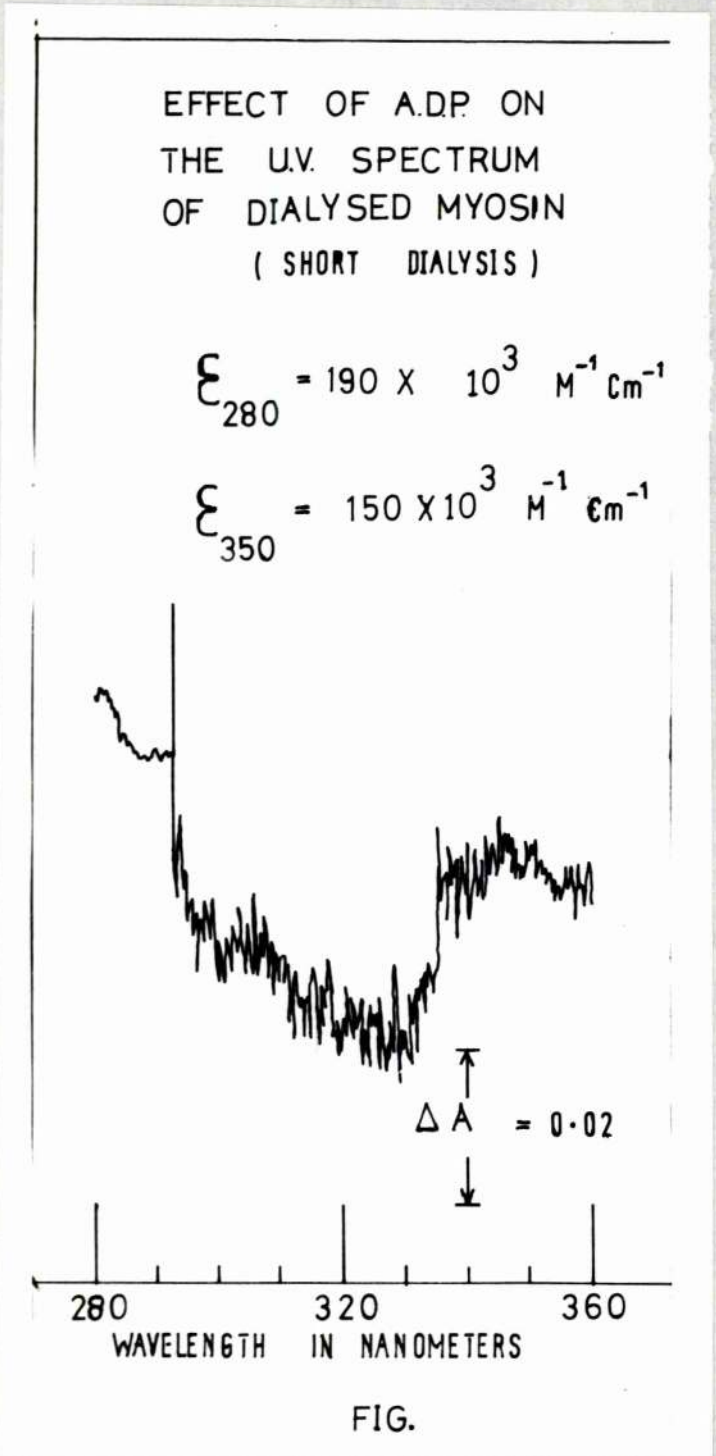


Fig. 34: The reference cell contained 80 µM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80 µg of myosin per ml., 80 µM ADP, 10.6 mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

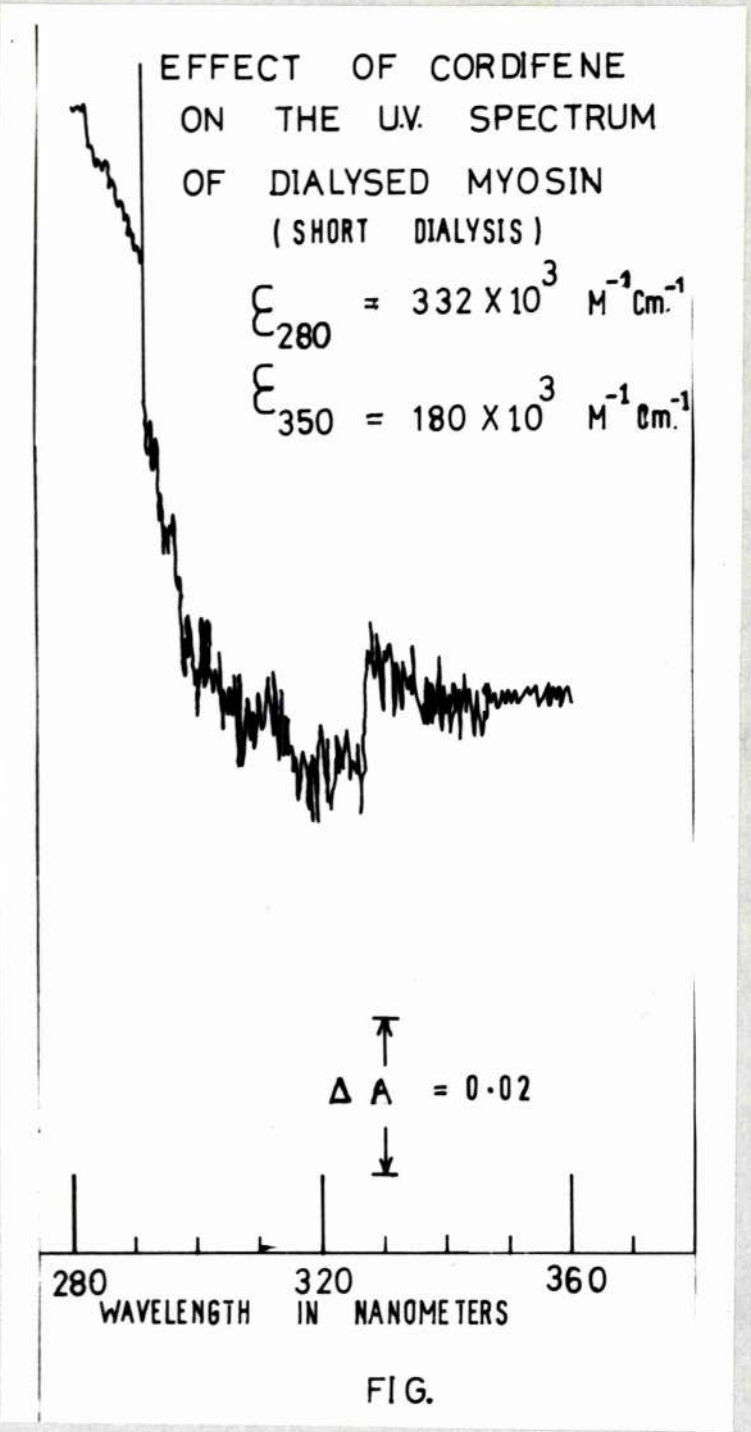


Fig. 35: The reference cell contained 20 µM cordifene, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80 µg of myosin per ml., 20 µM cordifene, 10.6 mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

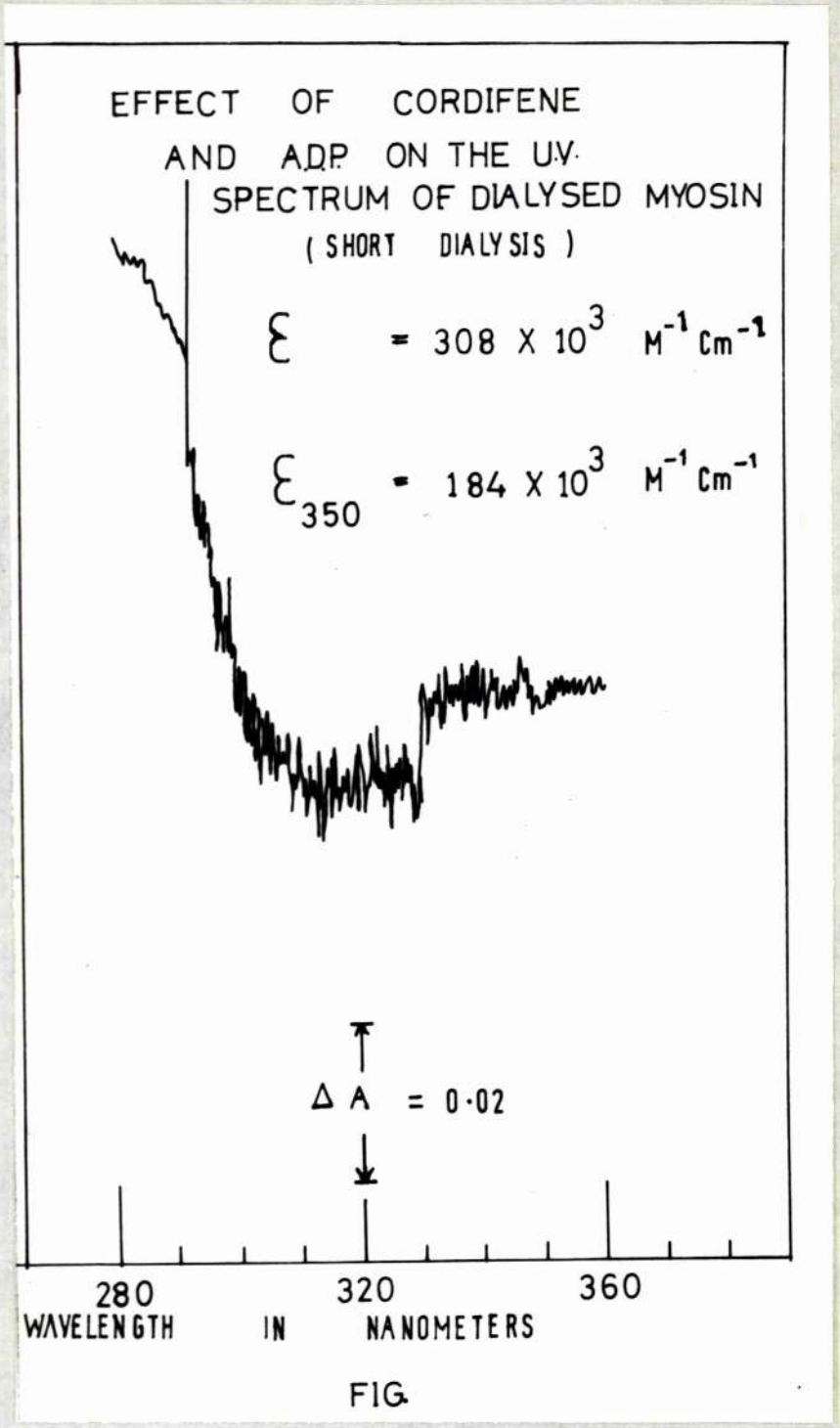


Fig. 36: The reference cell contained 20μM cordifene, 80μM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80μg of myosin per ml., 20μM cordifene, 80μM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

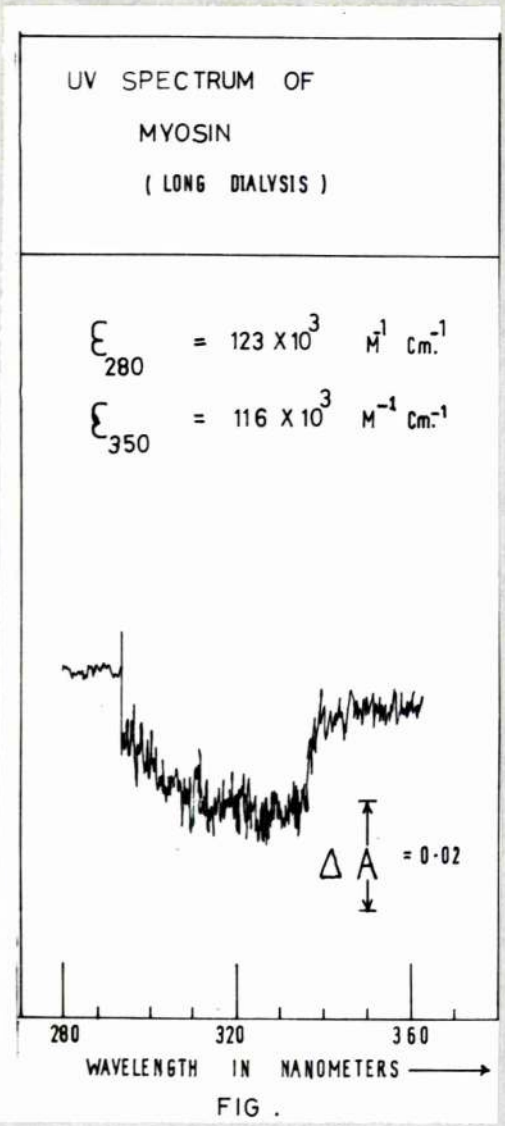


FIG .

Fig. 37: The reference cell contained 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80µg of myosin per ml., 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

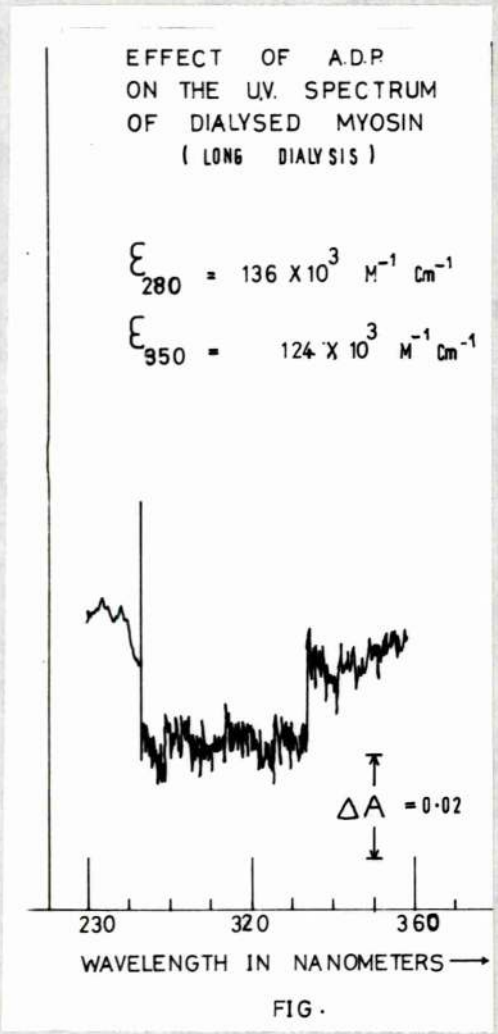


FIG.

Fig. 38: The reference cell contained 80μM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80μg of myosin per ml., 80μM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

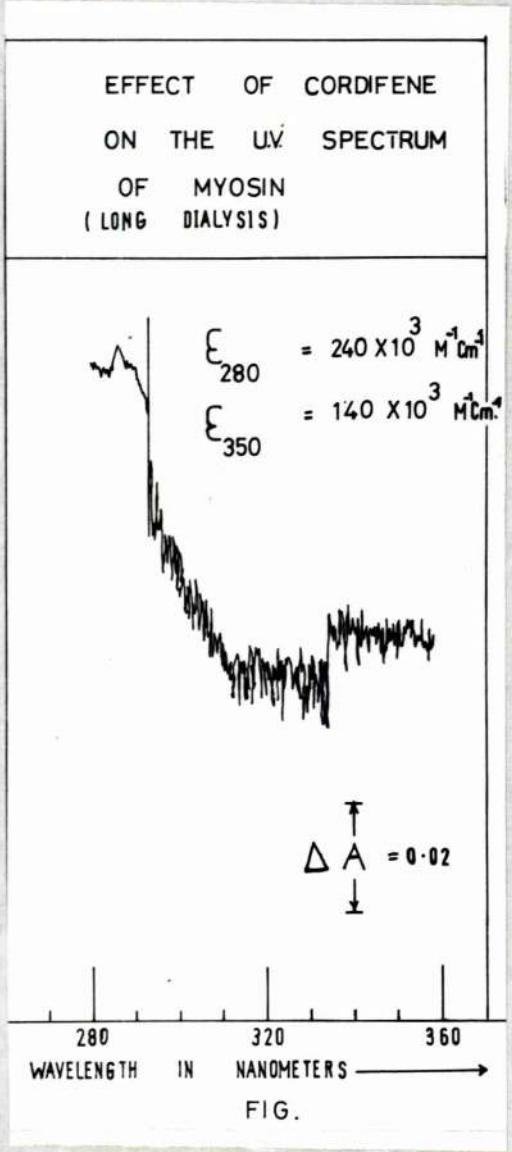


FIG.

Fig. 39: The reference cell contained 20µM cordifene, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80µg of myosin per ml, 20µM cordifene, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

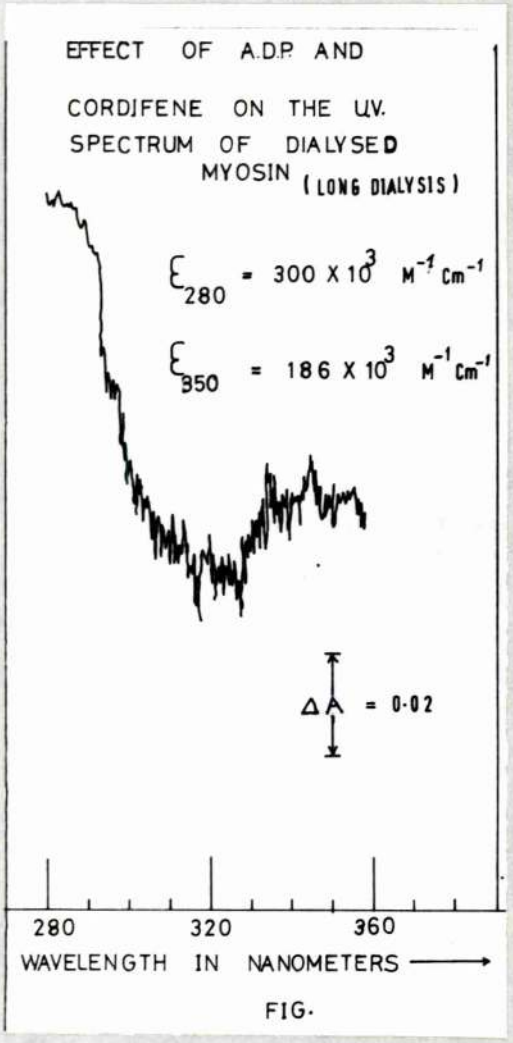


FIG.

Fig. 40: The reference cell contained 20μM cordifene, 80μM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8). The test cell contained 80μg of myosin per ml, 20μM cordifene, 80μM ADP, 10.6mM $MgCl_2$, 0.08M KCl, 0.07M Tris-HCl buffer (pH 8).

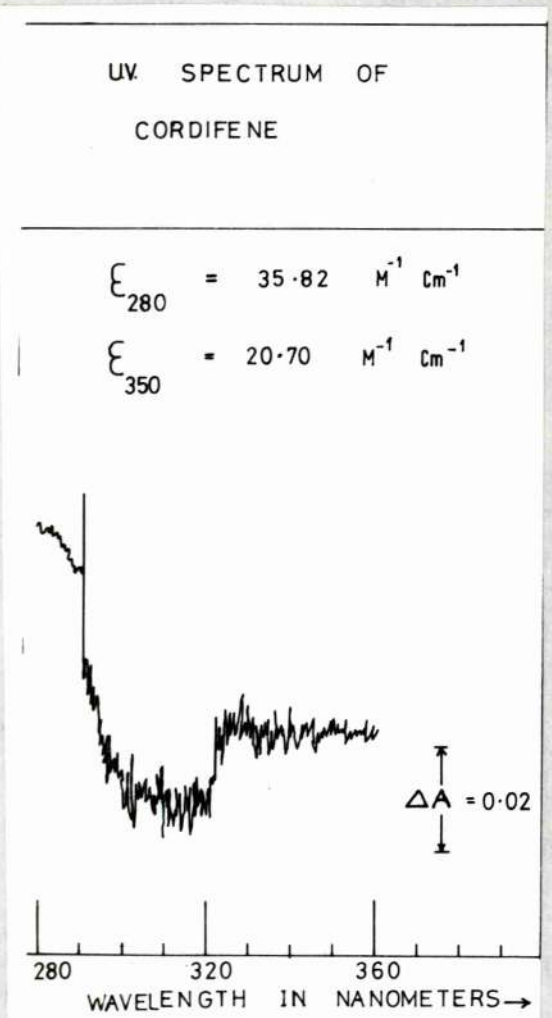


FIG.

Fig. 41: The reference cell contained methanol (analytical grade). The test cell contained methanol and 2mM cordifene.

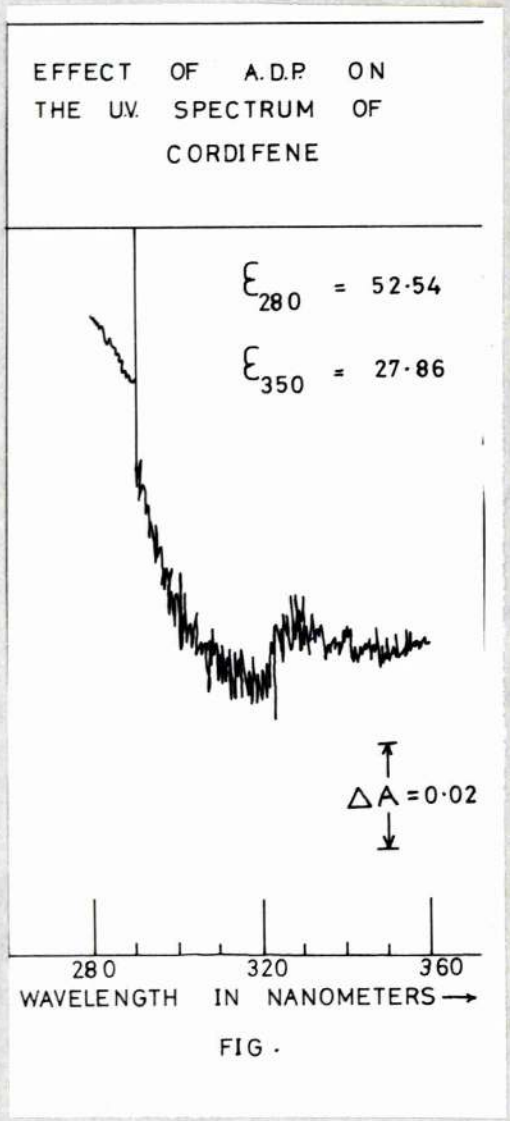


FIG.

Fig. 42: The reference cell contained methanol (analytical grade) and 80μM ADP. The test cell contained methanol (analytical grade), 80μM ADP and 2mM cordifene.

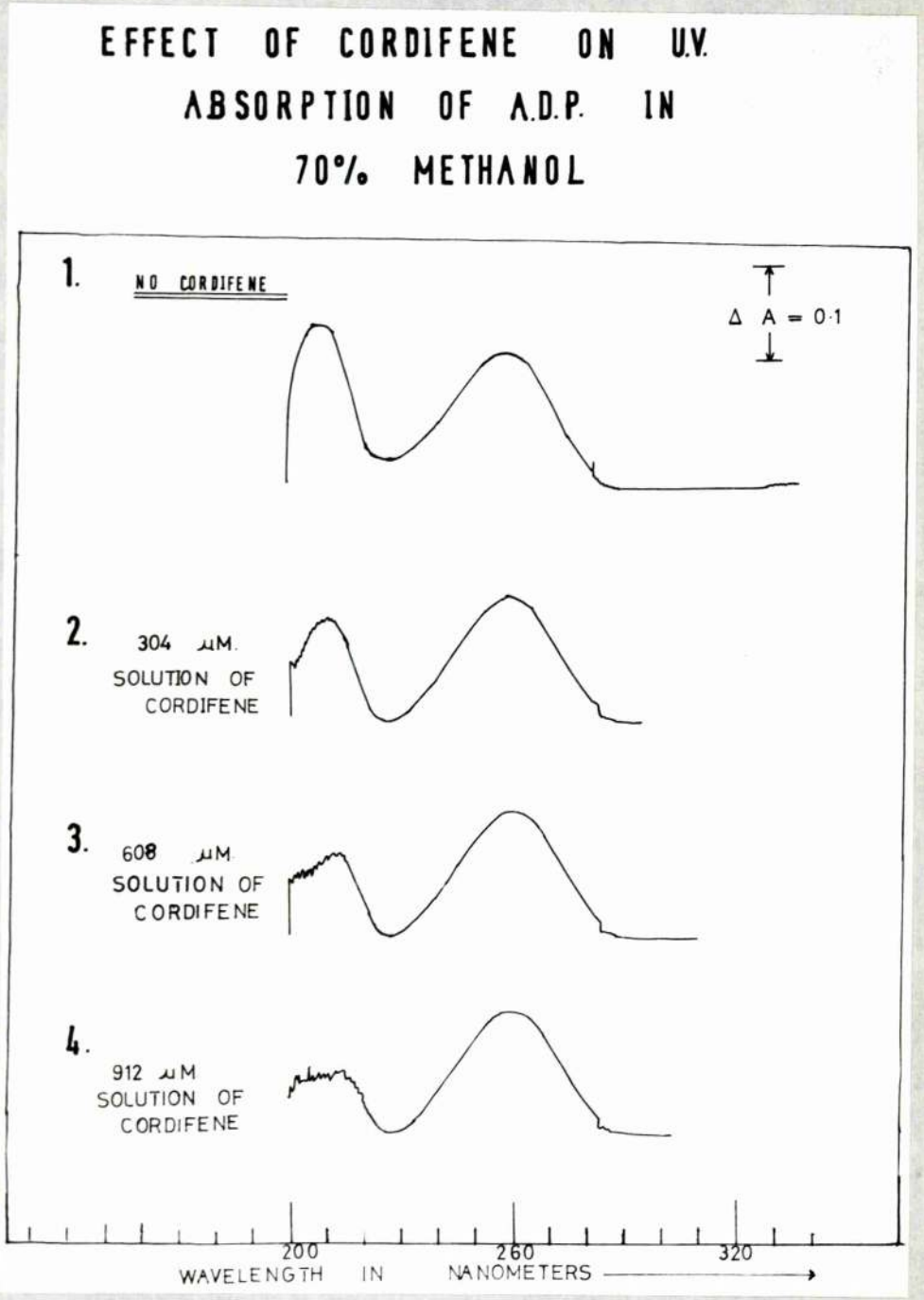
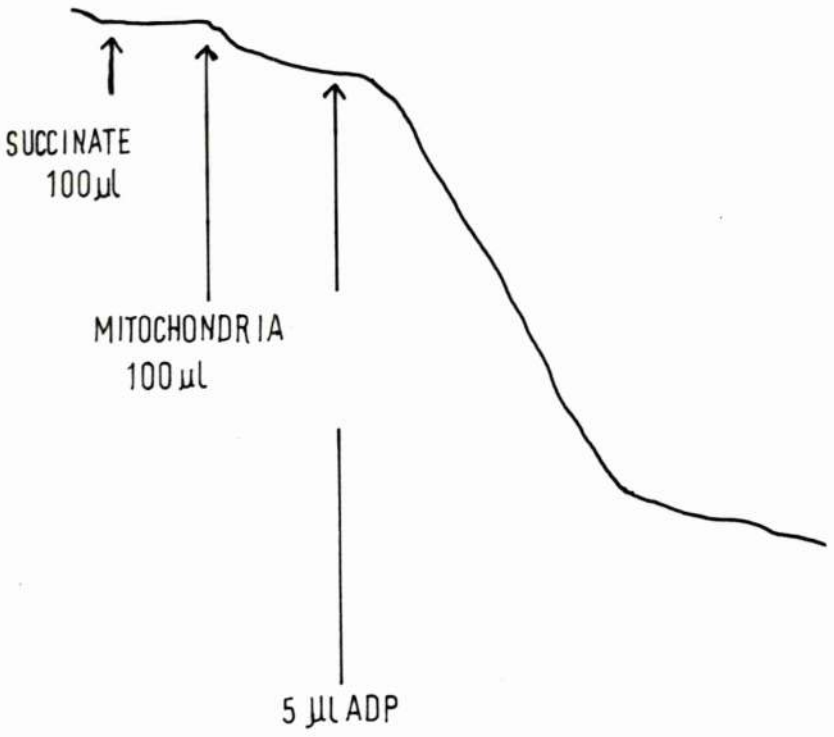


Fig. 43: The silica cuvette contained 2.5 ml of 1mg/ml solution of ADP in 70% methanol. Cordifene was added to make its final concentration 304μM (2), 608μM (3) and 912μM (4).

Fig. 44: Effect of ADP on oxygen uptake by coupled mitochondrial particles:

The assay was carried out as described in sec. 5.3.B.3. The assay contained 0.1ml of a 4mg/ml. mitochondrial protein, 15mM KCl, 50mM Tris-buffer pH 7.4, 5mM $MgCl_2$, 2mM EDTA, 20mM potassium phosphate pH 7.4 (made from a solution containg 400 ml 0.5M KH_2PO_4 and adjusting the pH to 7.4) 0.25mM ADP and 10mM succinate.



EFFECT OF ADP ON MITOCHONDRIAL RESPIRATION

Fig. 44

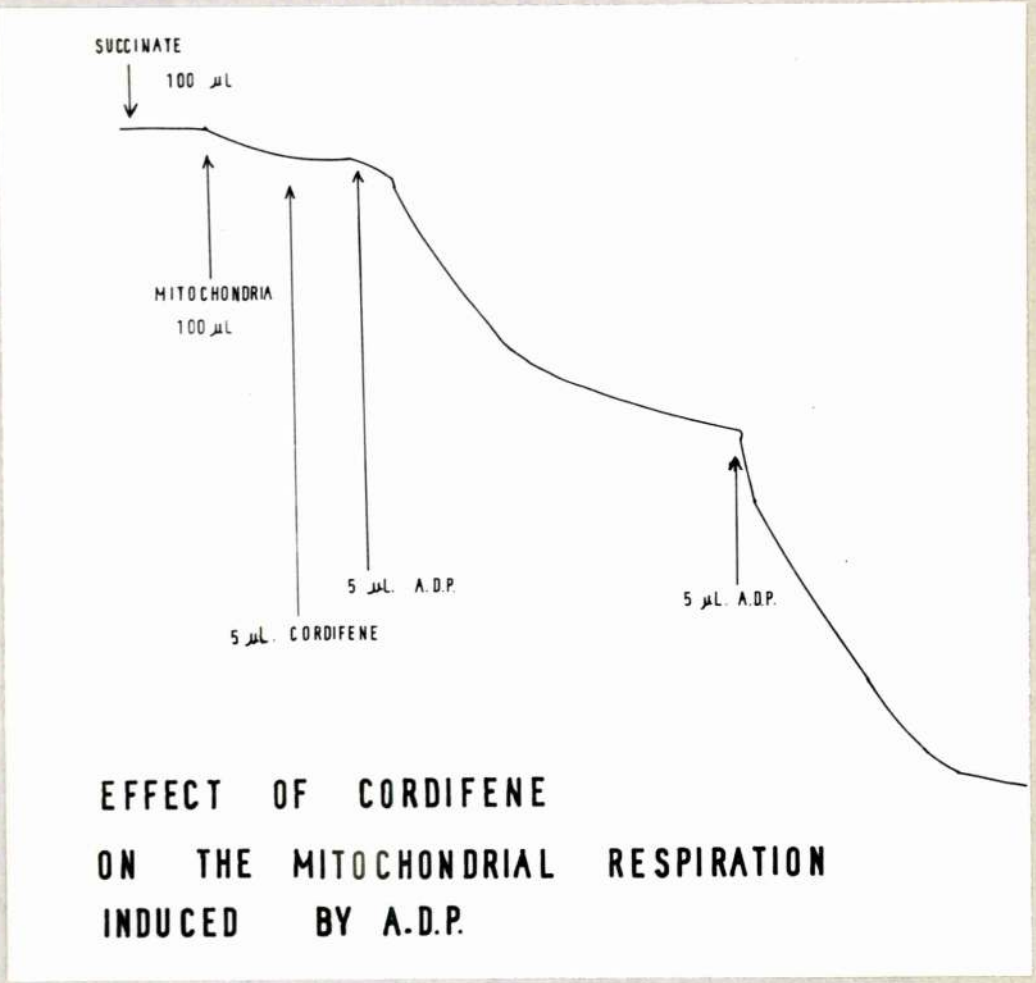


Fig. 45: Effect of cordifene on mitochondrial respiration induced by ADP. The assay was carried out as described in sec. 5.3.B.3. The assay contained 0.1ml. of a 4mg/ml mitochondrial protein, 15mM KCl, 50mM Tris- buffer pH 7.4, 5mM $MgCl_2$, 2mM EDTA, 20mM Potassium phosphate pH 7.4 (made from a solution containing 400ml 0.5M K_2HPO_4 and 200ml 0.5M KH_2PO_4 and adjusting the pH to 7.4), 0.25mM ADP and 60µl of 5mg/ml cordifene, 10mM succinate.

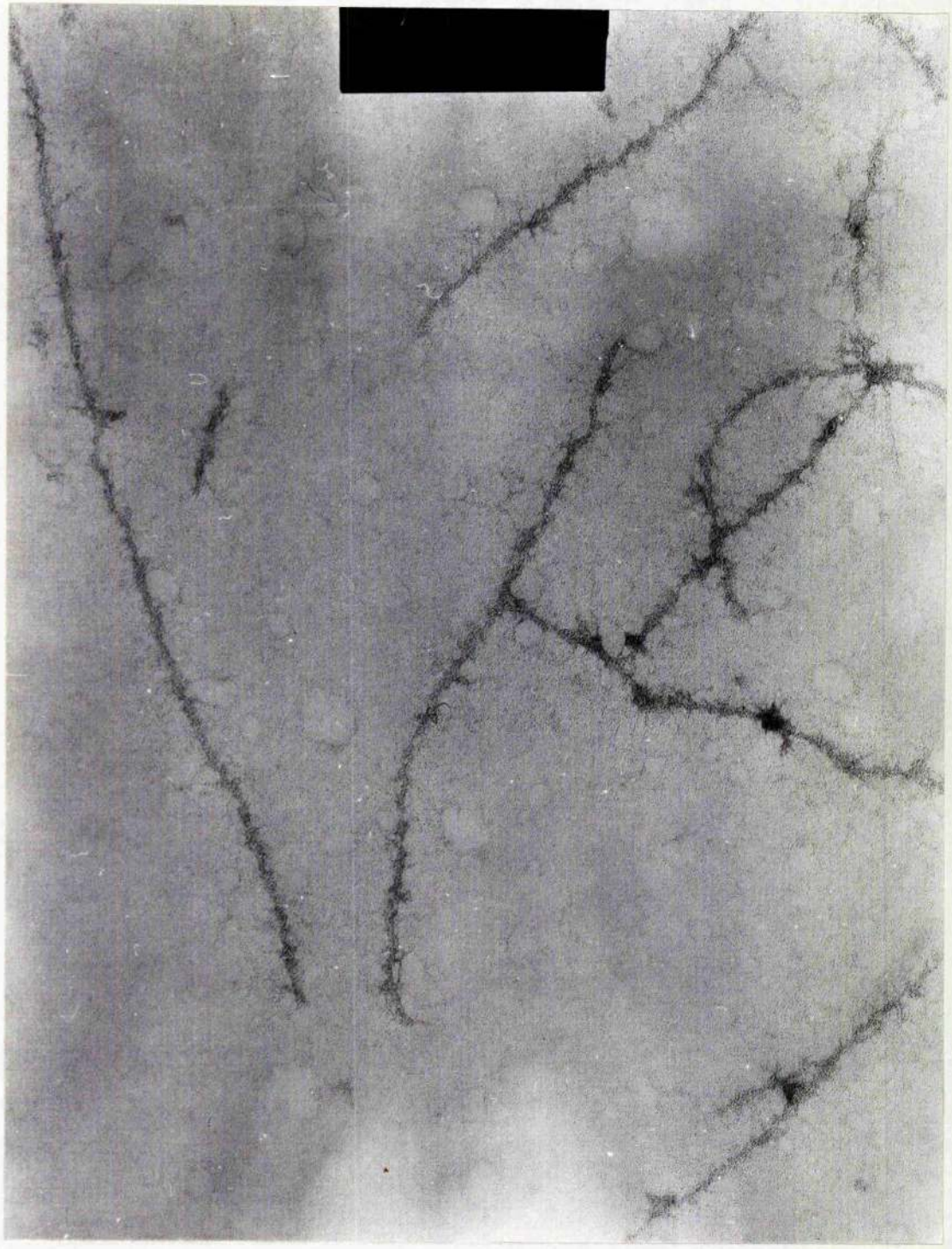


Fig. 46: Electronmicrograph of actomyosin molecules

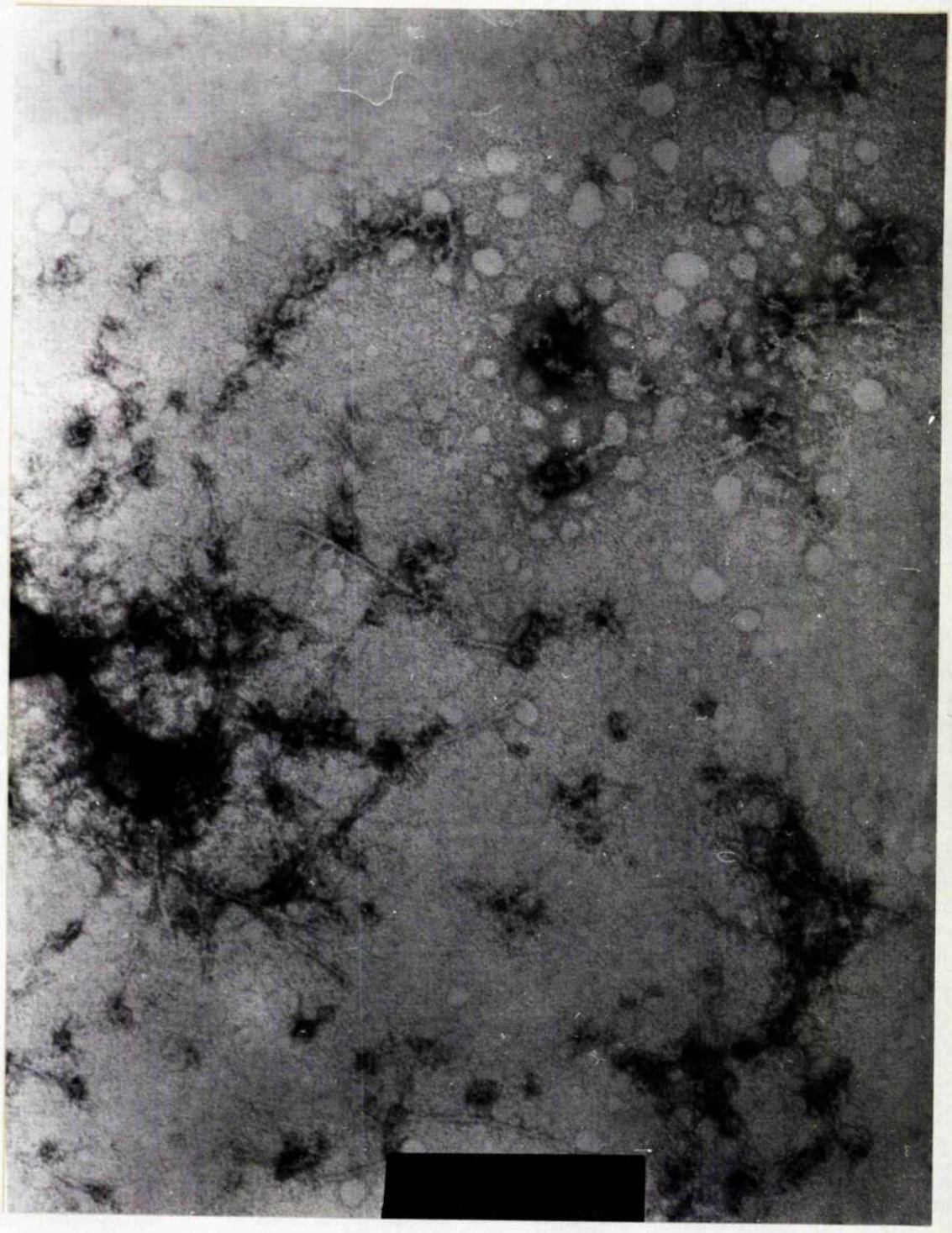


Fig. 47: Electronmicrograph of actomyosin molecules in the presence of cordifene

Table 1: Hydrolysis of acetylthiocholine by serum cholinesterase enzyme in the presence of cordifene. Conditions of the assay: Standard 1: 0.12M phosphate buffer (pH 8), 0.80 ml water, 0.4mM DTNB, 0.3mM cordifene, and 10 µl serum. Standard 2: 0.12M phosphate buffer (pH 8), 0.80 ml water, 0.8 mM acetylthiocholine, 0.4mM DTNB, and 10µl serum. Standard 3: 0.12M phosphate buffer (pH 8), 0.80 ml water, 0.8mM acetylthiocholine, 0.4mM DTNB, and 0.3mM cordifene. The test: 0.12M phosphate buffer (pH 8), 0.80 ml water, 0.8mM acetylthiocholine, 0.4mM DTNB, 0.3mM cordifene, and 10µl. serum. The reaction mixtures were made as described in section 4.2.4 and the reaction was followed spectrophotometrically at 412 nanometres.

Time in seconds	Standard 1	Standard 2	Standard 3	Test solution
0	0.020	0.08	0.69	0.77
30	0.028	0.15	0.69	0.85
60	0.033	0.21	0.69	0.94
90	0.035	0.27	0.69	0.98
120	0.036	0.33	0.69	1.04
150	0.038	0.39	0.69	1.10
180	0.039	0.45	0.69	1.17
210	0.040	0.51	0.69	1.22
240	0.040	0.56	0.69	1.28
270	0.041	0.61	0.69	1.32
300	0.041	0.66	0.69	1.39
330	0.041	0.72	0.69	1.43
360	0.042	0.77	0.69	1.47
390	0.043	0.82	0.69	1.50
420	0.044	0.87		
450	0.045	0.91		
480	0.047	0.96		
510	0.047	1.00		
540	0.047	1.03		
570	0.047	1.06		
600	0.047	1.16		
630	0.049	1.22		
660	0.050	1.22		
690	0.050	123		

Table 1: Spectrophotometric results of the hydrolysis of acetylthiocholine in the presence of cordifene. Optical density readings were carried out at 412 nanometres. Conditions of the assay was as described in the legends opposite page.

TABLE 2: Comparison of the rate of hydrolysis of acetylthiocholine by serum cholinesterase in the presence and absence of cordifene.

Time in seconds	Standard 2	ΔOD (Test cell-standard 3)
0	0.08	0.08
30	0.15	0.16
60	0.21	0.25
90	0.27	0.29
120	0.33	0.35
150	0.39	0.41
180	0.45	0.48
210	0.51	0.53
240	0.56	0.59
270	0.61	0.63
300	0.66	0.70
330	0.72	0.74
360	0.77	0.78
390	0.82	0.81

TABLE 3

Blank	Protein	Protein with cordifene
6090	5129	2834
5775	4700	2500
5900	4850	2645
6500	5450	2951
6350	5400	2850
6320	5290	2857
5990	5000	2750
5930	4975	2669
6000	5100	2775
Mean plus Standard error	6095 ± 80	5099 ± 83	2759 ± 45

A table of results showing the binding of $^{45}Ca^{2+}$ by myofibril protein in the presence of and the absence of cordifene.

TABLE 4

TABLE OF RESULTS SHOWING THE EFFECT OF CRUDE ERLANGIA CORDIFOLIA MATERIAL AND ALSO OF CORDIFENE ON THE HYDROLYSIS OF ATP BY MYOSIN ATPase (E.C. 3.6.1.3.)

Time in minutes	O.D. at 660 nanometres		
	A	B	C
0	0.04	0.08	0.08
10	0.17	0.28	0.20
20	0.22	0.30	0.25
30	0.23	0.31	0.27
40	0.24	0.32	0.29
50	0.25	0.32	0.305
60	0.26	0.32	0.315
70	0.27	0.32	0.32
80	0.28	0.32	0.32
90	0.29	0.32	0.32
100	0.30	0.32	0.32
110	0.31	0.32	0.32
120	0.32	0.32	0.32

The hydrolytic process was carried out as described in Hawk's Physiological Chemistry[39] and the determination of inorganic phosphate so produced was determined according to the method of Fiske and SubbaRow[47].

Column A: The absorbance of light measured at λ_{660} nanometres obtained by incubating ATP with actomyosin.

Column B: The absorbance of light measured at λ_{660} nanometres obtained by incubating ATP with actomyosin in the presence of crude Erlangia cordifolia material.

Column C: Shows the absorbance of light measured at λ 660 nanometres obtained by incubating ATP and actomyosin in the presence of cordifene.

TABLE 5: EFFECT OF ADDED ADP ON THE UV ABSORPTION OF MYOSIN

Duration of Dialysis of myosin in hours	Concentration of ADP in µM	UV absorbance	
		E_{280nM}	E_{350nm}
3	0	192×10^3	148×10^3
3	80	190×10^3	150×10^3
20	0	123×10^3	116×10^3
20	80	136×10^3	124×10^3

TABLE 6: EFFECT OF ADDED CORDIFENE ON THE UV ABSORPTION OF MYOSIN

Duration of Dialysis of Myosin in Hours	Concentration of ADP in µM	UV absorbance	
		E_{280nM}	E_{350nM}
3	0	192×10^3	148×10^3
3	20	332×10^3	180×10^3
20	0	123×10^3	116×10^3
20	20	240×10^3	140×10^3

CHAPTER 6:

DISCUSSION:

The extract of Erlangia cordifolia (S.Moore) compositae has been used traditionally by the Gikuyu people of Kenya for increasing myometrial contraction thereby facilitating parturition. Our experimentation with both crude extract and cordifene from the leaves of this herb has confirmed that the extract from this plant's leaves increases both amplitude of and the tension in a contracting muscle.

Muscle contraction can be increased by compounds that induce the increase of acetylcholine release at the motor-end plates in the sarcoplasmic reticulum. This is so because acetylcholine facilitates depolarization of the motor-end plate and the release of calcium into the cytoplasm of the muscle cell. Calcium ions are known to trigger the contractile process (3). This author's investigations into the possibility of the extracts of Erlangia cordifolia influencing the levels of acetylcholine at the motor-end plate either through effects on

nerve transmissions or the preservations of acetylcholine through inhibition of acetylcholinesterase showed that the extract from this plant did not have any such activities.

Crude Erlangia cordifolia extract was found to contain large quantities of calcium. However, it is likely that this calcium, on absorption, finds its way into the large calcium pool found in blood plasma. Investigation as to the possibility of cordifene facilitating the translocation of Ca^{2+} into the myofibrils (Section 5.1.) showed that, if anything, cordifene inhibits such a translocation to some extent.

This then means that Erlangia cordifolia extract manifests its effect on the contractile process by affecting processes within the muscle cell itself. Influences on muscle contraction by direct effect on the muscle cell itself are known to occur with chemical compounds like histamine and serotonin (5-hydroxytryptamine) (23). Erlangia cordifolia extract is water soluble and as such should have little difficulty in being absorbed through the gut wall and into the blood stream. That cordifene is one of the compounds extracted by water from the leaves of Erlangia cordifolia is shown by the facts that (a) the crude extract in methanol shows the characteristic peak of absorption for the unsaturated lactone at 213 nanometres and (b) by the fact that cordifene is soluble in 50% NaOH in the cold and lower con-

centrations of the alkali will dissolve it when hot. The basic salts in the leaves of the herb could alkalinize the extracting water thereby solubilizing cordifene in the water. The fact that when combined with myosin it becomes soluble in dilute concentrations of potassium chloride could also mean that it is most probably extracted by water in combination with other molecules most probably low molecular weight proteins or polypeptides.

Cordifene dissolves in 70% methanol but not in greater dilutions of methanol with water. This shows the compound to be hydrophobic to a great extent. Its hydrophobic nature in addition to its small molecular weight should facilitate its passing across the cell membrane. Cordifene was found to be the major organic constituent of the Erlangia cordifolia extractable material with methanol and was also found to reproduce the physiological activity of the crude extract. So cordifene can be taken as one of the major, if not the major, contributor to the physiological and therefore biochemical activity of Erlangia cordifolia extract.

Since Erlangia cordifolia extract effects contraction of muscle by acting directly on the contractile mechanism within the muscle cell itself, it achieves this by either potentiating myosin ATPase activity or by directly affecting the contractile elements within the myofibril in such a way that favourable configurational changes of these elements

result. Cordifene could potentiate myosin ATPase by either inducing favourable conformational changes on the protein (41) or by potentiating the removal of the enzyme inhibitors. The main inhibitor of myosin ATPase is ADP (11, 15, 49) and although some workers contend that at temperatures above 16°C the inhibition shifts from the enzyme-ADP complex dissociation to yet another unidentified step (50, 54) it is however conceivable that the mechanism of the obstetric "lazy" uterus could come about by failure of the dissociation of this complex. The removal of ADP from the cytoplasm could then decrease the enzyme-ADP association and facilitate dissociation of the complex. The author's results on the effects of cordifene on the UV absorption of ADP (Fig. 43) showed that the nucleotide peak around 210 nanometers is diminished by cordifene indicating complex formation. This then means that cordifene facilitates the enzyme-ADP complex dissociation.

However, this effect of cordifene and crude Erlangia cordifolia extract is bound to be limited in scope because a given amount of cordifene can only react with a given amount of ADP unless it is continually administered as in an intravenous drip to an actively contracting muscle. Since only a finite quantity of Erlangia cordifolia extract is administered orally and since ADP is continuously produced by a contracting muscle, it follows that the positive effect of cordifene in facilitating

myosin ATPase (E.C. 3.6.1.3) through the facilitation of the dissociation of myosin-ADP complex is limited in scope. If cordifene acted as an ADP carrier in between the myofibril and the mitochondrial particles surrounding it, then it could be feasible to argue that the removal of ADP from the myofibril is the effective means by which it potentiated muscle contraction. The author's investigation on the effect of cordifene on the rate of respiration of mitochondria in the presence of ADP (Section 5.3.B) showed that far from facilitating the entry of ADP into the mitochondria, cordifene bound ADP and kept it away from these particles.

Cordifene did not facilitate the binding of Ca^{2+} to the myofibrils. If anything it was found to inhibit such binding (see Table 3) most probably by binding onto sulphydryl groups that normally bind Ca^{2+} (37). This then means that the major contribution of cordifene to muscle contraction must be on a biochemical process occurring within the contracting elements themselves.

The most widely held view is that the thin filament through its actin components acts as a cofactor for the myosin ATPase (17). This means that conformational changes that are directly associated with the contraction of muscle are most likely to be found within the myosin molecule itself. Huxley (18) and Huxley and Brown (60) have shown that conformational changes do occur within the myosin head.

It is on the basis of these changes that Huxley based the sliding filament model of muscle contraction. By this model, it has been found that when maximally contracted, the sarcomere is shortened by anywhere from 20-50 percent (1). Yet it is a physiological fact that a muscle like the myometrium of a gravid uterus must contract to a size smaller than 30% of the original size if it is to expell the full grown foetus. This means therefore that the sliding filament theory can only be a partial explanation of the process of muscle contraction. Configurational changes in the main skeleton of the myosin filament would more than adequately explain the extra shortening of muscle inexplicable by the sliding filament theory.

Eisenberg et al. (14) has observed that only a small fraction of myosin molecules in a myofibril is bound to the thin filament at any one time. Hydrolysis of ATP on the heads of the attached myosin filaments followed by movement of these heads can hardly therefore alter the distances measurable by Optical Rotary Dispersion Spectra (1). While this fact agrees well with the findings of Huxley (18) and those of Huxley and Brown (60) it would hold equally well if major configurational changes on the main "body" of the myosin molecule were induced by the energy released from the hydrolysis of ATP on the attached myosin heads. This is so because the tension that could be generated by such configura-

tional changes occurring in some "pockets" of the myosin polymer would become dissipated along the length of the polymer. This means that major configurational changes occurring in some sections of the myosin polymer leave the Optical Rotary Dispersion Spectrum essentially the same as it was before the hydrolysis of the nucleotide. A major biochemical parameter cannot therefore be measured by present day available means because owing to lack of cofactor activity for the majority of myosin heads since these heads are not attached to the thin filament (14), only a few myosin molecules on the polymer hydrolyse ATP on their heads at any one time and therefore undergo the configurational change. This then means that if contraction in a few myosin molecules in the myosin polymer of the thick filament underwent contraction, extension in the other parts of the polymer would have, of necessity, to occur if the over-all length of the polymer is to remain virtually the same. In a polymer which is a doubly coiled double helix like myosin (1) the tension of contraction generated in some areas of the polymer would become evenly distributed throughout the polymer and the measurements of the band lengths would hardly show any changes. This means that the only measurable changes would be the configurational changes occurring at the actin-myosin head linkage.

It has been observed that a 50% decrease in

helical content of cardiac myosin (in 45% ethylene glycol)result in increase in the myosin's ATPase activity (20) and this huge conformationa change has been taken to emphasize the importance of the hydrophobic regions in the activation of this type of myosin. This is interesting considering that a great part of cordifene is hydrophobic from its chemical structure and therefore highly likely to be capable of reacting with the hydrophobic parts of myosin. The finding is also interesting from the point of view of the increase in the enzyme activity following the huge conformational change. Barany et al. (20) have obtained results which suggest that the ATPase site of gizzard myosin, a smooth muscle, undergoes a catalytically favourable alteration in the presence of low concentrations of urea as a result of configurational changes in regions of the myosin molecule removed from the ATPase site. All these findings agree well with the findings reported in this work that show that cordifene causes a huge configurational change in the actomyosin molecule and at the same time increases ATPase activity. And, in view of the fact that examples are known correlating speed of contraction of muscle and the ATPase activity of myosin (2), the correlation of conformational changes with ATPase activity discussed here and observed with cordifene must also correlate cordifene with the speed of contraction of muscle that has been observed by the Gikuyu people of Kenya.

SUMMARY

The wet leaves of Erlangia cordifolia are widely used for the production of an extract widely used in the Gikuyuland in Kenya for the purposes of facilitating the birth of a child in cases where the progress of the process of parturition is judged to be unsatisfactorily slow for the good of the mother and her child. The effect of the extract from the leaves of E. cordifolia on the contraction of myometrial strips from both rats and guinea-pigs has been confirmed. The extract has also been shown to increase the contractions of smooth muscles from the gastrointestinal tract and also those of striated muscles of rats and guinea pigs.

Chemical analysis of E. cordifolia leaves has revealed that the leaves contain an appreciable amount of calcium which, although found in the water extract from these leaves, does not play a significant role in the facilitation of muscle contraction. The same analysis has releaved an α-methylene-γ-lactone group containing organic compound which is the main compound that is extracted by methanol from such leaves. This compound has been

named cordifene by, the author and cordifene was found to reproduce the biological activity of the crude water extract.

Cordifene was also found to bind ADP very competitively and also to induce very marked configurational changes on myosin. The configurational changes were also accompanied by an increase in myosin ATPase activity.

Neither the crude extracts of E. cordifolia nor cordifene was found to have activity on the autonomic nervous system and for this reason the level at which these materials were judged to affect the process of muscle contraction was at the muscle cell itself. Since these materials were found to have no effect on the movement of calcium to and from the sarcoplasm reticulum, it was deduced that their point of activity must be at the contractile elements themselves. Therefore, in view of the conformational changes that were observed to be induced by cordifene and the fact that these conformational changes were found to be accompanied by increased myosin ATPase activity and in view of the fact that the speed of contraction of muscle has been correlated with ATPase activity, the correlation found in between ATPase activity and conformational changes in actomyosin induced by cordifene has enabled the author to correlate the conformational changes seen in actomyosin with the increased contractile activity induced by the E.cordifolia material and ultimately

with the biochemical basis for the use of Erlangia cordifolia material for augmenting myometrial contraction during parturition.

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